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A New Potent Kinin-inactivating Enzyme from the Mushroom *Psalliota hortensis*

Kazuyuki Kizuki* and Hiroshi Moriya

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya Funakawara-machi, Shinjuku-ku, Tokyo 162, Japan

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A new potent kinin-inactivating enzyme was purified from a kind of Japanese mushroom, *Psalliota hortensis* (Tsukuritake, in Japanese), by means of water extraction, ammonium sulfate fractionation, DEAE-Sephadex A-50 chromatography and Sephadex G-100 gel filtration. The final enzyme preparation had an activity of 2284 kininase U/E_{280}, which is the highest known among kininases derived from plants.

This enzyme cleaved Gly^4-Phe^5 and Phe^5-Ser^6 bonds of the bradykinin molecule. Its molecular weight was estimated to be 6.7×10^4 and the optimum pH for the degradation of bradykinin was 8.0. The enzymatic activity of this enzyme was inhibited by mercurials, diisopropyl-fluorophosphate (DFP) and a high concentration of ethylenediaminetetraacetic acid (EDTA), but tosyllysine chloromethyl ketone (TLCK), tosylphenylalanine chloromethyl ketone (TPCK), iodoacetic acid, Trasylol and sodium tetrathionate had no detectable effect.

Keywords——kinin-inactivating enzyme; bradykinin; kallikrein–kinin system; kinin destruction; mushroom; physiologically active peptide

The physiologically active peptides, the kinins, are considered to play important roles in the body and many possible physiological and pathological meanings of the kallikrein–kinin system have been reported by many investigators. However, we have not the sufficient evidence to support the true important meanings of the kallikrein–kinin system ever reported, and it should be actually realized that the physiological and pathological roles of the kallikrein–kinin system in the body are still not completely understood.

We have been working on kininases from various origins with the aim of blocking the kinin action in the body because the roles of the kallikrein–kinin system in the body should be revealed in the experiments in which the system is specifically blocked, and have found a useful enzyme, Shimeji kininase, from a kind of mushroom, *Tricholoma conglobatum* (Shimeji, in Japanese), after many screening studies.[1] On the other hand, we recently discovered a new enzyme from a different kind of mushroom, *Psalliota hortensis* (Tsukuritake, in Japanese), having much more potent kinin-inactivating activity than Shimeji kininase.

The present paper deals with the purification of a new potent kinin-inactivating enzyme from the mushroom *Psalliota hortensis* and some of its properties.

Table I summarizes the purification of the enzyme. The assay of kinin-inactivating activity was carried out by the Magnus method using an isolated guinea-pig ileum.[1a] One kininase unit was defined as the amount of enzyme that could hydrolyze 1 μg of synthetic bradykinin per min at 30°C, pH 8.0. The mushrooms (1.6 kg) were homogenized with 3 l of distilled water in a Waring blender for 5 min. After filtration through gauze, the filtrate was centrifuged for 20 min at 8000 rpm and the supernatant was obtained. Then, solid ammonium sulfate was added to make 90% saturation and the solution was left to stand for 10 h. The precipitate was collected by centrifugation (8000 rpm, 30 min), suspended in 200 ml of 0.01 M phosphate buffer, pH 7.4 and dialyzed against the same buffer for 20 h. The dialysate was centrifuged for 30 min at 10000 rpm and the supernatant was obtained. To this supernatant solid ammonium sulfate was added again and the precipitate formed between 60—70% saturation was collected and dialyzed as mentioned above. The specific activity of the dialysate was 186 kininase U/E_{280}. It was then applied to a DEAE–Sephadex A-50 column (3.3 × 15.0 cm)

which had been equilibrated with 0.01 M phosphate buffer, pH 7.4. The adsorbed proteins were eluted stepwise with NaCl (0.05, 0.2 and 1.0 M) in the same buffer, and the kininase fractions eluted with 0.2 M NaCl were pooled. After dialysis against 0.01 M phosphate buffer, pH 7.4, this material was again applied to a DEAE-Sephadex A-50 column (1.4×23.0 cm) equilibrated with the same buffer, and the adsorbed proteins were eluted stepwise with NaCl (0.05, 0.075, 0.1 and 0.2 M) in the same buffer. The active fractions eluted with 0.1 M NaCl were combined and concentrated to 5 ml. The concentrate was gel filtered on Sephadex G-100 (Fig. 1). The elution profile of kininase activity coincided with the main protein peak and fractions No. 44—50 were pooled as the active fraction. The specific activity of this prepara-

TABLE I. Purification of the Kinin-inactivating Enzyme from the Mushroom *Psalliota hortensis*

Treatment	Protein (E_{280})	Kininase activity (kininase U)	Activity recovery (%)	Specific activity (kininase U/E_{280})	P.F.[a]
Water extract	111920	334400	100	3.0	1
Dialysate of 90 % saturated $(NH_4)_2SO_4$ precipitate	76100	324500	97	4.3	1.4
Dialysate of 60—70 % saturated $(NH_4)_2SO_4$ precipitate	817	152000	45	186	62
1st DEAE-Sephadex A-50 eluate	120	142800	43	1190	397
2nd DEAE-Sephadex A-50 eluate	21	47200	14	2248	749
Sephadex G-100 gel filtrate	14	31980	9.6	2284	761

a) Purification factor.

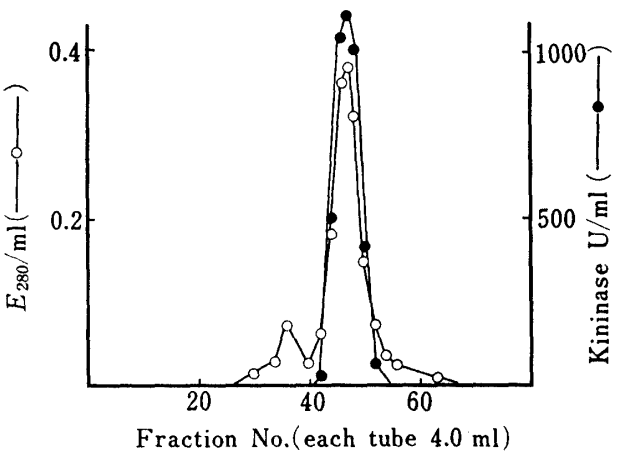

Fig. 1. Sephadex G-100 Gel Filtration of the Active Fraction from the 2nd DEAE-Sephadex A-50 Chromatography

The column (2.5×80 cm) was equilibrated and eluted with 0.05 M phosphate buffer, pH 7.5.

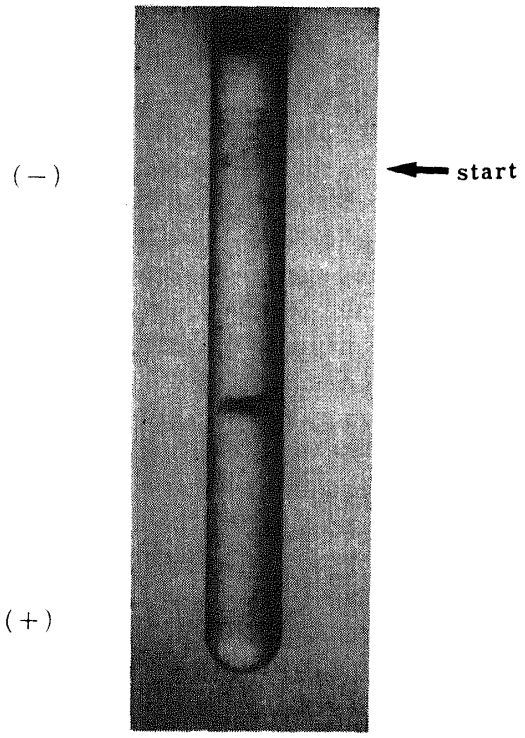

Fig. 2. Disc Electrophoresis of the Kinin-inactivating Enzyme from the Mushroom *Psalliota hortensis* at the Final Purification Step

Electrophoresis was carried out on 7 % (w/v) polyacrylamide gel with 0.05 M Tris-HCl buffer, pH 8.6. The gel was stained with amide black 10B for protein detection.

tion was 2284 kininase U/E_{280}, which is the highest value known among the kininases obtained from plants; it is about 5 times as potent as Shimeji kininase, which was the most potent plant kininase previously known.[1a)]

Figure 2 shows the disc-electrophorogram of the final preparation. The final preparation gave two bands; the main band and the almost imperceptible one migrating slightly more slowly than the main band. All of the above procedures were performed at 4°C or on ice.

The sites of action of this enzyme on the bradykinin molecule were examined by the same method as in our previous paper.[1a)] This enzyme formed 4 fragments (F1, F2, F3 and F4) from the bradykinin molecule having the *N*-terminal amino acids Arg, Phe, Ser, and Arg, respectively. The amino acid compositions of F1, F2, F3 and F4 were (Arg, Pro, Gly), (Phe, Ser, Pro, Arg), (Ser, Pro, Phe, Arg) and (Arg, Pro, Gly, Phe), respectively. Judging from these results, we concluded that the present enzyme cleaved the Gly^4–Phe^5 and Phe^5–Ser^6 bonds in the bradykinin molecule (Fig. 3), although a trace amount of contaminant was present in the kininase preparation. As shown in Fig. 2, the amount of this contaminant was extremely small, so it is unlikely to have affected the above results . The sites of action of this enzyme on the bradykinin molecule were identical with those of the thiol enzymes of plant origin, such as stem bromelain, ficin and papain, but different from those of Shimeji kininase.[1a)]

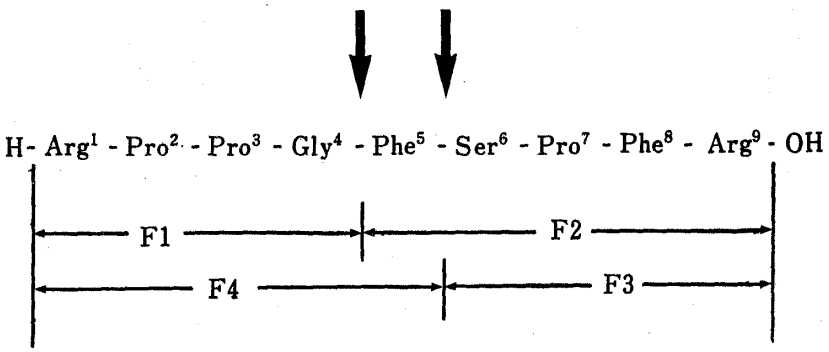

Fig. 3. Sites of Action of the Kinin-inactivating Enzyme from the Mushroom *Psalliota hortensis* on the Bradykinin Molecule

The optimum pH, the molecular weight of the present enzyme and the effects of various enzyme inhibitors on the kininase activity of the enzyme were also observed by the same methods as in our previous paper.[1b)] The maximum kinin-inactivation was found at pH 8.0 and the apparent molecular weight of the enzyme was estimated to be about 6.7×10^4 by Sephadex G-150 gel filtration. Co^{2+}, Ni^{2+}, Cu^{2+} and Mn^{2+} did not inhibit the enzymatic activity of this enzyme at the concentration of 1×10^{-3} M, while Fe^{3+} and Pb^{2+} inhibited the activity at this concentration. The mercurials, such as $HgCl_2$, phenylmercuric acetate and *p*-chloromercuribenzoic acid, strongly inhibited the activity at 1×10^{-4} M. DFP (1×10^{-3} M) weakly inhibited the enzyme. TLCK, TPCK (each 1×10^{-3} M), Trasylol (2500 KIU/ml), sodium tetrathionate (1×10^{-2} M), iodoacetic acid (1×10^{-3} M), cysteine and 2-mercaptoethanol (each 1×10^{-2} M) had no detectable inhibitory effect upon the enzymatic activity. EDTA and 8-hydroxyquinoline had no detectable inhibitory effect at the concentration of 1×10^{-3} M but a high concentration of EDTA (1×10^{-2} M) inhibited the activity.

These properties of the present enzyme differ from those of known kininases from plant or microbes, such as stem bromelain, ficin, papain,[2)] kininases from potato[3)] and red kidney bean,[4)] kininases from *Streptomyces kinoluteus*[5)] and *Pseudomonas aeruginosa*,[6)] *etc.* Thus, the present enzyme is a new potent kininase, having the highest kininase activity known among kininases from plants, and it may be a useful agent to block the kinin action like Shimeji kininase, although some of its properties, such as stability in the mammalian body and further effects on substances related to the kallikrein–kinin system in addition to kinin destruction, remain to be elucidated.

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References

1) *a*) K. Kizuki, C. Moriwaki, Y. Hojima, and H. Moriya, *Chem. Pharm. Bull.*, **24**, 1742 (1976); *b*) K. Kizuki, H. Moriya, and C. Moriwaki, *ibid.*, **27**, 654 (1979); *c*) *Idem*, *ibid.*, **27**, 662 (1979); *d*) *Idem*, *ibid.*, **27**, 1618 (1979); *e*) *Idem*, *ibid.*, **27**, 2424 (1979); *f*) *Idem*, *ibid.*, **29**, 2721 (1981); *g*) *Idem*, *ibid.*, **29**, 3037 (1981).
2) *a*) R. Arnon, "Methods in Enzymology," Vol. XIX, ed by G.E. Perlmann and L. Lorand, Academic Press, New York, 1970, pp. 226—244; *b*) I.E. Liener and B. Friedenson, *ibid.*, pp. 261—273; *c*) T. Murachi, *ibid.*, pp. 273—284.
3) *a*) Y. Hojima, M. Tanaka, H. Moriya, and C. Moriwaki, *Japanese J. Allergology*, **20**, 755 (1971); *b*) *Idem*, *ibid.*, **20**, 763 (1971).
4) Y. Hojima, H. Moriya, and C. Moriwaki, *Agric. Biol. Chem.* (Tokyo), **41**, 559 (1977).
5) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka, T. Takeuchi, and H. Umezawa, *Chem. Pharm. Bull.*, **17**, 714 (1969).
6) *a*) H.E. Rugstad, *Br. J. Pharmacol.*, **30**, 134 (1967); *b*) *Idem*, *ibid.*, **30**, 425 (1967); *c*) *Idem*, *ibid.*, **31**, 401 (1967).