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PURIFICATION AND PROPERTIES OF RAT STOMACH KALLIKREIN

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Summary

Kallikrein (EC 3.4.21.8) was purified from rat stomach by column chromatography on *p*-aminobenzamidine-Sepharose, DEAE-Sephadex A-50 and Sephadex G-150 and by isoelectric focusing, measuring its activities to hydrolyse L-prolyl-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide and to release kinin from heat-treated rat plasma. The purified stomach kallikrein showed a single band on polyacrylamide gel electrophoresis at pH 7.0.

Its molecular weight was calculated to be 29 000 by gel-filtration on a column of Sephadex G-50. The kallikrein was stable between pH 6—11 and hydrolyzed L-prolyl-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide optimally at pH 11.0. The L-prolyl-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide hydrolyzing activity of rat stomach kallikrein was inhibited by diisopropyl fluorophosphate and Trasylol, but not by trypsin inhibitors from soybean, lima bean and ovomucoid. These properties of rat stomach kallikrein are different from those of partially purified rat plasma kallikrein, but similar to those of glandular kallikreins from other species.

From these results, it was concluded that kallikrein is present in rat stomach and that it can be classified as a glandular kallikrein.

Introduction

In mammals, kinin-releasing enzymes are found in the plasma and in various tissues [1—4]. These enzymes can be classified into two types, plasma and glandular kallikreins (EC 3.4.21.8). The functions of glandular kallikreins have been suggested as being closely related to the function of the organs in which

Abbreviation: MCA, 4-methyl-coumaryl-7-amide.

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the enzymes are found. For example, excretion of kallikrein in the urine reflects the activity of renal kallikrein, which has been reported to regulate renal blood flow in cooperation with the renin-angiotensin system in kidney, and therefore, it takes part in regulation of blood pressure [5]. Kallikrein is also present in glands or cells of the gastrointestinal tract, where it has been suggested to influence transport of glucose and amino acids and absorption of water and electrolytes from the intestine [6].

Geiger et al. [7] recently suggested that kininogen is present in human gastric mucus, but the presence of kallikrein in the stomach has not been reported. It seemed interesting to examine whether the kallikrein-kinin system was present in the stomach, since secretion by the gastric mucosa is regulated by gastrin and secretin.

This paper reports the purification of kallikrein from rat stomach and presents evidence that rat stomach kallikrein has similar properties to other glandular kallikreins.

Materials and Methods

The following reagents were obtained commercially: ovalbumin, bovine serum albumin and γ-globulin (Nutritional Biochemical Corporation, Cleveland, OH, U.S.A.); DEAE-Sephadex A-50, Sephadex G-150, Sephadex G-50 and Sepharose 4B (Pharmacia Fine Chemicals Co., Uppsala, Sweden); soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid trypsin inhibitor and *p*-aminobenzamidine HCl (Sigma Chemical Co., U.S.A.); diisopropyl fluorophosphate (Katayama Chemical Co., Japan); Trasylol (Bayer AG., F.R.G.); Pro-Phe-Arg-MCA, Z-Phe-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, glutaryl-Gly-Arg-MCA, and Boc-Val-Leu-Lys-MCA (Protein Research Foundation, Osaka, Japan): *p*-chlorobenzylamine-Sepharose 4B was prepared by coupling ε-aminocaproyl-*p*-chlorobenzylamine (Protein Research Foundation, Osaka) with Sepharose 4B using CNBr.

Activated Factor XII was isolated from bovine plasma by the method of Fujikawa et al. [8]. Snake venom kininogenase from the venom of *Agkistrodon halys blomhoffii* was purified as reported previously [9]. Polyacrylamide gel electrophoresis at pH 7.0 using diethylbarbituric acid-Tris buffer was performed by the method of Williams and Reisfeld [10]. Isoelectric focusing was performed with a 110 ml column (LKB product) using Ampholine of pH 2.5—5. The pH optimum and stability of kallikrein were examined in Britton-Robinson buffer.

Homogenate of rat stomach. Male Wistar strain rats, weighing 150—200 g, were killed by decapitation and their stomachs were washed with ice-cold saline. Stomachs from 130 animals were homogenized with a Polytron PCU-2-110 in 3 vols. of 10 mM sodium phosphate buffer, pH 7.4, at 4° C.

Measurement of amidase activity. Peptidyl-4-methyl-coumaryl-7-amide was used for enzyme assay at 37° C in 0.5 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM substrate. The reaction was started by adding 20 μl of enzyme and the amount of 7-amino-4-methylcoumarin liberated after 30 min was measured in a Hitachi fluorescence spectrophotometer, model 650-10 M,

with excitation at 380 nm and emission at 460 nm, as described previously [11].

Bioassay of kinin. Aliquots of enzyme solution were added to a mixture of 0.15 ml of 50 mM Tris-HCl buffer, pH 9.0, containing 3 mM *o*-phenanthroline and 100 µl of heat-treated rat plasma (60° C, 30 min) and kinin was measured after incubation at 37° C. Kinin activity was measured by its ability to cause smooth muscle contraction of isolated rat uterus as described previously [12]. Standard bradykinin was a product of Sandoz Co. Ltd.

Preparation of p-aminobenzamidine-Sepharose 4B. ε-Aminohexanoic acid was first coupled to Sepharose 4B by the cyanogen bromide method [13]. *p*-Aminobenzamidine was attached to Sepharose 4B by 1-ethyl-3-(dimethylaminopropyl)-carbodiimide by the method of Holleman et al. [14].

Preparation of rat plasma kallikrein. Prekallikrein was partially purified from rat plasma by chromatography on columns of DEAE-Sephadex A-50, *p*-chlorobenzylamine-Sepharose 4B and Sephadex G-150, and activated with bovine activated Factor XII. Blood was withdrawn from the inferior cava of anesthetized male Wistar strain rats and treated with 3.8% sodium citrate to prevent coagulation. The plasma (160 ml) was collected by centrifugation and dialysed overnight against 1500 ml of 0.02 M Tris-HCl buffer, pH 8.0, containing 3 mM EDTA, 0.005% polybrene and 0.05% benzamidine. Then it was applied to a column (3.8 × 14 cm) of DEAE-Sephadex which had been equilibrated with the same buffer containing 0.02 M NaCl. The unadsorbed fraction was applied to a column (6.5 × 17 cm) of *p*-chlorobenzylamine-Sepharose which had been equilibrated with the same buffer. The column was washed with 500 ml of the same buffer, and then material was eluted with the same buffer containing 25% dioxane. The appropriate portion of the eluate was concentrated by ultrafiltration using a Diaflow membrane UM-10 and applied to a column (2 × 140 cm) of Sephadex G-150 which had been equilibrated with 0.02 M Tris-HCl buffer containing 0.15 M NaCl. Throughout the purification, prekallikrein was assayed after activation with bovine Factor XII, using Z-Phe-Arg-MCA as substrate. The partially purified prekallikrein obtained by gel filtration on a column of Sephadex G-150 was treated with the activated bovine Factor XII and applied to a column of Sephadex G-150.

Results and Discussion

Purification of rat stomach kallikrein

A homogenate of rat stomach prepared from 130 stomachs as described in the Materials and Methods was centrifuged at 105 000 × *g* for 60 min. Solid $(NH_4)_2SO_4$ was added to the supernatant with gentle stirring to 70% saturation. After 3 h, the precipitate was collected by centrifugation at 5000 rev./min for 20 min, and dissolved in 300 ml of cold 50 mM sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl.

After dialysis overnight against 50 mM sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl, the sample was applied to a column (3.0 × 24 cm) of *p*-aminobenzamidine-Sepharose equilibrated with the same buffer. The column was washed with 600 ml of the same buffer, and then material was eluted stepwise with 50 mM sodium phosphate buffer, pH 7.4/1.0 M NaCl. All the

Pro-Phe-Arg-MCA hydrolytic activity was found in the eluate, as shown in Fig. 1. Fractions containing kallikrein (Nos. 50—56) were pooled and dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl.

The sample was applied to a DEAE-Sephadex A-50 column (3.3 × 14 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl. The column was washed with 750 ml of the same buffer, and then elution was achieved with 2 l of a linear gradient of 0.05—0.5 M NaCl in the same buffer. As shown in Fig. 2, three peaks with Pro-Phe-Arg-MCA hydrolytic activity were separated, but only the second peak showed kinin-releasing activity on heat-treated rat plasma, as shown by the shaded bars. The fractions of eluate in this peak (Nos. 110—135) were pooled and concentrated to about 9 ml by ultrafiltration using a Diaflow membrane UM-10.

The sample was then applied to a Sephadex G-150 column (3.8 × 130 cm), equilibrated with 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. As shown in Fig. 3, the Pro-Phe-Arg-MCA hydrolytic activity was found in the fractions with a molecular weight of about 29 000 and kinin releasing activity was associated with the peak. The fractions in this peak (Nos. 180—220) were collected and subjected to isoelectric focusing using carrier ampholytes of pH 2.5—5.0. As shown in Fig. 4, the Pro-Phe-Arg-MCA hydrolytic activity was found to have a p*I* of 4.05 with a shoulder. Fractions containing kallikrein (Nos. 39—45) were pooled and their homogeneity was examined by polyacrylamide gel electrophoresis at pH 7.0. As shown in Fig. 5, a single protein band was obtained that stained with Coomassie brilliant blue.

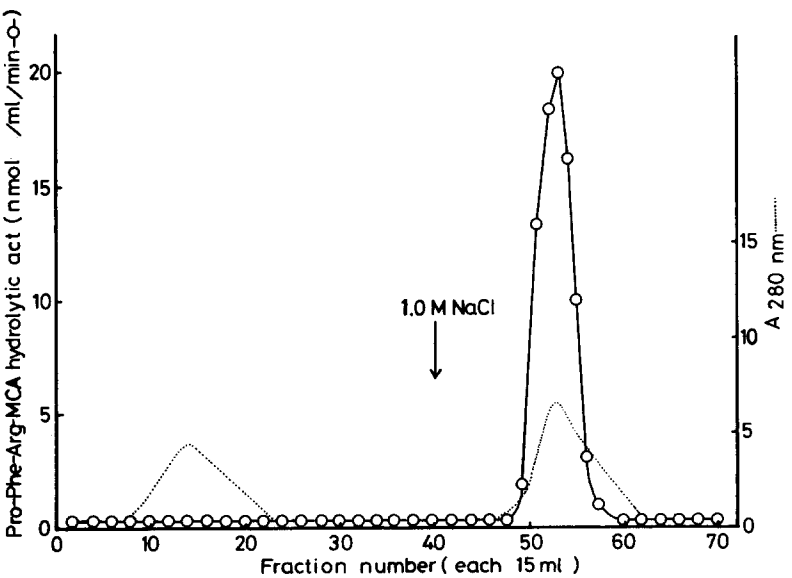

Fig. 1. *p*-Aminobenzamidine-Sepharose affinity chromatography of rat stomach kallikrein. The fraction of the homogenate of rat stomach precipitated with ammonium sulfate was applied to *p*-aminobenzamidine agarose equilibrated with 50 mM sodium phosphate buffer (pH 7.4)/containing 0.05 M NaCl. The column was washed with the same buffer, and then material was eluted stepwise with the same buffer containing 1 M NaCl from the point indicated by an arrow.

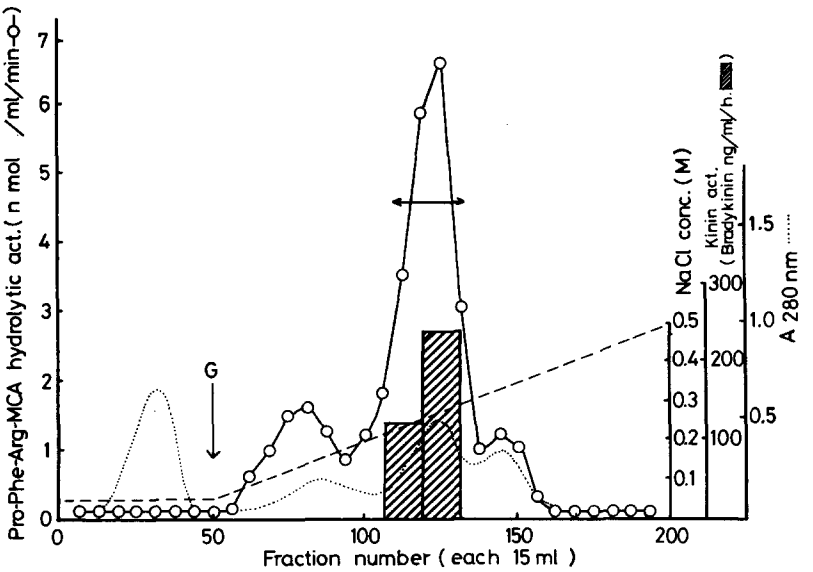

Fig. 2. DEAE-Sephadex A-50 chromatography of the kallikrein fraction from the *p*-aminobenzamidine-Sepharose and equilibrated with 50 mM sodium phosphate buffer, pH 7.4/0.05 M NaCl. From the point indicated by an arrow, material was eluted with linear gradient of 0.05—0.5 M NaCl in the same buffer.

Unstained gel was sliced into 0.28 cm wide sections and these were each extracted with 0.2 ml of 50 mM sodium phosphate buffer, pH 7.4. Fig. 5 also shows that Pro-Phe-Arg-MCA hydrolytic activity was found in the same position as the protein band.

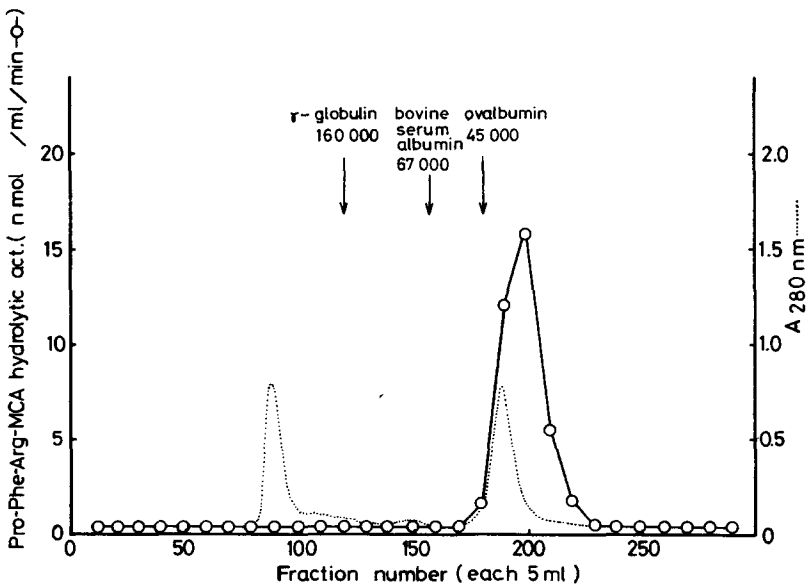

Fig. 3. Gel-filtration of the kallikrein fraction from the DEAE-Sephadex A-50 column with Sephadex G-150 in 50 mM sodium phosphate buffer, pH 7.4/0.15 M NaCl. The elution positions of γ-globulin, bovine serum albumin and ovalbumin are indicated in the figure.

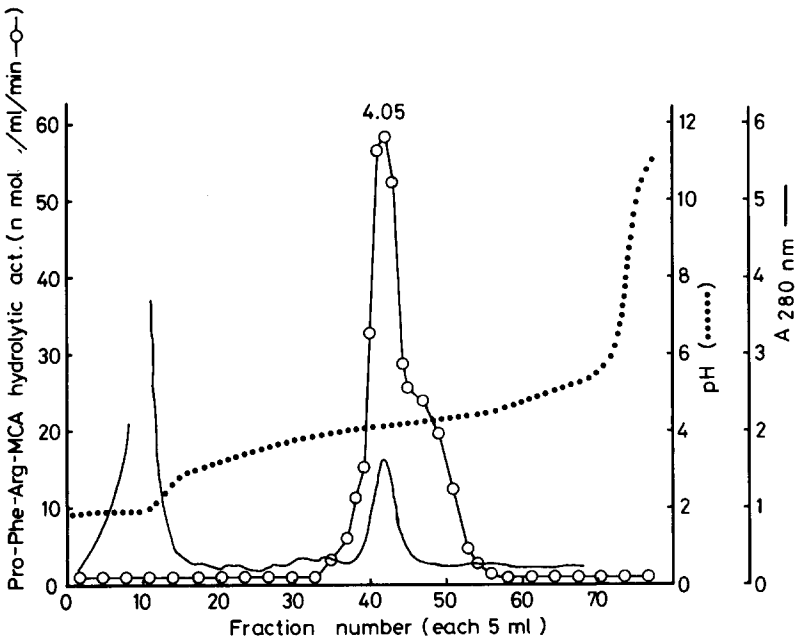

Fig. 4. Isoelectric focusing of rat stomach kallikrein. The kallikrein fraction obtained by gel-filtration was subjected to isoelectric focusing on Ampholine of pH 2.5—5.0.

Table I summarizes the purification procedures for rat stomach kallikrein. By this method, 10.2 mg of kallikrein was obtained from 130 stomachs with a yield of 13.6%. About 115-fold purification was achieved from the supernatant of the homogenate.

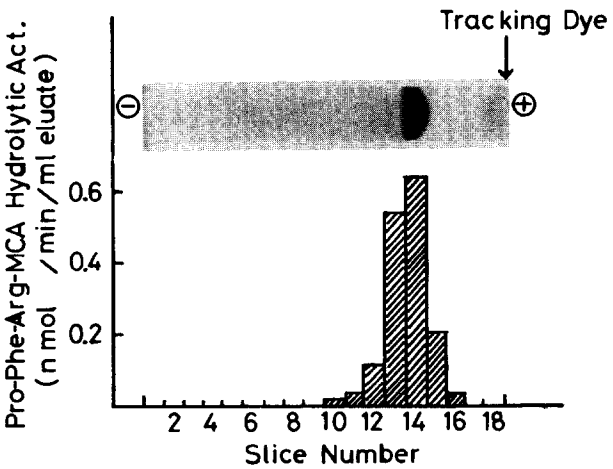

Fig. 5. Polyacrylamide gel electrophoresis of rat stomach kallikrein. 10 μg of purified rat stomach kallikrein was applied to an 8% polyacrylamide gel column and electrophoresis was carried out at pH 7.0. The upper half of the figure shows the protein band stained with Coomassie brilliant blue. Unstained gel was sliced. The Pro-Phe-Arg-MCA hydrolyzing activity in each segment is shown in the lower half of the figure.

TABLE I

PURIFICATON OF RAT STOMACH KALLIKREIN

Steps	Total protein (A_{280nm})	Total activity (nmol/min)	Specific activity (nmol/min per A_{280nm})	Purity	Yield (%)
1. 105 000 × *g* supernatant	8534	6609	0.77	1	100
2. $(NH_4)_2SO_4$ precipitation	4027	5911	1.47	1.9	89.4
3. *p*-Aminobenzamidine Sepharose	1576	5003	3.17	4.1	75.7
4. DEAE-Sephadex A-50	198	1711	8.64	11.2	25.9
5. Sephadex G-150	42.4	1600	37.74	49.0	24.2
6. Isoelectric focusing	10.2	900	88.24	114.6	13.6

In this purification procedure, affinity chromatography on *p*-aminobenzamidine-Sepharose column was very useful. However, when the homogenate of stomach was sonicated in the early stages of this study, affinity chromatography was not reproducible, and the Pro-Phe-Arg-MCA hydrolyzing activity sometimes appeared in the unadsorbed fraction. In such cases, the eluate with 1 M NaCl had an absorbance maximum at 260 nm. Thus sonication seemed to solubilize substances with higher affinity to *p*-aminobenzamidine-Sepharose. Therefore, we omitted sonication, and in this way achieved a higher capacity and higher reproducibility on the affinity column.

Optimum pH and stability of rat stomach kallikrein

When Pro-Phe-Arg-MCA was used as substrate, the optimum pH of rat stomach kallikrein was at pH 11, as shown in Fig. 6. Since the optimum pH of glandular kallikreins was reported to be about 8.0 with Bz-Arg-OEt as substrate, we examined the optimum pH of hog pancreatic kallikrein and human urinary kallikrein with Pro-Phe-Arg-MCA as substrate and found that they also showed abnormally alkaline optima of pH 11. It is unknown why rat stomach kallikrein has such an abnormal optimum pH, but it seems to be a common property of other glandular kallikreins. On the other hand, rat plasma kallikrein showed an optimum pH of 8.0 as shown in Fig. 6. Rat stomach kallikrein was most stable between pH 6 and 11. As shown in Fig. 6a, rat stomach kallikrein did not hydrolyse the substrate appreciably above pH 12, possibly due to inactivation of the enzyme as shown in Fig. 6b.

No spontaneous hydrolysis of Pro-Phe-Arg-MCA and no change of the extinction coefficient of 7-amino-4-methylcoumarin was observed until pH 13.0 under the conditions used.

Kinin-releasing activity and amidase activity of rat stomach kallikrein

50 μl of rat stomach kallikrein (A_{280} = 1.6) or 50 μl of snake venom kininogenase (A_{280} = 1.4) released kinin from heat-treated rat plasma. The maximum amount of kinin released by rat stomach kallikrein was lower than that by snake venom kininogenase. We have not yet identified the kinin released by rat stomach kallikrein, but the above result suggests that it may be kallidin, because it has been established that snake venom kininogenase releases brady-

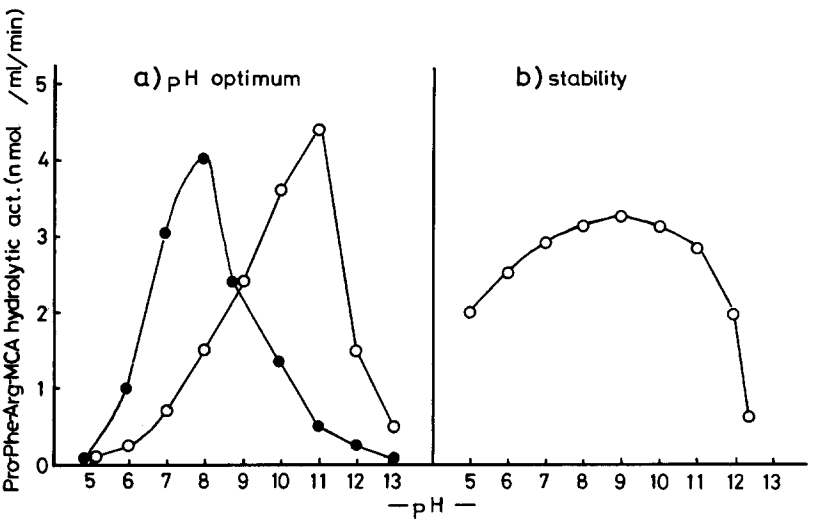

Fig. 6. pH optimum (a) and stability (b) for rat stomach kallikrein (○———○) and rat plasma kallikrein (●———●). Britton-Robinson's wide-range buffer (0.04 M) consisting of H_3PO_4-CH_3COOH-H_3BO_3-NaOH was used with Pro-Phe-Arg-MCA as substrate. (a) The solution of enzyme in buffer (0.5 ml) was incubated with 10 mM Pro-Phe-Arg-MCA (5 μl) at 37°C for 30 min at the indicated pH values. (b) The solution of enzyme in buffer (20 μl) was preincubated at 37°C for 30 min at the indicated pH values. Then 0.97 ml of 0.1 M sodium phosphate buffer, pH 7.4, and 10 μl of 20 mM Pro-Phe-Arg-MCA were added and incubation followed at 37°C for 30 min at pH 7.4.

kinin and that the contractile activity of kallidin is less than that of bradykinin.

Rat stomach kallikrein hydrolyzed Pro-Phe-Arg-MCA and Z-Phe-Arg-MCA, but scarcely hydrolyzed Boc-Val-Pro-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, Glutaryl-Gly-Arg-MCA or Boc-Val-Leu-Lys-MCA, which are specific fluorogenic substrates of thrombin, Factor Xa, urokinase and plasmin, respectively. It has been reported that hog pancreatic kallikrein and human urinary kallikrein preferably hydrolyze Pro-Phe-Arg-MCA to Z-Phe-Arg-MCA, while bovine plasma kallikrein preferably hydrolyzes Z-Phe-Arg-MCA. As shown in Table II, rat stomach kallikrein hydrolyzed Pro-Phe-Arg-MCA better than Z-Phe-Arg-MCA, whereas partially purified rat plasma kallikrein hydrolyzed Z-Phe-Arg-MCA, better than Pro-Phe-Arg-MCA. The Pro-Phe-Arg-MCA hydrolytic activity of rat stomach kallikrein was completely inhibted by Dip-F and Trasylol, but not by soybean trypsin inhibitor, lima bean trypsin inhibitor or ovomucoid trypsin inhibitor, as shown in Table II. Partially purified rat plasma kallikrein was inhibited by all these inhibitors. These results strongly indicate that the purified rat stomach kallikrein has similar substrate specificity to other glandular kallikreins. The susceptibility of rat plasma kallikrein to these inhibitors is different from these of human and bovine plasma kallikrein, but similar to that of guinea pig plasma kallikrein, which was purified recently by Yamamoto et al. (Yamamoto, T., personal communication).

Molecular weight of rat stomach kallikrein

The molecular weight of rat stomach kallikrein, was determined by gel-filtration on a column of Sephadex G-50, calculated to be 29 000. For comparison, the molecular weight of the partially purified rat plasma kallikrein was

TABLE II

PROPERTIES OF VARIOUS KALLIKREINS

Abbreviations: DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; OMTI, ovomucoid trypsin inhibitor; KIE, kallikrein inhibitor unit.

	Rat stomach	Rat plasma	Rat urinary	Porcine pancreas	Human urinary
Molecular weight	29 000	98 000	32 300 [15] 33 100 33 600 35 300	33 000 [16]	27 000 [17] 29 000
Inhibitor (% of inhibition)					
Trasylol	100 (500 KIE/mol)	100	+ [18]	+ [19]	+ [20]
DFP	100 (10^{-3} M)	100	+	+	+
SBTI	0 (100 μg/ml)	96	—	—	—
LBTI	0 (100 μg/ml)	80		—	—
OMTI	0 (100 μg/ml)	57	—	—	—
pI value	4.05		3.50 [15] 3.68 3.73 3.80	3.64 [21] 3.71 3.85 3.95 4.05	3.9 [17] 4.0 4.2
Substrate specificity					
Pro-Phe-Arg-MCA	1.0	1.0		1.0	
Z-Phe-Arg-MCA	0.23	1.57		0.2	
Specific activity (μmol Pro-Phe-Arg-MCA hydrolyzed/min per A_{280})	0.088	2.3		6.3 [11]	

determined by gel-filtration on a column of Sephadex G-150, calculated to be 98 000. The molecular weight of rat stomach kallikrein was clearly different from that of rat plasma kallikrein.

Table II summarizes the properties of rat stomach kallikrein in comparison with those of rat plasma kallikrein. The enzymes differed in molecular weight, susceptibility to protease inhibitors, optimum pH and substrate specificities. These results exclude the possibility that the kallikrein purified in this work was derived from plasma kallikrein. Contamination of the preparation with plasma can also be excluded, because rat stomach kallikrein did not hydrolyze casein or fibrin.

Table II also shows some properties of various glandular kallikreins (i.e., rat urinary, porcine pancreatic and human urinary kallikreins), which have been reported by other investigators.

The properties of rat stomach kallikrein are very similar to those of these glandular kallikreins. Thus it can be concluded that this enzyme is a typical glandular kallikrein. The location and function of the kallikrein in the stomach remain to be determined.

The high optimum pH of the kallikrein suggests that kallikrein may not function in the mucus membrane of the stomach. It would be interesting to know whether the kallikrein regulates the function of the stomach in some way in cooperation with gastrin and secretin.

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