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DEGRADATION OF PLASMA KININS BY AN ENZYME FROM *PSEUDOMONAS AERUGINOSA*

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Kinin-inactivating enzymes, kininases, are widely distributed in the mammalian organism. Kininase activity has also been demonstrated in other types of biological material—for example, microorganisms (for review see Erdøs, 1966; Rugstad, 1966). Most of these enzymes have not been available in a pure form and studies of their actions have been carried out with rather crude preparations. Studies of the breakdown of bradykinin, however, have also been performed with two purified, well-characterized enzymes, carboxypeptidase B and chymotrypsin, and it has been established that carboxypeptidase B hydrolyses the Phe^{8}-Arg^{9} bond (Erdøs, 1962), whereas chymotrypsin hydrolyses the Phe^{8}-Arg^{9} bond as well as the Phe^{5}-Ser^{6} bond (Elliott, Lewis & Horton, 1960). The degradation of bradykinin by several partly purified enzymes has also been investigated and the results have recently been reviewed by Erdøs (1966).

Previous publications (Rugstad, 1967a, b) describe the purification and properties of a kininase which is produced by *Pseudomonas aeruginosa*. In this work the activity of the enzyme was studied by examining its inactivation of synthetic bradykinin. The present investigation describes the action of this microbial kininase on kinins other than bradykinin and also an analysis of the breakdown products produced by its action on bradykinin.

METHODS

Kininase activity

This was measured as described in a previous paper (Rugstad, 1966) by the ability to destroy synthetic bradykinin as measured by bioassay on the rat uterus. As previously defined, 1 u. kininase denotes the amount of enzyme that will destroy 75% or more of 500 ng synthetic bradykinin in 11 min (but not in 6 min).

Enzyme preparation

The kininase from *Pseudomonas aeruginosa* cultures was purified as described by Rugstad (1967a). Unless otherwise stated, a stock solution containing 2,000 u./ml. was used. The enzyme was dissolved in 0.02 M Na-phosphate buffer of pH 7.5.

Synthetic kinins

The following kinins were used in the biological tests: Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9} (=bradykinin) (BRS 640, Sandoz), Lysyl-bradykinin (=kallidin) (KL 698, Sandoz), Bradykinyl-

isoleucyl-tyrosine (Farmitalia, Milano), Bradykinyl-isoleucyl-tyrosine-O-sulphate (=phyllokinin) (Farmitalia, Milano).

All these kinins will cause a contraction of the isolated rat uterus preparation (Anastasi, Bertaccini & Erspamer, 1966) and their inactivation can therefore be determined by bioassay.

Bradykinin triacetate (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was used in the degradation studies.

Thin-layer chromatography

This was carried out with Shandon thin-layer equipment (Shandon Scientific Company Limited, London, England). Glass-plates, 20×20 cm were layered with a 0.25 mm thick layer of Kiesel-gel G, obtained from E. Merck A.G., Darmstadt, West Germany. The amino acids and peptides were detected by spraying with ninhydrin and by the chlorine-tolidine test.

Solvents for thin-layer chromatography

Four different solvents were used for the chromatography of amino acids and peptides. Solvent 1: 96% Ethanol-water (70–30) (v/v), Solvent 2: N-propanol-34% ammonia solution (70–30) (v/v), Solvent 3: Methylethylketone-pyridine-water-glacial acetic acid (70–15–15–2) (v/v), Solvent 4: Phenol-water (75–25 (w/v), Solvent 5; Benzene-pyridine-glacial acetic acid (80–20–2) (v/v). A two-dimensional technique using solvent 1 in a first run and solvent 4 in a second run after rotating the plate through 90°, gave a good separation of the five different amino acids present in bradykinin. Solvent 2 was used for chromatography of acid- and water-soluble 2:4 dinitrophenyl-amino acids (DNP-amino acids), not extractable by ether. Solvent 5 was used for chromatography of acid-insoluble DNP-amino acids extractable by ether.

Reaction with 2:4 dinitrofluorobenzene (DNFB) was carried out as described by Brenner, Niederwieser & Pataki (1965), with the sole modification that triethanolamine, 1.5% in water (v/v), was used instead of trimethyl ammonium carbonate.

Reference amino acids

These were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. DNP-amino acids were obtained from Sigma (Sigma Chemical Company, St. Louis, Missouri, U.S.A.).

Peptides

The dipeptide, phenylalanylserine was obtained from Cyclo Chemical Corporation, Los Angeles, U.S.A. and hippuryl-L-lysine from Mann Research Laboratories, New York, U.S.A. Hippuryl-L-arginine was a present from Bayer, Leverkusen, W. Germany.

Electrophoresis

Electrophoresis on Whatman No. 1 paper and in 2 M acetic acid was carried out in a Shandon electrophoresis apparatus using a voltage gradient of 12.5 V/cm.

RESULTS

Inactivating of kallidin, bradykinyl-isoleucyl-tyrosine and phyllokinin

Five hundred ng of the kinin to be tested dissolved in 0.1 ml. saline were incubated at 37° C with 0.9 ml. buffered enzyme solution. In another tube 500 ng synthetic bradykinin in 0.1 ml. saline were incubated with the same amount of enzyme. Aliquots of 0.1 ml. of the two mixtures were simultaneously taken out at 5 min intervals for testing on each of two rat uterus preparations. The first tests were made after 1 min incubation. The enzyme solution contained 1 u. kininase/ml. and this apparently inactivated kallidin, bradykinyl-isoleucyl-tyrosine and phyllokinin at about the same rate as it inactivated the synthetic bradykinin (Fig. 1).

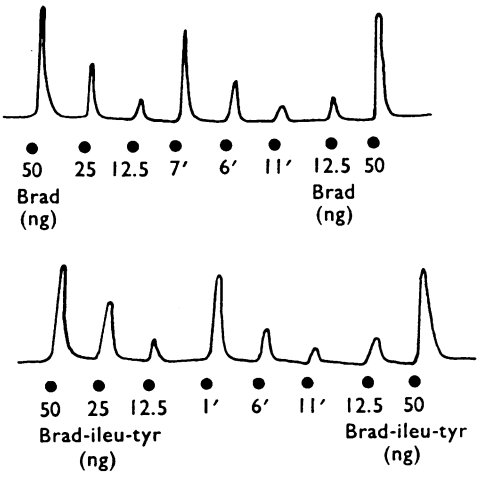

Fig. 1. Inactivation of synthetic bradykinin (upper tracing) and bradykinyl-isoleucyl-tyrosine (lower tracing) by a purified kininase produced by *Pseudomonas aeruginosa*. Kinin activity is assayed on the rat uterus preparation. Five hundred ng synthetic bradykinin (Brad) dissolved in 0.1 ml. saline were incubated at 37° C with 0.9 ml. buffered enzyme solution. In another tube 500 ng bradykinyl-isoleucyl-tyrosine (Brad-ileu-tyr) were incubated similarly with the same amount of enzyme. Aliquots of 0.1 ml. of the two mixtures were taken out at 5 min intervals for testing on each of two rat uterus preparations (see text).

Degradation of synthetic bradykinin by a kininase from Pseudomonas aeruginosa

Bradykinin triacetate, 0.5 mg, was dissolved in 0.2 ml. 0.02 M Na-phosphate buffer of pH 7.5, and the solution was incubated for 12 hr at 37° C with 0.04 ml. of a solution containing 2,000 u. kininase from *Pseudomonas aeruginosa* in the same phosphate buffer. After 18 hr of incubation samples of the mixture had no action upon the isolated rat uterus preparation. On thin-layer chromatography, three ninhydrin-positive spots were obtained, with all the solvent systems 1–4, either used separately, or with the two-dimensional technique in which chromatography with solvent 1 was followed by chromatography with solvent 2, 3 or 4. The results of chromatography of the reaction mixture in solvent 1 are shown in Fig. 2. The spots are called I, II and III. Spot I has the same R_f value as has arginine.

Incubation of synthetic bradykinin with the enzyme for other periods, varying from 30 min to 72 hr gave the same results. Lengthening the incubation did not produce any additional spots.

In one experiment 0.5 mg bradykinin triacetate was incubated with the enzyme as described. After 18 hr the digest was submitted to " micro-preparative " thin-layer chromatography with solvent system 1 by placing it on the plate as a streak. Only a small part of the plate was sprayed with ninhydrin and the bands corresponding to spots II and III were eluted from the part of the plate not sprayed with ninhydrin. Each eluate was then submitted to hydrolysis in a sealed tube with 6 N HCl at 110° C for 12 hr.

The hydrolysates were submitted to chromatography in solvent 1 and also to two-dimensional chromatography (solvent 1 followed by solvent 4). The hydrolysis of band III was found to yield three amino acids: serine, proline and phenylalanine. Hydrolysis

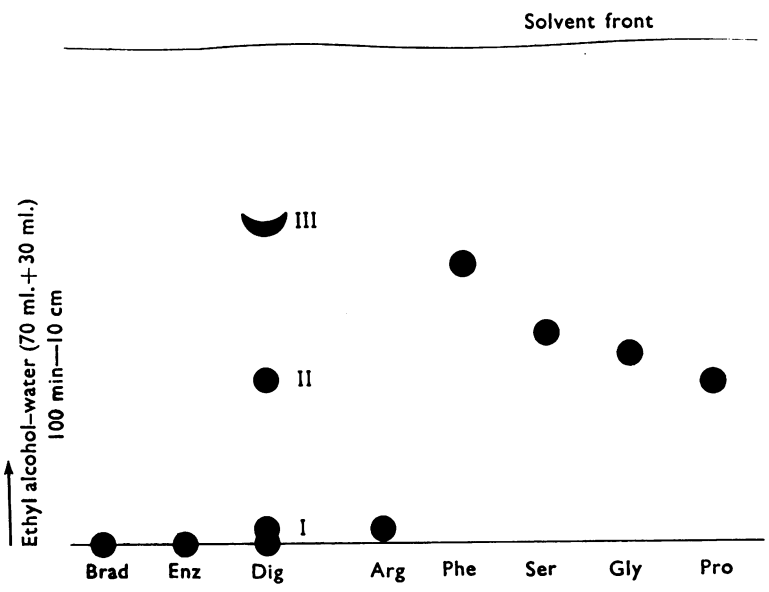

Fig. 2. Thin-layer chromatography of a mixture of bradykinin and the purified kininase produced by *Pseudomonas aeruginosa*; the enzyme, bradykinin and the five amino acids present in bradykinin were used as reference substances. Brad=synthetic bradykinin; Enz=purified enzyme; Dig=synthetic bradykinin and enzyme incubated at 37° C for 18 hr; Arg=arginine; Phe=phenylalanine; Ser=serine; Gly=glycine and Pro=proline. For details see Methods.

of band II was found to give four amino acids: arginine, proline, glycine and phenylalanine.

Dinitrophenol (DNP) derivatives were made of the products in the incubation mixture of bradykinin and enzyme. Free DNP-arginine was found after chromatography of the acid and water-soluble portion, but the ether-extractable material yielded no free DNP-amino acids.

In one set of experiments "micro-preparative" thin-layer chromatography in solvent 1 of the digest of enzyme and bradykinin was done as described above and 2:4 dinitrophenol derivatives were made of band II and band III separately after elution of these bands from the plate. After hydrolysis for 12 hr in 6N HCl, band II yielded DNP-arginine. No satisfactory results were obtained by similar treatment of band III.

Electrophoresis

Bradykinin triacetate, 0.5 mg, and enzyme were incubated for 18 hr at 37° C as described above. The reaction mixture was then submitted to electrophoresis on paper for 45 min (see Methods). Three ninhydrin-positive bands were seen. They had moved 3.3, 4.7 and 6.3 cm towards the cathode. The two bands which had moved the shortest distance were strongly coloured after the chlorine-tolidine test. The third band had moved the same distance as arginine, which was run simultaneously on a separate strip of paper.

Incubation of the enzyme with a dipeptide and some similar substances

Phenylalanyl-L-serine, hippuryl-L-arginine and hippuryl-L-lysine were also incubated with enzyme: 0.1 ml. of a solution of 0.02 M Na-phosphate buffer of pH 7.5 containing 2 mg of the peptide/ml. was incubated with 0.025 ml. enzyme (stock solution) at 37° C for 24 hr. None of the substances was hydrolysed as judged by thin-layer chromatography.

DISCUSSION

The results presented show that the kininase from *Pseudomonas aeruginosa* splits synthetic bradykinin into arginine and two peptides. The peptides of band II can yield DNP-arginine and must therefore have N-terminal arginine. The free arginine which the enzyme liberates from bradykinin is therefore most probably the arginine with the free carboxyl group. After hydrolysis band II yields arginine, proline, glycine and phenylalanine. It can therefore be concluded that the enzyme from *Pseudomonas aeruginosa* splits the Phe^5-Ser^6 bond and the Phe^8-Arg^9 bond of bradykinin.

The results presented here give no information as to which of the two bonds is split first. The enzyme apparently attacks these two bonds only in the bradykinin molecule, since with greatly prolonged incubation of the enzyme and bradykinin no additional amino acids or peptides are liberated. Whereas the enzyme splits the two peptide linkages in the bradykinin molecule which involves the carboxyl group of the phenylalanine, the similar bond in the dipeptide Phe-Ser is not attacked.

Of the two other purified bradykinin-splitting enzymes, chymotrypsin has previously been found to break the same bonds in the bradykinin molecule as the enzyme investigated here (Elliott *et al.*, 1960). The two enzymes are, however, differently affected by various inhibitors (Rugstad, 1967b).

Hippuryl-L-arginine and hippuryl-L-lysine, which are both very susceptible substrates for carboxypeptidase B (Neurath, 1960), were not split by the *Pseudomonas aeruginosa kininase.*

The three other kinins where the inactivation by the present enzyme has been investigated represent extensions of the bradykinin molecule at either the amino-end or the carboxyl-end. They are apparently inactivated at about the same rate as is bradykinin and it seems probable that they are attacked at the same peptide linkages.

SUMMARY

1. A purified enzyme from *Pseudomonas aeruginosa*, previously described, has been found to inactivate bradykinin, lysyl-bradykinin (kallidin), bradykinyl-isoleucyl-tyrosine and bradykinyl-isoleucyl-tyrosine-O-sulphate (phyllokinin) at about the same rates.

2. The products of the degradation of synthetic bradykinin by this enzyme have been analysed using thin-layer chromatography and electrophoresis. The enzyme apparently splits the Phe^8-Arg^9 and the Phe^5-Ser^6 bonds of bradykinin.

3. Hippuryl-L-arginine, hippuryl-L-lysine and the dipeptide Phe-Ser are not split by the enzyme.

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