PHYSICO-CHEMICAL AND BIOLOGICAL STUDIES ON ACTINOXANTHIN, AN ANTIBIOTIC FROM *ACTINOMYCES GLOBISPORUS* 1131

A. S. KHOKHLOV, B. Z. CHERCHES, P. D. RESHETOV, G. M. SMIRNOVA, I. B. SOROKINA, T. A. PROKOPTZEVA, T. A. KOLODITSKAYA and V. V. SMIRNOV

Institute for Chemistry of Natural Compounds, Ac. Sciences USSR, Moscow, USSR

S. M. NAVASHIN and I. P. FOMINA

Research Institute of Antibiotics, Moscow, USSR

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The isolation, some chemical and biological properties of a new antitumour antibiotic Actinoxanthin are described. It was found to be a low-molecular weight protein.

Actinoxanthin, an antibiotic produced by *Actinomyces globisporus* 1131 was described in 1957[1]. Its first preparations were isolated from the fermentation broth by two-fold precipitation with $(NH_4)_2SO_4$ followed with filtration through $BaCO_3$[1]. The actinoxanthin samples thus obtained, while exhibiting high antibacterial and antitumour activity, were shown by us to be a multicomponent mixture. The present paper describes the isolation of the homogeneous antibiotic and reports some of its properties.

Fermentation

To produce actinoxanthin we used the strain of *Actinomyces globisporus* 1131 described in 1958[2]. It was grown in 100-liter fermenters (60 liters of medium) at 28°C, and 750-ml flasks (150 ml of medium). Composition of nutrient medium (g/liter): corn steep extract, 10; $(NH_4)_2SO_4$, 3.5; NaCl, 5; $CaCO_3$, 4; glucose, 5; starch, 15. For the stock cultures the organism was shake-grown in flasks (210 r.p.m., 48 hours). The fermenter was inoculated with 1.5 liter of 24-hour fermentation broth, aerated at a rate of 60 liters per minute, as antifoam agent sperm whale oil was used. The incubation was stopped when cytoplasmic basophility went down, nuclear granularity seemed more pronounced, and the first pieces of decomposing mycelium were found afloat.

Isolation of Crude Actinoxanthin

All the procedures on isolation and purification of actinoxanthin were carried out at 4~5°C; specific activity of the preparation was assayed by serial two-fold broth dilution method on meat-peptone medium at pH 7.5 with *Staphylococcus aureus*

209P as test organism (number of cells, $2 \cdot 10^6$/ml) or by agar cup-dilution method with *Bacillus subtilis* as test culture.

To 77 liters of filtered cultural broth with activity 3.6×10^4 units/ml, 54 kg of $(NH_4)_2SO_4$ was added, the mixture was vigorously stirred for 30 minutes, the active precipitate was separated, dissolved in 7.7 liters of water and reprecipitated with 5.4 kg $(NH_4)_2SO_4$. The active precipitate was dissolved in 2 liters of water to give concentrate (I) with activity 128×10^4 units/ml. To the I thus obtained 30 ml of 50 % aqueous trichloroacetic acid was added under vigorous stirring, the in active sediment was quickly removed and the supernatant was brought to pH 6.5~6.8. To remove pigments the resulting 1,980 ml of concentrate (activity 128×10^4 units/ml) was twice stirred for an hour with 200 g portions of Dowex 1×2 (acetate form). Decolorized liquid of unchanged activity was stirred for 30 minutes with 1.4 kg of $(NH_4)_2SO_4$. The active precipitate was collected, dissolved in 200 ml distilled water, and treated with 3.7 ml of 50 % trichloroacetic acid. The precipitate was discarded, the active supernatant was neutralized to pH 6.5~6.8 and dialysed to negative test for sulphate ions. Resultant 430 ml of concentrate (II) with activity

Fig. 1. Acrylamide gel disc electrophoresis of actinoxanthin preparations

Experimental conditions: Orstein-Davis system (4); monomer concentration 15 %; charge, 400 μg; electric current, 5 MA; time, 60 minutes; dye, nigrosin (1.2 mg in 1,000 ml of ETOH : AcOH : H_2O 5 : 1 : 4)

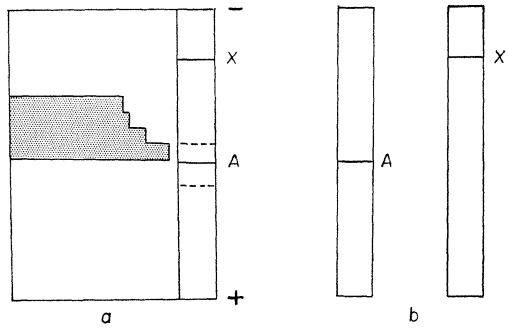


Fig. 2. Ion exchange chromatography of actinoxanthin

Column packing-DEAE Sephadex A-50 (medium) (3.5 × 80 cm); charge-2.9 g. Eluent mixture: mixing chamber, 2.5 liters of buffer pH 8.5 (AcOH - conc. NH_4OH - H_2O, 10 : 15.4 : 975); reservoir, 2.5 liters of buffer pH 8.5 (AcOH - conc. NH_4OH - H_2O, 20 : 30.8 : 950); fraction volume, 25 ml/20 minutes.

Characteristics: (1) optical density at 276 mμ; (2) activity against *B. subtilis*; (3) activity against *St. aureus* 209P

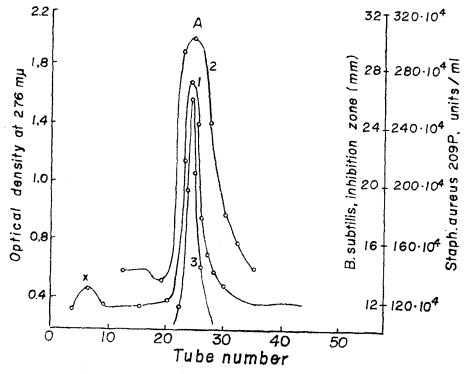
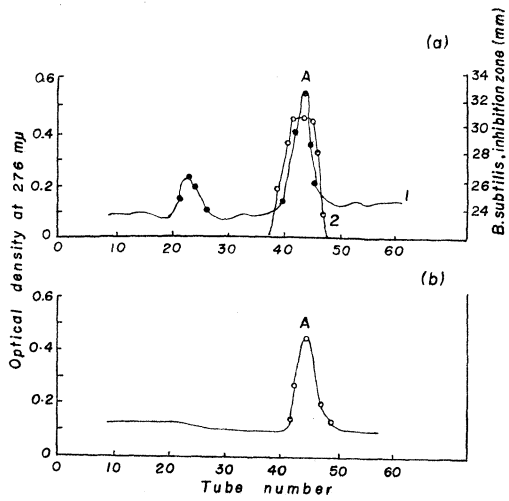


Fig. 3. Gel filtration of actinoxanthin on Sephadex G-75 (1.5×160 cm); charge, 40 mg of antibiotic in 1.5 ml of buffer; eluent solution, buffer at pH 8.2 (0.05 M ammonium bicarbonate); fraction volume, 4 ml/10 minutes.

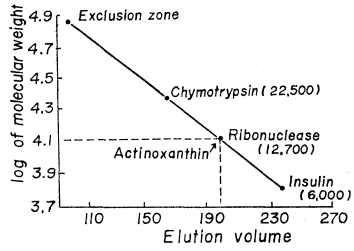
Characteristics: (1) optical density at 276 mμ, (2) activity against *B. subtilis*.



512×10^4 units/ml was freeze-dried to give 5.3 g of slightly coloured substance with activity 40×10^4 units/mg (80 % yield of initial activity). Disc electrophoresis in anionic system[3] showed the preparation to be inhomogeneous with two component named A and X predominating (Fig. 1a). The gel was cut into 20 pieces, which were extracted with 0.2 ml portions of water for 14~16 hours. The extracts were assayed by agar cup diffusion with *B. subtilis*. The antibacterial activity was associated with the zone A, which was then assigned to actinoxanthin (Fig. 1a).

Fig. 4. Actinoxanthin molecular weight determination

Column packing-Sephadex G-75 (1.5×160 cm); fraction volume, 4 ml/10 minutes.



Ion-exchange Chromatography and Gel Filtration of Actinoxanthin

The freeze-dried preparation (2.9 g), (activity 40×10^4 units/mg) in 50 ml of pH 8.5 buffer (AcOH - conc. $NH_4OH - H_2O$, 2.5 : 3.85 : 1,000) were applied to a DEAE-Sephadex A-50 column. The column was washed with the same buffer until the optical density of the eluate fell to 0.05~0.07 at 276 mμ. The actinoxanthin was eluted with an ionic strength gradient of the same buffer (Fig. 2).

Fractions containing actinoxanthin were dialyzed against distilled water and freeze-dried, to give 1.1 g. The preparation (activity 98×10^4 units/mg) though appearing homogeneous on acrylamide gel electrophoresis, (Fig. 1b) showed two peaks when applied to Sephadex G-75, (Fig. 3a). We subjected 41 mg of this preparation to gel filtration, and obtained 23 mg of pure actinoxanthin. This was proved to be homogeneous by repeated gel filtration, (Fig. 3b) disc electrophoresis, and N-terminal amino acid determination.

Properties

Molecular weight measurements carried out according to ANDREWS[4] in comparison with reference compounds are given in the Fig. 4. Actinoxanthin elution volume is equal to that of RNase, which makes it approach 13,000. Using TLC technique on silica gel, we identified alanine as an N-terminal amino acid[5]. Cystein was determined as cysteic acid upon oxidation with performic acid according to MOORE[6], a test for tryptophan according to OPIENSKA-BLAUTH[7] showed it to contain none. Antibiotic was hydrolyzed under standard conditions and its amino acid composition determined with BECKMAN analyzer Model 120 C as: Lys-1, His-1, Arg-1, Asp-9, Glu-5, Thre-9, Ser-16-17, Pro-6, Gly-15-16, Ala-20, Val-10, Ileu-1, Leu-5, Tyr-3, Phe-5, and 1/2 Cys-4 (determined as Cys SO_3H). Molecular weight calculated from these data (12,500) is in good agreement with that obtained by gel filtration.

Actinoxanthin is active against *Staph. aureus* 209 P at concentrations of 0.001 μg/ml; it inhibits also other Gram-positive organisms. Gram-negative organisms have been shown to be insusceptible to it. Actinoxanthin (concentrate II) in a dose of 13 μg/ml depresses HeLa cell growth 37 %, and at 130 μg/ml 64 %. By intraperitoneal injection LD_{50} for white mice is 0.24 mg/kg. A dose of 10~15 μg/kg depressed

a solid form of EHRLICH tumour 59~63 % when the treatment was begun on the 7 th day after transplantation. Preliminary data show adenocarcinoma 755 and leucosis La of mice C_{57}BL to be very susceptible to actinoxanthin too.

Actinoxanthin stored in solution and in freeze-dried state is very soon inactivated; its toxicity also decreases. (However its Rf-values on acrylamide gel electrophoresis suffer no change). It is more stable when stored suspended in saturated solutions of ammonium sulfate.

Discussion

Actinoxanthin properties, its amino acid composition, molecular weight, biological activity place it among acid in low molecular weight proteins that possess antitumour activity. Carzinocidin[8], marinamycin[9], macromomycin[10], and neocarzinostatin[11] also seem to belong to the same group. However, the description of their properties is very scant and controversial, and this makes it very difficult to compare them in detail. Neocarzinostatin is better investigated than the other ones; its amino acid composition was studied and has been found to be very similar to that of actinoxanthin. Nevertheless, it is quite clear, that the both antibiotics are not identical. Unlike actinoxanthin, neocarzinostatin does not contain histidine and has a molecular weight of about 9,000. Neocarzinostatin is degraded by trypsin, while actinoxanthin is resistant to this enzyme.

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