ISOLATION AND PROPERTIES OF A BLASTICIDIN S ACETYLATING ENZYME FROM A PRODUCER ORGANISM

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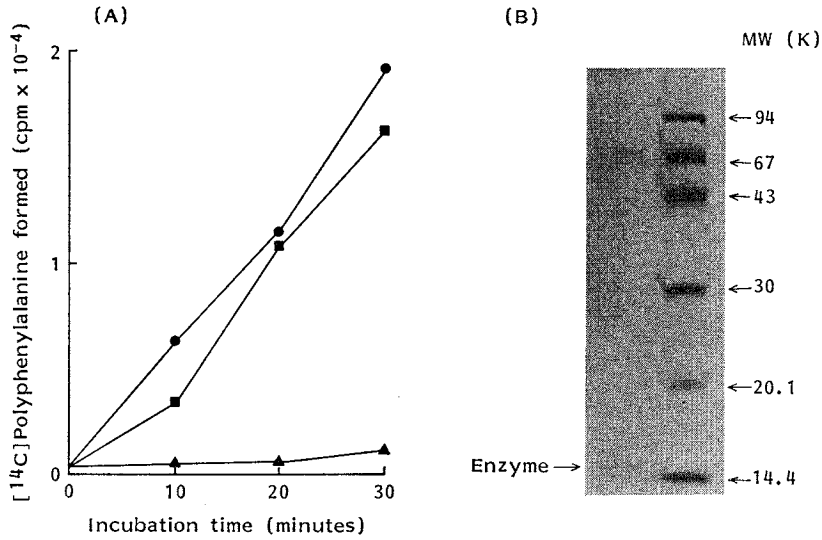
It is known that several antibiotics are inactivated by acetylation[1~5].

We have found that a blasticidin S (BS)-producing actinomycete possesses an enzyme activity which acetylated BS in the presence of acetyl coenzyme A (acetyl CoA). The modified drug was biologically inactive[6].

The present study involves the isolation and biochemical properties of the enzyme. *Streptoverticillium* sp. JCM 4673 (=*Streptomyces morookaensis* KCC S-0673) was used as a BS-producing microorganism. Spores of this strain maintained on a medium (fructose 10 g, Polypeptone 2 g, yeast extract 2 g, meat extract 1 g, agar 20 g, pH 7.0, per liter) were inoculated in 10 ml of GMP medium (glucose 10 g, NaCl 5 g, Polypeptone 4 g, yeast extract 2 g, meat extract 2 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, pH 7.0, per liter) and cultured for 48 hours at 28°C. This culture was transferred to the fresh medium in a jar fermentor (working volume 1.5 liters) and further grown for 24 hours at 28°C. The mycelium was harvested by centrifugation and washed with two buffer solutions described by SUGIYAMA *et al.*[7]. The washed cells (50 g, wet weight) were disrupted by grinding with quartz sand in an ice bath and extracted with 50 ml of buffer A (Tris - HCl 10 mM (pH 7.65), NH_4Cl 30 mM, magnesium acetate 10 mM, 2-mercaptoethanol 6 mM, Mg-titriplex 5 mM, phenylmethylsulfonyl fluoride 3.45 mM and diisopropyl fluorophosphate 0.2 mM) containing 2 μg DNase I/ml. The cell debris and quartz sand were removed by centrifugation at 18,000$\times g$ for 10 minutes. The supernatant was further centrifuged at 30,000 $\times g$ for 30 minutes and then 150,000$\times g$ for 3 hours. For fractionation, solid ammonium sulfate was added to 50 ml of the S-150 fraction to give 20% saturation and kept for 1 hour in an ice bath with gentle shaking. The resulting precipitate was removed by centrifugation at 30,000$\times g$ for 30 minutes. Subsequently, the concentration of ammonium sulfate in the supernatant was increased to 80%, and the precipitate was collected by centrifugation at 30,000$\times g$ for 40 minutes and dissolved in 9 ml of TMAP buffer (Tris - HCl 10 mM (pH 7.65), NH_4Cl 30 mM, magnesium acetate 10 mM, 2-mercaptoethanol 6 mM, and phenylmethylsulfonyl fluoride 0.345 mM) and dialyzed overnight against 1 liter of the same buffer. BS-inactivating enzyme activity was determined by two methods. One involves determination by bioassay of the decreased amount of BS after acetylation of the drug by the enzyme[6]. The other is essentially the same as the method established by SHAW and BRODSKY[8]. The dialyzed solution was applied to a gel filtration column (2.5$\times$102 cm) of Toyopearl HW50S (Tosoh MFG Co., Ltd.) equilibrated with TMAP buffer. The enzyme was eluted with TMAP buffer containing 0.2 M NaCl to avoid adsorption of the enzyme to this resin. The fraction (60 ml) with the enzyme activity were pooled and dialyzed against TMAP buffer. The dialyzed solution was applied to a column (2.2$\times$12 cm) of DEAE-Toyopearl 650S equilibrated with TMAP buffer. The column was washed with 150 ml of the TMAP buffer and then eluted with the same buffer containing 60 mM NaCl. The enzyme fraction was dialyzed overnight against 3 liters of TMAP buffer. The dialyzed solution was applied to an affinity column of AH-Sepharose 4B coupled with BS as a ligand. The enzyme was eluted with TMAP buffer containing 0.2 M NaCl. Then, a chromatofocusing column chromatography was carried out for further purification of the enzyme. A column (0.9$\times$15 cm) of PBE 94 resin (Pharmacia fine chemicals) was preliminary equilibrated with 25 mM Tris - acetic acid (pH 8.3) buffer and then washed with 5 ml of PA buffer (Polybuffer 96 - acetic acid (pH 6.0), Pharmacia chromatofocusing kit). Next, the sample solution was applied to the column and then eluted with PA buffer. The isoelectric pH of the enzyme was determined to be approximately 7.3 by elution profile of the column used above. SDS-PAGE of the enzyme solution from the final step revealed a main protein band together with less intense protein one. The

Fig. 1. Protection of polyphenylalanine synthesis from BS inhibition by BS-acetylating enzyme.



(A) Ten μl of a 500-μg/ml BS hydrochloride solution was incubated overnight at 28°C with the purified enzyme solution, in the presence or absence of 10 μl of 20 mM acetyl CoA. The reaction mixture was added to the polyphenylalanine-synthesizing system consisting ribosomes (60 μg) and S-150 fraction (80 μg as protein) from *Streptomyces griseus* PSR-2.

Additions are as follows: ● TMAP buffer without addition of BS and acetyl CoA, ■ the enzyme fraction incubated with BS and acetyl CoA, ▲ the enzyme fraction incubated with BS without addition of acetyl CoA.

(B) A photograph of SDS-PAGE of the purified enzyme.

molecular weight of the enzyme was estimated to be about 15,000 by SDS-PAGE (Fig. 1) and 13,000 by column chromatography of Toyopearl HW-50S (2.5×105 cm). The enzyme fraction thus purified to 120-fold catalyzed acetylation of BS and exhibited the *Km* values (determined by Lineweaver-Burk plots) for BS and acetyl CoA were 2 mM and 3 mM, respectively. Inhibition by CoA was competitive and *Ki* was 99 mM.

In the previous paper[6], we examined whether the cell extract from BS-producing microorganism could acetylate other antibiotics except BS. The results showed that the fraction inactivated strongly streptothricin and puromycin, in the presence of acetyl CoA. In addition, puromycin was completely inactivated by incubation with the S-150 fraction without addition of acetyl CoA. The purified enzyme fraction, however, did not inactivate puromycin and streptothricin, even in the presence of acetyl CoA. The results indicate that the BS-producing microorganism possesses some other enzyme(s) inactivating these two antibiotics.

It was of interest to know if the purified enzyme could protect protein synthesis against inhibition by BS. As shown in Fig. 1, polyphenylalanine-synthesizing system derived from *Streptomyces griseus* PSR-2 was prepared by the method of SUGIYAMA *et al.*[6] and turned out to be inhibited strongly by BS. This inhibition of protein synthesis, however, relieved when the enzyme fraction was added with acetyl CoA.

Based on these results, it seems likely that the BS-acetylating enzyme plays an important role in self-protection of the producing organism.

Determination of the site of acetylation and molecular cloning of a gene encoding the BS acetylating enzyme are in progress.

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References

1) SATOH, A.; H. OGAWA & Y. SATOMURA: Effect of sclerin on production of the aminoglycoside

antibiotics accompaired by salvage function in *Streptomyces*. Agric. Biol. Chem. 39: 1593~1598, 1975

2) SHAW, W. V. & D. A. HOPWOOD: Chloramphenicol acetylation in *Streptomyces*. J. Gen. Microbiol. 94: 159~166, 1976
3) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature 286: 525~527, 1980
4) KEERATIPIBUL, S.; M. SUGIYAMA & R. NOMI: Mechanism of resistance to streptothricin of a producing microorganism. Biotechnol. Lett. 5: 441~446, 1983
5) SUGIYAMA, M.; S.-Y. PAIK & R. NOMI: Mechanism of self-protection in a puromycin-producing micro-organism. J. Gen. Microbiol. 131: 1999~2005, 1985
6) SUGIYAMA, M.; A. TAKEDA, S.-Y. PAIK, O. NIMI & R. NOMI: Acetylation of blasticidin S by its producing actinomycetes. J. Antibiotics 39: 827~832, 1986
7) SUGIYAMA, M.; M. SAKAMOTO, H. MOCHIZUKI, O. NIMI & R. NOMI: Purification and characterization of streptomycin 6-kinase, an enzyme implicated in self-protection of a streptomycin-producing micro-organism. J. Gen. Microbiol. 129: 1683~1687, 1983
8) SHAW, W. V. & R. F. BRODSKY: Characterization of chloramphenicol acetyltransferase from chroramphenicol-resistant *Staphylococcus aureus*. J. Bacteriol. 95: 28~36, 1968