Kininogenase Activity of Tonin

MASAHARU IKEDA, M.D., AND KIKUO ARAKAWA, M.D.

SUMMARY Tonin, known for its specific and direct generation of angiotensin II, was highly purified from rat submaxillary gland and investigated for kininogenase activity. For the substrate, heat-treated plasma from ox blood, and highly purified low-molecular-weight (LMW) and high-molecular-weight (HMW) kininogens, were used. The reaction product formed at pH 8.0 well satisfied the characteristics of kinin, i.e., depressor and oxytocic activities and reactivity with antibradykinin antiserum. Kinin formed by tonin from purified LMW kininogen was identified with bradykinin in high performance liquid chromatography and radioimmunoassay. The results revealed tonin's new capability of forming kinin in addition to the hitherto known pressor angiotensin II, indicating tonin, too, is a member of the "kinin-tensin enzyme system." (Hypertension 6: 222-228, 1984)

KEY WORDS  tonin  kinin  kininogen  high performance liquid chromatography

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ONIN has been known not only to convert angiotensin I to angiotensin II, but also to generate angiotensin II from natural renin substrate and tetradecapeptide renin substrate.1-3

The substrate specificity against angiotensin and its analogs has been reported to be very strict.4 On the other hand, tonin has been shown to hydrolyze β-LPH, adrenocorticotropic hormone (ACTH), and substance P by cleaving some phenylalanyl and arginyl bonds.5-6 In addition, analysis of amino acid sequence and enzymological studies have revealed that tonin is an esteropeptidase of the serine protease family with trypsin- and chymotrypsin-like activity.7-10

In previous papers, we have reported that trypsin and kallikrein, both known as potent kininogenases, can generate angiotensin II and have proposed a "kinin-tensin enzyme system" for those having capability of forming both pressor and depressor substances.11-14

Taking into account that tonin belongs to the same serine protease family as trypsin and kallikrein, we investigated tonin's capability to form kinin.

Materials and Methods

Materials

Synthetic bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin were obtained from the Protein Research Foundation (Osaka, Japan). Activated CH-Sepharose 4B and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Polybrene was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin) and o-phenanthroline was from Katayama Chemical Industries Company Ltd. (Osaka, Japan). ¹²⁵I-[Tyr⁵]-bradykinin was obtained from New England Nuclear (Boston, Massachusetts). Aprotinin was a generous gift from Bayer (Leverkusen, Germany). Prolyl-phenylalanyl-arginyl-naphthylester (Pro-Phe-Arg-NE) and purified hog pancreas kallikrein were kindly supplied by Sanwa Kagaku (Nagoya, Japan). Purified high-molecular-weight (HMW) and low-molecular-weight (LMW) kininogens were kindly supplied by Doctor H. Kato,15 Faculty of Science, Kyushu University.

Purification of Tonin

Tonin was purified from rat submaxillary gland according to our method previously reported, with an additional step of an isoelectrofocusing technique.16 Submandibular gland homogenate equilibrated with 0.05 M sodium acetate buffer, pH 5.3, was applied on soybean trypsin inhibitor coupled with CH Sepharose 4B (1.5 × 40 cm) preequilibrated with the same buffer.

The pooled fractions with angiotensin I-hydrolyzing activity were concentrated, dialyzed against 0.02 M Tris-HCl buffer, pH 8.0, and applied to a DEAE cellul...
lose column (Whatman Ltd., Kent, England) (1.5 × 20 cm) pre-equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride (0–0.15 M) in the same buffer.

The fractions that contained tonin were pooled and concentrated, and preparative isoelectrofocusing was carried out on a 110 ml electrofocusing column (LKB 8101) with a pH gradient 5–7 using ampholine according to manufacturer's instruction. Tonin was focused at pH 6.18 with a symmetrical peak. The final step consisted of gel filtration on Sephadex G-100 (1.9 × 95 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Purity was ascertained by polyacrylamide gel electrophoresis.17

Preparation of Heated Ox Plasma

Bovine plasma used as substrate for kininogenase activity was prepared according to Moriwaki et al.18 and Alhenc-Gelas et al.,19 with a slight modification. Ox blood was collected in plastic bottles containing 0.5 g/liter of polybrene and 3 mM EDTA. The plasma was obtained by centrifugation at 3000 rpm for 15 minutes at 4°C and heated with gentle stirring at 60°C for 3 hours. The heated plasma was precipitated with ammonium sulfate between 30% and 50% saturation, and dialyzed against 0.02 M Tris-HCl buffer, pH 7.5 containing 0.15 M NaCl.

The dialysate was passed through aprotinin-coupled CH-Sepharose 4B (1.5 × 15 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl to eliminate kininogenases. Eluates were extensively dialyzed against distilled water, lyophilized, and used as a crude substrate for kininogenases.

The maximum kinin yield from 1 mg of crude substance for kininogenase by treatment with 0.5 mg of trypsin in 0.1 M Tris-HCl buffer, pH 8.0, containing 3 mM EDTA and 3 mM o-phenanthroline at 37°C for 30 minutes, was 120 ng bradykinin equivalent by radioimmunoassay. Rat plasma kininogen was prepared from heated plasma (60°C for 1 hour) by precipitation with ammonium sulfate between 30% and 50% saturation, dialyzed extensively against distilled water, and lyophilized.

Hydrolytic Activity of Pro-Phe-Arg-NE

The esterolytic activity for Pro-Phe-Arg-NE was determined in an incubation mixture containing 1.5 ml of 0.05 M sodium phosphate buffer (pH 7.5) containing 0.015% sodium dodecyl sulfate (SDS), 0.2 ml of Pro-Phe-Arg-NE (1.5 mM), and 0.1 ml of enzyme solution. Incubation was carried out at 37°C for 30 minutes. The enzymatic reaction was stopped by adding 0.02 ml of SDS and was cooled at 4°C for 3 minutes. Then 0.2 ml of 1% First Violet B was added. After 10 minutes, 2.0 ml of glacial acetic acid was added, and the absorbance was measured at 505 nm.20

The effect of aprotinin on their enzymatic activity was also studied. An aliquot of aprotinin solution (5000 units) was added to an incubation mixture in the same system, and the experiment was conducted in the same experimental procedure as the above. As a control, the mixture of substrate solution without enzyme solution was incubated. Enzymatic activity was expressed as the difference of absorbance between the complete system and the blank.

Radioimmunoassay of Kinin(s)

Antibodies were raised in male white rabbits immunized against bradykinin coupled to bovine thyroglobulin using ethyl carbodimide.21 Every 3 weeks for 4 months, an animal received 2 to 3 mg of bradykinin-coupled thyroglobulin with complete Freund's adjuvant. The antigen was injected intradermally on multiple sites in the backs of rabbits.

Antisera were collected and stored at −20°C. The antibody used showed 53% cross-reactivity with lysyl-bradykinin and 77% with methionyl-lysyl-bradykinin when bradykinin was used as the standard.125I-[Tyr*]-bradykinin was used as tracer. Ox LMW kininogen did not displace the tracer at doses up to 0.1 μg. Cross-reactivity of the antiserum with des-[Pro2]-bradykinin and des-[Arg9]-bradykinin was 4% and less than 0.01%, respectively.

Radioimmunoassay was carried out in a total volume of 0.6 ml in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2% BSA, 3 mM phenanthroline, 3 mM EDTA, and 0.02% sodium azide. Fresh dilutions of bradykinin as standards, ranging from 7.8 to 1000 pg/ml, were prepared for each experiment. The duration of antiserum required to bind 40% to 50% of125I-[Tyr*-bradykinin was used.

The reactants were added to 7 × 12 mm plastic tubes in the following order: 0.1 ml of standard or sample solution, 0.2 ml of diluted antiserum (1:70,000), 0.2 ml of the buffer, and 0.1 ml of125I-[Tyr*-bradykinin (about 6000 cpm). The tubes were mixed with a Vortex mixer and incubated 20 hours at 4°C. The separation of free and antibody-bound peptide was carried out by polyethylene glycol method.

After the addition of 0.1 ml of 2% bovine gamma globulin and 0.7 ml of 25% polyethylene glycol (6000) in phosphate buffered saline (pH 7.0), the tubes were mixed and centrifuged at 3000 rpm for 20 minutes. The supernatant was carefully aspirated, and the radioactivity of the precipitates was quantitated in a LKB gamma counter (Model 1270 Rack gamma, LKB-Produkter AB, Bromma, Sweden).

Kininogenase Activity

Effect of Time of Incubation

To test the effect of the time of incubation, 20 mg of the heat-treated ox plasma, 50 μg of LMW, or 50 μg of tonin in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 3 mM EDTA, and 3 mM o-phenanthroline were incubated at 37°C for each time of incubation. The reaction was terminated by the addition of ethanol, and the supernatant, after centrifugation at 3000 rpm for 10 minutes, was evaporated to dryness at 40°C by Savant Speed Vac Concentrator (New York, New York). The product was dissolved in 0.1 M phosphate...
buffer (pH 7.0) containing 0.1 M NaCl, 3 mM phenanthroline, 3 mM EDTA, 0.2% bovine serum albumin (BSA), and 0.02% sodium azide. The concentration of kinin was measured by radioimmunoassay.

**Effect of Concentration of Tonin**

To test the effect of the tonin concentration, 20 μg of heat-treated ox plasma was incubated with 30, 60, 90, and 120 μg of tonin in 0.6 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 3 mM EDTA, 3 mM o-phenanthroline at 37°C for 30 minutes. The reaction was terminated, and the samples were treated as described above.

**Effect of Proteinase Inhibitors**

The effect of proteinase inhibitors on kininogenase activity of tonin was examined. Soybean trypsin inhibitor, lima bean trypsin inhibitor, and aprotinin were dissolved respectively in concentration of 5 mg per milliliter of 0.1 M Tris-HCl buffer, pH 8.0. Diisopropyl fluorophosphate (DFP, 100 mM) and phenylmethyl sulfonyl fluoride (PMSF, 100 mM) were dissolved in isopropyl alcohol and ethyl alcohol respectively. Then 50 μg of tonin and 20 μg of heat-treated plasma were incubated in the presence of proteinase inhibitors in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), containing 3 mM EDTA and 3 mM o-phenanthroline at 37°C for 30 minutes. The reaction was terminated by the addition of ethyl alcohol, and the samples were treated as described above.

**Bioassay of Tonin-Generated Kinin**

For bioassay of tonin-generated kinin, 200 μg of highly purified LMW-kininogen were incubated with 200 μg of tonin in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 3 mM EDTA at pH 8.0. Disopropyl fluorophosphate (DFP, 100 mM) and phenylmethyl sulfonyl fluoride (PMSF, 100 mM) were dissolved in isopropyl alcohol and ethyl alcohol respectively.

Then 50 μg of tonin and 20 μg of heat-treated plasma were incubated in the presence of proteinase inhibitors in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), containing 3 mM EDTA and 3 mM o-phenanthroline at 37°C for 60 minutes. The reaction was stopped by adding 4 ml of ethanol. After centrifugation at 3000 rpm for 10 minutes, the supernatant was evaporated by vacuum centrifugal concentrator. The dried residue was dissolved in 2 ml of 0.15 M sodium chloride.

Depressor activity was examined by measurement of the blood pressure response of carotid artery of a female Wister rat (180 g) anesthetized with sodium amobarbital and ganglion-blocked with pentolium tartrate. Phenoxymenzamine was also administered. Then 0.1 ml of the sample, which contained 9.2 ng of kinin in bradykinin equivalent by radioimmunoassay, was injected into the femoral vein of the rat. Oxytotic activity using rat uterus was also examined in a 10 ml bath of De Jalon’s buffer solution. 0.1 ml of the sample, which contained the same amount, was administered in the bath.

**High Performance Liquid Chromatography**

Highly purified LMW-kininogen, 200 μg, was incubated with 200 μg of tonin in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 3 mM o-phenanthroline at 37°C for 60 minutes. The reaction was stopped by the addition of 4 ml of ethanol. After centrifugation at 3000 rpm for 10 minutes, the supernatant was evaporated. The dried residue was dissolved in 500 μl of distilled water, filtered through a millipore filter (0.45 μm), and applied on Waters Associates Model ALC/GPC 204 (Milford, Massachusetts).

High performance liquid chromatography (HPLC) was carried out in two different solvent systems on different columns. One of the HPLC systems was a μ-Bondapak phenyl column (0.4 × 30 cm), which was equilibrated with 19% acetonitrile in 0.1 M ammonium acetate (pH 7.1). Another was μ-Bondapak C18 column (0.4 × 30 cm), which was equilibrated with 0.04 M triethylamine-formate buffer (pH 3.25) containing 18% acetonitrile according to Powers and Nasjletti. In both cases, the samples, which contained 19.0 ng of kinin in bradykinin equivalent by radioimmunoassay and standards, were injected in a volume of 50 μl and eluted isocratically at a flow rate of 1.3 ml/min. The eluate was monitored at 254 nm and collected in 30-second fractions.

Synthetic bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin dissolved in water to a concentration of 100 μg/ml were used as standards to calibrate the HPLC column and monitored at 254 nm. Kinin content in the 30-second fractions was measured by radioimmunoassay, as described above.

**Results**

**Hydrolysis of Pro-Phe-Arg-NE by Tonin and Porcine Pancreatic Kallikrein**

The hydrolytic activity of tonin and porcine pancreatic kallikrein on Pro-Phe-Arg-NE at various concentrations and the effect of aprotinin on both enzymes are shown in Figure 1. The hydrolytic activity of both enzymes was directly proportional to the quantity of the enzymes under the condition employed. Tonin showed about one-eighth of the hydrolytic activity of porcine pancreatic kallikrein on a molar basis. The hydrolytic activity of porcine pancreatic kallikrein was completely inhibited by aprotinin (5000 units), whereas that of tonin was not.

**Kininogenase Activity of Tonin on Heated Ox Plasma**

**Effect of Time of Incubation**

Figure 2 shows a typical incubation curve of the reaction of tonin and the heated plasma substrate. The amount of kinin generated showed linearity with incubation time.

**Effect of Tonin Concentration**

The rate of formation of kinin as function of the concentration of purified tonin is shown in Figure 3. Under the condition employed, the concentration of kinin released was directly proportional to the quantity of tonin. Without the addition of tonin in control experiment, there was no appreciable amount of kinin formation.
Effect of Inhibitors on Kininogenase Activity of Tonin

Inhibition of the kininogenase activity of tonin by various trypsin-kallikrein inhibitors and serine protease inhibitors is summarized in Table I. Soybean trypsin inhibitor and aprotinin showed a potent inhibitory action on kininogenase activity of tonin, whereas lima-bean trypsin inhibitor did less. DFP and PMSF, chemical inhibitors of serine proteases, showed 38% and 55% inhibition, respectively, when 1400-fold excess was used on a molar basis.

Kinin Formation by Tonin

Kinin Formation by Tonin/Ikeda and Arakawa

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TABLE I. Effect of Various Inhibitors on Kininogenase Activity of Tonin

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Kinin formed (ng)</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>24.4</td>
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<tr>
<td>Soybean trypsin inhibitor</td>
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<tr>
<td>0.2 mg/ml</td>
<td>20.0</td>
<td>18</td>
</tr>
<tr>
<td>2.0 mg/ml</td>
<td>2.0</td>
<td>92</td>
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<td>Lima bean trypsin inhibitor</td>
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<tr>
<td>0.2 ml/mg</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>2.0 mg/ml</td>
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<td>79</td>
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<tr>
<td>Aprotinin</td>
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<td></td>
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<tr>
<td>0.2 mg/ml</td>
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<td>47</td>
</tr>
<tr>
<td>2.0 mg/ml</td>
<td>2.0</td>
<td>92</td>
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<tr>
<td>Diisopropyl fluorophosphate</td>
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<td>5 mM</td>
<td>15.2</td>
<td>38</td>
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<tr>
<td>Phenylmethanesulphonyl fluoride</td>
<td></td>
<td></td>
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<tr>
<td>1 mM</td>
<td>23.2</td>
<td>5</td>
</tr>
<tr>
<td>5 mM</td>
<td>10.9</td>
<td>55</td>
</tr>
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</table>

**Figure 4.** Vasodepressor activity of kinin formed by tonin from highly purified low molecular weight (LMW) kininogen. The LMW kininogen, 200 mg, was incubated with 200 μg of tonin in 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.0 containing 3 mM o-phenanthroline at 37°C for 60 minutes. After extraction by ethanol, the product was evaporated to dryness and dissolved in 2 ml of 0.15 sodium chloride. Then 0.1 ml of the sample, which contained 9.2 ng kinin in bradykinin equivalent by RIA (LMW-Kg + tonin), was injected into a rat. As a control, 200 μg of LMW kininogen (LMW-Kg) was incubated in the absence of tonin. The mode of depressor action by 10 ng of synthetic bradykinin (BK) was also shown (Synthetic).

Bioassay of Kinin Formed by Tonin

To verify the nature of the kinin formed, vasopressor activity was examined by measuring blood pressor response to an injection of 9.2 ng of kinin in bradykinin equivalent by radioimmunoassay; 10 ng of synthetic bradykinin was injected as a control. Depressor activity of the product was obtained, as shown in Figure 4. The mode of vasodepressor activity of the kinin formed by tonin was comparable to synthetic bradykinin. Incubation of tonin or LMW kininogen alone could not produce any vasoactive substances. Oxytocic activity was also shown in an isolated rat uterus preparation by tonin-generated kinin. No oxytocic activity was obtained from the incubation of tonin or kininogen alone.

Identification of Kinin Generated by Tonin

Displacement of Standard Dose Response Curve

The kinin generated by tonin displaced [125I-Tyr8]-bradykinin from antibradykinin antisera, and the slope of the displacement curve was identical to that of synthetic bradykinin, as shown in Figure 5.

High Performance Liquid Chromatography of Kinin Generated by Tonin

HPLC was carried out in two different solvent systems on different columns. The elution mode of synthetic lysyl-bradykinin, bradykinin, and methionyl-lysyl-bradykinin on a μ-Bondapak phenyl column equilibrated with 19% acetonitrile in 0.1 M ammonium acetate (pH 7.1) was monitored at 254 nm. The retention time of each kinin was 11.7, 14.7, and 21.2 minutes, respectively (Figure 6 upper). The peak of tonin-generated kinin, when collected from each 30-second fraction and radioimmunoassayed, appeared at the time corresponding to that of bradykinin (Fig. 6 lower). No immunoreactive kinin was detected in any other fraction corresponding to lysyl-bradykinin and methionyl-lysyl-bradykinin.

In another HPLC system on a μ-Bondapak C18 column equilibrated with 0.04 M triethylamine-formate buffer containing 18% acetonitrile (pH 3.25), the retention time of synthetic lysyl-bradykinin, methionyl-lysyl-bradykinin, and bradykinin was 9.2, 13.3, and 15.4 minutes, respectively. The peak of tonin-formed kinin appeared at the time corresponding to that of bradykinin, when detected from each 30-second fraction by radioimmunoassay. Consequently, bradykinin seemed to be the major kinin generated from LMW kininogen under the conditions employed.

Kinin-Forming Activity of Tonin from Purified LMW and HMW Kininogen

When 100 μg of highly purified LMW or HMW kininogen was incubated with 50 μg of tonin in the incubation buffer for 30 minutes at 37°C, 23 ng of immunoreactive kinin was formed from LMW kininogen, whereas 40 ng of kinin was generated from HMW kininogen. Incubation of tonin or LMW and HMW kininogen alone did not form any appreciable amount of kinin. Tonin generated 98.0 ng of kinin when 10 mg
of rat plasma kininogen was incubated with 50 μg of tonin at 37°C for 30 minutes.

**Discussion**

This study demonstrates a new capability of tonin, namely, tonin-formed depressor substances, which also possess oxytocic activity, from heat-treated plasma or highly purified kininogens of HMW or LMW. As investigated by radioimmunoassay and HPLC, the vasoactive substance was identical with bradykinin. When heat-treated plasma was used as substrate, there were two possible mechanisms of kinin formation. One possibility was that tonin directly generated kinin, and the other one was that tonin activated the kininogenase precursor that still existed in the plasma protein and secondarily released kinin from kininogen.

To elucidate the mechanism, highly purified ox LMW and HMW kininogens were used as the substrate. Tonin liberated kinin from both purified substrates, whereas the substrate alone did not. Thus, the results suggest that tonin directly formed kinin. Here, the purity of tonin is a big problem, especially because of the possible contamination by kallikrein. Kallikreins purified from rat submandibular gland were reported to release kinin equivalent to 35–55 μg of bradykinin min⁻¹ mg⁻¹ when incubated with rat kininogen. Tonin generated kinin equivalent to 65 ng of bradykinin min⁻¹ mg⁻¹ from heat-treated rat plasma. Thus, the kinin-forming activity of tonin is about 0.15% that of kallikrein. The preparation was proved free from any kallikrein contamination by the following criteria: 1) It was prepared by isoelectric focusing between pH 5 and 7, in addition to the previous method. Tonin was focused at pH 6.18, whereas the isoelectric point of kallikrein(s) from rat submandibular gland ranges from pH 3.87 to 4.16, far different from that of tonin. 2) The tonin used here showed a single protein band on polyacrylamide gel electrophoresis. 3) Aprotinin completely inhibited the hydrolytic activity of kallikrein on Pro-Phe-Arg-NE, but did not affect tonin’s esterase activity.

These results also suggest that the esterase activity might not be due to the contaminated kallikrein in tonin preparation. The discrepancy of this inhibitory effect of aprotinin on the hydrolysis of Pro-Phe-Arg-NE (Figure 1) and kininogen (Table 1) might be due to the different mechanisms of action, that is, esterolysis in the former case or proteolysis in the latter, or might be due to the difference in molecular size of the substrates.

So far, tonin has been known to liberate angiotensin II from angiotensin I, tetradecapeptide renin substrate, and plasmatic angiotensinogen, and has been reported to have a strict substrate specificity against angiotensin I and its analogs. However, Seidah et al. and Chretien et al. have reported that tonin hydrolyzed β-LPH, ACTH, and substance P by cleaving Arg-Tyr and Arg-Trp bonds, in addition to Phe-Arg, Phe-Lys, Phe-Phe, and Phe-Gly bonds. These findings suggest that tonin shares a similar substrate specificity as do trypsin and chymotrypsin. Recently, Thibault and Genest con-
cluded that tonin is an esteropeptidase of the serine protease family with trypsin- and chymotrypsin-like activity, since it can hydrolyze ester bonds of benzoyl-arginine methyl ester and tosyl-arginine methyl ester. Thus, tonin has been shown to cleave some phenylalanyl and arginyl bonds in peptides or proteins. Since cleaving of an arginine bond is essential for kinin release from substrates, kininogenase activity of tonin as evidenced here does not conflict with data previously reported. The possibility of kininase activity of tonin was suggested by Chrétien et al., but Pesquero et al. were not able to see bradykinin breakdown (kininase activity), even by extremely high concentration of tonin.

Our present study clarifies the kininogenase activity of tonin, and it is now apparent that tonin has the capability of generating not only angiotensin II, as hitherto known, but also bradykinin. We have proposed the term kinin-tensin enzyme system for those systems that can generate both pressor angiotensin II and depressor kinin(s) directly. Tonin now evidently belongs to this system, in addition to trypsin and kallikrein. Whether the kinin-tensin system, including tonin, plays some role in the regulation of regional perfusion remains to be elucidated.

Acknowledgments

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