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Production, Purification and Characterization of Two Proteinaceous Hen-Egg-White Lysozyme Inhibitors from *Pseudomonas aeruginosa* M-1001

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ABSTRACT

Two proteinaceous lysozyme inhibitors, hen-egg-white lysozyme inhibitors F-I and F-II, were isolated from the culture broth of a bacterial strain identified as *Pseudomonas aeruginosa* M-1001. Maximum lysozyme inhibitory activity was obtained when the bacterium was grown aerobically in a medium consisting of 0.25% glucose, 0.25% beef extract, 0.25% polypepton, 1.0% sodium L-glutamate, and 1.0% soluble starch (pH 7.0) at 37°C after 20-24 hrs. F-I and F-II were purified 20 and 7.5-fold, respectively, from the culture supernatant of *P. aeruginosa* M-1001 by ammonium sulfate fractionation, DEAE-Sepharose CL-6B column chromatography, and Sephacryl S-200 gel chromatography. The molecular weights of F-I and F-II were estimated to be about 57,000 and 33,000, by SDS-PAGE, respectively. F-I was stable in a pH range between 6 and 10 and below 50°C. F-II was stable in a pH range between 6 and 11 and below 40°C. Many Gram-positive bacteria were found to be inhibited by the crude lysozyme inhibitors.

Key Words: lysozyme; lysozyme inhibitor; antibiotic; *Pseudomonas aeruginosa*.

I. Introduction

The chemical structure of the bacterial cell wall is responsible for its rigid nature. The backbone of the cell wall is the macromolecule known as the peptidoglycan, consisting of two major subunits, amino sugars and amino acids. It is possible to destroy the cell wall by treating the cell with lysozyme, an enzyme that cleaves the chemical bonds between the N-acetylglucosamine subunits. Since Fleming found a lysozyme [EC 3.2.17] in 1922, similar enzymes have been shown to be widespread in animals (Jolles and Jolles, 1967; Jolles and Jolles, 1984; Leculier *et al.*, 1992; Osserman *et al.*, 1974; Buss, 1971; Canfield and McMurry, 1967; Fernandez-Sousa *et al.*, 1977a; Grinde *et al.*, 1988), plants (Audy *et al.*, 1988; Bernier, 1971; Ereifej and Markakis, 1980; Glazer *et al.*, 1969; Howard and Glazer, 1967; Meyer *et al.*, 1946), and microorganisms (Aoki *et al.*, 1985; Coyette and Shockman, 1973; Kamei *et al.*, 1988; Morita *et al.*, 1978; Osserman *et al.*, 1974). Lysozymes from different sources can be divided into the following classes: 1) primary muramidases with only slight chitinase activity (e.g., hen egg white lysozyme); 2) pure muramidases (e.g., goose egg white lysozyme); and 3) primary chitinase with slight muramidase activity (e.g., plant lysozyme) (Murao *et al.*, 1990; Osserman *et al.*, 1974). These enzymes, which react against living cells of microorganisms, have many potential uses as preservatives, medicaments, biochemical reagents, etc. (Aoard, 1966; Chander and Lewis, 1980; Fisher *et al.*, 1989; Garibaldi, 1960; Hughey and Johnson, 1987; Hughey *et al.*, 1989; Kim *et al.*, 1988; Murao *et al.*, 1990; Opel and Polzhofer, 1983; Razzakov *et al.*, 1988; Samuelson *et al.*, 1985; Vakil *et al.*, 1970; Vlasov *et al.*, 1988; Wasserfall and Teuber, 1979; Yajima *et al.*, 1968). Lysozyme is not only a hydrolysis enzyme, but also is important in physiological functions. Its physiological functions and control mechanisms was not yet known completely.

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Table 1. Media for Isolation

Medium A		Medium B	
Glucose	0.6%	Glucose	0.65%
Beef extract	0.4%	Beef extract	1.0%
Polypepton	0.4%	Polypepton	1.0%
NaCl	0.3%	Sodium L-glutamate	0.5%
Agar	2.0%	Agar	2.0%
pH	7.0	pH	7.0
Medium C		**Medium D**	
Beef extract	1.0%	Glucose	0.25%
Polypepton	1.0%	Beef extract	0.25%
NaCl	0.3%	Polypepton	0.25%
Agar	2.0%	Sodium glutamate	0.25%
pH	7.0	Agar	2.0%
		pH	7.0

Lysozyme inhibitors are not only useful in understanding their enzymatic mechanisms and physiological roles, but their pharmacological importance as well. Cellulose and chitin, both widespread organic compounds in nature, have similar structures with peptidoglycan, and are possibly hydrolyzed by same enzymatic reaction mechanisms as is lysozyme (Murao *et al.*, 1987). In this study, microorganisms in the soil of Taiwan were systematically screened to isolate proteinaceous lysozyme inhibitor producing strains. Identification of the isolated strain, medium composition, purification and characterization of the inhibitors were also investigated.

II. Materials and Methods

1. Isolation and Screening of Lysozyme Inhibitor Producing Bacterium

Microorganisms isolated from soils collected at three different locations in Taiwan were cultivated on medium A and B (Table 1) at 37°C for 2-3 days. Those colonies that grew well under such conditions were subcultured in medium D (Table 1) in shaken flasks at 37°C and 180 rpm. After incubation for 2 days, the culture broth was centrifuged, and the supernatants were collected for measurement of lysozyme inhibitor activities. Four strains out of 500 isolates showed high inhibitory activities in the culture broth. Among these four, the strain M-1001, which was isolated from soils collected from the central part of Taiwan, showed the highest capability of producing lysozyme inhibitor. This strain was maintained on medium C (Table 1) and used in the entire experiment for the production of lysozyme inhibitor.

2. Identification of Strain M-1001

After study of the morphological, cultural, biochemical and physiological characteristics of the organisms, identification was done in accordance with the method described in Bergey's *Mannual of Determinative Bacteriology* (Krieg and Holt, 1984).

3. Basal Medium Used for Optimal Medium Study

The basal medium used for optimal medium study consisted of 0.25% glucose, 0.25% beef extract, 0.25% polypepton and 0.25% sodium L-glutamate (pH 7).

4. Time Course of Inhibitor Production

Strain M-1001 was aerobically cultured at 37°C for 20-24 hrs on a rotary shaker in a 250 mL Erlenmeyer flask containing 100 mL of medium consisting of 0.25% glucose, 0.25% beef extract, 0.25% polypepton, 1.0% sodium L-glutamate, and 1.0% soluble starch, pH 7.0. The culture broth was centrifuged at 10,000 x *g* in a refrigerated centrifuge (J2-21M/°E, Backmen, U.S.A.) for 10 min, and the cell-free solution was used as the crude enzyme inhibitor.

5. Preparation of Substrates

Micrococcus lysodeikticus CCRC 11055 was aerobically cultured at 30°C for 24 hr on a rotary shaker in a 250 mL Erlenmeyer flask containing 150 mL of medium of 0.5% glucose, 1.0% beef extract, 1.0% polypepton, and 0.3% NaCl (pH 7.0). Cells were separated from the culture by centrifugation at 10,000 x *g* for 10 min. After washing twice with cold deionized water, the sedimented cells were lyophilized and stored at 4°C until used.

6. Enzymes

Hen egg white lysozyme was purchased from the Sigma Chemical Co., USA.

7. Assay System for Inhibitory Activity

Inhibitory activity was measured from the residual activity of hen-egg-white lysozyme, which was based on the decrease in turbidity due to lysis of *M. lysodeikticus* cells. Lyophilized cells of *M. lysodeikticus* were first suspended in 50 mM sodium phosphate buffer (pH 7.0) to give an initial O.D. of 1.7 at 660 nm using a spectrophotometer (Beckmen UD-70).

One-half mL of lysozyme solution (0.45 μg of lysozyme in 50 mM sodium phosphate buffer, pH 7.0) was added to 0.5 mL of inhibitor solution. This mixture was incubated at 37°C for 10 min. The enzymatic reaction was started by adding 1.5 mL of cell suspension, and the mixture was incubated at 37°C for 30 min. Inhibitory activity was calculated as a percentage of the inhibition of lysozyme activity by the following equation:

$$[(S-C)/(B-C)] \times 100(\%),$$

where S is the O.D. of the reaction mixture at 660 nm while C and B are O.D.'s for enzyme control and the substrate blank, respectively (Murao *et al.*, 1990). One unit of inhibitory activity was defined as the amount of inhibitor required to decrease the lysozyme activity by 50% under the assay conditions.

8. Molecular Weight

The molecular weight of the lysozyme inhibitor was estimated by SDS-PAGE using the method of Weber and Osborn (1969). The gels were stained with Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (5:1:5, v/v), and decolorizèd in 7% acetic acid.

9. Antibacterial Action of Lysozyme Inhibitors

The antibacterial action of lysozyme inhibitor was examine against both Gram-negative and Gram-positive bacteria. The crude lysozyme inhibitor solution was brought to 80% saturation with ammonium sulfate. The resultant precipitate was collected by centrifugation and then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7.0), followed by dialysis against the same buffer. The resultant dialyzate would be used as the lysozyme inhibitor for the measurement of growth inhibition, and buffer without dialyzate used also as a blank for the control experiment. The test bacteria used were *Lactobacillus lactis* CCRC 10791, *Staphylococcus aureus* CCRC 10451, *S. aureus* CCRC 10777, *Bacillus subtilis* CCRC 10029, *B. subtilis* CCRC 14689, *Escherichia coli* CCRC 11549, *Pseudomonas aeruginosa* K-187 and *P. aeruginosa* M-1001, which all were purchased from the Culture Collection and Research Center, Taiwan, except for *P. aeruginosa* K-187 (a strain isolated as a chitinase producer by Wang *et al.*, 1995) and *P. aeruginosa* M-1001 (this study).

III. Results

1. Identification of Strain M-1001

Table 2. General Characteristics of *P. aeruginosa* M-1001

Morphological characteristics	
Form	Rods, rounded ends
Size	0.3 – 1.0 × 1.5 – 3.0 μm
Mobility	Motile
Flagellum	Single polar
Gram stain	Negative
Spore	Non-sporulating
Fluorescein production	Positive
Culture characteristics	
Agar colonies	Circular, convex, smooth, entire, dull, brown
Agar slant	Echinulate to spreading
Broth	Turbid, pellicle, sediment
Gelatin stab	Infundibuliform liquefaction
Physiological properties	
BPC milk	coagulated, slightly acidic
Nitrate reduction	Nitrite and gas
Ammonia production	Negative
H2S production	Negative
Indole formation	Negative
Oxygen demand	Strictly aerobic
Catalase	Positive
Oxidase	Positive
Urease	Positive
Lecithinase	Positive
Arginine dihydrolase	Positive
Starch hydrolysis	Negative
Casein hydrolysis	Positive
Gelatin hydrolysis	Positive
Tween 80 hydrolysis	Positive
Acid is produced from	Arabinose, cellobiose, glucose, fructose, inositol, maltose, raffinose, xylose
Tyrosine utilization	Positive
Citric acid can be used as the sole source of carbon	
Malonic acid can be used as the sole source of carbon	

The bacteriological characteristics of strain M-1001 are shown in Table 2. Referring to the primary keys in Bergey's *Manual of Determinative Bacteriology*, strain M-1001 of this study was identified as a strain of *Pseudomonas aeruginosa*.

2. Effect of Carbon and Nitrogen Sources

Growth proceeded on basal medium containing either no additional carbon sources or with glucose, lactose, sucrose, soluble starch, carboxymethyl cellulose (CMC), pulp (toilet paper), molasses, rice bran, or sugar cane bagasse (a gift of the Taiwan Sugar Company, Chi-Hu, Taiwan, R.O.C.), respectively. The concentration of the carbon source used was adjusted to 0.25%. The flasks were removed after 2 days of incubation for inhibitor assays. The production of inhibitor by *P. aeruginosa* M-1001 was slightly enhanced by soluble starch incorporated into

Table 3. Effect of Carbon Source on Lysozyme Inhibitor Produced by *P. aeruginosa* M-1001

Carbon sources	Concentration (%)	Relative activity (%)
None	0	100
Glucose	0.25	95
Lactose	0.25	96
Sugar	0.25	91
Soluble starch	0.25	104
Carboxymethyl cellulose	0.25	78
Pulp	0.25	84
Molasses	0.25	8
Rice bran	0.25	30
Sugar cane bagasse	0.25	36

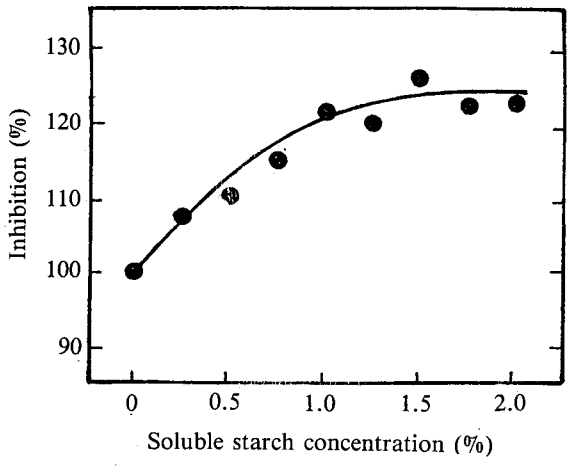

Fig. 1. Effect of soluble starch as a carbon source on lysozyme inhibitor produced by *P. aeruginosa* M-1001.

the medium. On the other hand, addition of other carbohydrates such as molasses, rice bran, or bagasse repressed inhibitor production tremendously (Table 3). A slightly increase in inhibitor production were observed by increasing the soluble starch from 0 to 1.0% (Fig. 1).

The effect of different nitrogen sources on inhibitor production was tested in basal medium containing 1.0% soluble starch. The nitrogen sources were applied at 0.25% of beef extract, polypepton, yeast extract, or sodium L-glutamate. The flasks were removed after 2 days of incubation for inhibitor assays. As shown in Table 4, the secretion of inhibitors was slightly influenced by the nitrogen source incorporated into the medium. Production of inhibitor was slightly higher in medium containing sodium L-glutamate or yeast extract. A slight increase in inhibitor production was observed by increasing the sodium L-glutamate from 0 to 1.0% (Fig. 2).

Table 4. Effect of Nitrogen Source on Lysozyme Inhibitor Produced by *P. aeruginosa* M-1001

Nitrogen sources	Concentration (%)	Relative activity (%)
None	0	100
Beef extract	0.25	98
Polypepton	0.25	93
Sodium L-glutamate	0.25	108
Yeast extract	0.25	107
Sodium nitrate	0.25	87

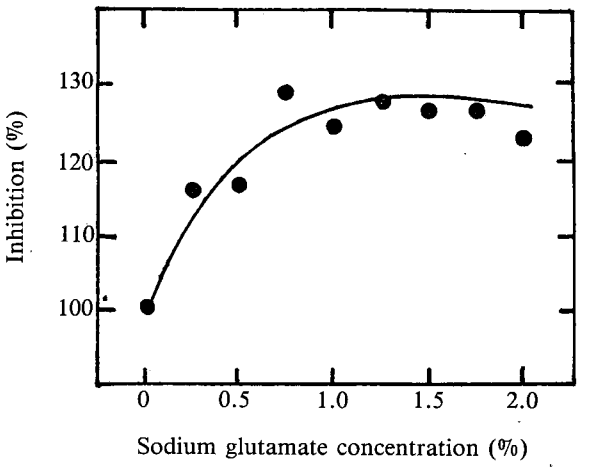

Fig. 2. Effect of Na-L-glutamate as a nitrogen source on lysozyme inhibitor produced by *P. aeruginosa* M-1001.

3. Effect of Initial pH and Cultivation Time

Growth was carried out on basal medium containing 1.0% soluble starch and 1.0% sodium L-glutamate. The media were adjusted with NaOH and HCl to pH 3-11. The flasks were removed after 12 hrs, 24 hrs, 36 hrs, 48 hrs, and 60 hrs incubation for assays of inhibitory activities. The results are shown in Table 5. The strain M-1001 produced the highest inhibitory activity when the initial pH and cultivation time were pH 7 and 24 hrs, respectively.

4. Effect of Temperature and Culture Volume

Growth occurred on the medium as described above (pH 7). The flasks were removed after 24 hrs of incubation at 24, 32, 35, 40, and 45°C for assays of inhibitory activities. Erlenmeyer flasks (250 mL) containing 50, 75, 100, 125, 150, 175, 200, and 225 mL of the culture media were incubated with shaking (180 rpm) at 37°C. The flasks were removed after 24 hrs of incubation for assays of inhibitory activities.

Table 5. Effect of Initial pH and Cultivation Time on Lysozyme Inhibitor Produced by *P. aeruginosa* M-1001

Initial pH	Relative activity (%)					
	0	12	24	36	48	60*
3	0	0	0	0	0	0
	(3.0)	(3.0)	(3.0)	(3.0)	(3.0)	[3.0]
4	0	0	0	0	0	0
	(3.7)	(3.7)	(3.7)	(3.8)	(3.9)	[3.9]
5	0	13	73	79	97	67
	(4.9)	(7.3)	(8.2)	(8.3)	(8.4)	[9.0]
6	0	50	96	99	99	69
	(4.8)	(7.6)	(8.3)	(8.5)	(8.3)	[9.2]
7	0	87	100	96	99	62
	(7.0)	(7.8)	(8.2)	(8.6)	(8.3)	[8.7]
8	0	60	92	99	98	45
	(7.8)	(7.7)	(8.3)	(8.6)	(8.2)	[9.0]
9	0	66	73	91	92	86
	(8.8)	(8.7)	(8.1)	(8.4)	(8.3)	[9.0]
10	0	18	51	58	89	70
	(9.7)	(9.4)	(9.3)	(9.2)	(8.3)	[8.6]
11	0	0	0	0	0	0
	(10.7)	(10.7)	(10.8)	(10.6)	(10.6)	[10.6]

( ): pH values at middle stage
[ ]: pH values at final stage
*: cultivation time (hours)

Table 6. Effect of Cultural Temperature on Lysozyme Inhibitor Produced by *P. aeruginosa* M-1001

Cultural temperature (°C)	Relative activity (%)
24	0
32	10
35	70
37	100
40	60
45	10

The basal medium consisted of 0.25% glucose, 0.25% beef extract, 0.25% polypepton, 1.0% sodium L-glutamate and 1.0% soluble starch (pH 7) in distilled water. The solution was autoclaved at 121°C for 20 minutes and cultured for 48 hrs on a rotary shaker (180 rpm).

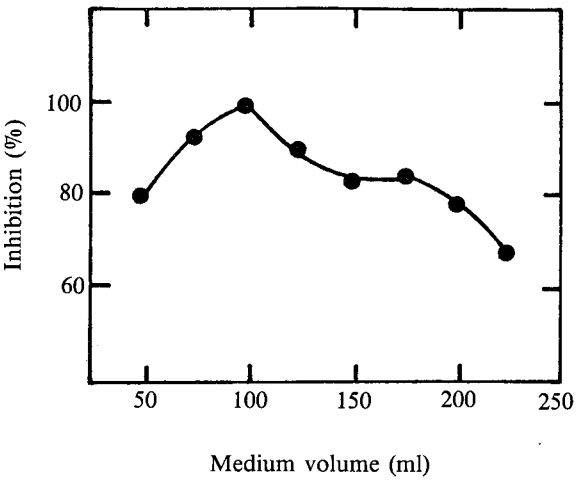

Fig. 3. Effect of the culture volume on lysozyme inhibitor produced by *P. aeruginosa* M-1001.

As shown in Table 6, the strain M-1001 produced the highest inhibitory activity at 37°C. The optimal culture volume for inhibitor production was 100 mL (Fig. 3).

5. Course of Inhibitor Production

P. aeruginosa M-1001 was grown aerobically in 100 mL of the optimum medium in a 250 mL Erlenmyer flask at 37°C. During the process of incubation, the lysozyme inhibitor, cell growth, and pH in the broth were measured every 12 hrs. A typical course of inhibitory activity formation is shown in Fig. 4; the activity reached a level maximum in 20-48 hrs of growth and dropped slowly thereafter. The pH increased steadily to a value of about 9.3. A comparison of inhibitory activity, cell growth and pH between the optimum and initial cultivation was also carried out. As shown in Fig. 4, the inhibitory activity increased from 1.63 U/mL to 1.87 U/mL after medium studied.

6. Purification of Inhibitor

Step 1: Precipitation with ammonium sulfate.

The crude enzyme inhibitor solution (2460 mL) was brought to 80% saturation with ammonium sulfate. This was followed by sedimentation overnight. The resultant precipitate was collected by centrifugation and then dissolved in 290 ml of 50mM sodium phosphate buffer (pH 7.0), followed by dialysis against the same buffer. The ammonium sulfate precipitate was difficult to redissolve in buffer after dialysis; therefore, a sample was collected after dialysis by centrifugation at 10,000 x *g* for 10 min. Because of the difficulty of recovering a sample in the dialysis step, the yield was low, and the supernatant fraction at this point was found to have 1232 units.

Step 2: DEAE-Sepharose CL-6B column chromatography.

The resultant dialyzate (360 ml) was divided into three equal portions. Each portion was put on a DEAE-Sepharose CL-6B (5 × 17 cm) which had been equilibrated with 50 mM sodium phosphate

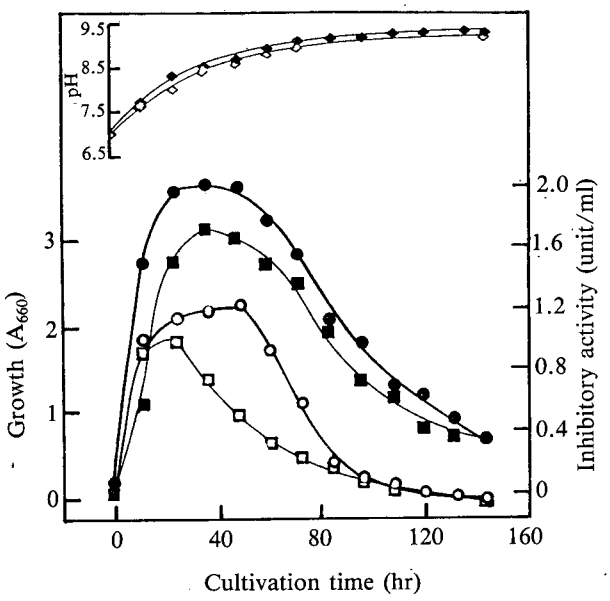

Fig. 4. A comparison of the inhibitory activity and microbe growing pH between optimun and initial cultivation.
●: Inhibitory activity of optimun cultivation.
○: Microbe growth of optimun cultivation.
◇: pH change of optimun cultivation.
■: Inhibitory activity of initial cultivation.
□: Microbe growth of initial cultivation.
◆: pH change of initial cultivation.

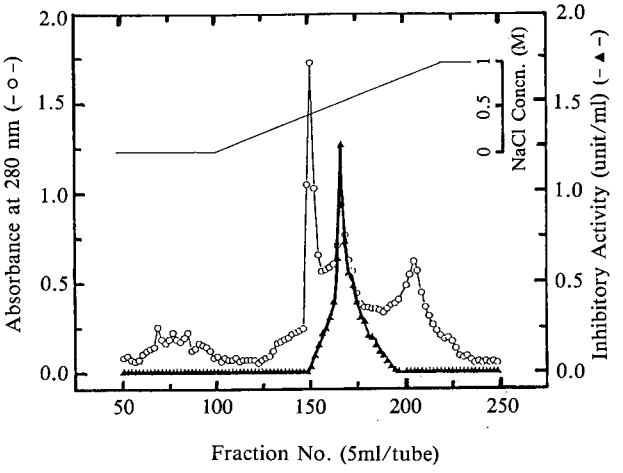

Fig. 5. A typical elution profile of the inhibitory activity measured by DEAE-Sepharose CL-6B column chromatography.

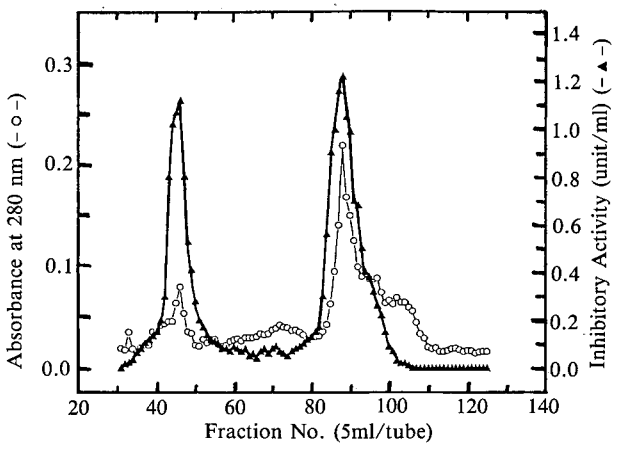

Fig. 6. A typical elution profile of the inhibitory activity measured by Sephacryl S-200 gel chromatography.

buffer (pH 7.0). The unadsorbed proteins were washed from the column with 50 mM sodium phosphate buffer (pH 7.0), and the inhibitor was then eluted with a linear gradient of 0 to 1.0 NaCl in the same buffer (300 mL in each reservoir). The effluent was fractionated into 5 mL portions. A typical elution pattern is shown in Fig. 5. The active fractions were combined, and ammonium sulfate was added to 80% saturation. The resultant precipitate was collected by centrifugation, dissolved in 17 mL of sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight.

Step 3: Sephacryl S-200 gel chromatography.

The resultant dialyzate (30 mL) was divided into three equal portions. Each portion was subjected to gel filtration on a column of Sephacryl S-200 (2.5×10^7 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Elution was made with the same buffer, and the effluent was fractionated into 5 mL portions. A typical chromatographic pattern obtained by gel filtration is illustrated in Fig. 6. The inhibitory activity presented at two fractions (F-I and F-II). The elution pattern of the gel filtration exibited a symmetrical protein peak accompanied by inhibitory activity in a completely constant ratio. The inhibitor fractions with equal specific activity were combined. The purification procedures are summarized in Table 7. The inhibitory activity recovered in the purified preparations of F-I and F-II were 4.0 and 9.3% of that present in the ammonium sulfate precipitate, with a 20- and 7.5-fold increase in specific activity, respectively. These purified inhibitors were used in the following experiments.

7. Molecular Weight

The molecular weights of F-I and F-II were estimated by SDS-PAGE to be about 57,000 and 33,000, respectively (Fig. 7).

Table 7. Summary of the Purification of Inhibitors from the Culture Supernatant of *P. aeruginosa* M-1001

Step	Volume (mL)	Total protein (A280)	Total activity (Unit)	Specific activity (Unit/A280)	Yield (%)
Culture supernatant	2,460				
$(NH_4)_2ASO_4$ ppt (80%)	290	5,178	3,778	0.72	100.0
Desalted (susp.)	360	1,325	1,232	0.93	33.1
DEAE-Sepharose CL-6B	364	257	455	1.77	12.1
$(NH_4)SO_4$ ppt (80%)	17	245	443	1.81	11.7
Desalted Sephacryl S-200	30	222	432	1.94	11.4
F-I	140	10	147	14.00	4.0
F-II	280	67	350	5.14	9.3

8. Thermal Stability

The thermal stability of F-I and F-II was measured by heating F-I and F-II at various temperatures for 10 min in a 50 mM sodium phosphate buffer (pH 7.0) solution. Residual inhibitory activity was measured as described in Materials and Methods. As shown in Fig. 8, F-I and F-II were stable up to 50°C and 40°C, respectively.

9. pH Stability

The pH stability of F-I and F-II was measured by incubating F-I and F-II in buffer solutions of various pHs at 37°C for 16 hrs. The buffer systems used were HCl-acetate (20 mM, pH 2-3), acetate (20 mM, pH 4-6), phosphate (20 mM, pH 7-8), and H_3BO_3-Na_2CO_3 (20 mM, pH 9-11). Residual inhibitory activity was measured at pH 7 as described in Materials and Methods. As shown in Fig. 9, F-I and F-II were stable between pH 6-10 and 6-11, respectively.

10. Antibacterial Activity of Lysozyme Inhibitors

The antibacterial activity of lysozyme inhibitor was examined against both Gram-negative and Gram-positive bacteria. The test bacteria used were *Lactobacillus lactis* CCRC 10791, *Staphylococcus aureus* CCRC 10451, *S. aureus* CCRC 10777, *Bacillus subtilis* CCRC 10029, *B. subtilis* CCRC 10051, *B. cereus* CCRC 14689, *Escherichia coli* CCRC 11549, *Pseudomonas aeruginosa* CCRC 11864, *P. aeruginosa* CCRC 11633, *P. aeruginosa* K-187 and *P. aeruginosa* M-1001. Cells

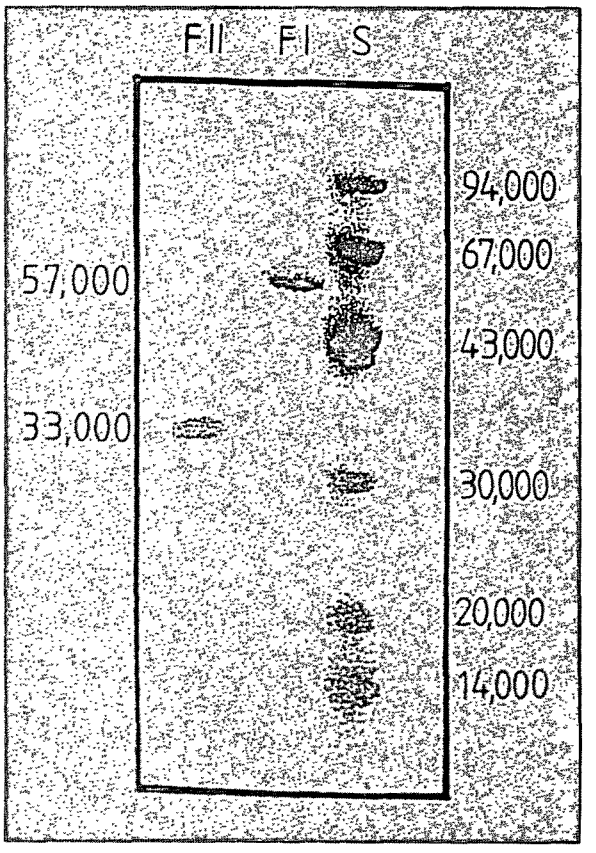

Fig. 7. SDS-PAGE analysis of purified inhibitors; lanes 1 to 3 (from right to left) are the low molecular weight standards (Pharmacia), F-I and F-II, respectively.

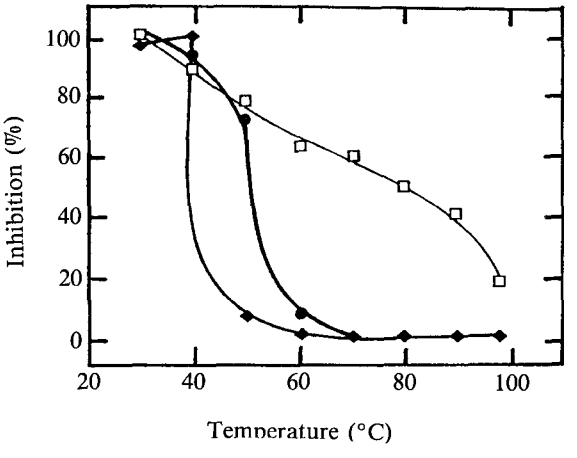

Fig. 8. The thermal stability of lysozyme inhibitors. □, crude inhibitor; ●, F-I; ◆, F-II.

of the organism were sprayed on molten nutrient agar medium after being poured into petri plates. Paper discs were placed onto the surface of the medium,

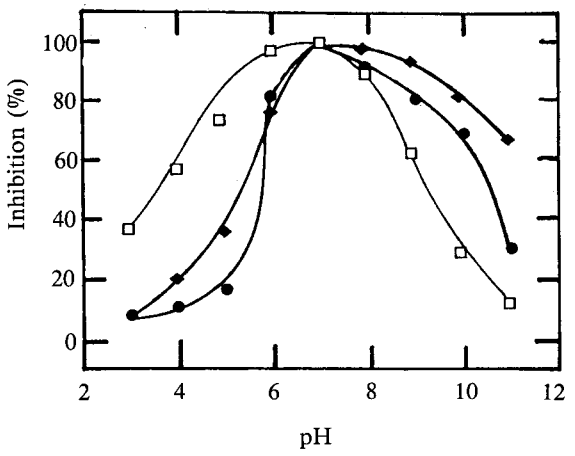

Fig. 9. The pH stability of lysozyme inhibitors. □, crude inhibitor; ●, F-I; ◆, F-II.

and lysozyme inhibitor to be assayed was pipetted into each disc. After incubation for 18 hrs, the susceptible cells grew uniformly in the medium except those where antibiotic had diffused into the medium. Antibacterial activity was indicated by clear zones of inhibition as the lysozyme inhibitors inhibited the growth of organisms. The Gram-positive bacteria were all found to be inhibited by the lysozyme inhibitor, but Gram-negative bacteria were not.

IV. Discussion

Although the substrate structure of lysozyme, cellulase or chitinase have been found to be very similar, only a few inhibitors of cellulase (Sinha *et al.*, 1981) or chitinase have been reported (Koga *et al.*, 1986; Sakuda *et al.*, 1986; Sakuda *et al.*, 1987). On the other hand, lysozyme has been known to be inhibited by pentane (Watanabe and Takesue, 1974), lipopolysaccharide (Ohno and Morrison, 1989), aminoglycosidic antibiotic (Ferrnandez-Sousa *et al.*, 1977b), histamine (Boller *et al.*, 1983), hyaluronic acid (Sela and Steiner, 1963), *Pneumococcus* polysaccharide Skarens and Watson, 1955), *E. coli* polysaccharide (Pryne *et al.*, 1969), teichoic acid (Mandelstam and Strominger, 1961), RNA (Wang *et al.*, 1991), DNA (Cattan and Bourgoin, 1968) (Wang *et al.*, 1990a), some copolymers of amino acids (Sela and Steiner, 1963; Wang *et al.*, 1991), surfactin (Murao *et al.*, 1990), imidazole and indole derivatives (Swan, 1972; Schinitzky *et al.*, 1966), gadolinium (Secemski and Lienhard, 1974) and Hewli (Murao *et al.*, 1990; Wang *et al.*, 1990a; Wang *et al.*, 1990b; Wang *et al.*, 1991). Except for Hewli (a lipopeptide) and the two inhibitors (FI and FII) of this study, almost all of the reported lysozyme inhibitors were prepared from the substrates of lysozyme.

The maximal inhibition by FI and FII of the *M. lysodeikticus* cell lytic activity of lysozyme were both 100% whereas hewli also inhibited the hen-egg-white lysozyme but maximal inhibition was only 45% (Murao *et al.*, 1990). Thus far, no protein-type lysozyme inhibitors have been found, and no lysozyme inhibitors have been reported to have antimicrobial action. Lysozyme inhibitor may be a novel antibiotic because of the antimicrobial mechanism which is different from those of penicillin and other antibiotics. Why the inhibitors had specific inhibition on Gram-positive bacteria rather than Gram-negative bacteria in our study is not clear. Different of cell wall compositions between Gram-positive and Gram-negative bacterias may have been the cause.

According to the optimal medium study of *B. subtilis* I-139 as described by Murao *et al.* (1990), maximum lysozyme inhibitory activity was obtained when the bacterium was grown aerobically in a medium consisting of 0.65% glucose, 1.0% beef extract, 4.0% polypepton, and 0.5% sodium L-glutamate at 37 after 20-24 hrs. When compared the optimal medium of strain M-1001 with *B. subtilis* I-139, it is interesting to see that both are highly concentrated carbon and nitrogen sources.

It is noteworthy that while *P. aeruginosa* M-1001, *B. subtilis* I-139 and *Staphylococcus aureus* M18 (Wadstrom and Hisasune, 1970) all produced autolysins in culture broth, the autolysins of the same producing strains were not inhibited by the lysozyme inhibitors. The existence of lysozyme inhibitor and autolysin in the same strain may play an important role in the biological control, cell division, and cell growth of these strains. Future study in this regard is needed.

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Pseudomonas aeruginosa M－1001所生產兩種溶菌酶抑制劑之純化及特性

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摘　要

由細菌M-1001之發酵上清液分離出兩種溶菌酶抑制劑F-I及F-II。此菌株經鑑定係爲*Pseudomonas aeruginosa*。於含0.25％葡萄糖，0.25％牛肉萃取物，0.25％蛋白腺，1.0％麩胺酸鈉，以及1.0％可溶性澱粉（pH 7.0）之培養基中，於37°C培養20-24小時可得較佳之抑制活性。取*P. aeruginosa* M-1001培養上清液，經硫酸銨沈澱，再以DEAE-Sepharose CL-6B陰離子交換層析及Sephacryl S-200膠體過濾層析進行抑制劑之分離，可純化出F-I及F-II兩種具抑制活性之蛋白質，F-I及F-II之純度分別提昇了20及7.5倍，分子量分別約爲57,000及33,000道耳頓，pH安定性分別爲6-10及6-11，熱安定性分別爲50°C及40°C。*P. aeruginosa* M-1001所生產抑制劑對測試之革蘭氏陽性菌具抑制效果。