LARVICIDAL TOXIN FROM *BACILLUS SPHAERICUS* SPORES

Isolation of toxic components

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1. Introduction

The utilisation of chemical pesticides in the control of tropical diseases vectors is facing many difficulties: the resistance of the vectors to the chemicals is increasing and the utilisation of pesticides hinders the ecosystem. The search for active agents in the control of vectors has led to the isolation of *Bacillus sphaericus* strains toxic to mosquito species [1,2], particularly to *Anopheles* larvae, vectors of malaria. In [3] we described a technique for the extraction of a crude fraction from *B. sphaericus* 1593 spores, which is highly toxic to *An. stephensi* larvae. We suggested that the toxic activity is due to one or many protein(s) or heteroprotein(s).

The aim of this study is to isolate the protein toxin.

2. Materials and methods

The crude extract and bioassays were prepared as in [3], but here the crude extract was filtered through 0.22 μm Millipore filters to eliminate all the toxic cell fragments. This filtration, which was followed by a decrease in toxicity, ensured that we were working with a soluble toxin only. Because of the low quantities of toxic material collected in the fractions, a micro-bioassay (5 larvae in 5 ml medium) was devised. These techniques do not permit determination of an LC_{50}, but only the mortality percent. Such a bioassay is sufficient to detect the active fractions.

Protein content was assessed by UV absorption or by Lowry protein assay [5]. Electrophoresis was performed on polyacrylamide gel (10% or 7.5%) according to Laemmli [6]. For migrations without SDS a Tris–glycine buffer (pH 8.8) was used.

3. Results and discussion

The toxicity of the crude fraction which contains 50–60% protein was not reduced by treatment with trypsin, papain or subtilisin, but was destroyed by treatment with pronase. This protein fraction seems to be highly heterogeneous. Slab electrophoresis on polyacrylamide gel (10%) of the protein fraction reveals ~15 bands which correspond to protein of 14 000–100 000 M_r (fig.1).

After treatment with mercaptoethanol, the proteins at >70 000 M_r disappeared. Meanwhile, the toxicity of the mercaptoethanol (50 mM) reduced fraction and the original fraction is similar. It should be noted that

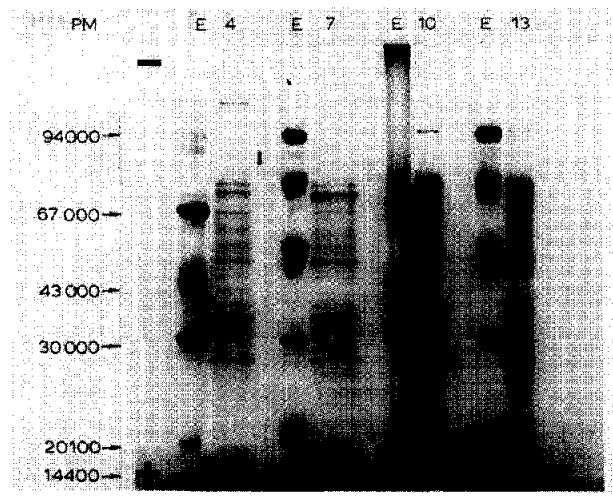

Fig.1. Polyacrylamide gel electrophoresis of the crude extract of *B. sphaericus* spores. The gel contained 10% polyacrylamide; 150 μg protein of the crude extract was used in each assay: no. 4, non-reduced extract; no. 7, extract reduced by mercaptoethanol; no. 10, extract heated 5 min, 100°C (SDS); no. 13, extract in SDS, mercaptoethanol and urea 8 M, heated 10 min, 60°C; E, control.

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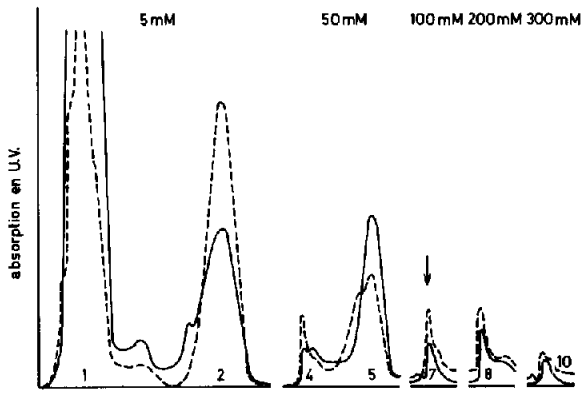


Fig.2. DE-52 Chromatography of the crude extract of *B. sphaericus* spores. Stepwise elution with increasing ionic strength of a sodium phosphate buffer: 5 mM, peaks 1 and 2; 50 mM, peaks 3 and 4; 100 mM, peak 7; 200 mM, peak 8; 300 mM, peak 10. No peak at 400 mM and 500 mM: (---) absorption at 280 nm; (——) absorption at 260 nm.

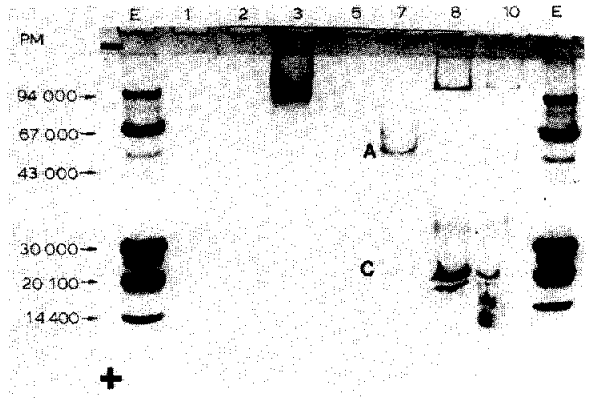


Fig.3. Polyacrylamide gel electrophoresis of the different fractions obtained from ion-exchange chromatography: Tris–glycine buffer (pH 8.8); 75 μg Protein of each sample was added to the gel containing 7.5% polyacrylamide. The number of the wells correspond to the number of the peaks obtained by DE-52 chromatography. Well E is a control.

direct extraction of the toxin by treatment of spores with mercaptoethanol produce a more active toxin [7].

We have detected ~8 or 9 enzymes (Api-Zym) in the crude toxin. The more active are: esterase; esterase–lipase; alkaline-phosphatase; phospho-amidase; cystine-aminopeptidase.

Because of poor results in the purification of this crude toxin by different Sephadex column chromatography, we used ion-exchange resin, particularly the DEAE-cellulose DE-52 (Whatman).

Toxic filtered crude extract (20 mg) was placed on the top of a pre-equilibrated column with sodium phosphate buffer (0.05 M, pH 8.0). Stepwise elution with the same buffer, of increasing ionic strength (5–500 mM), was performed. The elution profile is represented in fig.2.

Fractions of each peak were grouped and lyophilized before testing their activity. Only the fraction eluted by 100 mM buffer (pH 8.0) was found to be toxic (peak no. 7): protein at 6 μg/ml killed 50% larvae after 48 h exposure (100% after 3 days' exposure). In this fraction only these enzymes persisted: esterase; esterase–lipase; and phospho-amidase. We obtained 5 mg of this fraction (protein amount) from 240 mg of crude extract.

The electrophoresis on polyacrylamide gel (without SDS) of the fractions shows that the toxic fraction (peak no. 7) contains 2 bands: A (dense) specif to this fraction, corresponds to $M_r \sim 55\,000$; C (les stained) corresponds to $M_r \sim 25\,000$ (fig.3).

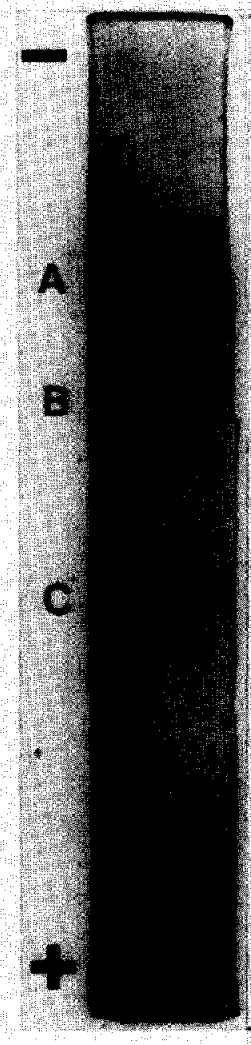
Preparative PAGE (7.5%) of 3 mg of this toxic fraction confirms this result and reveals a third band B (faintly stained), corresponding to M_r 43 000 (table 1). (Rapid staining technique with Coomassie brilliant blue G-250 [8] of a fragment of the slab.)

Each band was eluted by electrodialysis using an ISCO apparatus. After dialysis, the protein content of each fraction was determined. Band A gave 3 fractions: no. 5 which contains 50 μg protein/ml; no. 6, no detectable protein by UV absorption, or by Lowry assay; no. 7 which contains 25 μg protein/ml. The enzymatic activities corresponding to esterase, phospho-amidase and esterase–lipase have been recovered only in each of these 3 fractions.

Taking into consideration all the manipulations which have been performed on these protein fractions, and which can interfere with their toxic activity, we have preferred to use av. 25 μg protein/ml for each bioassay. This concentration is relatively high. These bioassays (table 1) have shown that toxicity is concentrated into bands no. 5–7, which correspond to the main band A. This protein A may be a protein complex, because band no. 6, apparently without detectable protein, produces the highest toxicity. However, it could be the result of an alteration of the slab segmentation. No toxic activity could be detected in the extracts corresponding to bands B and C.

Considering the micro-method employed we affirm that 20% mortality after 48 h exposure is not significant. Meanwhile we conclude that in the soluble spore crude extract there is at least one protein, or heteroprotein, present in small amounts, which is responsible

Table 1
Preparative electrophoresis of the toxic fraction obtained from DE-52 chromatography (peak no. 7)



N° of eluted bands	µg total protein recovered	BIOASSAYS mortality % (25 µg protein / ml)	
		24 h	48 h
1	0	0	20
2	7	0	0
3	0	20	20
4	0	20	20
5	350	0	80
6	0	20	100
7	175	20	80
8	0	0	0
9	0	0	0
10	0	–	–
11	0	0	0
12	0	0	0
13	267	0	0
14	107	0	0
15	0	0	0
16	0	0	0
17	20	20	20
18	0	0	0
19	128	0	0
20	Point of migration of Bromophenol Blue		

Peak no. 7 (3 mg) was added to the gel containing 7.5% polyacrylamide, running buffer, Tris–glycine HCl (pH 8.8); rapid stain procedure with Coomassie brilliant blue; bioassays with 5 second-instar larvae (*An. stephensi*) in 5 ml medium; protein was 25 µg/ml

for toxicity and corresponds to $M_r \sim 55\,000$. This toxin seems to be different from the soluble cytoplasmic toxin extracted from sporulating cells in [9] corresponding to $M_r \sim 100\,000$, its activity being destroyed by pronase and subtilisin.

This study has permitted us to establish a technique for the partial purification of the toxic extract in the process of characterizing the larvicidal toxin. It is the

first isolation of highly larvicidal toxin from *Bacillus sphaericus* spores. If the toxin (or one of its components) is an enzyme, a new assay of toxicity by enzymatic activity determination could be established.

Acknowledgements

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