ANTIPAIN, A NEW PROTEASE INHIBITOR ISOLATED FROM ACTINOMYCETES

Sir:

Specific enzyme inhibitors of microbial origin have been studied in the authors' laboratory and as reported in previous papers, leupeptins[1~5] inhibiting plasmin, trypsin, kallikrein and papain, pepstatin[6~9] inhibiting acid proteases, and chymostatin[10] inhibiting chymotrypsins have been isolated. These inhibitors were found in culture filtrates of various species of actinomycetes. In this paper, the isolation and properties of antipain are reported.

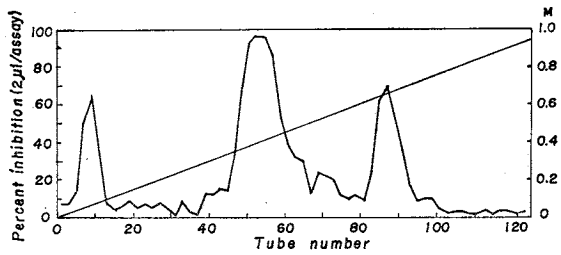
Antipain was found in culture filtrates of various species of actinomycetes and two strains were studied in detail: strain MB 561-C2 which resembles *Streptomyces michigaensis*[12~13]; strain MC829-AS1 which resembles *Streptomyces yokosukanensis*[14~15]. The same compound was also found by S. UMEZAWA *et al.*[11] in their study of SAKAGUCHI-positive products in a culture filtrate of strain KC84-AG13 which is closely related to *Actinomyces violascens*[16] and *Streptomyces mauvecolor*[17] and the structure was determined.

Activity of antipain was measured by the following method: one ml of 2.0 % casein solution, 0.5 ml of borate buffer (pH 7.4) and 0.3 ml of the same buffer with or without a test material were mixed and incubated for 3 minutes; then, 0.2 ml of a solution of papain (500 μg/ml, dissolved in the same buffer) was added. After the reaction mixture was incubated for 30 minutes at 37°C, 2.0 ml of 1.7 M perchloric acid was added and the extinction of the supernatant separated from the precipitate was read at 280 mμ; the percent inhibition was calculated by the formula $\frac{a-b}{a}\times 100$, wherein a is the absorbance of the reaction mixture without antipain, and b is the absorbance of the reaction mixture with antipain. Addition of 0.32 μg of antipain to the reaction mixture (that is 0.16 μg/ml) produced 50 % inhibition.

In a medium containing 2.0 % glucose, 1.0 % N-Z amine, 0.2 % yeast 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 antipain reached a maximum after 2~3 days in shake culture or 22~28 hours in tank fermentation. The pH of the broth and the percent inhibition of papain by 0.005 ml of the broth in an example were as follows: on 1st day, pH 7.7, 52 % inhibition; on 2nd day, pH 6.5, 87 %; on 3rd day, pH 6.5, 87 %, on 4th day, pH 8.3, 53 %, on 5th day, pH 8.6, 12 %; on 6th day, pH 8.5, 5 %; on 8th day, pH 8.8, 0 %.

When 6 liters of 72-hour cultured broth were inoculated in 300 liters of the medium described above in a 500-liter fermentor, after 25 hours at 28°C under aeration and stirring, the pH was 7.4 and 0.037 ml of a 10 times diluted culture filtrate produced 50 % inhibition. The filtrate was adjusted to pH 4.9 with 6 N HCl and passed through a column (48 liters, 20 cm in diameter) of Amberlite IRC-50 (H^+-form). After the column was washed with 150 liters of distilled water, antipain was eluted with 200 liters of 0.5 N HCl - 80 % MeOH. The active eluate (150 liters) was neutralized and concentrated and dried *in vacuo*. The dried active material was dissolved in 20 liters of methanol and the filtrate was concentrated *in vacuo* and lyophylized. The crude powder (30 g) thus obtained was dissolved in 300 ml of distilled water, and after adjusting to pH 7.0 this solution was subjected to the carbon column chromatography (1.2 liter), using 0.025 N HCl - 80 % MeOH. The active fraction was

Fig. 1. Chromatography on CM-Sephadex C-25.
Bed volume: 200 ml
Eluent: $HCOONH_4$ 0.01~1.0 M (linear)
Each fraction: 15 g



neutralized with Amberlite IR-45 (OH^--form) and evaporated *in vacuo*, yielding 5.0 g of a pale yellow powder. Addition of 7.5 μg of this powder to the reaction mixture produced 50 % inhibition of papain (ID_{50}=7.5 μg). The powder was further purified by CM-Sephadex C-25 column chromatography with a gradient of ammonium formate from 0.01 M to 1.0 M. Antipain appeared in the fraction obtained using 0.4 M ammonium formate concentration. Antipain in the eluate was adsorbed by carbon, and eluted with 0.05 N HCl - 80 % methanol. Evaporation of the eluate yielded antipain dihydrochloride.

Anal.: C 46.60, H 7.43, N 19.40, O 15.05, Cl 9.80.

Fig. 2. Ultraviolet absorption spectrum of antipain (1.16 mg/ml in water).

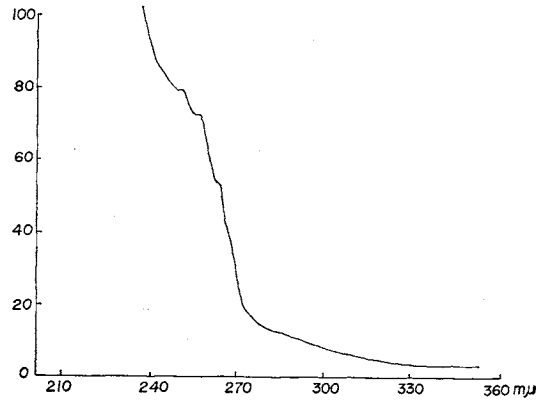
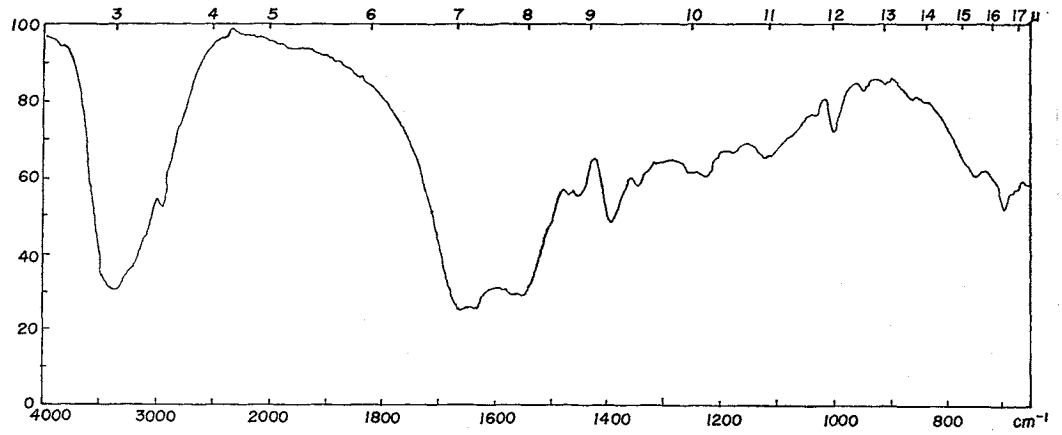


Table 1. Effect of leupeptin, pepstatin, chymostatin and antipain against various proteases

Enzymes	Substrates	ID_{50} (μg/ml)			
		Leupeptin	Pepstatin	Chymostatin	Antipain
Thrombokinase	Plasma	15	> 250	> 250	20
Thrombin	TAME[1]	10,000	> 250	> 250	> 250
Plasmin	Fibrinogen	8	> 250	> 250	93
Trypsin	Casein	2	> 250	> 250	0.26
Papain	Casein	0.5	> 250	7.5	0.16
Kallikrein	BAEE[2]	75	> 250	> 250	> 250
α-Chymotrypsin	Casein	> 500	> 250	0.15	> 250
β, γ and δ-Chymotrypsin	Casein	> 500	> 250	0.15	> 250
Pepsin	Casein	> 500	0.01	> 250	> 250
	Hemoglobin	> 500	0.0031	> 250	> 250
Proctase A	Casein	> 250	> 250	> 250	> 250
Proctase B	Casein	> 250	0.0072	26.5	190
Cathepsin A	Cb-Glut-Tyr[3]	1,680	> 125	62.5	
Cathepsin B	BAA[4]	0.44	> 125	2.6	
Cathepsin D	Hemoglobin	109	0.011	49.0	

1) α-N-(p-Toluenesulfonyl)-L-arginine methyl ester·HCl
2) α-N-Benzoyl-L-arginine ethyl ester·HCl
3) α-N-Carbobenzoxy-L-glutamyl-L-tyrosine
4) α-N-Benzoyl-L-arginine amide·HCl

Fig. 3. Infrared absorption spectrum of antipain hydrochloride (KBr).



Properties of antipain dihydrochloride are as follows: m. p. 170~177°C; $[\alpha]_D^{20}$ −10° (*c* 1, H_2O); maxima at 247 mμ ($E_{1cm}^{1\%}$ 7.09), 252 mμ ($E_{1cm}^{1\%}$ 6.83), 257 mμ ($E_{1cm}^{1\%}$ 6.29), 263 mμ ($E_{1cm}^{1\%}$ 4.64), 267 mμ ($E_{1cm}^{1\%}$ 3.48) in H_2O (Fig. 2). The IR spectrum is shown in Fig. 3. It is soluble in water, methanol and dimethylsulfoxide, less soluble in ethanol, propanol and butanol, insoluble in benzene, hexane, petroleum ether, ethyl ether and chloroform. It gives positive RYDON-SMITH, permanganate, SAKAGUCHI, and negative ninhydrin reactions. It gives the following Rf values in thin-layer chromatography: 0.4 by *n*-butanol - butyl acetate - acetic acid - water (4 : 2 : 1 : 1) on silica-gel G, 0.55 by *n*-butanol - ethanol - water (4 : 1 : 2) on cellulose (Avicel) and Rf 0.36 by *n*-butanol - ethanol - 17 % aqueous ammonia (3 : 1 : 2) on cellulose. It moves to the cathode in formic acid - acetic acid - water (25 : 75 : 900) under 3500 V electrophoresis for 15 minutes with an Rm value of 0.9 taking L-alanine as 1.0.

The activities of antipain inhibiting various proteases were examined using methods described in previous papers[2,8] and the results are shown in Table 1 together with the activities of leupeptin, chymostatin and pepstatin. In the structure reported by S. UMEZAWA *et al.* in another paper[11], an argininal moiety which has been found in leupeptin is also present in antipain. Comparing with leupeptin, antipain is more specific to papain and trypsin. Activity of antipain in inhibiting fibrinogenolysis by plasmin is much weaker than that of leupeptin. Antipain at 100 μg/ml showed no antibacterial and no antifungal activity. The intraperitoneal injection of 1.0 g/kg and the intravenous injection of 125 mg/kg to mice caused no death and the intravenous injection of 250 mg/kg caused death of all mice.

The effect of antipain on carrageenin edema was determined using the method described by WINTER *et al.*[18] Intraperitoneal injection of 50 mg/kg of antipain caused 40 % inhibition. Antipain, which inhibits thrombokinase, inhibits blood coagulation. This effect was studied in comparison with leupeptin and heparin. It showed a similar strength of inhibition as leupeptin and the type of inhibition is the same as leupeptin and different from heparin.

Isolation of leupeptin and antipain indicates that actinomycetes produce various types of protease inhibitors containing arginial.

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