

STUDIES ON MIKAMYCIN B LACTONASE

I. DEGRADATION OF MIKAMYCIN B BY *STREPTOMYCES MITAKAENSIS*

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Washed-cells or cell-free preparations of *Streptomyces mitakaensis*, the producing organism of mikamycins A and B, were found to inactivate mikamycin B by hydrolyzing its lactonic linkage. The reaction product was identified as mikamycin B acid. An enzyme catalyzing this reaction was named mikamycin B lactonase and its property was investigated by using intact cells.

Some intensive studies on the inactivation mechanisms of clinically important antibiotics such as penicillin¹⁾, chloramphenicol²⁾ and aminoglycosidic antibiotics^{3,4)} have been reported. Derivations and cleavages of these antibiotic molecules were the mechanisms of inactivation in resistant organisms. Another aspect of antibiotic inactivation so far studied is the action of soil microbes. ARGOUDELIS *et al.*⁵⁾ reported that several streptomycete species had the ability to inactivate lincomycin. With regards to the inactivation of depsipeptide antibiotics, WAKSMAN *et al.*⁶⁾ observed that the actinomycin complex was unstable in soil and KATZ⁷⁾ reported an isolation of an actinomycin-decomposing soil microorganism. The induction of lactonases in *Actinoplanes missouriensis* which hydrolyze actinomycin D and dihydrostaphylomycin S, was reported by PERLMAN *et al.*^{8,9)}

However, few efforts have been made to clarify the inactivation of antibiotics at the production stage. Mikamycin B, a depsipeptide antibiotic active against Gram-positive bacteria¹⁰⁾, is now being used widely as feed additives. This antibiotic is produced by *Streptomyces mitakaensis* (K-1839), but its accumulation curve fell down distinctively at mid-log to early stationary phase of cultivation (Fig. 1). In this connection, elucidation of this mechanism would be expected to be of help for increasing the production of mikamycin B. Therefore, we initiated an investigation to disclose factors involved in the degradation of mikamycin B during fermentation.

This report describes the enzymatic degradation of mikamycin B by *S. mitakaensis*, the producing organism of mikamycins A and B, the nature and reaction condition of the enzyme, and structural determination of the enzymatic degradation product.

Materials and Methods

Materials

Mikamycins A¹¹⁾ and B* were kindly supplied by Dr. WATANABE of Kanegafuchi Chemical Industrial Co., Ltd. All reagents used in this work were commercially available.

* Mikamycin B used in this experiments is 98% pure and remaining 1~2% consist of analogous members of mikamycin B (private communication from Dr. K. WATANABE).

Cultivation of *Streptomyces mitakaensis*

Streptomyces mitakaensis was maintained on BENNETT slants. Seed cultures were prepared by aseptic transfer of cells from a slant to 5 ml test tubes containing 15 ml of the following medium, each 10 g/liter of starch, polypeptone, meat extract and molasses. Inoculated tubes were submerge-cultured on a shaker at 27°C for 48 hours. Each 2 ml of this culture was transferred to 500 ml cotton-plugged Erlenmeyer flasks, containing 100 ml of the soybean meal-glycerol medium, which was composed of glycerol 20 g, soybean meal 10 g and sodium chloride 5 g per liter, and the flasks were cultured on a rotary shaker (280 rpm) at 27°C.

Chemical Preparation of Mikamycin B Acid

Mikamycin B acid was obtained according to the method reported by WATANABE¹²⁾.

Preparations of Washed-cells and Crude Enzyme System

A 4-day-old culture of *S. mitakaensis* was harvested and centrifuged at $3,800 \times g$ (5,000 rpm) for 10 minutes. The cell precipitate was washed 3 times with distilled water and suspended in 4 parts of 0.05 M phosphate buffer at pH 7.0 and this was used as a washed-cell preparation.

The cell suspension was subjected to sonic oscillation (10 KC) for 5 minutes under cooling at a temperature below 1°C, and centrifuged at $12,500 \times g$ (10,000 rpm) for 30 minutes. The supernatant solution was retained as a crude enzyme preparation.

Bioassay for Enzyme Activity

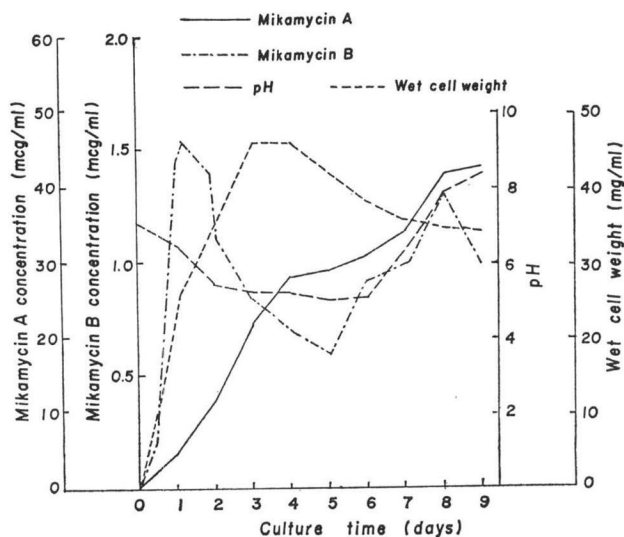
The standard reaction mixture contained 2 ml of the above washed-cell suspension or enzyme preparation and 0.5 ml of a mikamycin B solution (40 mcg/ml). The reaction mixture was incubated at 27°C for 60 minutes and then heated for 10 minutes at 80°C to terminate the reaction. The enzyme activity was measured by the determination of remaining mikamycin B activity, using *Staphylococcus aureus* FDA 209 P as a test organism by a cylinder plate method for mikamycins.¹³⁾

Results

Production of Mikamycin B

The time course of the production of mikamycin B is shown in Fig. 1. The mikamycin B production began at an earlier stage of the cultivation, and the decomposition of the antibiotic

Fig. 1. The time course of the production of mikamycins A and B



occurred at about 30 hours.

Enzymatic Degradation of Mikamycin B

When the standard reaction mixture of enzyme preparation was incubated at 27°C for different periods, amounts of mikamycin B in the reaction mixtures decreased in proportion to the incubation period until 2 hours. After 3 hours of incubation, approximately 100% of the antibacterial activity of mikamycin B disappeared.

Only the mycelial cells exhibited enzyme activity while the culture filtrates were inactive.

Influence of Mycelial Age on the Inactivation of Mikamycin B

The degrading activity of the mycelium was examined in relation to the growth phase of washed-cells.

It was observed that cells harvested at the late log or early stationary phase were most effective in the degradation of mikamycin B (Fig. 2).

Fig. 2. Influence of mycelial age of the organism on the inactivation of mikamycin B.

S. mitakaensis (K-1839) cultivated in the soy-bean meal-glycerol medium, was harvested at various intervals to determine the cell weights and enzyme activity. Equal amounts of washed cells (200 mg/ml) were suspended in 0.05 M phosphate buffer (pH 7.0) and incubated at 27°C for 3 hrs with 8 mcg/ml of mikamycin B.

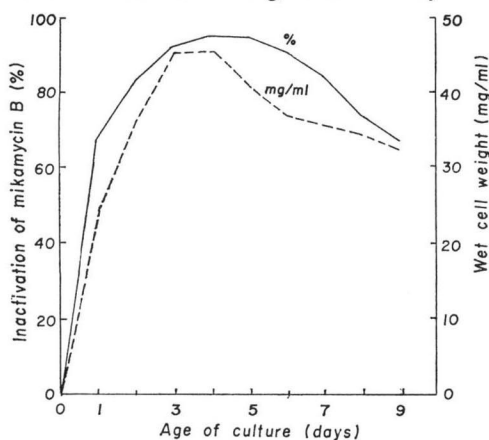
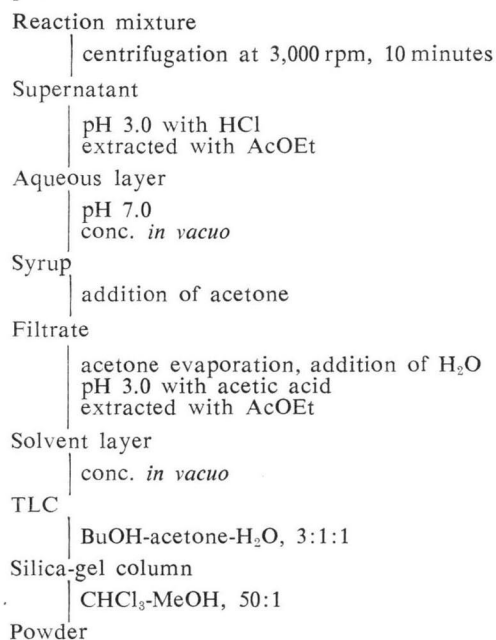


Chart 1. Purification of enzymatic degradation product



Purification and Identification of the Enzymatic Degradation Product from Mikamycin B

Six hundred grams of washed-cells harvested from a 4-day-old culture of *S. mitakaensis* were suspended in 2.4 liters of 0.02 M phosphate buffer (pH 7.0). Twenty milliliters of a mikamycin B solution containing 1.6 mg (total 48 mg) of mikamycin B was added to each 80 ml portion of the washed-cell suspension and incubated for 7 hours at 27°C.

The reaction product was purified from the reaction mixture by the procedure indicated in Chart 1.

Fig. 3. IR spectra of enzymatic degradation product from mikamycin B (a) and mikamycin B acid (b) (in CHCl_3)

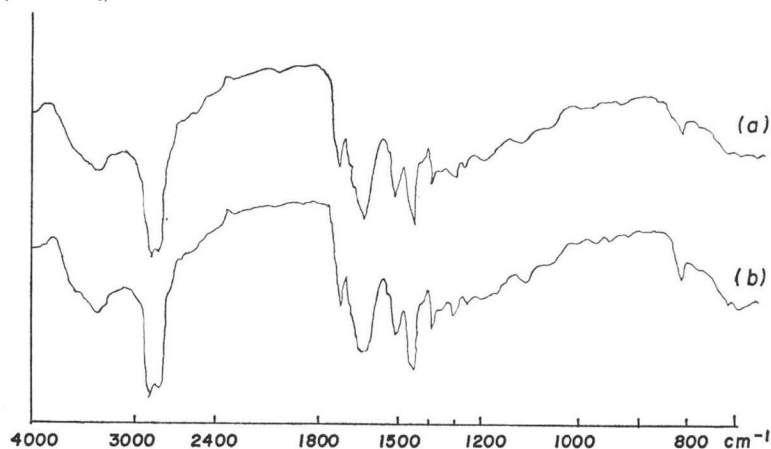


Table 1. Comparison of enzymatic degradation product of mikamycin B with mikamycin B-acid

Substance	Rf values on TLC			
	System A	System B	System C	System D
Mikamycin B	0.77	0.70	0.98	0.50
Enzyme product	0.45	0.43	0.28	0.10
Mikamycin B acid	0.45	0.43	0.28	0.10

Composition of solvent mixtures:

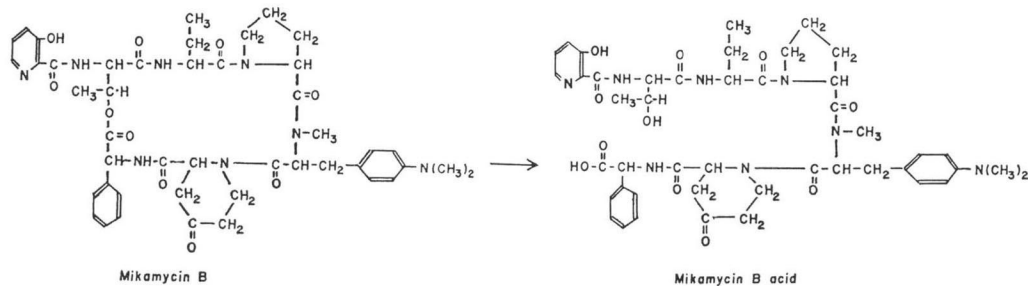
System A: butanol-acetone-water, 3:1:1

System C: chloroform-methanol, 4:1

System B: butanol-pyridine-water, 4:1:5

System D: chloroform-methanol, 10:1

Fig. 4. Reaction of mikamycin B lactonase



The purified enzymatic degradation product was compared with standard mikamycin B acid with the respect to UV, IR spectra and TLC. The UV and IR spectra (Fig. 3) of the enzymatic degradation product showed the same absorption peaks as reported for mikamycin B acid. The enzymatic degradation product was also compared with the chemically prepared mikamycin B acid in four different solvent systems by thin-layer chromatography. Both samples were shown to have identical Rf values as summarized in Table 1.

On the basis of these results, it was demonstrated that the enzymatic degradation product of mikamycin B was identical with mikamycin B acid (Fig. 4).

Some Properties of Washed-cells System

Effect of incubation time: The standard reaction mixture of washed-cell suspension was used in this reaction. The reaction mixture was incubated for different periods at 27°C and the inactivation of mikamycin B was determined. The degradation of mikamycin B increased linearly in proportion to the incubation time up to 120 minutes.

Effect of pH: The effect of pH on the enzymatic degradation of mikamycin B was studied over the range of 5.0~9.0 using acetate buffer (pH 5.0), phosphate buffer (pH 6.0 and 7.0), and tris-HCl buffer (pH 8.0 and 9.0). The optimum pH for degradation of mikamycin B by the washed-cell system appears to be 7.0 (Fig. 5).

Effect of temperature: The effect of incubation temperature on the degradation of mikamycin B was determined. Maximum rate of degradation occurred at 27°C.

Thermostability: The washed-cell suspension was treated over the range of 30~80°C for 10 minutes and the remaining degradation activity was then measured. The degradation activity was stable at 40°C for 10 minutes, but disappeared completely at 70°C for 10 minutes.

Discussion

In the course of mikamycin B fermentation, there was observed a marked depression of antibiotic accumulation in the fermentation beer. Mikamycin B is quite stable in this environmental condition and therefore no loss of antibiotic activity was expected by physical or chemical reasons.

When the antibiotic was incubated with washed-cell or a cell-free preparation of the producing organism, *S. mitakaensis*, disappearance of the antibiotic was noticed and mikamycin B acid was isolated from the incubation mixture as the sole degradation product. These observations suggested a participation of a microbial enzyme which catalyzed the hydrolysis at the lactonic linkage of mikamycin B.

This enzyme was named mikamycin B lactonase and was presumed to cause low titers of the antibiotic in its fermentation process. Degradation of a product by the producing organism might be the first example in the field of antibiotic production.

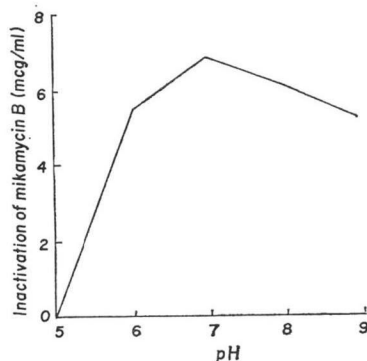
Purification and kinetic studies of this enzyme will be reported in successive papers.

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Fig. 5. Effect of pH on the inactivation of mikamycin B

The reaction mixture containing 2 ml of washed-cells suspension and 0.5 ml of mikamycin B solution (40 mcg/ml) was incubated at 27°C for 60 minutes.



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