รายงานฉบับสมบูรณ์

โครงการ การศึกษาโครงสร้างโปรตีนสารพิษชนิด Cry4A จากแบคทีเรีย *Bacillus thuringiensis* subsp. *israelensis*

Crystallographic structural investigation of the Cry4A mosquitocidal δ-endotoxin of *Bacillus thuringiensis* subsp *israelensis*

โดย

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มิถุนายน 2547

สัญญาเลขที่ TRG4580107

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ชื่อโครงการ การศึกษาโครงสร้างโปรตีนสารพิษชนิด Cry4A จากแบคทีเรีย *Bacillus thuringiensis* subsp. *israelensis*

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ระยะเวลาโครงการ 2 ปี

โปรตีนสารพิษ Cry4Aa ซึ่งผลิตจากแบคทีเรีย *Bacillus thuringiensis* subsp. *israelesis* เป็นโปรตีนที่ออกฤทธิ์ฆ่าลูกน้ำยุง โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษากลไกการออกฤทธิ์ฆ่าลูกน้ำยุงโดยการศึกษาโครงสร้างสามมิติของผลึกโปรตีนจากโปรตีนกลายพันธุ์ ของ Cry4Aa (R235Q) ผลึกโปรตีน Cry4Aa ถูกจำแนกอยู่ใน space group $C222_1$ ซึ่งมี cell parameters a=91.2, b=202.1, c=98.7Å และประกอบด้วย 1 โมเลกุลต่อ asymmetric unit ผลึกโปรตีนสามารถกระเจิงรังสีเอกซ์ได้ในระดับ 2.9 Å โดยการใช้ synchrotron radiation โครงสร้างสามมิติของโปรตีน Cry4Aa ได้ผ่านการศึกษาโดยวิธี molecular replacement โดยการใช้โครงสร้างสามมิติของโปรตีน Cry4Ba เป็นต้นแบบ และแสดงค่า R factor และ R free ในระดับ 20.9% และ 26.3% ตามลำดับ ในขั้นตอน Refinement โครงสร้างสามมิติของโปรตีน Cry4Aa ประกอบด้วย 3 domains โดย Domain I ประกอบด้วย โครงสร้างของ helical bundle Domain II ประกอบด้วยโครงสร้างของ β-sheet prism และ Domain III ประกอบด้วย โครงสร้างของ β-sheet sandwich ซึ่งโครงสร้างดังกล่าวนี้สามารถนำมาใช้อธิบายกลไกความจำเพาะเจาะจงและการทำลายเยื่อหุ้มเซลล์ของโปรตีนฆ่าลูกน้ำยุงได้ในอนาคต

Abstract

Project Code: TRG4580107

Project Title: Crystallographic structural investigation of the Cry4Aa mosquitocidal δ-endotoxin of *Bacillus thuringiensis* subsp. *israelensis*

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Project Period: 2 years

The Cry4Aa toxin, isolated from *Bacillus thuringiensis* subsp. *israelensis*, is specifically toxic to mosquito larvae. For a better understanding of the mechanism of toxicity, the 65-kDa functional form of the mosquito-larvicidal Cry4Aa-R235Q mutant toxin has been crystallised. The crystals belong to space group $C222_1$ with unit cell parameters a=91.2, b=202.1, c=98.7 Å and contain one molecule per asymmetric unit. The crystals diffract to about 2.9 Å using synchrotron radiation and a complete native data set has been collected. The structure has been solved using a molecular replacement method with the Cry4Ba toxin protein as a search model, and refined to an R-factor of 20.9 % and R_{free} of 26.3 %. The structure of the active Cry4Aa toxin consists of three domains: domain I, a α helical bundle; domain II, a β-sheet prism; and domain III, a β-sheet sandwich. The Cry4Aa structure therefore provides an invaluable database to investigate the structural basis of target specificity and membrane interaction of the toxins in this family.

Keywords: mosquito-larvicidal toxin, bio-insecticides, crystallization

1. Introduction

The naturally occurring crystal proteins of *Bacillus thuringiensis* (B.t.), known as endotoxins (Cry and Cyt toxins), are bioinsecticides considered safe for human and the environment. These proteins have been established as an alternative to chemical insecticides for the control of insect pests in agriculture and disease vectors of some serious tropical diseases such as malaria, dengue fever, and encephalitis. Bt Cry proteins are toxic against insect larvae of the orders Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Schnepf et al., 1998; de Maagd et al., 2001). The Bt crystal proteins are produced as inactive protoxin inclusions during sporulation which are subsequently dissolved upon ingestion under the alkaline conditions in the larval midgut lumen. The soluble protoxins are further activated by midgut proteases which remove C-terminal half of the toxins in addition to approximately 30 residues from the N-terminus to yield the active protease-resistant fragments. The active proteins then specifically bind to receptors on the brush-border membrane of midgut epithelium. So far, two families of putative protein receptors, a GPI-anchored aminopeptidase N (APN) (Knight et al., 1995; Masson et al., 1995; Sangadala et al., 1994) and a cadherin-like membrane protein (Vadlamudi et al., 1995) in lepidopteran insects have been characterized as receptor proteins of Cry1A toxins. Upon receptor binding, a subsequent conformational change allows the activated toxins to insert into the cell membrane to form pores that finally causes ion leakage, cell lysis, and eventual insect death (Schnepf et al., 1998).

X-ray crystal structures of Cry toxins representing different insect specificities have been elucidated, i.e. Cry3Aa (Li et al., 1991b), Cry3Bb (Galitsky et al., 2001), Cry1Aa (Grochulski et al., 1995), Cry1Ac (Li et al., 2001), and Cry2Aa (Morse et al., 2001). More recently, the X-ray crystallographic studies of Cry4Ba have been reported (Boonserm et al., 2003). All these Cry

toxins share the similar overall tertiary structure of the three domain organization. The N-terminal domain I, an amphipathic helical bundle, has been shown to be responsible for membrane insertion and pore formation (Walters et al., 1993; Von Tersch et al., 1994; Gazit et al., 1998; Puntheeranurak et al., 2004). Domain II, a three anti-parallel β-sheet domain containging apical hypervariable loops, has been demonstrated to participate in receptor binding and hence is involved in insect specificity (Smedley and Ellar, 1996; Rajamohan et al., 1996; Ballester, V et al., 1999; Jurat-Fuentes and Adang, 2001). The C-terminal domain III, a sandwich of two anti-parallel β-sheets, has been implicated to involve in structural integrity of toxin molecules, membrane permeabilisation (Masson et al., 2002) or receptor recognition (Lee et al., 1995; Burton et al., 1999). The detailed mechanism involving a series of events from receptor binding to membrane insertion remains unanswered.

Bacillus thuringiensis subsp. *israelensis* (Bti) has been effectively used as a biopesticide for mosquito and blackfly larvae control. The Bti mosquito-larvicidal activity is due to the production of four major crystal proteins composed of Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa (Ward et al., 1984). Synergism of these Bti toxins has been demonstrated based on the analysis of combination of cloned gene products (Angsuthanasombat et al., 1992). By comparison of the primary structures among Bti toxins, Cry4Aa and Cry4Ba toxins are most closely related which share about 55% amino acid sequence similarity in the N-terminal active protein portion (Ward and Ellar, 1988; Chungjatupornchai et al., 1988). Despite their relatively high sequence similarity, both toxins show variable levels of toxicity against mosquito species. The Cry4Ba exhibits highly toxic activity towards *Aedes* and *Anopheles* larvae (vectors of dengue fever, and malaria, respectively) but has no significant activity against *Culex* larvae (vectors of Nest Wile virus), while the Cry4Aa toxin shows high toxicity against all three mosquito larvae

(Angsuthanasombat et al., 1992). Interestingly, there is evidence of synergism *in vivo* between these two toxins against *Culex*, *Aedes*, and *Anopheles* larvae (Angsuthanasombat et al., 1992), however, the precise mechanism of synergistic interactions is still unknown. Recently, the X-ray crystal structure of the Cry4Ba Bti toxin has been revealed (Boonserm et al., in press). Hence, structural data of both Cry4Aa and Cry4Ba toxins will serve as critical information for addressing the larvicidal and synergistic mechanisms. For the rational design of Cry toxins in search of broadened insecticidal spectra, increased potency or prolonged toxin stability, structural-based protein engineering would be the highly effective approach for the Bt-based insect control.

Recently, we have reported protein crystallization and X-ray diffraction studies of the 65-kDa-active fragment of the Cry4Aa mutant (R235Q) (Boonserm et al., 2004). Here we determined the crystal structure of the active Cry4Aa-R235Q toxin from Bti which has been solved by molecular replacement using the homologous Cry4Ba protein as a model, and refined to 2.9 Å resolution.

2. Materials and Methods

2.1 Protein expression and solubilisation

E. coli JM109 cells harbouring a single amino-acid Cry4Aa mutant (see section 3.1 below) were grown at 30°C in a Luria-Bertani medium containing 100 μg ml^{-1} ampicillin until OD_{600} of the culture reached ~0.3-0.5. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 10 hrs and subsequently analysed by sodium dodecyl sulfate-(10% w/v) polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* cultures over-expressing the Cry4Aa single amino-acid mutant toxin as

cytoplasmic inclusions were harvested by centrifugation and resuspended in cold distilled water. Cell suspension was then disrupted by using a French Pressure Cell at 10,000 psi. After centrifugation at 8,000×*g* at 4°C for 15 min, the pellets were washed three times in cold distilled water and resuspended by sonication. Protein concentrations of the partially purified inclusions were determined using a protein micro-assay reagent (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard. Inclusions at a concentration of about 2 mg ml^{-1} were solubilised by incubation at 37°C for 1 hr in 50 mM Na_2CO_3 pH 10.0. Solubilised protoxins were then separated from insoluble materials by centrifugation at 12,000×g for 15 minutes.

2.2 Proteolytic activation and active toxin purification

The 130-kDa solubilised Cry4Aa protoxins were mixed with trypsin (*L*-1-tosylamide-2-phenylethyl chloromethyl ketone treated, Sigma) at an enzyme:protoxin ratio of 1:10 (w/w) and incubated at 37°C for 16 hrs. Proteolysis was stopped by adding 1 mM tosyl-lysine chloromethyl ketone (TLCK). After analysis by SDS-PAGE, the trypsin-activated fraction was concentrated at 4°C using a Centriprep ultrafiltration device with a 30-kDa molecular-weight cutoff (Amicon). The protein was further purified by size-exclusion chromatography on a FPLC system (Superdex^{TM} 200, Amersham Pharmacia Biotech) in 50 mM Na_2CO_3, pH 10.0 at a flow rate of 0.4 ml min^{-1}. In these conditions, the 65-kDa Cry4Aa toxin elutes as a monomer as shown by using BSA (67-kDa) as a marker. Eluted fractions containing the proteins were pooled and concentrated to 3-5 mg ml^{-1} by ultrafiltration as described above.

2.3 Crystallisation and data collection

Crystals were grown by using the hanging-drop vapour diffusion technique. 5µl of precipitant solution were mixed with an equal volume of the purified Cry4Aa at a concentration of 3 to 5 mg ml^{-1}. The drop was equilibrated against a reservoir containing 1 ml of the

precipitant solution at 23°C. The purified protein in 50 mM Na_2CO_3 (pH 10.0) was first mixed with solutions of 0.1 M Tris-Acetate at pH between 7 and 9. Several precipitants including salts, various MW of polyethylene glycol (PEG), glycerol, and 2-methyl-2,4-pentanediol (MPD) were tested. However, crystals only appeared in the conditions containing salts. Clusters of needle-liked crystals were found in 0.1 M Tris-Acetate pH 7.0 and 0.3-0.4 M Li_2SO_4. Microcrystals appeared after two weeks in 0.1 M Tris-Acetate pH 7.0, 0.2-0.3 M LiCl. However, in both cases crystals were too small and too poorly ordered for X-ray diffraction studies. Clusters of thin, plate-shaped crystals were successively obtained with a protein concentration greater than 5 mg ml^{-1} in the precipitant solution containing 0.1 M Tris-Acetate pH 7.0, 0.2-0.3 M KH_2PO_4. This condition was optimised by lowering the protein concentration to 3-5 mg ml^{-1}.

For data collection, crystals were briefly soaked in a cryoprotecting solution containing 20% 2-methyl-2,4-pentanediol (MPD), 10% polyethylene glycol (PEG) 400 and 0.1 M Tris-Acetate, 0.3 M KH_2PO_4 at pH 7.0, before being mounted in a cryoloop and cooled to 100 K in a nitrogen gas stream (Oxford cryosystem). Measurements were made at an X-ray wavelength of 0.976 Å at the ESRF beamline ID29 with an attenuated beam of dimensions 0.1 x 0.1mm^2. Diffraction intensities were recorded on an ADSC Quantum IV CCD detector. The crystal-to-detector distance was set to 250mm and the oscillation angle for each of the 180 images recorded was 1°. Integration, scaling and merging of the intensities were carried out using programs *MOSFLM* (Leslie, 1992) and *Scala* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994). Molecular replacement was carried out using *Amore* (Navaza, 1994).

2.4 Model Building and Refinement

The model building was done using the program *O* (Jones *et al.*, 1991) interspersed with cycles of electron density map improvement. Refinement was done using programs *arp-warp*,

CNS and *REFMAC* from the *CCP4* program suite (1994). *DALI* (Holm and Sander, 1993) was used to a three-dimensional structural similarity search and *MAPS* (see http://bioinfo1.mbfys.lu.se/TOP/webmaps.html) was used to perform the protein structural alignment.

3. Results and Discussion

3.1 Protein activation and purification

It was reported that the Cry4Aa protoxin is processed into two protease-resistant fragments of 20 and 45 kDa through the intramolecular cleavage of a 65-kDa intermediate and these two fragments need to associate to exert toxicity (Yamagiwa *et al.*, 1999). The cleavage site is after Arginine 235 (Angsuthanasombat *et al.*, 1993). Recently, we investigated the effect of the intramolecular cleavage on the toxicity of Cry4Aa toxin by constructing a Cry4Aa single amino-acid mutant (R235Q) which is devoid of this internal cleavage site and is thus resistant to proteolysis (Boonserm *et al.*, 2004). The R235Q single mutant still retains high toxicity against *Aedes aegypti* larvae at a level comparable to the wild type (Boonserm *et al.*, 2004), indicating that the mutation at this trypsin-cleavage site had no adverse effect on the Cry4Aa toxicity. The active R235Q mutant of Cry4Aa was used for the present crystallographic study. After trypsin treatment of the R235Q active mutant, a major proteolytic fragment of 65 kDa was obtained which was resistant to further proteolysis. N-terminal amino-acid sequencing after trypsin activation indicated the first residue to be Gln-5 which is putatively located before helix 1 in Domain I based on a sequence alignment with Cry3Aa. The trypsin-activated 65-kDa fragment was purified by gel filtration for subsequent crystallisation trials.

3.2 Crystallisation and data collection

The 68-kDa chymotrypsin-activated Cry4Ba toxin has been previously crystallised, and well-ordered crystals diffracting X-rays to 1.75 Å resolution were obtained (Boonserm *et al.*, 2003). By analogy with this study, a similar approach was used to promote crystal growth of Cry4Aa toxin. Salts were effective precipitants and plate-shaped crystals of Cry4Aa with approximate dimensions 0.15 x 0.15 x 0.005 mm (Fig. 1) were obtained using 0.1 M Tris-Acetate pH 7.0, 0.2-0.3 M KH_2PO_4 as a precipitant.

One such crystal was used to obtain a 100 % complete native data set to 2.95 Å resolution. Data collection statistics are summarized in Table 1. Assuming one Cry4Aa molecule per asymmetric unit, the V_M (Matthews, 1968) is 3.60 A^3/Dalton, giving a solvent content of 65%.

3.3 Solution of the structure

A preliminary model of the structure was found by molecular replacement using the program *AMoRe* (Navaza, 1994). The rotation function calculation was performed between the resolution limits of 20.0 Å and 4.0 Å using the Cry4Ba refined crystal structure as the search model (Li, personal communication), and a Patterson integration radius of 30 Å. This returned a weakly contrasted solution with a correlation coefficient for the structure factor amplitudes of 0.160 compared to 0.152 for the second highest solution (0.27 for the intensities compared to 0.24 for the second highest solution). This solution consistently appears as the first when varying the Patterson integration radius between 30 and 35 Å. The search model was then placed in the unit-cell using the Crowther-Blow translation function using data between 9.0 Å and 4.0 Å resolution. This returned a solution with a correlation coefficient of 0.157 and an R_{factor} of 49.8% compared to 0.117 and an R_{factor} of 51.0% for the second highest peak. As a control, a systematic search using the Crowther Blow translation function was carried out for the first 50 independent

peaks of the rotation function both in space group $C222$ and $C222_1$. This search unambiguously returned the same solution for space group $C222_1$ with the same relatively weak but significant contrast stated above. This is consistent with the rather low sequence identity between the search model Cry4Ba and Cry4Aa (35.6% for 516 aligned positions).

Rigid body refinement yielded a correlation coefficient of 0.17 and an R_{factor} of 49.6% for 7201 reflections between 9.0 Å and 4.0 Å resolution. Examination of the crystal packing did not reveal any steric hindrance with symmetry-related molecules. 1171 reflections randomly chosen between 20 to 2.9 Å (5% of the data) have been set aside to monitor the progress of the refinement. Further refinement with each of the three protein domains treated as three independent rigid bodies followed by one cycle of molecular dynamics in the space of torsion angles was carried out using program *CNS* (Brunger *et al.*, 1998). This returned an overall R_{factor} of 34.1% for 13814 reflections between 20 to 3.24 Å resolution and an R_{free} of 47.7%. Electron density maps generated with phases from the partial model (516 residues) show several indications compatible with the pattern of insertions and deletions as well as amino-acid substitutions between Cry4Aa and Cry4Ba. The refinement of the structure is now in progress using manual model building with the program *O* (Jones *et al.*, 1991) interspersed with cycles of electron density map improvement, and refinement with programs *arp-warp*, *CNS* and *REFMAC* from the *CCP4* program suite (1994). The current values for R_{factor} and R_{free} are 24.1 and 29.3% for all data between 7 to 2.9 Å.

3.4 Structure determination and refinement

The protein backbone was traced, and most of the side chains were fitted using the molecular replacement map as described in the Experimental procedures. The final R-factor and R-free are 20.9 % and 26.3 %, respectively, for 19646 unique reflections between 7.0 Å and 2.8

Å resolution. The root mean square deviation for the ideal for bond length is 0.0065 Å and for bond angles is 1.284°. There are 164 water molecules included in the final structure. The average B-factor of all atoms is 34.86 $Å^2$.

The Cry4Aa molecule is a three-domain structure. The final model comprises 611 amino acid residues. The electron density map showed a well-defined density for residues 68-679, but the electron densities for residues 235-246, corresponding to the loop connecting helices 5 and 6, and 483-488 ,corresponding to the loop connecting beta strands 9 and 10, are not visible, probably as a result of being highly disordered. Previous observation showed that helix 5-6 loop of Cry4Aa was highly susceptible to proteolysis by trypsin-like enzymes (Angsuthanasombat et al., 1993). Previously, we have demonstrated that the elimination of the tryptic cleavage site at Arg-235 in this loop by glutamine substitution (R235Q) had no effect on the toxicity against *Aedes aegypti* larvae (Boonserm et al., 2004). A protease-resistant fragment of 65-kDa Cry4Aa-R235Q protein was produced after trypsin digestion and was subsequently crystallized (Boonserm et al., 2004). The disordered density in this region may be due to the highly conformational flexibility of this loop rather than being cleaved by trypsin.

References

Abdullah,M.A., Alzate,O., Mohammad,M., McNall,R.J., Adang,M.J., and Dean,D.H. (2003). Introduction of Culex toxicity into Bacillus thuringiensis Cry4Ba by protein engineering. Appl. Environ. Microbiol. *69*, 5343-5353.

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. *73*, 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 Bacillus thuringiensis CryIVB delta-endotoxin. FEMS Microbiol. Lett. *111*, 255-261.

Aronson,A.I., Geng,C., and Wu,L. (1999). Aggregation of bacillus thuringiensis Cry1A toxins upon binding to target insect larval midgut vesicles. Appl. Environ. Microbiol. *65*, 2503-2507.

Ballester,V., V, Granero,F., de Maagd,R.A., Bosch,D., Mensua,J.L., and Ferre,J. (1999). Role of bacillus thuringiensis toxin domains in toxicity and receptor binding in the diamondback moth. Appl. Environ. Microbiol. *65*, 1900-1903.

Boonserm,P., Ellar,D.J., and Li,J. (2003). Crystallization and preliminary X-ray diffraction studies of a mosquito-larvicidal toxin from Bacillus thuringiensis subsp. israelensis. Acta Crystallogr. D. Biol. Crystallogr. *59*, 591-594.

Boonserm,P., Pornwiroon,W., Katzenmeier,G., Panyim,S., and Angsuthanasombat,C. (2004). Optimised expression in Escherichia coli and purification of the functional form of the Bacillus thuringiensis Cry4Aa delta-endotoxin. Protein Expr. Purif. *35*, 397-403.

Burton,S.L., Ellar,D.J., Li,J., and Derbyshire,D.J. (1999). N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a Bacillus thuringiensis insecticidal toxin. J. Mol. Biol. *287*, 1011-1022.

Cabiaux,V., Wolff,C., and Ruysschaert,J.M. (1997). Interaction with a lipid membrane: a key step in bacterial toxins virulence. Int. J. Biol. Macromol. *21*, 285-298.

Chungjatupornchai,W., Hofte,H., Seurinck,J., Angsuthanasombat,C., and Vaeck,M. (1988). Common features of Bacillus thuringiensis toxins specific for Diptera and Lepidoptera. Eur. J. Biochem. *173*, 9-16.

Coux,F., Vachon,V., Rang,C., Moozar,K., Masson,L., Royer,M., Bes,M., Rivest,S., Brousseau,R., Schwartz,J.L., Laprade,R., and Frutos,R. (2001). Role of interdomain salt bridges in the pore-forming ability of the Bacillus thuringiensis toxins Cry1Aa and Cry1Ac. J. Biol. Chem. *276*, 35546-35551.

de Maagd,R.A., Bakker,P.L., Masson,L., Adang,M.J., Sangadala,S., Stiekema,W., and Bosch,D. (1999). Domain III of the Bacillus thuringiensis delta-endotoxin Cry1Ac is involved in binding

to Manduca sexta brush border membranes and to its purified aminopeptidase N. Mol. Microbiol. *31*, 463-471.

de Maagd,R.A., Bravo,A., and Crickmore,N. (2001). How Bacillus thuringiensis has evolved specific toxins to colonize the insect world. Trends Genet. *17*, 193-199.

de Maagd,R.A., Kwa,M.S., van der,K.H., Yamamoto,T., Schipper,B., Vlak,J.M., Stiekema,W.J., and Bosch,D. (1996). Domain III substitution in Bacillus thuringiensis delta-endotoxin CryIA(b) results in superior toxicity for Spodoptera exigua and altered membrane protein recognition. Appl. Environ. Microbiol. *62*, 1537-1543.

de Maagd,R.A., Weemen-Hendriks,M., Stiekema,W., and Bosch,D. (2000). Bacillus thuringiensis delta-endotoxin Cry1C domain III can function as a specificity determinant for Spodoptera exigua in different, but not all, Cry1-Cry1C hybrids. Appl. Environ. Microbiol. *66*, 1559-1563.

Evans,L.J., Goble,M.L., Hales,K.A., and Lakey,J.H. (1996). Different sensitivities to acid denaturation within a family of proteins: implications for acid unfolding and membrane translocation. Biochemistry *35*, 13180-13185.

Falnes,P.O. and Sandvig,K. (2000). Penetration of protein toxins into cells. Curr. Opin. Cell Biol. *12*, 407-413.

Galitsky,N., Cody,V., Wojtczak,A., Ghosh,D., Luft,J.R., Pangborn,W., and English,L. (2001). Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of Bacillus thuringiensis. Acta Crystallogr. D. Biol. Crystallogr. *57*, 1101-1109.

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

Gerber,D. and Shai,Y. (2000). Insertion and organization within membranes of the delta-endotoxin pore-forming domain, helix 4-loop-helix 5, and inhibition of its activity by a mutant helix 4 peptide. J. Biol. Chem. *275*, 23602-23607.

Griffitts,J.S., Whitacre,J.L., Stevens,D.E., and Aroian,R.V. (2001). Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science *293*, 860-864.

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.

Holm,L. and Sander,C. (1993). Protein structure comparison by alignment of distance matrices. J. Mol. Biol. *233*, 123-138.

Jenkins,J.L., Lee,M.K., Sangadala,S., Adang,M.J., and Dean,D.H. (1999). Binding of Bacillus thuringiensis Cry1Ac toxin to Manduca sexta aminopeptidase-N receptor is not directly related to toxicity. FEBS Lett. *462*, 373-376.

Jenkins,J.L., Lee,M.K., Valaitis,A.P., Curtiss,A., and Dean,D.H. (2000). Bivalent sequential binding model of a Bacillus thuringiensis toxin to gypsy moth aminopeptidase N receptor. J. Biol. Chem. *275*, 14423-14431.

Johnson,P.E., Joshi,M.D., Tomme,P., Kilburn,D.G., and McIntosh,L.P. (1996). Structure of the N-terminal cellulose-binding domain of Cellulomonas fimi CenC determined by nuclear magnetic resonance spectroscopy. Biochemistry *35*, 14381-14394.

Jurat-Fuentes,J.L. and Adang,M.J. (2001). Importance of Cry1 delta-endotoxin domain II loops for binding specificity in Heliothis virescens (L.). Appl. Environ. Microbiol. *67*, 323-329.

Kanintronkul,Y., Sramala,I., Katzenmeier,G., Panyim,S., and Angsuthanasombat,C. (2003). Specific mutations within the alpha4-alpha5 loop of the Bacillus thuringiensis Cry4B toxin reveal a crucial role for Asn-166 and Tyr-170. Mol. Biotechnol. *24*, 11-20.

Killian,J.A. and von Heijne,G. (2000). How proteins adapt to a membrane-water interface. Trends Biochem. Sci. *25*, 429-434.

Knight,P.J., Knowles,B.H., and Ellar,D.J. (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor for Bacillus thuringiensis CryIA(c) toxin. J. Biol. Chem. *270*, 17765-17770.

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.

Lee,X., Thompson,A., Zhang,Z., Ton-that,H., Biesterfeldt,J., Ogata,C., Xu,L., Johnston,R.A., and Young,N.M. (1998). Structure of the complex of Maclura pomifera agglutinin and the T-antigen disaccharide, Galbeta1,3GalNAc. J. Biol. Chem. *273*, 6312-6318.

Li,J., Derbyshire,D.J., Promdonkoy,B., and Ellar,D.J. (2001). Structural implications for the transformation of the Bacillus thuringiensis delta-endotoxins from water-soluble to membrane-inserted forms. Biochem. Soc. Trans. *29*, 571-577.

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

Lijnzaad,P., Berendsen,H.J., and Argos,P. (1996). Hydrophobic patches on the surfaces of protein structures. Proteins *25*, 389-397.

Masson,L., Lu,Y.J., Mazza,A., Brousseau,R., and Adang,M.J. (1995). The CryIA(c) receptor purified from Manduca sexta displays multiple specificities. J. Biol. Chem. *270*, 20309-20315.