INTERCHAIN CROSSLINKS IN THE ENTOMOCIDAL *BACILLUS THURINGIENSIS* PROTEIN CRYSTAL

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1. Introduction

The bacterium *Bacillus thuringiensis* produces a parasporal body known as the δ-endotoxin which is toxic upon ingestion to the larval stage of many agriculturally important *Lepidoptera* insects [1,2]. This protein inclusion is formed in the bacterium during sporulation and the ultimate toxin is created by proteolytic digestion of the crystal in the alkaline midgut of the *Lepidoptera* larvae. Viable cells are not required for toxicity. However, the empirical use of this crystal as an insecticide has preceded definitive knowledge of its chemical structure.

In particular, no convincing reason has yet been proposed to explain why this protein crystallizes. The amino acid composition is not unusual [3], even though relatively high levels of hydrophobic (35%) and acidic (30%) amino acids are present. In this light, there are several reasons why a search for interchain crosslinks should prove profitable:

(i) They offer a potential explanation for the giant molecules (> 200 000 daltons) frequently observed [4,5] by SDS–PAGE;
(ii) They provide a feasible rationale for a crystal-specific pre-packaged protease [5]. If an unusual crosslink (e.g., ε-(γ-glutamyl)lysine) were present, it would not be cleaved by the larval gut proteases;
(iii) The solubility properties of the crystal [1] suggest that solubilization is accompanied by covalent modification of the crystal protein. Untreated crystals are insoluble at pH $\lesssim$ 11.5 [1,5]. However, once solubilized, the crystal protein can be precipitated at its isoelectric point (pH 4.4) whereupon it is readily soluble at neutral pH.

Here we present our studies on the identification of those crosslinks operative in the maintenance of crystal structure.

Abbreviations: DTT, dithiothreitol; GuHCl, guanidine hydrochloride; SDS, sodium dodecylsulfate; TLC, thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis

2. Materials and methods

The type strain *B. thuringiensis* var. *thuringiensis* (NRRL B-4039) was obtained from the Agricultural Research Service Culture Collection, Peoria, IL. The cells were grown in a glucose–yeast extract–salts medium [6] and, following sporulation, the crystals were purified by zonal gradient centrifugation on NaBr gradients [7].

2.1. *Potential ester bonds*

Alkaline hydroxylamine cleavage of potential ester bonds was assayed as follows [8]: The crystal suspension (1 ml at 1.0%) was incubated at 39°C for 4 h with 1 ml 2 M hydroxylamine (pH 10). The reaction was terminated by adjusting to pH 4.0 and the mixture was dialyzed overnight. The bound hydroxamate was determined by hydrolyzing 0.5 ml crystal with 0.5 ml 2 N HCl at 100°C for 3 h. After cooling, the mixture (0.5 ml) was incubated for 15 min with 0.5 ml 4 N NaOH and 2 ml 0.2% indole. The A_{400} band was developed by incubation for 30 min with 2.5 ml 4 N H_2SO_4.

Methanol would be produced by hydrolysis of methyl esters attached to the side chains of aspartic

or glutamic acid. The methanol assay [9] consisted of oxidizing the methanol to formaldehyde and quantitating the violet color formed on the addition of chromotropic acid.

2.2. *N-terminal amino acids*

The procedure employed was that of [10]. The crystals (250 μg) were dissolved in 50 μl 1% SDS containing 0.005 M β-mercaptoethanol and heated at 100°C for 2 min. After cooling, 50 μl *N*-ethylmorpholine and 75 μl dansylchloride (25 mg/ml in dimethylformamide) were added. After 60 min the crystal was precipitated with 0.5 ml acetone, pelleted by centrifugation, washed with 80% acetone, and dried. The protein was subsequently suspended in 100 μl 6.1 N HCl, sealed under N_2, hydrolyzed at 105°C for 16 h and analyzed via TLC on polyamide sheets [11].

2.3. *Measurement of ε-NH_2 lysine crosslinks*

In the method of [12] crystal protein (10 mg) was incubated with 30 μl triethanolamine and 0.3 ml acrylonitrile for 96 h at 37°C. The solution was dried and resuspended under N_2 in 0.5 ml 6 N HCl at 110°C for 24 h. The hydrolysis products were dried, dansylated, and resuspended in 0.1 ml acetone/acetic acid (3:2). The dansylated amino acids (2 μl) were identified via 2-dimensional TLC [11] on 5 × 5 cm polyamide sheets.

2.4. *Cysteine and cystine*

The crystal cysteine content was determined by reaction with Ellman reagent [13]. The 412 nm *p*-nitrophenol chromophore was assayed spectrophotometrically in 6 M GuHCl. The total cysteine plus cystine content was determined by two other methods:

(i) Taniguchi procedure [14] whereby protein sulfur is converted to ZnS and quantitated by conversion to H_2S;
(ii) The modification [15] of the Ellman procedure [13] wherein the crystal is reduced with 50 mM DTT (in 6 M GuHCl) prior to sulfydryl assay.

3. Results and discussion

3.1. *Ester bonds*

Crystal ester linkages are attractive for 3 reasons:

(i) These bonds are labile at pH 10, the pH at which crystals undergo solubilization in the larval gut;
(ii) Most proteases are also esterases [16];
(iii) Ester crosslinks between for instance, serine and glutamic acid could be formed from the side chains of the common amino acids in accordance with the absence of unusual amino acids in native crystals [3].

Quantitation [8] of the potential ester bonds (hydroxylamine-sensitive) in the crystal indicated 2.5 bonds/150 000 dalton subunit. However, this cleavage was accompanied by the appearance of newly introduced N-terminal glycine residues. Since alkaline hydroxylamine was recommended for the cleavage of especially labile asparagine-glycine peptide bonds [17], it is unlikely that the *B. thuringiensis* protein crystal contains interchain ester crosslinks.

An alternative source of ester bounds would be the aspartic and glutamic acid methyl esters in [18]. Hydrolysis of these bonds would release methanol as a potential toxin or toxin adjuvant. Not surprisingly, methanol is toxic to *Lepidoptera* larvae [19]. However, methanol assays following alkaline crystal solubilization have been consistently negative.

3.2. *ε-(γ-glutamyl) lysine crosslinks*

The presence of covalent crosslinks in insoluble proteins in unusual but by no means unique. The ε-(γ-glutamyl)lysine crosslink has been found in both human fibrin [12] and human skin [20], as well as in protein from hair and quills [21]. The preferred method [12] for the detection of these crosslinks involves reaction of the protein with acrylonitrile. Acrylonitrile reacts with free amino groups to form their acid-stable cyanoethyl derivatives. Thus, acid hydrolysis of the modified protein yields the carboxyethyl derivative of lysine whereas only those lysines participating in ε-crosslinks appear as underivatized lysine. However, following acid hydrolysis of the acrylonitrile-treated crystal, no free lysine or ornithine could be detected by TLC on polyamide sheets [11]. The *B. thuringiensis* crystal does not appear to contain ε-lysine or δ-ornithine crosslinks. Of course, the absence of free lysine proves the completeness of the acrylonitrile reaction.

3.3. *Disulfide bonds*

On the basis of solubility properties alone, one

would expect disulfide bonds to participate in the maintenance of crystal structure. Sulfhydryl reagents such as thioglycollate, mercaptoethanol, and DTT are essential for crystal solubilization at pH $\lesssim$ 11.5 [1,5], while the high hydroxyl ion concentrations at pH > 11.5 can also achieve disulfide cleavage [22]. However, there has been some discussion [1,23] whether sufficient disulfide bonds are present to account for the unique crystal properties. Table 1 lists the literature % cystine values for 8 strains of *B. thuringiensis*. Each report indicates between 1.1–1.9% cystine; sufficient for 7–12 disulfide bonds/150 000 dalton subunit. Additionally, each of the values in table 1 was determined by performic acid oxidation of cysteine to cysteic acid. This technique can easily underestimate the actual cystine present [24]. Accordingly, we determined the cysteine and total cysteine/cystine contents by alternative methods:

(i) No free sulfhydryls were detected (table 2) employing the procedure [13] on crystals boiled in 6 M GuHCl, to ensure the reagent unrestricted access to the crystal interior.

(ii) The alternative cystine assay techniques (table 2) [14,15] gave higher values than the previous cysteic acid reports (table 1)

Table 1
Representative literature values of the cystine content in *Bacillus thuringiensis* crystals

Variety name	% Cystine (g/100 g protein)	Ref.
Thuringiensis	1.1	[3]
Thuringiensis	1.35	[27]
Thuringiensis	1.32	[28]
Alesti	1.2	[29]
Alesti	1.45	[30]
Alesti	1.9	[3]
Kurstaki (HD-1)	1.4	[5]
Sotto	1.1	[27]
Sotto	1.2	[31]
Sotto	1.2	[3]
Galleriae	1.5	[3]
Entomocidus	1.4	[3]
Tolworthi	1.15	[32]
Anduze	1.34	[27]

Table 2
Sulfhydryl and disulfide content in *Bacillus thuringiensis* var. Thuringiensis crystals

Residue type	% (g/100 g protein)	no./150 000 dalton subunit
Sulfhydryl groups	0	0
Disulfide bonds [14]	1.85	11.9
Disulfide bonds [15]	1.99	12.4

These techniques indicate 12 cystines/150 000 dalton subunit.

In summary, we have eliminated the possibility that protein crystal structure is maintained via ester or ϵ-lysine peptide crosslinks. Instead, we have confirmed the original suggestions [25,26] regarding the importance of disulfide bonds. Of course, the mere presence of disulfide bonds does not prove that they are interchain disulfide bonds and experiments to determine the intrachain/interchain ratio are underway in our laboratory.

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