EFFECT OF PROTEASE INHIBITORS OF ACTINOMYCETES ON LYSOSOMAL PEPTIDE-HYDROLASES FROM SWINE LIVER

Sir:

Recently, AOYAGI, TAKEUCHI and UMEZAWA[1~7] have obtained from actinomycete fermentation three kinds of protease inhibitors, *i.e.*, leupeptins, chymostatin and pepstatin, which inhibit trypsin, chymotrypsin and pepsin, respectively.

In the present study, the effects of these inhibitors on cathepsins A, B and D (acid peptide-hydrolases locating in lysosomal particles of animal cell) are reported.

These acid peptidases were prepared from the acetone powder of lysosomal-mitochondrial pellet obtained from swine liver homogenate according to the method described by SNELLMAN[8]. Two hundred grams of fresh tissue of swine liver was cut into small pieces, homogenized with two volumes of 50 mM Tris buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and centrifuged at 700 *g* for 10 minutes. After removal of the precipitate (nuclear-debris), the supernatant was further centrifuged at 7,000 *g* for 60 minutes. To the resulting sediment (lysosomal-mitochondrial pellet) was added 200 ml of cold acetone, and the suspension was stirred for an hour at 4°C. After filtration of the acetone suspension, the filter cake was washed with cold acetone and dried *in vacuo*. The acetone powder thus obtained (yield 3.2 g) was used as a common source of cathepsins A, B and D. Before use, an appropriate amount of the acetone powder was extracted with five volumes of 0.1 M citrate, pH 5.0. The extract was dialyzed twice (first, 4-hour stirring; second, overnight) against the same buffer, and centrifuged. The supernatant was added to each assay system as an enzyme solution. The protein concentration of the enzyme solution was determined by the biuret method.

For the determination of cathepsin A, the method of IODICE *et al.*[9] was used with some modifications: To glass-stoppered test tubes were added 0.5 ml of 0.015 M α-N-carbobenzoxy-L-glutamyl-L-tyrosine (substrate), 0.4 ml of 0.2 M acetate buffer (pH 5.0) or inhibitor solution and 0.1 ml of the enzyme solution (protein: 1.25~1.65 mg). The reaction mixtures were incubated at 37°C for 2 hours. The reaction was stopped by the addition of 1 ml of 10% TCA. The TCA mixtures were heated for 10 minutes in a boiling water bath, then diluted with 8 ml of water and filtered. From the filtrates, 1 ml aliquots were withdrawn and used for ninhydrin determination[10] with the appropriate level of tyrosine as a standard. Controls without substrate or without the enzyme were treated in the same manner.

In the assay of cathepsin B, we used CONWAY's microdiffusion technique[11~13]. Reaction mixtures containing 1 ml of 0.1 M α-N-benzoyl-L-arginine amide (substrate), 0.5 ml of 0.1 M citrate buffer (pH 5.0) or inhibitor solution, 0.2 ml of 40 mM cysteine and 0.3 ml of the enzyme solution (protein: 6 ~ 6.45 mg) were incubated at 37°C for 2 hours. The reaction was stopped by the addition of 2 ml of 10% TCA. After additional incubation for 30 minutes, the TCA mixtures were filtered. From the filtrates, 0.1 ml aliquots were withdrawn and diluted with 3 ml of water. One ml of each diluted solution was pipetted into the outer compartment of a CONWAY's microdiffussion vessel. As the absorbent for NH_3, 1 ml of 0.01 N H_2SO_4 was added to the center compartment of the vessel. Immediately after 0.5 ml of saturated potassium carbonate was added to the outer compartment, the vessel was closely covered, shaken gently for mixing, and allowed to stand for an hour. After this diffusion process, 0.5 ml aliquot was withdrawn from the central compartment, and the absorbed NH_3 was determined colorimetrically by indophenol method of CHANEY and MARBACH[14], instead of titrimetric method. Controls without enzyme were treated in the same way.

In contrast to the assay of cathepsins A and B where the pH of the reaction mixtures were maintained at 5.0, the determination of the activity of cathepsin D was performed at pH 3.2 by using a modification of ANSON's

method.[15] For the preparation of the substrate of this proteolytic enzyme, 1 g of bovine hemoglobin was dissolved in 35 ml of water, mixed with 15 ml of N HCl and incubated for 30 minutes at 37°C. After incubation, the solution was adjusted to pH 3.2 with N NaOH, diluted with water to 90 ml, and mixed with 10 ml of 2 M acetate buffer, pH 3.2. The resulting solution of acid-denatured hemoglobin was used as the substrate in the assay system. In the assay of cathepsin D, the reaction mixtures containing 3 ml of substrate, 0.8 ml of 0.05 M acetate buffer or inhibitor solution and 0.2 ml of the enzyme solution (protein: 0.68~2.5 mg) were incubated for 30 minutes at 37°C. The enzyme reaction was stopped by the addition of 2 ml of 9% TCA. After further incubation for 30 minutes, the mixtures were filtered. The content of TCA-soluble peptide in each filtrate was measured in terms of optical density at 280 mμ. In the control runs, the enzyme was added to each reaction mixture after addition of TCA.

In evaluating the effect of inhibitors on these enzymes,[2] the per cent inhibition of peptidase reaction was calculated from the experiments described above, and the probit of the per cent inhibition was plotted against the logarithm of concentration of each inhibitor. The concentration of inhibitor required for 50% inhibition of enzyme activity, ID_{50}, was obtained from this plot and is shown in Table 1.

It is apparent from Table 1 that leupeptins strongly inhibit cathepsin B, a trypsin-like protease which splits α-N-benzoyl-L-arginine amide. Also cathepsin D, a pepsin-like acid protease, is specifically inhibited by pepstatin, while the other two inhibitors (leupeptins and chymostatin) are slightly inhibitory for this enzyme activity. It is interesting that chymostatin inhibits cathepsin B to an appreciable extent, while trypsin was not inhibited by this inhibitor[7]. Three inhibitors tested do not exhibit high degree of inhibition for the activity of cathepsin A which was shown to be a carboxypeptidase, although chymostatin seems to be slightly inhibitory against this enzyme.

Among the acid peptide-hydrolases (cathepsins A~E) present in lysosomal particles, cathepsin D plays a major role in proteolysis within the cells, and cathepsin B also seems to contribute to intracellular proteolysis under anaerobic conditions.[16] Iodice *et al.*[9] showed that the proteolysis by cathepsin D was considerably accelerated by the presence of cathepsin A. Thus three enzymes (cathepsins A, B and D) might be directly related to the intracellular digestion of protein. Cathepsin C, a dipeptidyl transferase, has a very narrow specificity as shown by Planta *et al.*[17] Its activity is restricted to the removal of N-terminal dipeptide units and the requirements for peptide donor and acceptor are very rigorous. According to Planta *et al.*[17], the high specificity of this enzyme renders its general role in intracellular protein catabolism unlikely. Cathepsin E is an acid protease which has a very low pH optimum (around 2.5)[13] that makes the physiological role of this enzyme less than cathepsin D.

Table 1.

Cathepsins	ID_{50} (μg/ml)		
	Leupeptins	Chymostatin	Pepstatin
A	1,680	62.5	>125
B	0.44	2.6	>125
D	109	49.0	0.011

For the reasons described above, we have investigated the effect of three inhibitors on cathepsins A, B and D. It should be noted that the lysosomal acid peptide-hydrolases are inhibited by the same inhibitors which inhibit extracellular peptide-hydrolases such as trypsin, chymotrypsin and pepsin.

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References

1) Aoyagi, T.; T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda & 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. Ishizaka, 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 synthesis of leupeptins Pr-LL and Ac-LL. Chem. Pharm. Bull. 17 : 1902~1909, 1969
5) Umezawa, H.; T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada & T. Takeuchi: Pepstatin, a new pepsin inhibitor produced by actinomycetes. J. Antibiotics 23 : 259~262, 1970
6) Morishima, H.; T. Takita, T. Aoyagi, T. Takeuchi & H. Umezawa: The structure of pepstatin. J. Antibiotics 23 : 263~265, 1970
7) Umezawa, H.; T. Aoyagi, H. Morishima, S. Kunimoto, M. Matsuzaki, M. Hamada & T. Takeuchi: Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. J. Antibiotics 23 : 425~427, 1970
8) Snellman, O.: Cathepsin B, the lysosomal thiol proteinase of calf liver. Biochem. J. 114 : 673~678, 1969
9) Iodice, A. A.; V. Leong & I. M. Weinstock: Separation of cathepsins A and D of skeltal muscle. Arch. Biochem. Biophys. 117 : 477~~486, 1966
10) Cocking, E. C. & E. W. Yemm: Estimation of amino acids by ninhydrin. Biochem. J. 58 : xii, 1954
11) Johnston, R. B.; M. J. Mycek & J. S. Fruton: Catalysis of transamidation reactions by proteolytic enzymes. J. Biol. Chem. 185: 629~641, 1950
12) Tallan, H. H.; M. E. Jones & J. S. Fruton: On the proteolytic enzymes of animal tissues X. Beef spleen cathepsin C. J. Biol. Chem. 194 : 793~805, 1952
13) Lapresle, C. & T. Webb: The purification and properties of a proteolytic enzyme, rabbit cathepsin E, and further studies on rabbit cathepsin D. Biochem. J. 84 : 455~462, 1962
14) Chaney, A. L. & E. P. Marbach: Modified reagents for determination of urea and ammonia. Clin. Chem. 8 : 130~132, 1962
15) Anson, M. L.; The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22 : 79~89, 1938
16) Ali, S. Y.: The presence of cathepsin B in cartilage. Biochem. J. 102 : 10c~11c, 1967
17) Planta, R. J.; J. Gorter & M. Gruber: The catalytic properties of cathepsin C. Biochem. Biophys. Acta 89 : 511~519, 1964