Amino acid substitution in α-helix 7 of Cry1Ac δ-endotoxin of *Bacillus thuringiensis* leads to enhanced toxicity to *Helicoverpa armigera* Hubner

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Abstract Insecticidal proteins or δ-endotoxins of *Bacillus thuringiensis* are highly toxic to a wide range of agronomically important pests. The toxins are formed of three structural domains. The N-terminal domain is a bundle of eight α-helices and is implicated in pore formation in insect midgut epithelial membranes. All the δ-endotoxins share a common hydrophobic motif of eight amino acids in α-helix 7. A similar motif is also present in fragment B of diphtheria toxin (DT). Site-directed mutagenesis of Cry1Ac δ-endotoxin of *B. thuringiensis* was carried out to substitute its hydrophobic motif with that of DT fragment B. The mutant toxin was shown to be more toxic to the larvae of *Helicoverpa armigera* (cotton bollworm) than the wild-type toxin. Voltage clamp analysis with planar lipid bilayers revealed that the mutant toxin opens larger ion channels and induces higher levels of conductance than the wild-type toxin.

Key words: δ-Endotoxins; α-Helix 7; Mutagenesis; Ion channel; *Bacillus thuringiensis*

1. Introduction

δ-Endotoxins are members of the family of insecticidal crystal proteins (ICPs) produced by *Bacillus thuringiensis* strains during sporulation [1]. ICPs are highly toxic to a wide variety of important insect pests as well as other invertebrates. Upon ingestion by a susceptible larva, these proteins are solubilized and proteolytically cleaved to a toxic fragment that binds to high affinity sites on the apical membrane of midgut epithelium cells before insertion into the membrane. The inserted toxins disturb the electrolyte balance by creating pores in the cell membrane, leading to cell lysis and finally to larval death [2].

The tertiary structures of Cry3Aa and Cry1Aa toxins have been determined [3,4]. The toxins are formed of three structural domains. The N-terminal domain I is a bundle of eight α-helices, with the central α-helix 5 surrounded by amphipathic helices. Domain II consists of three anti-parallel β-sheets and 1–2 short α-helices and domain III is a β-sandwich structure of two anti-parallel strands. Hofte and Whiteley [5] have identified five highly conserved regions among the ICP sequences. It has been proposed that the high degree of conservation of these blocks and their important structural location imply that all the toxins that possess these blocks would share similar structures [3]. The first two conserved regions have the highest degree of identity among the whole ICP family. Block II comprises the central α-helix 6, α-helix 7 and the β-strand of domain II [4]. All the ICPs share a common hydrophobic motif of eight amino acids in α-helix 7 of domain I [6], which emphasizes the importance of this motif in pore formation. Interestingly, a similar motif is also present at the C-terminal end of fragment B of diphtheria toxin (DT) which makes large pores in the mammalian cell membranes [7].

In the present study, we substituted the hydrophobic motif of α-helix 7 of Cry1Ac toxin of *B. thuringiensis* by a similar motif from DT fragment B by site-directed mutagenesis and studied the effect of the mutated toxin on neonate larvae of *Helicoverpa armigera* (cotton bollworm), an important pest on many crop plants. We also investigated the ion channel making properties of the wild-type (WT) and mutant (M) Cry1Ac toxins by using black lipid membranes (BLMs).

2. Materials and methods

2.1. Site-directed mutagenesis

The *cry1Ac* gene of *B. thuringiensis* cloned in the expression vector pKK 223-3 (pO5 4201) was used [8]. Site-directed mutagenesis of the gene was carried out using Chameleon site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The mutagenic primer 5′-AGAAGAGAATTAACAC-TAATTTCGTCGGATTCCATAGGCGTTCTTCCGAATTATGA-TAGTAGA-3′ contained the sequence from DT fragment B. The mutant clones were checked by DNA restriction analysis based on the presence of a *Hin*fI site in the mutagenic primer. The vector carrying the mutant gene was mobilized into *Escherichia coli* JM 103. The ICPs produced by overnight grown *E. coli* were purified according to Ge et al. [8]. Typically, the amount of ICP was about 40% of total *E. coli* protein [9]. Trypsin digestion and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out according to Lee et al. [10].

2.2. Insect bioassay

Insect bioassays were performed in 24 well Costar plates with six concentrations of the toxins. First and third instar larvae of *H. armigera* (one larva per well) were released on the artificial diet into which the toxins were incorporated [9]. Larval weight and mortality were recorded after 6 days and the data were subjected to probit analysis [11].

2.3. Reconstitution of Cry1Ac channels in BLMs

BLMs were formed across an aperture of 200 μm diameter in a Delrin cup (Warner instruments, Hamden, CT, USA) with diphytanoyl phosphatidyl choline (Avanti Polar Lipids, Birmingham, AL, USA) in *n*-decane (20 mg/ml). The bilayer was formed between symmetric bathing solutions containing 150 mM KCl, 10 mM Tris and 0.5 mM EDTA (pH 7.4 and 9.0). All salts used in the bathing solutions were ultrapure (Sigma).

Trypsin-cleaved Cry1Ac toxins prepared as previously described [9] were added to the aqueous solution and were allowed to incorporate

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into the bilayers spontaneously. The extracted channels were generally very active. Single channel currents were recorded with an Axopatch patch clamp amplifier (Axon Instruments, Foster City, CA, USA). The *cis* solution (voltage command side) was connected to the CV 201A head stage input and the *trans* solution was held at virtual ground via a pair of matched Ag-AgCl electrodes. Currents through the voltage-clamped bilayers were low pass-filtered at 10 kHz and recorded on video cassettes after digitization through an analog to digital converter (VR 10B, Instrutech, USA). Using standard voltage conventions, positive clamping potentials are quoted as potentials with respect to the ground (*trans* chamber) and positive currents are shown as upward traces.

2.4. Single channel recording and data analysis

Data were analyzed after filtration through an 8 pole Bessel filter (902 LPF, Frequency Devices, Haverhill, MA, USA) at 1–2 kHz using AxoData and AxoGraph software with additional standard non-linear fitting routines where necessary. Sampling was done using a ITC-16 (Instrutech, USA) at 5–20 kHz. Channel amplitudes were measured by fitting multiple Gaussian distributions to all point amplitude histograms. For optimum filtration, the filter's −3 dB point for each recording was set so that bilayer noise (in the absence of a channel) just failed to lead to the detection of false 'openings' to the lowest amplitude conductance level (determined after a preliminary amplitude analysis of a corresponding recording containing a channel). Under ideal conditions, the shortest directly measurable duration should theoretically have had a rise time of 0.3321/fc, where fc is the −3 dB point in kHz [12]. That is, use of a cut off frequency of 2 kHz should allow for the duration of events as short as 0.17 ms to be measured. The filter cut off frequencies are indicated in the figure legends.

3. Results and discussion

3.1. Site-directed mutagenesis

Site-directed mutagenesis of the *cry1Ac* gene was performed using the mutagenic primer comprising a sequence encoding hydrophobic motif (^{311}ISSDSIGVL^{319}) of fragment B of DT [7]. The M-Cry1Ac was expressed in *E. coli* and purified. The M- and WT-Cry1Ac proteins were electrophoresed to check for their integrity and pattern of trypsin digestion. The mutation did not affect the molecular weight and trypsin digestion pattern of the M-Cry1Ac protein (Fig. 1).

3.2. Insect bioassay

The WT- and M-Cry1Ac proteins were incorporated into the artificial diet of *H. armigera* at six concentrations and their effect on neonate larvae was studied. Table 1 shows that the M-Cry1Ac was eight times more effective than the WT-Cry1Ac in terms of larval mortality. The experiment was repeated with third instar larvae and essentially similar results were obtained.

The hydrophobic motif of DT fragment B is very similar to the motif present in α-helix 7 of Cry1Ac [6]. Fig. 2 depicts the hydropathic profiles of Cry1Ac (amino acids 188–319) and DT fragment B (amino acids 404–535) analyzed by the PC/Gene program with an interval of nine amino acids. The amino acids present in the hydrophobic motifs of Cry1Ac, DT-B

Table 1
Insect bioassay analysis of WT- and M-Cry1Ac proteins incorporated in the artificial diet of *H. armigera* larvae

δ-Endotoxin	LC_{50} (95% fiducial limits)	
	Neonate	III instar
WT-Cry1Ac	20.0 (12.5–36.3)	27.3 (15.2–42.7)
M-Cry1Ac	2.5 (1.0–5.0)	3.2 (0.8–5.5)

LC_{50} denotes the concentration of Cry1Ac protein (ng/cm^3) at which there is 50% larval mortality.

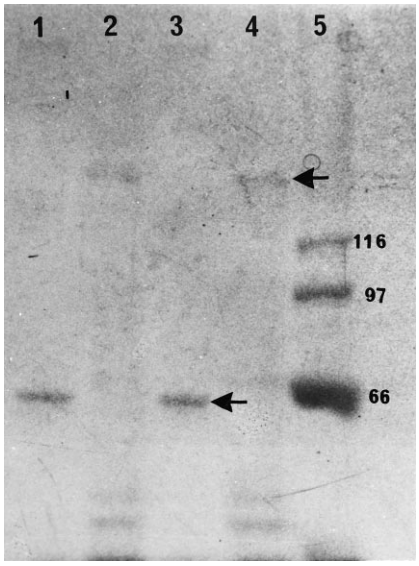

Fig. 1. Electrophoretic analysis of WT- and M-Cry1Ac proteins purified from *E. coli* and treated with trypsin. Lane 1: trypsinated WT-Cry1Ac, lane 2: untrypsinated Cry1Ac, lane 3: trypsinated mutant Cry1Ac, lane 4: untrypsinated mutant Cry1Ac, lane 5: molecular weight markers.

and Cry proteins of other classes are compared in Table 2. An interesting feature of this comparison is the presence of an aspartic residue (highly hydrophilic) in its middle part. α-Helix 7 of the Cry1A class of proteins is in contact with the first β-sheet of domain II, implicated in receptor binding. This helix is particularly important to the proposed insecticidal function, since it forms part of the conserved interface with domain II. It is also well-positioned for sensing receptor binding, thus being a likely candidate for initiating the membrane penetration needed to start pore formation [3]. Various models have been proposed to explain the mechanism of pore formation. Hodgman and Ellar [13] have proposed a 'penknife' model in which the strongly hydrophobic α-helices 5 and 6 joined by a loop at the top of the structure, open in a penknife fashion and insert into the membrane. In contrast, Li et al. [14] have surmized that the hydrophobic helical hairpin structure of α-helices 4 and 5 initiates pore formation (umbrella model). In a recent report, Schwartz et al. [15] suggested that α-helices 4 and 5 play a critical role in the formation of a functional pore. In another study which supported the 'umbrella model', Gazit et al. [16] suggested that α-helix 7 may serve as a binding sensor to initiate the structural rearrangement of the pore-forming domain. It was speculated that after receptor binding, the contacts between α-helices 5, 6 and 7 may assist the insertion of an α4-α5

Table 2
Comparison of the amino acid sequences of a hydrophobic motif (α-helix 7) present in the Cry protein family

Protein	Sequence of hydrophobic motif
Cry1Ac	^{239}TVLDIVALF
Cry1B	^{258}GVLDLVALF
Cry1C	^{238}TVLDIAAFF
Cry1D	^{238}SVLDIVAFF
Cry3A	^{283}TVLDLIALF
Cry4A	^{302}AVLDVVALF
DT-B	^{311}ISSDSIGVL

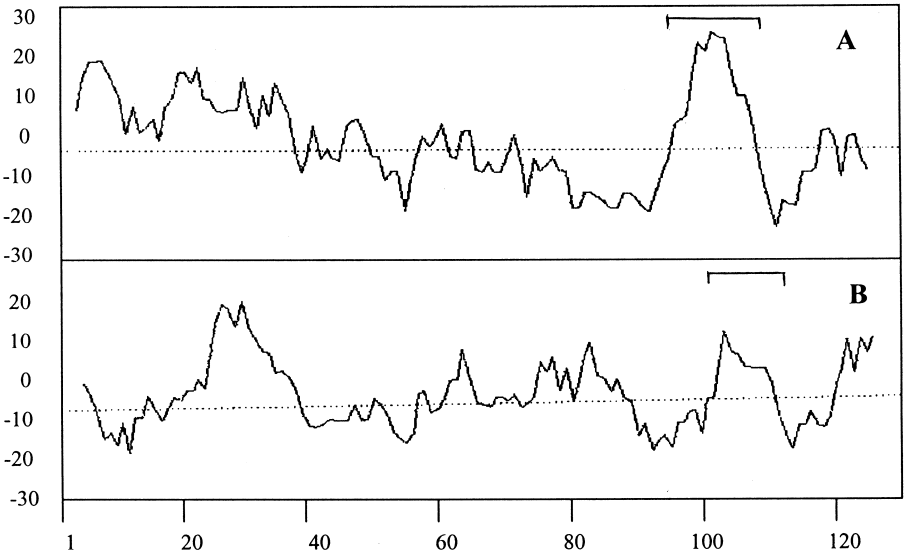

Fig. 2. Hydropathy profiles of Cry1Ac protein (A: amino acids 188–319) and DT fragment B (B: amino acids 404–535) derived by using the PC/Gene program with an interval of nine amino acids. Brackets indicate the hydrophobic peaks.

hairpin into the membrane by unpacking of the helical bundle that exists in the non-membrane-bound form of the toxin. Experiments with individual peptides and channel formation on PLBs have shown that in the presence of α-helix 5, α-helix 7 assembled and penetrated membranes better than did α-helix 5 complexes alone [17]. Our results show that the α-helix 7 is involved in the process of pore formation and substitution of its hydrophobic sequence by a similar motif from DT, which belongs to an entirely different class of pore-forming toxins, does not negatively affect its membrane insertion property. On the other hand, we observed an increase in the insecticidal activity of the M-Cry1Ac. Our findings support the previous observations that α-helix 7 has an important role to perform in the process of pore formation, presumably in conjunction with α-helix 5 [16,17]. To confirm our observation and also to test whether the amino acid substitution in α-helix 7 leads to better ion channel formation/stabilization, we have studied the ion channel induction properties of WT- and M-Cry1Ac proteins vis-a-vis BLM by voltage clamping analysis.

3.3. Voltage clamp analysis

The electrophysiological behavior of WT- and M-Cry1Ac

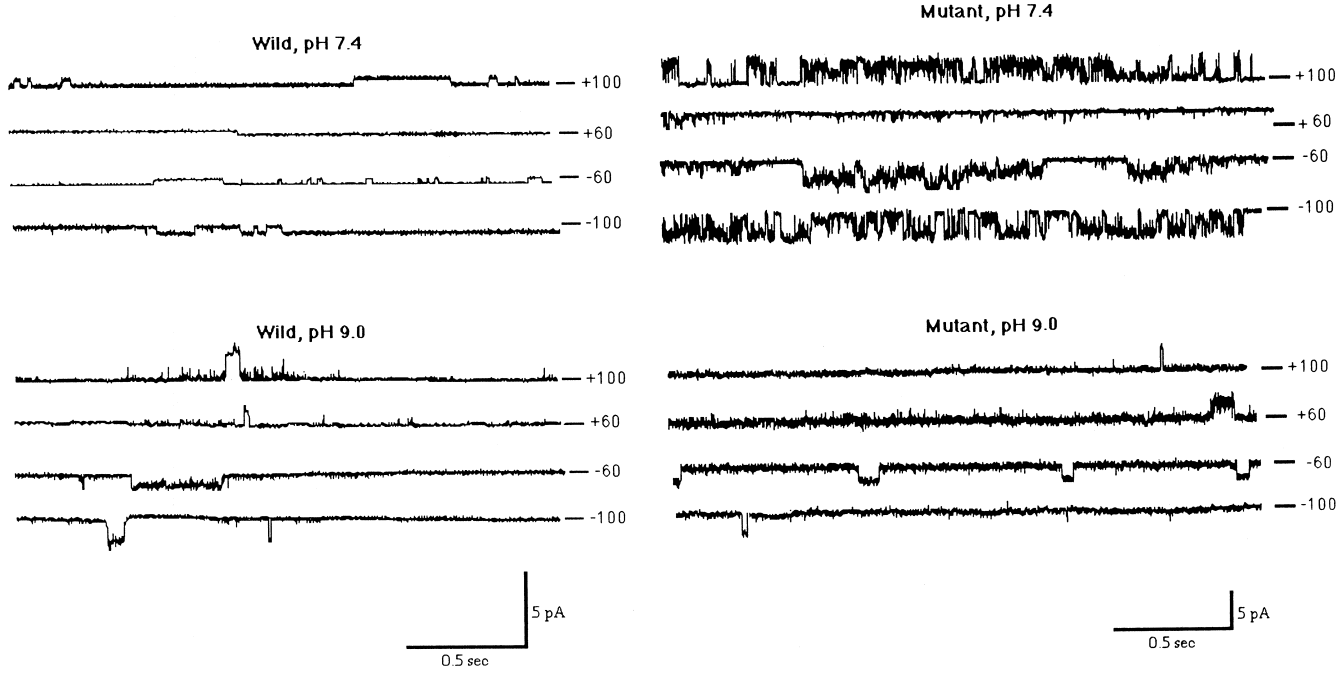

Fig. 3. Ion channels induced by WT and mutant forms of Cry1Ac. Current traces at different applied voltages and pH conditions are depicted. The bar at the side of each profile indicates the position of base line. Clamping potentials with respect to the ground are indicated along the left side of the figure. Data shown were filtered at 1 kHz and sampled at 5 kHz.

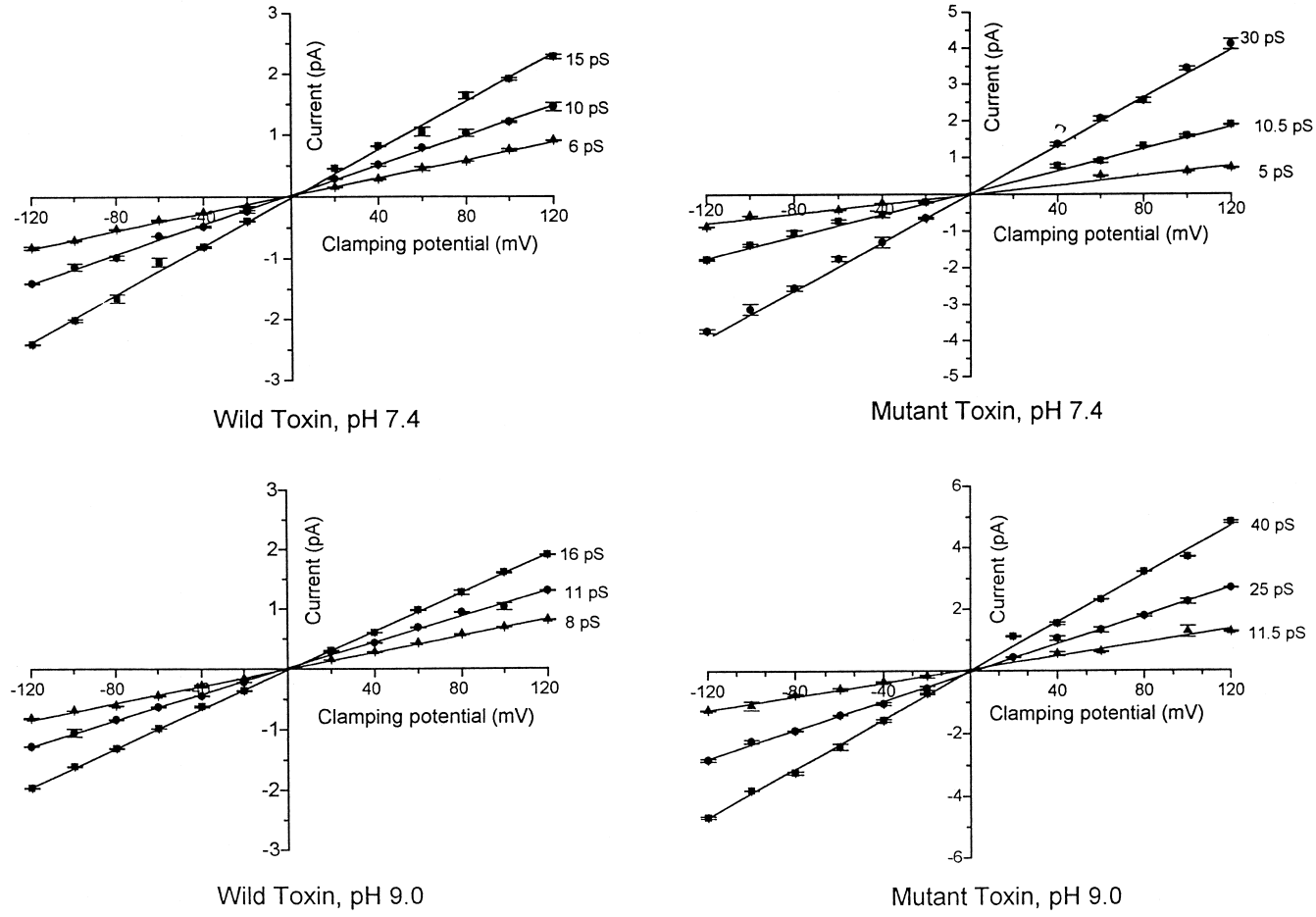

Fig. 4. Current-voltage relations of WT and mutant forms of Cry1Ac at pH 7.4 and 9.0. Toxin was incorporated into planar lipid bilayers composed of diphytanoyl phosphatidyl choline and cholesterol (4 mg/ml) in *n*-decane with a symmetrical bathing solution of 150 mM KCl, 0.5 mM EDTA and 10 mM Tris (pH 7.4 and 9.0). The conductance was calculated from the slope of the I-V curve. Values are the means of three independent observations.

proteins was compared at pH 7.4 and 9.0 in BLMs. Three levels of conductance were observed for both the mutant as well as the WT-Cry1Ac. M-Cry1Ac showed high channel conductance levels of 40, 25 and 11 pS as compared to those of WT-Cry1Ac (16, 11 and 8 pS) at pH 9.0 (Fig. 3). Slatin et al. [18] have reported much higher single channel conductance values (600 pS) for Cry1Aa, which may actually be the conductance of higher aggregates. Conductances of 6, 10 and 15 pS were observed at pH 7.4 when WT-Cry1Ac was used, whereas M-Cry1Ac elicited conductances of 5, 10.5 and 30 pS. These are integral multiples of 5 pS, indicating that these transitions may arise from aggregated channels which are operating in a cooperative manner. At a higher pH, the opening probability of the channel decreases significantly but the conductance of the most frequently observed transition increases to 40 pS. The higher effectivity of the M-Cry1Ac may probably be related to the pH dependence of its single channel properties. The most frequently observed M- and WT-Cry1Ac channels at some voltages are given in Fig. 4. The ability of the M-Cry1Ac to form bigger channels when reconstituted in BLMs suggests that the substitution of the hydrophobic motif in α-helix 7 of Cry1Ac has a positive effect. This is in line with the improved efficacy of M-Cry1Ac protein in insect bioassays. Voltage clamp experiments with midgut epithelial membranes or phospholipid bilayers into which larval brush border membrane vesicles have been incorporated may further support the findings.

Protein engineering helps in understanding the structure-function relationships of Cry toxin proteins and also in creating mutated proteins with an enhanced insecticidal activity. Schnepf et al. [2] reviewed the mutations in Cry toxins and their effects on receptor binding, pore formation and ion channel activity. Very few mutations resulted in positive effects on insecticidal activity. A mutation in α-helix 5 of Cry1Ac (domain I) caused a 2-fold increase in toxicity against *Manduca sexta* [19]. This was attributed to the rate of irreversible binding. In Cry1Ab, a combination of mutations in the α8 loop and loop 2 (domain II) resulted in a 32-fold increase in toxicity to *Lymantria dispar* [20]. The mechanism of increase in toxicity was correlated to improvement in initial binding affinity. Mutations in α-helices 3, 4 and 5 (domain I) generally resulted in negative effects and helped in understanding their role in membrane insertion [2]. The present study points towards the role of α-helix 7 in membrane insertion by exerting a positive effect on insecticidal activity and ion channel conductance in BLMs.

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