[Chem. Pharm. Bull. 27(3) 654—661 (1979)]

UDC 577.156.01.02.07 : 615.276.015.43.076.9

Kinin Inactivating Enzyme from Mushroom *Tricholoma conglobatum*. II. Some Enzymatic Properties of the Purified Enzyme[1]

Kazuyuki Kizuki, Hiroshi Moriya, and Chiaki Moriwaki

Laboratory of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo[2]

(Received August 28, 1978)

Some enzymatic properties of the new potent kinin inactivating enzyme (named as Shimeji kininase) which was purified from a mushroom *Tricholoma conglobatum* (Shimeji, in Japanese) were studied.

This enzyme inactivated bradykinin, kallidin and Met-Lys-bradykinin by the ratio of 1.0: 1.0: 1.5, respectively, calculated as the molar ratio, and its *K*m value for bradykinin hydrolysis was 1.9 μM.

Isoelectric point and molecular weight of the enzyme were 4.5 and 6.6×10^4, respectively.

Optimum pH of this enzyme was 7.4 and it was comparatively stable at pH 6—9.

This enzyme was thermolabile, *i.e.*, more than 50% of the activity vanished by heating at 45° for 10 min and completely vanished at 60° for 10 min.

The chelating agents, trasylol, SBTI and bradykinin potentiator B had no detectable influence upon the kininase activity of the enzyme. TLCK, TPCK and DFP weakly inhibited the enzymatic activity. The mercurials strongly inhibited the enzymatic activity of it and the inhibited activity was restored by adding excess amount of 2-mercaptoethanol. Accordingly, sulfhydryl groups in the enzyme molecule are requisite for its kininase activity and this enzyme may be classified as the thiol enzyme.

The enzyme hydrolyzed the amino terminal substituted peptides such as Bz- and Z-derivatives while N-terminal free di- or tri-peptides were hardly hydrolyzed by it.

Keywords——kininase from mushroom *Tricholoma conglobatum*; kinin destruction; kinin; properties of kininase; substrate specificity of kininase

The physiologically active peptides, kinins, have been interested in regard to some pathological rationale, such as inflammation, shock and so on. However, physiological and pathological meanings of the kallikrein-kinin system in the body are still obscure because we have not had the solid proof in which the kinin action should be specifically blocked. So that, the authors have been working on the kininases from various origins with a view to blocking the kinin action in the body by them.[3]

Recently, we found that one of the mushroom *Tricholoma conglobatum* (Shimeji, in Japanese) contained potent kininase activity, and this potent kinin inactivating enzyme, named as Shimeji kininase, was purified from it.[1] This enzyme cleaved Gly^4–Phe^5 and Pro^7–Phe^8 bonds of bradykinin molecule and had the most potent kininase activity of all the kininases obtained from many plants.[1] Therefore, its application to the kallikrein-kinin system would be greatly expected.

In the present paper, the authors describe about some enzymatic properties of this enzyme.

Materials and Methods

Materials——Bradykinin, kallidin, Met-Lys-bradykinin, bradykinin potentiator B and other peptides were supplied from Protein Research Foundation (Osaka) and papain (2 × crystallized) was obtained from

1) Part I: K. Kizuki, C. Moriwaki, Y. Hojima, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **24**, 1742 (1976).
2) Location: *Ichigaya Funagawaracho, Shinjuku-ku, Tokyo.*
3) *a*) Y. Hojima, H. Moriya, and C. Moriwaki, *Agric. Biol. Chem.* (Tokyo), **41**, 559 (1977); *b*) Y. Hojima, M. Tanaka, H. Moriya, and C. Moriwaki, *Japanese J. Allergology*, **20**, 755 (1971); *c*) *Idem, ibid.*, **20**, 763 (1971).

Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Silica gel H was purchased from E. Merck (Darmstadt, Germany) and Sephadex G-150 from Pharmacia Fine Chemicals (Uppsala, Sweden). 1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl), Trasylol and soybean trypsin inhibitor (SBTI) were products from Tokyo Kasei Kogyo Co. (Tokyo), Farbenfabriken Bayer A.G. (Leverkusen, Germany) and Sigma Chemical Co., respectively. DNS-bradykinin prepared in our laboratory was also used in this investigation.

Shimeji Kininase——Shimeji kininase was purified as described in our previous paper.[1] The final preparation had a kininase activity of 480 kininase U/E_{280}. One kininase unit was the amount of enzyme that could degrade 1 μg of synthetic bradykinin per min at 30°, pH 7.4. Kininase activity of the preserved enzyme solution was checked up just before the following experiments.

Kininase Activity Assay——This assay was carried out by Magnus method using isolated guinea pig ileum.[1] For the measurement of kininase activity, 0.15 ml of bradykinin solution (1 μg/ml in 0.05 M phosphate buffer, pH 7.4) was incubated with 0.15 ml of a sample solution for a certain minute at 30°, and the contractile response by 0.2 ml of this mixture (100 ng bradykinin eq.) was assayed. The kininase activity was calculated from the amounts of inactivated bradykinin. When the activity of a sample was so potent the sample was diluted with the same buffer.

Isoelectric Focusing Fractionation——The enzyme solution was applied to an Ampholine column (column size, 110 ml) using the LKB apparatus.[4] The pH range of carrier Ampholyte was 3—6 and electrophoresis was carried out for 40 hr at constant voltage (500 V) with a cooling system (4°). After measuring the pH's of the fractions obtained 2.5 ml of 0.2 M phosphate buffer, pH 7.4, was added to the each fraction (2.5 ml) and the absorbancy at 280 nm and the kininase activity were assayed.

Hydrolysis of Peptides——Substrate specificity of Shimeji kininase was examined by the following methods. A mixture of 1 ml of the aqueous enzyme solution (10 kininase U/ml) and 1 ml of 1/200 M substrate solution in 0.1 M triethylamine bicarbonate buffer, pH 7.4 was incubated for 20 hr at 30° and then lyophilized. The material was dissolved in 0.1 ml of 50% methanol and the aliquot of it was chromatographed on paper (Toyo filter paper No. 51, Toyo Roshi Kaisha, Ltd., Tokyo) with the following two solvents; (I) Phenol: H_2O (7:3), (II) *n*-BuOH: AcOH: H_2O (4:1:2). The digested substances were detected by spraying 0.2% ninhydrin solution. The digested substances were also detected by the next method. The above methanol solution was dried with blowing N_2 gas and dansylated according to the method of Gray and Hartley[5] with slight modification.[1] Then, it was applied to silica gel H thin-layer chromatography using 2PrOH: MeOAc: 28% NH_4OH (9:7:4). DNS-derivatives were detected by their yellow fuorescence under U.V.-light (3600 Å). Caseinolytic activity and esterolytic activity against N^{α}-benzoyl-L-arginine ethyl ester (BAEE) were also determined by the methods of Kunitz[6] and Schwert *et al.*,[7] respectively.

Effect of Enzyme Inhibitors on Kininase Activity——One ml of the enzyme solution (10 kininase U/ml in 0.05 M phosphate buffer, pH 7.4) was preincubated with 1 ml of inhibitor solution for 30 min at 30°, and then remaining kininase activity was assayed by Magnus method. Guinea pig ileum was sometimes damaged by some of the inhibitors at high concentrations, so that the inhibition of the enzyme by the inhibitors was made sure with the following experiments. A part of the above preincubated mixture (0.2 ml) was incubated with 10 μl of methanol solution of DNS-bradykinin (0.5 mg/ml) at 30°, and the aliquot of it was plotted on thin layer of silica gel H plate at the stated periods and developed with the solvent, 2PrOH: MeOAc: 28% NH_4OH (9:7:4). As Shimeji kininase cleaved Gly^4–Phe^5 and Pro^7–Phe^8 bonds of bradykinin molecule, fluorescent band of DNS-bradykinin gradually vanished and the fluorescent band of DNS-Arg-Pro-Pro-Gly, which had larger *Rf* value than DNS-bradykinin, was newly appeared on the plate of silica gel H as the result of the digestive action of the enzyme.[1] So that, inhibitory effects of the reagents on the activity of Shimeji kininase could be confirmed by the amounts of DNS-bradykinin and DNS-Arg-Pro-Pro-Gly detected on the silica gel H plate.

Results

Kinin Destruction

Table I shows the action of Shimeji kininase against three kinins, *i.e.*, bradykinin, kallidin and Met–Lys–bradykinin. These kinins were all inactivated by this enzyme. Kallidin was hydrolyzed by this enzyme in the same ratio as bradykinin, calculated as the molar ratio, while Met–Lys–bradykinin was more easily hydrolyzed than the other two peptides.

4) O. Vesterberg and H. Svenssorn, *Acta Chem. Scand.*, **20**, 820 (1966).
5) *a*) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 59p (1963); *b*) *Idem*, *ibid.*, **89**, 379 (1963).
6) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).
7) G.W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta* **16**, 570 (1955).

Table I. Action of Shimeji Kininase against Three Kinins

Substrate	Molecular weight	Hydrolyzed kinin by this kininase	
		μg/min/ml	nmol/min/ml
Bradykinin	1060	117	110(1.0)
Kallidin	1188	133	112(1.0)
Met-Lys-bradykinin	1319	222	168(1.5)

Kinin destructions by Shimeji kininase solution, 117 kininase U/ml, were determined by Magnus method[1] (see Methods) and compared between 3 kinins. For the assay of kininase activity against kallidin and Met-Lys-bradykinin, larger assay volume than 0.2 ml was necessary because the sensitivities of these kinins to the guinea pig ileum were weak as compared with that of bradykinin. The rate of kinin hydrolysis was shown in parenthesis based on bradykinin hydrolyzed, 1.0.

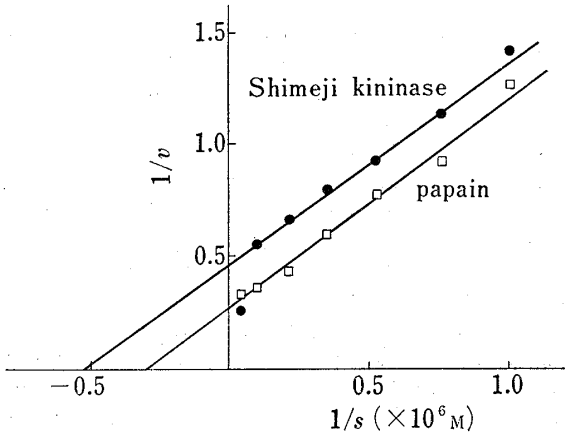

Fig. 1. Lineweaver-Burk Plots of Shimeji Kininase and Papain for Bradykinin Hydrolysis

Kininase activities of Shimeji kininase and papain were assayed by Magnus method at 30°, pH 7.4. Papain was used after the dilution with 0.05 M phosphate buffer, pH 7.4 containing 0.01 M of cystein to make active form. Each point in the figure was the average of 3 to 5 assays.

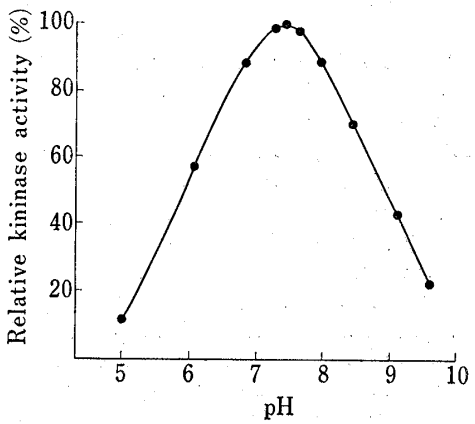

Fig. 2. Effect of pH on the Kininase Activity of Shimeji Kininase

The various pH's of 1/12.5 M Britton-Robinson's wide-range buffer (4 ml) was separately added to 1 ml of the enzyme solution (50 kininase U/ml) which was dialyzed against distilled water. Then, the kininase activity of them was assayed. If necessary, the sample was diluted with the same buffer. The substrate, bradykinin, was dissolved in saline. The relative kininase activity was expressed as percent of the activity at an optimal pH.

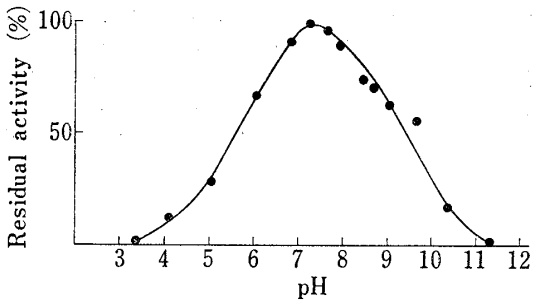

Fig. 3. pH Stability of Shimeji Kininase

The enzyme solution (336 kininase U/ml), 0.5 ml was incubated with 1.0 ml of 1/12.5 M Britton-Robinson's wide-range buffer at various pH for 20 hr at 4°. After that, 1.5 ml of 0.2 M phosphate buffer, pH 7.4 was added, and then the remaining kininase activity was assayed.

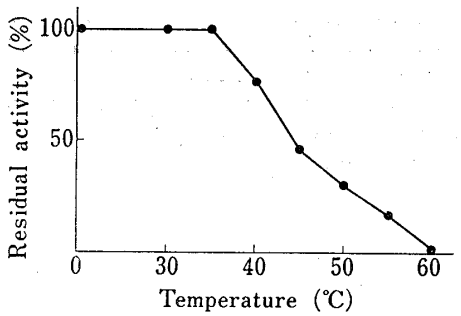

Fig. 4. Thermal Stability of Shimeji Kininase

The enzyme solution (12 kininase U/ml), 0.5 ml was incubated for 10 min at various temperature at pH 7.4. After that, samples were cooled with ice bath, then remaining kininase activity was assayed.

Effect of Substrate Concentration

Lineweaver-Burk plots of Shimeji kininase for synthetic bradykinin hydrolysis was linear (Fig. 1). From this result its *K*m value was apparently calculated to be 1.9 μM. On the other hand, that of papain, which was one of the most potent kininases, was 3.4 μM.

Optimum pH

The effect of pH on the kininase activity of the enzyme was investigated between pH 5.0—9.5 (Fig. 2). The optimum bradykinin hydrolysis was found at pH 7.4.

pH Stability

Figure 3 shows the pH stability of Shimeji kininase. This enzyme was comparatively stable at pH 6—9 at 4° for 20 hr but more than 80% of the activity was lost at pH 4 and 10.

Thermal Stability

As shown in Fig. 4 this enzyme was thermolabile. More than 50% of the activity was lost by the heat treatment at 45° for 10 min and completely vanished at 60° for 10 min.

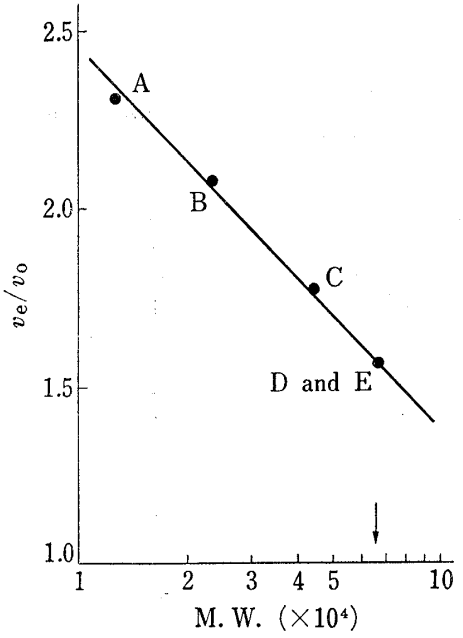

Fig. 5. Estimation of the Molecular Weight of Shimeji Kininase

Authentic proteins were bovine serum albumin (M.W., 6.7×10^4), ovalbumin (M.W., 4.45×10^4), trypsin (M.W., 2.38×10^4) and cytochrome c (M.W., 1.24×10^4). Blue dextran was also used as a marker for the estimation of void volume. One ml of each protein was separately applied to the column (1.5×73.0 cm) of Sephadex G-150 equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 0.45 M NaCl. The elution volume determined by measuring the absorbancy at 280 nm were recorded for bovine serum albumin, ovalbumin and trypsin, at 410 nm for cytochrome c and 620 nm for blue dextran. The esterase activity against BAEE was also assayed for trypsin. Shimeji kininase solution was subjected to gel filtration and the volume of the maximum activity was recorded. A: cytochrome c; B: trypsin,; C: ovalbumin; D: Shimeji kininase; E: bovine serum albumin.

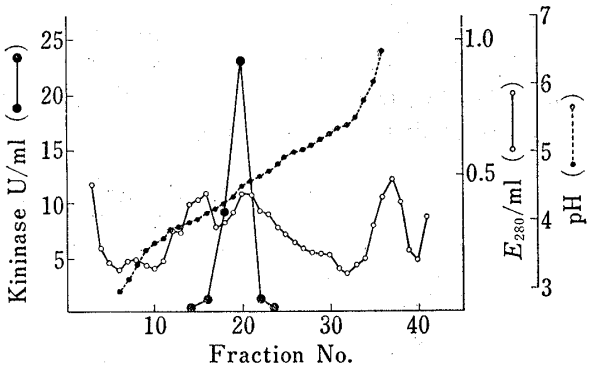

Fig. 6. Isoelectric Focusing Fractionation of Shimeji Kininase

The partially purified Shimeji kininase preparation which was the active fraction of the first DEAE-Sephadex A-50 chromatography[1] was dialyzed against distilled water for 10 min at 4°, and 15 ml of the dialyzed solution (210 kininase U/ml) was applied to the Ampholine column.

Table II. Action of Shimeji Kininase on Various Synthetic Substrates

Substrate	Degree of hydrolysis
H–Gly–Phe–OH	−
H–Gly–Gly–Gly–OH	−
H–Gly–Leu–OH	−
H–Gly–Pro–OH	−
H–Leu–Gly–Gly–OH	+
H–Arg–Gly–Asp–OH	−
H–Phe–OEt	−
H–Ser–Pro–Phe–OH	−
Bz–Gly–Arg–OH	+
Bz–Gly–Lys–OH	−
Z–Gly–Leu–OH	−
Z–Phe–Tyr–OH	+
Z–Gly–Phe–OH	±
Z–Glu–Tyr–OH	+
Z–Glu–Phe–OH	+
Z–Gly–Pro–Leu–Gly–OH	+
Z–Gly–Phe–NH_2	+
BAEE	−
Casein	+

Bz–: benzoyl, Z–: carbobenzoxy.
+: hydrolyzed, ±: weakly hydrolyzed, −: not hydrolyzed.

Molecular Weight

According to the method of Andrews,[8] the approximate molecular weight of Shimeji kininase was estimated to be about 6.6×10^4 (average of 3 experiments) by Sephadex G-150 gel filtration (Fig. 5).

Isoelectric Point

After preparative isoelectric focusing with Ampholyte (pH 3.0—6.0), the kininase activity of the enzyme could be detected in the eluate from the column and the isoelectric point of the enzyme was determined to be 4.5 (Fig. 6). However, the activity recovery was only 10.9% of the starting sample.

Substrate Specificity

The substrate specificity of Shimeji kininase was studied using synthetic derivatives of amino acids and peptides as shown in Table II. The enzyme would be considered to hydrolyze the N-terminal substituted peptides, while N-terminal free di- or tri-peptides were hardly hydrolyzed. Bz–Gly–Lys–OH which is a substrate of carboxypeptidase N (plasma kininase I) was not hydrolyzed by Shimeji kininase. H–Ser–Pro–Phe–OH was the peptide existing in bradykinin molecule.

Table III. Effects of Various Enzyme Inhibitors on the Activity of Shimeji Kininase

Inhibitor	Concentration (M)	Degree of inhibition
$CoCl_2$	1×10^{-3}	−
$NiSO_4$	1×10^{-3}	−
$CuCl_2$	1×10^{-3}	+
$MnCl_2$	1×10^{-3}	−
$FeCl_3$	1×10^{-3}	−
$HgCl_2$	1×10^{-5}	++
8-Hydroxyquinoline	1 mg/ml*	−
o-Phenanthroline	1×10^{-3}*	−
EDTA	1×10^{-3}**	−
	1×10^{-2}**	++
2,3-Dimercapto-1-propanol (BAL)	1×10^{-3}	−
$C_6H_5HgOCOCH_3$	1×10^{-5}	++
$ClHgC_6H_4COOH$	1×10^{-5}	++
CH_2ICOOH	1×10^{-4}*	++
$Na_2S_4O_6$	1×10^{-2}*	−
2-Mercaptoethanol	1×10^{-2}	−
Cysteine	1×10^{-2}	−
DFP[a]	1×10^{-2}	+
TLCK[b]	1×10^{-3}*	+
TPCK[c]	1×10^{-3}*	+
Trasylol	2500 KIU/ml*	−
SBTI	1 mg/ml*	−
Bradykinin potentiator B	100 μg/ml*	−

a) Diisopropyl-fluorophosphate.
b) Tosyllysine chloromethyl ketone.
c) Tosylphenylalanine chloromethyl ketone.
The degree of inhibitory effects was judged by the following experiments. The incubation mixtures of Shimeji kininase and each inhibitor and the control (the mixture of Shimeji kininase and distilled water) were severally diluted 3 times with 0.05 M phosphate buffer. After that, kininase activity of the diluted samples was assayed by Magnus method. The incubation with bradykinin was carried out for 3 min. The control completely inactivated 100 ng of bradykinin and the contractile response of bradykinin on guinea pig ileum was not detected. −: Kinin response was not detected, +: Kinin response was detected but very weak, ++: Kinin response was not affected or slightly reduced. The marks also mean that the DNS-bradykinin was not hydrolyzed or traces of the fluorescent band of the fragment of DNS-bradykinin was detectable (++), considerable amounts of fluorescence was detected at the position of the fragment (+) and DNS-bradykinin completely vanished (−). Assays were carried out by Magnus method (*), TLC method (**) and both methods (no mark).

8) P. Andrews, *Biochem. J.*, **91**, 222 (1964).

Effect of Enzyme Inhibitors on Kininase Activity

The effect of various inhibitors on the kininase activity of the enzyme is shown in Table III. Metal ions, Co^{2+}, Ni^{2+}, Mn^{2+} and Fe^{3+} had no detectable influence on the enzymatic activity at the concentration of 1×10^{-3} M but Cu^{2+} partially inhibited the enzyme at this concentration. The mercurials, such as $HgCl_2$, phenylmercuric acetate and *p*-chloromercuribenzoic acid, which inhibit enzymes containing sulfhydryl groups, strongly inhibited the enzyme at 1×10^{-5} M. Iodoacetic acid also strongly inhibited the enzyme at 1×10^{-4} M but one of the sulfhydryl reagent, sodium tetrathionate, had no inhibitory effect at 1×10^{-2} M. The chelating agents had no detectable effect at 1×10^{-3} M but EDTA strongly inhibited the activity at 1×10^{-2} M. TLCK and TPCK showed weak effects at 1×10^{-3} M. Trasylol and SBTI had no effect. DFP showed weak effect at 1×10^{-2} M but no effect at 1×10^{-3} M. Bradykinin potentiator B, which inhibits plasma kininase II (angiotensin-I converting enzyme),[9] had no detectable influence on the kininase activity of the enzyme.

Figure 7 shows the inhibition of Shimeji kininase by $HgCl_2$ and the restoration of the activity by 2-mercaptoethanol. The enzyme which was not treated with $HgCl_2$ completely inactivated 100 ng of bradykinin (Fig. 7-A-2) but the same amount of enzyme which was preincubated with $HgCl_2$ (1×10^{-5} M) did not inactivate bradykinin (Fig. 7-A-3). On the contrary, after adding 2-mercaptoethanol to the enzyme solution which was preincubated with $HgCl_2$, the enzyme again inactivated bradykinin (Fig. 7-A-4). However, the restoration of the enzyme activity was not perfect because weak contractile response due to the remaining bradykinin was detected (Fig. 7-A-4). The same results were also obtained by chromatographical analysis on thin layer of Silica gel H (Fig. 7-B). These results indicated that Shimeji kininase was strongly inhibited by $HgCl_2$ and the activity was restored by adding excess amount of 2-mercaptoethanol. The inhibition by high concentration of $HgCl_2$ ($1\times$

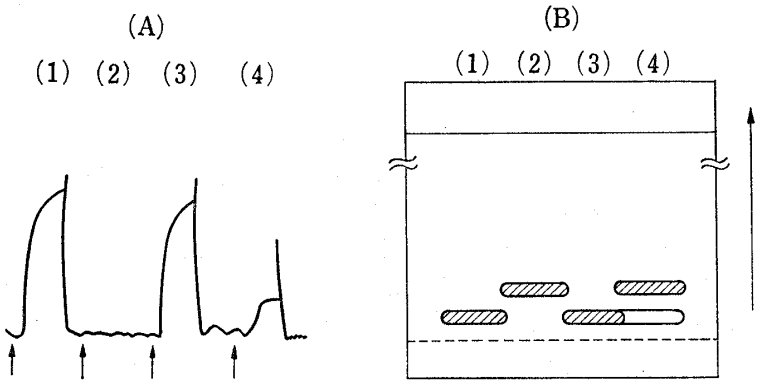

Fig. 7. Inhibition of Shimeji Kininase by $HgCl_2$ and Restoration of Activtiy by 2-Mercaptoethanol

Kinin destruction by Shimeji kininase was examined by Magnus method (A) and thin-layer chromatography (B).

(A): (1) bradykinin 100 ng; (2) bradykinin 100 ng was incubated with Shimeji kininase for 3 min; (3) bradykinin 100 ng was incubated with the enzyme solution preincubated with $HgCl_2$ at the concentration of 1×10^{-5} M for 3 min; (4) 2-mercaptoethanol was added to the enzyme solution used in (3) to make final concentration of 1×10^{-2} M and this solution was incubated with 100 ng of bradykinin for 3 min. The amount of enzyme incubated with bradykinin in (3) and (4) were the same as that of the enzyme in (2).

(B): (1) DNS-bradykinin; (2), (3) and (4) DNS-bradykinin was separately incubated with the enzyme solutions used in A-2, -3 and -4, respectively, for 30 min. The development was carried out with the solvent of 2PrOH: MeOAc: 28% NH_4OH (9:7:4).

9) *a*) Y.S. Bakhle, "Handbook of Experimental Pharmacology," Vol. XXXVII, ed. by I.H. Page and F.M. Bumpus, Springer-Verlag, Berlin-Heidelberg-New York, 1974, pp. 41—80; *b*) E. Ueda, T. Kokubu, H. Akutsu, and Y. Yamamura, *Experientia*, **27**, 1020 (1971).

10^{-3} M), however, was irreversible because the fluorescent band of the fragment of DNS-bradykinin shown in Fig. 7-B was not detected even after the addition of 2-mercaptoethanol.

Discussion

The plasma kinins, bradykinin, kallidin and Met–Lys–bradykinin are derived from their precursor proteins, the kininogens, by the action of various kininogenases. For example, kallidin is generally liberated by the action of glandular kallikreins while bradykinin is liberated by the action of plasma kallikrein and trypsin.[10] These kinins have the similar pharmacological properties, although there are some quantitative differences in their relative potencies on different test preparations. On the other hand, there are aminopeptidases which convert kallidin and Met–Lys–bradykinin to bradykinin in the mammalian body.[11] These facts suggest that there would be more than one kind of kinins naturally occurring in the body. Shimeji kininase, however, inactivated all the above three kinins. According to Hojima *et al.*, kininases from plant origin, such as ficin, papain, bromelain and potato kininase, hydrolyzed kallidin 1.5 to 5 times easier than bradykinin.[3b] However, Shimeji kininase hydrolyzed kallidin at almost the same rate as bradykinin, and Met–Lys–bradykinin was most rapidly hydrolyzed by this enzyme among the three kinins in our experimental conditions.

The optimum pH of this enzyme was 7.4 and this enzyme was stable near the neutral pH region. These properties of Shimeji kininase were very convenient for its application to the kallikrein-kinin system because the optimum pH of Shimeji kininase was almost the same as the pH of the body fluids of mammalians. Namely, if this enzyme is not inhibited by proteinase inhibitors in the mammalian body it would act at its optimum pH and would block the kinin action in the body for some period.

After the isoelectric focusing, big part of the activity of Shimeji kininase was lost (Fig. 6). It was reported that the Ampholyte had the chelating activity and loss of activities by isoelectric focusing occurred in some enzymes.[3b] However, the activity of Shimeji kininase was not inhibited by chelating reagents (Table III). In the isoelectric focusing, the enzyme was kept at pH 4.5 for a long time in the column. At this pH region, this enzyme was very unstable (Fig. 3), so that the loss of activity of Shimeji kininase would be explained by the property of pH stability of the enzyme.

The kininase activity of the enzyme was inhibited by sulfhydryl reagents such as mercurials and iodoacetic acid, and the inhibition by former chemicals was restored by adding excess amounts of 2-mercaptoethanol. These are the typical inhibitory properties of thiol enzymes.[12] Accordingly, sulfhydryl groups in the enzyme molecule are requisite for its kininase activity and this enzyme may be classified as the thiol enzyme. The potent kininases from plant origin, ficin, papain and bromelain are all thiol enzymes but the sites of action of them on bradykinin molecule are different from that of Shimeji kininase.[1] The molecular weight of Shimeji kininase was estimated to be 6.6×10^4 and pI was 4.5. These values are entirely different from those of papain (M.W. 2.07×10^4 and pI 8.8), ficin (M.W. 2.6×10^4 and pI 9.0) and stem bromelain (M.W. 3.3×10^4 and pI$>$9.6).[12] The molecular weight and pI of Shimeji kininase are similar to those of the kininases from potatoes *Solanum tuberosum* (M.W. 7.0×10^4 and pI 4.7)[3c] and red kidney beans *Phaseolus vulgaris* (M.W. 7.3×10^4 and pI 4.6).[3a] Optimum pH, pH stability and thermal stability of them are also

10) M. Schachter, *Physiol. Rev.*, **49**, 509 (1969).
11) *a*) J.A. Guimarães, D.R. Borges, E.S. Prado, and J.L. Prado, *Biochem. Pharmacol.*, **22**, 3157 (1973); *b*) D.R. Borges, J.L. Prado, and J.A. Guimarães, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **281**, 403 (1974).
12) R. Arnon, I.E. Liener, B. Friedenson, and T. Murachi, "Methods in Enzymology," Vol. XIX, ed. by G.E. Perlmann and L. Lorand, Academic Press, New York, 1970, pp. 226—284.

similar to those of Shimeji kininase. However, the action of them on various synthetic substrates and the behaviors to chelating agents are different from those of Shimeji kininase. Kinonase AI and AIII[13] which were purified from microbe *Streptomyces* cleave the same bonds on bradykinin molecule as Shimeji kininase and some properties such as optimum pH and thermal stability are closely resemble to those of Shimeji kininase. These enzymes, however, are different from Shimeji kininase in some of their substrate specificities, their behaviors to enzyme inhibitors. Furthermore, one of the most prominent differences among these enzymes was the stability to lyophylization. Shimeji kininase was very labile when lyophylized, so that we have not carried out lyophylization in the purification of the enzyme. On the contrary, kinonase AI and AIII seemed to be stable to lyophylization because the purification procedures of them contained four times of lyophylization. The kininase from microbe *Pseudomonas aeruginosa* (*Ps. aer.*) was also reported by Rugstad.[14] This enzyme was strongly inhibited by the metal binding reagents, *o*-phenanthroline and 2,3-dimercapto-propanol (BAL),[14b] while Shimeji kininase was not inhibited by them. Thermal stability of the kininase from *Ps. aer.* also differed from Shimeji kininase, *i.e.*, all the enzyme activity of the former enzyme remained after 10 min incubation at 65°,[14a] but the activity of Shimeji kininase was completely disappeared by heating at 60° for 10 min. The molecular weight of the kininase from *Ps. aer.* (M.W. 5.0×10^4) was also different from the present enzyme.[14b]

Thus, Shimeji kininase can be differentiated from the known kininases from plants or microbes.

13) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka, T. Takeuchi and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 714 (1969).

14) *a*) H.E. Rugstad, *Br. J. Pharmacol.*, **30**, 134 (1967); *b*) *Idem*, *ibid.*, **30**, 425 (1976).