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PROTEOLYSIS OF SALMINE BY HORSE URINARY KALLIKREIN*

CATHARINA M. W. BRANDI, JOSEFINA MENDES, A. C. M. PAIVA and ELINE S. PRADO

Laboratórios de Farmacologia e Bioquímica, Escola Paulista de Medicina† and Seção de Fisiologia Aplicada, Instituto Adolfo Lutz, São Paulo, Brasil

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Abstract—Purified horse urinary kallikrein preparations, free from hemoglobin and casein-splitting activities, were shown to hydrolyze protamine sulfate to a lesser extent than trypsin. This proteolytic activity is not inhibited by ovomucoid, whereas it is arrested by Trasylol. The proteolysis of salmine by this kallikrein parallels its kininogenic (kinin-liberating) activity. It is suggested that the presence of arginyl bonds in salmine may be significant for the observed proteolysis produced by this kallikrein.

THE kallikreins are enzymes that release, from a protein precursor in plasma, the kallidin peptides (Fig. 1). They possess esterolytic activity toward esters of benzoyl-

1	2	3	4	5	6	7	8	9	10
H. Lys.	Arg.	Pro.	Pro.	Gly.	Phe.	Ser.	Pro.	Phe.	Arg. OH

(kallidin-10; kallidin 2)

Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg. OH
(kallidin-9; kallidin 1; bradykinin)

FIG. 1. Amino acid sequences of kallidin peptides.

L-arginine and *p*-tosyl-L-arginine but do not hydrolyze lysine esters and benzoyl-L-argininamide[1–5]. Furthermore, it has been demonstrated that purified kallikrein preparations do not possess proteolytic activity on casein or hemoglobin.[5, 6]

In an attempt to characterize further the proteolytic activity of kallikreins, it seemed desirable to find suitable substrates. Since arginine appears in the C-terminal end of kallidin-10 and in both ends of kallidin-9 (Fig. 1), the protamines, owing to their high arginine content, appeared to be a promising kallikrein substrate. The present paper reports the demonstration of the proteolysis of one of the protamines (salmine) by horse urinary kallikrein.

EXPERIMENTAL

The purified kallikrein preparations used were obtained from horse urine as described in previous papers[5, 7] and had no detectable proteolytic activity on casein[5] and

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† Mailing address: Caixa Postal 12993, São Paulo, Brasil.

hemoglobin.[8] Three different kallikrein preparations, referred to as UK-1, UK-2, and UK-3, were used. On assaying the kinin-liberating (kininogenic) activities of these preparations, against a kallikrein standard with specific activity 1,[5] UK-1 and UK-2 had approximately the same specific activity (580 units/mg protein), whereas UK-3 was about 1·6 times less active (350 units/mg). It should be noted that when freshly passed through Sephadex, kallikrein UK-2 had a specific activity close to 800 which had decreased to 580 when used; this had been observed previously.[7]

The trypsin was a twice-crystallized salt-free product from Nutritional Biochemicals Corp. The salmine sulfate preparations were from Nutritional Biochemicals and Bios. Ovomucoid was from Worthington Biochemical Corp., and Trasylol is a product of Bayer.

Proteolysis

In order to follow the proteolysis of protamine by proteolytic enzymes, two methods were used: (a) a nephelometric assay based on the insolubility of protamine picrate; and (b) determination of liberated amino groups, as follows.

(a) Protamine solutions (1–4 mg salmine sulfate/ml) was diluted 16-fold with 2·5 mM picric acid and left at room temperature for 30 min, after which the turbidity was measured on a Coleman Jr. spectrophotometer at 480 mμ. When the conditions of the assay were held exactly the same, reproducible results were obtained which varied somewhat according to the protamine preparation used (Fig. 2). Only one

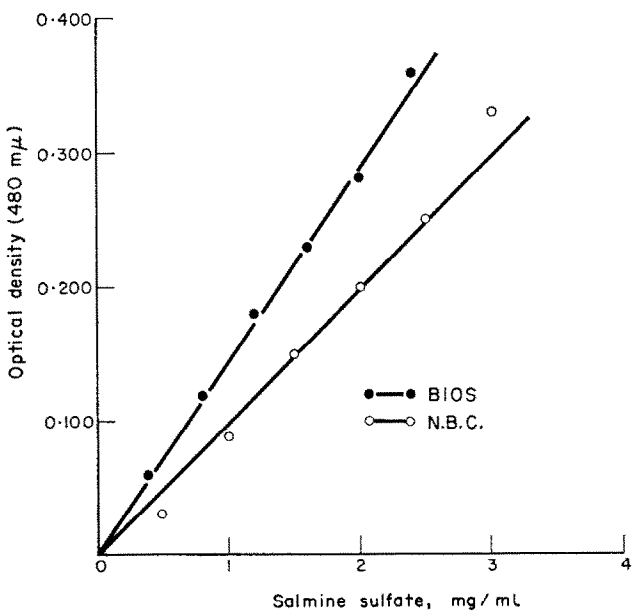

FIG. 2. Turbidimetric determination of protamine with picric acid; 0·2 ml salmine sulfate solutions was added to 3 ml of 2·5 mM picric acid, and optical densities read after 30 min at room temperature.

batch of each salmine preparation was used throughout the work. The action of trypsin and kallikrein upon salmine was studied by incubating a solution of substrate and enzyme in 0·05 M Tris buffer, pH 8·0, at 37°. The time course of the reaction was followed by withdrawing 0·2-ml aliquots immediately after mixing enzyme and

substrate and at regular intervals thereafter. In the incubations where the effect of enzyme or substrate concentrations was studied, a fixed time of incubation was chosen during which the time course of reaction was linear.

(b) Aliquots of the incubation mixtures of protamine sulfate and trypsin or kallikrein were removed at different periods of incubation and assayed with ninhydrin.[9] Blanks for the substrate and enzymes were also run, and the increase in absorption at 570 mμ for aliquots corresponding to 80 μg salmine sulfate was determined.

RESULTS

Action of trypsin and kallikrein on salmine

The turbidimetric method was tested in the determination of tryptic proteolysis of salmine. When crystalline trypsin was incubated with salmine, the concentration of the material precipitable by picric acid decreased rapidly, as was expected. The results of a typical experiment are shown in Fig. 3, which demonstrates also that no tryptic activity was detected when trypsin was preincubated for 20 min with 2·5 times its weight of ovomucoid at pH 8·0 and 37°.

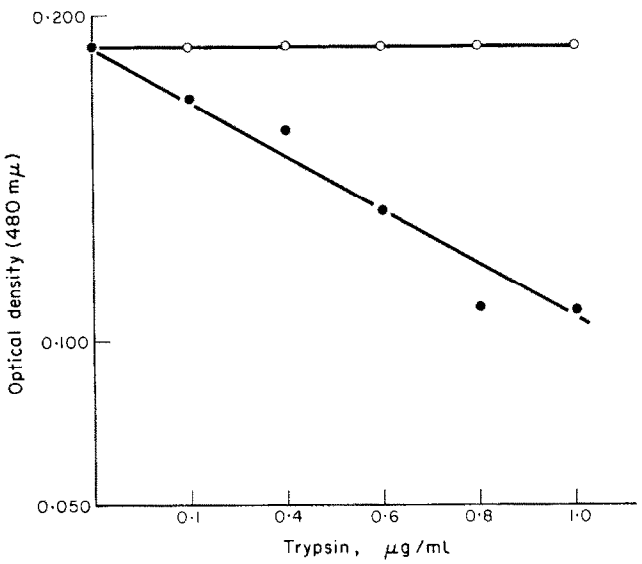

FIG. 3. Tryptic proteolysis of salmine sulfate (Nutritional Biochemicals Corp.) in presence and absence of ovomucoid. Substrate concentration, 2 mg/ml. A solution of trypsin containing 2 μg/ml was incubated with ovomucoid (5 μg/ml) during 20 min, 37°, pH 8. Aliquots of this mixture and aliquots of trypsin were incubated with salmine sulfate during 20 min at 37°, pH 8; ●—● trypsin; ○—○ trypsin + ovomucoid.

When the three kallikrein preparations were incubated with salmine, a rapid decrease of picric acid-insoluble material was observed. No significant difference between the activities of UK-1 and UK-2 was detected. On the other hand, UK-3 had an activity which was approximately 1·6 times less than that of UK-1 (Fig. 4). Thus, the relative salmine-splitting activities of the three kallikrein preparations nearly paralleled their relative kininogenic activities.

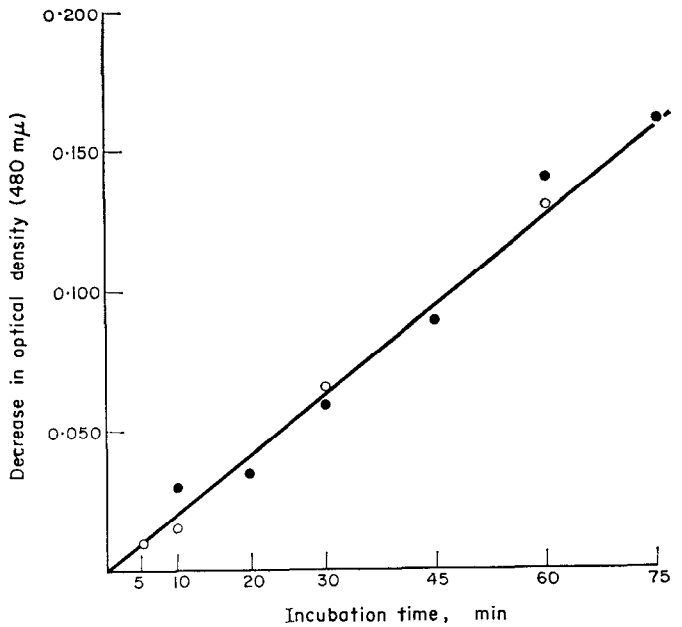

FIG. 4. Incubation of salmine sulfate (Bios) with two kallikrein preparations. Substrate concentration= 1 mg/ml. Incubation at 37°, pH 8; ●—● UK-3 (2·8 μg/ml); ○—○ UK-1 (1·7 μg/ml). The weight ratio UK-3/UK-1 was 1·6. The kininogenic activity ratio UK-1/UK-3 was 1·6.

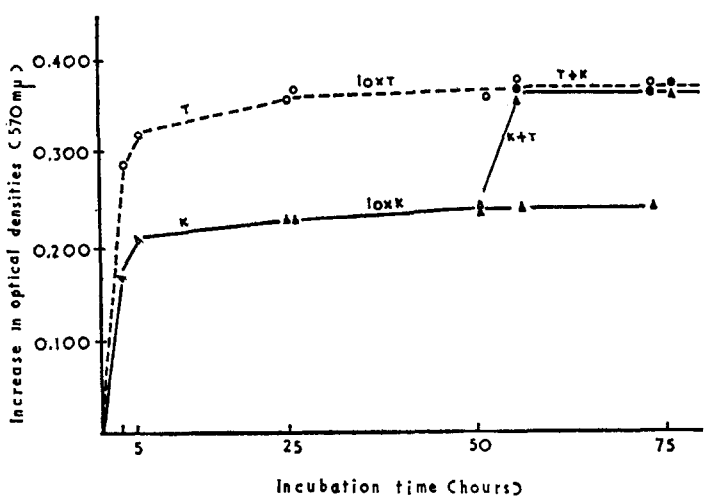

FIG. 5. Liberation of amino groups from salmine sulfate by trypsin (2 μg/ml, ○—○) and by kallikrein (10·6 μg UK-2/ml, △—△). Substrate concentration, 4 mg/ml. After 25-hr digestion (portions T and K of the graph), each enzyme concentration was increased 10-fold (10 × T and 10 × K). At the end of 50 hr, kallikrein (10·6 μg UK-2/ml) was added to the trypsin hydrolysate (T + K, ●—●); and trypsin (2μg/ml) was added to the kallikrein hydrolysate (K + T, ▲—▲).

The liberation of amino groups from salmine by trypsin and kallikrein is shown in Fig. 5. After a 25-hr incubation period, it may be seen that increasing each enzyme concentration 10-fold produced no further salmine hydrolysis during 25 hr. When kallikrein was now added to the trypsin hydrolysate (T + K, Fig. 5) no further increase of free amino groups was observed; however, when trypsin was added to the kallikrein hydrolysate (K + T, Fig. 5), a sharp augmentation of the ninhydrin color, to the level obtained with trypsin alone, was produced. This increase was not due to hydrolysis of horse kallikrein by trypsin since, in the blanks where horse urinary kallikrein was incubated with trypsin, no liberation of amino groups occurred. Furthermore, this kallikrein was not inactivated by trypsin,[8] a fact already known for other kallikreins.[10]

Effect of inhibitors

The effect of inhibitors upon the action of kallikrein on salmine was studied with ovomucoid and Trasylol and was based on the fact that kininogenic activity of horse kallikrein is not inhibited by ovomucoid, whereas it is inhibited by Trasylol.[7] A solution containing $6{\cdot}6 \times 10^{-3}$ mg of UK-1 and $6{\cdot}6 \times 10^{-1}$ mg of ovomucoid per ml was incubated at pH 8·0 (0·05 M Tris) and 37° for 60 min and then assayed for its activity upon salmine, by the nephelometric method. Solutions of UK-1 in 0·05 M Tris buffer, pH 8·0, were incubated with Trasylol in the proportions of 1 and 10 units of inhibitor for 1 μg UK-1, and then assayed by the same method. Figure 6 shows that

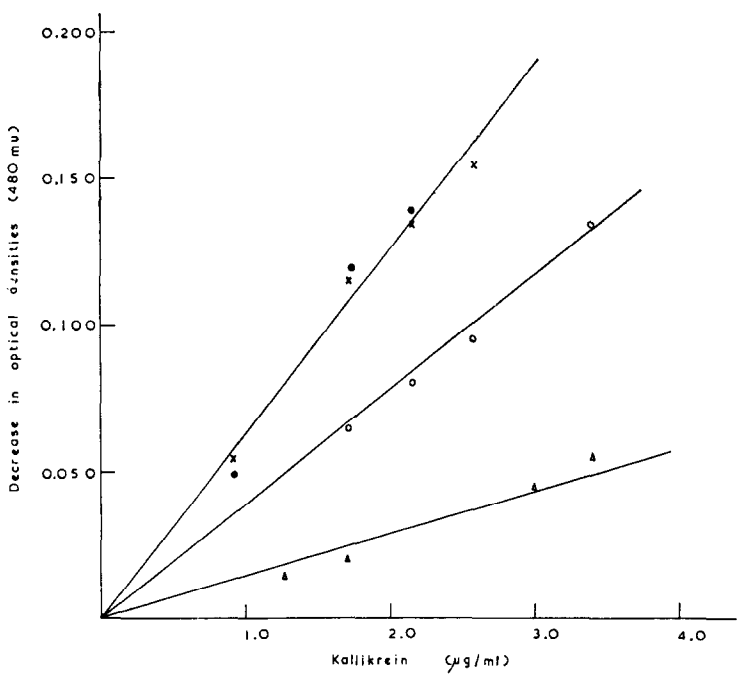

FIG. 6. Effect of ovomucoid and Trasylol upon salmine sulfate (Nutritional Biochemicals) splitting activity of kallikrein. Substrate concentrations 2 mg/ml. Solutions of kallikrein (UK-1) containing 6·6 μg/ml were incubated either with ovomucoid or with Trasylol during 60 min at 37°, pH 8. Aliquots of these mixtures were incubated with salmine; ●—● UK-1; x—x UK-1 + ovomucoid (660 μg/ml); ○—○ UK-1 + Trasylol ($6{\cdot}6 \times 10^{-3}$ units/ml); ▲—▲ UK-1 + Trasylol ($6{\cdot}6 \times 10^{-2}$ units/ml).

no inhibition of kallikrein by ovomucoid was detected, whereas Trasylol inhibited the action of kallikrein on salmine. The inhibition observed parallels the inhibition of the kininogenic activity. For instance, a UK-1 solution, in which preincubation with Trasylol had reduced the salmine-splitting activity by 85%, had its kininogenic activity reduced by 94%.

DISCUSSION

Salmine was shown to be a suitable substrate to demonstrate the proteolytic activity of purified horse urinary kallikrein preparations that had no detectable proteolytic activity toward casein and hemoglobin. Although one may consider that the proteolytic activity on salmine might be due to a contaminating proteolytic enzyme of the kallikrein preparations used, this possibility seems remote for two reasons: (a) highly purified horse urinary kallikrein was used, and the relative salmine-splitting activities of the three kallikrein preparations employed paralleled their relative kininogenic activities; (b) the inhibition of the proteolytic activities of our kallikrein preparations by Trasylol paralleled the inhibition of their kininogenic activities.

It has been reported[11] that hog pancreatic kallikrein (870 units/mg) produces a glycopeptide from kappa-casein; however, neither a correlation between this effect and kininogenic activity of the kallikrein used nor the effect of kallikrein inhibitors was tried. Moreover, much higher concentrations of kallikrein had to be used than in the present paper, which makes the observations more vulnerable to the possibility that a contaminating proteolytic enzyme was responsible for the observed proteolytic effect.

The fact that other proteins containing arginine are trypsin-hydrolyzable but are not attacked by kallikreins may indicate that these enzymes have a more strict specificity than trypsin. It was indeed observed that horse urinary kallikrein hydrolyzed salmine to a lesser extent than trypsin, and it was also shown that trypsin, when added to a plateaued kallikrein hydrolysate of salmine, produced still further hydrolysis; this is taken as evidence that horse urinary kallikrein is a proteolytic enzyme with a narrower specificity than trypsin. This specificity may be directed toward arginyl bonds that are so frequent in salmine.[12] Protamines are very poor or do not contain lysine,[12] and kallikreins do not attack lysine esters;[1-4] thus, ruptures of peptide bonds involving lysine in salmine seem improbable. Angiotensin, which has an arginyl–valyl bond, is inactivated by trypsin, whereas it resists horse urinary kallikrein; insulin, containing one arginyl–glycyl link is not hydrolyzed by this kallikrein; on the contrary, glucagon, with one arginyl–arginyl and one arginyl–alanyl bond, is split by horse urinary kallikrein.[8]

If the presence of arginyl residues is really a requisite for the proteolytic specificity of kallikreins,[13] these unpublished observations indicate that only some arginyl bonds are split by horse urinary kallikrein. Further work will be necessary to determine which are the structural features of the substrate molecule indispensable for the proteolytic action of this enzyme.

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