PROPERTY OF KININ-FORMING ENZYME IN RAT STOMACH

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Abstract—The effects of inhibitors on the kinin-forming enzyme (KFE) activity in the rat stomach were investigated at pH 4.8. The KFE activity was unaffected by trasylol (200 KIU/ml), soybean trypsin inhibitor (100 μg/ml) and p-tosyl-L-lysine-chloromethyl ketone (10^-3 M), but was inhibited by pepstatin A (10^-6 M) and chymostatin (1.5×10^-4 M). Each product from the rat plasma kininogen by the rat stomach KFE and the bovine spleen cathepsin D was eluted at the same retention time on the equilibrium chromatography on the SP-Sephadex C-25 column. The KFE activity in the rat stomach was considerably high compared with that in various regions of the intestine. These results suggest that the KFE is characteristically similar to cathepsin D, and the enzyme is probably relevant to the function of the stomach.

In a previous paper we reported the existence of kinin-forming enzyme (KFE), the optimum pH of which was observed at 4.8, in the rat stomach (1). Zeitlin (2, 3) noted that the optimum pH of rat intestinal kallikrein was observed at 8.5, and the enzyme activity was inhibited by trasylol, but not by soybean trypsin inhibitor (SBTI) and p-tosyl-L-lysine-chloromethyl ketone (TLCK). Greenbaum and Yamafuji (4) found that the bovine spleen preparation produced the kinin-like material, and the optimum pH for this reaction was observed at acidic pH. Chang et al. (5) demonstrated that the catheptic enzyme (leukokininogenases) of polymorphonuclear leukocyte (PMN) cells and macrophages could act on plasma proteins to release leukokinin. Greenbaum (6) has shown that the leukokininogenases were not inhibited by trasylol, but were strongly inhibited by pepstatin, a polypeptide which inhibits cathepsin D and pepsin.

The present report describes the effects of trasylol, SBTI, TLCK, pepstatin A and chymostatin on the KFE activity in the rat stomach, identification of the products from the rat plasma kininogen (KGN) by the rat stomach KFE and the bovine spleen cathepsin D, and the distribution of the KFE activity in the rat alimentary tract.

MATERIALS AND METHODS

Preparation of KFE from various regions in rat alimentary tract: Male Wistar rats were killed by a blow on the head. Blood was flushed from the alimentary tract by intra-vascular perfusion with 50 ml of heparinized (10 units/ml) Krebs-Hensleit solution followed by 50 ml of heparin-free solution, according to the method of Zeitlin (3, 7). The stomach, duodenum, jejunum, ileum, caecum and colon were removed, and homogenized separately in 10 volumes of cold distilled water. The homogenate was adjusted to pH 2.0 with 1 N HCl, and incubation was carried out for 20 min at 37°C to abolish the kininase activity, then pH was adjusted to 7.0 with 1 N NaOH. After
centrifugation at 30,000 g for 60 min, the supernatant fraction was lyophilized and used as KFE preparation.

**Preparation of KGN:** Blood was collected by heart puncture of ether-anesthetized rats into a polyethylene syringe containing 3.1% sodium citrate dihydrate solution (one part to nine parts of blood), through a siliconized needle. After centrifugation at 1,300 g for 30 min, the plasma was transferred to a polyethylene tube with polyethylene pipets, and heated for 30 min at 60°C. The denatured plasma was adjusted to pH 2.0 with 1 N HCl, incubated for 20 min at 37°C, after which the pH was adjusted to 7.0 with 1 N NaOH. After dialysis against distilled water for 48 hr at 4°C, the dialysate was centrifuged at 10,000 g for 30 min, then the supernatant fraction was lyophilized and used as KGN preparation. The KGN preparation was capable of yielding 200 ng of bradykinin (BK) equivalent per mg by trypsin treatment.

**Determination of KFE activity:** The incubation was carried out using the mixture of 4.0 ml of the KGN solution (4.0 mg/ml) dissolved in 0.1 M phosphate buffer (pH 4.8) and 0.4 ml of the solution of the KFE preparation (100 mg of wet tissue/ml) from various regions of the rat alimentary tract dissolved in the same buffer for 2 hr at 37°C. The reaction was terminated by boiling for 5 min, the pH adjusted to 8.0 with 1 N NaOH and the reaction mixture centrifuged at 700 g for 10 min. The supernatant fraction was assayed with the isolated rat uterus to determine the amount of kinin released. The activity of KFE was expressed in terms of ng BK equivalent formed per gram weight of wet tissue in 1 min.

To determine the effects of inhibitors on the KFE activity, the mixture of 4.0 ml of the KGN solution (4.0 mg/ml) dissolved in 0.1 M phosphate buffer (pH 4.8) and 0.2 ml of the same buffer with or without an inhibitor was incubated for 10 min at 37°C, then 0.2 ml of a solution of the KFE preparation (200 mg of wet tissue/ml) of the rat stomach dissolved in the same buffer was added. After incubation for 30 min at 37°C, the reaction was stopped by boiling for 5 min, and the reaction mixture was adjusted to pH 8.0 with 1 N NaOH and centrifuged at 700 g for 10 min. The supernatant fraction was assayed with the isolated rat uterus to determine the amount of kinin released. The result was expressed as per cent of the amount of kinin formed in the reaction with the inhibitor against that without the inhibitor.

**Extraction procedure:** The extraction of the products from the KGN preparation by the rat stomach KFE and the bovine spleen cathepsin D was carried out by the method described previously (8).

The reaction mixture containing the kinin, obtained in consequence of the determination procedure of the rat stomach KFE activity described above, was submitted to extraction. For the preparation of the kinin released with the bovine spleen cathepsin D, the mixture containing 8.0 ml of the KGN solution (10 mg/ml of distilled water) and 1.0 ml of the solution of cathepsin D from the bovine spleen (1.25 U/ml of distilled water) was adjusted to pH 4.0 with 1 N HCl, and incubated for 44 hr at 37°C. The reaction was terminated by boiling for 5 min, and the reaction mixture was submitted to extraction.

**Chromatographic procedure:** The preparations containing the kinins, obtained from the extraction procedure, were submitted to the gradient chromatography on SP-Sephadex C-25 for purification, and the equilibrium chromatography on SP-Sephadex C-25 for identification, carried out by the method described previously (8).

**Bioassay:** The test sample was assayed with the uterine tissue excised from virgin Wistar rats in induced estrus, and also tested on the rat duodenal tissue, against standard BK, by the method described previously (1).
Materials: SP-Sephadex C-25 Fine was purchased from Pharmacia Fine Chemicals (Sweden). BK, methionyl-lysyl-bradykinin (MLBK), kallidin, pepstatin A and chymostatin were obtained from Protein Research Foundation (Osaka, Japan). Cathepsin D from bovine spleen, trypsin from bovine pancreas Type III (2×crystallized), Tris-aminomethane and SBTI were from Sigma Chemical Company (U.S.A.). Atropine sulfate was purchased from E. Merck AG (Germany). Trasylol was obtained from Bayer AG (Germany). Dibenamine HCl and TLCK were from Nakarai Chemicals LTD. (Kyoto, Japan).

RESULTS

Effects of inhibitors on KFE activity in rat stomach: As shown in Fig. 1, the KFE activity in the rat stomach was unaffected by trasylol (200 KIU/ml), SBTI (100 μg/ml) and TLCK (10⁻³ M), but was inhibited by pepstatin A (10⁻⁸ M) and chymostatin (1.5×10⁻⁴ M).

Chromatography of products from KGN by rat stomach KFE and bovine spleen cathepsin D: The panel A of Fig. 2 shows the elution pattern of standard BK, MLBK and kallidin, on the equilibrium chromatography on the SP-Sephadex C-25 column, detected by assay with the isolated rat uterus. The products of the rat stomach KFE and the bovine spleen cathepsin D, contracted the

Fig. 1. Effects of inhibitors on KFE activity in rat stomach. The reaction was carried out at pH 4.8. Results are expressed as means of four experiments±S.E. *, Significant difference from control (p<0.01)

Fig. 2. Chromatography of products from KGN by rat stomach KFE and bovine spleen cathepsin D. The elution pattern of the following materials is shown in each panel. A: standard kinins, BK, MLBK and kallidin. B: kinin released from the KGN with the rat stomach KFE. C: kinin released from the KGN with the bovine spleen cathepsin D. Column size: 0.6 x 9 cm. Elution buffer: 0.05M Tris-HCl buffer (pH 8.0) containing 0.08 M NaCl. Flow rate: 9.0 ml/hr. Fraction volume: 1.5 ml. Fractions were assayed with the isolated rat uterus suspended in De Jalon's solution containing atropine sulfate (10⁻⁶ g/ml) and dibenamine HCl (10⁻⁷ g/ml).

Fig. 3. Distribution of KFE activity in various regions of rat alimentary tract. The enzyme activity was estimated at pH 4.8. Results are expressed as means of four experiments±S.E.
isolated rat uterus and relaxed the isolated rat duodenum, were eluated at the same retention time (Fig. 2, Panel B and C, respectively), and at the different retention time from that of BK, MLBK and kallidin.

**Distribution of KFE activity in rat alimentary tract:** The KFE activity was estimated at pH 4.8. As shown in Fig. 3, the KFE activity in the stomach was much higher than that in various regions of the intestine.

**DISCUSSION**

We have already reported the existence of KFE, the optimum pH of which was observed at 4.8, in the rat stomach (1). In the present work, we found that the KFE activity of the rat stomach was unaffected by trasylol, SBTI and TLCK, but that the activity was inhibited by pepstatin A and chymostatin (Fig. 1). Each product from the same KGN by the rat stomach KFE and the bovine spleen cathepsin D was eluated at the same retention time on the SP-Sephadex C-25 column (Fig. 2).

It has been noted that kallikrein, the optimum pH of which was 8.5, exists in gastrointestinal tissue, and that the kallikrein activity of the rat ileum was inhibited by trasylol, but not by SBTI and TLCK (2, 3). Seki et al. (9) have shown that the kallikrein activity originating from human, monkey or dog colon was inhibited by not only trasylol, but also by SBTI. The bovine spleen preparation produced the kinin-like material at acidic pH, and the optimum pH for this reaction was shown to be at around 5.0 (4). The catheptic enzymes of PMN cells and macrophages released leukokinins (5), and their enzyme activities, although not inhibited by trasylol, were inhibited by pepstatin (6). It has been demonstrated that MLBK was liberated in pepsin digestion of plasma protein, and the maximal rate of kinin release occurs around pH 1.0 (10).

Trasylol inhibits plasma, salivary and urinary kallikreins, plasmin, trypsin, papain and chymotrypsin, but does not inhibit rat pancreatic kallikrein (3, 11). SBTI inhibits plasma kallikrein, trypsin and chymotrypsin (3, 11). TLCK has been shown to be a specific inhibitor of trypsin (12). Pepstatin A shows no inhibition against kallikrein, plasmin, trypsin, papain, chymotrypsin, thrombokinase and thrombin, but is a specific inhibitor of acid proteases, cathepsin D and pepsin (11). Chymostatin is an inhibitor towards papain, chymotrypsin, cathepsin A, B and D, but it has no appreciable effect on pepsin (11).

We propose that the KFE in the rat stomach is characteristically similar to cathepsin D which is distinct from the gastro-intestinal kallikrein reported by Zeitlin (2, 3), and pepsin. Zeitlin (7) reported that the kinin release could well be one of the factors controlling the normal vasomotor tone, permeability and motility of the intestine. It has been demonstrated that the kallikrein content in the rat stomach was lower than that in any other region of the alimentary tract (7, 13). In the stomach, the content of the KFE, the optimum pH of which was 4.8, was much higher than that in any region of the intestine (Fig. 3). Thus, the KFE may function as a possible substitute for kallikrein, the optimum pH being 8.5, in the stomach.

**REFERENCES**

1) Kobayashi, M., Shikimi, T., Miyata, S. and Ohata, K.: Studies on kinin-forming enzyme in rat stomach. Japan. J. Pharmacol. 29, 947-950 (1979)
2) Zeitlin, I.J.: Pharmacological characterization of kinin-forming activity in rat intestinal tissue. Brit. J. Pharmacol. 42, 648P-649P (1971)
3) Zeitlin, I.J.: Rat intestinal kallikrein. Vasopeptides, Edited by Back, N. and Sicuteri, F., p. 289-296, Plenum Press, New York (1972)
4) Greenbaum, L.M. and Yamafuji, K.: The in vitro inactivation and formation of plasma kinins by spleen cathepsins. Brit. J. Pharmacol. 27, 230-238 (1968)
5) Chang, J., Freer, R., Stella, R. and Greenbaum, L.M.: Studies on leukokinins-II, studies on the
formation, partial amino acid sequence and chemical properties of leukokininins M and PMN. Biochem. Pharmacol. 21, 3095–3106 (1972)

6) Greenbaum, L.M.: Leukocyte kininogenases and leukokinins from normal and malignant cells. Am. J. Pathol. 68, 613–623 (1972)

7) Zeitlin, I.J.: Kinin release associated with the gastrointestinal tract. Bradykinin and Related Kinins, Edited by Sicuteri, F., Rocha e Silva, M. and Back, N., p. 329–339, Plenum Press, New York (1970)

8) Kobayashi, M., Shikimi, T., Miyata, S. and Ohata, K.: A bradykinin-like substance in rat stomach. Japan. J. Pharmacol. 30, 701–710 (1980)

9) Seki, T., Nakajima, T. and Erdös, E.G.: Colon kallikrein, its relation to the plasma enzyme. Biochem. Pharmacol. 21, 1227–1235 (1972)

10) Guimaraes, J.A., Pierce, J.V., Hial, V. and Pisano, J.J.: Methionyl-lysyl-bradykinin, the kinin released by pepsin from human kininogens. Adv. exp. Med. Biol. 70, 265–268 (1976)

11) Wingender, W.: Proteinase inhibitors of microbial origin, a review. Proteinase Inhibitors, Edited by Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., p. 548–559, Springer-Verlag, Berlin, Heidelberg, New York (1974)

12) Pudles, J. and Bachellerie, D.: Studies on proteolytic inhibitors. III. Stability of trypsin in trypsin-inhibitor complexes to specific chemical inactivating agents. Arch. Biochem. Biophys. 128, 133–141 (1968)

13) Frankish, N.H. and Zeitlin, I.J.: The assay of tissue kallikrein in rat intestine. Brit. J. Pharmacol. 59, 517P (1977)