BIOLOGICAL ACTIVITY OF PEPSTATINS, PEPSTANONE A AND PARTIAL PEPTIDES ON PEPSIN, CATHEPSIN D AND RENIN

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The inhibition of pepsin, cathepsin D and renin by pepstatins, pepstanone A and their partial peptides is described. A new method using a nonapeptide containing ^{3}H-Val as the substrate was devised for determination of renin activity. The pepstatin partial peptides valyl-valyl-4-amino-3-hydroxy-6-methylheptanoic acid (Val-Val-AHMHA), isovaleryl-valyl-valyl-4-amino-3-hydroxy-6-methylheptanoic acid (IVA-Val-Val-AHMHA) and carbobenzoxyvalyl-valyl-4-amino-3-hydroxy-6-methylheptanoic acid (Z-Val-Val-AHMHA) weakly inhibited proteolysis by pepsin and did not inhibit renin. Pepstatins B,C,E and G were as active as pepstatin A against pepsin and were slightly more active against renin than pepstatin A. Pepstanone A was as active as pepstatin A against pepsin but slightly less active against renin.

As reported in previous papers[1,2,3], pepstatins A,B,C,E and G, and pepstanone A are produced by the pepstatin A-producing streptomyces. Pepstatin A has been shown to be a specific inhibitor of acid proteases and shows a strong protective effect against stomach ulcer in pylolusligated rats[4]. It also inhibits cathepsin D, a pepsin-like acid protease[5].

A new method was devised for the determination of renin activity. It is based on the cleavage of a nonapeptide, His-Pro-Phe-His-Leu-Leu-(^{3}H-Val)-Tyr-Ser by renin to yield a pentapeptide His-Pro-Phe-His-Leu and a tetrapeptide Leu-(^{3}H-Val)-Tyr-Ser. The latter is separated from the reaction mixture by Dowex 50-X8 column chromatography and determined by a soft beta-counting spectrometer.

In this paper, the effects of pepstatins, pepstanone A and partial peptides on pepsin, cathepsin D and renin are reported.

Materials and Methods

Enzymes: Porcine pepsin (3,200 units/mg) was purchased from Sigma Chemical Co., U.S.A., and hog kidney renin (1 dog unit/mg) was purchased from General Biochemicals, U.S.A. Both were used without further purification. Porcine pepsin (40 μg/ml) was dissolved in 0.001 N HCl, and hog kidney renin (1 mg/ml) was dissolved immediately before use in pH 7.5 0.05 M phosphate buffer containing 0.5% polyvinylpyrrolidone (PVP). Cathepsin D was prepared from the acetone powder of lysosomal-mitochondrial pellets

obtained from swine liver homogenate as described previously[5].

Substrates: Casein was purified according to the procedure described by NORMAN[6] and a 0.6% casein solution in 0.75% lactic acid was used as a pepsin substrate. Bovine hemoglobin was purchased from Nutritional Biochemical Co., U.S.A. and a 1% hemoglobin solution in 0.2 M acetate buffer (pH 3.2) was used as a substrate for cathepsin D. His-Pro-Phe-His-Leu-Leu-(^{3}H-Val)-Tyr-Ser (abbreviated as ^{3}H-Val-nonapeptide, 2.43×10^6 cpm/mg) was synthesized by the method described below and a stock solution of ^{3}H-Val-nonapeptide was dissolved in 0.01 N HCl. This solution was adjusted carefully to pH 4.0 by the addition of 0.5 N NaOH and diluted to 0.6% concentration with water. The stock solution was diluted with 0.05 M phosphate buffer, pH 7.5, containing 0.05% PVP, to give the final concentration of 0.06%.

Pepstatin A,B,C,E and G, and Pepstanone A: They were prepared from fermentation broths of *Streptomyces testaceus* as described in previous papers[1,3]. Their structures are described later.

Synthesis of partial peptides of pepstatin: AHMHA (4-amino-3-hydroxy-6-methylheptanoic acid) used for synthesis was obtained by hydrolysis of pepstatin A. All amino acids used had the L-configuration.

Ala-AHMHA: Carbobenzoxyalanine (Z-Ala) and AHMHA were coupled by an active ester method. Succinimide ester of carbobenzoxyalanine was prepared by the method of ANDERSON *et al*[7]., and the protective group was removed by catalytic hydrogenation. m.p. 194~195°C (dec.), $[\alpha]_D^{23}-43.0°$ (*c* 1, water). Anal. Calcd. for $C_{11}H_{22}N_2O_4$: C 53.64, H 9.00, N 11.37. Found: C 53.05, H 8.89, N 10.90.

IVA-Val-Val: Carbobenzoxyvaline (Z-Val) and methyl ester of valine were coupled by the dicyclohexylcarbodiimide (DCC) method, and valylvaline (Val-Val) was obtained by alkaline saponification followed by catalytic hydrogenation. Isovaleryl-valyl-valine (IVA-Val-Val) was prepared from Val-Val and isovaleryl chloride by the SHOTTEN-BAUMANN reaction. m.p. 142~143.5°C, $[\alpha]_D^{22}-38.4°$ (*c* 1, methanol). Anal. Calcd. for $C_{15}H_{28}N_2O_4$: C 59.97, H 9.40, N 9.33. Found: C 59.79, H 9.14, N 9.40.

IVA-Val-Val-AHMHA: The synthesis was described in a previous paper[8].

Val-AHMHA: Z-Val was condensed with the *p*-nitrobenzyl ester of 4-amino-3-hydroxy-6-methylheptanoic acid *p*-toluensulfonate[8] in the presence of one equivalent of triethylamine by a mixed anhydride method, and from the reaction product, Val-AHMHA monohydrate was obtained by catalytic hydrogenation. $[\alpha]_D^{25}+13.0°$ (*c* 0.92, water). Anal. Calcd. for $C_{13}H_{26}N_2O_4\cdot H_2O$: C 53.40, H 9.65, N 9.58. Found: C 53.42, H 9.50, N 9.24.

Z-Val-Val-AHMHA: This compound was prepared by reaction of succinimide ester of carbobenzoxyvaline with Val-AHMHA monohydrate in aqueous dioxane: m.p. 199.5~200°C, $[\alpha]_D^{22}-47.9°$ (*c* 1.3, acetic acid). Anal. Calcd. for $C_{26}H_{42}N_3O_7$: C 61.39, H 8.32, N 8.26. Found: C 61.19, H 8.02, N 8.27.

Val-Val-AHMHA: This compound was obtained from Z-Val-Val-AHMHA by catalytic hydrogenation. $[\alpha]_D^{20}-27.7°$ (*c* 0.65, water). Anal. Calcd. for $C_{18}H_{35}N_3O_5$: C 57.88, H 9.45, N 11.25. Found: C 57.44, H 9.28, N 10.81.

Synthesis of His-Pro-Phe-His-Leu-Leu-(^{3}H-Val)-Tyr-Ser: All amino acids used had the L-configuration. The synthesis of the protected nonapeptide-resin was carried out according to the usual solid phase procedure[9] starting from Boc-Ser(Bz)-resin (4.0 g; 0.40 mmole of ser/g, Boc; *tert*-butyloxycarbonyl, Bz; benzyl). Each Boc-amino acid to be added was used in 3-fold excess. The extra functional groups of Boc-Tyr and -His were protected with benzyl and tosyl, respectively. DCC was used for coupling (3-fold excess). Boc-groups were removed with 1 N HCl in acetic acid. Boc-^{3}H-Val was prepared from the reaction of *tert*-butyl azidoformate and ^{3}H-Val (purchased from New England Nuclear Corporation) with Val (0.234 g) as a carrier in the usual method. After Boc-^{3}H-Val was coupled to the dipeptide-resin followed by successive washing with dichloromethane, ethanol and dichloromethane, the resin was allowed to react with Boc-Val in the usual manner[10]. The rest

of the synthetic sequence was then carried out. The yield of the protected nonapeptide-resin was 5.63 g. Cleavage of the protected nonapeptide from the resin (5.60 g) together with removal of all protecting groups was achieved in one step by treatment with anhydrous HF in the presence of anisole for 60 minutes at 0°C. The crude product was extracted with a large quantity of 1 N acetic acid and the extract was applied to an Amberlite CG-50 (H^+ form, type 1) column which was throughly washed with 2 N acetic acid. The product was eluted with 90% acetic acid and the eluate was lyophilized. The nonapeptide diacetate weighed 0.597 g; m.p. 203°C (dec.); $[\alpha]_D^{29}$ −39.8° (*c* 0.5, acetic acid); radioactivity 2.43×10^6 cpm/mg. On paper electrophoresis (25 V/cm for 50 minutes, 0.2 M pyridine - acetic acid, pH 4.8), the peptide was homogeneous, moving to the cathode with an Rm value of 0.63 compared to L-histidine as 1.0. On thin-layer chromatography using Silica Gel G (E. Merck), the peptide gave a single spot at Rf 0.61 with butanol - acetic acid - water - pyridine (15 : 3 : 12 : 10) compared to L-histidine with Rf 0.34. Amino acid ratios were obtained by automatic ion-exchange chromatography of hydrolysates prepared by heating the peptides in 6 N HCl at 110°C in evacuated tubes for 48 hours: His, 1.89; Pro, 1.03; Phe, 1.05; Leu, 2.01; Val, 0.98; Tyr, 1.02; Ser, 1.00.

Reaction system for hydrolysis of casein by pepsin: One ml of 0.6% casein solution, 0.8 ml of 0.02 M KCl-HCl buffer (pH 2.0) and 0.1 ml of the same buffer with or without an inhibitor were mixed and incubated for 3 minutes at 37°C. Then, 0.1 ml of a solution of porcine pepsin (40 μg/ml) was added. After incubation at 37°C for 30 minutes, 2.0 ml of 1.7 M perchloric acid (PCA) was added and the mixture was kept for 1 hour at room temperature. It was then centrifuged and the extinction of the acid-soluble fraction was read at 280 mμ[4].

Reaction system for hydrolysis of hemoglobin by cathepsin D: Three ml of 1.0% hemoglobin solution, 0.8 ml of 0.05 M acetate buffer (pH 3.2) with or without an inhibitor and 0.2 ml of the enzyme solution (protein : 0.68~2.5 mg) were mixed. After incubation at 37°C for 30 minutes, 2.0 ml of 9% trichloroacetic acid (TCA) was added and the mixture was kept for 30 minutes at 37°C. The mixture was filtered and the extinction at 280 mμ was measured as described above.

Reaction system for hydrolysis of ^{3}H-Val-nonapeptide by renin: The nonapeptide His-Pro-Phe-His-Leu-Leu-(^{3}H-Val)-Try-Ser was used as the substrate. Five tenth ml of 0.06% ^{3}H-Val-nonapeptide, 0.3 ml of 0.05 M phosphate buffer, containing 0.05% PVP (pH 7.5) and 0.1 ml of the same buffer with or without an inhibitor were mixed and incubated for 3 minutes at 37°C. Then, 0.1 ml of a solution of hog kidney renin (1 mg/ml) was added. After incubation at 37°C for 60 minutes, the reaction mixture was put in a boiling water bath for 1 minute. Then, it was passed through a column of Dowex 50-X8 (NH_4^+ form, 200~400 mesh, 0.5×5.0 cm) at room temperature and followed by 1 ml of distilled water. The radioactivity of Leu-(^{3}H-Val)-Tyr-Ser in the eluate and the wash was determined in a Beckman Liquid Scintillation System LS-250 using 8 ml of BRAY's scintillation solution[11]. The reaction was also carried out in the reaction mixture containing boiled enzyme solution and the result was taken as the blank value.

Estimation of percent inhibition on proteolytic reactions: The percent inhibition of an enzymatic reaction was calculated from the experimental data obtained by the methods described above, and the probit of the percent inhibition was plotted against the logarithmic concentration of each inhibitor. The concentration of inhibitor required for 50% inhibition (ID_{50}) of enzyme activity was obtained from this plot as described in a previous peper[4].

Results and Discussion

Pepstatin A is a specific inhibitor of acid proteases [1,4,12] and the hydrophobicity of its structure seems to play an important role in binding of pepstatin to acid proteases. On the other hand, two hydroxyl groups of pepstatin are thought to be

Fig. 1. Effect of the renin concentration on hydrolysis of ^{3}H-Val-nonapeptide

The indicated amounts of renin were incubated in the standard assay procedure.

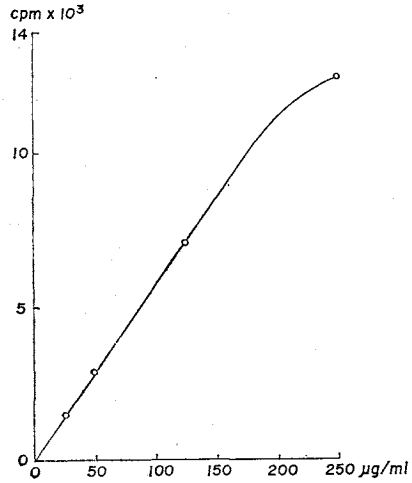


Fig. 2. Time course of hydrolysis of ^{3}H-Val-nonapeptide by renin

Renin (125 μg) was added to 1 ml of a reaction system similar to that described in the standard assay procedure.

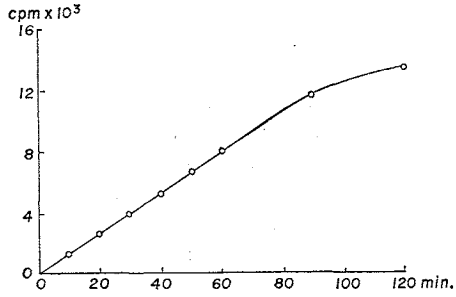


Table 1. Biological activity of pepstatin and its partial peptides

Compounds	ID_{50} (μg/ml)	
	Pepsin	Renin
AHMHA	>250	>250
Ala-AHMHA	>250	>250
Val-AHMHA	>250	>250
IVA-Val-Val	>250	>250
Val-Val-AHMHA	18	>250
IVA-Val-Val-AHMHA	0.52	>250
Z-Val-Val-AHMHA	0.2	>250
Pepstatin	0.01	4.5

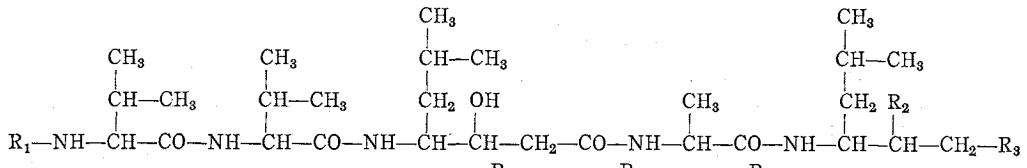
involved in the binding of pepstatin A with pepsin because dehydroacetyl pepstatin, in which the hydroxyl group of dehydropepstatin is acetylated, is 150 times less active than pepstatin. We have also suggested that the 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA) moiety in pepstatins and pepstanone is the most important moiety for binding with pepsin.

For the determination of renin activity, methods including biological assay[13], radiochemical assay[14] and an automatic chemical determination[15] have been reported. We established a new assay method of renin activity, using a ^{3}H-Val-nonapeptide and counting the radioactivity of a reaction product.

The effect of the renin concentration on the hydrolysis of ^{3}H-Val-nonapeptide was examined. The reaction rate was linear up to an enzyme concentration of 200 μg (Fig. 1). The reaction rate was linear for 60 minutes but thereafter gradually slowed down (Fig. 2).

The effect of pepstatins, pepstanone A and their partial peptides and ^{3}H-Val-nonapeptide on hog kidney renin is shown in Table 1. It is apparent from Table 1 that pepstatins strongly inhibit pepsin and renin. Against pepsin and renin, AHMHA, Ala-AHMHA, Val-AHMHA and IVA-Val-Val showed no inhibition even at the high concentration of 250 μg/ml. However, as shown in the table, Val-Val-AHMHA, IVA-Val-Val-AHMHA and Z-Val-Val-AHMHA weakly inhibited proteolysis by pepsin. The results suggest that an increase of hydrophobic property may enhance the inhibition.

The structure of pepstatin A,B,C,D,E,F,G and H, and pepstanone A are as follows:



	R_1	R_2	R_3
Pepstatin A	iso-valeryl	$<^{H}_{OH}$	-COOH
Pepstatin B	*n*-caproyl	$<^{H}_{OH}$	-COOH
Pepstatin C	iso-caproyl	$<^{H}_{OH}$	-COOH
Pepstatin D	*n*-heptanoyl	$<^{H}_{OH}$	-COOH
Pepstatin E	iso-heptanoyl	$<^{H}_{OH}$	-COOH
Pepstatin F	anteiso-heptanoyl	$<^{H}_{OH}$	-COOH
Pepstatin G	*n*-capryl	$<^{H}_{OH}$	-COOH
Pepstatin H	iso-capryl	$<^{H}_{OH}$	-COOH
Pepstanone A	iso-valeryl	=O	-H

The activity of pepstatins and pepstanone A in inhibiting pepsin, cathepsin D and renin are shown in Table 2. Pepstatins B, C, E and G were equally active against pepsin as pepstatin A, but their activity against renin and cathepsin D was slightly greater. The activity of pepstatins against the latter enzymes depended on the number of carbon atoms in the fatty acid moiety, especially with renin. The larger the carbon number the stronger the activity. Pepstanone A in which the C-terminus was not carboxyl was equally or slightly less active against pepsin than pepstatin A. It indicates that free carboxyl and hydroxyl groups in the C-terminal amino acid are not essential for the activity. However, as shown in Table 2, the activity of pepstanone A on the hydrolysis of nonapeptide by renin was about one-sixth that of pepstatin A, suggesting an involvement of the terminal carboxyl and hydroxyl groups in the inhibition of renin.

Table 2. Biological activity of pepstatins

	ID_{50} (μg/ml)		
	Pepsin	Renin	Cathepsin D
Pepstatin A	0.01	4.5	0.011
Pepstatin B	0.01	3.0	0.0003
Pepstatin F	0.01	1.8	0.002
Pepstatin G	0.01	1.2	0.003
Pepstanone A	0.013	25	—

—: not examined.

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