Journal of Biochemistry and Molecular Biology, Vol. 34, No. 2, March 2001, pp. 150-155

JOURNAL OF
Biochemistry and Molecular Biology

Redesign of an Interhelical Loop of the *Bacillus thuringiensis* Cry4B delta-endotoxin for Proteolytic Cleavage

Chartchai Krittanai, Panida Lungchukiet, Sarinthip Ruangwetdee, Tipparut Tuntitippawan, Sakol Panyim, Gerd Katzenmeier and Chanan Angsuthanasombat*

Laboratory of Molecular Biophysics, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhonpathom 73170, Thailand

Received 10 October 2000, Accepted 13 January 2001

The mosquito-larvicidal Cry4B protein from *Bacillus thuringiensis* subsp. *israelensis* was expressed in *Escherichia coli*. Upon activation by trypsin, the 130-kDa protoxin was processed into the 65-kDa active toxin containing two polypeptide fragments of ca. 47 and ca. 20 kDa. These two polypeptides are products of internal cleavages on the exposed loop connecting helices 5 and 6 in the seven-helical bundle domain. PCR-based mutagenesis was employed to introduce an additional cleavage site into the loop connecting helices 3 and 4. A series of amino acid changes were introduced into the targeted loop, resulting in seven mutant protoxins. Upon digestion with trypsin, a group of mutants with arginine introduced into the loop (EPRNQ, EPNRNQ, EPRNP, ESRNP and SSRNP) produced polypeptide products similar to those of the wild type (EPNNQ). When the loop, SSRNP, was expanded by an insertion of either asparagine (NSSRNP) or valine (VSSRNP), an additional cleavage was detected with proteolytic products of 47, 12 and 6 kDa. This cleavage was confirmed to be at the introduced arginine residue by N-terminal sequencing. The mosquito larvicidal assay against *Aedes aegypti* demonstrated a relatively unchanged toxicity for the mutants without cleavage and reduced toxicity for those with an additional cleavage.

Keywords: *Bacillus thuringiensis*, δ-Endotoxin, Proteolytic cleavage, Interhelical loop, Protein engineering

Introduction

Cry4B is a δ-endotoxin produced by *Bacillus thuringiensis (Bt)* subsp. *israelensis* as a crystalline inclusion during sporulation (Hofte and Whiteley, 1989). This protein has been shown to be toxic to mosquito larvae, but safe to the environment (Feitelson, 1992). The crystal structures of Cry3A and Cry1Aa revealed that an active Cry toxin is composed of three distinct domains (Li *et al.*, 1991; Grochuski *et al.*, 1995; Crickmore *et al.*, 1998). The seven-helical bundle domain (I) on the N-terminal has been studied extensively with a possible function in membrane insertion and channel formation (Ahmed and Ellar, 1990; Von Tersch *et al.*, 1994; Chen *et al.*, 1995; Gazit and Shai, 1995; Uawithya *et al.*, 1998; Gazit *et al.*, 1998; Masson *et al.*, 1999; Sramala *et al.*, 2000). The variable domain (II) with three anti-parallel β-sheets has been suggested to play roles in receptor binding and specificity determination (Lee *et al.*, 1992; Wu and Dean, 1996). The domain III is a β-sandwich with a proposed function in maintaining toxin structure or receptor binding (Ge *et al.*, 1991; Chen *et al.*, 1993; Lee *et al.*, 1995).

It is generally accepted that the native *Bt* δ-endotoxins are initially synthesized as inactive protoxins. After ingestion by susceptible insect larvae and dissolution, the solubilized protoxin is converted into active toxins by trypsin-like proteases. The active toxins, which are relatively resistant to further proteolysis, then form ion channels (Slatin *et al.*, 1990; English *et al.*, 1991; Walters *et al.*, 1993; Gazit *et al.*, 1994). Shortly after ingestion, the midgut cells swell and lyse, resulting in extensive damage to the midgut and eventually larval death (Knowles, 1994).

The proteolytic activation of the protoxin seems to be an important step for toxin function. It acts like a switch in turning on the toxin activity. Studies have shown that the C-terminal half, and a few residues from the N-terminus of the 130-kDa protoxin, are removed during proteolytic activation *in vitro*, yielding the 65-kDa N-terminal half as the active domain (Chesterkhina *et al.*, 1985; Li *et al.*, 1991). Within the active molecule, there is an additional cleavage detected in the exposed interhelical loop between α3-α4 of Cry2A (Nicholls *et al.*, 1989) and Cry3A (Carroll *et al.*, 1989), and in the α5-α6 loop of Cry4A and Cry4B (Angsuthanasombat *et al.*,

*To whom correspondence should be addressed.
Tel: +66-2-441-9003; Fax: +66-2-441-9906
E-mail: stcas@mahidol.ac.th

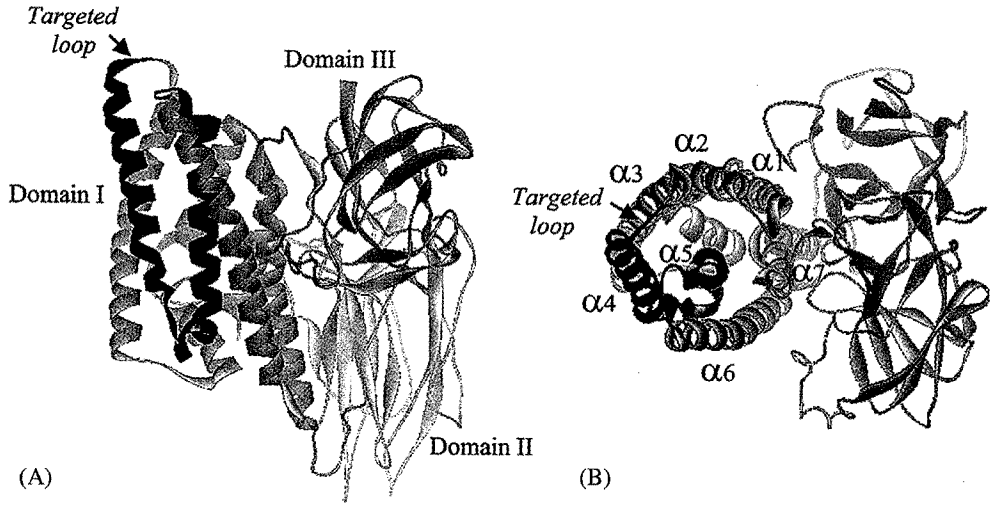

Fig. 1. Ribbon structure of the wild type Cry4B toxin generated from homology modeling. (A) Side view and (B) top view of the 65-kDa trypsin-activated toxin comprised of three distinct domains. Helices α4 and α5 of the domain I are colored in black. The targeted loop connecting helices α3 and α4 is indicated by an arrow.

Table 1. Modified amino acid sequences, and their oligonucleotide primers used for site-directed mutagenesis. (F) and (R) indicate forward and reversed primers, respectively. Codons directing for substitution or insertion of amino acids are shown in bold letters. The restriction sites are underlined.

EPNNQ → EPRNQ	
(F) 5′-GAGAGCCT**AGG**AACCAGTCCTATAGAACAGCTGTAATAACTC-3′	*Pvu*II and *Avr*II
(R) 5′-GAGTTATTACAGCTGTTCTATAGGACTGGTT**CCT**AGGCTCTC-3′	
EPRNQ → EPRNP	
(F) 5′-GAGAGCCTAGGAAC**CCG**TCCTATAGAACAGCTGTAATAACTC-3′	-
(R) 5′-GAGTTATTACAGCTGTTCTATAGGA**CGG**GTTCCTAGGCTCTC-3′	
EPRNP → ESRNP	
(F) 5′-GGAAAAGAGAG**TCT**AGGAACCCGTCCTATAGAACAGCAGTAATAAC-3′	*Hin*fI
(R) 5′-GTTATTACTGCTGTTCTATAGGACGGGTTCCT**AGA**CTCTCTTTTCC-3′	
EPRNP → SSRNP	
(F) 5′-GGAAAAGA**TCTTCT**AGGAACCCGTCCTATAGAACAGCAGTAATAAC-3′	*Bgl*II
(R) 5′-GTTATTACTGCTGTTCTATAGGACGGGTTCCT**AGAAGA**TCTTTTCC-3′	
EPNNQ → EPNRNQ	
(F) 5′-GAGAGCCTAAT**AGG**AACCAGTCCTATAGAACAGCTGTAATAACTC-3′	*Pvu*II *and Nla*I
(R) 5′-GAGTTATTACAGCTGTTCTATAGGACTGGTT**CCT**ATTAGGCTCTC-3′	
SSRNP → NSSRNP	
(F) 5′-CACTTGGAAAAGA**AAT**TCTTCTAGGAACCCGTCCTATAGAACAGCAGTAATAAC-3′	*Bgl*II and *Xba*I
(R) 5′-GTTATTACTGCTGTTCTATAGGACGGGTTCCTAGAAGA**ATT**TCTTTTCCAAGTG-3′	
SSRNP → VSSRNP	
(F) 5′-CACTTGGAAAAGA**GTA**TCTTCCAGGAACCC-3′	*Bst*NI
(R) 5′-GGGTTCCTGGAAGA**TAC**TCTTTTCCAAGTG-3′	

1992). We previously demonstrated that elimination of the cleavage site within the α5-α6 loop affects the larvicidal activity of the Cry4B toxin (Angsuthanasombat *et al.*, 1993).

Current evidence suggest that the helical hairpin, containing α4 and α5, is the most likely machinery for membrane insertion and channel formation (Duche *et al.*, 1994; Gazit and Shai, 1995; Schwartz *et al.*, 1997; Gazit *et al.*, 1998; Uawithya *et al.*, 1998; Masson *et al.*, 1999; Sramala *et al.*, 2000). Thus, proteolytic cleavages on the interhelical loops of α3-α4 and α5-α6 may enhance a conformational flexibility for the α4/α5 hairpin to penetrate into the membrane and form ion channels.

To investigate the possibility of introducing an additional proteolytic cleavage site in the loops, we employed site-directed mutagenesis to modify the loop sequence located between helices 3 and 4 of Cry4B into a series of arginine-containing sequences (Fig. 1). The results reveal the significance of the loop length by demonstrating that the arginine-containing sequences on the targeted loop cannot be cleaved by trypsin, unless the loop length is expanded. In addition, the nature of the sequence is also important for the tryptic cleavage, since not all the mutants with an expanded loop were susceptible to the cleavage. The effects of this additional cleavage on the mosquito-larvicidal activity against *Aedes aegypti* were also determined and discussed.

Materials and Methods

Construction and expression of mutant toxins Mutant plasmids were constructed by the PCR-based method using the Stratagene's QuickChange mutagenesis kit. Synthetic oligonucleotides (Table 1) that direct each mutation were purchased from Bio-Synthesis (USA) and GenSet (Singapore). The pMU388 plasmid containing the *cry4B* gene (Angsuthanasombat *et al.*, 1987) was used as a template. PCR was performed using high fidelity *Pfu* DNA polymerase for a primer extension (Lundberg *et al.*, 1991). The products were treated with *Dpn* I endonuclease at 37°C for 1 hour before transformation into *E. coli* JM109. The transformants were screened by plasmid extraction together with a number of restriction enzymes analysis. DNA sequences were confirmed by an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, USA). Proteins were expressed in an incubation with IPTG (0.1 mM) at 37°C for 4 hours. Cytoplasmic inclusions were collected from cell suspension lysed with a French Press Cell, and washed several times in distilled water or a tris-HCl buffer, pH 7.5.

Proteolytic processing of toxin Protoxin inclusions were solubilized in 50 mM Na_2CO_3, pH 9.0 at 37°C for 1 hour. Protein concentrations were determined by a dye-binding method (Bradford, 1976) using bovine serum albumin as a standard. Proteolytic processing was performed by incubating the 130-kDa protoxin with TPCK-treated trypsin (1 : 20 w/w of enzyme:toxin) at 37°C for 16 hours. The polypeptide products were analyzed by SDS-PAGE and blotted onto a Problott™ PVDF membrane using a semi-dry blot apparatus (Bio-Rad). Their N-terminal sequences were determined on an ABI 492 (Applied Biosystems) automated sequencer. Molecular mass of the polypeptide fragment was determined on an ABI 365 Electrospray Ionization Liquid Chromatography Mass Spectrometer (ESI-LC/MS) equipped with a C18 reverse-phase column.

Mosquito-larvicidal assay 2-day old larvae of *Aedes aegypti* were incubated with *E. coli* JM 109 expressing for wild type or mutant toxins. The percentage of mortality was determined after a 24-hour incubation using a total of 100 larvae for each *E. coli* sample. Data were collected from 4 independent experiments for each toxin. The cell concentrations used were 10^8 cells/ml (approximately 2 μg of expressed toxin/ml). *E. coli* cells carrying plasmid with (pMU388) and without the *cry4B* gene (pUC12) were used as positive and negative references in all assays.

Results

Construction and expression of mutant toxins A numbers of mutant Cry4B toxins have been constructed previously in our laboratory to investigate the structural and functional relationships of the toxin, especially with mutations in the helices of domain I. In this work, the PCR-based site directed mutagenesis of our template plasmid containing the *cry4B* gene produced an approximated 6.3-kb PCR product. A screening of plasmids from *E. coli* transformants by restriction enzymes yielded a number of *E. coli* clones with the expected digestion patterns. Automated DNA sequencing data confirmed that the nucleotide sequences of plasmids correspond to the expected mutation of amino acids on the targeted loop. The wild type and mutant protoxins were expressed in the form of cytoplasmic inclusions after 4 hours of 0.1 mM IPTG induction. The protoxin appeared as an intense band at 130 kDa on SDS-PAGE. The expression levels of the five mutant protoxins, T1 (EPRNQ), T2 (EPRNP), T3 (ESRNP), T4 (SSRNP) and T5 (EPNRNQ) were found to be similar to that of the wild type (EPNNQ). The other two mutants, T6 (NSSRNP) and T7 (VSSRNP), were found expressed at a lower level. Solubilities of mutant and wild type protoxins in 50 mM Na_2CO_3, pH 9.0 were found to be the same. An immunoblotting experiment confirmed that these expressed products cross-reacted with Cry4B antisera (data not shown).

Proteolytic processing of toxins After a 16-hour incubation of protoxins with trypsin, the T1, T2, T3, T4 and T5 mutants produced a digestion pattern identical to that of the wild type on SDS-PAGE. The digestion products were identified as ca.

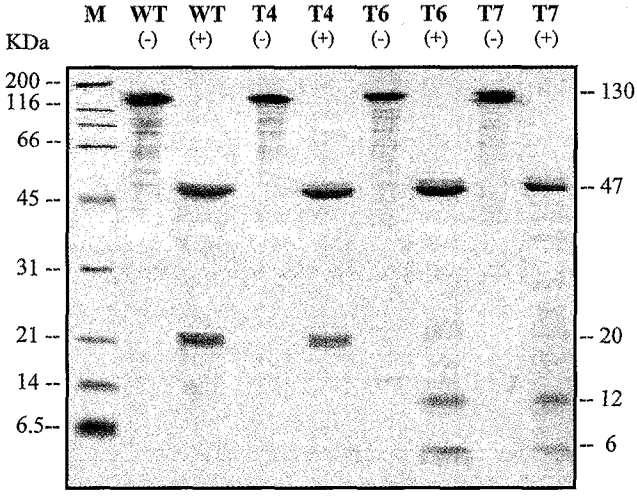

Fig. 2. A Coomassie Brilliant Blue-stained SDS-PAGE analysis of the 130-kDa protoxins with (+) and without (-) trypsin treatment. Lane M, standard protein marker; lane WT, the wild type Cry4B; lane T4, T4 (SSRNP) mutant; lane T6, T6 (NSSRNP) mutant; lane T7, T7 (VSSRNP) mutant.

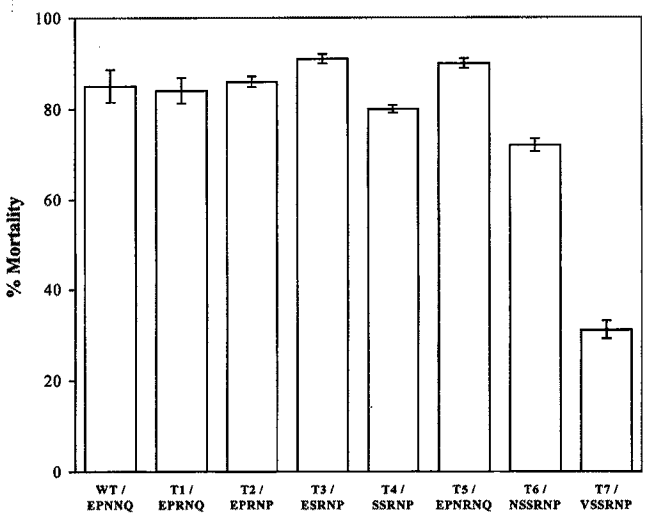

Fig. 3. Larvicidal activity of *E. coli* cells expressing wild type and mutant protoxins against 2-day-old *Aedes aegypti* larvae. Data were recorded after 24 hours of incubation. Error bars represent standard error of the mean (SEM) from four independent experiments.

47- and ca. 20-kDa polypeptide fragments. However, the T6 and T7 mutants produced a distinct digestion pattern containing polypeptide products around 47, 12 and 6 kDa. A comparison of digestion patterns between the wild type protoxin, the mutant T4 (SSRNP), and the mutants containing additional cleavage site, T6 (NSSRNP) and T7 (VSSRNP), is illustrated in Fig. 2. The N-terminal sequencing of the 12- and 6-kDa fragments reveal the amino acid sequences NPSYRTAVI and NTNYKD, respectively. These amino acid sequences suggest that the two polypeptide fragments were generated by trypsin cleavage in the targeted loop.

Mosquito-larvicidal activity of mutant toxins The activity of the toxins *in vivo* against 2-day old *Aedes aegypti* larvae, using *E. coli* cell expressing the wild type and mutants toxins, was recorded after 24 hours of incubation. Data from 4 independent experiments for each mutant are shown in Fig. 3. The results showed a comparable activity among the T1, T2, T3, T4, and T5 mutants ranging from 80.0 to 91.0% mortality. However, the mortality rates in the T6 and T7 mutants decreased, ranging from 31.0 to 72.0%. The control experiment using *E. coli* cells expressing the wild type toxin consistently demonstrated 80-90% mortality in all experiments.

Discussion

An alignment of amino acid sequences from several Cry toxins, and the construction of a homology model based on a crystal structure of Cry3A, allowed an accurate identification of structural elements and loops in the structure of Cry4B, especially within the conserved region of the helical bundle domain. According to the model, the relatively hydrophobic helix 5 is located in the center of the bundle, surrounded by six other amphipathic helices (Fig. 1). The interhelical loop between helices 5 and 6 (containing arginine-203, which is exposed to the solvent) is cleaved by a typsin-like protease during the activation of protoxins (Li *et al.*, 1991; Angsuthanasombat *et al.*, 1993). The loop of sequence E_{135}PNNQ, joining helices 3 and 4, is pointing above the helical bundle. This loop sequence contains no lysine or arginine residues as a cleavage site for typsin. Substitution of asparagine-137 in the wild type sequence (EPN$_{137}$NQ) with arginine yielded the T1 (EPRNQ) mutant. Cleavage within the mutated loop resulted from treatment of this mutant with trypsin. We then constructed a series of mutants by modifying the loop sequence to make it similar to the loop sequence (NPVSSRNP) joining helices 3 and 4 of Cry3A (Li *et al.*, 1991). This loop sequence in Cry3A is susceptible to the tryptic cleavage during the proteolytic activation *in vitro* (Carroll *et al.*, 1989).

Upon IPTG induction, all mutants, except T6 and T7, expressed the 130-kDa protoxin at a level similar to that of the wild type. All of mutant and wild type protoxins can be solubilized in a carbonate buffer pH 9.0, suggesting a native-like folding for these mutant proteins. Trypsin digestion of the Cry4B protoxin produced two stable products of 20 and 47 kDa, corresponding to the helices α1-α5 fragment, as well as the rest of the active molecule, respectively. From the T1, T2, T3 and T4 mutants with amino acid sequence changes from wild type EPNNQ to EPRNQ, EPRNP, ESRNP and SSRNP (all contain an amino acid arginine as the cleavage site) it is apparent that the targeted loop cannot be cleaved by trypsin. For the three other mutants, T5 (EPNRNQ), T6 (NSSRNP) and T7 (VSSRNP) with one additional amino acid inserted into the loop, we detected the additional cleavage only in T6 and T7. The trypsin digestion pattern for these two mutants contains the polypeptide bands located around 47, 12 and 6 kDa. According to an amino acid sequence of the smallest fragment, its calculated mass, based on this cleavage site, is 7.8 kDa. To clarify this discrepancy, the 6-kDa fragment was characterized further by a mass spectrometer. The accurate mass obtained for this fragment is 5,914.72 Da. The N-terminal sequencing of this polypeptide suggested that a 1.8-kDa difference is due to a truncation at some other cleavage sites on the c-terminus. This suggestion was supported by the fact that the molecular mass calculated from its N-terminus to arginine-190 is 5,914 Da.

Results from this work demonstrate that the number of amino acids on the engineered loop is important for the cleavage. Since all of the four mutants with the loop containing five amino acids (T1, T2, T3 and T4) were not susceptible to the additional cleavage, this targeted loop would need at least six amino acids on the loop structure to get an arginine residue properly exposed for the cleavage. However, the nature of the amino acid sequence residing on the loop also has a significant role for cleavage, since the digestion occurred on NSSRNP and VSSRNP, but not the EPNRNQ

sequence.

In larvicidal activity assays the T1, T2, T3, T4 and T5 mutants, which all give the same trypsin-activated products of 47 and 18 kDa like the wild type protoxin, exhibited relatively the same toxicity as the wild type against *Aedes aegypti* larvae. The T6 mutant showed a slight decrease in larvicidal activity, but T7 exhibited a dramatic reduction in toxicity. Maybe the reduction in toxicity is due to the possibility that the activated toxins generated with the additional cleavage site are unstable and degraded more rapidly in the larval gut environment. However, at this stage there is no clear explanation for the dissimilar activity between the T6 and T7 mutants.

In summary, we succeeded in generating an additional proteolytic cleavage site into a pore-forming domain of wild type toxin. The results reveal that the engineered loop sequence between helices 3 and 4 needs to be expanded to have at least six amino acid residues in length to expose the susceptible cleavage site. Moreover, the nature of the amino acid sequence of the targeted loop is critical for the cleavage, based on the required sequence of (N/V) SSRNP.

Acknowledgments We would like to thank C. Boonchuay and S. Leetachewa for their technical assistance in DNA and amino acid sequencing. This work is supported by the National Science and Technology Development Agency (NSTDA), and the Thailand Research Fund (TRF).

References

Ahmed, W. and Ellar, D. J. (1990) Directed mutagenesis of selected regions of a *Bacillus thuringiensis* entomocidal protein. *FEMS Microbiol. Lett.* **56**, 97-104.

Angsuthanasombat, C., Chungjatupornchai, W., Kertbundit, S., Luxananil, P., Settasatian, C., Wilairat, P. and Panyim, S. (1987) Cloning and expression of 130-kd mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var *israelensis* in *Escherichia coli*. *Mol. Gen. Genet.* **208**, 384-389.

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1992) Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. *FEMS Microbiol. Lett.* **94**, 63-68.

Angsuthanasombat, C., Crickmore, N. and Ellar, D. J. (1993) Effects on toxicity of eliminating a cleavage site in a predicted interhelical loop in the *Bacillus thuringiensis* CryIVB δ-endotoxins. *FEMS Microbiol. Lett.* **111**, 255-262.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation for microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Caroll, J., Li, J. and Ellar, D. J. (1989) Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* **261**, 99-105.

Chen, X. J., Lee, M. K. and Dean, D. H. (1993) Site-directed mutagenesis in a highly conserved region of *Bacillus thuringiensis* delta-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. *Proc. Natl. Acad. Sci. USA* **90**, 9041-9045.

Chen, X. J., Curtiss, A., Alcantara, E. and Dean, D. H. (1995) Mutation in domain I of *Bacillus thuringiensis* delta-endotoxin CryIAb reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. *J. Biol. Chem.* **270**, 6412-6419.

Chesterkhina, G. G., Zalunin, I. A., Bormatova, M. E., Klepikova, F. S, Khodova, O. M. and Stepnov, V. M. (1985) Structural feature of crystal forming proteins produced by *Bacillus thuringiensis* subsp. *isaelensis*. *FEBS Lett.* **190**, 345-348.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum. J. and Dean, D. H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 807-813.

Duche, D., Parker, M. W., Gonzales-Mana, J. M., Pattus, F. and Baty, D. (1994) Uncouple steps of the colicin A pore formation demonstrated by disulfide bond engineering. *J. Biol. Chem.* **269**, 6332-6339.

English, L. H., Readdy, T. L. and Bastian, A. E. (1991) Delta-endotoxin-induced leakage of $^{86}Rb^+/K^+$ and H_2O from phospholipid vesicles is catalyzed by reconstitution midgut membrane. *Insect Biochem.* **21**, 177-184.

Feitelson, J. S., Payne, J. and Kim, L. (1992) *Bacillus thuringiensis*: insect and beyond. *Biol. Technol.* **10**, 271-275.

Gazit, E., Bach, D., Kerr, I. D., Sansom, M. S., Chejanovsky, N. and Shai, Y. (1994) The alpha-5 segment of *Bacillus thuringiensis* delta-endotoxin: *in vitro* activity, ion channel formation and molecular modeling. *Biochem. J.* **304**, 895-902.

Gazit, E., Rocca, P. L., Sansom, M. S. P. and Shai, Y. (1998) The structure and organization within the membrane of the helices composing the pore-forming domain of *Baillus thuringiensis* delta-endotoxin are consistent with an umbrella-like structure of the pore. *Proc. Natl. Acad. Sci. USA* **95**, 12289-12294.

Gazit, E. and Shai, Y. (1995) The assembly and organization of the alpha 5 and alpha 7 helices from the pore-forming domain of *Bacillus thuringiensis* delta-endotoxin: Relevance to a functional model. *J. Biol. Chem.* **270**, 2571-2578.

Ge, A. Z., Rivers, D., Milne, R. and Dean, D. H. (1991) Functional domain of *Bacillus thuringiensis* insecticidal crystal proteins. *J. Biol. Chem.* **266**, 17954-17958.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R. and Cygler, M. (1995) *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol.* **254**, 447-464.

Hofte, H. and Whiteley, H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242-255.

Knowles, B. H. (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. *Adv. Insect Physiol.* **24**, 276-307.

Lee, M. K., Milne, R. E., Ge, A. Z. and Dean, D. H. (1992) Location of a *Bombyx mori* receptor binding region on a *Bacillus thuringiensis* delta-endotoxin. *J. Biol. Chem.* **267**, 3115-3121.

Lee, M. K., Young, B. A. and Dean, D. H. (1995) Domain III exchanges *of Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. *Biochem. Biophys. Res. Commun.* **216**, 306-312.

Li, J., Carroll, J. and Ellar, D. J. (1991) Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature* **353**, 815-821.

Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J.

M., Sorge, J. A. and Mathur, E. J. (1991) High fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **31**, 53-60.

Masson, L., Tabashnik, B. E., Liu, Y. B., Brousseau, R. and Schwartz, J. L. (1999) Helix 4 of the *Bacillus thuringiensis* CryIAa toxin lines the lumen of the ion channel. *J. Biol. Chem.* **274**, 31996-32000.

Nicholls, C. N., Ahmad, W. and Ellar, D. J. (1989) Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. *J. Bacteriol.* **171**, 5141-5147.

Schwartz, J. L., Juteau, M., Grochulski, P., Cygler, M., Prefontaine, G., Brousseau, R. and Masson, L. (1997) Restriction of intramolecular movements within the CryIAa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. *FEBS Lett.* **410**, 397-402.

Slatin, S. L., Abrams, C. K. and English, L. H. (1990) Delta-endotoxins form cation-selective channels in planar lipid bilayers. *Biochem. Biophys. Res. Commun.* **169**, 765-772.

Sramala, I., Uawithya, P., Chanama, U., Leetachewa, S., Krittanai, C., Katzenmeier, G., Panyim, S. and Angsuthanasombat, C. (2000) Single proline substitutions of selected helices of the *Bacillus thuringiensis* Cry4B toxin affect inclusion solubility and larvicidal activity. *J. Biochem. Mol. Biol. Biophys.* **4**, 187-193.

Uawithya, P., Tuntitippawan, T., Katzenmeier, G., Panyim, S. and Angsuthanasombat, C. (1998) Effects on larvicidal activity of single proline substitutions in α3 and α4 of the *Bacillus thuringiensis* Cry4B toxin. *Biochem. Mol. Biol. Int.* **44**, 825-832.

Von Tersch, M. A., Slatin, S. L., Kulesza, C. A. and English, L. H. (1994) Membrane-permeabilizing activities of *Bacillus thuringiensis* coleopteran-active toxin CryIIIB2 and CryIIIB2 domain I peptide. *Appl. Environ. Microbiol.* **60**, 3711-3717.

Walters, F. S., Slatin, S. L., Kulesza, C. A. and English, L. H. (1993) Ion channel activity of the N-terminal fragments from CryIA(c) delta-endotoxin. *Biochem. Biophys. Res. Commun.* **196**, 921-926.

Wu, S. J. and Dean, D. H. (1996) Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA delta-endotoxin. *J. Mol. Biol.* **255**, 628-640.