PEPSTATIN, A NEW PEPSIN INHIBITOR PRODUCED BY ACTINOMYCETES

Sir:

A specific inhibitor of an enzyme might be useful for the analysis of certain pathogenic phenomena and also perhaps for the treatment of certain diseases. It was postulated that microorganisms because they produce catobolic enzymes may also produce inhibitors of these enzymes. The authors therefore started to search for enzyme inhibitors in microbial culture filtrates and, as reported in previous papers[1~4] leupeptins inhibiting plasmin, trypsin and papain were found in culture filtrates of various species of actinomycetes. In this paper, the isolation and properties of pepstatin, a pepsin inhibitor, are reported.

Pepstatin was found in culture filtrates of various species of actinomycetes and three strains were studied in detail. Two of them (forming spirals with spores having a smooth surface; pale yellow, yellow, or pale brown growth; thin grayish or light gray aerial mycelium; reddish brown growth on a potato slice; nonchromogenic type; strong proteolytic action) were compared with *Actinomyces longisporus-flavus*[5,6]. The latter species resembled most closely these strains but because of differences the strains were designated as a new species named *Streptomyces testaceus* HAMADA *et* OKAMI. Another strain was found to resemble *Streptomyces argenteolus*[7,8] and, though differences were found in utilization of carbohydrates and liquefaction of gelatin, it was designated *Streptomyces argenteolus* var. *toyonakensis*.

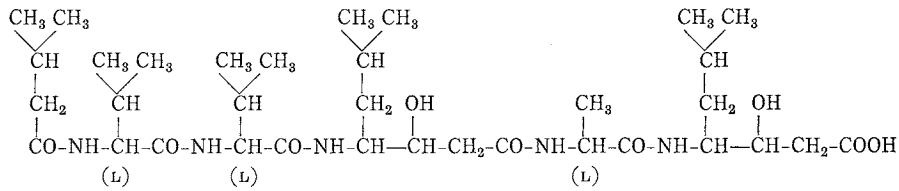
For the determination of pepstatin, the method described by ANSON[9] was modified as follows: To 1.0 ml of 0.6 % casein (Wako Chemical Co., Osaka) in 0.08 M lactic acid (pH 2.2), 0.7 ml of 0.02 N HCl - 0.02 M KCl buffer (pH 2.0) and 0.2 ml of the same buffer with or without test material were added. After 3 minutes at 37°C, 0.1 ml of 4 μg of pepsin in 0.02 N HCl - 0.02 M KCl buffer (pH 2.0) was added and the reaction mixture incubated for 30 minutes at 37°C. The optical density at 280 mμ of the supernatant after separation from the precipitate one hour after addition of 2.0 ml of perchloric acid (1.7 M) was read. From the standard curve of the percent inhibition *versus* the concentration, the amount of pepstatin in the test material could be determined. By this method, addition of 0.02 μg of pepstatin (0.01 μg/ml in the reaction mixture) showed 50 % inhibition.

Pepstatin was produced in shake culture and fermentation tanks in media containing various kinds of carbon sources and nitrogen sources. The typical medium used for production contained 1.0 % glucose, 1.0 % starch, 0.75 % peptone, 0.75 % meat extract, 0.3 % NaCl, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$, 0.0002 % $ZnSO_4 \cdot 7H_2O$. Production of pepstatin reached a maximum after 3~5 days in shake culture or 60~70 hours in a tank fermentation.

Pepstatin can be obtained from the culture filtrate by adsorption processes using activated carbon but is more easily isolated by extraction with an organic solvent. As an example, pepstatin was obtained by the following procedure: 2,700 liters of culture filtrate containing 420 g of pepstatin was extracted with 1,400 liters of *n*-butanol at pH 8.2. The butanol extract was concentrated under reduced pressure yielding 10 liters. This was collected, dissolved in 16 liters of methanol at 40°C, and treated with 1,500 g of carbon. Filtration and cooling yielded crystals of pepstatin. After recrystalization from methanol, the trotal yield was 221 g. Pepstatin, which is insoluble in water, can also be obtained from the mycelium cake by extraction with methanol. If necessary, silica gel column chromatography using *n*-butanol - acetic acid - water - butyl acetate (4 : 1 : 1 : 4) may be used for purification.

Pepstatin is obtained as colorless needles, m. p. 228~229°C. *Anal.* calcd. for $C_{34}H_{63}N_5O_9$: C 59.53, H 9.25, N 10.21, O 20.99; found: C 59.02, H 9.27, N 10.11, O 21.41. Mass spectrography of the methyl ester (the parent peak at 699) of pepstatin and the methyl ester (the parent peak at 783) of

diacetylpepstatin supported the formula. It is optically active, $[\alpha]_D^{27}$ −90° (0.288 % in methanol). Pepstatin shows no maximum at 220~400 mμ except for end absorption. It gives positive RYDON-SMITH and permanganate tests but negative ninhydrin, SAKAGUCHI and EHRLICH reactions. It is soluble in methanol, ethanol, acetic acid, pyridine and dimethylsulfoxide, but is slightly soluble or insoluble in ethyl acetate, ether, benzene, chloroform and water. As reported in the next paper[10], the following structure is proposed for pepstatin:



As would be expected from the structure, pepstatin can form metal salts, and sodium pepstatin (m. p. 250~252°C, dec.; calcd. for $C_{34}H_{62}N_5O_9Na$: C 57.68, H 8.82, N 9.89, O 20.34, Na 3.24; found: C 57.97, H 8.92, N 9.96, O 20.26, Na 2.98), magnesium pepstatin (m. p. 257~258°C, dec.; calcd. for $C_{34}H_{62}N_5O_9\frac{1}{2}Mg$: C 58.58, H 8.96, N 10.04, O 20.65, Mg 1.74; found: C 58.30, H 9.05, N 9.94, O 20.84, Mg 1.49), and calcium pepstatin (m. p. 262~263°C, dec.; calcd. for $C_{34}H_{62}N_5O_9\frac{1}{2}Ca$: C 57.93, H 8.86, N 9.93, O 20.42, Ca 2.84; found: C 57.92, H 8.98, N 9.62, O 20.07, Ca 2.62) were prepared. These salts, especially the sodium salt, were more soluble in water than the acid form of pepstatin. These salts showed pepsin inhibition at almost the same concentration as pepstatin. Treatment of pepstatin in methanol with diazomethane gives the methyl ester of pepstatin (m. p. 249~251°C, dec.; calcd. for $C_{35}H_{65}N_5O_9$: C 60.05, H 9.36, N 10.00, O 20.57; found: C 59.88, H 9.35, N 9.74, O 20.69) and treatment of pepstatin in ethanol with sulfuric acid gives the ethyl ester of pepstatin (m. p. 190~193°C, dec.; calcd. for $C_{36}H_{67}N_5O_9$: C 60.56, H 9.45, N 9.80, O 20.16; found: C 60.96, H 9.65, N 9.43, O 19.70). Treatment of the methyl ester of pepstatin in methanol with ammonia gives pepstatin amide (m. p. 229~231°C, dec.; calcd. for $C_{34}H_{64}N_6O_8$: C 59.62, H 9.41, N 12.27, O 18.68; found: C 59.69, H 9.45, N 12.17, O 18.24). Treatment of pepstatin or its methyl ester in pyridine with acetic anhydride gives their mono or diacetyl derivatives. The esters, the amide and the acetyl derivatives of pepstatin showed 50 % inhibition of pepsin using the method described above at the following concentrations: pepstatin 0.01 μg/ml; the methyl ester 0.008 μg/ml; the ethyl ester 0.01 μg/ml; the *p*-bromophenacyl ester 0.01 μg/ml; the amide 0.027 μg/ml; the monoacetyl derivative 1.1 μg/ml; the diacetyl derivative 4.2 μg/ml; the methyl ester of the diacetyl derivative 2.26 μg/ml. Kinetic studies have been done on the effect of pepstatin using both a natural protein and a synthetic dipeptide as substrates. Uncompetitive inhibition was observed in the system of pepsin and hemoglobin, but noncompetitive inhibition was observed in the system of pepsin and N-acetyl-L-phenylalanyl-L-tyrosine as determined by a LINEWEAVER-BURK plot. However, detailed studies showed the occurrence of multiple binding of the inhibitor to pepstin which was dependent on the concentration of pepstatin.

The effect of pepstatin is specific on acid protease. At 3.6×10^{-4} M (250 μg/ml), pepstatin showed no inhibition of the following substrate-enzyme reactions[2]: *p*-toluenesulfonyl-L-arginine methyl ester-thrombin, fibrinogen-plasmin, casein-trypsin, α-N-benzoyl-L-arginine methyl ester-kallikrein, casein-α-chymotrypsin, casein-papain. With the following substrate-acid protease reaction systems pepstatin showed a 50 % inhibition at the following concentrations: casein-pepsin at 1.5×10^{-8} M (0.01 μg/ml); hemoglobin-pepsin at 4.5×10^{-9} M (0.0031 μg/ml); casein-proctase[11] (an acid protease produced by *Aspergillus niger* var. *macrosporus*) at 2.9×10^{-8} M (0.02 μg/ml), hemoglobin-proctase at 6.2×10^{-9} M (0.0042 μg/ml); casein-acid protease of *Trametes sangui-*

Table 1. Effect of pepstatin on ulceration and gastric secretion in pylorus ligated rats (Shay rats)

Dose mg/kg (oral)	No. of rat	Gastric secretion				Grade of ulcer**				
		Volume (ml)	pH	Total acid	Pepsin* μg/ml	−	+	++	+++	++++
50	4	11.6	2.3	22.3	0	4/4	0/4	0/4	0/4	0/4
10	5	12.5	2.1	27.0	0.84	5/5	0/5	0/5	0/5	0/5
1	9	14.3	1.6	39.7	0.92	7/9	2/9	0/9	0/9	0/9
0.5	4	15.9	1.3	49.0	25.6	2/4	1/4	1/4	0/4	0/4
Control	10	11.3	1.7	41.8	493.0	0/10	0/10	0/10	5/10	5/10

Rat: male Wistar
Body weight: 200 g±15 g
Fasted: 48 hours
Administration route: oral
Pepstatin Treatment: 15 minutes after pyloric ligation
Total acid: N/10 NaOH ml per gastric secretion 100 ml
Pyloric ligation: 22 hours
* Pepsin activity corresponding to pepsin μg/ml
** −; No ulceration: +; A single ulceration: ++; Some points of ulceration: +++; Generalized ulceration: ++++; Perforated ulceration

nea[12] at 5.2×10^{-8} M (0.035 μg/ml), hemoglobin-acid protease of *Trametes sanguina* at 2.6×10^{-8} M (0.018 μg/ml).

Pepstatin effectively prevents stomach ulceration of the pylorus in ligated rats (Shay rats). Data of such an experiment are shown in Table 1. Free sialic acid and bound sialic acid in the gastric juice of the Shay rats were also determined, and when pepstatin was given a marked decrease of the latter was observed.

The effect of pepstatin on carrageenin edema was determined using the method described by WINTER *et al.*[13] In these experiments even low doses of pepstatin showed an inhibition of edema formation. Intraperitoneal injection of 1.25 mg/kg of pepstatin caused a 30 % inhibition. This effect suggests that enzymes which are inhibited by pepstatin may play a role in carrageenin edema.

Pepstatin has a very low toxicity. LD_{50} values by the intraperitoneal route were 1,090 mg/kg for mice, 875 mg/kg for rats, 820 mg/kg for rabbits and 450 mg/kg for dogs. Oral LD_{50} values were >2,000 mg/kg for all species. Daily oral administration of 250 mg/kg to rats for 180 days caused no toxic signs and the mice grew at an equal rate as the controls. Daily oral administration of 1,000 mg/kg to rats for 180 days slightly lowered the weight gain in the treated group. The control group after 90 days had an average weight of 404 g and the treated 380 g. No indication of toxicity could be histologically observed in the organs of these rats. Daily oral administration of 800 mg/kg, 400 mg/kg or 200 mg/kg to monkeys (each group consisting of 4 monkeys) for 180 days caused no toxic symptoms. Thus pepstatin was found to be a compound having very low toxicity. Pepstatin caused no irritation of smooth muscle such as the rabbit ileum, rat intestine, rat stomach, guinea pig trachea, rat uterus and rabbit aorta.

Using tritiated pepstatin, it was found that more than 90 % of the pepstatin orally administered was excreted in the faeces. However, after intraperitoneal injection of 50 mg/kg of pepstatin to rats or dogs levels of 5~10 μg/ml were found in the serum 6~24 hours after injection in rats and 2~24 hours in dogs. Pepstatin was found in the urine of rats up to 72 hours after injection. Such a long retention period is thought to be due to low solubility of pepstatin in the intraperitoneal liquid.

As described above, by screening for enzyme inhibitors produced by microorganisms pepstatin was discovered. It is the first specific pepsin inhibitor. Pepstatin has very low toxicity and has been tested clinically. Treatment of patients with stomach ulcer should give useful information on the role of pepsin in stomach ulcers.

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Reference

1) Aoyagi, T.; T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada & H. Umezawa: Leupeptins, new protease inhibitors from actinomycetes. J. Antibiotics 22 : 283～286, 1969
2) Aoyagi, T.; S. Miyata, M. Nanbo, F. Kojima, M. Matsuzaki, M. Ishizuka, T. Takeuchi & H. Umezawa: Biological activities of leupeptins. J. Antibiotics 22 : 558～568, 1969
3) Kondo, S.; K. Kawamura, J. Iwanaga, M. Hamada, T. Aoyagi, K. Maeda, T. Takeuchi & H. Umezawa: Isolation and characterization of leupeptins produced by actinomycetes. Chem. Pharm. Bull. 17 : 1896～1901, 1969
4) Kawamura, K.; S. Kondo, K. Maeda & H. Umezawa: Structures and syntheses of leupeptins Pr-LL and Ac-LL. Chem. Pharm. Bull. 17 : 1902～1909, 1969
5) Shirling, E. B. & D. Gottlieb: Cooperative description of type cultures of Streptomyces III. Additional species descriptions from first and second studies. Inter. J. Syst. Bacteriol. 18 : 342, 1968
6) Waksman, S. A.: The Actinomycetes. Vol. II, 237, Williams & Wilkins Co., 1961
7) Shirling, E. B. & D. Gottlieb: Cooperative description of type cultures of Streptomyces III. Additional species descriptions from first and second studies. Inter. J. Syst. Bacteriol. 18, 294, 1968
8) Waksman, S. A.: The Actinomycetes. Vol. II, 175～176, Williams & Wilkins Co., 1961
9) Anson, M. L.: The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22 : 79～89, 1938
10) Morishima, H.; T. Takita, T. Aoyagi, T. Takeuchi & H. Umezawa: The structure of pepstatin. J. Antibiotics 23 : 263～265, 1970
11) Koaze, Y.; H. Goi, K. Ezawa, Y. Yamada & T. Hara: Fungal proteolytic enzyme. Part I. Isolation of two kinds of acid proteases excreated by *Aspergillus niger* var. *macrosporus*. Agr. Biol. Chem. 28 : 216～223, 1964
12) Tomoda, K. & H. Shimazono: Acid protease produced by *Trametes sanguinea*, a wood-destroying fungus. Part I. Purification and crystallization of the enzyme. Agr. Biol. Chem. 28 : 770～773, 1964
13) Winter, C. A.; E. A. Risley & G. W. Nuss: Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. Proc. Soc. Exp. Biol. & Med. 111 : 544～547, 1962