A BLEOMYCIN-INACTIVATING ENZYME IN MOUSE LIVER

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(Received for publication March 13, 1974)

Affinity chromatography of a bleomycin-inactivating enzyme was studied on Sepharose 4B-bleomycin A_5 and Sepharose 4B-lysinamide. Mouse liver was homogenized, and the enzyme extracted by ammonium sulfate precipitation and Sephadex G-200 chromatography. Affinity chromatography of the extract on Sepharose 4B-lysinamide gave 25-fold purified enzyme. Further purification was not successful because of instability of the enzyme. It hydrolyzed L-lysinamide, L-lysyl-β-naphthyl-amide, L-arginyl-β-naphthylamide, L-lysyl-L-lysine and lysyl-bradykinin, but not leucinamide or L-leucyl-β-naphthylamide. Hydrolysis of L-lysyl-β-naphthylamide and L-arginyl-β-naphthylamide by the enzyme was competitively inhibited by bleomycin B_2. The bleomycin-inactivating enzyme of rat liver was separated from a known aminopeptidase B.

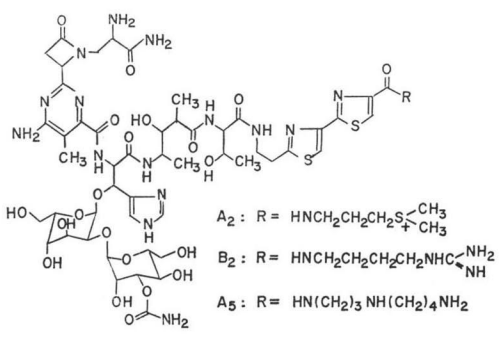
In previous papers[1,2], we described the presence of a bleomycin-inactivating enzyme in various animal tissues. The content of this enzyme was significatly lower in squamous cell carcinoma in mouse skin than in sarcoma, both of which were induced by 20-methylcholanthrene. This enzyme obtained from mouse liver hydrolyzed the carboxamide group of the β-amino-alanine moiety of bleomycin molecule (Fig. 1). In this paper, we report the extraction of this enzyme by affinity chromatography and its relation to known enzymes.

Materials and Methods

Materials:

Cooper-free bleomycins B_2 and A_5 which were purified by CM-Sephadex C-25 chromatography with a gradient of ammonium formate from 0.05 M to 1.0 M or with a gradient of sodium chloride from 0.05 M to 1.0 M were employed throughout the experiments. Repeated chromatography yielded only single peaks. Lysinamide was prepared in our institute, and L-lysyl-β-naphthylamide and L-arginyl-β-naphthylamide were purchased from Mann Research Laboratories, N. Y.. L-Leucyl-β-naphthylamide was purchased from Tokyo Kasei Kogyo Co., Ltd. L-Lysylglycine, L-lysyl-L-leucine, L-lysyl-L-phenylalanine, and L-lysyl-L-lysine were purchased from Research Divison of Miles Laboratories, U. S. A. and lysyl-bradykinin was purchased from Protein Research Foundation, Osaka, Japan. Fast Garnet GBC salt (O-aminoazatoluene diazonium salt) was purchased from Sigma Chemical Company, U.S.A.

Fig. 1. Structure of bleomycins.



Inactivation of bleomycin B_2 and determination of the residual bleomycin B_2:

In the reaction mixture which contained 0.1 ml of the enzyme solution and 0.3 ml of

1/15 M phosphate buffer, pH 7.2, the reaction was started by addition of 100 μg (0.14 mM) of bleomycin B_2 in 0.1 ml of distilled water. The reaction was continued for 60 minutes at 37°C and terminated by addition of 0.5 ml of methanol. The residual bleomycin B_2 was determined by disc assay using *Bacillus subtilis* PCI 219 as the test organism.

Determination of hydrolysis of lysinamide and leucinamide:

The reaction mixture contained 0.2 ml of 10 mM lysinamide or leucinamide, 0.2 ml of the enzyme soultion and 0.1 ml of 1/15 M phosphate buffer, pH 7.2. After 60 minutes at 37°C the reaction was stopped by addition of 0.5 ml of methanol. After centrifugation, the supernatant was spotted onto filter paper and subjected to high voltage electrophoresis at 3,500 V, in pH 1.8 buffer of formic acid-acetic acid-water (25:75:900 in volume) for 15 minutes. The lysine or the leucine liberated were determined colorimetrically at 570 nm by ninhydrin reaction after extraction with 70% ethanol.

Determination of hydrolysis of L-lysyl-β-naphthylamide, L-arginyl-β-naphthylamide and L-leucyl-β-naphthylamide:

A buffered stock substrate solution was prepared by mixing 0.1 M tris HCl buffer, pH 7.0 (3 parts), 1 mM L-arginyl-β-naphthylamide, L-lysyl-β-naphthylamide or L-leucyl-β-naphthylamide (1 part) and distilled water (1 part) and was stored at 0°C. To 1.25 ml of this solution, 0.1 ml of the enzyme solution and 0.15 ml of distilled water were added, and the reaction was carried out at 37°C for 60 minutes. After the reaction, the tube was placed in a ice bath and immediately 0.5 ml of a solution of the stabilized diazonium salt Garnet GBC (1 mg/ml) in 1 M pH 4.2 acetic acid buffer containing 10% Tween 20 was added. The amount of β-naphylamine liberated was determined from the absorbance at 530 nm.

Affinity chromatography using Sepharose 4B-bleomycin A_5:

Cyanogen bromide-activated Sepharose 4B (1.5 g) purchased from Pharmacia Fine Chemicals AB, Upsala, Sweden, was washed successively with 200 ml of 1 mM hydrochloric acid, 150 ml of distilled water and 70 ml of 100 mM sodium bicarbonate in 500 mM sodium chloride, The washed material was added to 10 ml of 100 mM sodium bicarbonate in 500 mM sodium chloride which contained 188 mg of bleomycin A_5 and the suspension was gently stirred at 4°C overnight. Sepharose 4B-bleomycin A_5 thus prepared was washed with 70 ml of 100 mM sodium bicarbonate in 500 mM sodium chloride, and treated with 45 ml of 1 M monoethanolamine hydrochloride (pH 8.0). It was further washed with 15 ml of 100 mM acetate buffer (pH 4.0) in 1 M sodium chloride, 15 ml of 100 mM borate buffer (pH 8.0) in 1 M sodium chloride and 150 ml of distilled water, successively. The residual bleomycin A_5 in the filtrate and the washed solution was determined by disc assay using *B. subtilis* as the test organism or by absorption at 292 nm which is characteristic of all bleomycins. In this reaction, it was confirmed that 53 mg of bleomycin A_5 bound with 1.5 g of the activated Sepharose 4B. The Sepharose 4B-bleomycin A_5 was suspended in 10 mM phosphate buffer, pH 7.2, and placed in a column (10 mm in diameter).

Mouse liver was homogenized with 3 times its weight of 0.25 M sucrose and the homogenate was centrifuged at 105,000 g for 60 minutes.

Affinity chromatography using Sepharose 4B-lysinamide:

The activated Sepharose 4B (1.5 g) was reacted with lysinamide (300 mg) at 4°C overnight and treated by the method discribed above. The 105,000 g supernatant (5 ml) of mouse liver which was prepared as described above was applied to the Sepharose 4B-lysinamide column.

Results and Discussion

The hydrolysis of the carboxamide group by the bleomycin-inactivating enzyme suggested a similarity of the inactivating enzyme to leucine aminopeptidase. However, leucine aminopeptidase which was purchased from Tokyo Kasei Kogyo Co., Ltd. did not inactivate bleomycin.

Arginase which was described by MUELLER *et al.*[3] to inactivate bleomycin did not inactivate bleomycins in our experiments.

Among the various bleomycins, bleomycin A_5 was considered suitable for affinity chromatography, because it could be found by the terminal amine, the spermidine moiety which is far from the carboxamide group. Therefore, bleomycin A_5 was bound to *S*epharose 4B by the usual method of cyanogen bromide activation.

The bleomycin-inactivating enzyme was very unstable, and we could not elute the enzyme from Sepharose 4B-bleomycin A_5 with a gradient of sodium chloride. Therefore, elution was carried out by stepwise increase of the concentration of sodium chloride in 10 mM phosphate buffer. Fractions of 1.7 g were taken and examined for optical density at 280 nm, and hydrolytic activity on lysinamide, leucinamide and bleomycin B_2. The results are shown in Fig. 2. The bleomycin-inactivating enzyme was eluted with 0.05 M sodium chloride, and this fraction hydrolyzed lysinamide and leucinamide. During storage, hydrolysis activity against bleomycin B_2 and lysinamide were equally reduced.

In affinity chromatography with Sepharose 4B-lysinamide, the bleomycin-inactivating enzyme was eluted with 0.1~0.2 M sodium chloride in 10 mM phosphate buffer, when the concentration

Fig. 2. Sepharose 4B-bleomycin A_5 affinity chromatography of bleomycin-inactivating enzyme.

Five ml of 105,000 *g* supernatant of the homogenate obtained from mouse liver with the 3 times volume of 0.25 M sucrose was chromatographed on an affinity column prepared from 1.5 g cyanogen bromide-activated Sepharose 4B and 188 mg of bleomycin A_5 (53 mg of A_5 was bound). The bleomycin-inactivating enzyme was eluted by stepwise increase of NaCl in 20 mM phosphate buffer. The eluate was collected in 1.7-g fractions, and tested for bleomycin-inactivating activity, lysinamide-hydrolyzing activity and leucinamide-hydrolyzing activity.

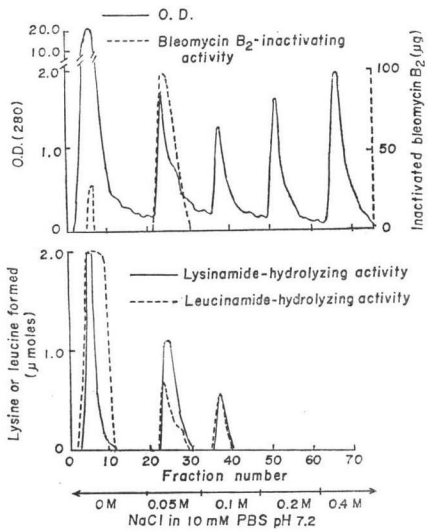


Fig. 3. Purification of bleomycin-inactivating enzyme by affinity chromatography with Sepharose 4B-lysinamide.

The enzyme (41.5 mg protein) prepared by Sephadex G-200 gel filtration was chromatographed on an affinity column which was preparared from 2.0 g of cyanogen bromide activated Sepharose 4B and 450 mg of lysinamide. The bleomycin-inactivating enzyme was eluted by stepwise increase of NaCl concentration in 10 mM phosphate buffer. The eluate was collected in 2.0-g, fractions and tested for bleomycin-inactivating activity and arginyl-β-naphthylamide and leucyl-β-naphthylamide-hydrolyzing activity.

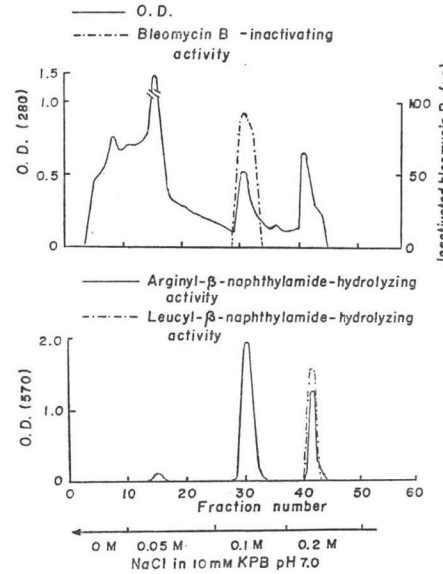


Fig. 4. Competitive inhibition of L-lysyl-β-naphthylamide hydrolysis by bleomycin B_2.

Reaction mixture contained 100 μmoles of tris-HCl buffer, pH 7.2, various concentrations of L-lysyl-β-naphthylamide, enzyme preparation, and distilled water in a final volume of 1.5 ml.

The reaction was carried out at 37°C for 30 minutes.

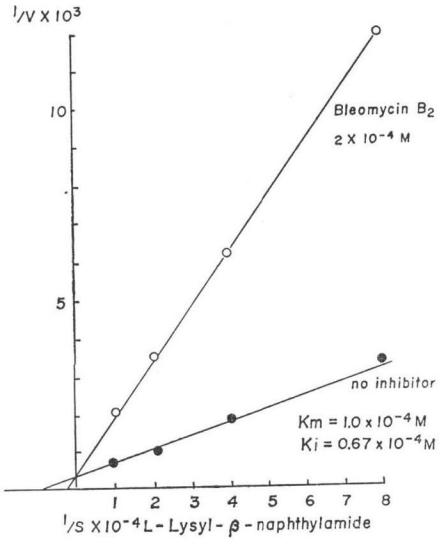
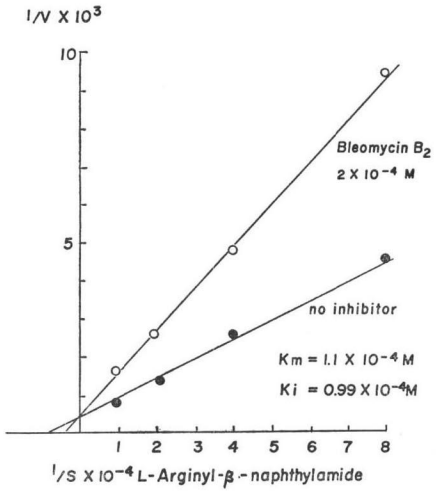


Fig. 5. Competitive inhibition of L-arginyl-β-naphthylamide hydrolysis by bleomycin B_2.

Reaction mixture contained 100 μmoles of tris-HCl buffer, pH 7.2, various concentrations of L-arginyl-β-naphthylamide, enzyme preparation, and distilled water in a final volume of 1.5 ml.

The reaction was carried out at 37°C for 30 minutes.



of sodium chloride was increased in steps as follows: 0, 0.05 M, 0.1 M, 0.2 M and 0.4 M. The fraction which inactivated bleomycin B_2 showed much stronger activity in hydrolyzing lysinamide than leucinamide. The bleomycin-inactivating enzyme was extracted from mouse liver by ammonium sulfate precipitation and Sephadex G-200 chromatography as described in a previous paper[1,2], and this fraction was used for Sepharose 4B-lysinamide chromatography. The results are shown in Fig. 3. The fraction which inactivated bleomycim B_2 hydrolyzed lysinamide and L-arginyl-β-napethylamide but not leucinamide or L-leucyl-β-naphthylamide. By this method, as shown in Table 1, 27-fold purification was accomplished.

The action of the bleomycin-inactivating enzyme thus purified on L-lysyl-β-nayhthylamide and L-arginyl-β-naphthylamide was studied, and it was found that the fraction which contains the bleomycin-inactivating enzyme hydrolyzed these dipeptides. Moreover, this enzyme fraction hydrolyzed L-lysylglycine, L-lysyl-L-phenylalanine, L-lysyl-L-leucine, L-lysyl-L-lysine and lysyl-bradykinin.

In a reaction mixture which contained 0.1 ml of purified enzyme solution (64 μg protein) and 0.07 mM bleomycin B_2 in 1/15 M phoshate buffer, pH 7.2, L-lysyl-β-naphthylamide and L-arginyl-β-naphthylamide at 0.7 mM showed 69 % and 68 % inhibition of bleomycin B_2 inactivation.

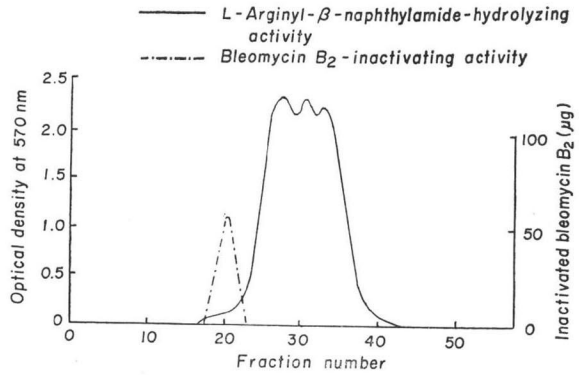
In the same reaction mixture, 0.1 mM bleomycin B_2 showed 49 % and 45 % inhibition of hydrolysis of L-lysyl-β-naphthylamide and L-arginyl-β-naphthylamide respectively. The inhibition by 0.2 mM bleomycin B_2 in the same reaction mixture was 65 % with L-lysyl-β-naphthylamide and 61 % with L-arginyl-β-naphthylamide.

Table 1. Extraction and purification of bleomycin-inactivating enzyme from mouse liver.

	Volume (ml)	Total protein(mg)	Total* units	Specific activity	Yield (%)	Grade of purification
105,000 *g*	19.0	361.0	353.8	0.98	100	1.0
$(NH_4)_2SO_4$ 0.3~0.6 sat.	5.2	137.8	246.7	1.79	69.7	1.8
Sephadex G-200	21.0	41.5	193.6	4.66	54.7	4.8
Sepharose 4B-lysinamide chromatography	6.0	1.92	51.1	26.6	14.4	27.1

* One unit is the activity which inactivates 1 μg bleomycin B_2 per minute under the condition described in Materials and Methods.

Fig. 6. Separation of the bleomycin-inactivating enzyme from aminopeptidase B of rat liver.



As shown in Figs. 4 and 5, bleomycin showed competitive inhibition of the hydrolysis of these peptides.

The results described above suggest that the same enzyme is involved in bleomycin inactivation (bleomycin hydrolysis) and hydrolysis of lysinamide, L-lysyl-β-naphthylamide, L-arginyl-β-naphthylamide. Thus, this enzyme was tentatively named bleomycin hydrolase. Among known enzymes, aminopeptidase B obtained from rat liver is most closely related to bleomycin hydrolase. Aminopeptidase B was extracted from rat liver by the following method described by HOPSU *et al.*[4]: Rat liver (6.0 g) was homogenized in 12 ml of 0.1 M tris-hydrochloride buffer (pH 7.0) with a glass homogenizer at 4°C; the homogenate was immediately centrifuged at 105,000 *g* for 60 minutes; the supernatant was precipitated with ammonium sulfate (30~50 % saturation). The precipitate was dissolved in 1.2 ml of 0.1 M tris-hydrochloride and subjected to Sephadex G-200 chromatography. Then, as shown in Fig. 6, the bleomycin B_2-inactivating activity was separated from the main peak of the enzyme which hydrolyzed L-arginyl-β-naphthylamide, providing that the bleomycin-inactivating enzyme in rat liver is different from aminopeptidase B. The bleomycin-inactivating enzyme of rat liver was far more unstable than aminopeptidase B, and was not activated by chloride ion. We have confirmed that bleomycin B_2 inactivated by rat liver enzyme behaves similarly in CM-Sephadex C-25 chromatography as that inactivated by the mouse enzyme and thus the rat enzyme was shown to hydrolyze the carboxamide group. However, it is not completely certain that the bleomycin-inactivating enzyme of rat liver has activity in hydrolyzing lysinamide and the other peptides described above.

We have not yet succeeded in confirming the induction of bleomycin hydrolase in mouse liver and rat hepatoma cells. However, bleomycin hydrolase which hydrolyzes L-lysyl-L-lysine and lysyl-bradykinin may play a physiological role in hydrolysis of lysyl-badykinin and basic proteins.

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