Determination of Exotoxin in *Bacillus thuringiensis* Cells

K. Horská *, J, Vanková **, and K. Šebesta *

Institute of Organic Chemistry and Biochemistry *, Czechoslovak Academy of Sciences, Prague
and
Institute of Entomology **, Czechoslovak Academy of Sciences, Prague

The presence of exotoxin in *Bacillus thuringiensis* was demonstrated and its quantity in the cells determined. The concentration of exotoxin in the producing microorganism is approximately half the concentration of ATP. Exotoxin is produced at such a rate that the cell excretes 1/5 to 1/4 of its exotoxin content into the medium per minute.

B. thuringiensis has been known predominantly as a producer of endotoxin, a crystalline inclusion of protein character, which specifically acts on Lepidoptera caterpillars [1]. This property of endotoxin has been exploited also for industrial purposes. Special attention has been devoted during the past few years to exotoxin which is excreted by *B. thuringiensis* into the culture medium [2-5]. The elucidation of the chemical structure of the exotoxin has shown that this compound contains glucose, allaric acid, and a phosphoric acid residue in addition to adenosine [6]. The unusual chemical structure of this compound, containing elements not encountered in nature so far (an ether bond between glucose and ribose, allaric acid), is paralleled also by an interesting mechanism of its effect. Exotoxin, which is strongly toxic both for insects and mammals [7], can play the role of inhibitor in the reaction synthetizing RNA by DNA-dependent RNA polymerase. Exotoxin specifically competes during this reaction with one of the substrates, namely with ATP (ref. [8, 9]).

The biosynthesis of exotoxin has not been studied as yet. It was therefore of interest to determine whether and in what quantity exotoxin is present in the bacterial cell.

Materials and Methods

Chemicals

Carrier-free [^{32}P]orthophosphate was supplied from the GDR. [^{32}P]Labelled exotoxin was prepared by the method described earlier [10]. [^{14}C]ATP was

a product of the Institute of Research, Production, and Use of Radioisotopes, Prague. Ecteola cellulose ET 11 Whatman was used for ion-exchange chromatography. Triethylammonium bicarbonate (TEA) was prepared by saturation of a triethylamine solution with carbon dioxide to pH 7.8. Active charcoal was boiled with dilute hydrochloric acid before use and washed free of chloride ions by distilled water.

Cultivation of bacteria

In all experiments, *B. thuringiensis var. gelechiae* was used; it was cultivated both in a low P medium to which [^{32}P]orthophosphate had been added (600 μCi/50 ml of culture medium) and in a high P medium, as described earlier [10].

Qualitative proof of exotoxin in cells

Bacteria cultivated in 50 ml of low P medium containing [^{32}P]orthophosphate were centrifuged at different time intervals (10, 12, 24 hours). Only the bacteria were treated further; the supernatants were discarded. The bacteria were washed with 0.9% NaCl (3 × 60 ml, 0 °C) and extracted with perchloric acid (0,24 M, 2 × 2.5 ml, 0 °C, 10 min). The acid extracts were immediately neutralized by 7 M KOH. Active charcoal (100 mg) was added to the neutral extracts after the separation of $KClO_4$. The suspensions were allowed to stand for 30 min and then centrifuged at 3000 × g, 20 min (the centrifugation of all extracts described below was carried out under identical conditions). The sediments were washed with distilled water (2 × 20 ml), then extracted with 50% aqueous ethanol (2 × 1.5 ml), and with a mixture of 25% aqueous ethanol and 0.5% ammonia (1 × 1.5 ml). The pooled ethanolic extracts were evaporated to dryness. The dry residues were dissolved in 1 ml of distilled water and 150 μl aliquots of the solution subjected to separation by paper electrophoresis

(on Whatman 3 MM paper bands 3.6 cm wide, in 0.05 M primary sodium citrate, pH 3.8, 25 V/cm, 90 min); a mixture of ATP, ADP, AMP and exotoxin served as a standard. The electropherograms were scanned in an automatic Frieseke-Hoepfner Radioactivity Scanner. Zones corresponding to radioactivity peaks were eluted and buffer was removed from the radioactive products by charcoal treatment. The products eluted from charcoal were chromatographed (Whatman No. 1 paper) in two systems: *n*-propanol-ammonia-water (6:3:1) and isobutyric acid-ammonia-water (66:1.5:33).

Determination of exotoxin and ATP in cells

Both compounds were determined in the *B. thuringiensis* cells by the method of isotope dilution [10]. Bacteria cultivated in the high P medium were centrifuged at chosen time intervals (9, 12, 16 hours). Twenty-four flasks containing each 50 ml of medium were used for each time interval. Samples were withdrawn for turbidity measurement, dry weight determination (3×10 ml of bacterial suspension, 120 °C, 24 hours), and for checking the culture purity and growth-stage in phase contrast. The sediment of the bacteria was washed with 0.9% NaCl (3×150 ml) and then extracted with perchloric acid (0.24 M, 2×30 ml, 0 °C, 10 min). [^{32}P]Exotoxin (93 μg, 1.56 μCi) and [^{14}C]ATP (106.2 μg, 0.532 μCi) were added to the pooled perchloric acid extracts. After neutralization by 7 M KOH and separation of $KClO_4$, charcoal was added to the neutral extracts (time 9 hours, 180 mg; time 12 and 16 hours, 300 mg). The suspensions were centrifuged 30 min afterwards. The charcoal was then extracted with 50% aqueous ethanol (3×25 ml) and with a solution of 25% aqueous ethanol and 0.5% ammonia (30 ml). The samples were concentrated to a small volume and applied onto the ion-exchange column.

The quantity of exotoxin excreted into the culture medium was determined simultaneously for each time interval. This determination was carried out also by the method of isotope dilution. [^{32}P]Exotoxin (93 μg, 1.56 μCi) was added to 40 ml (or to 80 ml for a 9 hour culture) of supernatant after the separation of bacteria, adjusted to pH 7. Exotoxin was adsorbed from the solution to 200 mg charcoal. The latter was washed three-times with distilled water and exotoxin desorbed by 50% aqueous ethanol (2×20 ml). This extract was concentrated to 2 ml and shaken with phenol saturated with water (2×2 ml). The rest of phenol was removed by extraction with ether and the solution applied onto the ion-exchange column.

Samples of exotoxin from both the cells and the medium were chromatographed on modified Ecteola cellulose equilibrated with triethylammonium bicarbonate buffer. Exotoxin samples from cells were applied onto columns 0.65×25 cm and eluted by a linear gradient of TEA solution (80 ml of 0.05 M + 80 ml of 0.5 M TEA, flow rate 1.2 ml/10 min). Exotoxin samples from the supernatant were chromatographed on the same columns, and eluted by a steeper gradient (75 ml of 0.05 M + 75 ml of 0.9 M TEA). The UV-absorbance, refraction, and radioactivity of the fractions were measured. Peak fractions corresponding both to ATP and exotoxin were pooled and TEA removed. The final purification was effected by chromatography in the mixture *n*-propanol-ammonia-water (6:3:1), (Whatman No. 3 MM paper, 96 hours). After the chromatograms had been scanned for radioactivity, the radioactive spots of exotoxin and ATP were eluted; the samples were subjected to UV-absorbance and radioactivity measurement and their specific radioactivity was determined. The quantity of exotoxin present in the samples was calculated according to a formula reported earlier [10].

Results and Discussion

In preliminary experiments aimed at the detection of exotoxin in the bacterial cell, the low molecular weight pool was examined after extraction of *B. thuringiensis* at different stages of growth in low P medium containing ^{32}P. A fraction of free nucleotides heavily labelled with ^{32}P was obtained from cold perchloric acid extracts of bacteria by adsorption to charcoal; this fraction was subsequently resolved by paper electrophoresis. The results, which are essentially identical for all the time intervals chosen (time 10 hours is given by way of example in Fig. 1), show that the pool contains, in addition to other components, also products whose positions coincide with those of adenosine mono-, di-, and triphosphate and exotoxin added as markers. The analysis of the radioactive peaks from electrophoresis by paper chromatography in two systems showed, however, that except for the peak near to the origin and representing AMP, all the remaining peaks are mixtures of two to four components. Exotoxin was found as one of the components of the peak corresponding to the position of the exotoxin sample on electrophoresis.

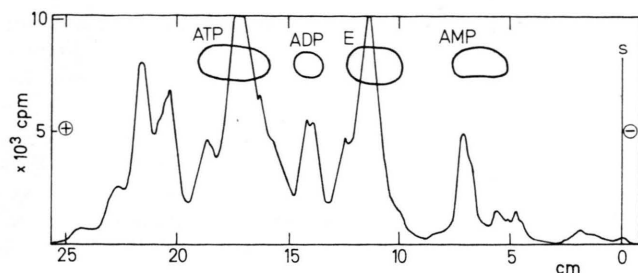In view of these results, two compounds only, exotoxin and ATP, were chosen for quantitative

Fig. 1. Paper electrophoresis of charcoal-purified acid soluble extract of B. thuringiensis. Curve-radioactivity; spots designate positions of markers separated simultaneously.

determination of their concentration in the cell. For these experiments bacteria were cultivated in the high P medium where the production of exotoxin is higher than in the low P medium [10]. The determination was effected by the method of isotope dilution using [^{32}P]exotoxin and [^{14}C]ATP. The radioactive compounds were added to perchloric axid extracts of bacteria before their neutralization by KOH. It may therefore be assumed that the results obtained by this method approach closely the actual values of ATP and exotoxin in the extracts.

Extracts to be used as a source of pure exotoxin and ATP for specific radioactivity determination were purified on charcoal and subjected to chromatography on Ecteola cellulose. The elution profile of the column indicates a satisfactory resolution of ATP and exotoxin (Fig. 2) which corresponds to the separation of a model column. Fractions correspodning to the two peaks were subjected to paper chromatography in order to prevent contamination of the compounds by each other and to remove the possible UV-absorbing impurities. The concentration of ATP and exotoxin, calculated from the specific radioactivity values determined, was based on the dry weight of the bacteria (Fig. 3).

The results show that exotoxin is present in B. thuringiensis cells at a concentration which approximates the concentration of ATP. The dependence of the concentration of exotoxin on the culture age follows a trend similar to that of ATP. The decrease of
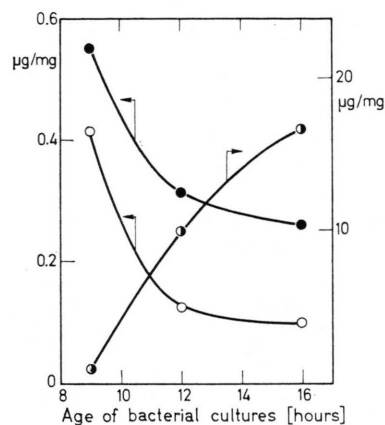

Fig. 3. Concentration of exotoxin and ATP in bacterial cells and of exotoxin in culture medium in μg/mg of dry weigt of B. thuringiensis. ●—● ATP; ○—○ exotoxin in bacterial cells; ◑—◑ exotoxin in culture medium.

exotoxin content in the cells with increasing age of the culture suggests that exotoxin determined in the cells had not been contaminated by exotoxin from the medium; if this were the case the trend would necessarily be the opposite (see quantity of exotoxin in the medium which increases with the increasing age of the culture and exceeds the quantity inside the cells by approximately two orders, Fig. 3). The data on the amounts of exotoxin inside the cell and on the quantity excreted into the medium permit us to deduce the rate of synthesis of exotoxin. The rate of synthesis corresponds to the
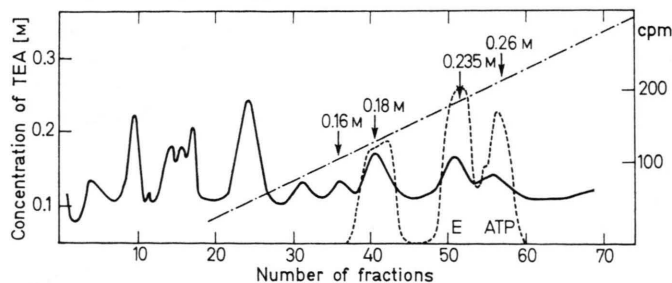

Fig. 2. Fractionation of charcoal-purified acid soluble extract of B. thuringiensis on Ecteola column. ———— UV-absorbance; — — — radioactivity; — · — · — concentration of eluent. The conditions of the separation are given in the text.

excretion of $1/5 - 1/4$ of the quantity of exotoxin contained in the cells per minute. This value is identical for both intervals examined (9 – 12 hours, 12 – 16 hours).

From the discovery of exotoxin in *B. thuringiensis* cells certain new problems emerge. First, there is a question of compartmentalization of exotoxin synthesis in the cell. The biosynthesis of exotoxin must evidently be separated from the DNA-dependent RNA polymerase which exotoxin strongly inhibits even in *B. thuringiensis* cells [11, 12]. Second,

there is a problem of exotoxin excretion or, more accurately, of the transport system responsible for the transport of this strongly acidic compound across the bacterial cell wall. Both these problems would require an independent investigation with special regard to the complicated architecture of the cell wall of the producing microorganism.

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