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Kinin Inactivating Enzyme from Mushroom *Tricholoma conglobatum*. I. Purification and the Sites of Action on Bradykinin Molecule

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Potent kininase activities were found in Japanese mushrooms. Especially *Tricholoma conglobatum* (shimeji, in Japanese) contained 40—334 kininase units/g and the enzyme was purified by water extraction, ammonium sulfate fractionation, diethylaminoethyl (DEAE)-Sephadex A-50 chromatography and Sephadex G-100 gel filtration. The final preparation gave a single band in disc electrophoresis and its kininase activity, that was expressed in terms of μg bradykinin degraded in 1 min at 30°, was 480 units/E_{280}. This value was extremely potent, so this enzyme could be expected as a useful agent on the clinical purposes or other investigations of kallikrein-kinin system.

The sites of action of this enzyme on bradykinin molecule were investigated by examination of 1-dimethylaminonaphthalene-5-sulphonyl (DNS)-modified products, which were liberated from bradykinin by this enzyme, on thin layer chromatography. It cleaved Gly^4-Phe^5 and Pro^7-Phe^8 bonds and the former bond was split more easily than the latter one.

Physiologically active peptides, the kinins, are liberated from their precursor proteins, the kininogens, with kininogenases and cause various actions such as vasodilation, permeability increase on the capillaries and so on. On the other hand there are kinin inactivating enzymes, the kininases, in the mammalian organs and similar enzymes are also found in some sorts of plants or microbes.[2] Bromelain [EC 3.4.4.24],[3] ficin [EC 3.4.4.12],[4] papain [EC 3.4.4.10][5] and kinonase (AI and AIII)[6] are known as the enzymes which cleave various bonds in bradykinin molecule and give anti-inflammatory effect.[7] Besides these proteases the authors found a new kininase in potatoes,[8] and in the course of further survey of kininase in various plants, very potent activities were found in the mushrooms, especially in *Tricholoma conglobatum* (shimeji, in Japanese).

The present paper deals with purification of Shimeji kininase and the sites of action of the enzyme on bradykinin molecule. A new quantitative kininase activity assay method was also established in this investigation.

Materials and Methods

Materials——The mushroom, *Tricholoma conglobatum*, and other plants were commercially obtained. Synthetic bradykinin was supplied from Protein Research Foundation (Osaka), and γ-chymotrypsin (53

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2) E.G. Erdös and H.Y.T. Yang, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, Berlin-Heiderberg-New York, 1970, pp. 289—324.
3) *a*) T. Enomoto, S. Mineshita, S. Ohishi, and T. Shigei, *Jap. J. Pharm.*, **17**, 331 (1967); *b*) T. Murachi and T. Miyake, *Physiol. Chem. Physics.*, **2**, 97 (1970).
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units/mg) was obtained from Worthington Biochemical Co. (U.S.A.). Diethylaminoethyl (DEAE)-Sephadex A-50 and Sephadex G-100 from Pharmacia (Sweden), DNS–Cl (1-dimethylaminonaphthalene-5-sulphonyl chloride) from Tokyo Kasei Kogyo Co. (Tokyo), DNS-amino acids and polyamide layer from Seikagaku Kogyo Co. (Tokyo) were also used in this investigation.

Kininase Activity Assay——An isolated guinea pig ileum segment was suspended in a 10 ml bath in which Mg^{2+} free Tyrode solution was aerated at 30°. Synthetic bradykinin or assay sample was added into the bath and the contractile response of the ileum was recorded isometrically for 45 sec. Assays were carried out with 4 min interval. The stock solution of bradykinin (100 μg/ml) was made with saline and reserved at −20°. From this stock solution, various concentrations of bradykinin were made with 0.02 M phosphate buffer, pH 7.4 and kept in polyethylene containers in an ice bath during the assay. Silicon coated glass wares were used to avoid adsorption of bradykinin on glass wall.

For measurement of kininase activity, 0.15 ml of bradykinin solution (1 μg/ml) was incubated with 0.15 ml of a sample for several minutes at 30°, and the contractile response by 0.2 ml of this mixture (100 ng bradykinin eq.) was assayed. When the activity of a sample was so potent that more than 70% of bradykinin was inactivated, the sample was diluted with the same buffer to give at least 30 ng bradykinin remain after 3—5 min incubation. One kininase unit was defined as the amount of enzyme that could degrade 1 μg of synthetic bradykinin in 1 min at 30°, pH 7.4.

Preparation of Dansylated Bradykinin (DNS-Bradykinin)——DNS-bradykinin was prepared by the method of Gray and Hartley[9] with slight modification. About 1.5 mg of synthetic bradykinin in 0.5 ml of 0.1 M triethylamine bicarbonate buffer, pH 8.5—9.0, and 0.5 ml of 0.5% DNS–Cl dissolved in dioxane were mixed. This clear yellow solution was stood for 3—5 hr at room temperature and it was dried under N_2 gas. After reconstitution in a small volume of MeOH, it was applied to an activated silica gel H plate and firstly developed with acetone and then with a mixture of *n*-BuOH–AcOH–H_2O (4: 1: 5). Detecting the DNS-derivatives by their yellow fluorescence under UV-light (3600 Å), DNS-bradykinin which located close to the origin was scrapped off from the plate, eluted out from silica gel with a mixture of acetone–H_2O–pyridine–AcOH (50: 50: 1: 3) and the solvent was evaporated with N_2 gas. Then DNS-bradykinin was dissolved in about 3 ml of MeOH and stored in refrigerator.

Hydrolysis of DNS-Bradykinin by the Shimeji Kininase——About 100 μl of the above DNS-bradykinin solution was taken in a test tube and MeOH was evaporated. Into the test tube 30—40 μl of the enzyme solution (25 units/ml) was added and evaporated to dryness with N_2 gas after 3 hr reaction at room temperature. The residue dissolved in 10 μl of the triethylamine bicarbonate buffer was reacted with 10 μl of DNS–Cl solution as described above. The DNS-sample was dried again, dissolved in a small volume of MeOH and chromatographed on a silica gel H plate with a solution of 2-PrOH–methylacetate–28%NH_4OH (9: 7: 4). After development, the fluorescent bands were separately scrapped off from the plate and eluted with about 2 ml of acetone–H_2O–pyridine–AcOH (50: 50: 1: 3). Each of the eluates was dried with N_2 gas and the residue were then hydrolyzed with 100 μl of 6 N HCl in a sealed tubes at 90° for 16 hr. After that HCl was blown off with N_2 gas until the yellow fluorescence was detected again. For determination of N-terminal amino acid of each fragment, the residue was dissolved in a small volume of MeOH and chromatographed on a polyamide layer plate with the following two kinds of solvents: benzene–AcOH (9: 1) and H_2O–formic acid (100: 1.5).[10] The hydrolysates were further treated with DNS–Cl and the amino acid compositions were examined by polyamide layer chromatography.

Results

Assay of Kininase Activity

Fig. 1 shows a typical result of this kininase assay. With a 10 times diluted kininase preparation, 33, 49 and 68 ng of bradykinin were inactivated in 2, 3 and 4 min, respectively, and the similar inactivations were found by treatment with the 20 times diluted preparation for 4, 6 and 8 min. In these two cases, the amounts of inactivated bradykinin were proportional to the amounts of kininase and the incubation periods, and the activity of this kininase preparation was calculated to be 1.63—1.65 units/ml. The activity of this preparation could not be determined accurately at higher concentration than 10 times dilution because degradation of bradykinin was too fast and such proportionality could not be observed. Furthermore, when more than 70 ng of bradykinin was inactivated, the proportionality was no longer observed. Based on these data, the kininase activity assay was carried out within a range of 30—70 ng bradykinin inactivation.

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10) K.R. Woods and K.T. Wang, *Biochim. Biophys. Acta*, **133**, 369 (1967).

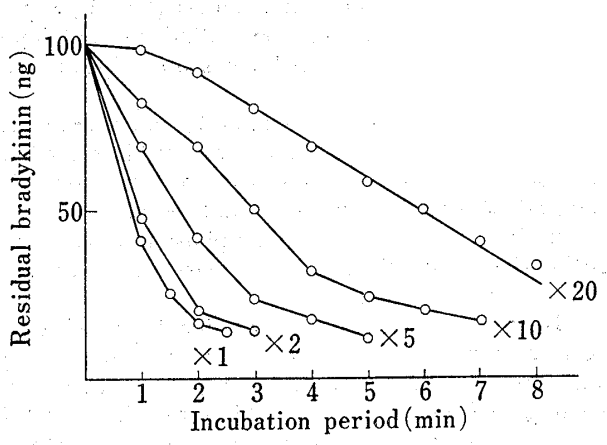

Fig. 1. Assay Results of Shimeji Kininase Preparation under Different Incubation Periods and Sample Dilution

Numbers in the figure show dilution factor.

Kininase Activities in Various Plants

Water extracts of various plants were prepared and their kininase activities were determined (Table I). Two kinds of muschrooms, *Tricholoma conglobatum* and *Psalliota hortensis* (tsukuritake, in Japanese), contained potent kininase activities, however, other mushrooms, *i.e. Callybia velutipes* (enokitake), *Armillaria Matsutake* (matsutake), *Cortinellus edodes* (shiitake) and *Pholiota Nameko* (nameko) possessed less kininase than the above two mushrooms. In these two mushrooms, the cap portion contained twice as much of kininase in the stalk. The activities in other vegetables and fruits were very weak or negligible.

Table I. Kininase Activities of Various Plants

	Units/g		Units/g
Mushrooms		Lily bulb	0.3—0.7
Tricholoma conglobatum	40 —334	Ginger	0.1—0.4
Psalliota hortensis	31 —115	Yam	0.1—0.2
Collybia velutipes	13 — 17	Radish	0.9—1.5
Armillaria Matsutake	12 — 13	Onion	0.1—0.4
Cortinellus edodes	5.4— 6.4	Pumpkin	0.5—3.9
Pholiota Nameko	1.6— 1.7	Cucumber	2.1—2.2
White potato	0.5— 0.9	Cabbage	0.1—0.8
Sweet potato	0.6— 2.1	Tomato	0.0—0.1
Young taros	0.2— 0.3	Apple	0.2—0.4
Spanish paprika	0.3— 0.5	Persimmon	0.0—0.3

Each of vegetables or fruits were minced and homogenized by the Waring blender with 5—10 times volume of water, and the homogenate was centrifuged after adjusting pH to 7.4. The kininase activity of the supernatant was determined. Each value shows the minimum and the maximum value of 5 preparations.

Purification of the Kininase from *Tricholoma conglobatum*

The results of the purification of the kininase were summarized in Table II. All of the following procedures were performed at 4° or ice cold.

Table II. Purification of the Kininase from Mushroom *Tricholoma conglobatum*

Procedure	Protein (E_{280})	Kininase activity (Total units)	Specific activity (Units/E_{280})
Water extraction	131000	197000 (100)[a]	1.5 (1)[b]
Dialysate of 90% saturated $(NH_4)_2SO_4$ precipitate	29500	148000 (75)	5.0 (3.3)
Dialysate of 50—70% saturated $(NH_4)_2SO_4$ precipitate	5240	96200 (49)	18 (12)
DEAE-Sephadex A-50 eluate	185	36500 (19)	197 (131)
Sephadex G-100 gel filtration	81	26100 (13)	322 (215)
DEAE-Sephadex A-50 eluate	42	20100 (10)	479 (319)

a) activity recovery (%)
b) Purification factor is shown in parenthesis.

Water Extraction——The mushrooms (2.5 kg) were homogenized with 6 liter of distilled water by Waring blender for 5 min. Insoluble residue in the homogenate was removed by centrifugation and the supernatant was adjusted to pH 7.4 with 5N NaOH (1.5 units/E_{280}).

First Ammonium Sulfate Precipitation——Solid ammonium sulfate was added to the solution to make 90% saturation, and after standing overnight it was centrifuged at 8000 rpm for 30 min. The precipitate was suspended in 0.01M phosphate buffer, pH 7.4, and dialyzed against the same buffer. The dialysate was centrifuged at 10000 rpm for 30 min and the supernatant was obtained (5.0 units/E_{280}).

Second Ammonium Sulfate Precipitation——Solid ammonium sulfate was again added to this supernatant, and the precipitate formed between 50—70% saturation was separated and dialyzed as mentioned above. The activity recovery in this step was about 65% and the specific activity increased to 18 units/E_{280}. The activities of other protein fractions were almost negligible.

First DEAE-Sephadex A-50 Chromatography——The dialysate was applied on a column of DEAE-Sephadex A-50. The kininase was eluted out with 0.01M phosphate buffer containing 0.3M NaCl in the fractions from No. 55—68, and those of No. 56—64 were pooled (Fig. 2). This procedure was so effective that the specific activity of the pooled fraction elevated more than 10 times in comparison with the preceding step. Most of the brownish substances in the extract remained on the top of the column, so they could be removed easily from the kininase fraction.

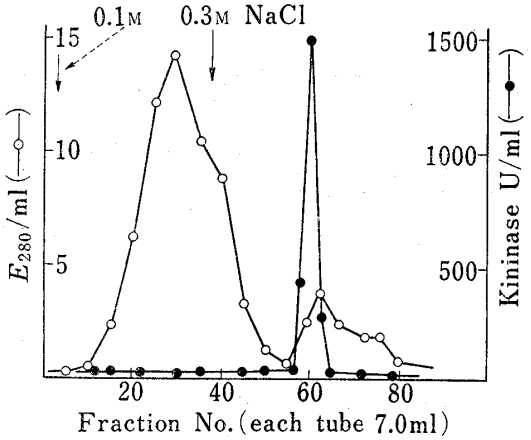

Fig. 2. DEAE-Sephadex A-50 Chromatography following Ammonium Sulfate Fractionation

The column (2.5×39.0 cm) was equilibrated with 0.01 M phosphate buffer, pH 7.4 and eluted out with 0.1 and 0.3 M NaCl in the same buffer.

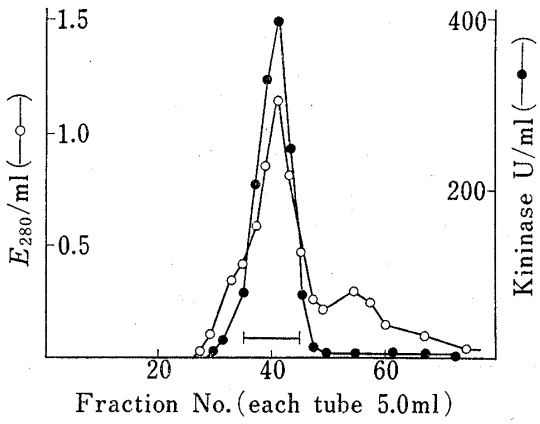

Fig. 3. Sephadex G-100 Gel Filtration of the Active Fraction from the DEAE-Sephadex A-50 Chromatography

The column (2.5×85.5 cm) was equilibrated with 0.01 M phosphate buffer, pH 7.4.

Sephadex G-100 Gel Filtration——The pooled sample was concentrated to 5 ml and gel filtrated with Sephadex G-100 (Fig. 3). Fractions No. 35—45 were pooled and dialyzed against 0.01M phosphate buffer. The specific activity increased to 322 units/E_{280}, but this preparation still gave a few protein bands in disc electrophoresis.

Second DEAE-Sephadex A-50 Chromatography——The dialyzed sample was rechromatographed with DEAE-Sephadex A-50 (Fig. 4). The kininase activity was found in the fractions of No. 33—52 which were eluted out with 0.01M phosphate buffer containing 0.15M NaCl, and those of No. 37—44 were pooled. This active fraction was dialyzed against distilled water and stored at −20°. This final preparation had a specific activity of 479 units/E_{280} so 319 fold purification could be achieved. It gave a single band in disc electrophoresis (Fig. 5) and used in the following experiments as the kininase preparation.

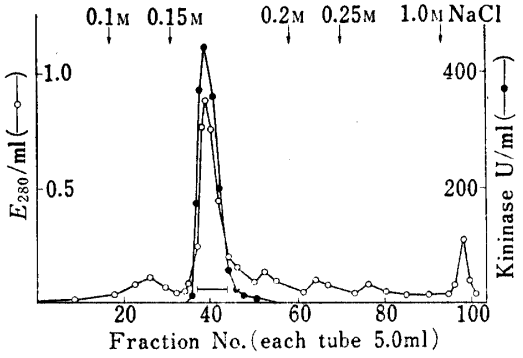

Fig. 4. DEAE-Sphadex A-50 Chromatography following Sephadex G-100 Gel Filtration

column: 1.5×26.0 cm

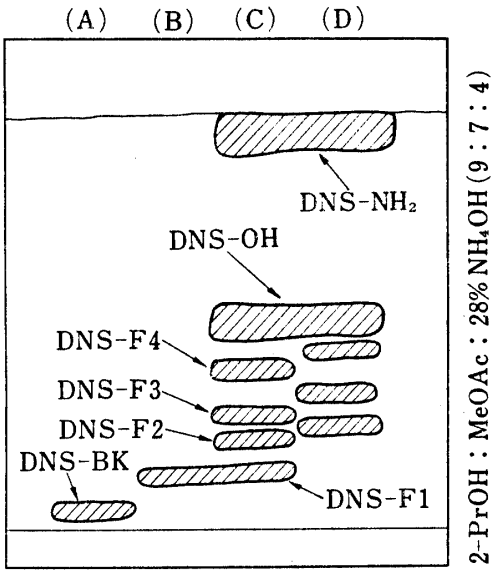

Fig. 6. Thin-Layer Chromatography of DNS-Bradykinin and DNS-Fragments Liberated from Bradykinin by Shimeji Kininase and γ-Chymotrypsin on Silica gel H

A: DNS-bradykinin
B: DNS-bradykinin+shimeji kininase
C: B treated with DNS-Cl
D: DNS-bradykinin+γ-chymotrypsin, treated with DNS-Cl

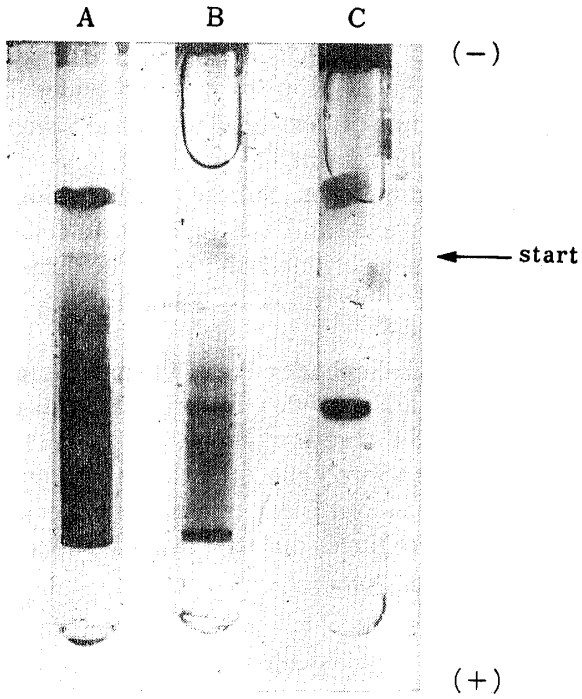

Fig. 5. Disc Electrophoresis of Shimeji Kininase at Each Purification Step

Electrophoresis was carried out on 7% (w/v) polyacrylamide gel with 0.05 M Tris buffer pH 8.6. The gel was stained with amide black 10 B for protein detection.
A: water extract
B: active fraction in the 1st DEAE-Sephadex A-50 chromatography
C: final preparation

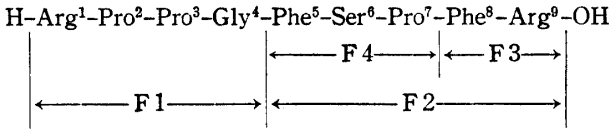

Fig. 7. Sites of Action of Shimeji Kininase on Bradykinin Molecule

TABLE III. Amino Acid Analysis of the Fragments Liberated from DNS-Bradykinin by Shimeji Kininase

Fragments	N-Terminal amino acids	Amino acid compositions
DNS-F1	Arg	Arg, Pro, Gly
DNS-F2	Phe	Phe, Ser, Pro, Arg
DNS-F3	Phe	Phe, Arg
DNS-F4	Phe	Phe, Ser, Pro

Sites of Action of the Kininase on Bradykinin Molecule

The incubated mixture of DNS-bradykinin and the kininase preparation was treated with DNS-Cl and chromatographed on a Silica gel H plate as described in Methods. Four yellow fluorescent bands were obtained other than DNS–NH_2 and DNS–OH (Fig. 6-C), and they were named as DNS-F1, -F2, -F3 and -F4 from the origin side. DNS-F1 gave stronger fluorescence than the other 3 bands. The fluorescence of DNS-F3 and -F4 gradually became intense by prolonged incubation with the kininase, while that of DNS-F2 disappeared. DNS-F1 was obviously the N-terminal peptide derived from DNS-bradykinin by the enzyme digestion because it had been detected before the treatment with DNS-Cl (Fig. 6-B). These 4 fluorescent bands were quite distinct from the bands formed from DNS-bradykinin by the action of γ-chymotrypsin (Fig. 6-D). It was reported that chymotrypsin cleaved Phe^5-Ser^6 and Phe^8-Arg^9 bonds,[11] so the 3 fluorescent bands in Fig. 6-D were DNS-Arg, DNS-Arg-Pro-Pro-Gly-Phe and DNS-Ser-Pro-Phe, respectively, from the origin side. These results suggest that the kininase cleaves 2 or 3 peptide bonds in bradykinin, and the sites of action of this enzyme were different from those of chymotrypsin.

These 4 bands were separately eluted out from the Silica gel H plate. Each peptide was hydrolyzed and chromatographed on a polyamide layer plate to determine its N-terminal amino acid. Yellow fluorescent band from the hydrolysate of DNS-F1 was coincident with authentic DNS-Arg and those from other 3 fragments were all DNS-Phe. This result means that N-terminal amino acids of DNS-F1 is Arg which is situated in the N-terminus of bradykinin and those of the others are Phe.

Those hydrolysates were further treated with DNS-Cl and amino acid composition of each fragment was examined. From the results shown in Table III, the amino acid sequences of DNS-F1, -F2, -F3 and -F4 are decided as Arg-Pro-Pro-Gly, Phe-Ser-Pro-Phe-Arg, Phe-Arg and Phe-Ser-Pro, respectively. In consequence of these experiments it could be concluded that the kininase could split the 2 peptide bonds, *i.e.* Gly^4-Phe^5 and Pro^7-Phe^8, in bradykinin molecule as shown in Fig. 7, and that Gly^4-Phe^5 bond would be more easily cleaved than Pro^7-Phe^8 bond.

Discussion

Kinin inactivating enzymes are distributed widely in nature. Some of these enzymes have been investigated thoroughly, and the sites of action of them on bradykinin molecule have been clarified.[2] Most of the activity assay methods of these enzymes are spectrophotometrical determination of degradation of various synthetic substrates,[2] but these methods can not be applied to a new kininase of which enzymatic properties are unknown. Hence, bradykinin is often used as the substrate in kininase assay and the amount of residual bradykinin is measured by its smooth muscle contractile response. However, the definite method has not been established, and most investigators determine the activity by the time necessary to inactivate 50 or 75% of a certain amount of bradykinin and express the activity by their own units, for example, Rugstad defined 1 kininase unit as the amount of enzyme that could destroy 75% of 500 ng of synthetic bradykinin in 11 min.[12] In the present investigation, the kininase activity was determined by the amounts of bradykinin inactivated at 30°, and the unit was defined in accordance with the recommendation of IUB, in which the unitage of an enzyme should based on μmoles of its substrate being degraded in 1 min under its optimal standard condition. The molecular weight of bradykinin is 1060, so as an arbitrary unit we determined the activity not by μmole but by μg of bradykinin degraded. Our method is simple and the kininase activity can be determined accurately.

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12) H.E. Rugstad, *Br. J. Pharmac. Chemother.*, 31, 401 (1967).

As the kininase from plant origin, bromelain, papain and ficin have been reported, and the authors found a new kininase in potato, *Solanum tuberosum*, besides the kallikrein inhibitors.[8] Stem bromelain, papain and ficin are SH-enzyme and they cleave Gly^4-Phe^5 and Phe^5-Ser^6 bonds of bradykinin molecule, but purified stem bromelain cleaves Phe^5-Ser^6 bond only.[3b] The bond hydrolyzed by potato kininase was not clarified yet. The present kininase from mushroom was found to be an endopeptidase which cleaved Gly^4-Phe^5 and Pro^7-Phe^8 bonds of bradykinin and the former bond was split easier than the latter one. The both bonds hydrolyzed with this enzyme were the amino side of phenylalanine. Kinonase AI and AIII, which were the kininases in microbes, *Streptomyces*, can cleave the same bonds in bradykinin molecule,[6] but such enzyme has not been found in plant or mammalian organs.

As shown in Table I, the crude water extracts of mushrooms contained extremely potent kininase activity in comparison with the other plants. Purified stem bromelain, papain, ficin-A, -B, -C, -D and potato kininase gave 3.3, 456, 180, 7.5, 5.6, 0.3 and 5.0 kininase units/mg, respectively, in our assay system,[4,8a] while the activity of our final preparation was 479 units/E_{280}, the highest among those plant enzymes.

Recently, anti-inflammatory effect on carrageenin induced edema in rats has been observed in some plant kininases. Therefore, this enzyme might be a useful agent in the study of kallikrein-kinin system, and its clinical application on inflammation, shock or allergic diseases are also expected.

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