

In vitro binding of *Bacillus thuringiensis* var. *israelensis* individual toxins to midgut cells of *Anopheles gambiae* larvae (Diptera: Culicidae)

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Abstract Midguts from *Anopheles gambiae* fourth instars were dissected and processed for immuno-light microscopy. Cloned insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* var. *israelensis* (*Bti*) were individually expressed in crystal-negative strains of *Bacillus thuringiensis*. Tissue sections of *A. gambiae* were incubated in vitro with each solubilized and trypsin-activated ICP. Immunodetection of CryIVA, CryIVB, CryIVD and CytA toxins on sections was performed using purified rabbit IgG directed against *Bti* ICPs, in combination with an anti-rabbit IgG/peroxidase. CryIVA, CryIVB, CryIVD and CytA toxins were detected on the apical brush border of midgut cells, in the gastric caecae and posterior stomach. CytA was also detected, to a lesser extent, on microvilli of anterior stomach cells.

Key words: *Bacillus thuringiensis* var. *israelensis*; δ -Endotoxin; Binding; *Anopheles gambiae*; Receptor

1. Introduction

The high and specific toxicity of *Bacillus thuringiensis* var. *israelensis* (*Bti*) [1,2] to mosquito and blackfly larvae [1,3] is linked to the synthesis of crystals containing four major polypeptides with apparent molecular weights of 125, 135, 68 and 28 kDa. These insecticidal crystal proteins (ICPs) are referred to as CryIVA, CryIVB, CryIVD, and CytA, respectively, according to the nomenclature of Höfte and Whiteley [4]. After cloning and expression of individual *cryIV* and *cytA* genes either in heterologous or homologous bacterial hosts, it has been shown that the in vivo larvicidal activity on *Anopheles* spp. larvae is mainly related to the presence of CryIVA, CryIVB and/or CryIVD toxins [6–10]. It has been previously shown that the in vitro non-specific cytolytic activity is due to CytA [5,6]. Nevertheless, the contribution of CytA in the expression of the toxicity remains unclear. Indeed, it has been previously demonstrated that CryIVD and CytA were immunologically detected, both bound on apical microvilli of *A. gambiae* larval midgut cells, after in vivo intoxication with purified *Bti* crystals [11]. Other *B. thuringiensis* ICPs have also been shown to bind specifically, in vivo and in vitro, to midgut brush border membranes of Lepidoptera and Coleoptera larvae [12–15].

We investigated the ability of CryIVA, CryIVB, CryIVD and

CytA ICPs to bind in vitro to tissue sections of healthy mosquito larvae. We used immunological detection to determine whether and where each polypeptide bound on midgut cells.

2. Materials and methods

2.1. Preparation and activation of ICPs

Bti strain 4Q2-72 is a crystal-producing strain harbouring only the 72 MDa plasmid which encodes all the *Bti* ICPs. Strains 4Q2-81 and 4Q7 are non-toxic crystal-negative strains, used as recipient strains. Genes encoding ICPs originated either from *B. thuringiensis* var. *israelensis* [16] or *B. thuringiensis* var. *morrisoni* [17], as summarized in Table 1.

Bacteria were grown in UG liquid medium [18] supplemented (except strain 4Q2-72) with 25 μ g/ml erythromycin, with shaking, at 30°C until cells lysed. Spores and crystals were harvested by centrifugation, incubated for 30 min in 1 M NaCl, then washed twice with deionized water and stored in aliquots at –20°C. After thawing, aggregates were separated by ultrasonic treatment (Branson Sonifier; large tip, 40% output scale, 25% duty cycle, for 4 min). Crystals were purified by ultracentrifugation on pre-formed sucrose gradients (67/72/79%, w/v); purified crystals (localized at the 72–79% sucrose interface) were collected and extensively washed with deionized water, and stored in aliquots at –20°C. ICPs were solubilized in 50 mM NaOH for 90 min at 30°C, neutralized using diluted HCl, and extensively dialyzed against 10 mM sodium phosphate buffer at pH 8.5, then activated by trypsin (EC 3.4.21.4; Serva; 1/50 trypsin vs. protein ratio, w/w) for 2 h at 37°C. Proteolysis was prevented by the addition of 0.1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma). Protein concentrations were measured with the Bio-Rad assay [19] using bovine serum albumin as a standard.

2.2. Preparation of immunoglobulins against *Bti* ICPs

Purified crystals from *Bti* strain 4Q2-72 were solubilized in alkali, injected into rabbits and the immunoglobulins (IgGs) purified from the collected antisera using ammonium sulphate precipitation and anion-exchange chromatography as previously described [11]. Non-specific binding to mosquito midgut cells was reduced by adsorbing the IgG solution on freshly ground fourth instar larvae of *A. gambiae*, as previously described [11].

2.3. Preparation of mosquito larvae sections

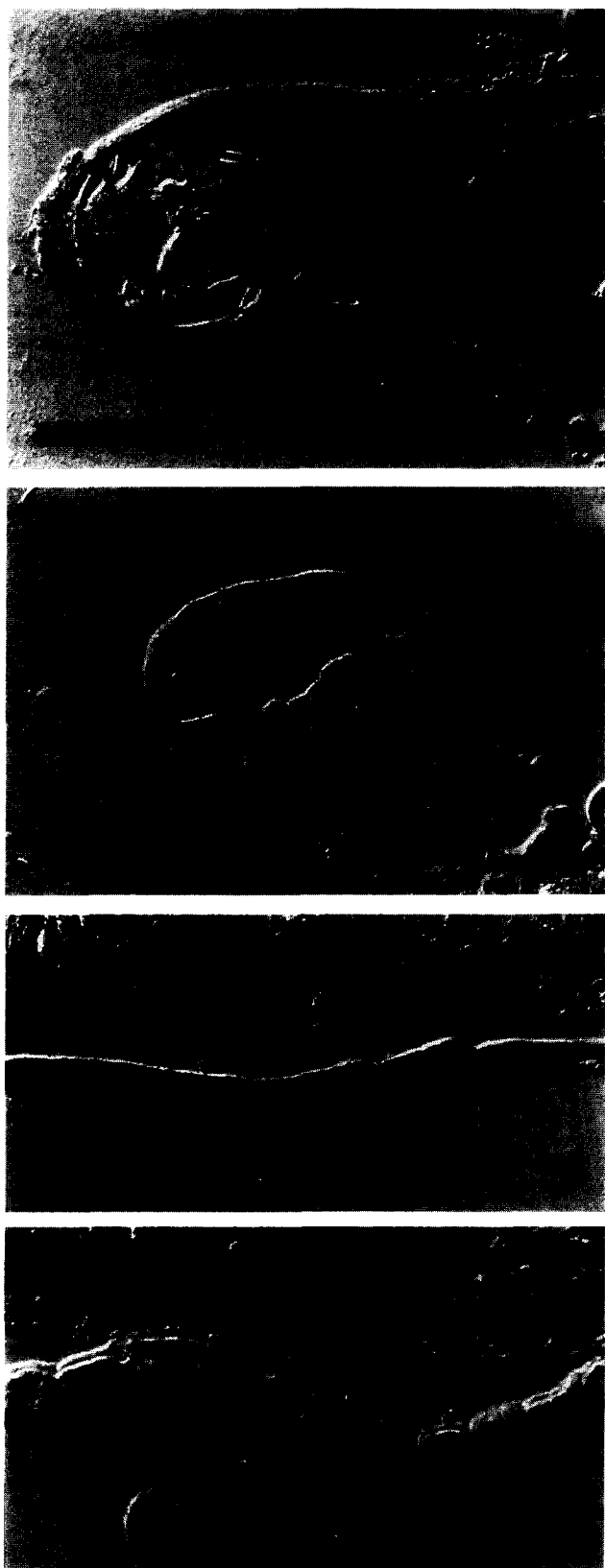
Young fourth instar larvae of *A. gambiae* were chilled on ice and midguts with their contents dissected out in ice-cold 0.1 M sodium phosphate buffer at pH 7.4 containing 2% paraformaldehyde and 0.1% glutaraldehyde, then incubated at room temperature for 4 h. The samples were partially dehydrated in a series of ethanol baths, infiltrated and embedded at 38°C in polyester wax [20], stored for at least 12 h at 4°C, longitudinally sectioned into 7.5- μ m slices using a refrigerated microtome set at 10°C, and attached on mounting glass coated with gelatin (1 mg/ml) in 0.4% chromium potassium sulphate.

2.4. In vitro binding assay and immunological detection

After deparaffination in ethanol and hydration in sodium phosphate buffered saline at pH 7.4 (PBS), tissue sections were blocked in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100 (PBS/BSA/Tx), incubated with the activated ICP at 5 μ g/ml in PBS/BSA/Tx (1 h, room temperature) and rinsed twice with PBS/BSA/Tx. Subsequently, sections were covered with the purified rabbit IgGs (1 h, room temperature), at the following concentrations: 90, 22 or 9 μ g/ml in

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PBS/BSA/Tx for sections incubated with CryIVB, CryIVA and CytA, or CryIVD, respectively. Following removal of excess IgGs with PBS/BSA/Tx, sections were incubated with horseradish peroxidase-conju-

Fig. 1. Longitudinal sections through *A. gambiae* fourth instars, probed with rabbit IgGs directed against *Bti* ICPs, then with the peroxidase-conjugated anti-rabbit IgGs (negative control). Light microscopy, Nomarski interferential contrast. Double bar in A corresponds to 100 μ m; B–D are at the same magnification. (A) Cardia cells; (B) gastric caeca; (C) anterior stomach; (D) posterior stomach. L, midgut lumen; mv, apical microvilli; PM, peritrophic membrane.

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gated goat anti-rabbit IgGs (Bioss; 10 μ g/ml in PBS/BSA/Tx, 30 min, room temperature). Unbound secondary antibody was washed out with PBS/BSA/Tx, then sections were incubated in 3,3'-diaminobenzidine (DAB; Sigma; 0.5 mg/ml 50 mM Tris buffer at pH 7.6) in the presence of H_2O_2 . Finally, sections were mounted in Mowiol 4-80 (Hoescht) and viewed with a Nikon photomicroscope equipped with Nomarski interferential contrast.

Sections treated under the same conditions but incubated in the absence of either the toxin or the first antibody were used as negative controls to exclude false-positive results.

3. Results

The main structural differences between the cardia, gastric caeca, the anterior stomach and the posterior stomach have been previously shown [11,21,22]: gastric caeca and the posterior stomach harbour very long and thin apical microvilli, whereas in the cardia and in the anterior stomach cells they are shorter.

Longitudinal sections from the cardia, gastric caeca, anterior and posterior stomach of control *A. gambiae* larvae midgut epithelium at different levels are shown Fig. 1. They did not show any positive (brown-coloured) reaction whichever incubation step was omitted, indicating the absence of non-specific binding.

No positive reaction was found in the cytoplasm of the cells, whatever the *Bti*-activated protein used for binding on tissue sections. In the cardia cells, there was no visible labelling. In addition, the peritrophic membrane was not labelled.

CryIVA, CryIVB, CryIVD and CytA were strongly detected on the brush border membrane of midgut cells, associated to microvilli of posterior stomach cells (Figs. 2C, 3C, 4C and 5C, respectively). A signal, less intense, was also detected on microvilli of gastric caeca cells (Figs. 2A, 3A, 4A and 5A) and, to a lesser extent, on basolateral membranes. A very-low intensity signal was also found on microvilli of the anterior stomach (Figs. 2B, 3B and 4B). In addition, for CytA, there was a clear labelling of the microvilli of the anterior stomach cells, visible as a regular brown-coloured line at the apical end of cells (Fig. 5B).

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Figs. 2–5. Longitudinal sections through *A. gambiae* fourth instars, probed with CryIVA (Fig. 2), CryIVB (Fig. 3), CryIVD (Fig. 4) and CytA (Fig. 5), then with rabbit IgGs directed against *Bti* ICPs, and finally with the peroxidase-conjugated anti-rabbit IgGs. Nomarski interferential contrast, set under low contrast to reduce the shadows. Double bars in panels A correspond to 100 μ m; panels B and C are at the same magnification. (A) Gastric caeca; (B) anterior stomach; (C) posterior stomach. The brown-coloured labelling is indicated with arrowheads. The different aspect of the gastric caeca in Fig. 2A and 5A are due to the longitudinal presentation of the caeca, while the others (Figs. 3A and 4A) are transversally sectioned. In Fig. 2B, the dark line on the apical part of the cells, right of the arrowhead, is a shadow. L, midgut lumen; PM, unlabelled peritrophic membrane.

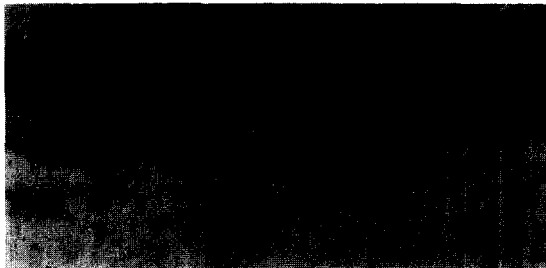
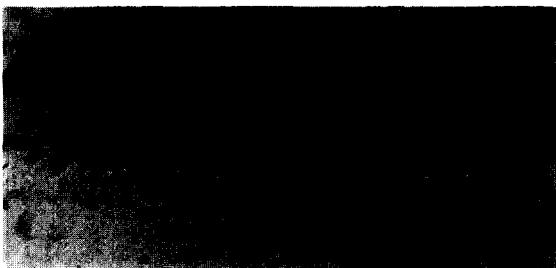
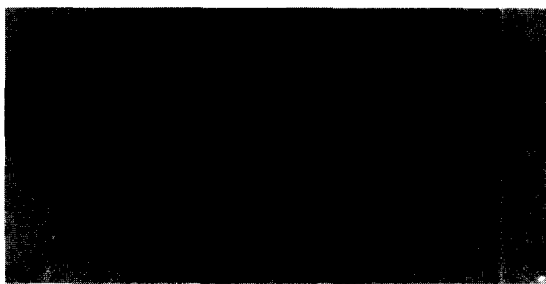
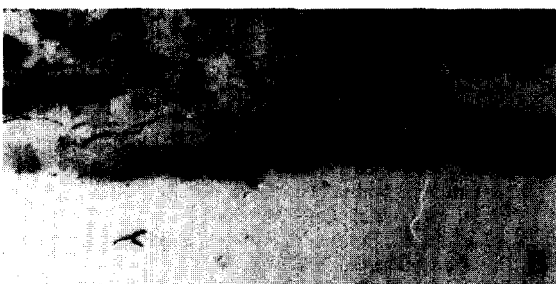
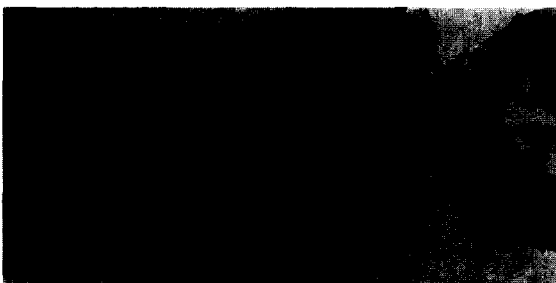
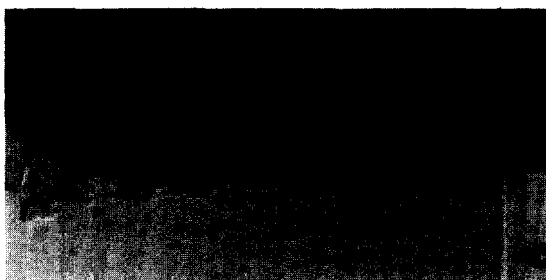
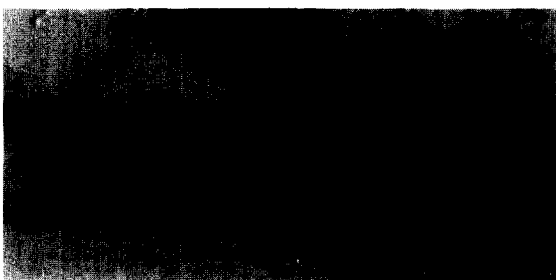
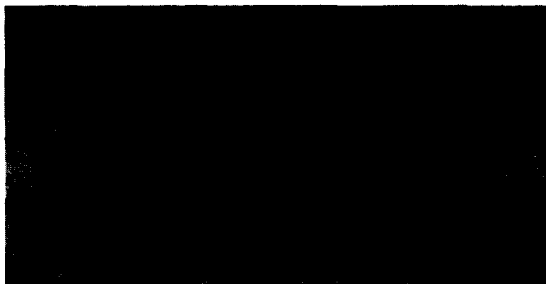
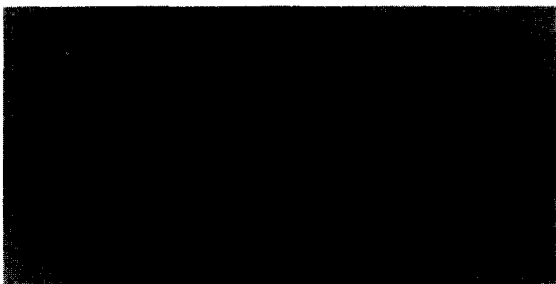
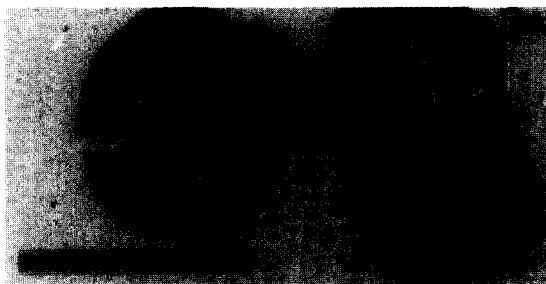
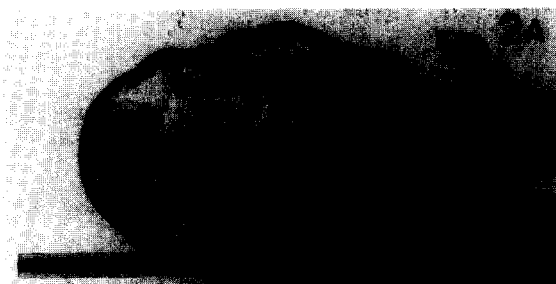


Table 1
Source of recombinant insecticidal crystal proteins

<i>Bt</i> ICP	Gene origin	Transformant	Reference	Provided by
Whole crystals	<i>Bti</i> strain 4Q2-72		[26]	Dr. D.H. Dean, Ohio State University, Columbus, OH, USA
CryIVA	<i>Bti</i> strain 4Q2-72	4Q2-81 (pHT606)	[8]	Dr. A. Delécluse, Institut Pasteur
CryIVB	<i>Bti</i> strain 4Q2-72	4Q2-81 (pHT611)	[8]	Dr. A. Delécluse, Institut Pasteur
CryIVD	<i>Btm</i> strain PG-14	4Q2-81 (pHT640)	[9]	Dr. A. Delécluse, Institut Pasteur
CytA	<i>Btm</i> strain PG-14	4Q7 (pWF45)	[27]	Dr. B.A. Federici, University of California, Riverside, CA, USA

4. Discussion

In this study we present an analysis of *in vitro* binding of the four major *Bti* proteins to tissue sections of *A. gambiae* mosquito larvae. The method chosen for a good antigenicity preservation of putative membrane receptors was successful, as the immunological detection of activated toxins bound on membranes gave positive and specific signals.

All CryIV toxins bound strongly to microvilli from both posterior stomach cells and gastric caecae, although giving a lower signal in the latter, while only a very low signal was found on apical membranes of anterior stomach cells. This might reflect the presence of specific receptors on posterior stomach microvilli with a higher affinity for the toxin and/or a higher concentration than on the gastric caecae microvilli. In addition, the low binding to apical microvilli of anterior stomach cells could be due to either the lack of receptors on these cells, or a low concentration or affinity of these receptors, resulting in low-detectable binding.

CytA was detected on the microvilli of all midgut cells, cardia cells excepted. This detection, also found in anterior stomach cells, could be related to the ability of CytA to induce pores through the biological membranes without requiring the participation of any specific receptor. The fact that cardia cell microvilli were not labelled might be related to the secretion of the peritrophic membrane by those cells, as previously described [23,24], the presence of which could block the access of CytA to apical membranes.

There are some differences between the detection of *Bti* toxins on sections of intoxicated larvae after *in vivo* ingestion of crystals containing all the *Bti* ICPs [11], and the detection of individual toxins after binding to tissue sections from healthy larval insects. In particular, CytA was not detected on the apical microvilli of the anterior stomach in the former study, while it was clearly detected in the latter. This could be due to a different conformation of the CytA polypeptides when other ICPs are present, leading to a different behaviour. Alternatively, the gene origin used to produce CytA and CryIVD in both experiments were not exactly the same (Table 1). Indeed, for CryIVD, the gene sequence from *Btm* PG-14 differs from the one of *Bti* strain 4Q2-72 by one substitution, but the resulting codon encoded the same amino acid, so that sequences of the encoded proteins are identical [25]. In contrast, the nucleotide sequences of *Btm* PG-14 and *Bti* 4Q2-72 differ by one base substitution, resulting in the occurrence of alanine rather than proline in position 82 of the CytA from strain *Btm* PG-14 [17] that we used in the present study. Such a change might also induce a different ICP conformation. This explanation is, nevertheless, only putative, since polyclonal antibodies were used for the detection of bound toxins. Another possibility is that the conformation of binding sites may be different in the *in vivo*

and in the *in vitro* binding: molecules onto which CytA does not bind *in vivo* may have undergone alterations during preparation of the tissue sections, causing them to interact with CytA under *in vitro* conditions.

Observations on binding of other *B. thuringiensis* ICPs CryIA(b), CryIA(c), CryIB and CryIIIA to lepidopteran and coleopteran midgut cells indicated that these δ -endotoxins bind *in vitro* to peritrophic membranes, but without any correlation with toxicity [12,13]; in contrast, the *in vitro* binding of these toxins to apical midgut microvilli correlated well with the susceptibility of the target insect to specific toxins [12]. In our study, no binding to the peritrophic membrane was observed, while the *in vitro* binding seems to be limited to some cellular types (especially the posterior stomach and the gastric caecae), except for CytA which also bind to the anterior stomach. The binding of CryIV δ -endotoxins, especially to apical membranes of midgut cells, is consistent with the involvement of specific receptors in the binding of the toxin on midgut cells, which is assumed to be the necessary step in the mode of action of the entomopathogenic toxins.

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