ISOLATION AND PROPERTIES OF A PUROMYCIN ACETYLTRANSFERASE FROM PUROMYCIN-PRODUCING *STREPTOMYCES ALBONIGER*

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Puromycin 2″-*N*-acetyltransferase was isolated from cell extracts of puromycin-producing *Streptomyces alboniger* KCC S-0309 by ammonium sulfate fractionation, heat treatment to eliminate contaminant proteins and chromatography on DEAE-Toyopearl 650S. After PAGE (polyacrylamide gel electrophoresis) of the final fraction, a single protein band corresponding to puromycin 2″-*N*-acetyltransferase was detected. The molecular weight of the enzyme determined by SDS-PAGE and Sephadex G-150 chromatography was about 21,000 and 85,000, respectively, suggesting that the enzyme consisted of four subunits. The isoelectric point and the optimum pH for reaction were 6.2 and 7.7, respectively. The K_m values for puromycin and acetyl coenzyme A were 40 μM and 67 μM, respectively. The enzyme was thermostable up to 70°C for 12 minutes.

It was shown, by using an *in vitro* protein synthesizing system from a puromycin-susceptible organism *S. flavotricini* subsp. *pseudochromogenes* V-13-1, that the isolated puromycin 2″-*N*-acetyltransferase could protect polyphenylalanine synthesis from inhibition by puromycin.

Puromycin inhibits bacterial protein synthesis by binding to both the 30S and 50S ribosomal subunits[1~3]. Since a puromycin-producing microorganism is inherently tolerant to its own antibiotic product, the organism must have some puromycin-resistant mechanism.

The organism was reported to express an enzyme activity which acetylated puromycin using acetyl coenzyme A[4]. We demonstrated recently that protein synthesis in extracts of puromycin-producing *Streptomyces alboniger* was inhibited by puromycin as a result of binding of the drug to both the 30S and 50S subunits of the ribosomes, but that the organism could be protected from puromycin by the action of puromycin 2″-*N*-acetyltransferase (PAT) which inactivated puromycin[5]. In addition, it was also indicated that the site of acetylation was the 2″-NH_2 group of the *O*-methyltyrosine moiety of puromycin[5].

The present study involves the isolation and physico-chemical properties of the enzyme.

Materials and Methods

Microorganisms

S. alboniger KCC S-0309[5] and *S. flavotricini* subsp. *pseudochromogenes* V-13-1[6] were used as a puromycin producer and a puromycin-susceptible strain, respectively.

Preparation of Ribosomes and S-150 Fraction

The ribosomes and S-150 fraction were prepared according to the method of SUGIYAMA *et al.*[7]

using mycelia grown to approximately 250 Klett units in GMP medium (glucose 1%, NaCl 0.5%, peptone 0.4%, yeast extract 0.2%, meat extract 0.2% and $MgSO_4 \cdot 7H_2O$ 0.025%, pH 7.0) at 28°C with shaking.

Determination of Protein

The protein in eluates from column chromatography was monitored by following the absorbance at 280 nm. The protein in enzyme solution was determined by the LOWRY method[8] using bovine serum albumin as a standard.

Assay of PAT

PAT activity was determined by two methods. Method A involved determination by bioassay of the decreased amount of puromycin after acetylation of the drug by the enzyme using *Bacillus subtilis* IFO 3134 as a test organism. Method B is essentially the same as that of SHAW and BRODSKY[9] which determined acetyl coenzyme A used by the enzyme for acetylation of puromycin. The reaction mixture (1 ml) consisted of 10 mM Tris-HCl (pH 7.65), 0.2 mM acetyl coenzyme A, 1 mM DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) and enzyme solution (10 to 20 μl), and then 0.1 mM puromycin was added immediately before the start of reaction. The reaction was carried out at 28°C for 15 minutes and increase in absorbance at 412 nm was measured. Acetylated puromycin was estimated from increase in the absorbance using a molecular extinction coefficient of 13,600[9] for liberated thionitrobenzoic acid. One unit of enzyme was defined as that activity which acetylated 1 μmol of puromycin in 1 minute.

Purification of the Enzyme

All procedures were carried out at 0°C to 6°C and when necessary, enzyme solutions were stored at −20°C.

(a) Preparation of S-30 Fraction: The whole cells collected by centrifugation (about 50 g, wet weight) were washed twice with buffer I [10 mM Tris-HCl (pH 7.65), 1 M KCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 5 mM Mg-Titriplex (EDTA-K_2-Mg salt)] and twice more with buffer II [10 mM Tris-HCl (pH 7.65), 30 mM NH_4Cl, 6 mM 2-mercaptoethanol, 10 mM magnesium acetate, 5 mM Mg-Titriplex, 3.45 mM phenylmethylsulfonyl fluoride and 0.2 mM diisopropyl fluorophosphate]. The washed cells were ground with quartz sand in an ice bath and extracted with buffer II containing 2 μg DNase I/ml. The cell debris and quartz sand were removed by centrifugation at 18,000×g for 10 minutes. The supernatant was further centrifuged at 30,000×g for 30 minutes and the resulting supernatant (S-30 fraction) was used in the following step of purification.

(b) First Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to 50 ml of the S-30 fraction to give 30% saturation and kept for 1 hour in an ice bath with stirring, then centrifuged at 30,000×g for 20 minutes. The supernatant containing PAT activity was used for the next step.

(c) Heat Treatment: The enzyme solution from step (b) was kept at 60°C for 10 minutes under gentle shaking. After cooling, the precipitate was removed by centrifugation at 30,000×g for 15 minutes.

(d) Second Ammonium Sulfate Fractionation: The concentration of ammonium sulfate in the supernatant fluid from step (c) was increased to 80%, the precipitate was collected by centrifugation at 40,000×g for 30 minutes and then dissolved in TMAP buffer [10 mM Tris-HCl (pH 7.65), 0.6 mM 2-mercaptoethanol, 30 mM NH_4Cl, 10 mM magnesium acetate and 0.345 mM phenylmethylsulfonyl fluoride]. The solution was dialyzed against one liter of the same buffer for 2 hours.

(e) DEAE-Toyopearl 650S Chromatography: The dialyzed solution was applied to a column (1.8 cm×30 cm) of DEAE-Toyopearl 650S (Toyo soda MFG, Co., Ltd.) equilibrated with TMAP buffer. The column was washed with 100 ml of the same buffer and then with 100 ml TMAP buffer containing 0.2 M NaCl, before being eluted with 400 ml of TMAP buffer containing 0.2 to 0.4 M NaCl (linear gradient of concentration). The enzyme was recovered in the fractions containing about 0.3 M NaCl in TMAP buffer. The fractions with enzyme activity were pooled and dialyzed against TMAP buffer. The dialyzed solution was used for physico-chemical characterization of the enzyme, and stored at −20°C when necessary.

Disc Gel Electrophoresis

Enzyme purity was determined by disc gel electrophoresis at pH 8.0 using a 7.0% (w/v) polyacrylamide gel (0.5 cm×0.5 cm stacking gel and 0.5 cm×5 cm separating gel)[10]. Coomassie brilliant blue R-250 [0.2% (w/v) in 50% methanol containing 7.5% acetic acid] was used for staining protein bands and 20% methanol containing 7.5% acetic acid was used for destaining.

Isoelectric Focusing

The isoelectric pH of the enzyme was determined by using polyacrylamide gel containing carrier Ampholine (pH 3.5 to 10, LKB, Sweden). Marker proteins used for pH gradient determination were from a pI-marker kit (pI 4.1~10.6) consisting of cytochrome c and five acetylated derivatives of cytochrome c (Oriental Yeast Co., Ltd., Japan). Cathode and anode solutions were 1 M NaOH and 0.02 M H_3PO_4, respectively. Isoelectric focusing was performed for 5 hours at 200 V, and then the protein was stained for 2 hours with Coomassie brilliant blue G-250 after fixing the gel with 3.5% (w/v) perchloric acid. Destaining was as described above.

Molecular Weight Determination

Molecular weight was determined by SDS-PAGE according to the method of LAEMMLI[11] and by Sephadex G-150 column chromatography.

Assay of Protein Synthesis *In Vitro*

Polyuridylate-directed polyphenylalanine synthesis was performed according to the method of SUGIYAMA *et al.*[7].

Results and Discussion

Purity of the Enzyme

The results of enzyme purification are summarized in Table 1. PAGE of the enzyme solution from the final step (e) revealed a single protein band which corresponded to the PAT activity when measured in gel slices by method A (data not shown). This indicate that the enzyme preparation is substantially homogeneous and also that the major protein in the preparation expresses the puromycin-acetylating activity.

Substrate Specificity and Kinetics of the Enzyme

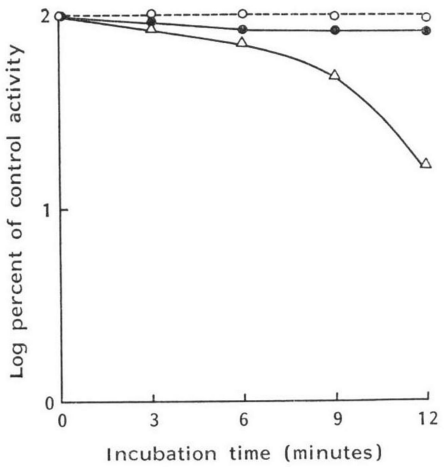
The purified enzyme catalyzed acetylation of puromycin and the K_m values determined by Lineweaver-Burk plots for puromycin and acetyl coenzyme A were 40 μM and 67 μM, respectively. It was shown in the previous paper[5] that streptothricin was inactivated by an S-150 fraction from *S. alboniger* in the absence of acetyl coenzyme A and that the inactivating mechanism was not clear. Since streptothricin did not serve as a substrate of the present purified enzyme, the S-150 must contain

Table 1. Purification of puromycin 2″-*N*-acetyltransferase from *Streptomyces alboniger*.

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
(a) S-30 Fraction	800	1,225	1.53	1	—
(b) First $(NH_4)_2SO_4$	588	1,080	1.84	1.2	88.2
(c) Heat treatment	365	941	2.58	1.7	76.8
(d) Second $(NH_4)_2SO_4$	240	886	3.69	2.4	72.3
(e) DEAE-Toyopearl 650S	3	162	54.0	35.2	13.2

One unit of enzyme is that activity which acetylates 1 μmol of puromycin in 1 minute.
Condition of heat treatment is described in the text. PAT activity was determined by method B.

Fig. 1. Heat stability of PAT.
PAT activities were determined by method B at 60°C ○, 70°C ● and 80°C △.



other enzyme(s) which inactivated streptothricin. As already reported, neomycin, kanamycin and chloramphenicol were not substrate for PAT.

Heat Stability

The PAT activity from *S. alboniger* was stable up to 70°C for 12 minutes (Fig. 1) and this property proved useful in the elimination of contaminant proteins from the enzyme preparation[5].

With respect to other antibiotic acetyltransferases, it has been reported elsewhere[9] that a chloramphenicol acetyltransferase from *Staphylococcus aureus* was also heat stable up to 75°C for 10 minutes, whereas rapid inactivation of the *Escherichia coli* enzyme was observed at 75°C.

Fig. 2. Estimation of the molecular weight of PAT by SDS-PAGE (a) and by Sephadex G-150 column chromatography (b).

Marker proteins used in SDS-PAGE were as follows: 1; Hexamer, 2; tetramer, 3; trimer, 4; dimer and 5; monomer (M_r 12,400) of cytochrome c. Marker proteins loaded onto a Sephadex G-150 column (1.6 cm × 76 cm) together with the enzyme solution were as follows. 6; Rabbit muscle aldolase (M_r 158,000), 7; bovine serum albumin (M_r 68,000), 8; hen egg albumin (M_r 45,000), 9; bovine pancreas chymotrypsinogen A (M_r 25,000). The column was eluted with TMAP buffer at a rate of 21.0 ml/hour and 3.15 ml-fractions were collected. A 10 μl portion of each fraction was assayed for PAT activity by method B. Position of marker protein was established by their absorbance at 280 nm.

Arrow: The position of PAT.

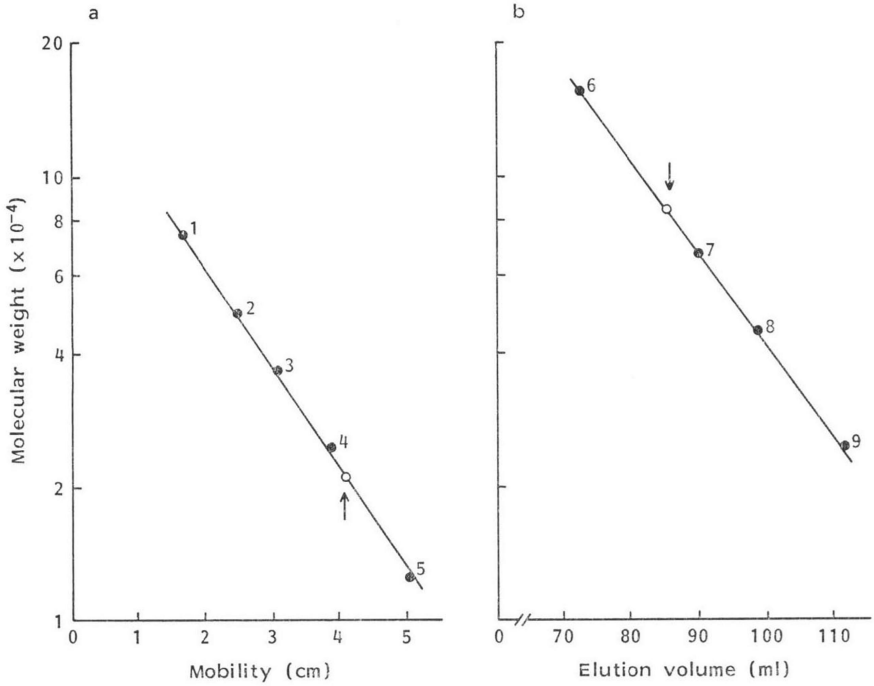
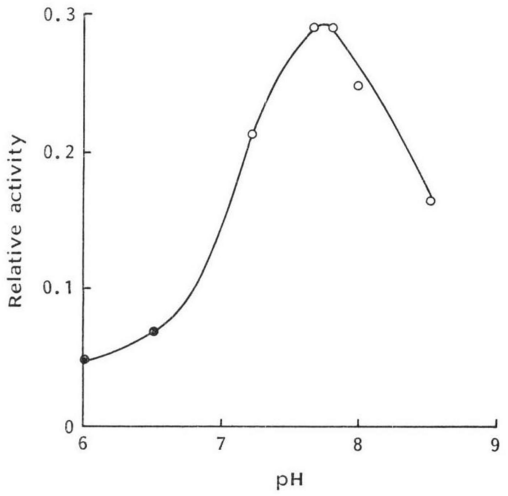


Fig. 3. Effect of pH on PAT activity.

The activities were assayed by method B using Tris - maleate - NaOH buffer ● (pH 6~6.5) and Tris-HCl buffer ○ (pH 7.2~8.5).



Molecular Weight, Isoelectric Point and pH Optimum

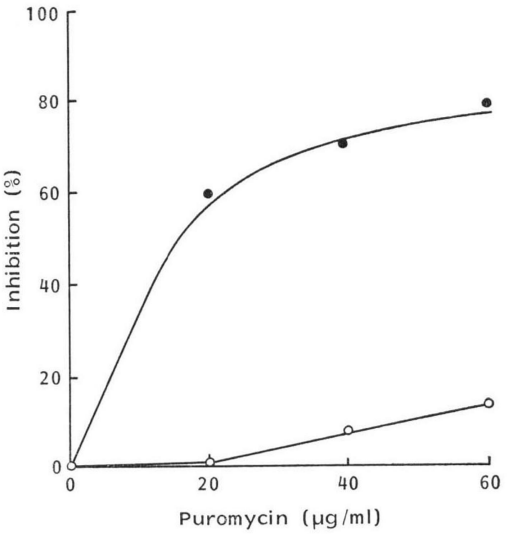
The molecular weight of PAT was estimated to be about 21,000 by SDS-PAGE and about 85,000 by chromatography on Sephadex G-150 which suggests that the enzyme consists of four subunits (Fig. 2). The isoelectric pH of the enzyme and optimal pH for acetylating activity (Fig. 3) were 6.2 and 7.7, respectively.

Fig. 4. Protection of polyphenylalanine synthesis from puromycin inhibition by PAT.

Percentage of inhibition is expressed as the ratio of the level of polyphenylalanine synthesis in the presence of puromycin to that in the puromycin-free reaction mixture.

Amount of polyphenylalanine synthesized in the puromycin-free reaction mixture was about 3.5×10^4 cpm in the presence of L-[*U*-^{14}C]phenylalanine (100 μCi/μmol) and 60 μg (about 22 pmol) ribosomes from strain V-13-1.

To reaction mixtures was added either ○, a 20 μl (10.9 μg) portion of the isolated enzyme solution from step (e) and 2 mM acetyl coenzyme A or ●, the same volume of TMAP buffer.



Inhibition Studies

PAT activity from *S. alboniger* was inhibited by *p*-chloromercuribenzoate (0.1 mM) and iodoacetic acid (1 mM).

Protection of Protein Synthesis by the Isolated PAT against Inhibition by Puromycin

It was necessary to examine if the isolated PAT could protect protein synthesis against inhibition by puromycin. *S. flavotricini* subsp. *pseudochromogenes* V-13-1 was susceptible to puromycin *in vivo* and, *in vitro*, polyphenylalanine synthesizing systems from this organism were strongly inhibited by puromycin at 20 μg/ml. This inhibition of protein synthesis was, however, relieved when PAT was added with acetyl coenzyme A to the reaction mixture (Fig. 4).

Judging from these results and the previous observation[5], we conclude that PAT plays an important role in self-protection of puromycin producer.

It is of interest that the puromycin acetyltransferase described here was similar to some chloramphenicol acetyltransferases with respect to pH optimum, heat stability, molecular weight, number of

subunits (four subunits) and enzyme inhibitors[12], though there is a difference in that former enzyme modifies -OH groups and latter one alters an $-NH_2$ group.

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