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Hydrolysis of Lysine Peptides by Plasmin[1]

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The syntheses of L-leucyl-L-lysine amide, glycyl-L-lysyl-L-lysine, glycyl-L-lysyl-L-lysine amide, glycyl-L-lysyl-L-lysyl-L-arginine and L-lysyl-L-lysyl-L-arginine were described.

All dipeptides and dipeptide amides examined were resistant to plasmin, while glycyl-L-lysine amide and L-leucyl-L-lysine amide were cleaved by trypsin although dipeptides were not.

Tetrapeptide, glycyl-L-lysyl-L-lysyl-L-arginine, was hydrolyzed by plasmin to glycyl-L-lysine and L-lysyl-L-arginine, as well as by trypsin. L-Lysyl-L-lysyl-L-arginine was cleaved on lysyllysine bond by plasmin, however, no reaction occurred by trypsin. The other hand, L-lysyl-L-lysyl-L-lysine was hydrolyzed by trypsin to L-lysyl-L-lysine and L-lysine, although this tripeptide was not attacked by plasmin. Also glycyl-L-lysyl-L-lysine amide was not to be the substrate for plasmin, while this amide was attacked by trypsin, yielding glycyl-L-lysine and L-lysine amide in addition to glycyl-L-lysyl-L-lysine, ammonia and L-lysine.

The work of many laboratories has made it clear that trypsin (EC 3.4.4.4) and plasmin (EC 3.4.4.14) as well as thrombin (EC 3.4.4.13) and certain other proteases have descended from a common ancestral type.[3,4] Plasmin is similar in specificity to trypsin in that it catalyze the hydrolysis of α-amino substituted lysine and arginine esters.[5] It also, like trypsin, cleaves the Arg_{22}–Gly and Lys_{20}–Ala peptide bonds of the β-chain of oxidized bovine insulin.[6] In addition, plasmin will cleave only lysine and arginine peptide bonds in human S-sulfofibrinogen. Only 50% of the available arginine and lysine peptide bonds of S-sulfofibrinogen are cleaved while trypsin cleaves 80% of them.[7] In the amino terminal part of the human fibrinogen Aα-chain (cyanogen bromide fragment), plasmin cleaves one lysine peptide bond whereas trypsin cleaves all six lysine and arginine bonds.[8,9] Another investigators reported that plasmin hydrolyzed the peptide linkages including lysine or arginine such as Lys–Lys or Arg–Trp in the polypeptide molecule.[10]

A better understanding of the mode of plasmin action on the high molecular weight peptide might be gained by a study of the action of the enzyme on a series of lysine peptides. In the present article, we report the behaviour of the following substrates towards plasmin in comparison with that towards trypsin: Lys–Leu, Lys–Tyr, Lys–Lys, Gly–Lys–NH_2, Leu–Lys–NH_2, Gly–Lys–Lys–Arg, Lys–Lys–Arg, Lys–Lys–Lys and Gly–Lys–Lys–NH_2.

1) The amino acids except glycine are L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochemistry*, **5**, 2485 (1966). The following abbreviations used: NO_2-nitro; TsOH–toluenesulfonic acid; AcOH–acetic acid.
2) Location: *Nanakuma, Nishi-ku, Fukuoka.*
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Experimental

Materials

Plasminogen——This was prepared directly from human plasma euglobulin fraction by affinity chromatography following the procedure of Liu and Mertz,[11] and stored as a lyophilized powder. When activated by 1000 streptokinase units/casein unit of plasmin, it had a 7.2 casein units per mg protein. The plasmin casein unit is defined as the amount of enzyme which will liberate 450 μg of trichloroacetic acid-soluble tyrosine in 1 hour under the conditions of the assay.[12]

Streptokinase——Commercial varidase (Lederle), one vial consisted to 100000 units of streptokinase and 25000 units of streptodornase, was used in activation of plasminogen.

Trypsin——Bovine pancreas trypsin (Böehringer-Mannheim), 9000 units per mg protein, was used through out.

Synthesis of Peptides

Z-Leu-Lys(Z)-OMe (I)——This compound was prepared by coupling of Lys(Z)-OMe·HCl (3.3 g) with the mixed anhydride derived from Z-Leu (2.65 g) and ethyl chloroformate in chloroform, according to the procedure of Vaughan and Osato.[13] Recrystallization from ether–petroleum ether gave 3.6 g of Z-Leu-Lys(Z)-OMe, mp 91—92°. *Anal.* Calcd. for $C_{29}H_{39}O_7N_3$: C, 64.31; H, 7.26; N, 7.76. Found: C, 63.85; H, 7.91; N, 7.68.

Z-Leu-Lys(Z)-NH_2 (II)——A solution of I (2.3 g) in anhydrous methanol was saturated with NH_3 at 0° and kept in a tightly closed vessel for 20 hr at room temperature. 2.2 g of the amide was obtained after evaporation of solvent and recrystallization from methanol–ether, mp 137—138°. *Anal.* Calcd. for $C_{28}H_{38}O_6N_4$: C, 63.86; H, 7.27; N, 7.76. Found: C, 63.38; H, 7.53; N, 7.52.

Leu-Lys-NH_2·2HCl——Compound II (1.41 g) was hydrogenated with Pd-black in methanol containing 3.3N HCl (1.52 ml) for 3 hr. The solution was filtered and concentrated to dryness under vacuum. Purification was effected by precipitation from a concentrated methanolic solution by means of glacial acetic acid and ether. Yield, 78%. Dried *in vacuo* at 80° over KOH and P_2O_5 before analysis. *Anal.* Calcd. for $C_{12}H_{28}O_2N_4Cl_2$: C, 43.50; H, 8.52; N, 16.91; Cl, 21.40. Found: C, 43.12; H, 8.81; N, 16.97; Cl, 21.85.

Z-Gly-Lys(Z)-Lys(Z)-OEt (III)——This compound was prepared from Z-Gly-Lys(Z) (3.81 g) and Lys(Z)-OEt·*p*-TsOH (2.64 g), in tetrahydrofuran, according to the procedure of Vaughan and Osato.[11] Recrystallization from methanol–ether–petroleum ether gave 3.7 g of compound III, mp 166—168°. *Anal.* Calcd. for $C_{40}H_{51}O_{10}N_5$: C, 63.06; H, 6.75; N, 9.19. Found: C, 63.51; H, 7.10; N, 9.08.

Z-Gly-Lys(Z)-Lys(Z) (IV)——A solution of compound III (1.52 g) in methanol was treated with 2N NaOH (1 ml) for 4 hr at room temperature. The solution was evaporated to a small volume under reduced pressure. Acidification of the residual solution with HCl led to the deposition of an oil. The latter was separated by decantation, dissolved in ethyl acetate, and the resulting solution was dried over Na_2SO_4 and evaporated to dryness. The desired product was recrystallized from ethyl acetate–ether. Yield, 1.3 g, mp 89—92°. *Anal.* Calcd. for $C_{38}H_{47}O_{10}N_5$: C, 62.20; H, 6.46; N, 9.54. Found: C, 62.51; H, 6.63; N, 9.71.

Gly-Lys-Lys·2HCl——Compound IV (1.1 g) was hydrogenated with Pd-black in methanol containing a few drops of glacial acetic acid for 3 hr. The solution was filtered and dried down under vacuum. Gly-Lys-Lys·2AcOH was converted to it's dihydrochloric acid salt to permit crystallization, and 0.57 g of tripeptide dihydrochloride was obtained. It showed single spot on paper chromatogram and amino acid analysis after acid hydrolysis of it gave the following uncorrected ratios: Gly 0.96, Lys 2.08. *Anal.* Calcd. for $C_{14}H_{31}O_4N_5Cl_2$: N, 17.32; Cl, 17.54. Found: N, 17.52; Cl, 17.61.

Z-Gly-Lys(Z)-Lys(Z)-NH_2 (V)——This compound was prepared in a 63% yield from III analogously to the procedure of II. After recrystallization from ethanol–ether, it had mp 148—151°. *Anal.* Calcd. for $C_{38}H_{48}O_9N_6$: C, 62.28; H, 6.60; N, 11.47. Found: C, 62.60; H, 6.83; N, 11.52.

Gly-Lys-Lys-NH_2·3HCl——Compound V (0.44 g) was hydrogenated in methanol analogously to the preparation of Gly-Lys-Lys·2HCl. The desired product was further purified by precipitation from a concentrated methanolic solution with glacial acetic acid and ether, it was obtained in 70% yield. Amino acid ratios in acid hydrolysate: Gly 1.07, Lys 1.97, NH_3 0.95. *Anal.* Calcd. for $C_{14}H_{33}O_3N_6Cl_3$: N, 19.11; Cl, 24.18. Found: N, 19.02; Cl, 24.23.

Z-Gly-Lys(Z)-Lys(Z)-Arg(NO_2) (VI)——Compound IV (1.46 g) was converted to the mixed anhydride and coupled with Arg(NO_2)-OEt·*p*-TsOH (0.92 g), analogously to the preparation of III. The resulting tetrapeptide ethyl ester was light yellow hygroscopic powder and it was saponified without further purification. The saponified product was recrystallized from ethyl acetate–ether. Recrystallization gave 1.2 g of compound VI, mp 83—85°. *Anal.* Calcd. for $C_{44}H_{58}O_{13}N_{10}$: C, 56.52; H, 6.25; N, 14.98. Found: C, 56.92; H, 6.81; N, 14.72.

Gly-Lys-Lys-Arg·3HCl——Compound VI (0.93 g) was hydrogenated until H_2 gas was no longer taken up, following the procedure described in Gly-Lys-Lys-NH_2. Yield of the tetrapeptide was 65%, after

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12) L.F. Remmert and P.P. Cohen, *J. Biol. Chem.*, **181**, 431 (1949).
13) J.R. Vaughan, Jr. and R.L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

repeated precipitation from concentrated methanolic solution it showed single spot on paper chromatogram. Amino acid ratios in acid hydrolysate: Gly 1.00, Lys 2.06, Arg 0.95. *Anal.* Calcd. for $C_{20}H_{44}O_5N_9Cl_3$: N, 21.12; Cl, 17.82. Found: N, 20.80; Cl, 18.13.

Z-Lys(Z)-Lys(Z)-Arg(NO_2) (VII)——Z–Lys(Z)–Lys(Z)–Arg(NO_2)–OEt was obtained as oily syrup from Z–Lys(Z)–Lys(Z) (1.35 g) and Arg(NO_2)–OEt·*p*–TsOH (0.92 g) according to the procedure described at the synthesis of compound VI. The oily ester was hydrolyzed with NaOH in methanol and a yield of 0.78 g of compound VII, mp 100—103°, was obtained. Recrystallization from ethanol–ether raised the melting point to 106—107°. *Anal.* Calcd. for $C_{42}H_{55}O_{12}N_9$: C, 57.46; H, 6.20; N, 14.36. Found: C, 57.65; H, 6.57; N, 14.12.

Lys-Lys-Arg·3HCl——The benzyloxycarbonyl group of compound VII were removed by catalytic hydrogenation. The free tripeptide trihydrochloride isolated from the reaction mixture was pure chromatographically. Amino acid ratios in acid hydrolysate: Lys 2.04, Arg 0.89. *Anal.* Calcd. for $C_{18}H_{41}O_4N_8Cl_3$: N, 20.75; Cl, 19.70. Found: N, 21.11; Cl, 19.25.

Other peptides were prepared according to Waley and Watson[14] and Greenstein and Winitz.[15]

Methods

Paper chromatography was used tc identify the hydrolysis products produced by the hydrolytic action of plasmin and trypsin on the synthetic peptides.

The concentrations of enzymes used were as follows: four casein units of plasmin activated by streptokinase per ml of 0.1M phosphate buffer, pH 7.6, and 2 mg of trypsin per ml of 0.1M phosphate buffer, pH 7.6.

Enzymatic Hydrolysis of Dipeptides——A weighed amount of substrate (10 μM of peptide) was dissolved in 0.2 ml of 0.1M phosphate buffer, pH 7.6, and 0.2 ml of an enzyme solution (containing 0.8 casein unit of plasmin or 0.4 mg of trypsin) in the same buffer was added. The mixture was incubated at 37°, aliquots (10 μl) were withdrawn at given intervals and applied to paper chromatography. Chromatographic analyses were carried out according to Waley and Watson,[14] *n*-butanol, acetic acid, pyridine and water (30: 6: 20: 4 by volume) being used as chromatographic solvent.

Enzymatic Hydrolysis of Tri- and Tetrapeptides——A weighed amount of substrate (25 μM of peptide) was dissolved in 0.5 ml of 0.1M phosphate buffer, pH 7.6, and 0.5 ml of an enzyme solution (containing 2 casein units of plasmin or 1 mg of trypsin) in the same buffer was added. The mixture was incubated at 37°, aliquots (30 μl) were withdrawn at given intervals for chromatographic analysis. A chromatographic solvent system of *n*-butanol, acetic acid, pyridine and water (4: 1: 1: 2 by volume) was used.

Amino Acid Analysis——Tri- and tetrapeptides were hydrolyzed with 6N HCl, for 24 hr at 110° and analysis of the hydrolysate was carried out by using automatic amino acid analyzer (JLC-5AH type of JEOL).

Results

Enzymatic Hydrolysis of Dipeptides

Under our experimental conditions, neither plasmin nor trypsin significantly hydrolyzed any of the free dipeptides. Within 3 hr no reaction products could be detected chromatographically, the spots of dipeptides remaining unchanged. In the experiments with dipeptide

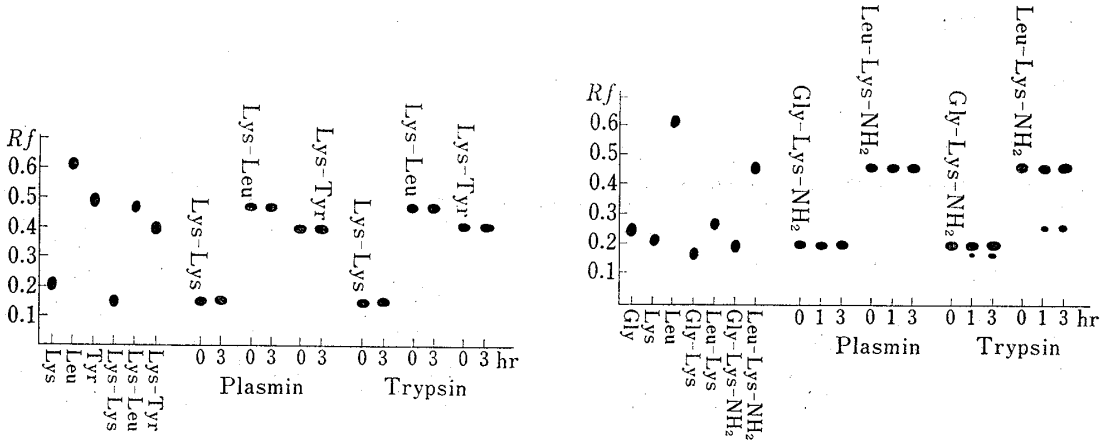

Fig. 1. Chromatograms Showing the Actions of Plasmin and Trypsin on Dipeptides and Dipeptide Amides

14) S.G. Waley and J. Watson, *Biochem. J.*, 55, 328 (1953).
15) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, Wiley, New York, N.Y., 1961.

amides, plasmin did not hydrolyze the amides, in contrast, trypsin hydrolyzed them to ammonia and free dipeptide as shown in Fig. 1.

Enzymatic Hydrolysis of Tetrapeptide

The actions of plasmin and trypsin on Gly–Lys–Lys–Arg are given in Fig. 2. The chromatogram revealed that Gly–Lys–Lys–Arg gives on incubation with plasmin or trypsin Gly–Lys and Lys–Arg, however, the produced two dipeptides could not be separated by paper chromatography. A large quantity of a substance with an *Rf* equal to that of Gly–Lys was therefore separated from a 5 hr enzymatic mixture by paper chromatography, the spot obtained was eluted with 0.1N acetic acid and analyzed by paper electrophoresis. Two distinct spots corresponding to Gly–Lys and Lys–Arg were obtained. The latter moved towards the cathode considerably faster than the former. (Paper electrophoresis was carried out with a potential gradient of 10 V/cm. A 0.1M acetate buffer, pH 4.0, was used and the electrical current passed for 2 hr.)

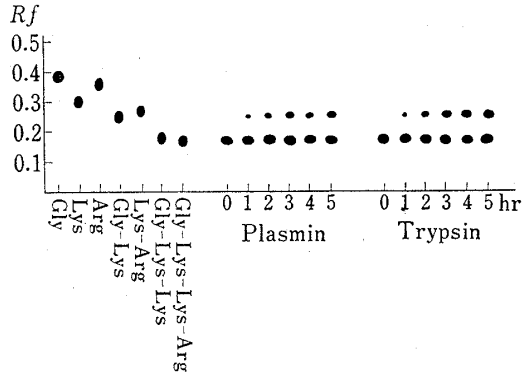

Fig. 2. Chromatogram Showing the Actions of Plasmin and Trypsin on Gly–Lys–Lys–Arg·3HCl

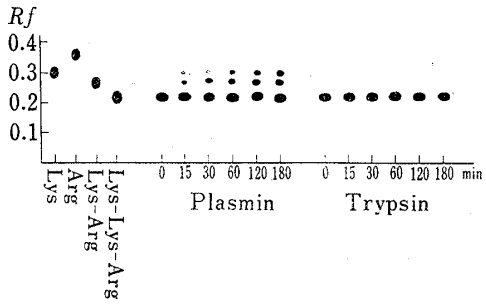

Fig. 3. Chromatogram Showing the Actions of Plasmin and Trypsin on Lys–Lys–Arg·3HCl

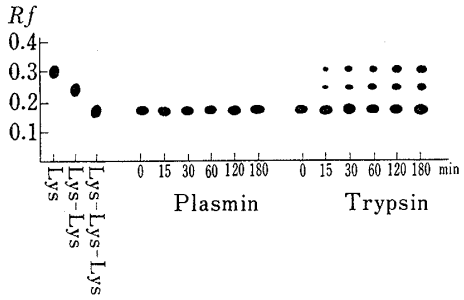

Fig. 4. Chromatogram Showing the Actions of Plasmin and Trypsin on Lys–Lys–Lys·4HCl

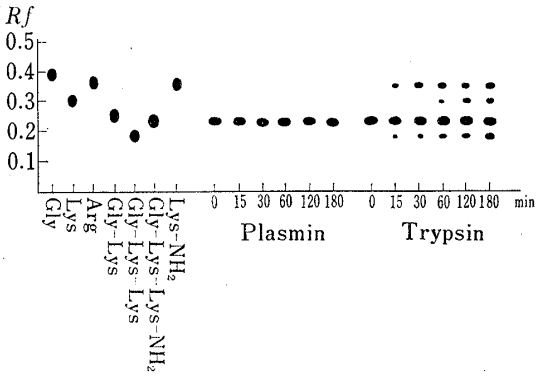

Fig. 5. Chromatogram Showing the Actions of Plasmin and Trypsin on Gly–Lys–Lys–NH_2·3HCl

Enzymatic Hydrolysis of Tripeptides

The course of reactions are shown in Fig. 3, 4 and 5. In the experiment of Lys–Lys–Arg, as shown in Fig. 3, after 15 min of incubation with plasmin the chromatogram revealed distinct spots of lysine and Lys–Arg, the amount of which increased with time. However, within 3 hr of incubation with trypsin significant hydrolysis could not be detected chromatographically. In contrast, Lys–Lys–Lys could not be hydrolyzed by plasmin but be hydrolyzed by

trypsin as shown in Fig. 4. Our observation on the incubation of Lys–Lys–Lys with trypsin was in disagreement with the finding that tetra-, penta- and hexa-lysine appear in an incubation mixture of Lys–Lys–Lys and trypsin within a few minutes.[16] On the other hand, Fig. 5 shows that Gly–Lys, Lys–NH_2, Gly–Lys–Lys and lysine appear in an incubation mixture of Gly–Lys–Lys–NH_2 and trypsin within 15 min, and their concentrations increase during 3 hr, while plasmin does not effect towards this tripeptide amide.

Discussion

Weinstein and Doolittle[17] have supported general notions, from the hydrolysis rates of the methyl esters of lysine and arginine, that thrombin has a natural preferential specificity for arginyl side chain, plasmin for the lysyl group and trypsin an approximately equal affinity for both. The specificity of plasmin for the lysyl side chain had been inferred from the widely observed inhibitory effect of ε-aminocaproic acid, a lysine analogue, on fibrinolysis.[18] Furthermore, amino acid sequence determinations performed on plasmin produced fragments of sulfitolyzed fibrinogen, have yielded oligopeptides containing internal arginines, but for the most part, having lysine at their carboxyl terminal ends.[9] Under the conditions employed in the present study, plasmin could not hydrolyzed any of the lysyl dipeptides and aminoacyllysine amides. The observation that trypsin catalyzed the hydrolysis of α-aminosubstituted lysine amide is in accord with the observation by other investigators in analogous study.[16]

From the data presented in this paper it is readily seen that plasmin catalyzes the hydrolysis of lysyllysine bond in Lys–Lys–Arg and Gly–Lys–Lys–Arg, which are including in the molecule of ACTH.[19] In these digestions, plasmin could not be inhibited by a free α-amino group adjacent to the hydrolyzable bond of a substrate.

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17) M.J. Weinstein and R.F. Doolittle, *Biochim. Biophys. Acta*, **258**, 557 (1972).
18) H. Lukasiewicz and D. Niewiarowski, *Thromb. Diath. Haemorrh.*, **14**, 587 (1968).
19) I.A. Mirsky, G. Perisutti and N.G. Davis, *J. Clin. Invest.*, **38**, 14 (1959).