SP127, A BACTERIAL PROTEIN ENHANCING THE ACTIVITY OF MACROLIDE ANTIBIOTICS AGAINST *PSEUDOMONAS AERUGINOSA*: ISOLATION AND CHARACTERIZATION

MASAKAZU KIKUCHI and YOSHIO NAKAO

Microbiological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

(Received for publication November 29, 1976)

Some strains belonging to the genera *Bacillus* and *Pseudomonas* were found to produce in the culture broth, a protein which synergistically enhanced the activity of macrolide antibiotics against *Pseudomonas aeruginosa*. The protein produced by *Bacillus brevis* ATCC 8185, designated SP127, was isolated in a pure state by means of ammonium sulfate fractionation, CM-cellulose column chromatography and gel filtration on Sephadex G-100. The sedimentation coefficient and the molecular weight of SP127 were 1.8 s and 15,000, respectively according to the analytical ultracentrifuge. The isoelectric point of SP127, obtained by electrofocusing experiments, was over 10.0. Half cystine and histidine were absent in the molecule.

It is well recognized that hospital-acquired infections due to *Pseudomonas aeruginosa* present difficult problems in management. The main reason is attributed to the resistance of *P. aeruginosa* against various chemotherapeutic agents. To overcome the difficulties, many trials have been carried out. From an interest in combined antibiotic effect, we screened substances which enhanced anti-*Pseudomonas* activity of macrolide antibiotics. In addition to the relatively small side effect of macrolide antibiotics, the finding of enhancing substances would be helpful in widening the spectrum of the antibiotics and decreasing the dosage.

In the experiments, some strains of *Bacillus* and *Pseudomonas* were found to produce in the culture broth the protein which answered our purpose. The present paper reports the isolation and the physicochemical properties of the protein SP127 produced by *Bacillus brevis* ATCC 8185.

Materials and Methods

Culture medium and conditions for screening

The medium for screening was composed of glycerol 2.5%, meat extract 0.5%, Polypepton 0.5%, yeast extract 1.0%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05% and $CaCO_3$ 0.35% (pH 7.0). Various microorganisms, isolates from soil and stock cultures, were grown in 20 ml of the medium in a 200-ml flask at 28°C for 4~8 days with shaking. The supernatant obtained was subjected to assay.

Method for screening the substances enhancing the antibiotic activity of erythromycin against *P. aeruginosa*

Active substances were detected and measured by means of an agar diffusion assay (Fig. 1). Bouillon agar was seeded with *P. aeruginosa* IFO 3080 and then mixed with a sufficient quantity of a sterile solution of erythromycin (EM) to give a concentration of EM in the agar of 10 μg/ml. The agar was then poured into a petri-dish(A). A petri-dish(B) contained the buoillon agar medium seeded with *P. aeruginosa* IFO 3080. After solidification of the agar, equally spaced cylindrical holes (6 mm) were made in the layer of agar by using a cork borer. The sample (50 μl each) to be tested was introduced into the holes of (A) and (B), and the petri-dishes were incubated at 37°C for 18~20 hours.

Clear circular zones of inhibition of growth of the test organism were formed around the holes containing the active substances. Samples were selected which formed the inhibition zones around the holes of (A), but not of (B). Activity is shown with the diameter of inhibition zone in the text.

Protein estimation

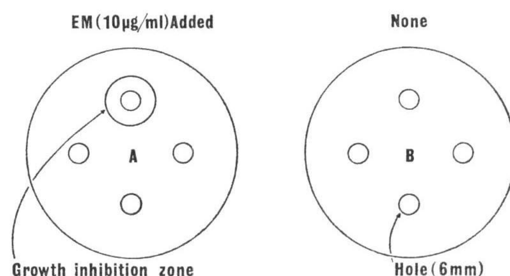
Protein concentration was estimated spectrophotometrically from the absorbance at 280 nm.

Ultracentrifugation

Centrifugation was performed using a Beckman-Spinco model E Ultracentrifuge. Prior to centrifugation SP127 preparation was dialyzed overnight at 5°C against 0.1 M NaCl. Sedimentation velocity experiments were done using schlieren optics at a rotor speed of 59,680 rpm and at 20°C. The molecular weight was determined by the method of ARCHIBALD[1].

Fig. 1. Screening method

The sample (50 μl each) to be tested was introduced into holes (A) and (B), and the petri-dishes were incubated at 37°C for 18~20 hours. Clear circular zones of inhibition of growth of *P. aeruginosa* IFO 3080 were formed around the holes containing the active substances. Details are in the text.



Polyacrylamide gel electrophoresis

Disc gel electrophoresis was performed by the method of DAVIS.[2] Electrophoresis was done for 60 minutes at 5 mA per tube (pH 4.3 gel[3]) and protein bands were detected by staining with amido schwartz.

Isoelectric focusing

The isoelectric focusing[4] of SP127 was performed for 48 hours at 700 V using the carrier ampholites(LKB-Produkter AB[5]).

Molecular weight determination by gel filtration

Molecular weight determination was done on a Sephadex G-100 column according to the method of ANDREWS[6].

Amino acid analysis

SP127 was hydrolyzed with 6 N HCl in the evacuated sealed-tube at 105°C for 24, 48 and 72 hours. Analysis was performed with a Hitachi Model KLA-3B automatic amino acid analyzer. Tryptophan was determined spectrophotometrically according to the method of SPIES and CHAMBERS[7,8].

Materials

For determination of molecular weight by the gel filtration method, we used non-enzymatic protein molecular weight markers produced by Schwarz/Mann.

Results

Screening of the Substances which Enhance the Antibiotic Activity of EM against *P. aeruginosa*

Among 7,078 strains examined, 30 strains produced the active substance. From precise studies on the active substance-producing strains, producers were restricted to strains belonging to the genera *Bacillus* and *Pseudomo-*

Table 1. Microorganisms producing active substances

Strain		Diameter of inhibition zone (mm)
Bacillus brevis	ATCC 8185	15.5
Bacillus brevis	ATCC 8186	16.5
Bacillus brevis	ATCC 10068	15.0
Bacillus brevis	IFO 12334	13.0
Bacillus brevis	IFO 12374	16.0
Pseudomonas aeruginosa	IFO 3812	12.0
Pseudomonas aeruginosa	IFO 3919	16.0
Pseudomonas aeruginosa	IFO 12630	15.0
Pseudomonas polycolor	IFO 3918	10.0

nas (Table 1). In this report, SP127 produced by *Bacillus brevis* ATCC 8185 was isolated and its physicochemical characteristics were elucidated. The culture filtrate containing SP127 was not antibiotic against *Staphylococcus aureus* FDA 209P, *Bacillus subtilis* PCI 219, *Sarcina lutea* PCI 1001, *Escherichia coli* IFO 3044, *Proteus vulgaris* IFO 3045, *P. aeruginosa* NCTC 10490 and *Candida albicans* IFO 0583.

Isolation of SP127

B. brevis ATCC 8185 was grown in 100 liters of the screening medium in a 200-liter fermentor at 28°C for 2 days (aeration 50%, agitation 280 rpm).

Step 1. Filtration

The culture broth was mixed with 1% of Hyflo Standard Super-Cel and then filtered with the filter-press. In 60 liters of the filtrate, 50 μg/ml of SP127 was contained.

Step 2. Ammonium sulfate fractionation

The filtrate was brought to 25% saturation by addition of solid $(NH_4)_2SO_4$. After 30 minutes, the suspension was centrifuged and $(NH_4)_2SO_4$ was added to the supernatant solution to 50% saturation. After 30 minutes, the precipitate was gathered by centrifugation.

Step 3. Chromatography on CM-cellulose

The precipitate was dissolved in 0.01 M Tris-maleate buffer at pH 6.0 and dialyzed against 3 liters of the same buffer, with four changes. The dialyzed solution was applied to a column (2.4 × 74.5 cm) of CM-cellulose which was previously equilibrated with the same buffer. After the column was washed adequately with the equilibrating buffer, SP127 was eluted with the same buffer containing 0.2 M NaCl. Active fractions were concentrated with ammonium sulfate fractionation and dialyzed as described in Step 2. The dialyzed solution was applied again to a column (2.4 × 74.5 cm) of CM-cellulose according to the above procedure. After the column was washed with 0.1 M NaCl added buffer, SP127 was eluted with a linear gradient of NaCl (Fig. 2), formed by placing in the mixing chamber, 600 ml of the 0.1 M NaCl supplemented buffer and 600 ml of 0.22 M NaCl added buffer in the reservoir. The flow rate was 5 g/10 minutes. Active fractions were pooled and SP127 was precipitated by adding $(NH_4)_2SO_4$.

Step 4. Gel filtration

The precipitate was dissolved in a small amount of 0.025 M phosphate buffer at pH 7.0 and applied to a column (5 × 70 cm) of Sephadex G-100 previously equilibrated with the same buffer. SP-127 was eluted as shown in Fig. 3 and fractions of 10 g were collected. The flow rate was 10 g/10 minutes. Active fractions were pooled and SP127 was precipitated by adding $(NH_4)_2SO_4$. The precipitate was dissolved in distilled water and dialyzed against distilled water and lyophilized. White SP127 (757 mg) was obtained.

Physicochemical Properties of SP127

Homogenity

SP127 gave a single band on acrylamide gel electrophoresis at pH 4.3 (Fig. 4). SP127 showed symmetrical peaks in sedimentation velocity runs, as in Fig. 5. The sedimentation coefficient was extrapolated to zero protein concentration and 1.8 s was obtained as $S_{20,w}$ at 0.9% concentration.

Molecular weight

The molecular weight of SP127 was determined by the method of ANDREWS[6]. As a result shown

Fig. 2. CM-cellulose column chromatogram
The column (2.4×74.5 cm) was equilibrated with 0.01 M Tris-maleate buffer (pH 6.0).

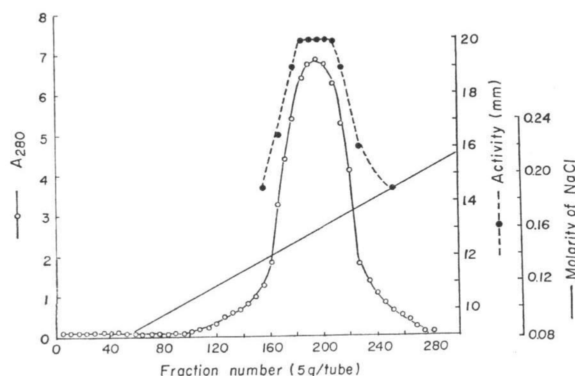
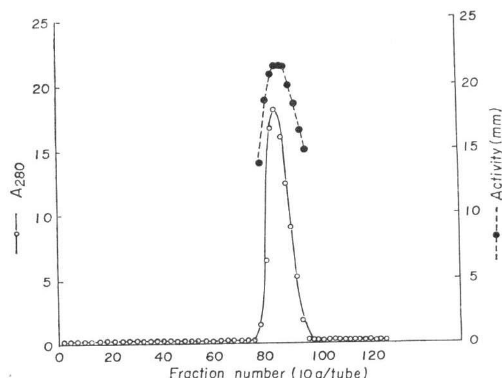


Fig. 3. Gel filtration of SP127 on Sephadex G-100
The column (5×70 cm) was equilibrated with 0.025 M phosphate buffer (pH 7.0).



in Fig. 6, the elution volume of SP127 gave an apparent molecular weight of 14,000±1,000. The molecular weight of SP127 calculated from sedimentation experiments was 15,000.

Isoelectric point

As a result of analytical isoelectric focusing experiments, SP127 had an isoelectric point of over 10.0 at 5°C as shown in Fig. 7.

Amino acid composition

Table 2 presents the amino acid composition of SP127. The number of residues per molecule of SP127 was 117, calculated for a molecular weight 15,000. The half cystine and histidine were absent in SP127.

Absorption spectrum

The dissolved SP127 had an absorption peak at 282.5 nm as shown in Fig. 8. The extinction coefficient of SP127 at 282.5 nm, $E^{1\%}_{1cm}$, was 22.1.

Fig. 4. Disc gel electrophoretic pattern of SP127
SP127 (50 μg) was subjected to electrophoresis for 60 minutes at 5 mA per tube (pH 4.3).

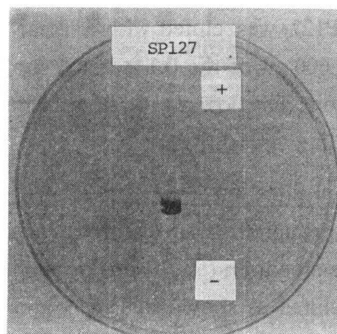
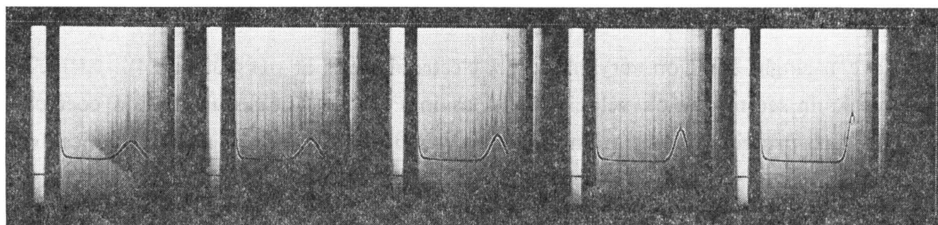


Fig. 5. Schlieren patterns of SP127
The photographs were taken 10, 30, 50, 70 and 90 minutes after reaching 59,680 rpm, at a bar angle of 70°. The sample cell contained 0.9% SP127 in 0.1 M NaCl.



Stability

SP127 was stable between pH values 3~9 when left standing for 24 hours at 2°C. Above 80°C, SP127 was rapidly inactivated. When kept for 10 minutes at 30°~80°C, SP127 was gradually inactivated parallel with temperature increase.

Table 2. Amino acid composition of SP127

Amino acid	Content[a)]	Number of residue[b)]
Asp	0.56	8
Thr	0.49	7
Ser	0.69	10
Glu	0.65	10
Pro	0.50	7
Gly	0.77	12
Ala	0.51	8
Half CyS	0	0
Val	0.89	13
Met	0.16	2
Ileu	0.44	6
Leu	0.40	6
Tyr	0.40	6
Phe	0.16	2
Lys	0.67	10
His	0	0
Arg	0.31	5
Try	0.36	5

a) Extrapolated to zero time of hydrolysis (μmole/mg of SP127)

b) Nearest integer per mole of SP127, based on a molecular weight of 15,000.

Fig. 6. Estimation of the molecular weight of SP127 by Sephadex G-100 gel filtration

The column (2.4×50 cm) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing KCl (0.1 M).

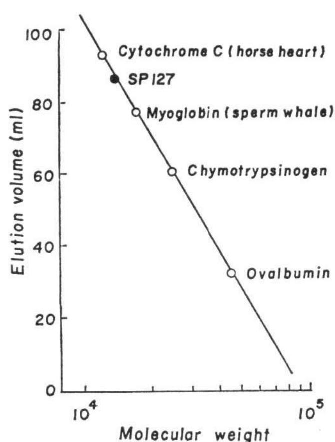


Fig. 7. Isoelectric focusing of SP127

The isoelectric fractionation of SP127 (10 mg) was performed for 48 hours at 700 V.

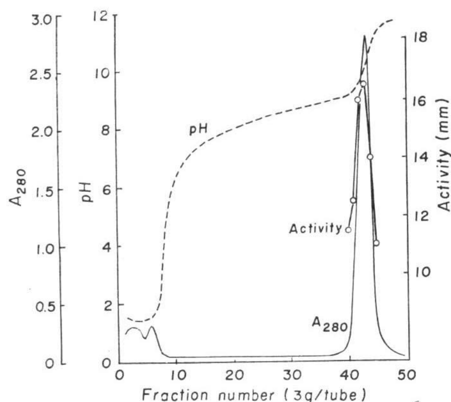
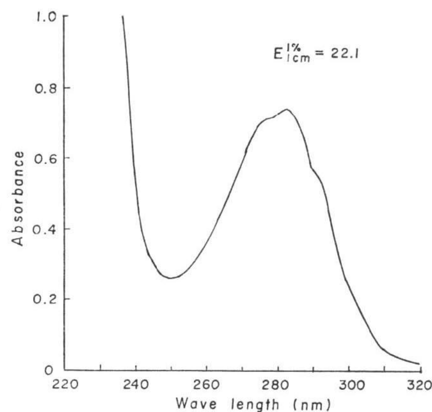


Fig. 8. Ultraviolet absorption spectrum of SP127

The sample cell contained 321 μg of SP127 in 1 ml of 0.01 M phosphate buffer (pH 7.0).



Discussion

With the widespread use of antimicrobial agents, there has been an emergence of drug resistance in anaerobic bacteria. On the other hand, infections caused by gram-negative bacilli, especially ***P.***

aeruginosa have become a major problem among hospitalized patients.

To obtain agents with greater activity and a broader spectrum, the search for new effective antimicrobial agents and the modification of naturally occurring antibiotics have been continued. The protein SP127 obtained in our experiments arouses our interest because it has no antibiotic activity in itself, but enhances the activity of macrolide antibiotics against *P. aeruginosa*.

SP127 was obtained as the homogeneous state which has an isoelectric point of over 10.0 and is lacking in half cystine and histidine. Although there have been many examples of synergy between antibiotics, synergism of an antibiotic with non-antibiotic protein has scarcely been known. From a consideration of the diverse functions of protein, this synergism is interesting physiologically and chemotherapeutically.

The present screening method would possibly select substances of the following groups. Group I includes inhibitors of enzymes which inactivate macrolide antibiotics, provided the target organisms have such enzymes. Group II includes the substances promoting the uptake of macrolide antibiotics. Group III includes substances which show lytic action in the presence of macrolide antibiotics. Group IV includes other substances showing synergism with macrolide antibiotics.

Until now, substances of groups I, II and III are as yet unknown. The target organisms used in the experiments lack inactivating enzymes of macrolide antibiotics, so SP127 is not an inhibitor of the enzymes. Much further work is needed to clarify which group SP127 belongs to, however, the finding of substances like this, is important to clarify the behavior of microorganisms against chemotherapeutic agents.

Acknowledgements

We wish to thank Drs. E. OHMURA, M. ISONO and H. FUKUDA for their interest and encouragement, and Dr. A. KAKINUMA and Mr. H. SUGINO for helpful suggestions in the analysis of protein. We also thank Mr. F. KIKUMOTO for his technical assistance.

References

1) ARCHIBALD, W. J.: A demonstration of some new methods of molecular weights from the data of the ultracentrifuge. J. Phys. & Colloid Chem. 51: 1204~1218, 1947
2) DAVIS, B. J.: Disc electrophoresis-II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404~427, 1964
3) REISFELD, R. A.; U. J. LEWIS & D. E. WILLIAMS: Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281~283, 1962
4) VESTERBERG, O. & H. SVENSSON: Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta Chem. Scand. 20: 820~834, 1966
5) VESTERBERG, O.; T. WADSTRÖM, K. VESTERBERG, H. SVENSSON & B. MALMGREN: Studies on extracellular proteins from *Staphylococcus aureus*. I. Separation and characterization of enzymes and toxins by isoelectric focusing. Biochim. Biophys. Acta 133: 435~445, 1967
6) ANDREWS, P.: Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91: 222~233, 1964
7) SPIES, J. R. & D. C. CHAMBERS: Chemical determination of tryptophan. Study of color-forming reactions of tryptophan, *p*-dimethylaminobenzaldehyde, and sodium nitrite in sulfuric acid solution. Anal. Chem. 20: 30~39, 1948
8) SPIES, J. R. & D. C. CHAMBERS: Chemical determination of tryptophan in proteins. Anal. Chem. 21: 1249~1266, 1949