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COLON KALLIKREIN, ITS RELATION TO THE PLASMA ENZYME*

T. SEKI,† T. NAKAJIMA and E. G. ERDÖS‡

Department of Pharmacology, University of Oklahoma School of Medicine, Oklahoma City, Okla., U.S.A.

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Abstract—A prekallikrein was extracted from colon wall and activated with Sepharose-bound trypsin complex. Kininogenase activity was also found in a particulate (microsomal) fraction of normal human colon and of adenocarcinoma. The active kallikrein originating from human, monkey or dog colon was subsequently purified. Colon kallikrein liberated bradykinin from human kininogen. Its molecular weight and inhibition pattern also suggested that it is different from glandular kallikrein and similar to plasma kallikrein.

KALLIKREINS (EC 3.4.4.21) or kininogenases are enzymes that release kinins from the plasma precursor globulin kininogen. Many properties distinguish plasma kallikrein[1] from glandular[2] and urinary kininogenases.[3] For example, the plasma enzyme liberates the nonapeptide bradykinin, while tissue or urinary kallikrein releases the decapeptide kallidin. In the blood several different plasma kallikreins[1,4,5] may exist simultaneously, even if some authors have found only one type of inactive pre-enzyme,[6] prekallikrein. A variety of enzymes and blood clotting factors may activate this prekallikrein. Although the participation of plasma kallikreins in various pathological processes has been frequently mentioned in the literature,[7,8] and blood-borne kallikreins have been studied by many investigators, the site of their origin is still not known. Our experiments showed that the properties of a kininogenase purified from colon wall[9,10] are very similar to those of a plasma kallikrein, and this is taken as an indication that a plasma enzyme may originate from this tissue.§

MATERIALS AND METHODS

Intestines of rat, mongrel dog and rhesus monkey were collected from laboratory animals. Swine intestine was obtained from a slaughterhouse. Human colon and adenocarcinoma were removed from surgical subjects during colotomy, and the tissues were stored frozen at — 20°. Human low molecular weight kininogen was prepared by Dr. J. V. Pierce of NHLI, NIH. It was partially purified by batchwise extraction on DEAE-cellulose.[11] The kininogen preparation, however, still contained an aminopeptidase that converted kallidin to bradykinin.[12] In order to destroy this

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‡ To whom the reprint requests should be sent.

§ Parts of our results were presented at meetings held in Fiesole, Italy[9] in 1969 and in Key Biscayne, Fla.[10] in 1971.

contamination, the pH of the solution was adjusted to 3 with formic acid for 10 min, and neutralized afterward with NH_4OH.

Pancreatic kallikrein (1000 U/mg) was a gift of Professor E. Werle. (2,3 Proline-^{14}C)-bradykinin and (3,4 proline-^{14}C)-kallidin were obtained through the support of the Radioactive Peptide Program of NHLI, NIH.

In initial exploratory studies, tissues were homogenized in 0·25 M sucrose solution (1:10, w/v), using a Polytron homogenizer. In subsequent experiments, the portions of human, monkey and dog colon which were easily scraped off with a siliconized glass slide and consisted mainly of mucosa and submucosa, were used. They were suspended in a 0·25 M sucrose solution containing 0·05 M Tris buffer of pH 8 and 0·01 % hexadimethrine (1:2 w/v). The tissues were homogenized in a Potter–Elvehjem homogenizer and centrifuged twice for 20 min at 9000 *g* in a Sorvall refrigerated centrifuge. The precipitate was discarded and the supernatant was the source of enzyme. Microsomal fractions were prepared in a Spinco L2-65 ultracentrifuge by centrifugation at 104,000 *g* in 60 min.

Prekallikrein present in the homogenate was activated[9] by adding 1 ml of settled volume of water-insoluble Sepharose–trypsin complex to a 3-ml sample of homogenate. The insoluble trypsin was prepared by coupling about 3 mg of trypsin to 1 ml Sepharose 4B. The mixture was incubated at 37° for 15 min in a shaking bath. The insoluble trypsin was removed by two centrifugations at 3000 *g* for 10 min. The Sepharose–trypsin complex did not release any soluble trypsin after centrifugation. The supernatant containing active kallikrein was dialyzed against 7·5 mM phosphate buffer of pH 8 overnight in the cold.

The activated kallikrein was separated in DEAE-cellulose chromatography on a 1·5 × 26 cm column using the phosphate buffer mentioned with a gradient increasing from 0 to 1 M NaCl for elution. The molarity of the eluent solution was established in a conductivity meter. Sephadex G-200 gel filtration was done on a 2·5 × 35 cm column in the phosphate buffer. The collected active fractions containing kininogenase were pooled and lyophilized. The protein content of the extracts was estimated by assaying the optical density at 280 nm or by using the technique of Lowry.[13]

The molecular weight of human colon kallikrein was estimated on a Sephadex G-200 column according to the instructions of the manufacturer (Pharmacia). Water-insoluble derivatives of dog colon kaliikrein and trypsin were prepared by coupling them covalently to Sepharose 4B.[9] Two-mg samples of dog colon kallikrein purified 12–25 times and lyophilized were coupled to 1 ml settled volume of Sepharose 4B each.

Kallikrein activity was assayed on the isolated rat uterus[7] by measuring the amount of kinin released from purified kininogen from the initial steady rate of the reactions. Samples were taken from the incubation mixture every 5 min. To prevent the inactivation of kinins by contaminating kininases, 1,10-phenanthroline (1×10^{-3} M) was added to the mixture.[12] Routinely, 5 mg kininogen was incubated with 10–20 mg crude homogenate or 1–2 mg activated homogenate in a 0·08 M tris buffer of pH 8. In control studies, human plasma heated at 62° for 30 min, or boiled in 0·1 N acetic acid for 20 min, was the substrate. The esterase activity of the extracts was measured with benzoylarginine ethyl ester (BAEe) substrate in a Cary recording u.v. spectrophotometer.[12]

When inhibitors were used, kallikrein was preincubated with the inhibitor for 15 min at 37° in a 0·08 M tris buffer of pH 8, then incubated with 5 mg of kininogen

for 45 min. Samples were withdrawn at 5-min intervals, and the kinin content was assayed on the isolated rat uterus. The inhibitors used were: soybean trypsin inhibitor (SBTI), ovomucoid trypsin inhibitor (OMTI), lima bean trypsin inhibitor (LBTI) or Trasylol.

The liberated kinin was separated by gel filtration on a Sephadex G-15 column and subsequently purified on a CM-Sephadex C-25 microcolumn. During the latter step, the peptide was eluted and separated with a linear gradient of ammonium formate.[14,15] The radioactivity of added labeled bradykinin or kallidin was measured in a liquid scintillation counter.[15] The mean recovery of added bradykinin was 41 per cent and that of kallidin 57 per cent.

RESULTS

Homogenates of intestines. We observed no kinin release when homogenates of small intestines were incubated with kininogen. In initial screening tests, intestinal tissues of rat, dog and swine did not release any significant amounts of kinin even after preincubation with trypsin. Homogenates of swine or rat colon wall liberated a kinin only after activation by trypsin. Homogenized mucosa and submucosa of human, monkey and dog colon released, however, a small amount of kinin even before activation by trypsin. Adding the Sepharose–trypsin complex activated a prekallikrein in the colon homogenates. The kininogenase content of colon of man and animals increased in the following order: rat, swine, monkey, dog and man. 47 μg of bradykinin equivalent (g/hr) was released by human colon, 6·0 μg by monkey and 34 μg by dog tissue. When the prekallikrein in the colon homogenate was not preincubated with trypsin, it had less than 10 per cent activity of the activated extract, indicating that over 90 per cent of the enzyme is present in the tissue as prekallikrein.

Human colon released approximately the same amount of bradykinin equivalent from 62° heated human plasma as from the partially purified kininogen, but 61 per cent less from plasma denatured by boiling in acetic acid. A particulate fraction obtained from homogenized dog colon and from homogenized human adenocarcinoma that sedimented at 104,000 *g* also contained kininogenase activity. Interestingly, the 104,000 *g* sediment of the adenocarcinoma preparation contained active kallikrein instead of prekallikrein. Unfortunately, the microsomal fractions contained very potent kininases[16] that destroyed most of the kinin released and prevented the accurate estimation of the amount of peptide liberated. Crude homogenate of human adenocarcinoma, obtained by surgical resection of the colon, also showed kininogenase activity which was of the same order of magnitude as that of normal colon tissue.

Purification. Colon kallikrein was purified from human and dog tissues three times and from monkey tissue once by the following procedure. The mucosa and the submucosa were scraped off from the colon wall and then homogenized. The prekallikrein present in the crude homogenate was activated with an insoluble Sepharose-trypsin complex.[9] The active kallikrein was absorbed on a DEAE-cellulose column and eluted with a linearly increasing NaCl gradient (Fig. 1). The active fraction from human tissue was further concentrated by gel filtration in a Sephadex G-200 column. (Dog and monkey colon kallikreins were filtered through Sephadex G-150 columns.) Table 1 shows a representative purification experiment with the human enzyme. Here the enzyme activity was determined by measuring the amount of kinin released and by assaying the amount of BAEe hydrolyzed per 280 nm adsorbing material.

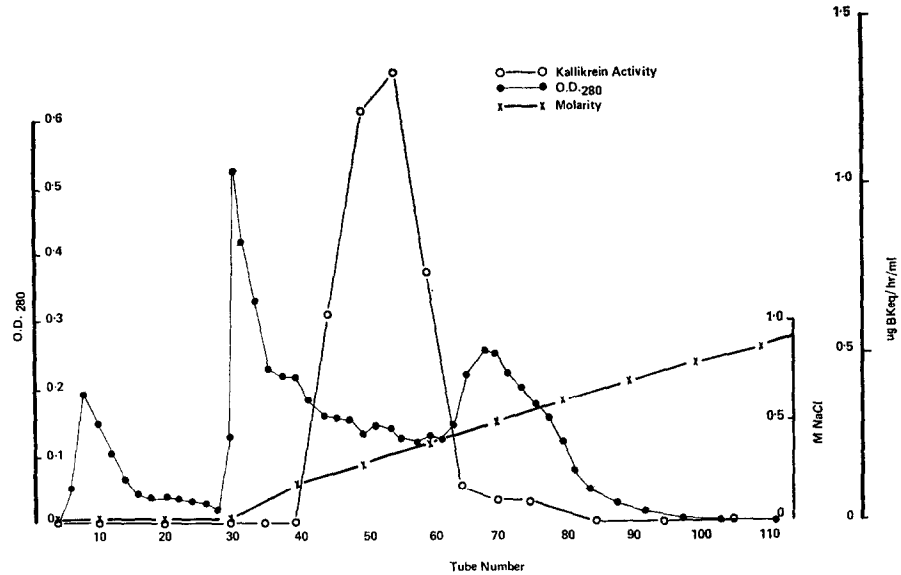

FIG. 1. Purification of kallikrein from human colon on a DEAE-cellulose column (26 × 1·5 cm) Protein (140 mg) in 10 ml, was applied to the column. Gradient elution was carried out with 600 ml of 7·5 mM phosphate buffer of pH 8 in the mixing vessel and 12·5 mM phosphate buffer with 1 M NaCl in the reservoir. The flow rate was 35 ml/hr. Ordinates: concentration of 280 nm absorbing materials, amount of bradykinin equivalent released and concentration of NaCl used for elution in a linear gradient. Abscissa: test-tube number.

Activation by trypsin increased the kininogenase activity 8·5-fold, but the esterase activity in the homogenate increased only about 50 per cent. The purification of the kininogenase in the selected pooled fractions was 116-fold over the activated homogenate and 969-fold over the crude homogenate. The esterase activity increased only 12- and 17-fold respectively. Obviously only a fraction of the esterase activity of the colon can be attributed to kallikrein.

TABLE 1. PURIFICATION OF COLON KALLIKREIN

Step	Specific activity*	Esterase activity†	Purification	Yield (%)	O.D. (280 nm)
Crude homogenate	0·13	6·5	(0·12)	—	283
Activation by Sepharose-trypsin	1·1	9·2	1	100	203
DEAE-cellulose column chromatography	14·7	16·8	14	84	13
Gel filtration Sephadex G-200 column	126	112‡	116	48	0·8

* Micrograms bradykinin equiv. released by 1·0 unit of 280 nm absorbing material per hour.

† 1 mU at 253 nm (see Trautschold in Ref. 1, p. 52).

‡ 1 mU (nmole) per minute per milligram protein = 14.

Other purification experiments yielded a 20- to 28-fold purification of human, monkey and dog colon kallikrein after pooling various active fractions obtained in gel filtration.

The molecular weight of human colon kallikrein was estimated by means of gel filtration in a Sephadex G-200 column. Human kallikrein from two different batches had a mol. wt. of 71,000.

Dog colon kallikrein was stabilized by rendering it insoluble by covalent coupling to Sepharose-4B.[9] About 50 per cent of the 280 nm absorbing material was complexed with Sepharose. The insoluble enzyme retained about 10 per cent of its kininogenase action.

Characterization of the kinin released. Kallikrein[2] originating from glandular tissues or from urine releases kallidin, while plasma kallikrein liberates bradykinin from kininogen. Our data indicate that, similar to the plasma enzyme, human, monkey and dog colon kallikrein release mainly bradykinin from human kininogen substrate. This was concluded from the following experiments.

The kinins released were separated from the incubation mixture by gel filtration on a Sephadex G-15 column. The collected active fraction was adsorbed on a CM-Sephadex column and subsequently eluted and separated with a linear gradient of ammonium formate.[14,15] Usually the first peak of activity eluted with 0·17–0·21 M

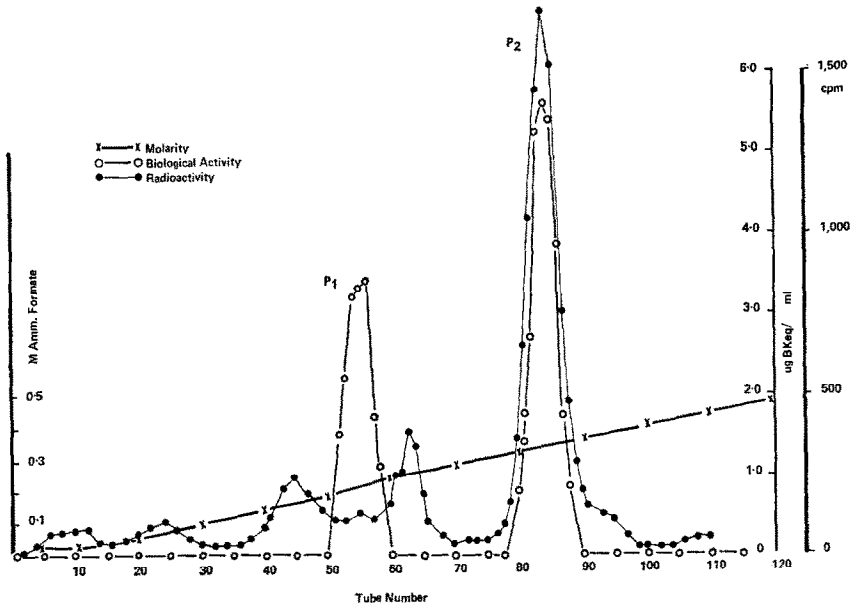

FIG. 2. CM-Sephadex column (8 × 0·9 cm) chromatography was used to show that colon kallikrein released bradykinin. Radioactive (3,4 proline-^{14}C)-kallidin (0·2 μC) was added to the kinin released by colon kallikrein. The flow rate was 35 ml/hr. The biological activity was measured on the rat uterus. The peptide was eluted with a gradient of ammonium formate increasing from 0·05 M (pH 5) to 0·5 M (pH 7·5). The kinin liberated by the colon enzyme (P_1) was eluted with an ammonium formate concentration typical for bradykinin. The second peak (P_2) represents the elution of added radioactive kallidin at a higher ammonium formate concentration. Ordinates: molarity of the ammonium formate eluent, amount of bradykinin equivalent released and count per 0·5 ml/min. Abscissa: tube number.

ammonium formate was due to bradykinin; the second one eluted with 0·29–0·35 M ammonium formate contained kallidin. When synthetic labeled bradykinin and labeled kallidin were used as tracers and added before chromatography to the naturally released peptide, (2,3 proline-^{14}C)-bradykinin emerged with the first peak and (3,4-proline-^{14}C)-kallidin with the second peak. Human colon kallikrein released a mixture of kinins that consisted of 86% bradykinin and 14% kallidin. Figure 2 shows an experiment, and Table 2 summarizes the results. For the sake of comparison, purified pancreatic kallikrein was also added to the kininogen substrate used. This enzyme liberated mainly kallidin (Table 2). The ratio of bradykinin to kallidin in the kinin release by monkey colon was 9 to 1, and dog colon kallikrein released bradykinin only. In control studies, no conversion of added, labeled, synthetic kallidin to bradykinin was observed in the incubation mixture; thus colon kallikrein indeed released bradykinin from the kininogen. We did not detect the liberation of Met-Lys-bradykinin either in any of the experiments.

TABLE 2. TYPE OF KININ RELEASED BY COLON KALLIKREIN FROM A HUMAN KININOGEN PREPARATION

Source	Peptide (% of total liberated)		
	Bradykinin	Kallidin	Met-Lys-bradykinin
Dog colon	100	0	0
Monkey colon	90	10	0
Human colon	86	14	0
Pancreatic kallikrein	34	66	0

Inhibitors. The inhibition patterns of plasma and glandular kallikreins are different. For example, SBTI or OMTI inhibit some plasma kallikreins, but not urinary or pancreatic kallikreins.[3,17] Trasylol, the protease inhibitor of the bovine lung, on the other hand, inhibits all three types of kallikreins, but it is less active against the dog enzyme.[3] In our experiments SBTI inhibited the kininogenase and esterase activity of kallikrein coming from the three sources of colons used (Table 3). OMTI on the other hand inhibited only the activity of human colon kininogenase, but not the hydrolysis of BAEe or the release of bradykinin by monkey and dog colon kallikrein. LBTI was ineffective against the kininogenase action, but inhibited the esterase activity of the human enzyme. These data also indicate a lack of correlation between the kinin release and ester hydrolysis.

DISCUSSION

Werle and Vogel[18] found first in the homogenate of colon wall a substance that was activated by trypsin. Injection of the substance, i.v., lowered the blood pressure of the dog, thus it acted as a kallikrein. This effect of the agent was inhibited by SBTI. Amundsen and Nustad[19] reported that mucosa from various parts of the gastrointestinal tract, including the colon, released a kinin from kininogen. Our experiments showed that the colon wall of man and animals contains a prekallikrein with properties similar to those of a plasma pre-enzyme and different from glandular

TABLE 3. PER CENT INHIBITION OF KININOGENASE (K) AND ESTERASE (E) ACTIVITIES OF COLON KALLIKREIN

	Source of enzyme			
	Man		Monkey	Dog
Inhibitor	K	E	K	K
SBTI	47	40	35	49
OMTI	67	12	0	9
LBTI	10	60	0	17
Trasylol†	100	100	100	62

* Concentration of inhibitor: man, 100 μg/ml; monkey, 260 μg/ml; and dog 260 μg/ml.
† Trasylol: man, 10–100 μg/ml; dog and monkey, 260 μg/ml.

kallikreins. Colon prekallikrein can be activated by trypsin, and the activated enzyme releases bradykinin from a kininogen substrate. This action in man can be inhibited by SBTI, OMTI and Trasylol. In contrast, kallikreins originating from glandular tissues release kallidin.[3] The literature on the inhibition of kallikreins by trypsin inhibitors is controversial,[17] but generally SBTI and OMTI do not inhibit glandular kallikreins. The reported[1,6] molecular weights of plasma kallikreins vary from 86,000 to 97,000, while that of glandular kallikreins[2] range from 24,000 to 33,000. The molecular weight of human colon kallikrein was 71,000 which is much higher than that of the glandular enzyme.

In most of our experiments, so-called low molecular weight kininogen substrate was used. This type of kininogen was recently employed as substrate of a plasma kallikrein by Jahrreiss and Habermann.[20] Colon kallikrein releases much less kinin from a denatured substrate than from native kininogen, while trypsin acts the opposite way.[20]

The presence of prekallikrein in the microsomal-ribosomal fraction of the homogenized colon indicates that the pre-enzyme indeed originates from this tissue. Homogenates and particulate fractions of the homogenized adenocarcinoma of the colon also contain a kininogenase in active form. In contrast to these results, extracts of small intestines showed insignificant traces of kininogenase activity in our hands.

Among the various species tested, human colon had the highest prekallikrein content, while monkey and dog tissues had less. Swine and rat colon had very little kininogenase. Undoubtly the results of the experiments were influenced by the very high kininase content of the extracts. Thus some of the kinin released may have been inactivated even in the presence of the kininase inhibitor *o*-phenanthroline.

The use of an insoluble complex of trypsin for the activation of prekallikrein was of great advantage[9] because the activated kallikrein has many properties in common with trypsin.[1–3] The contamination of the extract by trypsin during activation could be avoided by short centrifugation which removes added insoluble trypsin. Dog colon kallikrein, after purification, can be covalently bound to water-insoluble carrier,[9] and will still release a kinin in this stabilized form. The application of an isotope dilution technique[15] established that most of the kinin released was bradykinin. That colon kallikrein did not liberate kallidin, which could have been converted *in vitro* to bradykinin via cleavage of the N-terminal lysine by a contaminating aminopeptidase,[16] was

shown by adding labeled synthetic kallidin. The labeled kallidin was not converted to bradykinin in the incubation mixture as demonstrated in CM-Sephadex chromatography experiments. The release of a small amount of kallidin by human and monkey colon kallikrein may have been due to contamination by glandular kallikrein coming from the same organ.

The BAEe esterase activities of the extracts cannot be correlated with their kallikrein content. An active esterase was already present in the crude homogenate prior to the activation of prekallikrein, when the free kininogenase activity was very low. Table 2 also indicates that the two enzymic activities were inhibited differently.

The fact that some trypsin inhibitors were ineffective against the kininogenase studied and that trypsin and the colon kallikrein had the opposite effects on denatured substrate also indicate that the colon enzyme is not identical with trypsin.

If the colon is the site of origin of a prekallikrein, this may be of special importance in pathological conditions. The proteolytic inhibitor Trasylol has been used in pancreatitis and in endotoxin shock,[3] but its mode of action is not yet fully understood. The effect of Trasylol can be attributed partially to the inhibition of an increased plasma kallikrein activity.[3] The area of the splanchnic circulation is of special interest in shock and may involve the kinin system. For example, increased blood kallikrein content was found in the portal circulation during hemorrhagic shock.[21] Kallikrein by causing the release of kinin may aggravate the condition of man and laboratory animals during shock.[7] Damage to the colon wall in a variety of conditions such as shock or during other impairments of the circulation presumably can lead to an increase in apparent plasma kallikrein level by the liberation of colon kallikrein. The rise in the concentration of this potent hypotensive agent could deteriorate the circulation further.

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