Communications to the editor

EFFECT OF ANTIPAIN ON LYSOSOMAL PEPTIDE-HYDROLASES FROM SWINE LIVER

Sir:

Since AOYAGI, TAKEUCHI and UMEZAWA[1~8] discovered several proteinase inhibitors in the culture filtrates of actinomycetes, the chemical properties of these inhibitors and the mode of action have been studied.

The effect of antipain, one of these inhibitors with the structure, [(S)-1-carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valyl-argininal, on various proteinases has been studied.[7,8]

In the previous report[9] the authors examined the effect of leupeptin, pepstatin and chymostatin on cathepsins A, B and D. In the present study, the effect of antipain on cathepsins was investigated in comparison with the inhibitors mentioned above. In addition, the effect of a partial degradation product (AN-HCl) of antipain was also examined. AN-HCl is (S)-1-carboxy-2-phenylethyl carbamoyl-L-arginine.

These acid peptidases were prepared from the lysosomal-mitochondrial pellet of swine liver, according to the method of SNELLMAN.[10] Fresh swine liver (1 kg) was cut into small pieces and homogenized with 2 liters of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. From the homogenate, the nuclear-debris fraction was removed by centrifugation of $700\times g$ for 10 minutes. The supernatant was further centrifuged at $7{,}000\times g$ for an hour. The resulting sediment (lysosomal-mitochondrial pellet) was suspended in 1 liter of cold acetone and dried *in vacuo*. Ten grams of the acetone powder was suspended in 50 ml of 0.1 M citrate buffer (pH 5.0) and dialyzed overnight against the same buffer. The dialyzed suspension was centrifuged and the supernatant was used as enzyme solution for the assay system.

For the determination of cathepsin A activity, the method of IODICE *et al.*[11] was used with some modifications: To glass-stoppered test tubes were added 0.5 ml of 0.015 M N^{α}-carbobenzoxy-L-glutamyl-L-tyrosine (substrate), 0.4 ml of 0.2 M acetate buffer (pH 5.0) or antipain (or AN-HCl) solution and 0.1 ml of the enzyme solution (protein: 0.66 mg). The reaction mixtures were incubated at 37°C for 2 hours. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (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 From the filtrates, 1 ml aliquots were withdrawn and used for ninhydrin determination[12] with appropriate level of tyrosine as a standard.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[13~15]. Reaction mixtures containing 1 ml of 0.1 M N^{α}-benzoyl-L-arginine amide (substrate), 0.5 ml of 0.1 M citrate buffer (pH 5.0) or antipain solution (protein: 4.31 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, 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 CONWAY's microdiffusion 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, a 0.5 ml aliquot was withdrawn from the central compartment, and the absorbed NH_3 was determined colorimetrically by the indophenol method of CHANEY and MARBACH[16]. Controls without enzyme were treated in the same way.

The determination of the activity of cathepsin D was performed at pH 3.2 using a modification of ANSON's method[17]. 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 1 N HCl and incubated for 30

minutes at 37°C. After incubation, the solution was adjusted to pH 3.2 with 1 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. The reaction mixtures containing 3 ml of substrate, 0.8 ml of 0.05 M acetate buffer (pH 3.2) or antipain solution and 0.2 ml of the enzyme solution (protein: 2.5 mg) were incubated for 30 minutes at 37°C. The enzyme reaction was stopped by the addition of 2 ml of 90% TCA. After further incubation for 30 minutes, the mixture was 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. The inhibitory effect of antipain and AN-HCl on cathepsins was expressed in terms of ID_{50}, the concentration of inhibitor that causes 50% inhibition of enzymatic hydrolysis, shown in Table 1.

It is apparent from the Table 1 that antipain strongly inhibits cathepsin B, a trypsin-like proteinase, as might be expected from the results with trypsin and papain. On the other hand, cathepsin D, a pepsin-like proteinase, is not inhibited to an appreciable extent. Interestingly, antipain inhibits the activity of cathepsin A, a carboxypeptidase, while other proteinase inhibitors from actinomycetes have no significant effect on cathepsin A. In this respect, antipain differs from leupeptins, which have no appreciable effect on cathepsin A. For comparison, the effects of AN-HCl on some proteinases were examined. The results are shown in Table 2. The tested proteinases are not significantly inhibited by AN-HCl. This substance has a structural similarity to carbobenzoxy-L-glutamyl-L-tyrosine, a substrate of cathepsin A, in that a free carboxyl group of an aromatic amino acid residue is present in both molecules of AN-HCl and the substrate. However, the activity of cathepsin A is not inhibited to an appreciable extent (ID_{50} = >125 μg). Thus it is concluded that the whole molecule of antipain is needed for the inhibition of cathepsin A.

Table 1. Effect of antipain and AN-HCl against cathepsins

Cathepsins	ID_{50} (μg/ml)	
	Antipain	AN-HCl
A	1.19	>125
B	0.595	>125
D	>125	>125

Table 2. Effect of antipain and AN-HCl against various proteases

Proteases	ID_{50} (μg/ml)	
	Antipain	AN-HCl
Plasmin	93	>250
Trypsin	0.26	>250
Papain	0.16	>250
α-Chymotrypsin	>250	>250
β,γ,δ-Chymotrypsin	>250	>250
Pepsin	>250	>250
Proctase A	>250	>250
Proctase B	190	>250
Cathepsin A	1.19	>125

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