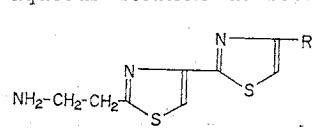
PREPARATION OF BLEOMYCINIC ACID: HYDROLYSIS OF BLEOMYCIN B_2 BY A *FUSARIUM* ACYLAGMATINE AMIDOHYDROLASE

Sir:

The structure of the bleomycins was reported in a previous paper.[1] The various bleomycins produced by *Streptomyces verticillus* differ in the terminal amine moiety, with the bleomycinic acid moiety present in all bleomycins.[2] Therefore, bleomycinic acid is useful for preparing semi-synthetic bleomycins. In this paper, an enzymatic hydrolysis of bleomycin B_2 to bleomycinic acid and agmatine is reported.

We searched for microorganisms which hydrolyze bleomycin B_2. A culture of microorganisms was inoculated to 10 ml of a sterilized nutrient medium in a 50-ml shaking flask and shake-cultured at 26°C. The medium contained 0.5% corn steep liquor, 5% glucose, 0.4% Polypeptone, 0.03% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.01% NaCl, 0.01% $CaCl_2$, and 0.001% $FeCl_3 \cdot 6H_2O$. The pH was adjusted to 6.5 before sterilization. The mycelium was harvested after 72 hours of the shaking culture, and washed twice with 0.02 M phosphate buffer, pH 7.0. The washed cells were used as the enzyme source. One ml of the reaction mixture contained 1 mg of copper-chelated bleomycin B_2, 50 μmoles of pH 7.5 phosphate buffer, 200 mg of the wet washed cells, and 1 drop of toluene. After 15-hour incubation of the reaction mixture at 37°C, the residual antibacterial activity was assayed by a cylinder plate method using *Mycobacterium* 607. Reaction mixtures in which the antibacterial activity of bleomycin B_2 was reduced were examined by thin-layer chromatography on silica gel using methanol - 10% ammonium acetate - 10% ammonia (10 : 9 : 1). A strain of *Fusarium anguioides* SHERBAKOFF was thus found to liberate agmatine, and another product was detected at Rf 0.78 by spraying with 0.2% rubeanic acid in ethanol solution. The Rf value of bleomycin B_2 was 0.66.

The mycelial cells of *F. anguioides* obtained by the shake culture were disrupted in a French press in 0.02 M phosphate buffer at pH 6.9. The homogenate thus prepared was centrifuged in the cold successively at 19,000 *g* for 20 minutes and at 105,000 *g* for 60 minutes. The enzyme was found in the 105,000 *g* supernatant. Five grams of Cu^{++}-chelated bleomycin B_2 was inactivated in the following reaction mixture: 1.0 M phosphate buffer at pH 7.5 125 ml, the enzyme solution prepared from 200 g wet mycelium as described above, and toluene 10 ml, made up to 2,500 ml with distilled water. The reaction mixture was kept at 37°C for 18 hours. Eighty % of the bleomycin was hydrolyzed during the incubation. After filtration the product of Rf 0.78 was adsorbed on Amberlite IRC-50 (H^+-form) column and eluted with 0.1 N HCl. The eluate was neutralized and then adsorbed on Amberlite XAD-2 column. It was eluted with 0.002 N HCl and methanol (1 : 1). Further purification was carried out by CM-Sephadex C-25 column chromatography using 0.05 M ammonium chloride solution followed by treatment with Amberlite XAD-2 column. Thus, 3.41 g of the purified decomposition product was obtained by lyophilization: a blue-colored amorphous powder decomposing at 224~227°C, $[\alpha]_{436}^{27}$ −84.6° (*c* 0.1, H_2O), $\lambda_{max}^{H_2O}$ ($E_{1cm}^{1\%}$): 246 nm(148), 292.5 nm(145); a weak antimicrobial activity against *Mycobacterium* 607, 159 u/mg compared to bleomycin A_2 free base as 1,000 u/mg. The product of Rf 0.78 was confirmed to be copper-chelated bleomycinic acid. It was hydrolyzed with 6 N HCl at 105°C for 20 hours. Two dimensional paper electrophoresis (acetic acid - formic acid - water, 75 : 25 : 900) and ascending paper chromatography (*n*-propanol - pyridine - acetic acid - water, 15 : 10 : 3 : 12) of the hydrolyzate indicated the presence of all amine components of bleomycin B_2 except agmatine. The product of Rf 0.78 was esterified with methanol and conc. HCl (100 : 1) at 27°C for 43 hours. The methyl ester was reduced with sodium borohydride in aqueous solution at room temperature



VI: R = COOH
VI': R = CH_2OH

for 5 hours. After decomposition of excess reagent and desalting, the product was hydrolyzed with 6 N HCl at 105°C for 20 hours. Two dimensional paper chromatography indicated the presence of a new ninhydrin-positive chromophore (**VI'**), instead of compound **VI** which was obtained by hydrolysis of bleomycins as described in previous papers.[3,4]

Finally, we could prepare bleomycin B_2 from bleomycinic acid obtained as above. To the aqueous solution of bleomycinic acid (95 mg/2.4 ml) were added 5 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 10 equivalents of agmatine sulfate under ice cooling. The reaction mixture was adjusted to pH 7.0 and kept at 5°C overnight. Thereafter it was charged on a CM-Sephadex C-25 column in 0.05 M ammonium chloride solution. The chromatogram was developed by a linear gradient of ammonium chloride from 0.05 to 1.0 M. The fraction eluted with 0.63~0.65 M ammonium chloride solution showed strong activity against *Mycobacterium* 607. The active principle was isolated. The yield was 8 mg. The antimicrobial activity was 3,015 u/mg, while bleomycin B_2 showed 3,094 u/mg. The chromatographic behavior and the analytical data of the acid hydrolyzate were exactly the same as those of bleomycin B_2.

The enzyme which hydrolyzed bleomycin B_2 was extracted from the 105,000 *g* supernatant at 0°C by the following successive steps: treatment with protamine sulfate (775 mg added to 500 ml), precipitation by 55~75 % $(NH_4)_2SO_4$ saturation, dialysis against 0.02 M phosphate buffer at pH 6.9. Using this enzyme solution, the results described below were obtained.

The optimum pH for hydrolysis of bleomycin B_2 was 8.0 in phosphate buffer and 7.5 in Tris-HCl buffer. Unexpectedly the action of this enzyme was specific to bleomycin B_2: other bleomycins as shown in Table 1 were not hydrolyzed. These results suggested that action of the enzyme would be specific to agmatine, but not bleomycinic acid moiety. Really, the enzyme was confirmed to be an acylagmatine amidohydrolase as shown in Table 2. Acetyl, propionyl and benzoyl agmatines were hydrolyzed. As far as we know, it is the first observation of enzymatic hydrolysis of acylagmatines.

Table 1. Substrate specificity of the enzyme for various bleomycins

Bleomycin	Terminal amine	Enzyme activity (nmol/min/mg)
B_2	NH_2-$(CH_2)_4$-NH-C(=NH)-NH_2	135
artificial	NH_2-$(CH_2)_3$-NH-C(=NH)-NH_2	0
artificial	NH_2-$(CH_2)_2$-NH-C(=NH)-C_2NH_2	0
B_4	NH_2-$(CH_2)_4$-NH-C(=NH)-NH-$(CH_2)_4$-NH-C(=NH)-NH_2	0
A_2	NH_2-$(CH_2)_3$-S^+-$(CH_3)_2X^-$	0
A_5	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2	0
A_2'-a	NH_2-$(CH_2)_4$-NH_2	0
A_2'-b	NH_2-$(CH_2)_3$-NH_2	0

Experimental condition: The reaction mixture contained substrate 1 mg, phosphate buffer (pH 8.0) 50 μmoles, the enzyme solution 0.1 ml in 1.0 ml; incubated at 37°C for 1 hour; assayed by antimicrobial activity against *Mycobacterium* 607.

Table 2. Kinetic constants of acylagmatine amidohydrolase from *F. anguioides*

Substrate	km (M)	Vm (μmol/min/mg)
Bleomycin B_2	8.0×10^{-4}	0.029
Acetyl agmatine	13.3×10^{-4}	0.168
Propionyl agmatine	20.0×10^{-4}	0.242
Benzoyl agmatine	2.2×10^{-4}	0.279

Experimental condition: The reaction mixture contained phosphate buffer (pH 8.0) 50 μmoles, the enzyme solution 0.1 ml, varied amounts of substrates in 1.0 ml. Incubation time for bleomycin B_2 was 1 hour and for others 10 minutes. The residual bleomycin was determined by activity against *Mycobacterium* 607. In other cases agmatine produced was determined by ninhydrin spectroscopy.

Hamao Umezawa
Yasushi Takahashi*
Akio Fujii*
Tetsushi Saino*
Tadashi Shirai*
Tomohisa Takita

Institute of Microbial Chemistry,
3-14-23, Kamiosaki, Shinagawa-ku,
Tokyo, 141 Japan,
* Research Laboratory,
Nippon Kayaku Co., Ltd.,
3-31-12, Shimo, Kita-ku,
Tokyo, 115 Japan

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