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# Prediction-based protein engineering of domain I of Cry2A entomocidal toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects

**Chandi C. Mandal[1,3], Srimonta Gayen[1], Asitava Basu[1], Kalyan S. Ghosh[2], Swagata Dasgupta[2], Mrinal K. Maiti[1] and Soumitra K. Sen[1,4]**

[1]IIT – BREF Biotek, Indian Institute of Technology, Kharagpur-721 302, India and [2]Department of Chemistry, Indian Institute of Technology, Kharagpur-721 302, India
[3]Present address: Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78 229, USA

[4]To whom correspondence should be addressed.
E-mail: sk_sen55@yahoo.co.in

**Issues relating to sustenance of the usefulness of genetically modified first generation Bt crop plants in the farmer's field are of great concern for crop scientists. Additional biotechnological strategies need to be in place to safeguard the possibility for yield loss of Bt crop by other lepidopteran insects that are insensitive to the Cry1A toxin, and also against the possibility for emergence of resistant insects. In this respect, Cry2A toxin has figured as a prospective candidate to be the second toxin to offer the required protection along with Cry1A. In the present study, the entomocidal potency of Cry2A toxin was enhanced through knowledge-based protein engineering of the toxin molecule. Deletion of 42 amino acid residues from the N-terminal end of the peptide followed by the replacement of Lys residues by nonpolar amino acids in the putative transmembrane region including the introduction of Pro resulted in a 4.1–6.6-fold increase in the toxicity of the peptide against three major lepidopteran insect pests of crop plants.**

*Keywords*: *Bacillus thuringiensis* δ-endotoxins/enhancement of insecticidal property/insect resistance management/protein engineering of Cry2A/transmembrane domain

## Introduction

Genetically modified (GM) crops that express insecticidal crystal (Cry) proteins produced by *Bacillus thuringiensis* (Bt) during sporulation are an integral part of modern agriculture. Suitable Cry1A toxins based on their specificity and potency against some of the lepidopteran major insect pests of crops such as cotton, corn and rice have been deployed in the making of first-generation insect-resistant GM crops. As a result, controlling predation of crop plants by insect pests has significantly increased productivity of crop plants in the farmer's field. It was however experienced that additional protection is urgently required as other lepidopteran insects that are insensitive against the Cry1A toxin cause sufficient yield loss. Furthermore, it was realized that protection through single entomocidal *cry1A* gene expression might not prevent crop damage in the long run due to emergence of resistant insects (Heckel *et al*., 2007). Thus, deployment of a second generation transgenic plant incorporated with a different toxin having an altered mode of action and broader spectrum of sensitivity to lepidopteran insects than that of Cry1A, is necessary. In fact a second toxin, Cry2Ab of Bt, has been deployed in the making of the *BollgardII* version of GM cotton of Monsanto, offering protection against damages caused by cotton insect pests. Similarly, deployment of Cry2Aa toxin in GM rice has also been found to be suitable (Chen *et al*., 2005). Genes coding for the Cry2A group of proteins contain limited sequence homology with the genes coding for the Cry1 series of crystal proteins. Moreover, the Cry2A group of toxins do not share similar receptor binding sites toward insect epithelium cell membrane proteins with the Cry1A toxin (English and Slatin, 1994, Karim *et al*., 2000) and are effective against a broader range of lepidopteran insect pests (Wolfersberger *et al*., 1987; Donovan *et al*., 1988; Jellis *et al*., 1989; Morse *et al*., 2001; Zhao *et al*., 2005). However, the entomocidal potency of Cry2A toxin is not often as high as the Cry1A toxin. Highly potent Cry toxins play a major role in transgenic approaches since a high expression of the bacterial *cry* gene in plants is difficult to achieve. Thus, enhancement of the toxicity potential of Cry2A toxin was considered desirable.

The insecticidal mechanisms that result in the toxicity of Cry proteins toward lepidopteran insects are fairly well understood. Insertion of domain I of the toxin peptide into the insect brush border membrane (Ge *et al*., 1989; Ahmad and Ellar, 1990; Wu and Aronson, 1992; Walters *et al*., 1993) and presence of membrane spanning domains in the active Cry toxin molecule are important contributory factors for effective pore channel formation that cause toxicity (Schnepf *et al*., 1998). Additionally, refolding of a hydrophobic motif primed by decrease in pH or contact with the cytoplasmic membrane primarily plays a crucial role in the pore formation, as has been viewed in other bacterial toxins, such as colicins as well as cholera, pertussis, and anthrax toxins (Evans *et al*., 1996; Cabiaux *et al*., 1997; Falnes and Sandvig, 2000). It has been suggested that the organization of domain I of Cry toxins is reminiscent of organization of other pore-forming proteins composed of α-helices, such as colicin A (Parker *et al*., 1989; Gazit *et al*., 1998; Pardo-Lopez *et al*., 2006; Jiménez-Juárez *et al*., 2007). Studies on the orientation of the membrane-bound state of the seven helices comprising the pore-forming domain I of Cry toxins showed that insertion of hydrophobic α-4 and α-5 helices into the membrane causes toxicity (Gazit *et al*., 1998; Kantintrokul *et al*., 2003). This has been exploited for the enhancement of toxic efficacy in domain I of various Cry toxins (Wu and Aronson, 1992; Angsuthanasombat *et al*.,

1993; Aronson *et al*., 1995; Wu and Dean, 1996; Jiménez-Juárez *et al*., 2007).

In view of the above, *in silico* analysis of the Cry2A toxin molecule revealed that the putative transmembrane (TM) segment present in the N-terminal region of the Cry2A toxin is unusually short, compared with other insecticidal Cry toxins. This led us to initiate engineering of the domain I of the Cry2Aa toxin peptide. As the outcome of this effort, an engineered Cry2A toxin was designed, which contained enhanced entomocidal potency against three major polyphagous lepidopteran insect pests of Indian agriculture.

## Methods

### *Bacterial strains and plasmids*

A local isolate of *B. thuringiensis*, BRL 43, was used for the isolation of a *cry2Aa* gene. *Escherichia coli* strain DH10B and plasmid pUC18 were used for cloning and the *E. coli* strain BL21 (DE3) pLysE and plasmid pRSETA were used for over expression of the recombinant *cry2Aa* gene.

### *Isolation of a cry2Aa gene from* B. thuringiensis

The *cry2A* gene was amplified from genomic DNA of a local isolate of *B. thuringiensis*, strain BRL 43 through PCR, using degenerate primers designed from the complete coding sequences of the published nucleotide sequence of the *cry2A* gene (Accession numbers: M31738; AF047038; AJ132464; AJ132465; X55416; X57252; AF200816). The primer sequences used are as follows: forward primer 5′-CGGCGGATCCATGAATAA(G)CTGTATTGAATAG(A/T)CGG-3′ and reverse primer 5′-GCGCGCGAATTCTTAATAAAGTGGTGA(G)AAT(G)ATTAG-3′. The underlined sequences are indicative of the restriction sites (*Bam*HI and *Eco*RI, respectively) that were introduced in the primers for facilitating the subsequent cloning process. The PCR thermal profile followed was initial heat denaturation at 95°C for 4 min, followed by 30 cycles at 94°C for 40 s, 60°C for 40 s, 75°C for 2 min, followed by final extension at 75°C for 7 min. The PCR-amplified product (1.9 kb) was cloned in the bacterial vector, pUC18, sequenced (Sanger *et al*., 1977) using T7 Sequenase/7-deaza-dGTP sequencing kit (USB) and deposited to the Gene Bank (Accession number, DQ064596). From the sequences, it was ascertained that the isolated gene was *cry2Aa*. All molecular cloning techniques were carried out according to the method proposed by Sambrook *et al*. (1989).

### *Bioinformatics analyses*

DNA sequence alignment and structural analysis were performed using the ClustalW algorithm (http://www.ebi.ac.uk/clustalw/) and SWISS-pdb Viewer v3.1 (Guex and Peitsch, 1997). Many algorithms designed to identify putative TM helices in the primary amino acid sequence have been developed over the years and identification of ~90–95% of all true TM segments is currently possible. TM prediction analysis was carried out using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0). A probability >0.5 for helices is indicative of a TM segment. The probability has been calculated on the basis of the hidden Markov model (Durbin *et al.*, 1998; Krogh *et al*., 2001). In TMpred (http://www.ch.embnet.org/), helices are considered as TM helices when the score is ≥500 (Hofmann and Stoffel 1993). Hydrophobicity of the predicted TM segment was estimated using the PC/GENE software (Bairoch, 1993). Protein sequence alignment was carried out using the pfam algorithm (Chen *et al*., 2003). Contact map analysis of various segments of the N-terminal region with functional domains was carried out using SPACE (http://www.atlantis.weizmann.ac.il/cma/). The SOSUI server was used to identify the helices of putative TM segments (http://www.sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html). The CSU server (Sobolev *et al*., 1999) was used to study the interaction of residues (http://www.ligin.weizmann.ac.il/cgi-bin/lpccsu/LpcCsu.cgi). The accessible surface area was calculated using the GETAREA server (http://www.pauli.utmb.edu/cgi-bin/get_a_form.tcl).

### *Generation of deletion mutations*

Deletion mutants D42 and D70 were generated by PCR using the isolated wild-type *cry2Aa* gene as template. For the generation of D42 and D70 (deletion of the corresponding nucleotides of 42 and 70 amino acid residues from the N-terminal end of Cry2Aa, respectively), the following primers were used: 5′-CGGCGGATCCATGACAGATCATAGTTTATATGTAGCTCC-3′ and 5′-CGGCGGATCCATGAAAAGGATATTGAGTGAATTATGG-3′, respectively, where ATG was kept as the translational initiation codon. 5′-GCGCGCGAATTCTTAATAAAGTGGTGAAATATTAG-3′ was used as the reverse primer in each case. The underlined sequences are restriction sites (*Bam*HI for forward primers and *Eco*RI for reverse primer) The PCR conditions were similar as described earlier. The truncated *cry2Aa* fragments generated were cloned in pUC18.

### *Generation of point mutations*

The point(s) mutants such as D42/$K_{63}$F, D42/$K_{64}$F, D42/$K_{63}$F/$K_{64}$F and D42/$K_{63}$F/$K_{64}$P were introduced in the generated D42 deletion mutant using the Quick Change XL site-directed mutagenesis kit (Stratagene), following instructions from the manufacturer. The primers used are included in the Supplementary data. Mutants were confirmed by double-stranded DNA sequencing.

### *Bacterial over expression of wild type and mutants of* cry2Aa *toxin gene*

For bacterial over expression, mutant *cry2Aa* and wild type genes were recloned into the T7 promoter-based bacterial expression vector pRSET-A through *Bam*HI/*Eco*RI sites. *E. coli* clones were grown in terrific broth (Sambrook *et al*., 1989), containing chloramphenicol (34 mg/l) and ampicillin (100 mg/l). Expression of recombinant genes was induced at a culture density of 0.6 absorbance units at 600 nm with 1 mM isopropyl thiogalactoside (IPTG) and cells were harvested after 6 h at 37°C. Inclusion bodies were harvested by cell lysis using lysis buffer (25 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0) and washed repeatedly with 0.5 M NaCl followed by water. The resulting pellet was solubilized in 50 mM $Na_2CO_3$ (pH 11.0) with 10 mM dithiothreitol and dialyzed against 50 mM $Na_2CO_3$ (pH 10.5). The integrity and solubility were checked by SDS–PAGE. Secondary structure analyses of the protein and mutants were performed by circular dichroism (CD) experiments. CD measurements were made on a Jasco-810 automatic recording spectrophotometer, using a path length of 1 mm at 25°C.

The spectra were recorded in the range of 190–240 nm with a scan rate of 50 nm/min and a response time of 4 s. For baseline correction, CD spectra of buffer (50 mM sodium carbonate, pH 10.5) were collected and were subtracted from each sample spectra. The protein concentration was ~66 μg/ml. Secondary structures were determined using DICHROWEB, an online server for protein secondary structure analyses from CD spectroscopic data using the SELCON 3 analysis program (Whitmore and Wallace, 2004).

### *Test for stability of the toxin peptide*

The stability of the recombinant toxins was tested by chymotrypsin digestion. Digestion was carried out in 50 mM $Na_2CO_3$ buffer (pH 10.5) at 37°C for 2 h. Reactions were stopped by boiling with protein-loading dye and the products were monitored on a 10% SDS–PAGE as discussed by Laemmli (1970).

### *Insect feeding assays*

Insect feeding assays were carried out with first instar larvae of cotton leafworm (*Spodoptera littoralis*; CLW), cotton bollworm (*Helicoverpa armigera*; CBW) and black cutworm *(Agrotis ipsilon*; BCW) reared on semi-synthetic diet at 28°C, 70% relative humidity with a photoperiod of 16 h light/8 h darkness (Nayak *et al*., 1997). Toxins at different concentrations were mixed thoroughly with the artificial diet. Thirty larvae of insect species were used for each set of experiments. Bioassays were performed in triplicate in all cases. Insect mortality was checked after 5 days and the data were subjected to probit analysis (Finney, 1972) to obtain the medium lethal concentration ($LC_{50}$) at 95% confidence limits.

### *Brush border membrane vesicle purification and ligand blotting*

Brush border membrane vesicles (BBMVs) were isolated from CBW and CLW insect larvae following the protocol of Wolfersberger *et al.* (1987). The pellet containing the BBMVs was resuspended in a solubilization buffer containing 8 mM $Na_2HPO_4$, pH 7.4, 2 mM $KH_2PO_4$ (Karim *et al*., 2000) and kept on ice. Protein concentrations were determined using the Bradford assay (Bradford, 1976) with BSA as a standard, and the specific activity of alkaline phosphatase was measured to assess the purity of BBMV. The membrane proteins were subjected to PAGE and transferred onto polyvinylidene fluoride membranes. After membrane blocking, the toxin was added (20 μg/ml) in fresh blocking buffer and incubated overnight at 4°C with gentle shaking followed by three washes with washing buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% Tween20). Immunological detection was carried out using the BM Chromogenic western blotting kit (Boehringer Mannheim, Germany), following instructions from the vendor.

## Results

### In silico *analysis of the putative TM segment in the N-terminal pore-forming domain I region of the* Cry2A *toxin*

Most pore-forming toxins contain TM domain(s). These TM domains may reside either at the C-terminal region or at the N-terminal region (like in case of lethal factor, LF of *Bacillus anthrax*,) as detected by different TM prediction servers. In *B. anthrax*, the LF toxin showed lower membrane association following deletion of the N-terminal end (1–32 amino acids) and Colicin Ia showed lower pore formation when the hydrophobic segment (550–592 amino acids) was removed from the C-terminal end (Raffy *et al.,* 2004; Zhang *et al*., 2004). A fresh analysis with TM prediction servers indicated that in both cases the TM potency had been dramatically reduced (Table I). A recent report of the toxin YopB (*Yersinia pseudotuberculosis*) where the protein sequence was analyzed by the TMHMM prediction server showed inhibition of membrane translocation and pore formation to the host membrane on deletion of the TM segment (Ryndak *et al*., 2005).

Other *in silico* analyses of the primary structure of several groups of Cry toxins for the detection of TM domains using TMpred, TMHMM and DAS programs indicated that most Cry toxins have well-defined TM segments in the N-terminal region of domain I, varying in length from 18 to 24 residues (Table I; Fig. 2). An exception has been the case of Cry2A toxin, where the presence of a short TM segment comprising 12 amino acids (spanning residues 51–62) was detected. The TM score and probability of Cry2A resembled that of the mutated Cry1Ab F50K (Table I), which showed lower pore formation and reduced toxicity potential (Ahmad and Ellar, 1990).

### *N-terminal deletions of Cry2Aa toxin molecule*

On the basis of the *in silico* analysis, it was comprehended that the low probability and short span of the N-terminal TM segment of the Cry2Aa molecule may contribute toward its low toxicity. The SOSUI server indicated that the deletion of the N-terminal region beyond amino acid position 42 from wild type Cry2Aa toxin molecule led to a loss of the putative N-terminal TM segment, whereas the deletion of N-terminal residues till #42 had no such effect. Contact Map analysis of the structure of Cry2Aa toxin using the SPACE program additionally revealed that the N-terminal region formed two major contacts. The loop region comprising residues 1–25 is in contact with domain II. The hydrophilic helix (spanning residues 26–42) interacts with the predicted TM segment (Fig. 1) suggesting a potential negative effect on membrane insertion of the N-terminal TM domain. Thus, two deletion mutants, D42 (residues 1–42 deleted) and D70 (residues 1–70 deleted, where the proposed TM has also been eliminated), of Cry2Aa toxin peptides were generated. The D42 and D70 mutant lines produced smaller toxins, when expressed in *E. coli*, compared with the wild type (wild type, ~72 kDa; D42, ~68 kDa; D70, ~65 kDa). Insect feeding assays against the three insects under test, CBW, CLW and BCW, revealed that the D42 mutant showed a 2–3-fold enhanced toxicity compared with the wild-type Cry2Aa toxin, whereas D70 showed lower toxicity than that of D42 (Table II).

### *Amino acid substitution in the putative TM segment of the D42 mutant*

To improve the toxic potential of Cry2Aa further, alignment of the N-terminal pore-forming domain I sequences of different Cry group toxins on the basis of the pfam algorithm was carried out. The analysis revealed that all Cry toxins except Cry2A have either Phe or Val and Pro at sites corresponding

**Table I.** *In silico* analysis of different pore-forming toxins for the presence of putative N-terminal transmembrane (TM) segments including other engineered Cry2A toxins

| Pore forming toxins | TM segment | | | Functions |
|---|---|---|---|---|
| | Length | Probability | Score | |
| Colicin Ia | 38, 32 | ~1,~1 | 1982 | Deleted colicin [Del (550–592)] showed impaired membrane insertion (Raffy *et al*., 2004). Deleted colicin shows lower probability, score and length of TM. |
| Lethal factor (LF) | 22 | 0.9 | 1440 | Deleted LF(1–32) of *Bacillus anthrax* showed impairment to form pore ( channel) to host plasma membrane (Zhang *et al*., 2004). Deleted LF shows lower probability, score and length of TM. |
| YopB | 22, 22 | 0.97, 0.95 | 2417, 1256 | Protein sequence of YopB (*Y. pseudotuberculosis*) was analyzed by TMHMM prediction server and TM-deleted YopB showed inhibition of membrane translocation and pore formation (Ryndak *et al*., 2005). |
| Cry1A | 18 | 0.60 | 1642 | Mutated Cry1Ab(F50K) showed impaired pore formation and reduces toxicity (Ahmad and Ellar, 1990). |
| Cry1Ab(F50K) | 13 | 0.18 | 1232 | |
| Cry3A | 24 | 0.60 | 1765 | Function not reported. |
| Cry2A | 12 | 0.07 | 1210 | Function not reported, although possibility of TM formation is very low in compared with other toxins. |
| D42 | 12 | 0.26 | 1241 | Toxicity of these engineered Cry2Atoxins has been tested in this study. |
| D42/$K_{63}$F | 18 | 0.76 | 1764 | |
| D42/$K_{64}$F | 19 | 0.76 | 1636 | |
| D42/$K_{63}$F/$K_{64}$F | 21 | 0.97 | 2326 | |
| D42/$K_{63}$F/$K_{64}$P | 19 | 0.94 | 1922 | |

The effect of amino acid substitutions or deletion on its length, probability and score using different prediction algorithms has been mentioned.

to positions 63 and 64, respectively (Fig. 2). Owing to the presence of Lys residues at positions 63 and 64, the N-terminal hydrophobic region of the Cry2Aa molecule is limited to 12 residues. This implies that alterations made in conformity to other Cry toxins in the N-terminal hydrophobic region of Cry2A toxin could make up for at least 19 amino acids with the substitution of Lys with a nonpolar amino acid. This would result in a TM helix of optimal length for the expected regular function of the TM region (Reithmeier, 1995). On considering the hydrophobicity of possible amino acids, it was decided that Phe would replace Lys. The relative TM properties of different mutant Cry2A toxins are given in Table I.

It was also observed that if Phe replaced both Lys residues there would be a further enhancement of the hydrophobicity and probability of TM helix formation (Table I). As with other Cry group of toxins, it was envisaged that replacement of Lys at position 64 by Pro would create a molecular hinge that may favor a membrane environment (Cordes *et al.,* 2002). Interestingly, structural analysis through the SOSUI server predicted that the N-terminal TM helix (residues 47–69) resulting from the alteration would promote the formation of a secondary TM helix (residues 73–95).

### *Functional validation for the amino acid substitution of the Cry2Aa D42 toxin*

Site-directed mutagenesis generated two single amino acid replacement mutant lines such as D42/$K_{63}$F and D42/$K_{64}$F and two double mutants such as D42/$K_{63}$F/$K_{64}$F and D42/$K_{63}$F/$K_{64}$P (Fig. 3). The engineered toxins were expressed in the bacterial expression system and the solubility of inclusion bodies for different mutant toxins by $Na_2CO_3$ buffer (pH 10.5) was estimated and found to be similar to the wild-type *Cry2Aa* (see Supplementary data, Fig. S1). The stability of the expressed recombinant proteins was tested against chymotrypsin digestion. SDS–PAGE analysis revealed that digestion of the recombinant proteins with chymotrypsin for 2 h produced the same 52 kDa as the Cry2Aa D42 deletion line (Fig. 4), indicating their unaltered stability and protein-folding pattern.

Functional activities in terms of entomocidal property of the mutant Cry2Aa toxins were then tested and compared with the wild type Cry2Aa toxin. The effect of the modifications brought in through protein engineering of the Cry2Aa peptide followed the predicted pattern of changes in hydrophobicity and TM propensity of the native Cry2Aa toxin. The D42 deletion led to a 2–3-fold decrease in $LC_{50}$, and the replacement of the Lys residues at positions 63 and 64 resulted in another 1.5–2-fold increase in toxicity (Table II). Replacement of the Lys residue at position 63 by Phe shows a negative effect whereas a slight enhancement in toxicity is encountered in the case of the same replacement at position 64. The most active toxin variant was the combination of D42 deletion with the $K_{63}$F/$K_{64}$P substitution, which had a 4.1–6.6-fold greater toxicity than Cry2Aa. However, the time course of insect mortality caused by all toxin variants remained nearly the same (Fig. 5). CD spectral analysis of different mutant toxins indicted that there was no major change in the secondary structure compared with the wild-type Cry2Aa for the mutants. The most significant change observed is a 7% increase in the $\alpha$-helical content for the D42/$K_{63}$F/$K_{64}$P mutant, which shows increased toxicity (see Supplementary data, Fig. S3).

### *Recognition of the receptor protein by the mutant toxin*

The confirmation that the D42/$K_{63}$F/$K_{64}$P mutant toxin binds to specific protein(s) present in the BBMVs of the CLW and CBW mid-gut epithelial cells was proved through ligand blot analysis. The engineered peptide, like the wild type Cry2Aa toxin, recognized the 130 kDa receptor protein in case of CLW (lanes F and G, Fig. 6) and the 240 kDa receptor in case of CBW (lanes C and D, Fig. 6). The Cry1Ac

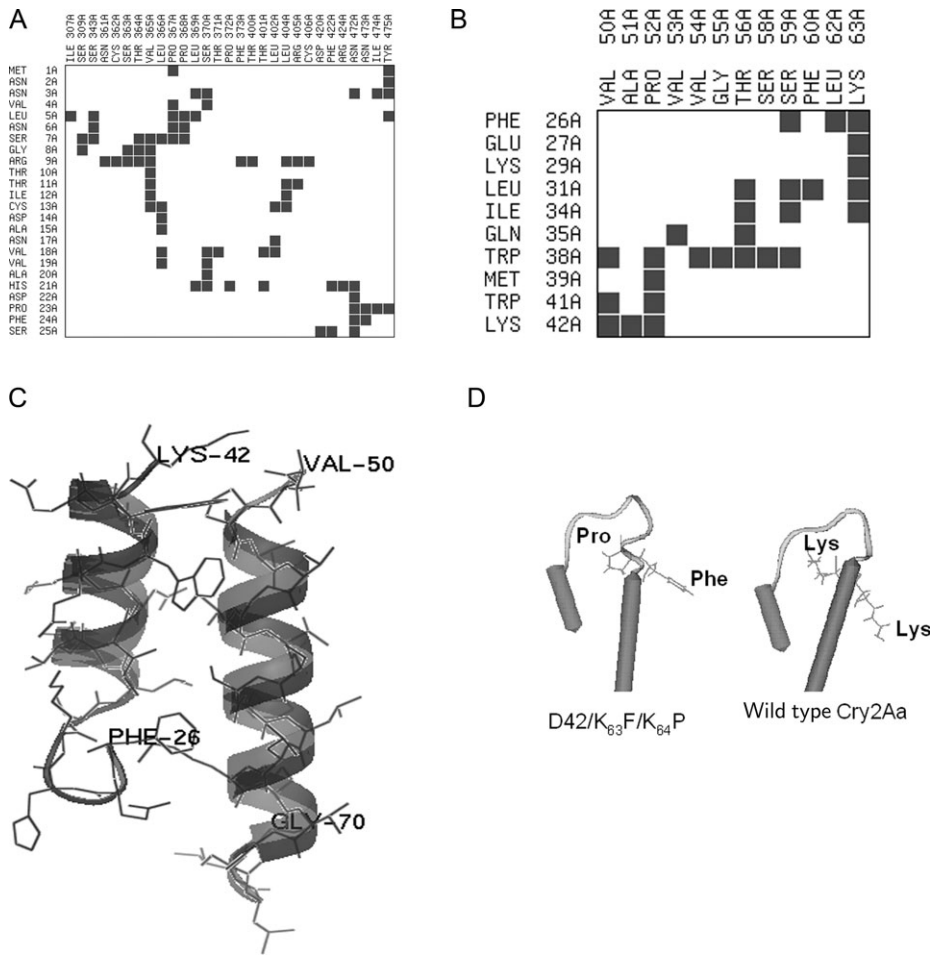


**Fig. 1.** Interaction of the N-terminal loop region (residues 1–25) and the hydrophilic helix (26–42 residues) of Cry2Aa with domain II and the proposed transmembrane (TM) segment. (**A**) Contact map with ordinate as N-terminal coil (residues 1–25) and abscissa as interacting residues of domain II (residues 307–475). (**B**) Contact map with ordinate: N-terminal helix (residues 26–42) and abscissa: interacting residues of predicted TM region in domain I (residues 50–63). (**C**) Cartoon representation of the possible interaction of the N-terminal helix (residues 26–42) with the proposed TM region of domain I (residues 50–70). (**D**) Representation of the loop regions at the C-terminal end of the proposed TM region of Cry2Aa and D42/$K_{63}$F/$K_{64}$P toxins. In case of D42/$K_{63}$F/$K_{64}$P, the loop region extends from residue 63 instead of residue 67, as observed in the wild type Cry2Aa toxin.

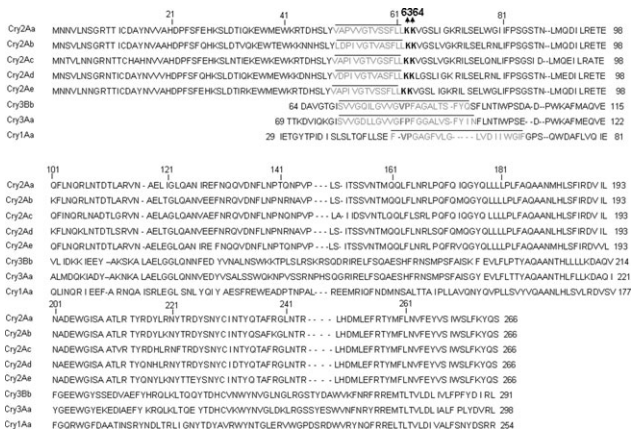


**Fig. 2.** Protein sequence alignment with pfam of N-terminal pore forming domain I of Cry2Aa, Cry3Bb, Cry3Aa and Cry1Aa toxins including different Cry2A group of toxins. Protein sequences of known three-dimensional structure from PDB: Cry2Aa (1I5P), Cry3Bb (1JI6), Cry3Aa (1DLC), Cry1Ac (1CIY) and Cry2Ab, Cry2Ac, CryAd and Cry2Ae from accession numbers M23 724, X57252, AF200816, AAQ52362, respectively. The predicted TM segment of all cry toxins has been underlined.

toxin, on the other hand, recognized the 120 kDa receptor protein in case of CBW (lane A, Fig. 6). The results indicate that the recombinant toxin D42/$K_{63}$F/$K_{64}$P maintained the same specificity for the recognition of its cognate receptor, as the wild type toxin, Cry2Aa.

## Discussion

The present study has led to the generation of a modified Cry2Aa of *B. thuringiensis* toxin with enhanced entomocidal property through protein engineering. This knowledge-based experimental approach took advantage of the results of the comparative analysis of the primary structure of the Cry2Aa toxin with that of other entomocidal Bt crystal (Cry) toxins. The toxicity mechanism of Cry toxins operates after ingestion by the sensitive insect followed by solubilization and proteolytic activation of the toxin core in the gut juice. Thereafter, a cascade of events leading to binding of the active toxin to receptors present in the sensitive insect midgut epithelial cells, followed by pore-formation through insertion and oligomerization of the hydrophobic domain I of the Cry toxin result in cell death by osmotic shock (Knowles, 1994; Schnepf *et al*., 1998, Jiménez-Juárez *et al*.,

**Table II.** Comparative toxicity analysis of wild type and various mutant Cry2Aa toxins tested on first instar larvae of cotton leaf worm (CLW), cotton bollworm (CBW) and black cutworm (BCW)

| Toxins | CLW | | CBW | | BCW | |
|---|---|---|---|---|---|---|
| | $LC_{50}$ (ng/ml) | Toxicity (-fold) | $LC_{50}$ (ng/ml) | Toxicity (-fold) | $LC_{50}$ (ng/ml) | Toxicity (-fold) |
| Cry2Aa (wild type) | 40.09 (33.74–47.36) | 1.00 | 299.88 (255.70–359.20) | 1.00 | 251.33 (218.45–287.96) | 1.00 |
| D42 | 14.06 (11.16–17.32) | 2.85 | 149.95 (123.25–183.09) | 1.99 | 87.32 (3.02–103.65) | 2.87 |
| D70 | 46.39 (38.8–56.28) | 0.86 | 346.44 (300.00–412.83) | 0.86 | 264.70 (30.00–304.55) | 0.94 |
| D42/$K_{63}$F | 20.31 (16.6–24.26) | 1.97 | 246.18 (210.56–286.70) | 1.21 | 140.44 (16.69–166.20) | 1.78 |
| D42/$K_{64}$F | 10.19 (8.44–12.17) | 3.93 | 116.01 (96.64–138.68) | 2.58 | 76.63 (3.87–91.21) | 3.27 |
| D42/$K_{63}$F/$K_{64}$F | 8.88 (5.24–11.02) | 4.51 | 103.19 (85.09–124.52) | 2.90 | 64.92 (3.49–78.50) | 3.86 |
| D42/$K_{63}$F/$K_{64}$P | 6.09 (5.12–7.23) | 6.58 | 72.37 (59.95–86.33) | 4.14 | 51.33 (1.54–62.22) | 4.89 |

To determine the fold alteration, the $LC_{50}$ values are normalized to the $LC_{50}$ obtained with wild-type toxin.

2007). *In silico* analysis carried out in the present study revealed that the hydrophobic segment at the N-terminal end of the Cry2A toxin is unusually short compared with other Cry toxins. Unlike other Cry toxins with longer N-terminal TM segments, this short region was assumed to be inefficient in forming an efficient TM segment. The functional role of the N-terminal TM segment on the entomocidal potency of a Cry2A toxin has been investigated in the present study for the first time.

It was observed that deletion of the N-terminal region resulting in D42 indicated that the extra N-terminal end to the putative TM segment played a negative regulatory role on the toxicity of the Cry2Aa peptide since the mutant showed a 2–3-fold enhanced toxicity potential compared with the wild type Cry2Aa toxin. This extra N-terminal end is composed of an extra coil (1–25 amino acids) and an extra hydrophilic helix comprising 26–42 amino acid residues. Information based on a Contact Map analysis indicated that the N-terminal extra coil (1–25 amino acids) interacted with the receptor-binding domain II (Fig. 1A). It was further speculated that this might possibly interfere in the receptor binding process (Morse *et al*., 2001). The possible interaction of this hydrophilic helix with the putative TM domain I (Fig. 1B and C) may be responsible in interfering with the traversing process of the TM domain into the hydrophobic membrane. The accessible hydrophobic surface area of the TM segment was found to increase when the N-terminal 42 residues were eliminated (see Supplementary data, Fig. S1). The low receptor-binding affinity of Cry2Aa toxin (Karim *et al*., 2000; Morse *et al*., 2001) is also attributed to the presence of the extra N-terminal region. The domain I–II contact area has been found earlier to be larger for Cry2Aa compared with the other Cry group of toxins (Boonserm *et al*., 2005). Reduction in the size of contacts between the helical and sheet domains due to deletion of the N-terminal extra coil and helix may contribute towards operational ease in unfolding around a hinge region, linking domains I and II. A similar developmental feature in Cry1Aa toxin has been speculated to be essential for pore formation (Schwartz *et al*., 1997).

The propensity for TM helix formation depends on various structural and physiochemical factors where hydrophobicity remains the major contributing factor. It has been demonstrated in the past that positional hydrophobicity promoted peptide aggregation in solution and high local concentration of the peptide inserted into the membrane improved the permeabilization (Guerrero *et al*., 2004). Thus, alignment of Cry toxin sequences of the N-terminal region of the toxin was carried out in order to identify the sites where substitution of residues with nonpolar amino acids would possibly increase the hydrophobic nature of the region. On the basis of these analyses two single site mutants, such as D42/$K_{63}$F and D42/$K_{64}$F, were generated. Insect feeding tests documented enhancement of toxicity in case of D42/$K_{64}$F, but not in case of D42/$K_{63}$F. This demonstrated that positional hydrophobicity played a crucial role in toxicity modulation. Intramolecular contact analysis of D42/$K_{63}$F indicated that Phe at position 63 interacted with more hydrophilic residues in remote regions compared with other mutants (data not shown). Functionally, it was also documented that enhancement of toxicity in case of D42/$K_{63}$F/$K_{64}$F was achieved through the generation of a pair of helices (data not shown). Helices are known to be major contributory elements for membrane insertion of pore forming toxins *(*Parker *et al*., 1989). The present results support the notion that critical levels of hydrophobicity determine the helical conformations

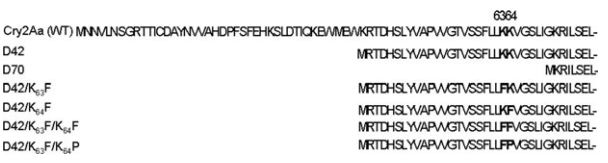


**Fig. 3.** N-terminal amino acid sequences of wild type (WT) and mutant Cry2Aa toxins. The N-terminal methionine (M) was introduced into the Cry2Aa mutants during PCR-based deletion.

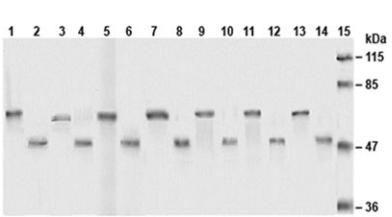


**Fig. 4.** SDS-PAGE (10%) of chymotrypsin digestion products of wild-type and mutant Cry2Aa toxins. Undigested toxins: lanes 1, 3, 5, 7, 9, 11 and 13; lanes 2, 4, 6, 8, 10, 12 and 14 represent chymotrypsin-digested toxins in the order: wild type, D70, D42, D42/$K_{63}$F, D42/$K_{64}$F, D42/$K_{63}$F/$K_{64}$F and D42/$K_{63}$F/$K_{64}$P toxins, respectively. Lane 15: molecular weight markers (in kDa).

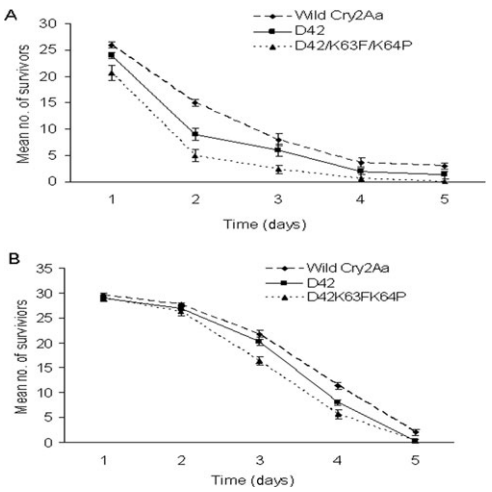


**Fig. 5.** Graphical representation of the susceptibility patterns with the gradual elapsing of days in (**A**) CLW and (**B**) CBW under a particular toxin concentration at which 90–100% mortality was observed after 5 days.

of peptides in the membrane environment (Liu *et al*., 1996). It was also suggested earlier that the presence of Pro in channel pore-forming TM segments play a crucial role in helix packing through the extension of loops (Fig. 1D) which may serve as molecular hinges (Cordes *et al*., 2002). Thus, the introduction of Pro in lieu of Phe at position 64 of D42/$K_{63}$F/$K_{64}$F was attempted. The hydrophobicity and TM probability of the engineered Cry2Aa molecule, D42/$K_{63}$F/$K_{64}$P was not affected because of this alteration. CD studies did not reveal any major change in the secondary structure of the mutants compared with the wild type. The D42 $K_{63}$F/$K_{64}$P mutant that showed increased toxicity resulted in a 7% increase in the $\alpha$-helical content. Insect-feeding tests demonstrated that D42/$K_{63}$F/$K_{64}$P contained maximum insecticidal potency against all the three insects in comparison with all other recombinant toxins tested. The enhancement was 4.1–6.6 times higher than the wild type Cry2Aa toxin. This had quite likely resulted through membrane–toxin effective interaction facilitated by the position-dependent presence of Pro in the TM domain. Interestingly, the time course of insect mortality caused by all toxin variants remained the same (Fig. 5). Our present study demonstrated that knowledge-based structural understanding of the toxic property of a Cry toxin protein conforms to the actual functional property of the toxin.

In summary, enhancement of the insecticidal potency of Cry2Aa toxin could be attained through a prediction-based three-stage protein engineering process. First, the deletion of N-terminal 42 residues led to a 2–3-fold increase in toxicity, presumably by abrogating their inhibitory interactions with domains I and II. Secondly, the replacement of Lys residues in domain I by nonpolar amino acids increased the toxicity by another 50%, presumably by extending the length of the TM region. Thirdly, the introduction of Pro in the hydrophobic region led to a further enhancement in toxicity to another 40%, possibly by providing a molecular hinge to optimize helix packing. The aggregate enhancement of activity by these three changes ranged between 4.1- and 6.6-fold.

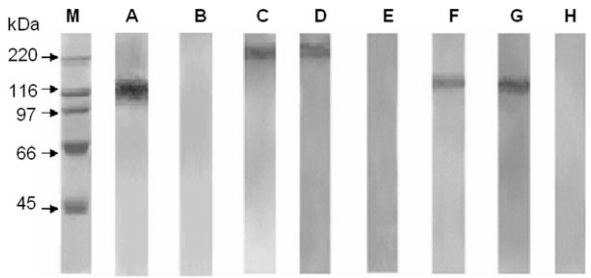


**Fig. 6.** Ligand blot of the insect brush border membrane vesicle (BBMV) proteins against Cry2Aa, D42/$K_{63}$F/$K_{64}$P and Cry1Ac. 50 μg of total BBMV proteins was run in each lane on an 8% PAGE and electroblotted on a PVDF membrane for ligand blotting. Lane M: molecular weight markers (in kDa); lane A: Cry1Ac toxin against CBW; lane B: BBMV of CBW without Cry1Ac toxin, lane C: wild-type Cry2Aa against CBW BBMV; lane D: D42/$K_{63}$F/$K_{64}$P toxin against CBW BBMV; lane E: BBMV of CBW where no Cry2A toxin was added; lane F: wild-type Cry2Aa against CLW BBMV; lane G: D42/$K_{63}$F/$K_{64}$P against CLW BBMV; lane H: BBMV of CLW without Cry2A toxin.

## Supplementary data

Supplementary data are available at *PEDS* online.

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