PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF STREPTOTHRICIN ACETYLTRANSFERASE CODED BY THE CLONED STREPTOTHRICIN-RESISTANCE GENE OF *STREPTOMYCES LAVENDULAE*

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Streptothricin acetyltransferase was purified from *Streptomyces lividans* harboring a plasmid which carried the streptothricin-resistance gene cloned from a streptothricin-producing strain, *Streptomyces lavendulae* No. 1080. Some properties of the enzyme were determined and the reaction product was identified to be N^{β}-acetylstreptothricin by NMR spectroscopy.

The streptothricin group of antibiotics produced by various *Streptomyces* strains are potent inhibitors of prokaryotic protein synthesis with a broad antibacterial spectrum. Chemical acetylation of the β-amino group of the β-lysine moiety in streptothricins caused loss of their antibacterial activity[1]. Acetyltransferase activity encoded by a plasmid-born streptothricin-resistance gene was detected in *Escherichia coli*[2], however, neither purification of the enzyme nor structural identification of the acetylated product has yet been performed.

The presence of the acetyltransferase activity in streptothricin-producing *Streptomyces* strains was reported[3,4]. We have cloned a streptothricin-resistance gene from *Streptomyces lavendulae* No. 1080, one of the streptothricin-producing microorganisms, into *Streptomyces lividans*[4] and constructed a cloning vector pKST2 containing the gene as a selective marker[5]. This paper deals with purification and characterization of the streptothricin acetyltransferase (STAT) encoded by the cloned resistance gene and identification of its reaction product, acetylstreptothricin F.

Materials and Methods

Bacterial Strains and Culture Conditions

S. lividans TK21 containing pKS7, a multicopy plasmid carrying the streptothricin-resistance gene from *S. lavendulae* No. 1080[4,5], was grown aerobically in CE medium (Casamino acids (Difco) 0.5%, yeast extract (Difco) 0.25%, $NaNO_3$ 0.3%, KH_2PO_4 0.1%, KCl 0.05% and $CaCO_3$ 0.5%) containing 20 μg/ml streptothricin at 30°C for 3 days with rotary shaking. *Bacillus subtilis* ATCC 6633 was used for bioassay of streptothricin.

Preparation of Crude Extract

Mycelia harvested from 9 liters of culture were washed in 10 mM Tris-HCl (pH 7.2). Washed mycelia were frozen and disrupted by grinding with alumina at 4°C. Five hundred ml of buffer E consisting of 10 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol was added and alumina was

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removed by centrifugation. One mg of DNase I was added to the supernatant and the cell debris was removed by centrifugation at 100,000×*g* for 1 hour. The supernatant was used as a crude preparation of STAT.

Bioassay for STAT

One ml of the reaction mixture of 20 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mg/ml streptothricin, 0.34 mM *S*-acetyl coenzyme A (CoA) was incubated at 30°C for 1 hour. Fifty μl of the reaction mixture was loaded onto an 8-mm paper disc which was placed on a nutrient broth plate overlayed with soft agar containing *B. subtilis* ATCC 6633. After incubation at 30°C overnight, streptothricin inactivating activity was detected by measuring the diameter of the growth inhibition zone of *B. subtilis*

Colorimetric Assay for STAT

STAT activity was assayed by measuring CoA production with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[6]. Two ml of a reaction mixture consisting of 50 mM Tris-HCl (pH 7.8), 0.34 mM *S*-acetyl CoA, 0.2 mg/ml streptothricin, 200 mM NaCl, 1 mM DTNB and various concentrations of STAT was incubated at 30°C and the change in the absorbance at 412 nm was followed. One unit of the enzyme represents the activity to produce 1 μmol of CoA per minute. Kinetic measurement of the STAT reaction was performed using this colorimetric assay.

Purification of STAT

The crude extract from 9 liters of the culture was brought to 30% saturation with ammonium sulfate, and centrifuged. The supernatant was then brought to 60% saturation with ammonium sulfate. The precipitate was collected and dissolved in buffer E. The solution was dialyzed against the same buffer containing 50 mM NaCl. The resulting solution was applied to a DEAE-cellulose (Whatman DE52) column (3.5×20 cm) equilibrated with buffer E containing 50 mM NaCl. After washing the column with the same buffer, elution was performed with a linear gradient of 50~400 mM NaCl in 500 ml of buffer E. Enzymatically active fractions detected by bioassay were directly applied to a Blue Sepharose CL-6B column (2×20 cm) and washed with buffer E containing 100 mM KCl. Fractions were eluted with a linear gradient of KCl from 100 mM to 1 M in 500 ml of buffer E and STAT activity was assayed colorimetrically. The active fractions were dialyzed against buffer E containing 100 mM KCl, concentrated in a collodion bag and further purified by gel filtration on Sephadex G-75 (1.6×50 cm) using the same buffer. Fractions (2.5 ml each) were pooled and assayed colorimetrically. STAT eluted between fractions 25 and 35 was concentrated in a collodion bag. All purification procedures were carried out at 4°C.

Preparation of Streptothricin F

Streptothricin F was purified from the streptothricin mixture by essentially the same method as previously described[7]. Streptothricin mixture (150 mg) was applied to a cellulose column (2.6×65 cm) and eluted with propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12). Fractions producing a spot of Rf 0.38 on cellulose TLC (Merk) using the same solvent were collected and dried with a rotary evaporator. Spots on TLC were visualized by ninhydrin. Samples were dissolved in water, passed through Dowex 1-X8 (Cl^- form) and freeze-dried. The powder was dissoved in 10% methanol and freeze-dried. The powder was dissolved in 10% methanol and further purified by gel filtration on Sephadex LH-20 (1.4×29 cm) equilibrated with 10% methanol. The purification resulted in 50 mg of streptothricin F trihydrochloride. The purified preparation gave a single spot on cellulose TLC (Rf 0.38 in the previous solvent system).

Purification of the Product of STAT Reaction

Streptothricin F (15 mg) was acetylated with purified STAT in 5 ml of 50 mM Na-phosphate buffer (pH 7.8) containing 10 mM $MgCl_2$, 34 μmol *S*-acetyl CoA at 30°C until the spot of streptothricin F on cellulose TLC (Rf 0.38) disappeared. The reaction mixture was passed through a Dowex 1-X8 (Cl^- form) column, concentrated by freeze-drying and purified by gel filtration on Sephadex LH-20 equilibrated with 10% methanol. The fractions which gave a single spot on cellulose TLC (Rf 0.46) were collected and freeze-dried.

Results

Purification of STAT

Streptothricin inactivation was catalyzed by the crude cell-free extract of *S. lividans* (pKS7) only in the presence of *S*-acetyl CoA[4]. No inactivation occurred with the extract of *S. lividans* without the plasmid. STAT was purified from the cells of *S. lividans* (pKS7) as described in Materials and Methods. A single peak of STAT activity was eluted between 170 and 400 mM NaCl from the DEAE-cellulose column (data not shown) and between 500 and 750 mM KCl from the Blue Sepharose CL-6B column (Fig. 1). SDS-Polyacrylamide gel electrophoresis[8] of the final preparation showed a single major band with less than 10% contaminants. We examined the following enzymatic properties with this purified STAT.

Properties of STAT

Molecular Weight

The molecular weight of STAT was estimated to be 24,000 in dalton by SDS-polyacrylamide gel electrophoresis (data not shown).

Reaction by STAT

Incubation of the streptothricin mixture with the purified STAT preparation in the presence of *S*-acetyl CoA caused rapid inactivation of the antibiotic, which was accompanied by liberation of free CoA. The initial reaction velocity of CoA liberation was dependent on the amounts of STAT added (Fig. 2).

Optimum pH

STAT activity was measured at various pH using 50 mM Na-phosphate buffer (pH 5.4~7.5) and 50 mM Tris-HCl buffer (pH 6.9~9.1). The pH optimum of the reaction was determined to be 7.8 (Fig. 3).

Fig. 1. Elution profile of streptothricin acetyltransferase (STAT) from a Blue Sepharose CL-6B column. Solid line; A_{280}, dotted line; KCl concentration (mM) and open columns; STAT activity (μmol CoA formed/minute), respectively.

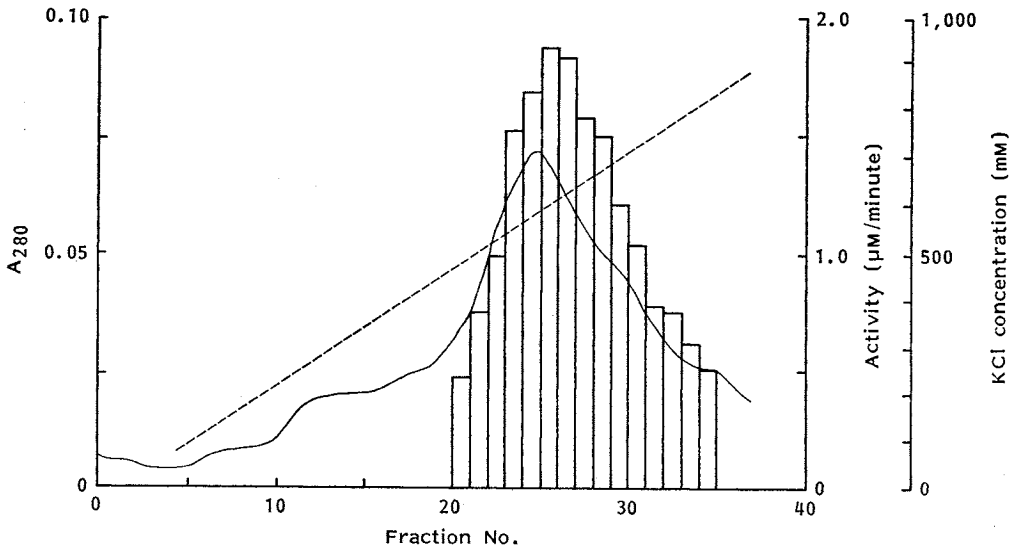


Fig. 2. Time course of coenzyme A liberation catalyzed by various amounts of STAT.

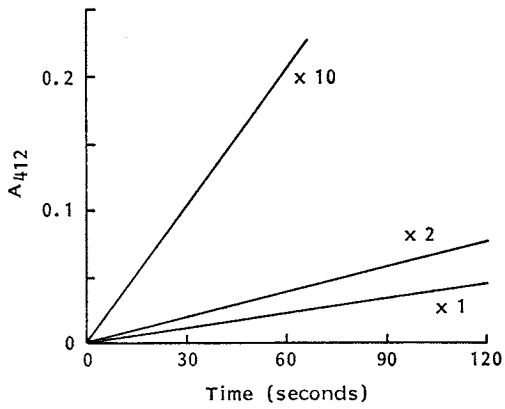


Fig. 3. Effect of pH on streptothricin acetyltransferase (STAT) activity.

Na-Phosphate buffer was used between pH 5.4 and 7.5 (●), and Tris-HCl buffer was used between pH 6.9 and 9.1 (○). STAT activity is shown as μM of CoA reduced per minute.

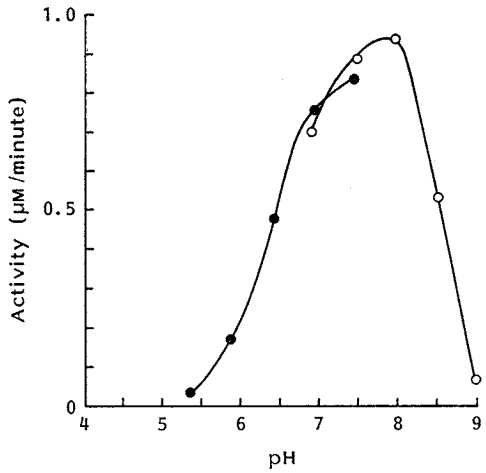


Fig. 4. Thermostability of streptothricin acetyltransferase (STAT).

Effect of temperature on STAT activity was measured as described in the text using 2 units of the enzyme in each incubation.

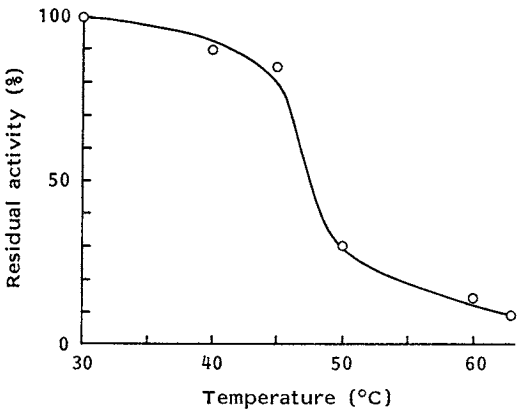


Table 1. Effect of $MgCl_2$ and NaCl on STAT activity.

Metals	Concentrations (mM)	Relative activity (%)
$MgCl_2$	0	100
	5	100.6
	10	116.0
	20	127.7
NaCl	0	100
	50	126.2
	100	140.5
	200	152.4
	300	138.1

Thermal Stability

The purified STAT was incubated in 50 mM Tris-HCl buffer (pH 7.8) at various temperatures for 3 minutes and the residual activity was measured. The enzyme activity was stable at 35°C but rapid inactivation occurred at 50°C (Fig. 4).

Effect of $MgCl_2$ and NaCl

The STAT preparation was dialyzed against buffer E without $MgCl_2$ after which the enzyme activity was measured in the standard reaction mixture containing various concentrations of $MgCl_2$ and NaCl (Table 1). STAT activity was slightly increased by the addition of $MgCl_2$ up to 20 mM, however, no significant effect was observed, suggesting that Mg^{2+} is not essentially required for STAT activity. The optimum NaCl concentration was determined to be 200 mM.

Fig. 5. Estimation of *Km* values for *S*-acetyl coenzyme A (CoA) (A) and for streptothricin F (B) by Lineweaver-Burk plot.

The initial rate of streptothricin acetyltransferase (STAT) activity was measured using various concentration of *S*-acetyl CoA or streptothricin F.

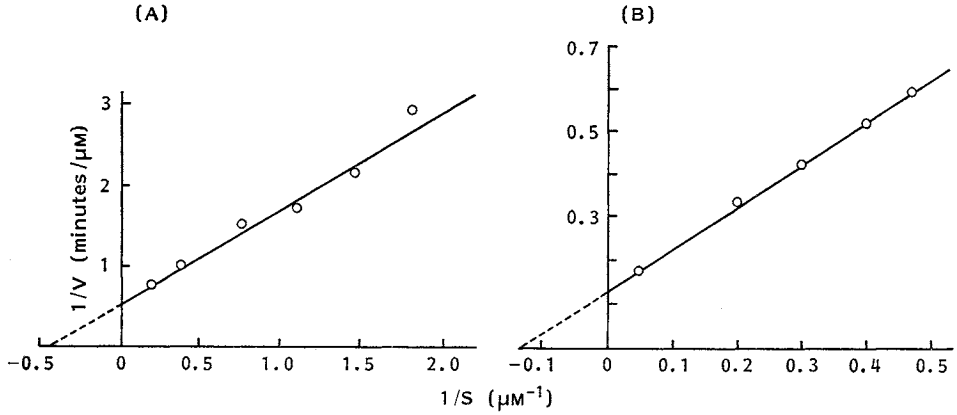
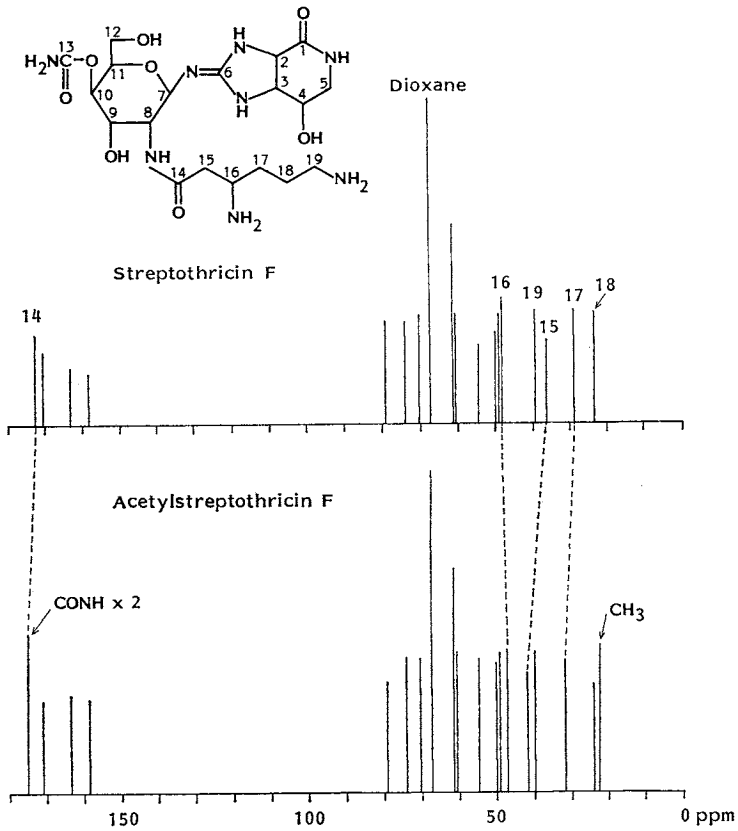


Fig. 6. ^{13}C NMR spectra of streptothricin F and acetylstreptothricin F (in D_2O, 25 MHz).



Km Values

The *Km* values for *S*-acetyl CoA and streptothricin F were determined to be 69 μM and 2.3 μM, respectively (Fig. 5).

Identification of Acetylstreptothricin as the Reaction Product

Starting with 15 mg of the purified streptothricin F as a substrate, we obtained almost the same amount of the reaction product giving a single spot of Rf 0.46 on cellulose TLC. ^{1}H NMR (in D_2O, 100 MHz) and ^{13}C NMR (in D_2O, 25 MHz) spectra of the purified product were measured and compared with those of streptothricin F. One sharp singlet signal of the acetyl protons appeared at 1.92 ppm in the ^{1}H NMR spectrum, indicating that streptothricin F was mono-acetylated at a single site. The ^{13}C NMR spectra of streptothricin F and the reaction product (Fig. 6) were very similar to each other except that two extra signals, assigned as acetyl carbon atoms, appeared at 22.9 ppm and 174.4 ppm for the product. In addition, the signals of the 14, 15 and 17 carbon atoms of the β-lysyl residue in the product shifted in comparison with those of streptothricin F (Table 2). These results indicate that the mono-acetylation occurred at the β-amino group of the β-lysyl moiety.

Table 2. ^{13}C NMR chemical shift of β-lysine side chain of streptothricin F and acetylstreptothricin F.

No.	Streptothricin F	Acetylstreptothricin F
14	172.8	174.4
15	37.0	41.9
16	49.1	47.6
17	29.8	31.8
18	23.7	24.1
19	39.7	40.0
CH_3	—	22.9
CONH	—	174.4

Discussion

The streptothricin-inactivating enzyme encoded by the cloned resistance gene of *S. lavendulae* required *S*-acetyl CoA for inactivation and produced acetylstreptothricin which was identified by ^{1}H and ^{13}C NMR spectroscopy. N^{β}-Acetylstreptothricins were isolated from a culture filtrate of a *Streptomyces* strain[9] and its spectral data coincide completely with those of the reaction product reported here (T. ANDO, personal communication).

STAT of *S. lavendulae* showed a low *Km* value of 2.3 μM for streptothricin F, which seems to be sufficient to overcome the potent inhibitory effect of the antibiotic (for example, 10 μM of the antibiotic is required for the complete growth inhibition of *S. lividans*). STAT may play a major role in self-resistance of the streptothricin-producing organisms.

The NH_2-terminal amino acid sequence of the purified STAT is consistent with that determined by our recent DNA sequencing. This along with the nucleotide sequence of the STAT gene will soon be reported elsewhere[10].

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