Biochemical Pharmacology, Vol. 39, No. 3, pp. 549–553, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

RELEASE OF [HYDROXYPROLINE^3]-KININS BY TISSUE KALLIKREINS OF PIG, RAT AND MAN

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(*Received* 29 *March* 1989; *accepted* 3 *September* 1989)

Abstract—To determine the susceptibility of kininogens containing the recently described [Hyp^3]-bradykinin moiety to cleavage by tissue kallikreins, we have studied the release of [Hyp^3]-kinins from heat inactivated human plasma by purified tissue kallikreins. Kallikreins from man and pig were employed and compared with purified rat urinary kallikrein which is known to have a different cleavage specificity. Kinins released were separated by a modified reversed phase HPLC method and quantitated by bioassay and radioimmunoassay. Human urinary kallikrein and hog tissue kallikreins released 85–90% of the total kinins as Lys-bradykinin and 10–15% as [Hyp^3]-Lys-bradykinin. In contrast, rat urinary kallikrein released 77% as bradykinin, 22% as [Hyp^3]-bradykinin and negligible amounts of [Hyp^3]-Lys-bradykinin from the identical substrate source indicating that rat tissue kallikreins prefer the Lys-Arg-bond within both unhydroxylated and hydroxylated kininogens. Therefore, hydroxylation of human kininogens apparently does not affect their ability to serve as substrates for tissue kallikreins with different cleavage specificities.

Kinins are a family of potent vasodilator peptides released by limited proteolysis from their plasma precursor proteins—high molecular weight and low molecular weight kininogens—by enzymes known as kallikreins [1]. Tissue kallikreins (EC 3.4.21.35) which are found in a number of organs such as the kidney, pancreas and salivary glands and in their secretions have been characterized to release primarily Lys-bradykinin from both high and low molecular weight kininogens [2]. However, rat tissue kallikreins from the urine [3, 4], the colon [5], the vasculature [6] and the pars intermedia of the neurointermediate lobe of the pituitary gland [7] *in vitro* predominantly generate bradykinin from kininogen substrates of several species and bradykinin is apparently the major kinin in rat urine [3]. These data indicate species differences in the substrate specificity among tissue kallikreins.

In addition to being the kinin precursors, kininogens also serve as a cofactor in contact activation of the Hageman factor-dependent pathways [8] and as thiol protease inhibitors [9, 10]. Recently, we and others [11–13] have described the presence in human plasma of modified kininogens containing hydroxyproline instead of proline in position three of the bradykinin moiety ([Hyp^3]-Lys-bradykinin). These data have been interpreted to indicate posttranslational hydroxylation of human kininogens, the physiological significance of which is unknown. However, it could be speculated that such a hydroxylation might influence the versatile functions of kininogens [11].

The present study was therefore designed to qualitatively assess the property of hydroxylated human kininogens in serving as substrates for tissue kallikreins. A single human substrate source known to contain hydroxylated kininogens‡ and well characterized tissue kallikreins purified from man and the hog were used. The release of [Hyp^3]-kinins by these tissue kallikreins was studied and compared to that of a rat tissue kallikrein known to exhibit a different cleavage specificity [3–7].

MATERIALS AND METHODS

Bradykinin triacetate (BK§), Des-Arg^1-bradykinin (Serva Feinbiochemica, Heidelberg, F.R.G.); Lys-bradykinin, Met-Lys-bradykinin, Ile-Ser-bradykinin and Des-Arg^9-bradykinin (Sigma Chemical Co., St Louis, MO); Centricon 10-filter (Amicon B.V., Oosterhout, The Netherlands); acetonitrile HPLC grade (J.T. Baker Chemical B.V., Deventer, The Netherlands); trifluoroacetic acid and triethylamine (Pierce Chemical Co., Rockford, IL) were obtained as indicated. Synthetic [Hyp^3]-Lys-bradykinin was obtained from the Max-Planck-Institute for Biochemistry in Martinsried, F.R.G. [11]. [Hyp^3]-bradykinin was prepared from [Hyp^3]-Lys-bradykinin by a one step N-terminal Edmann degradation and the product was characterized after HPLC purification by amino acid composition, amino acid sequence analysis and Fab-mass spectrometry [11]. All other reagents were of the highest quality available.

Purification and characterization of enzymes. Hog

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‡ This study was presented in part at the International Congress Kinin '87 Tokyo in December 1987.

§ Abbreviations: HIP: heat inactivated human plasma; BK: bradykinin triacetate; HPLC: high performance liquid chromatography, RIA: radioimmunoassay.

tissue kallikreins from urine, pancreas and submandibular gland were purified to homogeneity by the method described previously for hog urinary kallikrein [14]. The kallikrein from pig pituitary glands [15] and human urinary kallikrein [1] were purified as previously described. Rat urinary kallikrein was purified from 3–4 L batches of rat urine by the procedure used for the purification of human urinary kallikrein [1].

The purified enzymes were characterized by alkaline disc gel electrophoresis [16], sodium dodecyl sulfate gel electrophoresis [17], and determination of enzyme kinetic parameters [1, 4]. The protein concentration of the purified enzymes was determined by the procedure of Lowry [18]. Kallikreins from the urine, submandibular gland and pituitary gland of the pig, and those from human and rat urine were isolated as homogenous single-chain proteins, while the pig pancreatic kallikrein preparation contained active kallikrein A and B, some of which in their two-chain beta-conformation [2]. Upon trypsin treatment of the purified enzymes [4] their specific activities did not increase indicating that they were obtained in their active enzymatic form. The pig tissue kallikreins showed a line of complete identity against a rabbit anti-hog urinary kallikrein IgG [14] when analysed in an Ouchterlony double immunodiffusion experiment [19] and their activities were inhibited in a dose-dependent fashion by the IgG.

Assay method. Kinins generated by tissue kallikreins upon incubation with heat-inactivated human plasma (HIP) as substrate source and the biological activity of kinins eluted from the HPLC-column were quantitated by the rat uterus bioassay [20] using synthetic bradykinin as a standard and expressed as bradykinin equivalents. Heat inactivated human plasma which contains no residual kallikrein, prekallikrein, kallikrein inhibitors or kininase activity was prepared from a single donor as described [20] and a single batch was used throughout this study to guarantee an identical substrate source for the different enzymes. An excess of purified hog urinary kallikrein had the capacity to liberate 675 μg bradykinin equivalents from 1000 μL HIP after incubation for 5 min at 37° as assayed on the rat uterus bioassay. Kinins were also quantitated by radioimmunoassay [21] employing a commercially available antibody (anti-bradykin IgG-fraction; Protogen, Switzerland) and (8-tyrosin)-[^{125}I]-bradykinin triacetate (New England Nuclear, Boston, MA) as tracer. The sensitivity limit of the radioimmunoassay was 5 pg of standard bradykinin.

Incubation conditions. The purified enzymes (20 μL, approximately 20–60 ng) were incubated with HIP (380 μL) for 30 min at 8.5 [1] and room temperature, diluted to 2 mL with 100% of solution B and centrifuged. After lyophilization of the supernatant, the samples were dissolved in 200 μL of solution A and filtered through Centricon (cut off 12,500). After washing the filter with 100 μL of solution A, 190–250 μL of the combined effluent were subjected to high pressure liquid chromatography (HPLC).

HPLC analysis of kinins generated. Separation of the kinins generated from human HIP by the purified enzymes was achieved by reversed phase HPLC using a micro-Bondopak C_{18}-HPLC column (Waters Associates, Milford, MA) as described by Proud [22] with slight modifications [11]. Solution A consisted of 0.05% trifluoroacetic acid and 0.025% triethylamine in distilled water, solution B of 80% acetonitrile in solution A. The column was run at a flow rate of 1 mL/min and the effluent was collected in fractions of 0.5 mL. Kinins were separated isocratically at 84% of solution A and 16% of solution B and identified by comparing their respective retention time, biological activity and immunoreactivity with those of the kinin standards. Between 44 and 60% of their individual biological activity and immunoreactivity, respectively, was recovered and the losses were not markedly different from one kinin to another. Kinins were not detected by incubation of HIP or the enzymes alone and neither Lys-bradykinin nor Met-Lys-bradykinin standards were converted to bradykinin upon incubation with HIP or the enzymes. Furthermore, no evidence for degradation of the bradykinin standard upon incubation with HIP or with the purified enzymes was seen. Therefore, determination of biological and immunological activity of *in vitro* generated kinins and their respective retention times upon HPLC compared to the retention times and respective activities of the standard kinins allows identification of kinins released from HIP by the enzymes used. The distribution of biologically active or immunoreactive kinins released by a particular enzyme was calculated by comparing the activity of a particular kinin eluted to the total amount of kinin activity eluted from the column. Data are expressed as mean ± SE.

Under the conditions described, however, [Hyp^3]-bradykinin and Lys-bradykinin had the same retention time and were not separated. To achieve separation an additional HPLC procedure was therefore developed using a Delta-Pak C-18 column (3.9 mm × 15 cm, spherical 5 μ; Nihon Waters Ltd, Japan) and identical conditions. Using this procedure, [Hyp^3]-bradykinin and Lys-bradykinin could be separated by 1.5 min. Biologically active and immunoreactive kinins eluting from the μ-Bondopak column at the position of the Lys-bradykinin standard were therefore pooled, lyophilized, rechromatographed on the Delta-Pak column and analysed accordingly.

RESULTS

A typical elution profile upon HPLC separation of the kinin standards incubated with heat inactivated human plasma and prepared as described is shown in Fig. 1. In the amounts used, [Ala^3]-Lys-bradykinin, des-Arg^1-bradykinin and des-Arg^9-bradykinin have no biological activity on the rat uterus. In the radioimmunoassay, des-Arg^1-bradykinin shows 1/400 and des-Arg^9-bradykinin 1/80 the activity of bradykinin (retention times 9.9 and 53.3 min, respectively). Lys-bradykinin and bradykinin and their respective hydroxylated analogues exhibited a similar biological activity in the bioassay and produced identical displacement curves to bradykinin in the radioimmunoassay.

Upon incubation of purified human urinary kallikrein with HIP, 45–62% of biologically active and

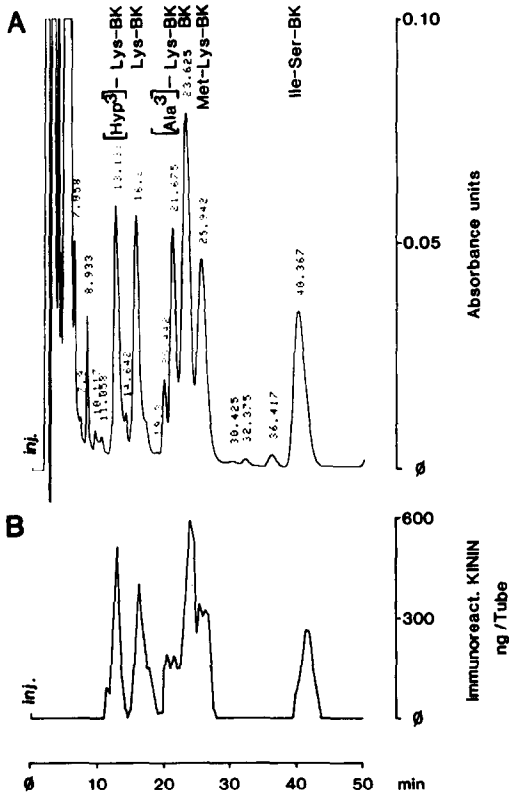

Fig. 1. Separation of standard kinins by reversed phase HPLC after incubation with HIP. Panel A shows the absorbance at 214 nm and the respective retention times, panel B the kinin immunoreactivity.

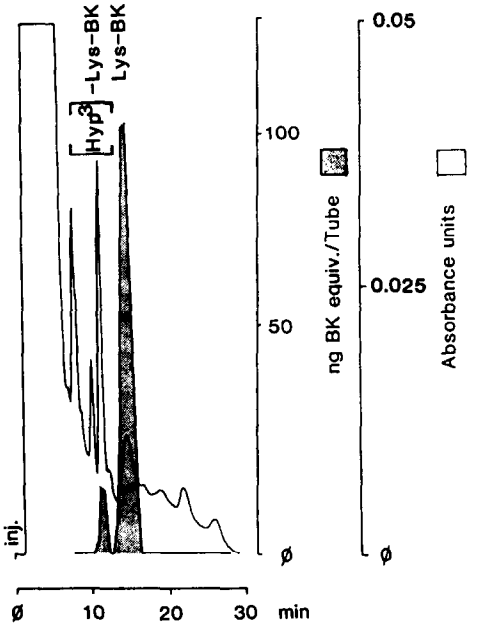

Fig. 2. Kinins generated upon incubation of HIP (380 μL) with human urinary kallikrein (20 ng) and separated by reversed phase HPLC. Kinins were determined by RIA and by the rat uterus bioassay (ng BK equivalents per tube) and identified by comparison with standard kinins.

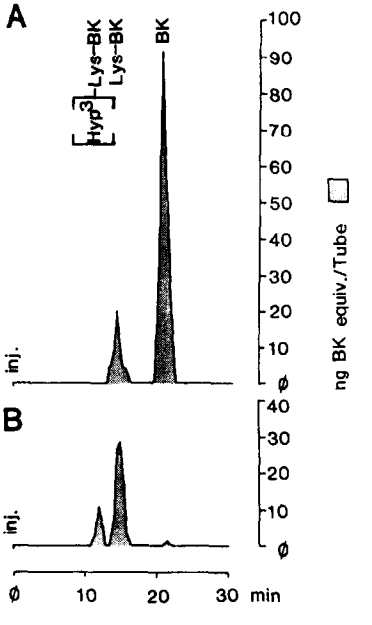

Fig. 3. Kinins generated upon incubation of HIP (380 μL) with rat urinary kallikrein (40 ng, panel A) and pig pituitary kallikrein (60 ng, panel B), separated by reversed phase HPLC. Biological activity of kinins is expressed as ng of bradykinin equivalents per tube.

immunoreactive kinins generated could be recovered in the HPLC-eluate. The majority of the biological and immunological activity (83.2–89.9%) eluted at a retention time corresponding to the Lysyl-bradykinin standard (Fig. 2). A small amount of biological and immunological activity (10.0–15.4%) eluted earlier the retention time of which corresponded to that of the [Hyp³]-Lys-bradykinin standard. When the kinin corresponding to the Lys-bradykinin standard was rechromatographed on the Delta-Pak column, the activities again eluted at the position of Lys-bradykinin (not shown). A similar distribution of kinins was found when the kallikreins from the pig pituitary (Fig. 3B), pig urine, submandibular gland and pancreas were incubated with human HIP and the reaction products separated by HPLC. The distribution of kinins obtained with purified pig urinary kallikrein was [Hyp³]-Lys-bradykinin (14.4%) and Lys-bradykinin (85.6%); with purified pig pancreatic kallikrein: [Hyp³]-Lys-bradykinin 4.3%, Lys-bradykinin 87.8% and bradykinin 7.9%; and with purified pig submandibular kallikrein, 10.9% [Hyp³]-Lys-bradykinin and 89.1% Lys-bradykinin were found. No other immunoreactive or biologically active kinin or inactive kinin could be identified.

In contrast, a significantly different distribution of kinins was found upon incubation of purified rat urinary kallikrein with the same human HIP (Fig. 3A). The majority of kinins released eluted at the position of bradykinin (69.1–87.1%), while 12.8–30.9% eluted at a position corresponding to the Lys-bradykinin standard. In one of four experiments some activity corresponded to [Hyp³]-Lys-bradykinin (0.3%). The kinin eluting at the position of Lys-bradykinin was also subjected to rechroma-

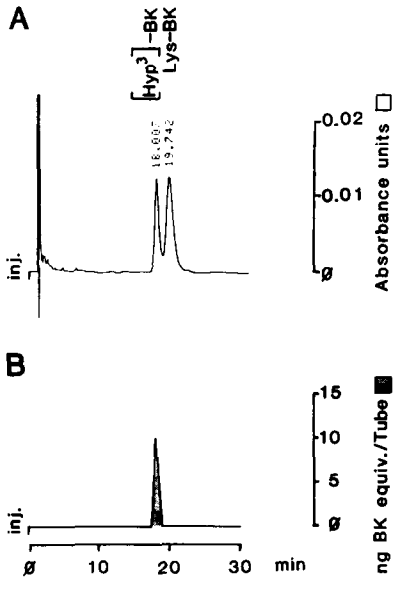

Fig. 4. Separation of [Hyp³]-bradykinin and Lys-bradykinin standards upon reversed phase phase HPLC using a Delta-Pak column (panel A). The kinin peak obtained with rat urinary kallikrein eluting at the position of Lys-bradykinin from the μ-Bondopak column (in Fig. 3, panel A) was rechromatographed and identified as [Hyp³]-bradykinin (panel B).

tography on the Delta-Pak column. In contrast to the tissue kallikreins from hog and man, all of the particular activity generated by rat urinary kallikrein consistently eluted at the position of [Hyp³]-bradykinin (Fig. 4). Based on these results Table 1 shows the distribution of kinins generated by tissue kallikreins of the three species studied.

DISCUSSION

Tissue kallikreins purified from the pig and man release Lys-bradykinin and [Hyp³]-Lys-bradykinin from a single, identical substrate source, e.g. heat inactivated human plasma obtained from a single donor (Table 1). This confirms and extends earlier data reported for tissue kallikreins from several species [2, 3, 12]. The release of [Hyp³]-Lys-bradykinin was also reported from human plasma Cohn's fraction IV-4 by pig pancreatic kallikrein [12], from human plasma by human urinary kallikrein [13] and from human high and low molecular weight kininogens by purified human urinary kallikrein [11]. Together, these results indicate that the cleavage specificity of tissue kallikreins for the Met-Lys bond in kininogens [2] is not affected by hydroxylation of proline in position 3 of the bradykinin moiety.

In contrast to the tissue kallikreins of pig and man, purified rat urinary kallikrein releases bradykinin and [Hyp³]-bradykinin from the same substrate source (Fig. 4; Table 1) indicating its preference for cleavage at the Lys-Arg-bond within both unhydroxylated and hydroxylated human kininogens. Bradykinin is also the main product generated from bovine, rat and dog substrates by tissue kallikreins of the rat [3, 4, 23]. [Hyp³]-bradykinin, on the other hand, could be released from human plasma by trypsin [23] and is present in human tumor ascites [24]. In contrast to a previous report [23] almost no Lys-bradykinin or [Hyp³]-Lys-bradykinin was generated by rat urinary kallikrein in our study. This difference might be explained by the use of a μ-Bondopak HPLC column [23], which does not separate [Hyp³]-bradykinin from other kinin standards as demonstrated in this study (Fig. 3A). However, using the same buffer system and isocratic conditions, [Hyp³]-bradykinin and Lys-bradykinin can be clearly separated on a Delta-Pak-C-18 column (Fig. 4).

Our results might have been influenced by the use of heat inactivated plasma as a source of kininogens. However, in our control experiments neither of the kinin standards was converted or degraded into smaller fragments upon incubation with heat inactivated plasma indicating that kininases were destroyed during heating. Furthermore, the tissue kallikreins from man and hog release the same type of kinins (Lys-bradykinin and Hyp³-Lys-bradykinin)

Table 1. Distribution of biologically active kinins generated from human heat inactivated plasma

	Kallikreins		
Kinins	Human urine	Rat urine	Pig pituitary
Total kinins (ng)	347.2 ± 12.1	358.6 ± 16.2	194.5 ± 5.5
[Hyp³]-Lys-BK (%)	13.1 ± 1.6	0.1 ± 0.1	11.1 ± 2.4
Hyp³-BK (%)	—	22.4 ± 3.0	—
Lys-BK (%)	86.3 ± 1.8	—	88.5 ± 2.5
BK (%)	—	77.4 ± 3.0	0.3 ± 0.7
Met-Lys-BK (%)	—	—	—
Ile-Ser-BK (%)	—	—	—

Kinins were separated by reversed phase HPLC after incubation of the respective purified enzymes with a single batch of heat inactivated human plasma. The results are given as the mean ± SE of at least three experiments employing at least three different preparations of the respective enzymes. Kinins were quantitated by the rat uterus bioassay and identified by comparing their respective retention time with those of kinin standards. The total amount of kinins generated was determined in the incubation mixture and is given in bradykinin equivalents.

from heat inactivated plasma as those released from inhibitor treated native plasma by human urinary kallikrein [13], from Cohn's fraction IV-4 by hog pancreatic kallikrein [12] and from purified low and high molecular weight human kininogens by human urinary kallikrein [11]. This indicates that different human substrate preparations including heat inactivated plasma contain both hydroxylated and non-hydroxylated kininogens the susceptibility of which to cleavage by tissue kallikreins is not altered by the way the substrates were prepared. Whether Hyp^3-bradykinin and bradykinin or Hyp^3-Lys-bradykinin and Lys-bradykinin are being released, however, depends on the cleavage specificity of the particular tissue kallikrein. In addition, the percentage of [Hyp^3]-kinins released may depend on the amount of specifically hydroxylated kininogens available.

Using heat inactivated plasma as the single substrate source containing both hydroxylated and unhydroxylated kininogens and using tissue kallikreins with different cleavage specificities we conclude that hydroxylation of human kininogens appears not to affect their susceptibility to specific cleavage by a particular tissue kallikrein. However, analysis of the rate at which [Hyp^3]-kinins are released from the respective kininogen precursors must await selective purification of [Hyp^3]-bradykinin containing kininogens.

Acknowledgements—This work was supported in part by the Austrian Fund for the Promotion of Scientific Research, Project No. P6357. The authors thankfully acknowledge the patience and excellent secretarial assistance of Mrs H. Hitschmann.

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