CHYMOSTATIN, A NEW CHYMOTRYPSIN INHIBITOR PRODUCED BY ACTINOMYCETES

Sir:

Specific enzyme inhibitors of microbial origin have been studied in the authors' laboratory and as reported in previous papers, leupeptins inhibiting plasmin, trypsin, kallikrein and papain, and pepstatin[5,6] inhibiting pepsin have been isolated. Now we report the isolation from culture filtrates of actinomycetes of chymostatin which inhibits chymotrypsin and papain.

Actinomycetes produce proteases in their culture filtrates and inhibitors of these enzymes are obtained from various species, for instance, leupeptins were obtained from more than 11 species and pepstatin from *Streptomyces testaceus*, *Streptomyces argenteolus* var. *toyonakensis* and others. We have now isolated chymostatin from culture filtrates of 7 strains of actinomycetes, among which one was classified as *Streptomyces hygroscopicus* (the strain No. MC521-C8) and another as *Streptomyces lavendulae* (the strain No. MC524-C1).

For the determination of chymostatin, the method described by KUNITZ[7] was modified as follows: To 1.0 ml of 2 % casein (Wako Chemical Co., Osaka) in 0.025 M borate buffer of pH 7.4 (pH is adjusted to 7.4 with 1 N NaOH during dissolution of casein), 0.05 ml of 0.1 M $CaCl_2$ and 0.85 ml of the same buffer with or without a test material were added; after 3 minutes at 37°C, 0.1 ml of 5 μg of α-chymotrypsin in the same buffer was added and the reaction mixture incubated for 30 minutes at 37°C; 2.0 ml of 1.7 M perchloric acid was added, and after 1 hour at room temperature the extinction of the supernatant of the centrifuged reaction mixture was read at 280 mμ; from the standard curve of percent inhibition *versus* concentration, the amount of chymostatin in the test material was obtained. By this method, addition of 0.3 μg of chymostatin (0.15 μg/ml in the reaction mixture) showed 50 % inhibition.

Chymostatin was produced in shake culture and fermentation tanks in medium containing various carbon sources and nitrogen sources. Glycerol and glucose are examples of carbon sources and peptone, meat extract, yeast extract and soy-bean meal are examples of nitrogen sources suitable for production. The typical medium used for production contained 2.5 % glycerol, 0.5 % meat extract, 0.5 % peptone, 1.0 % yeast extract, 0.2 % NaCl, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % K_2HPO_4, 0.32 % $CaCO_3$ (pH 7.0). Production of chymostatin reached a maximum after 48~60 hours in shake culture or 24~40 hours in tank fermentation.

Chymostatin can be obtained from the culture filtrate by adsorption processes using activated carbon, but it can be more easily isolated by extraction with *n*-butanol. Chymostatin is also contained in the mycelium and in large amounts when the fermentation yield is high and can be extracted with methanol. For example, chymostatin was obtained by the following procedure: 1.5

Fig. 1. Ultraviolet absorption spectra of chymostatin (80 μg/ml in acetic acid)

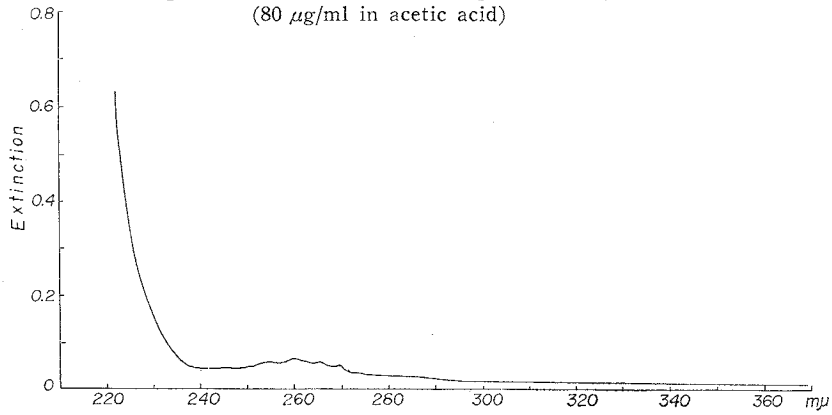
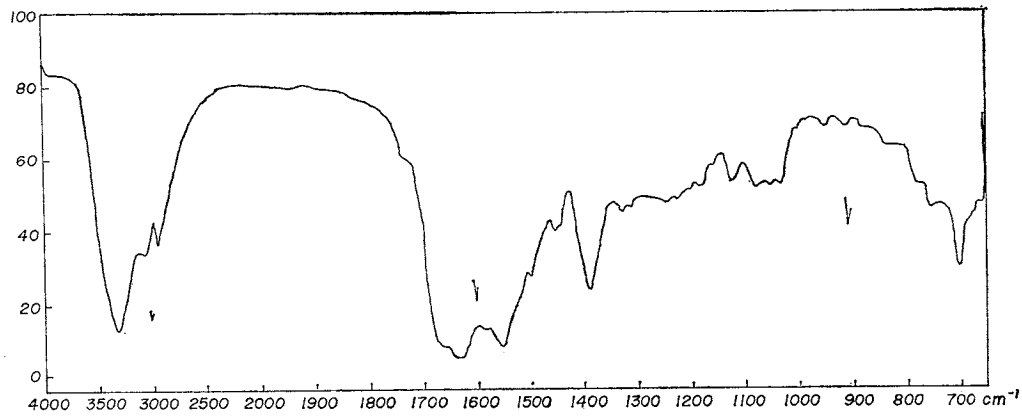


Fig. 2. Infrared absorption spectra of chymostatin (KBr)



liters of 27 hours cultured broth were inoculated to 140 liters of medium in a 200-liter fermenter; after 24 hours at 27°C under aeration and stirring, the pH was 4.8 and 0.05 ml of the 100 times diluted filtrate showed 38 % inhibition; the cultured broth was filtered with 3 % diatomaceous earth (Dicalite) and the cake was washed with 8 liters of water; the filtrate and the water used for washing were combined and extracted with 80 and 60 liters of *n*-butanol successively; the butanol extract, after washing with distilled water, was concentrated under reduced pressure and dried to a syrup, 200 g. The syrup was washed with 2 liters of ethyl acetate and dried, yielding 40 g of a crude powder of chymostatin (1.0 μg showed 50 % inhibition). Chymostatin was further purified by silica gel chromatography: 0.75 g of the powder (0.6 μg showed 50 % inhibition) was placed on a silica gel column (silica gel 70 g in butanol : water in the volume ratio of 9 : 1, diameter 2 cm) and eluted with the same solvent system. The eluate was fractionated into 7 g samples. The concentration and evaporation of No. 16~62 fractions yielded 0.4 g of a light yellow powder of chymostatin (0.4 μg showed 50 % inhibition). The colored impurity was removed by anion-exchange resin chromatography: 0.4 g of the powder (0.4 μg showed 50 % inhibition) was adsorbed on a column of Dowex 1 Cl-form (40 ml), and eluted with distilled water. The effluent was cut into each 5 g fraction. The evaporation of No. 20 ~70 fractions yielded 0.15 g of pure chymostatin. It was crystallized from methanol.

Table 1. Effect of chymostatin against proteases

Enzymes	Substrates	Chymostatin ID_{50} (μg/ml)
Thrombokinase	Plasma	>250
Thrombin	TAME[1]	>250
Plasmin	Fibrinogen	>250
Trypsin	Casein	>250
Papain	Casein	7.5
	BAA[4]	>250
Kallikrein	BAEE[2]	>250
Pepsin	Casein	>250
α-Chymotrypsin	Casein	0.15
	ATEE[3]	0.29
β-Chymotrypsin	Casein	0.15
γ-Chymotrypsin	Casein	0.15
δ-Chymotrypsin	Casein	0.2

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

Chymostatin was obtained as white crystals, m.p. 205~207°C (dec.). Anal.: C 57.95, H 7.19, N 15.13, O 17.11. It is optically active: $[\alpha]_D^{22}$ +9° (*c* 0.25, acetic acid). The ultraviolet absorption spectrum showed very weak maxima at 253 mμ, 260 mμ, 265 mμ and 270 mμ in acetic acid solution as shown in Fig. 1. The infrared absorption spectrum (Fig. 2), indicates a peptide nature.

It was soluble in acetic acid and dimethylsulfoxide, slightly soluble in water, methanol, ethanol, *n*-propanol, *n*-butanol, ethylene glycol, and insoluble in ethyl acetate, butyl acetate, ether, hexane, petroleum ether, chloroform and benzene. It gave a positive RYDON-SMITH reaction, but negative EHRLICH, naphthoresorcin, anisaldehyde-sulfuric acid

and ninhydrin reactions. Rf value on thin-layer chromatography on silica gel with butanol – methanol – water (4 : 1 : 2) was 0.45. On high-voltage paper electrophoresis (3,500 V, 15 minutes, formic acid – acetic acid – water 25 : 75 : 900), it moved toward the cathode, with an Rm value 0.40 (taking L-alanine as 1.0).

Chymostatin at 200 μg/ml showed no antibacterial and no antifungal activity except weak inhibiton of a strain of *Pseudomonas aeruginosa*. The intraperitoneal injection or the oral administration of 500 mg/kg caused no toxic reactions in mice.

Chymostatin, when tested by the method described above, showed strong inhibition against α, β, γ and δ-chymotrypsin with the concentration needed for 50 % inhibition shown in Table 1. The effects of chymostatin on trypsin, plasmin, thrombin, thrombokinase, kallikrein, pepsin and papain were tested by methods described in a previous paper[1,2]. As shown in Table 1, it showed 50 % inhibition of papain at a concentration of 7.5 μg/ml. Chymostatin at 250 μg/ml did not inhibit the other enzymes, except for a slight inhibition of kallikrein.

Chymotrypsin is known to hydrolyze bradykinin. However, its inhibitor, chymostatin reduces carrageenin edema[8]. The result of an experiment is shown in Table 2.

Since a peptide having the properties of chymostatin has not been previously reported, we believe that chymostatin is a new compound. The amino acid analysis of chymostatin indicated that valine, isoleucine, leucine, arginine and phenylalanine are present. The results varied with different samples and therefore it is possible that chymostatin is a mixture of compounds differing in amino acid content.

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Table 2. Inhibition by chymostatin of carrageenin-induced edema in the rat hind paw

Dose (mg/kg)	No. of rats	Route	% inhibition of edema			
			1 hr	3 hrs	5 hrs	24 hrs
100	5	i. p.	69.6	77.5	64.1	38.1
25	10	i. p.	30.0	49.7	41.3	30.4
12.5	5	i. p.	40.4	53.3	48.6	22.7
6.25	10	i. p.	17.5	50.2	41.9	16.5
3.1	10	i. p.	36.4	36.8	35.3	22.8
1.5	10	i. p.	30.1	31.5	29.2	11.8
0.78	5	i. p.	17.4	18.8	12.0	6.8
0.4	5	i. p.	10.9	9.5	7.4	−1.1

Rat : Wistar, Male ; Body weight 140~150 g.
Carrageenin : 0.1 ml of the 1 % solution.
ED_{30} = 1.5 mg/kg.
Volume increase of the control (15 rats) : 1 hr. 0.39 ml, 3 hrs. 0.88 ml, 5 hrs. 0.97 ml, 24 hrs. 0.65 ml.

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