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IDENTIFICATION OF THE PEPTIDES OF THE CRYSTALS OF *BACILLUS THURINGIENSIS VAR ISRAELENSIS* INVOLVED IN THE MOSQUITO LARVICIDAL ACTIVITY

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Tryptic digestion of the proteins from the purified crystals of *B.thuringiensis var israelensis* resulted in the decline of high molecular weight peptides without the loss of mosquito larvicidal activity, measured after immobilization of the digests with DEAE- Sephadex A 50 beads. Amongst the peptides generated (<44 kDa), a 21kDa peptide was immunoreactive to the crystal antiserum. Analysis of the peptides released from spores of the toxic (Cry+) and non-toxic (Cry-) strains has revealed a pattern in which only the 26kDa peptide was missing in the Cry- strain. Sporulation and crystal formation were dissociated by the addition of the antibiotic netropsin, which could also inhibit the crystal assembly, without considerable decrease of the larvicidal activity and retention of the 26kDa peptide. These results implicate the 26kDa peptide in the larvicidal action.

The mosquito larvicidal toxin(s) of *B.thuringiensis* var *israelensis* reside in a proteinaceous parasporal crystal formed during sporulation (1). In addition, cytotoxicity to mosquito cell lines and hemolytic activity are exhibited by the crystals solubilized with alkali(5), which produce a complex pattern of polypeptides (2-7). Conflicting reports have appeared on the roles played by two major peptides of the crystals, i.e.26kDa (4,6,7) and 66kDa (8,9) in the larvicidal action. In the present report, by SDS-PAGE and Western blot analysis of the peptides that retain larvicidal activity, we provide evidence in support of the role played by the 26kDa peptide in the biological activity. Furthermore, by the use of the antibiotic

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netropsin, we show that the process of sporulation and crystal formation can be dissociated.

MATERIALS AND METHODS

B.thuringiensis var *israelensis* H14 was obtained from Dr.H.de Barjac, Pasteur Institute, Paris. Media and growth conditions were described earlier (10). Pure spores and crystals (99% purity as estimated by phase contrast microscopy) were obtained by repeated centrifugation through a sorbitol gradient. Crystals banded at 40 to 50% interface and spores at 50 to 60% interface. The crystals were solubilized by 0.05N NaOH for 1h at 30 C, and the insoluble materials were removed by centrifugation at 10,000xg for 5min.The pH of the supernatant was adjusted to 8 with 1M acetate buffer (pH 4.0). This extract was used for antibody production in rabbit and peptide analysis in SDS-PAGE (13). Cells were ground with glass powder and alkali followed by precipitaion with 1M acetate buffer. For Western blots (14) horse radish peroxidase linked to anti-rabbit globulin and diamino benzidine as substrate were employed. Protein was estimted by the method of Lowry *et al* .(15). Toxicity assays were carried out as described earlier (10). The antibiotic netropsin (obtained from American Cyanamide), was added at the mid logarithmic growth phase and 8 hours after the end of vegetative growth, the cultures were heated at 80 C for 10 minutes and plated for spore counts. For immobilization of soluble toxin, the extracts (1 mg protein) were absorbed to DEAE Sephadex A50 beads (10 mg), dried at room temperature for 1h and the absorbed toxin was diluted and assayed.

RESULTS

1.EFFECT OF TRYPTIC DIGESTION OF THE CRYSTAL PEPTIDES ON THE LARVICIDAL ACTIVITY:

The pattern of peptides obtained from the alkali extracts of the purified crystals is in close agreement with the previous reports (2-7). In order to limit the size of the active peptide components, the solubilized crystals were treated with trypsin at a ratio of 1:4. From the SDS- Polyacrylamide gels of the tryptic digests (Fig.1A), it is evident that all the high molecular weight peptides as well as the 66kDa and 26kDa peptides, previously implicated in the larvicidal action are digested and a new set of peptides (21, 35 and 40kDa) appear. Western blot

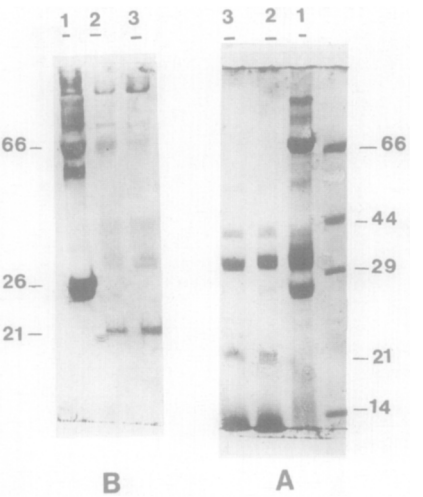

Fig.1 Tryptic digestion of the peptides of crystals from B.thuringiensis var israelensis .

Alkali solubilized peptides from the crystals were digested with trypsin for the indicated periods as outlined in methods. A) Coomassie blue stained gel. B) Western blot of this gel. Lanes: 1-crystals; 2- with trypsin for 3 hrs. 3- with trypsin for 5 hrs.

analysis of this gel revealed that only the newly appeared 21kDa peptide is immunoreactive with the crystal antiserum at a significant level (Fig.1B).

The biocidal activities of the tryptic digests were low (LC_{50} =1-10ug/ml). However, after their immobilization with DEAE-Sephadex A50 beads, the potency rose to 100ng/ml, a value comparable to that of the solubilized crystals treated similarly (LC_{50} =10-50ng/ml). Similar observations on the enhancement of potency of the solubilized crystals upon immobilization with latex beads have been made (16).

2.CORRELATION OF THE POLYPEPTIDES RELEASED FROM SPORES AND LARVICIDAL ACTIVITY:

The spores of cry+ strains of B.thuringiensis var israeliensis are toxic (3), while the

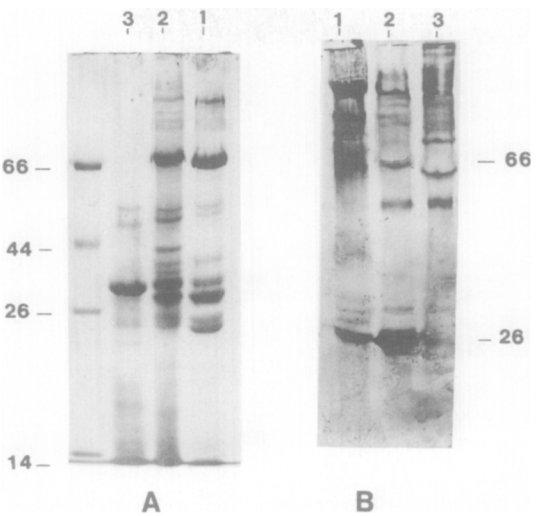

Fig.2. Pattern of peptides released from spores of cry+ and cry- strains of B.thuringiensis var israelensis .

Alkali soluble peptides of crystals and spores were analysed in A) 10% SDS-PAGE B) Western blot of this gel. Lanes: 1-Pure crystals; 2- Spores of Cry+ strain; 3- Spores of cry- strain.

spores from the cry- are not toxic(10). The pattern of the peptides released from the spores of the cry+ strain is similar to that of the purified crystals(Fig.2A). The predominant peptides are 26kDa and 66 kDa. In the spores of the cry- strain, these peptides were seen only in trace amounts. However, Western blot analysis of these peptides (Fig.2B) revealed that only the 26kDa peptide was absent in the spores of Cry- variants, thus indicating that this peptide confers toxicity.

3.EFFECT OF NETROPSIN ON CRYSTALLOGENESIS IN B.THURINGIENSIS VAR ISRAELENSIS :

It has been earlier observed that the antibiotic netropsin inhibits the process of sporulation, without affecting the vegetative growth in B.subtilis (11) and B.polymyxa (12). Addition of netropsin (1-3 ug/ml) to the vegetative cells of B.thuringiensis var israelensis

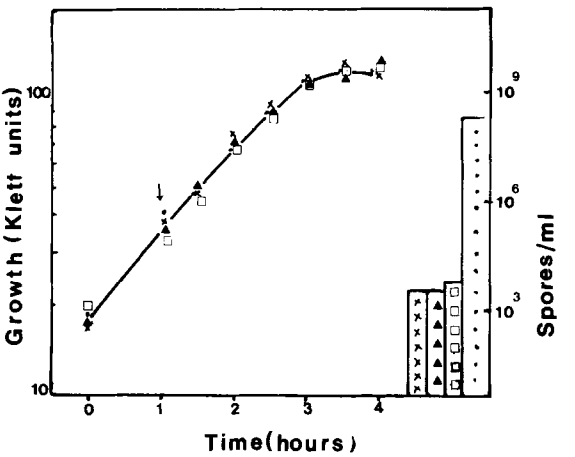

Fig.3 Effect of netropsin on growth and sporulation of B.thuringiensis var israelensis .

Netropsin at different concentrations was added to growing cultures as indicated by the arrow. Optical density measurements were made for the different cultures and the spore counts were determined at the end of eight hours after stationary phase of growth. Control (•—•); Netropsin at 1ug/ml(□—□); 3ug/ml (▲—▲); 7ug/ml (×—×).

has a similar inhibitory effect on sporulation (Fig.3). At these levels of the antibiotic, the formation of the crystalline inclusions in the cells was unaffected (data not shown), nor was there any loss of larvicidal activity (Table 1). At higher concentrations of netropsin (3-7 ug/ml),the

Table.1 Effect of netropsin on the biocidal potency of B.thuringiensis var israelensis

Netropsin	LC50 (ng protein/ml)	
ug/ml	1 Hr	6 Hrs
0	50	50
1	250	42
3	1000	50
5	1500	450
7	1250	400

Cultures were assayed for biocidal potency at the end of 8 hours after the stationary phase growth. The hours indicated are the larval assay periods.

presence of visible crystalline inclusions were absent, but only a 10-fold reduction of the biocidal potency of the cultures was observed (Table 1), which at this dose range (50-400ng protein/ml) is not considered to be a drastic decline of potency.

SDS-PAGE analysis of the polypeptide composition of the alkali soluble fractions obtained from the cells, treated with different concentrations of netropsin is shown in fig.4A. In the cells in which the crystal formation was not affected (lanes 2,3&4) the pattern was similar to that obtained from crystals (lane 1). In cells in which the crystals were absent (lane 5 & 6), two

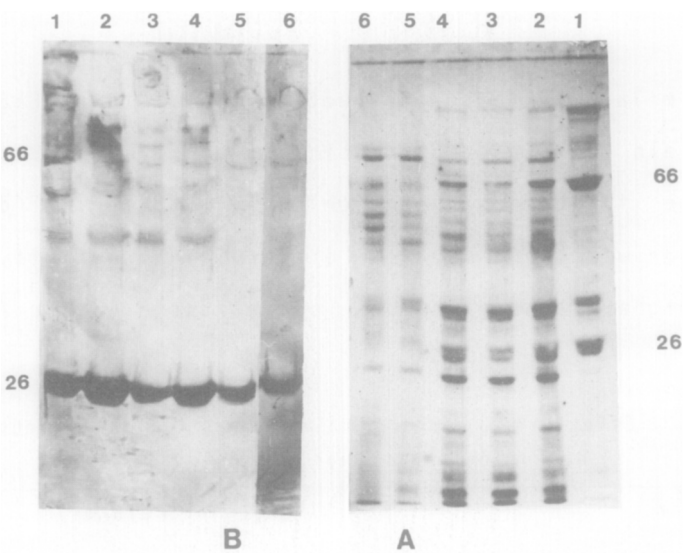

Fig.4 Alkali extracts of cells of B.thuringiensis var israelensis treated with netropsin.

Cultures in presence of netropsin at the concentrations indicated were extracted with alkali and the peptide composition was determined by A) Coomassie Blue stained SDS-polyacrylamide gel. B) Western blot of this gel.
Lanes: 1-Crystals; 2-Control cells without netropsin; 3-Cells with 1ug netropsin/ml; 4-3 ug netropsin/ml; 5-5 ug netropsin/ml; 6-7 ug netropsin/ml.

high molecular weight peptides (100kDa range) were missing. In addition, there was a decrease of all other peptide components. Western blot analysis of these gels revealed that a progressive loss of crystal assembly was accompanied by a decrease of most of the high molecular weight peptides (Fig.4B), suggesting that they may be involved in crystal assembly. However, in all cases the 26kDa peptide was present and was strongly immuno-reactive with the antiserum, thus indicating that the presence of this peptide bestows larvicidal activity to the cells. This observation contradicts the reports that attribute this activity to the 66kDa peptide (Lane 5 & 6), which is detected in netropsin treated cells only in trace amounts.

DISCUSSION

Earlier studies on the identification of the peptide responsible for the larvicidal activity of the crystals from different strains of *B.thuringiensis* var *israelensis* have ascribed this role to 26-28 kDa peptides (4-7). Our studies also support this conclusion. The demonstration of the larvicidal activity of the soluble form of the toxin (such as with tryptic digests of crystals or with the cells treated with netropsin) which had only trace amounts of high molecular weight peptides, suggest that these may be involved in the aggregation of the 26 kDa toxic peptide. Thus the failure by Lee *et al* (8) to detect the larvicidal activity with fractions containing the 26 kDa peptide may be due to the presence of the toxin in a soluble form, although this did not affect the hemolytic activity.

Armstrong *et al* have observed that both the cytotoxic and larvicidal effects are exhibited by a single peptide of 25 kDa, derived by proteolysis of 26-28

kDa peptides of the crystal(7). The difference in the molecular weights of the trypsin resistant crystal antigenic peptide reported here (21 kDa) and the trypsin and proteinase K resistant peptide (25 kDa) reported by Armstrong et al (7) may be due to different proteolytic treatment employed or to the strain difference.

Studies with netropsin have demonstrated that the processes of crystal formation and sporulation can be dissociated. In addition, the crystal assembly can also be differentially inhibited , without affecting very much the potency of the cultures and retaining the 26kDa peptide. The absence of this peptide in the alkali extracts of the spores of Cry- strain, which harboured all the other crystal components, including the 66kDa peptide (Fig.2B) further lend support to the conclusion that the 26kDa peptide is essential for larvicidal action of the crystals.

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