COMPLESTATIN, A POTENT ANTI-COMPLEMENT SUBSTANCE PRODUCED BY *STREPTOMYCES LAVENDULAE*

I. FERMENTATION, ISOLATION AND BIOLOGICAL CHARACTERIZATION

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A new potent inhibitor of complement system, named complestatin, was isolated from the mycelium of *Streptomyces lavendulae* SANK 60477. Complestatin ($C_{61}H_{45}N_7O_{15}Cl_6$, MW 1,325) was a peptide compound having two unusual amino acids, D-(−)-4-hydroxyphenylglycine and D-(−)-3,5-dichloro-4-hydroxyphenylglycine. This compound inhibited the hemolysis of sensitized sheep erythrocytes (EA) mediated by guinea pig and human complement 50% at concentrations of 0.4 and 0.7 μg/ml, respectively, but did not trypsin and α-chymotrypsin activities at 200 μg/ml. When complestatin was administered intravenously to the sensitized guinea pigs, it strongly inhibited the systemic anaphylactic shock elicited by the antigen probably by blocking generation of anaphylatoxins (C3a and C5a).

Complement system in classical and alternative pathways plays an important role in host defense system against bacteria, viruses, parasites and other injurious stimuli[1]. Sequential activation of complement in both pathways results in not only deposition of C3b and membrane attack complexes on targets but also generation of biological active peptides (anaphylatoxins C3a and C5a) which stimulate chemotaxis of phagocytic cells, vascular permeability and release of various chemical mediators[2,3]. These biological activities of complement, however, are likely to be disadvantageous in some diseases such as systemic anaphylactic shock, systemic lupus erythematosus and glomerulonephritis, especially when antibodies are directed to self antigens[2~4]. One can expect the reduction of such allergic reactions by a specific inhibitor of complement system. For this purpose, various type of inhibitors of complement system have been investigated, which include a large number of protease inhibitors[5~8], non-steroidal anti-inflammatory agents[9], a microbial product K-76[10] and cobra venom factor[11]. Of these compounds, cobra venom factor and K-76 have been reported to be selective in inhibiting experimental allergic reactions[12,13].

During the course of searching for new microbial metabolites that inhibit complement system, a highly active principle, designated complestatin, was found to be produced by a strain of *Streptomyces lavendulae*. In this paper, the fermentation, isolation, physico-chemical and some biological properties of complestatin are described. The structural elucidation of complestatin and more details of the anti-complement activity will be published elsewhere.

Materials and Methods

Materials

Trypsin and α-chymotrypsin were obtained from Sigma Chemical Company. Leupeptin-1 and chymostatin were kindly supplied by DRS. T. AOYOGI and T. TAKITA, Institute of Microbial Chemistry

(Tokyo). Gelatin glucose veronal buffer containing Ca^{2+} and Mg^{2+} ($DGVB^{++}$) was prepared according to RAPP and BORSOS[14]. Fresh guinea pig serum and normal human serum which had been stored at −80°C were used as whole complement components.

Sheep erythrocytes (E) were obtained from Nippon Bio-Supply Center (Japan). Rabbit anti-sheep erythrocytes antibody (A) was obtained from Cordis Laboratories, Inc., (U.S.A.). The sensitized sheep erythrocytes (EA) were prepared as described previously[15].

Assay for Inhibition of Complement Mediated Hemolysis of EA

Complement activity in classical pathway was measured as follows. Guinea pig serum (0.1 ml) diluted at 200~500-fold in $DGVB^{++}$ (sufficient to cause approximately 63% hemolysis (Z=1)) was mixed with 0.1 ml of $DGVB^{++}$ with or without samples in glass test tubes. After preincubation for 10 minutes at 30°C, 1×10^7 EA in 0.1 ml $DGVB^{++}$ were added, and the incubation was continued for an additional 60 minutes at 37°C. After addition of 1.6 ml saline and following centrifugation for 5 minutes, the average number of hemolytic sites per cell (Z) was calculated by measuring A_{414} nm of the supernatant. Data were expressed by Z/Z_0 where Z_0 indicated the hemolytic sites obtained in control without samples, and one inhibitory unit was defined as amounts to inhibit by 50% ($Z/Z_0=0.5$) of control activity (Z_0). In some experiments, normal human serum was used instead of guinea pig serum.

Assay for Inhibition of Trypsin and α-Chymotrypsin Activities

The activities of trypsin and α-chymotrypsin were assayed by a modified ANSON's method[16]. Briefly, the reaction mixture (1.5 ml) containing 7.5 mg of casein and 15 μg of enzyme was incubated in the presence or absence of samples. After 30 minutes of incubation at 37°C, 1.5 ml of 10% TCA was added to each tube and the mixture was kept for 20 minutes at room temperature, followed by centrifugation at 1,000×*g* for 10 minutes. The supernatant was incubated with Folin reagent and A_{660} nm was measured. Enzyme activities were expressed as number of units that developed the color equivalent to 1 μmol of tyrosine per minute under standard conditions.

Results and Discussion

Fermentation

S. lavendulae SANK 60477 isolated from a soil sample in Shizuoka Prefecture, Japan, was used in this experiment. A loopful of well-grown agar slant culture of this strain was inoculated into a medium (100 ml) containing soluble starch 3%, meat extract 1%, Pharmamedia 1.5%, corn steep liquor 2% and Nissan Disfoam CB442 0.01% (anti-foaming agent) at pH 7.0 before sterilization in a 500-ml baffled Erlenmeyer flask, and cultured at 25°C on a rotary shaker at 220 rpm for 72 hours. The seed cultures were then transferred at the rate of 2% to 15 liters of the same medium in a 30-liter jar fermentor, and the cultivation was carried out at 25°C with aeration of 30 liters/minute and agitation at 310 rpm. A typical time course of the jar fermentation is shown in Fig. 1. Mycelial growth was expressed as a packed cell volume percent after centrifugation at 3,000 rpm for 10 minutes. Anti-complement activities (U) of culture broth (25 μl aliquot) was determined as described in Materials and Methods. The total inhibitory activities rapidly increased during late logarithmic phase of growth with a concomitant increase of pH in the medium and then reached a maximum level at 72~96 hours.

Isolation and Purification

After 4 days of incubation (15 liters), the time when the active principles were produced maximally, the mycelia (3.1 kg) was collected by filtration. The mycelia was extracted with 30 liters of 70% aqueous acetone with stirring, and the extract was concentrated *in vacuo* to 500 ml. The solution was acidified by conc HCl to pH 3.0, and the active principles were extracted with 500 ml of ethyl acetate.

Fig. 1. Time course of complestatin production in a 30-liter jar fermentator.

■ Packed cell volume, ○ pH, ● anticomplementary activity (u) of 25 μl of culture broth as measured in Materials and Methods.

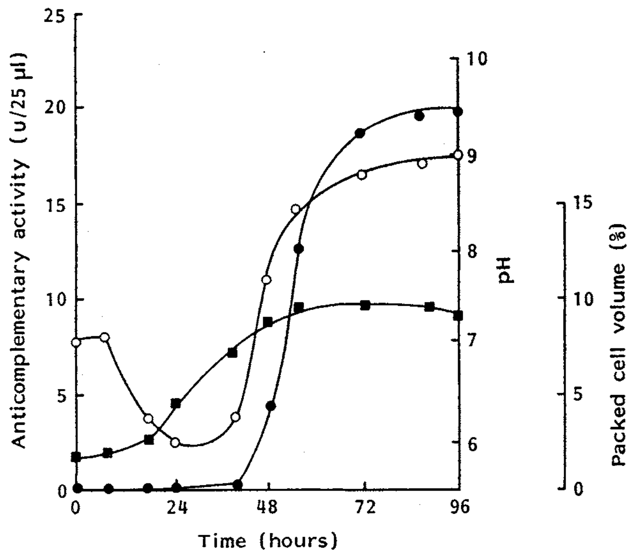
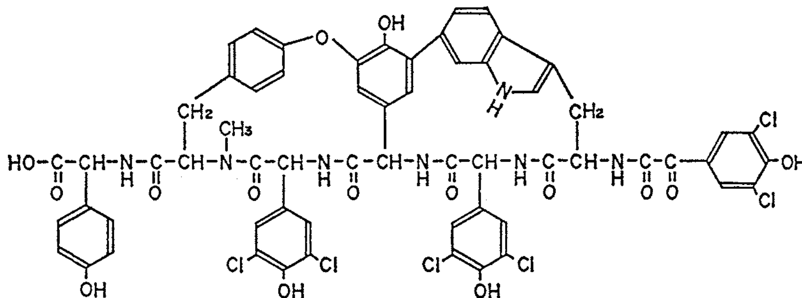


Fig. 2. Structure of complestatin.



The solvent layer was evaporated to dryness, and the dried materials were washed with chloroform and methanol successively. The resulting residue (363 mg) was applied to a column of silica gel (170 g, 3×50 cm) in ethyl acetate - methanol (5 : 1). Active principles were then eluted with ethyl acetate - methanol (1 : 1), and concentrated *in vacuo* to dryness. The residue (192 mg) dissolved in 0.01 M $NaHCO_3$ buffer (pH 8.3) was submitted to chromatography on Diaion HP-20 (4.5×26 cm) equilibrated with the same buffer. After washing, the main active fractions eluted with the mixture of acetone - 0.01 M $NaHCO_3$ (30 : 70) were precipitated at pH 2.0 by adding 10% H_3PO_4, giving 125 mg of complestatin as a yellow powder. This material was shown to be homogenous by TLC on a silica gel plate in ethyl acetate - methanol (2 : 1) and by a HPLC (ODS, H-2151, Senshu) in 45% aqueous CH_3CN - 0.2% TFA.

Physico-chemical Properties

Complestatin was acidic, stable to 100°C for 30 minutes at pH 7.0, and soluble in aqueous solution at alkaline pH, slightly in lower alcohols, acetone, pyridine, dimethyl sulfoxide and dimethylformamide but hardly in chloroform. The structure and physico-chemical properties of complestatin were shown

Table 1. Physico-chemical properties of complestatin.

Nature	Yellow powder
MP	>300°C (dec)
$[\alpha]_D^{24}$	+24.5° (*c* 0.13, MeOH - 0.01 N NaOH, 2:1)
UV λ_{max} nm (ε)	
MeOH, 0.01 N HCl - MeOH	282 (13,780), 292 (13,250)
0.01 N NaOH - MeOH	292 (10,600), 355 (12,190)
IR (KBr) cm^{-1}	3400, 1650, 1510, 1490
Molecular formula	$C_{61}H_{45}N_7O_{15}Cl_6$ (MW 1,325)
HRFAB-MS (*m/z*) M^+	
Calcd for:	1,325.1110
Found:	1,325.1060
Elemental analysis	
Calcd for:	C 55.14, H 3.41, N 7.38, Cl 16.01.
Found:	C 54.13, H 3.81, N 7.27, Cl 15.45.
Color reaction	
Positive:	Ehrlich, Liebermann
Negative:	Molisch, ninhydrin

HRFAB-MS: High-resolution fast atom bombardment mass spectra.

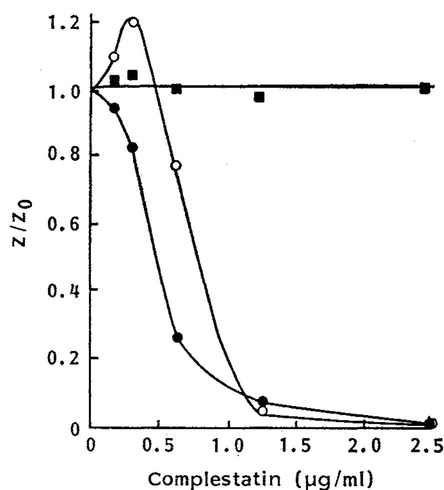
Table 2. Effect of complestatin on trypsin and α-chymotrypsin activities.

	Concentration (μg/ml)	Protease activity (% of control)
Trypsin:		
Complestatin	0	100.0
	10	109.4
	100	106.0
	200	106.2
Leupeptin-1	40	24.1
α-Chymotrypsin:		
Complestatin	0	100.0
	10	105.2
	100	110.1
	200	110.6
Chymostatin	20	14.2

Trypsin and α-chymotrypsin activities were determined by using casein as substrate as described in Materials and Methods. The data were expressed as percentage of control activity of each enzyme. Control activities of trypsin and α-chymotrypsin were 136 and 610 U (pmol tyrosine/minute/mg protein), respectively. Each value represents the average of duplicates.

in Fig. 2 and Table 1. The compound was characterized by UV spectrum at 355 nm in alkaline solution and by the constituents of unusual amino acids, 1 mol of D-(−)-4-hydroxyphenylglycine and 2 mol of D-(−)-3,5-dichloro-4-hydroxyphenylglycine, and a new compound, 3,5-dichloro-4-hydroxybenzoformic acid (Fig. 2).

Fig. 3. Inhibition by complestatin of hemolysis of EA mediated by classical pathway activation of guinea pig and human complement.



Guinea pig serum (●) diluted at 500-fold or normal human serum (○) diluted at 200-fold in 0.1 ml DGVB^{++} was preincubated for 10 minutes at 30°C with 0.1 ml DGVB^{++} containing various doses of complestatin, followed by addition of EA (1×10^7) in 0.1 ml DGVB^{++}. The incubation was continued for 60 minutes, and the hemolytic activity (Z) was determined as described in Materials and Methods. The hemolysis by H_2O (■) was obtained by using 0.1 ml H_2O instead of the serum dilution. Each value was the average of duplicate assays.

Biological Activities

Complestatin inhibited the hemolysis of EA mediated by guinea pig and human complement in classical pathway in a dose related manner, giving 50% inhibition at 0.4 and 0.7 μg/ml, respectively (Fig. 3). Thus, complestatin was far more potent compaired with known inhibitors such as flufenamic acid and leupeptin-1, assessed under the same conditions (Table 2). Complestatin had no direct hemolytic activity nor inhibitory effects on the hemolysis caused by distilled water (Fig. 3).

When guinea pig whole serum was treated for 10 minutes at 37°C with 10 μg/ml of complestatin, the treated serum, but not control serum, lost its hemolytic activity almost completely even after removal of free complestatin by gel filtration. Preincubation of guinea pig serum with complestatin produced a time dependent enhancement of the inhibitory activity. The results indicate that this substance irreversibly inhibited the complement activity in fluid phase.

Unlike anticomplementary protease inhibitors, complestatin produced no inhibitory effects on trypsin and α-chymotrypsin activities at concentrations where complement activity was completely bloked (Table 2), suggesting a specific action of complestatin. It is also unlikely that complestatin exerts its inhibitory effect through chelating divalent cations, Mg^{2+} and Ca^{2+}, since neither of them in molar excess reversed the inhibition. Extensive studies on the inhibitory action indicated that complestatin primarily blocked formation of classical C3 convertase (EAC14b+C2→EAC142a) (to be published elsewhere). Complestatin has been also shown to inhibit the alternative pathway of complement by blocking formation of the amplification C3 convertase, C3, Bb, P[17].

Although there are some similarity in the structures of complestatin and aglycone portions of glycopeptide antibiotics such as vancomycin and chloropolysporin[18,19], these typical antibiotics were not shown to be anticomplementary. Complestatin had no significant bactericidal activities.

Acute toxicity (LD_{50}) of complestatin was about 20~30 mg/kg of body weight when administered intravenously to guinea pigs, mice and rats, and more than 2,000 mg/kg when administered orally to these animals.

Table 3 shows the inhibitory effects of complestatin on systemic anaphylactic shock in sensitized guinea pigs after a single intravenous administration; Hartley male guinea pigs, weighing about 300 g, were sensitized with bovine serum albumin (BSA) as an antigen at a dosage of 20 mg/kg, subcutaneously. After 3 weeks when the anti-BSA antibodies reached a maximum level, complestatin in PBS was administered intravenously to the sensitized guinea pigs 15 minutes before intravenous injection of the same antigen (20 mg/kg). Under these conditions, most of control guinea pigs died within 5 minutes after injection of BSA antigen, accompanied with typical systemic anaphylactic shock (coughing, convulsion and cyanosis) which are primarily produced by anaphylatoxins C3a and C5a[2,3].

Table 3. Effect of complestatin on systemic anaphylactic shock in the sensitized guinea pigs.

Dose (mg/kg, iv)		Behavior		Mortality
		Coughing	Convulsion	
Complestatin	0	5/5	5/5	5/5
	0.1	4/5	2/5*	1/5**
	1.0	4/5	2/5*	1/5**

Complestatin in 0.1 ml PBS at doses indicated was injected into a peripheral vein of one hind leg. After 15 minutes, BSA antigen (20 mg/ml) was injected into a vein in the other leg, and their behavior in first 10 minutes were observed. Mortality was determined 24 hours after the antigen injection. The data were expressed as incidences/treated.

* $P<0.05\%$, ** $P<0.01\%$ in chi-square test.

Complestatin at doses of 0.1 and 1.0 mg/kg strongly prevented the animals from massive convulsion and death in systemic anaphylactic shock probably by blocking generation of the anaphylatoxins (Table 3). It should be noted that the effective dose was 200 times less than the toxic dose. Similar inhibitory effects were obtained on active cutaneous anaphylaxis in guinea pigs. Complestatin, however, had no significant effects on carrageenin-induced edema, a nonspecific inflammation model, in rats.

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