Structural features of crystal-forming proteins produced by *Bacillus thuringiensis* subspecies *israelensis*

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Entomocidal crystals produced by *Bacillus thuringiensis* ssp. *israelensis* are formed by two proteins with molecular masses of 130 and 28 kDa, whereas the protein with a molecular mass of 70 kDa appears as a result of 130 kDa protein limited proteolysis by admixtures of bacterial proteinases in the course of its dissolution. The comparison of the N-terminal sequences of the protein with molecular mass of 70 kDa (Met-Glu-Asn-Xaa-Pro-Leu-Asp-Thr-Leu-Ser-Ile-Val-Asn-Glu-Thr-Asp) and that of 28 kDa (Met-Glu-Asn-Leu-Asn-[Phe]-[Trp]-Pro-Leu-Gln-Asp-Ile-Lys-Val-Asn-Pro) reveals only marginal similarity between them (only 4 identical residues among 16 aligned). Both *B. thuringiensis israelensis* crystal-forming proteins appear hardly related to those contained in the crystal produced by other *B. thuringiensis* subspecies, e.g. *kurstaki*. It might be concluded that at least some of the entomocidal proteins found in the crystalline inclusion bodies of various *B. thuringiensis* subspecies revealed rather strong variations in their primary structures that facilitate their adaptation to different hosts.

Bacillus thuringiensis *ssp.* israelensis *δ-Endotoxin* *Entomocidal crystal* *Insecticide* *Mosquito*

1. INTRODUCTION

Bacillus thuringiensis ssp. israelensis was described in 1977 [1]. It is distinguished from the other subspecies of these bacteria by its pathogenicity for larvae of many blood-sucking Diptera, whereas it acts only weakly on Lepidoptera larvae. The entomocidal action of *B. thuringiensis israelensis* is caused by crystal-like protein inclusions formed within the cell in the course of its sporulation.

According to the literature data, *B. thuringiensis israelensis* crystals contain proteins of different molecular masses, the main component being a rather small protein of about 28 kDa. The content of the 130 and 70 kDa proteins is somewhat lower [2–6]. The origin of the latter component remains unclear. Some authors believe this protein to be coded by a separate gene [6,7], although the possibility of its formation by limited proteolysis of a high-molecular-mass precursor (e.g. 130 kDa protein) cannot be excluded. Admixtures of bacterial proteinases, invariably present in preparations of the crystals, might lead to partial hydrolysis of the main protein components which explains the appearance of minor fragments of 35, 40, 50 and 25 kDa.

Rather contradictory data are available on the toxicity of the 28 kDa component to Diptera larvae [12–14] whereas its cytotoxic activities are well established [12,13,15]. The 70 kDa protein is toxic for mosquito larvae, but does not possess cytotoxic activity [12,14]. So far the 130 kDa protein has not been purified and its biological activity remains unknown.

The very fact that *B. thuringiensis israelensis* crystals appear to contain several protein components that strongly differ in their chemical and toxicological characteristics indicates that their origin, evolutionary relations and their position among other *B. thuringiensis* endotoxins require

Abbreviations: DTT, dithiothreitol; ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis

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elucidation. This induced us to isolate these proteins and to study their structural features.

2. MATERIALS AND METHODS

B. thuringiensis ssp. *israelensis* strain B-2395, kept in the bacterial collection of this institute, was used throughout this work. The bacteria cultivation and isolation of crystals by two-phase distribution in a *p*-xylene-water mixture have been described [16]. The separated crystals were successively washed with 1 M NaCl, H_2O, suspended in 0.01 N HCl (1 mg/ml) and stirred for 3 h at 20°C. Then the crystals were centrifuged and washed with water until neutral pH was attained. The crystals thus treated could be completely dissolved by 8 M urea and 1% ME in 20 mM Tris-HCl buffer, pH 7.0.

To dissolve selectively the 28 kDa protein, the crystals (5 mg/ml) were treated with 50 mM sodium carbonate buffer, pH 9.5, that contained 10 mM DTT and 1 mM Na_2EDTA, for 1 h at 20°C and then the solution was separated by centrifugation. Subsequent treatment of the insoluble residue with 50 mM NaOH and 10 mM Na_2EDTA, pH 12.0, resulted in the extraction of 130 kDa protein.

The 70 kDa protein remained in the residue collected after extraction with 100 mM DTT of the 130 and 28 kDa proteins from the crystals not treated with HCl. This protein dissolved in 0.05 M NaOH was essentially pure for sequencing.

Electrophoresis in the presence of 0.1% SDS was performed in a 10×20 cm slab of 7.5% polyacrylamide gel at 150 mA and 150 V; the protein bands were stained with Coomassie R-250.

For amino acid analysis the lyophilized samples were hydrolysed with 5.7 N HCl for 24 h at 105°C in sealed tubes in vacuo, and then the amino acid content was assayed using a Biotronic C 5001 analyzer.

N-terminal amino acid sequences were determined by the automated Edman procedure in the presence of 1 M Quadrol using a Beckman 890 C sequencer. About 30 nmol protein was used for each run; PTHs were identified by TLC.

3. RESULTS AND DISCUSSION

The solution of *B. thuringiensis israelensis* crystals in 8 M urea and 1% ME at pH 7.0 contained, as judged by SDS-PAGE, comparable quantities of the 2 main components, the 130 and 28 kDa proteins. It has to be stressed that this rather simple pattern could only be observed after the crystals' treatment with dilute HCl (pH 2.0) which was necessary to inactivate the bacterial proteinases sorbed by them [16]. When this treatment was omitted, in addition to these components, the 70 kDa protein appeared in the solution. Apparently, this component was formed by limited proteolysis of the 130 kDa protein. SDS-PAGE also showed the appearance of less intense bands that corresponded to molecular masses of 35, 40 and 50 kDa.

The level of proteolytic activity found in the various preparations of *B. thuringiensis israelensis* crystals was rather high [4,9,10] and substantially varied depending on the strain, culture medium and method chosen for the crystal isolation. It appears likely that these factors might be responsible for the contradictions in the literature data on the protein composition of the *B. thuringiensis israelensis* crystals.

The differences in solubility of the components allowed one to isolate selectively 3 proteins, those

Table 1

Amino acid composition of crystal proteins from *B. thuringiensis israelensis* (mol/100 mol amino acids)

Amino acid	Proteins		
	28 kDa	70 kDa	130 kDa
Aspartic acid[a]	15.4	14.9	15.8
Threonine	8.4	8.0	10.2
Serine	7.2	8.4	8.7
Glutamic acid[a]	12.0	11.3	10.5
Proline	4.4	5.6	3.8
Glycine	6.4	7.7	6.2
Alanine	8.5	7.7	6.3
Valine	8.3	5.9	5.2
Isoleucine	6.1	6.7	5.1
Leucine	9.2	9.9	8.7
Tyrosine	2.6	3.0	4.1
Phenylalanine	3.9	5.3	3.7
Histidine	1.2	2.8	2.2
Lysine	3.0	2.0	4.9
Arginine	1.9	4.6	3.4

[a]Aspartic and glutamic acid values include asparagine and glutamine, respectively

with molecular masses of 130, 70 and 28 kDa (see section 2).

The amino acid composition of the 28 kDa protein we isolated (table 1) was similar to that determined by Tyrell et al. [2] for the analogous protein from *B. thuringiensis israelensis* crystals.

It deserves to be mentioned that the preliminary treatment of *B. thuringiensis israelensis* crystals with HCl prevented its proteolytic conversion into the component of 25 kDa [5,6].

The amino acid composition of the 130 and 70 kDa proteins has been found to be similar to those of δ-endotoxins produced by other *B. thuringiensis* subspecies, although some differences might be indicated, e.g. in histidine, arginine and lysine content. The N-terminal sequences of 16 amino acid residues were determined for the 28 and 70 kDa components. The sequences thus established (BTI-28K and BTI-70K, respectively) are compared below with the sequence found by Armstrong et al. [5] for the fragment of 25 kDa (BTI-25K) obtained by limited proteolysis of the *B. thuringiensis israelensis* component of 28 kDa. For comparison the N-terminal sequence of *B. thuringiensis* sp. *kurstaki* δ-endotoxin derived from the gene sequence [18] is shown (BTK).

A good deal of information has been collected recently on the structure of *B. thuringiensis* δ-endotoxins produced by subspecies *sotto* and *kurstaki* [18,19]. These data confirm earlier suggestions on the structural similarities of δ-endotoxins synthesized by some *B. thuringiensis* subspecies [2]. *B. thuringiensis israelensis* crystal-forming proteins differ substantially from this group of related δ-endotoxins as judged by their antigenic properties and peptide mapping [2,8,20]. Armstrong and co-workers have not found any similarity between the subspecies *kurstaki* δ-endotoxin sequence and the segment of the 28 kDa protein presumably located close to its N-terminus. We observed only very faint homology between the N-terminal sequences of the 28 kDa protein or the 70 kDa component and the N-terminal sequence of *kurstaki* δ-endotoxin. It corresponds to 25% for the 70 kDa component/*kurstaki* δ-endotoxin pair, which is a very low value, especially if one takes into account that the coincidence of the N-terminal methionines should be considered as rather trivial.

Hence, the protein components of the *B. thuringiensis israelensis* crystal, those with molecular masses of 28 and 70 kDa, appear to be only remotely related to one another. One may suggest

BTI-28K	M E N L N F W P L Q D I K V N P
BTI-70K	M E N x P L D T L S I V N E T D
BTI-25K	R V E D P N E I N N L L S I N E I D N P N Y I L Q A I S I A
BTK	M D N N P N I N E C I P Y N C L S N P E V E V L G G E R I E T G Y T P I D I S L S L T Q F L L S E F V P G A G F V L G L

F and W identification in BTI-28K needs addtional data. For the N-terminal sequences of the *B. thuringiensis israelensis* 28 and 70 kDa components only marginal homology (4 amino acid residues from 16 compared, 25%) was observed. This value hints at rather faint structural homology of these 2 proteins, which is in accordance with the immunological data [8]. Anyway, it is clear that the 28 kDa component could not be a degradation product of the 70 kDa protein. It appears that limited proteolysis of the 28 kDa *B. thuringiensis israelensis* component described by Armstrong et al. [5] leads to the removal of the N-terminal segment containing about 25–30 amino acids. Therefore, it might be assumed that the sequence found by these authors (BTI-25K) follows that established by us (BTI-28K, see above), both being separated by a dozen amino acids.

that the same holds for the 130 kDa protein that is presumed to be the precursor of the 70 kDa component.

The relationships between *B. thuringiensis* ssp. *israelensis* crystal-forming proteins and δ-endotoxin produced by other *B. thuringiensis* subspecies remain unclear, although one might suspect a certain degree of similarity in the structural organization of the *B. thuringiensis israelensis* 130 kDa protein and its presumed 70 kDa degradation product and the proteins with molecular masses of about 130 kDa and their proteolysis-stable domains (~70 kDa) found in the crystals produced by a number of *B. thuringiensis* subspecies [17]. In any case, these proteins are very distantly related, which corroborates with the drastic differences in the spectra of their entomocidal activity.

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