ESTIMATION OF EXOTOXIN PRODUCTION BY DIFFERENT STRAINS OF *Bacillus thuringiensis* USING ^{32}P-LABELLED EXOTOXIN

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Employing *Bacillus thuringiensis var. gelechiae* its exotoxin was labelled with ^{32}P which was added to the culture. With the aid of the labelled exotoxin using isotopic dilution, the actual concentration of exotoxin during cultivation of several strains of *B. thuringiensis* was determined. The results made it possible to estimate the yield of the individual steps of the isolation procedure and to show that greatest losses occur during purification on charcoal.

Isolation, characterization and the mechanism of action of *B. thuringiensis* exotoxin have been studied here for a number of years[1-5]. The isolation method developed here and based on the adsorption of exotoxin on charcoal and its purification by ion-exchange chromatography is not quantitative just as the other methods described for the isolation of the substance[1,6,7]. It was therefore attempted to establish a method for quantitative estimation of exotoxin and, using this method, to determine the true concentration of exotoxin in the cultivation medium. In view of the fact that the preparation of ^{32}P-labelled exotoxin is relatively simple it was decided to use for this purpose the method of isotopic dilution. The literature references on exotoxin production during cultivation are not unequivocal and there do not seem to exist any comparative data for the various strains[8-11]. It was hence desirable to obtain the required data by using various strains of *Bacillus thuringiensis* and a strain of *B. cereus* as a control.

EXPERIMENTAL

Bacterial strains. The following strains were used, all of them from the Culture Collection of Entomogenous Bacteria (CCEB), Institute of Entomology, Czechoslovak Academy of Sciences: *Bacillus thuringiensis var. gelechiae* CCEB 555 (isolated by Metalnikov from *Gelechia gossypiella*), *B. thuringiensis var. thuringiensis* CCEB 056 (isolated by Mattes from *Ephestia kühniella*), *B. thuringiensis var. thuringiensis* CCEB 601 (isolated by Krieg from *Galleria mellonella*), *B. thuringiensis var. thuringiensis* CCEB 653 (isolated by Leskova from *Apis mellifera*), *B. finitimus* CCEB 460 (isolated by Heimpel and Angus from *Malacosoma distria*, differing from *B. thuringiensis* in that its sporangium does not lyze, and the spore and the parasporal body do not separate), *B. cereus* CCEB 615.

Cultivation. Bacteria were cultivated in a medium according to Cantwell and coworkers[12]: 0·5% K_2HPO_4, 0·5% KH_2PO_4, 0·1% $NaNH_4PO_4$, 0·3% tertiary sodium citrate, 1·0% casein hydrolyzate (Casamino acids, Difco), 0·00002% thiamine hydrochloride, 0·005% $MgSO_4$, 0·003% $MnSO_4$, 0·001% $FeSO_4$ and 0·005% $CaCl_2$ (high P medium). For the preparation of ^{32}P-labelled exotoxin, the following medium was used: 0·7% KCl, 0·05% NaCl, 0·05% NH_4Cl, 0·3% tertiary sodium citrate, 1·0% casein hydrolyzate, 1·2% Tris, 0·00002% thiamine hydrochloride, 0·005% $MgSO_4$, 0·003% $MnSO_4$, 0·001% $FeSO_4$ and 0·005% $CaCl_2$ (low-P medium). As inoculum, a 24-h culture from agar slant sporulation media was used. Inoculated 300 ml flasks with 50 ml medium were agitated on a Dubnoff shaker at 29°C at 105 strokes/min for 12 h to obtain the vegetative inoculum. Flasks containing 50 ml medium were inoculated with 8% vegetative inoculum and cultivated under the same conditions as above for different periods of time. Bacterial growth was assayed on the basis of absorbance of the suspension at 575 nm, by determination of bacterial dry weight (120°C, 24 h) and by measuring the pH of bacterial supernatant. Morphology was followed microscopically on Gram-stained preparations.

Biosynthetic labelling of exotoxin. Exotoxin labelled with ^{32}P was prepared from *B. thuringiensis var. gelechiae* grown in a low-phosphorus medium for 28 h under the conditions shown above. Flasks containing 50 ml medium were inoculated directly from agar slants. After 30 min of cultivation, carrier-free ^{32}P-orthophosphate was added (700 μCi/50 ml cultivation medium; because of the presence of inorganic phosphate in the medium, due to contamination of the casein hydrolyzate, the specific radioactivity of phosphorus in the medium was 195 μCi/mg P). Exotoxin was isolated from the supernatant after centrifugation of bacteria as described before[1]. After phenol extraction, the exotoxin solution was evaporated to dryness *in vacuo*. The exotoxin was purified by preparative electrophoresis in a citrate buffer (0·05M primary sodium citrate, pH 3·8, 25 V/cm, 90 min; exotoxin corresponding to 50 ml of bacterial medium was placed on 18 cm of a Whatman 3MM paper).

Spectrophotometric estimation of exotoxin production. Exotoxin was isolated from the supernatants of bacterial suspensions obtained at appropriate time intervals[1]. Each supernatant (40 ml) was made to pH 7 with 1M-HCl and mixed with 200 mg charcoal. After 30 min, the suspension was centrifuged at 3000 *g* for 20 min (with all the subsequent extractions, centrifugation was done under the same conditions). The supernatant was discarded and the sediment was further washed 3 times with 20 ml water. The sediment was then extracted twice with 20 ml 50% ethanol for 30 min. The combined ethanolic extracts were concentrated to 2 ml in a rotary evaporator and the crude preparation of exotoxin was extracted 3 times with 2 ml 90% phenol and 3 times with 2 ml ether. The purified exotoxin extract was evaporated *in vacuo* to dryness. The last purification step was preparative electrophoresis in a citrate buffer for 120 min. According to the expected amount of exotoxin, aliquots of the various samples corresponding to 10 or 20 ml starting bacterial supernatant were placed on 8 cm strips of Whatman 3MM paper. Bands corresponding to exotoxin were eluted from the electrophoreogram with water, and eluate spectra were estimated. The amount of exotoxin was calculated on the basis of an extinction coefficient of 14200 and of known molecular weight of exotoxin.

Determination of exotoxin production by the isotopic dilution method. The supernatants after centrifugation of bacteria were made to pH 7 and mixed with a known amount of ^{32}P-exotoxin of known specific radioactivity (about 105000 c.p.m.). Exotoxin was then isolated from the supernatants in the previously described way. The spectra and radioactivities of the eluates after electrophoresis were estimated. The amount of exotoxin per sample was calculated from the formula $x = (b - ac)/c$, where a is the amount of radioactive exotoxin added to the sample in μg, b is the radioactivity of the added exotoxin, c is the specific radioactivity of the analyzed sample and x is the amount of exotoxin in the analyzed sample in μg.

RESULTS AND DISCUSSION

In previous work, Cantwell's medium with phosphate buffer (high-P medium) was used for the production of exotoxin. To attain the maximum specific radioactivity of exotoxin labelled with ^{32}P it was necessary to use a medium where the concentration of cations would be maintained but that of phosphate would be reduced to a minimum. Such a medium was obtained by replacing alkaline phosphates with chlorides (low-P medium). In comparison with Cantwell's medium it contains only 4·3% P. The course of culture growth in this medium was principally similar to that in Cantwell's medium (Fig. 1) but the maximum growth of bacteria was about 25% lower. Morphologically, there were clear differences between the cultures from the two media. In Cantwell's medium one may observe indications of spores within cells between 12 and 20 h of cultivation; from 24 h on, the spores are released together with parasporal inclusions from the sporangia and after 48 h, 70–90% spores and inclusions are free. In the low-P medium, sporulation was lower and was accompanied by the formation of filamentous forms. After 48 h, only 20% spores and inclusions were liberated.

By using a low-P medium with carrier-free radioactive orthophosphate added, exotoxin was prepared at a specific radioactivity identical with that of the radioactive phosphorus in the medium, *i.e.* 212 μCi/mg P (6·64 μCi/μmol exotoxin). The ^{32}P-labelled exotoxin was used for determination of the true concentration of exotoxin by the method of isotopic dilution. This method yields useful results irrespective of losses during isolation and its accuracy depends in principle on the purity of the resulting exotoxin. In the present work, exotoxin was purified by adsorption on charcoal, removal of ballast substances by extraction of the eluate with phenol and, as a final

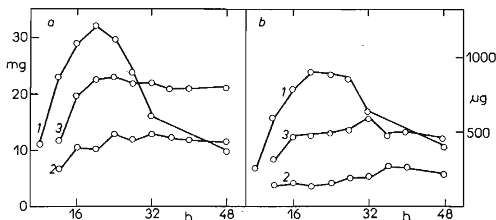

FIG. 1

Exotoxin Production by *B. thuringiensis var. gelechiae* in High- and Low-P Medium

a High-P, *b* low-P, *1* dry matter in mg per 10 ml bacterial suspension (average of 3 samples); *2* exotoxin production in μg per 10 ml of bacterial supernatant as estimated spectrophotometrically and *3* by isotope dilution. Time of cultivation in hours.

step, by paper electrophoresis. The exotoxin band is clearly separated; in view of the fact that with some strains, a fluorescent band of an unknown compound appears near the exotoxin, in all cases only the centres of the exotoxin bands with maximum absorbance and radioactivity were eluted.

Comparison of exotoxin production in the low-P medium and Cantwell's medium was done for *B. thuringiensis var. gelechiae* both by the isotopic dilution method and by spectrophotometry as used in previous work[1] (Fig. 1). In both media the production of exotoxin is proportional to culture growth. Comparison of the two

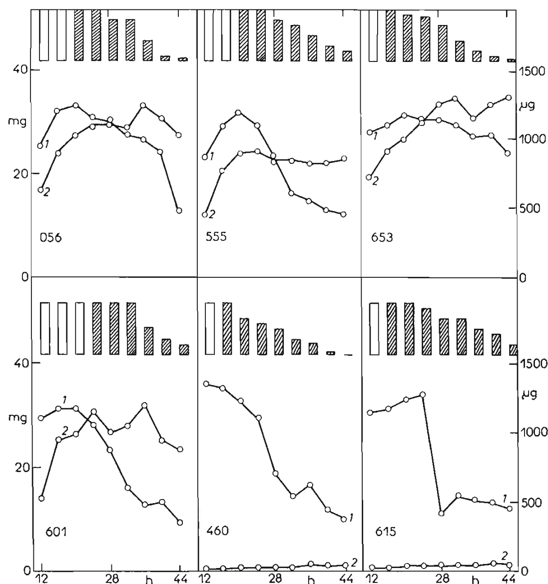

Fig. 2

Exotoxin Production by Different Bacterial Strains Estimated by Isotope Dilution

The various strains are denoted by their number in CCEB. *1* Dry matter in mg per 10 ml of bacterial suspension; 2 exotoxin in μg per 10 ml of bacterial supernatant. *t* Time of cultivation in hours. The columns indicate the morphological state of the culture. Empty columns – vegetative cells; full columns – spores formed inside bacteria; diminishing of full columns is proportional to the number undegraded cells.

TABLE I

Losses of Exotoxin During Purification on Charcoal

The per cent yield refers to the radioactivity of the bacterial supernatant prior to addition of charcoal.

Bacterial strain	Incubation h	Not adsorbed	Not desorbed	Bacterial strain	Incubation h	Not adsorbed	Not desorbed
555	16	3·3	46·7	056	24	6·8	43·2
	20	2·0	48·2	460	24	2·5	41·1
	24	2·6	44·7	601	24	4·8	40·6
	28	2·9	44·9	615	24	1·8	41·9
	32	3·2	48·6	653	24	6·4	45·5
	36	5·0	47·3				
	40	10·1	33·2				

methods shows that spectrophotometric estimation of exotoxin production is subject to considerable error (about 50%, or 70% for the low-P medium) but the error is rather standard.

The method of isotopic dilution made it possible to establish the source of this error by estimating the balance of the individual isolation steps. Losses during adsorption on charcoal are minimal while losses during desorption of exotoxin from charcoal lie between 30 and 50% for Cantwell's medium and are relatively independent of the bacterial strain used (Table I). On the other hand, the further isolation steps, *i.e.* extraction of ballast compounds with phenol and electrophoresis of the isolated exotoxin, are not accompanied by losses. Similarly, during isolation of exotoxin from the low-P medium, incomplete desorption of exotoxin from the charcoal was observed, the losses at this isolation stage being 50–70%.

Results obtained by the method of isotopic dilution can be taken as the first reliable data on the concentration of exotoxin in the medium. The previous results were based either on yield estimation[1] which is subject to serious error, or on a calculation proceeding from toxicity for insects[7]. These last-named results are to be evaluated with caution since the effect of a pure substance is being compared with the effect of the crude extract. Even if in the case quoted the effect was diminished considerably by frequent control experiments one cannot on the basis of toxicity alone determine generally the exotoxin concentration, particularly with strains displaying a low exotoxin production.

In further work the method was applied to the production of exotoxin by some strains in dependence on morphology and culture growth (Fig. 2). With most strains it was found that exotoxin is released into the medium in the course of maximum growth in such a way that its production is practically terminated at the time when the culture begins to sporulate. Different behaviour was observed only with strain

653. The synthesis of exotoxin proceeds with this strain even during the sporulation phase and at the beginning of spore release into the medium. This different type of exotoxin production agrees with the pattern of culture growth. All the strains of *B. thuringiensis var. thuringiensis* are similar as to the overall production of exotoxin (an average of 120 mg/l culture) whereas with *B. thuringiensis var. gelechiae* the production is by about 25% lower. The values found for *B. finitimus* and *B. cereus* are identical and confirm the existing reports that *B. finitimus* does not produce exotoxin but only the endotoxin[13,14]. The minute amount of exotoxin found in the two strains indicates simultaneously the error of the method.

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