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# KALLIDIN (LYSYLBRADYKININ), THE KININ FORMED FROM HORSE PLASMA BY HORSE URINARY KALLIKREIN*

ELINE S. PRADO, MARION E. WEBSTER† and J. L. PRADO

Department of Biochemistry and Pharmacology, Escola Paulista de Medicina and Section of Applied Physiology, Institute Adolfo Lutz, São Paulo, Brasil

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**Abstract**—Horse urinary kallikrein when incubated with horse plasma formed kallidin (lysylbradykinin) from the kininogens in the plasma. Horse plasma, like human plasma, was found to contain an aminopeptidase capable of converting kallidin to bradykinin. No evidence, however, could be found that the plasma contained an aminopeptidase capable of converting Met-Lys-bradykinin to kallidin, thus eliminating the possibility that the kallikrein had released Met-Lys-bradykinin which was converted to kallidin during the 1–5 min incubations. The method used for identification of the kinins is rapid, gives a good recovery and requires small amounts of plasma and enzyme.

THE NATURE of the kinins liberated from kininogens or their kinin-containing fragments by the kallikreins may vary according to the enzyme and also with the substrate used. Thus, the plasma kallikreins, e.g. those derived from human plasma[1] and hog serum,[2] formed bradykinin from human and bovine kininogens respectively. The glandular kallikreins, e.g. those derived from human urine,[1,3] hog submaxillary gland[4] and hog pancreas,[2] formed kallidin (lysylbradykinin) from human and bovine kininogens. On the other hand, when fragments of the kininogen molecule were employed as substrates, hog serum kallikrein still liberated bradykinin[5] while hog pancreatic kallikrein no longer gave kallidin but rather Met-Lys-bradykinin.[5,6]

In these studies it was found that horse urinary kallikrein liberates kallidin, rather than bradykinin, from horse plasma. The use of plasma as a source of kininogen is complicated by the fact that it contains kininases which rapidly destroy the kinins and the likelihood that it will contain an aminopeptidase which converts kallidin to bradykinin.[1] In the present study, the addition of 1,10-phenanthroline to the plasma inhibited the kininases[7] and it was found that the aminopeptidase in horse plasma, provided the incubation period was not prolonged, transformed only a small quantity of kallidin into bradykinin. No evidence could be found for the presence in horse plasma of another aminopeptidase capable of converting Met-Lys-bradykinin to kallidin, thus eliminating the possibility that the kallikrein had formed Met-Lys-bradykinin which was then converted to kallidin by this aminopeptidase. A method

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† Present address: Experimental Therapeutics Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014.

was developed for the rapid identification of the kinins which were recovered in good yields from small amounts of plasma and enzyme.

## MATERIALS AND METHODS

*Formation and preparation of kinins for chromatography.* Horse blood was collected in 1:50 20% sodium citrate–blood (v/v) in silicone-coated glassware and the plasma separated by centrifugation at room temperature. The plasma was distributed in 5·0-ml aliquots and frozen once at −20°. Repeated freezing and thawing was avoided. To 0·5 ml $3 \times 10^{-2}$ M 1,10-phenanthroline (pippeted from a solution prepared by warming in a boiling water bath) was added 5·0 ml of plasma and the mixture incubated at 37° for 5 min. The enzyme reaction was initiated by the addition of 2 μl (18 KL units, 54 μg) of a horse urinary kallikrein preparation which had a specific activity of 330 KL units/mg and had been obtained by purification through step 4 of the procedure previously described.[8,9] One min later 20 ml of a boiling mixture of 0·01 M ammonium formate, pH 4·7, containing 0·0175 M formic acid was added to stop the enzymatic reaction. The mixture was heated in a boiling water bath for 15 min, and the coagulated proteins were removed by centrifugation. The clear supernatant which contained the kinins could be bioassayed on the guinea-pig ileum and applied directly to the column described below for separation of the kinins.

Silicone-coated glassware was employed throughout these procedures, both to prevent glass activation of the plasma and adsorption of the kinins to the glass surfaces during preparation and chromatography.

*Separation of the kinins.* The kinins, i.e. bradykinin, kallidin and Met-Lys-bradykinin, could be separated by chromatography on CM-cellulose employing a procedure similar to that described by Habermann and Blennemann.[2] In these experiments, essentially all (i.e. about 20 ml) of the plasma supernatant prepared as described above was added directly to a column (0·4 × 15 cm) of CM-cellulose (Whatman CM-32, 1·0 m-equiv./g) which had been washed, as described by Peterson and Sober,[10] equilibrated with 0·05 M ammonium formate, pH 4·7, and just before use suspended in this same buffer, allowed to stand for 1–2 min and decanted to discard the large particles. After the addition of the sample at a flow-rate of approximately 2·0 ml/hr, the column was developed with 10 ml 0·05 M ammonium formate, pH 4·7, and a gradient of 0·05 M to 0·3 M ammonium formate, pH 4·7 (constant volume mixing chamber of 105 ml) was initiated. Use of a steeper gradient (mixing chamber of 30–50 ml) gave a poorer resolution of Met-Lys-bradykinin and kallidin. The column was developed at room temperature (12–24°) and 1·0-ml fractions were collected in a refrigerated (4°) fraction collector at a flow-rate of 2 ml/hr. These fractions were assayed for conductivity; for biological activity employing the guinea-pig ileum[9] with the corresponding kinin standard; and for radioactivity by counting 0·2 ml of each fraction diluted with 10 ml Bray's solution[11] in a Beckman scintillation counter (LS-100). The use of the scintillation counter of the Department of Physiology (Dr. G. Malnick), University of São Paulo, is gratefully acknowledged.

(3,4-L-proline-$^{14}$C) kallidin was obtained from Schwarz BioResearch (Lot No. 6901, sp. act. 215 mc/m-mole), bradykinin triacetate was obtained from the New England Nuclear Corp. (Lot No. 307–98), and Met-Lys-bradykinin (Schering A. G.) was obtained through the support of the Radioactive Peptide Program of the National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. Kallidin

(KL-698) was a gift from Sandoz Pharmaceuticals. Trypsin B grade, essentially freed of chymotrypsin, was purchased from Calbiochem.

## RESULTS

*Separation of the kinins.* In earlier studies[2,3] a variety of methods were used to purify the kinins prior to their separation on CM-cellulose chromatography. These methods resulted in losses of kinin activity and made it necessary to use large volumes of substrate and enzyme in a time-consuming procedure. However, if the bulk of the plasma proteins are removed by heating at pH 4·7, the residual proteins in the clear supernatant do not interfere with the separation of the kinins. As shown in Fig. 1, when

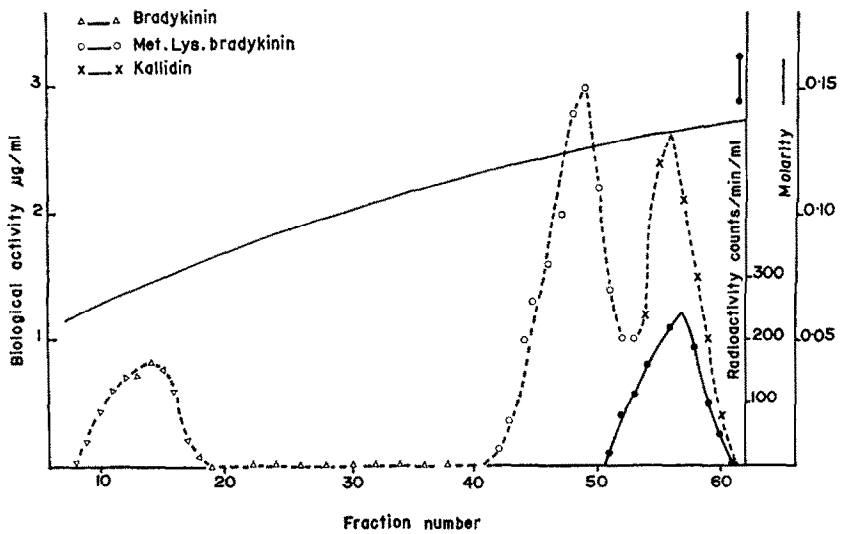


FIG. 1. Chromatography on CM-cellulose of a mixture of bradykinin (5 µg), Met-Lys-bradykinin (20 µg) and kallidin (10 µg) dissolved in 20 ml of a plasma supernatant. (3,4-L-proline-$^{14}$C) kallidin (50 ng) was used as a marker. The biological activity of the eluates was assayed on the guinea-pig ileum against the corresponding kinin standard. Volume of fractions: 1·0 ml.

5 µg bradykinin, 20 µg Met-Lys-bradykinin, 10 µg kallidin and 50 ng labeled kallidin were added to 20 ml of the supernatant from 5·0 ml of plasma containing 1,10-phenanthroline, three peaks of biological activity were obtained. The third peak could readily be identified as kallidin by comparing the biological activity and the radioactivity. The second peak was identified as Met-Lys-bradykinin by the increase in kinin activity found after digestion with trypsin.[12] For this test, appropriate aliquots of fractions 43–60 were adjusted to pH 8·0 by the addition of an equal volume of 0·2 M tris–HCl, pH 8·0, and incubated at 37° with 100 µg trypsin for 15 min. An increase in the guinea-pig ileum response when compared to control aliquots was found only in fractions 43–53. These results when compared to those with the labeled kallidin would indicate that both polypeptides are found in fractions 51–53. The first peak had been previously shown to be bradykinin by means of added radioactive peptide. Also, it was found that the supernatant obtained from the plasma and 1,10-phenanthroline mixtures contained a red pigment. This red pigment was retained on the columns and eluted in the same fractions as bradykinin. Thus, it served not only as a marker for

bradykinin, but from its distribution the column efficiency could be assessed. Bradykinin, Met-Lys-bradykinin and kallidin were recovered in this experiment in yields of 100, 85 and 108 per cent, respectively, as measured by bioassay.

*Conversion of other kinins to bradykinin.* That the aminopeptidase which converts kallidin to bradykinin is present in horse plasma was shown by the following experiment. Kallidin (20 $\mu$g) was incubated for 5 min at 37° with 10 ml of horse plasma in the presence of 1,10-phenanthroline. Chromatography of 43 ml of the plasma supernatant corresponding to 17 $\mu$g of kallidin gave a recovery of 2·1 $\mu$g bradykinin and 9·4 $\mu$g kallidin showing that 18 per cent of the recovered polypeptide had been transformed into bradykinin. More prolonged incubation (2 hr) resulted in an 80 per cent conversion of kallidin to bradykinin.

Experiments were also conducted to detect the presence in horse plasma of an enzyme which would convert Met-Lys-bradykinin to kallidin. However, as shown in Fig. 2, when 10$\mu$g Met-Lys-bradykinin was incubated for 1 min with 5·0 ml horse

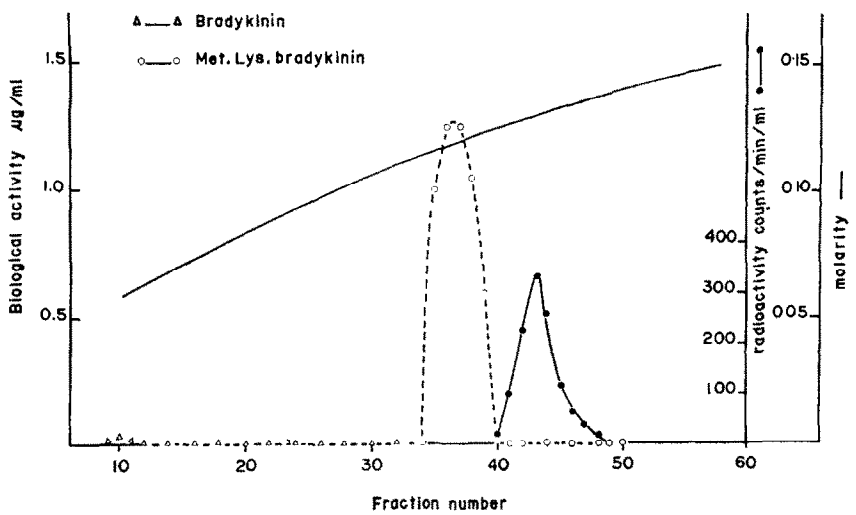


FIG. 2. Chromatography on CM-cellulose of 21 ml of the supernatant resulting from 1-min incubation of Met-Lys-bradykinin (10 $\mu$g) and horse plasma (5·0 ml). Same conditions as in Fig. 1. Volume of fractions: 1·2 ml.

plasma in the presence of 1,10-phenanthroline, no kallidin and only small traces of bradykinin could be detected in the eluates. The recovery of Met-Lys-bradykinin in this experiment was 86 per cent. Similar results were obtained when the incubation period was increased to 5 min. Also, when 10 $\mu$g Met-Lys-bradykinin was added to a mixture of 5·0 ml horse plasma and 50 KL units horse urinary kallikrein and incubated for 1·0 min in the presence of 1,10-phenanthroline (Fig. 3), it was not converted to kallidin and could still be detected even in the presence of the relatively large concentration of kallidin generated by the enzyme reaction.

*Formation of kallidin by horse urinary kallikrein.* Previous studies [13,14] had suggested that horse urinary kallikrein might form bradykinin from the kininogens in horse plasma. However, in these experiments the plasma and enzymes were incubated in the presence of 1,10-phenanthroline for 90 min and an elaborate purification procedure

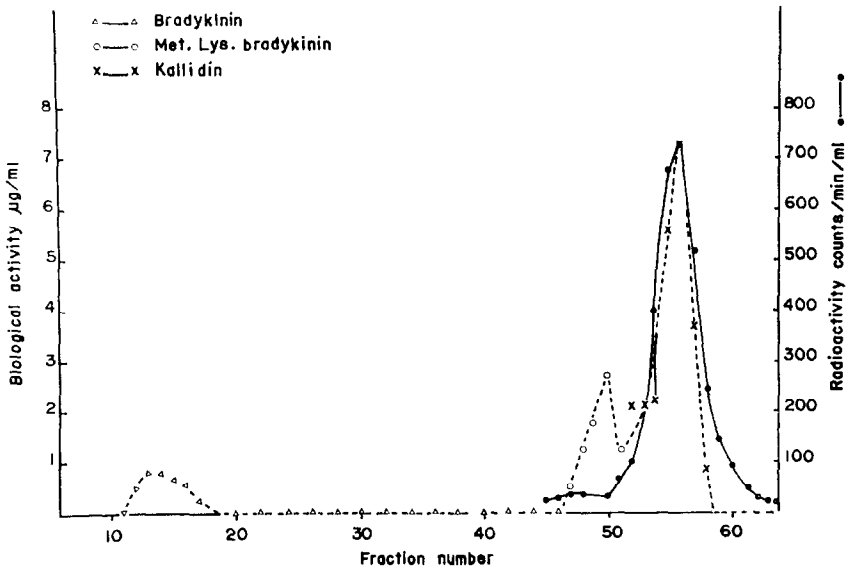


FIG. 3. Chromatography on CM-cellulose of 20 ml of the supernatant resulting from 1-min incubation of Met-Lys-bradykinin (10 $\mu$g), horse plasma (5·0 ml) and horse urinary kallikrein (50 KL units). (3,4-L-proline-$^{14}$C) kallidin (200 ng) was used as a marker. Other conditions similar to Fig. 1. Volume of fractions: 0·8 ml.

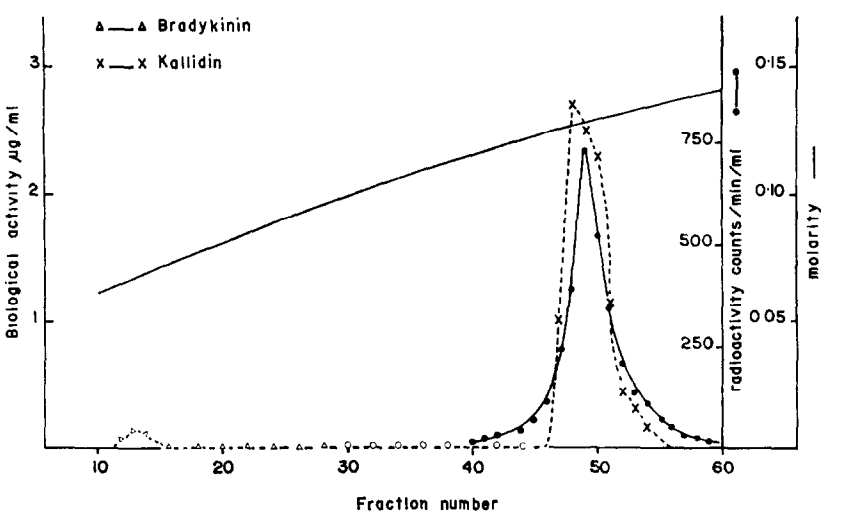


FIG. 4. Chromatography on CM-cellulose of 20 ml of the supernatant resulting from 1-min incubation of horse plasma (5·0 ml) and horse urinary kallikrein (18 KL units). Other conditions similar to Fig. 3. Volume of fractions: 1·0 ml.

employed with consequent losses of activity and, therefore, uncertainty as to the kinins formed in the starting incubation mixture. In the present experiments, when horse urinary kallikrein was incubated with 5·0 ml plasma in the presence of 1,10-phenanthroline for only 1 min, 11 $\mu$g of kinin (calculated as kallidin) was found in the supernatant from this reaction. Chromatography of 20 ml of this supernatant (Fig. 4)

showed that only traces of bradykinin could be found and kallidin was recovered quantitatively. When an incubation time of 5 min was employed, the yield of bradykinin increased to 15 per cent. Control experiments without kallikrein showed that neither bradykinin nor kallidin was formed.

## DISCUSSION

Our data indicate that horse urinary kallikrein liberates only kallidin from the kininogens in horse plasma. This enzyme, therefore, has an unusual specificity since in order to form kallidin it must cleave both a Met-Lys bond and an Arg-X (Arg-Ser?) bond. That the Met-Lys bond is present in horse kininogen has been shown by Henriques *et al.*,[15] who isolated Met-Lys-bradykinin from incubates of plasmin with low molecular weight kininogen(s).

The presence of traces of bradykinin in our incubates was not because of activation of plasma prekallikrein, as bradykinin could not be detected when plasma was incubated in the absence of kallikrein. The bradykinin found after only 1 min of incubation is most likely because of the partial conversion of kallidin into bradykinin by an aminopeptidase in the plasma. In fact, prolonging the incubation time or incubating plasma with kallidin results in the conversion of kallidin into bradykinin, demonstrating that horse plasma, like human plasma,[1] contains an aminopeptidase capable of splitting the Lys-Arg- bond.

The demonstration that kallidin is the kinin liberated by horse urinary kallikrein does not favor the classification of this kallikrein as a bradykinin-forming enzyme, as proposed by Diniz *et al.*,[16] who based their analogy on its inhibition by competitive trypsin inhibitors. Hochstrasser and Werle[6] isolated from peptic digests of bovine plasma fractions IV and IV-4 two peptides with the sequence Gly- or Ser-Arg-Met-Lys-bradykinin. One would be tempted to suppose that these sequences are also present in kininogens from other species and to postulate, from what is known about the specificity of this kallikrein,[17–19] that horse urinary kallikrein would split the Arg-Met bond liberating first Met-Lys-bradykinin; this peptide would then be transformed into kallidin by an enzyme contaminating either the substrate or the enzyme. However, neither horse plasma nor horse urinary kallikrein[19] was capable of converting Met-Lys-bradykinin into kallidin. It is likely, therefore, that this enzyme does indeed split two such dissimilar bonds as Met-Lys and Arg-X. The former bond is susceptible so far only in the undenatured kininogen molecule. Considering the rapidity with which horse urinary kallikrein releases kallidin from kininogen and the dissimilarity of the two peptide bonds split, it seems probable that this enzyme molecule contains two active sites.

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