SOME PROPERTIES OF SF-973 B SUBSTANCE, THE ENZYME CATALYZING THE CONVERSION OF DIHYDROABIKOVIROMYCIN TO ABIKOVIROMYCIN

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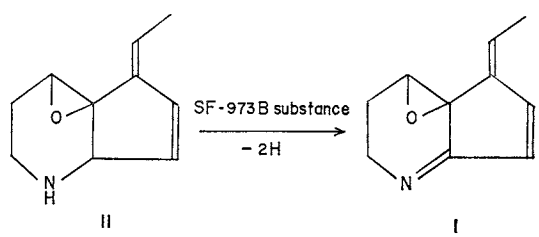
Three components, SF-973 A, B, and C were obtained from a *Streptomyces olivaceus* SF-973. SF-973 B catalyzed the converison of inactive SF-973 C (dihydroabikoviromycin) to bioactive SF-973 A (abikoviromycin). Purification of SF-973 B was conducted by sequential chromatographies on columns of DEAE-cellulose, Sephadex G-75, and Sephadex G-100. The enzyme SF-973 B was most active at around pH 7 and 40~45°C in temperature range. The molecular weight was about 36,000. SF-973 B showed oxidoreductase activity and was named dihydroabikoviromycin dehydrogenase.

In the course of isolation of a bioactive component from the culture broth of a *Streptomyces*, strain SF-973, we encountered an unusual phenomenon. Although a bioactive substance could be extracted with ethyl acetate under alkaline condition, solvent extraction resulted in a considerable loss of the total bioactivity originally found in the culture filtrate. However, when the aqueous layer and the solvent extract were combined, most of the antibiotic activity of the original broth was recovered. This phenomenon was explained as synergistic action of two biologically inactive substances, *i.e.* SF-973 B, a large molecular compound which remained in the aqueous layer, and SF-973 C, a small molecular compound which was solvent-soluble. This strain also produced the third component (SF-973 A) as a minor component which displayed the antibiotic activity by itself and was solvent-soluble under alkaline condition. Through structural study, the low molecular components, SF-973 A and C were identified as abikoviromycin and dihydroabikoviromycin respectively[1]. The synergistic action between SF-973 B and SF-973 C was interpreted in terms of the enzymatic dehydrogenation of SF-973 C (dihydroabikoviromycin) to SF-973 A (abikoviromycin) by the catalyst of SF-973 B, as briefly reported in the previous paper[1].

In this paper, we wish to report the isolation and the enzymatic properties of SF-973 B in more detail.

Scheme 1



II I

Materials and Methods

Organism

Streptomyces strain SF-973 which produces SF-973 A, B, and C was isolated from a soil sample collected at Hiroshima

Table 1. Characteristics of *Streptomyces olivaceus* SF-973

	Characteristics
Morphology : Spore chain	Open spirals are common, but sometimes closed or imperfect spirals are also observed. This morphology is seen on inorganic salts-starch agar, oatmeal agar and sucrose nitrate agar.
Spore surface	Smooth
Color of colony	Aerial mass color is grayed olive to yellowish gray on inorganic salts-starch agar, glucose asparagine agar, oatmeal agar and sucrose nitrate agar.
Reverse side of colony	Color of growth is usually grayed yellow on most media. On sucrose nitrate agar, dark brown to black color is produced. Substrate pigment is not a pH indicator.
Color in medium	Melanoid pigments are not formed in peptone-yeast-iron agar and tyrosine agar. No pigment is found in medium in synthetic agar.
Carbon utilization	D-Glucose, L-arabinose, D-mannitol, rhamnose and sucrose are utilized for growth. No growth or only trace of growth on raffinose, D-xylose, *i*-inositol and D-fructose.

in Japan. Important characteristics of this strain were summarized in Table 1, and, based on the taxonomic study, the organism was named *Streptomyces olivaceus* SF-973 by the authors.

Assay methods of the enzymatic activity of SF-973 B

Method A :

Dihydroabikoviromycin (SF-973 C, II) showed a UV maximum at 244 mμ ($E^{1\%}_{1cm}$ 850) and abikoviromycin (SF-973 A, I) at 246 mμ ($E^{1\%}_{1cm}$ 540) and 290 mμ ($E^{1\%}_{1cm}$ 480) in neutral or alkaline water. The activity of the enzyme (SF-973 B), then could be assayed by measuring an increment of the optical density at 290 mμ which is proportional to the amount of I derived from II by dehydrogenation.

The standard system contained 0.1 M phosphate buffer (pH 8.0), 0.25 μM dihydroabikoviromycin (SF-973 C) and enzyme (SF-973 B) in a total volume of 4.0 ml. The reaction was carried out at 30°C. One unit of the enzymatic activity was defined as the amount that induces an increase in the absorbancy at 290 mμ of 0.001 in one minute under the above condition.

Method B :

Since dihydroabikoviromycin (II) or SF-973 B alone shows no antibacterial property, the enzymatic activity of SF-973 B could be indirectly estimated by the antibiotic activity of I formed from II as the dehydrogenation proceeds. *Klebsiella pneumoniae* was used as a test organism, and a paper disc plate method was employed. To the medium consisting of 1.0 % glucose, 0.5 % NaCl, 0.3 % K_2HPO_4, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.1 % $NH_4H_2PO_4$ and 1.5 % agar (pH 7.0), compound II was added in the concentration of 100 mcg/ml. After spraying the test organism, a paper disc dipped in solution containing SF-973 B was put on the plate and incubated at 28°C for 20 hours.

The content of SF-973 B in a test sample was assayed from the inhibition diameter, relative to that of the standard SF-973 B with the highest purity so far obtained. This method has an advantage of simpler operation than Method A and preferably was used in the course of purification of the enzyme SF-973 B.

Determination of protein and polysaccharide content

The method of LOWRY *et al.*[2] was used to determine protein concentrations of various fractions obtained in the course of purification. The phenol-sulfuric acid reaction[3] was employed for estimation of polysaccharides, which was contaminated in the crude preparation of SF-973 B. Quantitation was made by utibzing absorption at 490 mμ, with D-glucose as a standard.

Results and Discussion

Production and Isolation of SF-973 B

A strain SF-973 was grown in submerged culture in a 300-liter jar fermenter at 28°C in a medium containing 2.5% glucose, 2.0% wheat embryo, 0.5% soluble vegetable protein, and 0.25% NaCl (pH 7.0). After 48-hour cultivation, the fermented broth was filtered at pH 7.0. To the filtrate (150 liters), 80 kg of ammonium sulfate were added with constant stirring. After standing overnight at 5°C, the precipitate containing SF-973 B was collected on a Büchner funnel.

Solvent extraction of the filtrate free of SF-973 B followed by countercurrent distribution gave 300 mg of SF-973 A (I) and 5.3 g of SF-973 C (II) as crystals. Dihydroabikoviromycin has been prepared by chemical reduction of abikoviromycin[4], but the direct formation in a culture broth has not been reported.

The precipitate containing SF-973 B was dissolved in 3 liters of 0.1 M phosphate buffer (pH 8.0) and was dialyzed against 20 liters of 0.01 M phosphate buffer (pH 8.0) for 2 days. Dialysis was repeated two times. To the dialysate (5 liters, solid 120 g), 80 g of $BaCl_2$ were added, and colored precipitate was removed by filtration.

Colored impurities still adhering to SF-973 B were further precipitated by the addition of 760 g of ammonium sulfate (20% saturation), and, to the filtrate were added 1,800 g of ammonium sulfate to precipitate SF-973 B. The precipitate was collected, dissolved in 1 liter of 0.01 M borate buffer (pH 6.8) and was dialyzed against 0.01 M borate buffer (pH 6.8) for 24 hours. The dialyzed solution (1.8 liters, pH 6.6) was loaded on a column of DEAE-cellulose (10×14 cm) which was equilibrated with 0.01 M borate buffer of pH 6.5. After washing with 1 liter of 0.01 M borate buffer (pH 6.5), the column was eluted with 0.1 M borate buffer (pH 9.0). The activity of SF-973 B was assayed by Method B, and a chromatographic pattern was shown in Fig. 1. The active fractions (Fract. vol. 4,000~5,800 ml, 1,800 ml) were combined and ammonium sulfate was added to the combined solution to a final concentration of 60% saturation. The resulting precipitate was dissolved in 200 ml of 0.1 M phosphate buffer (pH 7.5) and dialyzed against 0.003 M phosphate buffer (pH 7.5) for 24 hours. The dialyzed solution was freeze-dried to give 5 g of the light yellowish powder. When analyzed by the phenol-sulfuric acid method, the preparation contained 23% of polysaccharide as impurity. This crude powder was dissolved in 80 ml of 0.1 M phosphate buffer (pH 7.5), and subjected to column chromatography on Sephadex G-75 (7 liters), which was equilibrated with 0.1 M phosphate buffer containing 0.1 M NaCl, pH 7.5. The column was developed with the same buffer and collected in 20-ml fractions. The active

Fig. 1. DEAE-Cellulose column chromatography of crude preparation of SF-973 B
A. Protein concentration (FOLIN-LOWRY method ; absorption at 500 mμ)
B. Activity of SF-973 B estimated by Method B

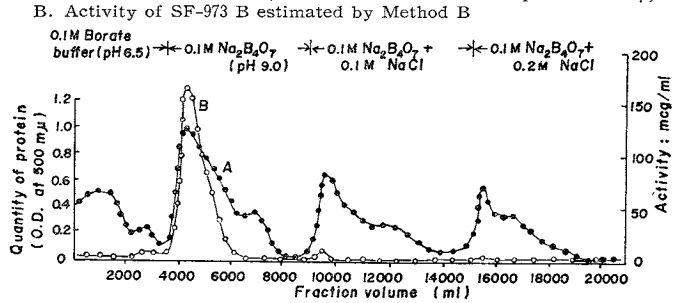
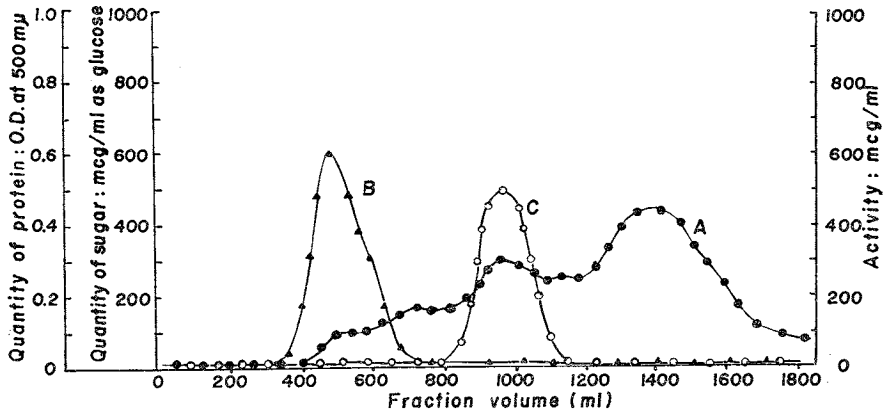
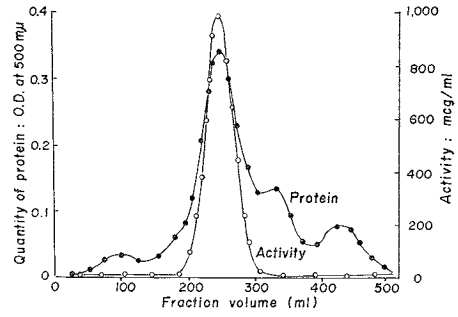


Fig. 2. Column chromatography of crude preparation of SF-973 B on Sephadex G-100.
A. Protein concentration (FOLIN-LOWRY method; absorption at 500 mμ)
B. Polysaccharide concentration (Phenol-sulfuric acid method)
C. Activity of SF-973 B estimated by Method B



fractions (Fract. vol. 4,000~5,000 ml, 1,000 ml) were gathered and concentrated utilizing the Diaflow filtration method. The concentrate (15 ml) was charged to a column of Sephadex G-100 (1,700 ml), which was equilibrated with the buffer solution as described above and developed with the same buffer, fractionating in 20 ml. The representative chromatographic pattern is illustrated in Fig. 2. By this procedure, a great majority of inert protein and polysaccharide contaminants were removed. The fractions containing the enzyme SF-973 B (Fract. vol. 840~1,300 ml) were collected (460 ml), concentrated to about 10 ml by the Diaflow filration method, and re-chromatographed over Sephadex G-100 (400 ml). The chromatographic condition was the same as that described above, and 18-ml fractions were collected.

Fig. 3. Re-chromatography of SF-973 B preparation over Sephadex G-100.
A. Protein concentration (FOLIN-LOWRY method; absorption at 500 mμ)
B. Activity of SF-973 B estimated by Method B.



As shown in Fig. 3, good correlation was observed between the activity and protein peaks. Active fractions (Fract. vol. 210~280 ml, 70 ml) free of impure peaks were collected and concentrated to about 20 ml by the Diaflow filtration method. The concentrate was dialyzed with a Visking cellophane tube and freezed dry. The SF-973 B preparation thus obtained was a white powder (80 mg), and 120-fold more potent than that of the first ammonium sulfate precipitate. This final preparation of SF-973 B was not a completely pure enzyme but further purification was difficult due to a very low production of the enzyme in the culture broth. The specific activity of the final preparation was 18,200 u/mg N. On cellulose acetate electrophoresis in 0.1 M tris buffer at pH 8.0, SF-973 B migrated 1.3 cm toward an anode under the electric current of 1.5 mA/cm for 1 hour. Spots were visualized by staining with amido black or nigrosin, and two minor extra bands were observed in addition to a main band of SF-973 B.

Table 2. Synergism of SF-973 B and compound II

Test organisms	Innhibitory diameter (mm)			Media
	SF-973 B 500 mcg/ml	compound II 500 mcg/ml	SF-973 B (100 mcg/ml) + compound II (100 mcg/ml)	
Klebsiella pneumoniae	0	0	16.6	1
Escherichia coli	0	0	16.7	1
Proteus vulgaris	0	0	17.2	1
Shigella dysenteriae	0	0	17.1	1
Bacillus subtilis ATCC 6633	0	0	16.4	1
Staphylococcus aureus FDA 209 P	0	0	13.1	1
Sarcina lutea	0	0	16.8	1
Mycobacterium smegmatis 607	0	0	12.0	2
Candida albicans	0	0	15.8	3
Torula utilis	0	0	17.4	3
Saccharomyces cerevisiae	0	0	16.1	3
Penicillium chrysogenum	0	0	16.7	4
Aspergillus niger	0	0	13.2	4
Piricularia oryzae	0	0	18.9	4
Fusarium oxysporum	0	0	15.4	4

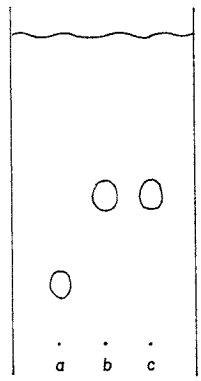
Paper disc plate method
Medium : 1. Bouillon agar, 2. Glycerine-bouillon agar, 3. SABOURAUD's agar, 4. Malt extract-yeast extract agar

Properties of SF-973 B

As briefly reported in a previous paper[1], copresence of SF-973 C (II) and SF-973 B showed bioactivity as a result of catalytic conversion of SF-973 C (dihydro-abikoviromycin, II) to SF-973 A (abikoviro-mcyin, I). Table 2 illustrates the synergism of SF-973 B and II as determined by Method B.

The catalytic property of SF-973 B in the dehydrogenation reaction was demonstrated by the following experiment. One hundred mg of II hydrochloride in 30 ml of 0.1 M phosphate buffer (pH 7.0) was incubated with 20 mg of SF-973 B at 30°C. The reaction was followed by silica gel TLC shown in Fig. 4.

Fig. 4. Silica gel TLC of the reaction mixture of S-973 B and compound II
a ; Compound II. b ; Ethyl acetate extract of the solution after 30-minute incubation. c ; Compound I.
Solvent system ; ethyl acetate-MeOH (10 : 1)
Detection ; 10% sulfuric acid followed by heating.



Immediately after addition of SF-973 B, there appeared a new spot of Rf 0.5 with concomitant decrease of the spot of Rf 0.2 (II). After 30 minutes, the spot of II disappeared completely and a single bioactive spot of Rf 0.5 was observed.

The incubated solution was extracted with ethyl acetate at pH 9, and the solvent layer was washed with water, and then reextracted with acidified water (pH 3 by sulfuric acid). Ethyl-ether extraction of the aqueous layer at pH 9 and addition of sulfuric acid gave 30 mg of the crystalline sulfate. This was identified as abikoviromycin by comparison of UV, NMR and antibacterial spectrum[1]. In a blank experiment, compound II or SF-973 B alone was incubated, but no formation of I was

Fig. 5. Effect of pH on the enzymatic activity of SF-973 B

Reaction mixture contained 2 ml of 0.001% SF-973 B solution and 2 ml of 0.0025% Compound II in buffer solution at various pH values. The buffers used were 0.1 M McIlvaine (pH 2~7) and 0.1 M glycine-NaCl-NaOH (pH 7~12). The reaction was carried out at 30°C and the activity was estimated by Method A.

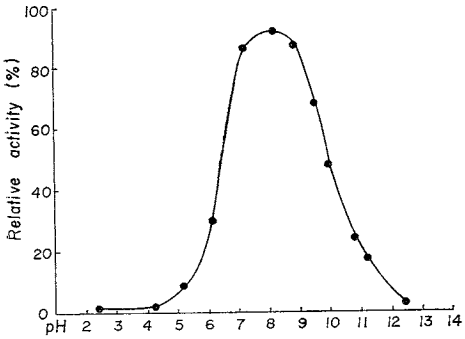


Fig. 6. Effect of pH on stability of the enzyme SF-973 B

Two ml of 0.01% SF-973 B solution was incubated for 20 hours at various pH values and 35°C. The remaining activity was assayed by Method B. The buffers used were 0.1 M McIlvaine (pH 2~7) and 0.1 M glycine-NaCl-NaOH (pH 7~12).

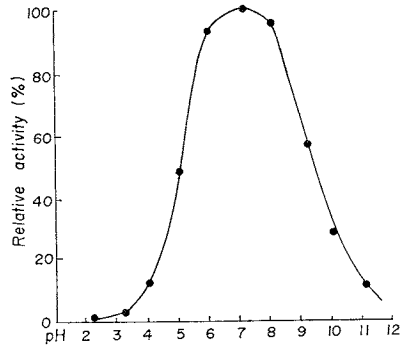


Fig. 7. Effect of temperature on the enzymatic activity of SF-973 B

Seventy μg of compound II hydrochloride in 3.15 ml of 0.1 M phosphate buffer (pH 8.0) was placed in a quartz cuvette with a temperature-regulating equipment. Twenty μg of the enzyme SF-973 B in 0.35 ml of the same buffer was added and incubation was done at an ambient temperature, and the activity after 4 minutes was estimated by Method A.

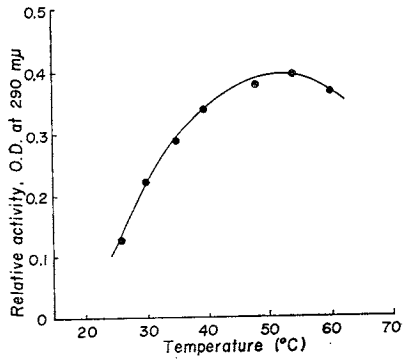
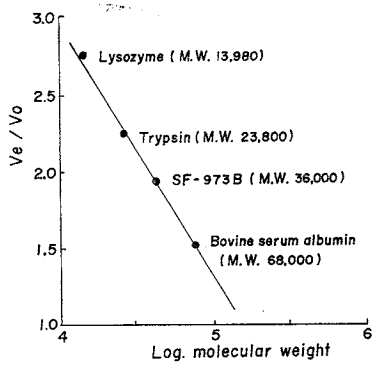


Fig. 8. Approximate molecular weight of the enzyme SF-973 B estimated by gel filtration.

A column of Sephadex G-100 (1.2×100 cm) which was equilibrated with 0.03 M phospate buffer containing 0.3 M NaCl (pH 7.5), was developed with the same buffer. The flow rate was 15 ml/hr and peaks of each protein was determined by absorbancy at 280 mμ.



recognized.

The conversion of II to I involves the dehydrogenation of -CH-NH- to -C=N- group. Therefore, the enzyme SF-973 B is an oxidoreductase, and may be named dihydroabikoviromycin dehydrogenase.

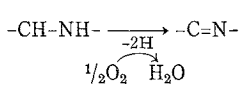
The enzyme SF-973 B has the maximum calalytic activity at around pH 7.0 as shown in Fig. 5. It is stable in an aqueous solution over a pH range of 6.5~8.5 at 35°C for 20 hours (Fig. 6), but at higher temperature such as at 80°C, the activity is almost completely lost within 10 minutes, even at pH 7.0.

The optimum temperature for the catalyst was found to be relatively high, about 45~55°C (Fig. 7).

The molecular weight of SF-973 B was estimated by gel filtration over Sephadex G-100. Fig. 8 illustrates the ratio of elution volume to void volume plotted against

logarithm of molecular weights of the marker proteins. From the calibration curve, the molecular weight was estimated to be about 36,000.

Scheme 2



The catalytic reaction of SF-973 B was not accelerated by the addition of coenzymes such as flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine nucleotide phosphate (NADP).

The reaction was not inhibited by the addition of $MnCl_2$, $CaCl_2$, $Pb(CH_3COO)_4$, $ZnSO_4$, $FeSO_4$ and urea in the concentration of 3×10^{-2} M, but completely inhibited by 10^{-2} M mercaptoethanol.

The reaction did not occur when the reaction mixture was free from oxygen as shown by the following experiment. Twenty mg of compound II in 4 ml of 0.1 M phosphate buffer (pH 7) was placed in a Thunberg tube. In a sidearm was added 10 mg of the enzyme SF-973 B dissolved in 3 ml of the same buffer. After de-gassing by vacuum pump (10 mmHg), both solutions were mixed and incubated at 30°C under the oxygen-free condition. After 2 hours, the incubated solution was extracted with ethyl acetate at pH 9 and submitted to silica gel TLC. No conversion of compound II into I was recognized. These results suggested that SF-973 B may be an enzyme catalyzing autoxidation of dihydroabikoviromycin and that the last acceptor of two hydrogens liberated in the reaction is oxygen as shown in Scheme 2.

In this study, it was not possible to determine a favorable condition for the reverse hydrogenation reaction from I to II.

The simultaneous production of compounds I and II, as well as SF-973 B in the culture broth of *Streptomyces* strain SF-973 suggested that the last step of the biosynthesis of abikoviromycin (I) may be dehydrogenation of dihydroabikoviromycin (II) by the enzyme SF-973 B.

Acknowledgement

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