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Action of horse urinary kallikrein on synthetic derivatives of bradykinin*

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PREVIOUS experiments indicated that horse urinary kallikrein (UK) hydrolyzes salmine[1, 2] and polyarginine,[3] but not polylysine.[3] This paper reports the action of UK on bradykinyl-serine, methionyllysyl-bradykinin and lysyllysyl-bradykinin (Fig. 1).

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-OH
(Bradykinyl-Ser)

H-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
(Met-Lys-bradykinin)

H-Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
(Lys-Lys-bradykinin)

FIG. 1. Structure of the peptide substrates, bradykinyl-serine, methionyllysyl-bradykinin and lysyllysyl-bradykinin.

MATERIALS AND METHODS

Enzymes. Partially purified horse urinary kallikrein (UK-160) with sp. act. of 160 units/mg was prepared as previously described.[4, 5] Trypsin (Worthington Biochemical Corp.) was a twice-crystallized, salt-free preparation.

Substrates and Standards. Synthetic Met-Lys-bradykinin and Lys-Lys-bradykinin were most kindly supplied by Dr. E. Schröder, Schering A. G., Berlin, Germany; synthetic bradykinyl-Ser, by Prof. E. Habermann, Giessen, Germany; synthetic bradykinin and kallidin, by Dr. E. D. Nicolaides, Parke Davis & Co., Ann Arbor, Mich. U.S.A.

Column chromatography. Carboxymethyl (CM)—cellulose microcolumns, prepared as described by Habermann and Blennemann,[6] were used to separate hydrolysis products of Met-Lys-bradykinin and Lys-Lys-bradykinin. A mixing flask containing 50 ml of eluent was used to obtain a gradient of ammonium formate buffer, pH 4·8, from 0·01 M to 0·3 M.

Molarity. The molarity of the buffer in the fractions collected was established by measuring the conductivity of 100-fold diluted samples.

Biological activity. Aliquots (about 0·2 ml) from the eluates were assayed on the isolated guinea pig ileum preparation by using synthetic peptides as standards.

Thin layer chromatography (TLC). Dansyl (DNS) derivatives[7, 8] of standard amino acids and peptides and of the products of hydrolysis of bradykinyl-Ser were separated by TLC. Chromatograms on plates coated with silica gel H (25 × 25 × 0·025 cm) were developed for about 1 hr with 2-propanol:methyl acetate:concentrated ammonium hydroxide (90:70:40, v/v) and examined under u.v. light. To obtain the dansylated derivatives, 20 μl of an acetone solution of 1-dimethyl-aminonaphthalene-5-sulfonyl chloride ($1{\cdot}9 \times 10^{-2}$ μmole/μl), 5 μl of the peptide solution ($2{\cdot}4 \times 10^{-3}$ μmole/μl) and 5 μl of 0·1 M triethylamine-CO_2 buffer, pH 8, were incubated for 3 hr at room temperature.

RESULTS

Hydrolysis of Bradykinyl-Serine by UK. Synthetic bradykinyl-Ser ($8{\cdot}7 \times 10^{-2}$ μmole) in 0·1 M triethylamine-CO_2 buffer, pH 8, was incubated at 37° for 2 hr with 50 μg UK-160. *o*-Phenanthroline

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in a final concentration of 3×10^{-3} M was added to protect bradykinyl-Ser from any action by the kininase[9] present in the UK-160 preparation. A control mixture containing benzamidine, which was shown to be an inhibitor of the proteolytic activities of UK,[2, 3] was also run. The following further control mixtures were also incubated (a) substrate and enzyme controls (b) substrate and trypsin, which is known[10] to split the Arg-Ser bond in bradykinyl-Ser. The final volume of each incubation mixture was 0·35 ml. The dansyl derivatives obtained with 5 μl ($1{\cdot}2 \times 10^{-2}$ μmole) from every incubation mixture as well as from synthetic bradykinin and from serine were prepared. A thin-layer plate was spotted with 3-μl aliquots and developed. Fig. 2 is a diagram of the pinkish fluorescent spots in u.v. light. It may be seen that (1) the dansyl derivatives of bradykinin, bradykinyl-Ser and Ser migrated at different speeds and separated well from each other (2) trypsin (Try) completely split Ser from bradykinyl-Ser, the bradykinyl-Ser spot having disappeared (trypsin alone, in the amounts used, gave no spot) (3) UK, as shown, remained at the origin and, like trypsin, digested bradykinyl-Ser completely into Ser and bradykinin (however, when benzamidine was present, no splitting occurred). Secondary products, DNS-NH_2 and DNS-OH, resulting respectively from reactions between DNS-Cl and either NH_2 group or water are also shown on the top rows, and had respectively yellowish and green fluorescences, different from the pinkish main spots.

The action of kallikrein on Met-Lys- and Lys-Lys-bradykinin. In order to study the action of kallikrein on these substrates the following preliminary observations were made (1) A mixture of 5 μg bradykinin, 5 μg kallidin and 50 μg Lys-Lys-bradykinin was separated into its components by using a microcolumn ($0{\cdot}4 \times 9{\cdot}0$ cm) of CM-cellulose and gradient elution with ammonium formate buffer. The molarities of the eluates corresponding to the peaks of biological activity were constant on different columns run under exactly the same conditions and corresponded in these experiments to 0·12 (bradykinin), 0·16 (kallidin) and 0·20 (Lys-Lys-bradykinin). (2) By using a mixture of 5 μg bradykinin and 50 μg Met-Lys-bradykinin, a distinct separation by the same procedure was also obtained. The eluate molarity corresponding to Met-Lys-bradykinin was 0·15. (3) From the mixture of kallidin plus Met-Lys-bradykinin, however, a partial overlapping of the two peaks occurred. The increase of guinea pig ileum activity resulting from trypsin digestion was employed as an identifying test for Met-Lys-bradykinin.

The incubation conditions for Met-Lys-bradykinin and Lys-Lys-bradykinin with UK-160 were the same: 2·5 mg UK-160 in 0·2 ml of 0·05 M ammonium carbonate buffer, pH 8, was preincubated for 30 min at 37° with 0·2 ml of $2{\cdot}5 \times 10^{-2}$ M *o*-phenanthroline. Twenty μl of a water solution of the substrate (5 mg/ml) was used. The final volume was made up to 0·5 ml with the buffer. The reaction was interrupted after 2 hr of incubation by acidification (pH 3) with 0·25 ml of 0·5 N formic acid and boiling for 5 min. The pH was adjusted to 4·8, the solution was cooled to 4° and chromatographed on a CM-cellulose microcolumn in a cold room at 4°.

Two control experiments were run for each substrate: in one the incubation was interrupted at zero time, and in the other benzamidine was added to the incubation mixture in a final concentration of 1×10^{-1} M.

When Met-Lys-bradykinin was the substrate, two biologically active peaks were obtained in the chromatography. The first peak, with an eluate molarity of 0·08, whose activity remained constant when incubated with trypsin, corresponded to bradykinin and not to kallidin, which is eluted at higher molarity, partially overlapping the position of Met-Lys-bradykinin. The second peak, which contained most of the activity, eluted at a molarity of 0·12, showed an increase in activity after trypsin digestion and corresponded to undigested Met-Lys-bradykinin.[11] In these experiments, an accelerated elution of the peptides relative to the standard columns, similar to that described by Habermann,[10] was observed; it may depend on the different composition of the solutions being added to the microcolumns.

Chromatography of the control mixtures gave the following results (1) the zero time incubation mixture gave a single peak of activity at an eluent molarity of 0·12; the active fractions when incubated with trypsin had their activity increased, meaning that they corresponded to undigested Met-Lys-bradykinin (2) the presence of benzamidine (a kallikrein inhibitor) in the incubation mixture reduced to traces the amount of active material in the elution zone corresponding to bradykinin, proving that the presence of bradykinin in the kallikrein digest was really due to the hydrolysis of Met-Lys-bradykinin by that enzyme.

Similar results were obtained when Lys-Lys-bradykinin was used as substrate. The even smaller hydrolysis of this peptide under the effect of UK also disappeared entirely in the presence of benzami-

dine. When Lys-Lys-bradykinin was incubated with trypsin, there was an increase of activity, probably due to the formation of bradykinin, since it is known that trypsin does not cleave the peptide bond next to the *N*-terminal amino acid.

DISCUSSION

The Arg-Ser bond in bradykinyl-Ser is hydrolyzed by horse urinary kallikrein with formation of bradykinin and free serine. This effect is blocked by benzamidine, which is known[2, 3] to inhibit the proteolytic effect of this kallikrein. According to Habermann,[10] this proteolytic specificity is common to pig serum kallikrein, trypsin, *C. adamanteus* venom and carboxypeptidase A, but not to pig pancreatic kallikrein.

Results obtained with Met-Lys-bradykinin and Lys-Lys-bradykinin suggest that the bond Lys-Arg in these peptides is slowly split by horse urinary kallikrein as compared to the Arg-Ser bond hydrolysis in bradykinyl-Ser by UK. This may be said since in the 2-hr incubation period a 3·5-fold higher enzyme concentration produced only incomplete splitting of the Lys-Arg bond. This relative resistance to UK action is higher in Lys-Lys-bradykinin than in Met-Lys-bradykinin, suggesting that the second Lys residue hindered the enzyme action further. The Lys-Lys bond in Lys-Lys-bradykinin seems resistant to UK because no kallidin peak appeared in the eluates; this is in agreement with results on polylysine.[3]

From the comparison of previous results on other arginine and lysine peptides, including polyarginine and polylysine,[3] with current findings, it may be said that horse urinary kallikrein is an endopeptidase, which splits bonds involving the carboxyl end of arginine preferentially to those involving the carboxyl side of lysine. In the polypeptides studied, the bonds Arg-Arg, Arg-Ser and Lys-Arg are susceptible in different degrees to the enzyme, while the Lys-Lys bond is resistant to it.

Dipeptides of Arg and Lys are not split[3] even when the amino group is blocked by acetylation.[12] The hydrolysis of the bond Arg-Lys in polypeptides has not yet been studied with horse urinary kallikrein.

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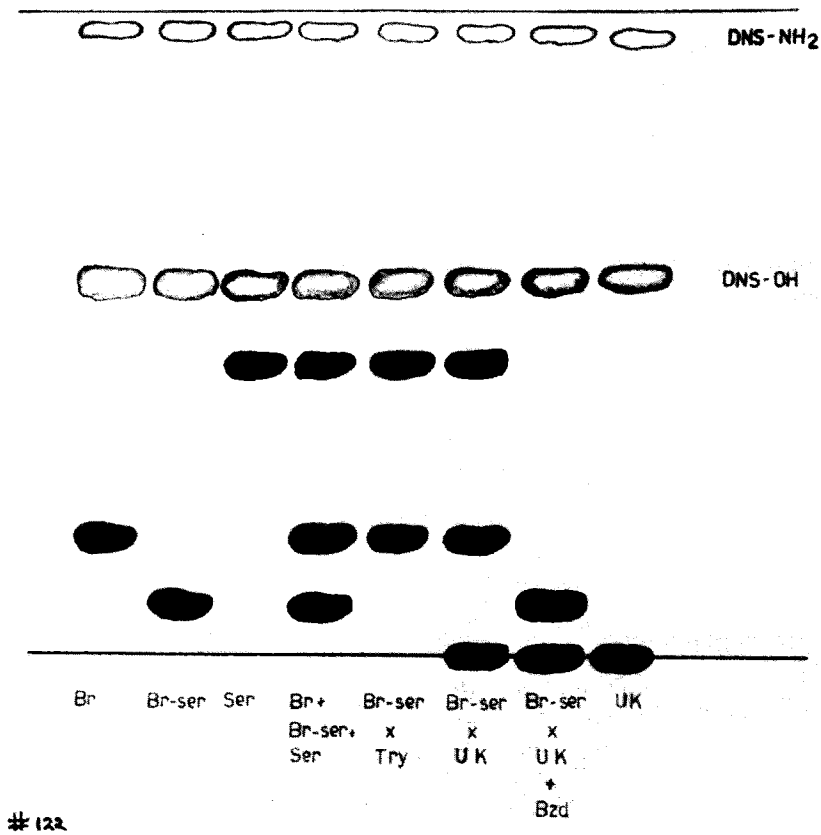

FIG. 2. Diagram of thin-layer chromatogram examined under u.v. light showing separation of the DNS derivatives of bradykinin (Br), bradykinyl-Ser (Br-ser) and Ser from each other and the effect of either trypsin (Try) or horse urinary kallikrein (UK) on bradykinyl-Ser.

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