Chapter 5A

Construction of hybrid Cry toxins & ssOE-PCR
5A.1 Introduction

Cry toxins of *Bacillus thuringiensis* have been successfully used for decades to control pests in agriculture, forestry and insect vectors of human and animal disease [Arora et al, 2007; Schnepf et al, 1998]. *Bt* transgenics expressing Cry toxins have made it easier to control pest and thereby reduce the chemical pesticide usage [Qaim 2003].

However, a few setbacks to Cry toxins have been encountered in last decade. Development of field level resistance in insects poses a great threat to transgenic crops and requires major concern [Tabashnik and Carriere 2003]. Many Cry toxins are ineffective to target more than one type of insects due to narrow spectrum of toxicity. *P. xylostella* and *H. armigera* showed variation in susceptibility to Cry toxins [Gujar et al, 2007; Siegfried et al, 2000]. It thus necessitates use of novel or modified toxins with improved toxicity and broadened spectrum of activity.

Hybrid Cry toxins with different domain combinations from a variety of Cry toxins have been constructed. Fusion of domain I-II of Cry1Ab toxin with domain III from Cry1C resulted into increased toxicity to *S. exigua* compared to the parental toxins [de Maagd et al, 1996a]. Combination of domain I-II of Cry1la and domain III of Cry1Ba led to a change in specificity of the hybrid toxin towards a different order Coleoptera [Naimov et al, 2001]. Combination of domain I-II Cry1Ba with domain III Cry1Ca broadened the spectrum of activity towards *S. exigua* and *M. sexta* [de Maagd et al, 2000]. Similar results were obtained with other hybrid toxins [Karlova et al, 2005; Sakai et al, 2007]. In some hybrid toxins, however a decrease in toxicity was also observed [de Maagd et al, 1996a; Karlova et al, 2005; Rang et al, 2001]. Nevertheless, hybrid Cry toxins were successfully constructed to achieve desired results of increase in toxicity, change of specificity as well as broadening the spectrum of action. Effect of domain swapping among other toxins has been described in table 5A.1.
A large pool of Cry toxins specific for Lepidopterans are available which can be tested using different permutation and combinations to construct hybrid toxins with increased toxicity, changed specificity and/or broaden spectrum of activity. Development of resistance in insects towards Cry toxins being the major trouble in recent times, hybrid toxins can be constructed for insect resistance management [Bosch et al, 1994]. Domain swapping could be helpful to understand function of domain III, which remains an ambiguity among different toxins, at the same time detailed mode of action of Cry toxin can be explored. Domain swapping between different Cry toxins was helpful to understand the role of inter-domain interactions on the overall toxicity of the protein [Rang et al, 1999]. Domain swapping has also provided a deeper insight into the structure function relationship of each domain. Hybrid toxins displayed change in receptor recognition than the parental toxins and thus it can be used to explore the role of different receptors in the mode of action of Cry toxins [de Maagd et al, 1996b].

Construction of hybrid cry genes by exchanging regions coding for either domain I, II or III among various cry genes has been performed previously by using different techniques. The method of in vivo recombination as described by Bosch et al, 1994 is more random and requires screening for desired site of recombination. Generation of restriction sites by site directed mutagenesis becomes time consuming. The in vitro method of recombination described by Knight et al, 2004 is better for construction of library of chimeric genes but requires use of 5' phosphorylated primers for amplification and use of λ exonuclease to generate single strands of DNA segment. As a result, it becomes cumbersome when handling more number of cry genes. Henceforth, an appropriate method allowing exchange of specific domain coding region among multiple cry genes is need of the time.

The method of single strand OE-PCR described in present chapter is a much simpler and convenient method for the generation of the desired cry gene
recombinants. In the present study; we have generated cry gene hybrids with a method of gene recombination by Overlap Extension PCR using overlapping sequence from conserved blocks (Fig. 5A.1). We have generated hybrids of more closely related cry1Ac and cry1Ab genes with a 233bp overlap sequence and distantly related cry1Ac and cry1Ea genes with an 18 bp overlap.

Figure 5A.1: Schematic representation of five conserved blocks positions [de Maagd et al, 2001]
## Table 5A.1: Domain swapping of Cry toxins and their effects

<table>
<thead>
<tr>
<th>Hybrid toxin &amp; Reference</th>
<th>Details</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cry1E - Cry1C</td>
<td>G 27: Domain I &amp; II of Cry1E and domain III of Cry1C</td>
<td>Higher toxicity than parental Cry1Ca and comparable to Cry1Ea towards <em>S. exigua</em> and <em>Mamestra brassicae</em></td>
</tr>
<tr>
<td></td>
<td>F26: Domain I &amp; II of Cry1C and domain III of Cry1E</td>
<td>Toxic to <em>Manduca sexta</em> but no toxicity towards <em>S. exigua</em> and <em>M. brassicae</em></td>
</tr>
<tr>
<td>2. Cry1Ab - Cry1C</td>
<td>H 04: Domain I &amp; II of Cry1Ab and domain III of Cry1C</td>
<td>Highly toxic towards <em>S. exigua</em> than Cry1Ab and Cry1C</td>
</tr>
<tr>
<td></td>
<td>H 205: Domain I &amp; II of Cry1C and domain III of Cry1Ab</td>
<td>Low activity to <em>S. exigua</em> and <em>M. sexta</em>, but bind strongly to BBMV</td>
</tr>
<tr>
<td>3. Cry1Ac - Cry1C</td>
<td>H 130: Domain I &amp; II of Cry1Ac and domain III of Cry1C</td>
<td>Did not bind to BBMV of <em>S. exigua</em></td>
</tr>
<tr>
<td></td>
<td>H 201: Domain I &amp; II of Cry1C and domain III of Cry1Ac</td>
<td>Did not bind to BBMV of <em>S. exigua</em></td>
</tr>
<tr>
<td>4. Cry1Ab - Cry1C</td>
<td>H 265: Domain I of Cry1Ab and domain II&amp;III of Cry1C</td>
<td>No increase in toxicity level compared to Cry1C toxin towards insect cell line Sf9</td>
</tr>
<tr>
<td>5. Cry1Ac - Cry1C</td>
<td>Domain I of Cry1Ac and domain II&amp;III of Cry1C</td>
<td>No increase in toxicity level compared to Cry1C toxin towards insect cell line Sf9</td>
</tr>
<tr>
<td>6. Cry1C - Cry1E</td>
<td>Domain I of Cry1C and domain II&amp;III of Cry1E</td>
<td>Not much effective against insect cell line Sf9</td>
</tr>
<tr>
<td></td>
<td>Domain I of Cry1E and domain II&amp;III of Cry1C</td>
<td>Not much effective against Sf9 cell line</td>
</tr>
<tr>
<td>7. Cry3Aa-Cry1Ac</td>
<td>pUIBI-2: Domain I-II of Cry3Aa and domain III of Cry1Ac</td>
<td>Hybrid showed 88% mortality to <em>L. texana</em></td>
</tr>
<tr>
<td>Carmona and Iberra 1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Cry1Ab - Cry1Ca</td>
<td>H04: Domain I-II of Cry1Ab domain III of Cry1Ca</td>
<td>Activity 26 fold higher than parental toxin Cry1Ab</td>
</tr>
<tr>
<td>de Maagd et al, 2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hybrid Toxins Description</td>
<td>Activity/Specificity</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>9.</td>
<td>Cry1Ac - Cry1Ca&lt;br&gt;H130: Domain I-II&lt;br&gt;Cry1Ac domain III&lt;br&gt;Cry1Ca</td>
<td>Activity 25 fold higher than parental toxin Cry1Ac</td>
</tr>
<tr>
<td>10.</td>
<td>Cry1Ba - Cry1Ca&lt;br&gt;BBC 13: Domain I-II&lt;br&gt;of Cry1Ba and entire domain III of Cry1Ca</td>
<td>Not more active against <em>S. exigua</em> but highly active against <em>M. sexta</em> than Cry1Ba and comparable to Cry1Ca.</td>
</tr>
<tr>
<td></td>
<td>BBC 16: Domain I-II&lt;br&gt;of Cry1Ba and domain III of Cry1Ca (has more C-terminal crossover point)</td>
<td>Significant activity against <em>S. exigua</em>, but less than Cry1Ca. Higher activity against <em>M. sexta</em> than Cry1Ba and comparable to Cry1Ca. (Broaden spectrum)</td>
</tr>
<tr>
<td>11.</td>
<td>Cry1Da - Cry1Ca&lt;br&gt;DDC 49 &amp; DDC 51: Domain I-II of Cry1Da and domain III of Cry1Ca</td>
<td>Both the hybrid toxins did not show any significant activity against <em>S. exigua</em> and <em>M. sexta</em></td>
</tr>
<tr>
<td>12.</td>
<td>Cry1Fa - Cry1Ca&lt;br&gt;Domain I-II of Cry1Fa and domain III of Cry1Ca</td>
<td>Hybrid toxin was 5.5 times more toxic than Cry1Fa and comparable to that of Cry1Ca against <em>S. exigua</em></td>
</tr>
<tr>
<td>13.</td>
<td>Cry1Ba - Cry1La&lt;br&gt;Naimov et al, 2001&lt;br&gt;SN 15: Domain I-II of Cry1La and domain III of Cry1Ba</td>
<td>It was 2.5 times more toxic than Cry1La and 7.5 times more toxic than Cry1Ba against CPB (Change in specificity)</td>
</tr>
<tr>
<td></td>
<td>SN 16: Domain I-II of Cry1Ba and domain III of Cry1La</td>
<td>It showed very low activity against CPB</td>
</tr>
<tr>
<td></td>
<td>SN 19: Domain I &amp; III of Cry1Ba and domain II of Cry1La</td>
<td>Showed highest activity against CPB from all three hybrid toxins</td>
</tr>
<tr>
<td>14.</td>
<td>Cry1Ac - Cry1C&lt;br&gt;Rang et al, 2001&lt;br&gt;Domain I of Cry1Ac and domain II-III of Cry1C</td>
<td>Both hybrid toxins showed less than 5% mortality against <em>S. exigua</em> and <em>P. xylostella</em> compared to 100% mortality with parental Cry1Ca</td>
</tr>
<tr>
<td>15.</td>
<td>Cry1C - Cry1E&lt;br&gt;Rang et al, 2001&lt;br&gt;Domain I of Cry1E and domain II-III of Cry1C</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Cry1C - Cry1A&lt;br&gt;Sakai et al, 2007&lt;br&gt;GTS-CC1A: Domain I &amp; II of Cry1C and domain III of Cry1A</td>
<td>Showed higher activity against cell line Sf9 than Cry1C and Cry1A</td>
</tr>
<tr>
<td></td>
<td>GTS-CC4A: Domain I &amp; II of Cry1C and domain III of Cry4A</td>
<td>Showed higher activity than hybrid GST-CC1A, Cry1C and Cry4A toxins</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>17. CrylBa - CrylAc</th>
<th>pRK6: Domain I-II of CrylBa and domain III of CrylAc</th>
<th>Showed toxicity 16 fold higher than CrylBa to <em>H. virescens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Karlova et al 2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. CrylCa - CrylAc</td>
<td>RK15: Domain I-II of CrylCa and domain III of CrylAc</td>
<td>115 times higher than CrylCa to <em>H. virescens</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. CrylDa - CrylAc</td>
<td>RK7: Domain I-II of CrylDa and domain III of CrylAc</td>
<td>No toxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. CrylEa - CrylAc</td>
<td>RK8, 9, 10: Domain I-II of CrylEa and domain III of CrylAc</td>
<td>No toxicity</td>
</tr>
</tbody>
</table>

### 5A.2 Materials and Methods

#### 5A.2.1 Primer designing

Using Clustal W (www.ebi.ac.uk/Tools/msa/clustalw2), multiple sequence alignment of different *cryl* genes sequences was performed to locate the homologous regions. Internal primers were designed from these homologous regions so as to generate overlapping ends in the amplification products for recombination by OE-PCR. Conservation of ORF after OE-PCR was considered during primer designing. All primers were obtained from MWG-Bioron, Bangalore, India as listed in Table 5A.2.

#### 5A.2.2 PCR amplification of domain coding regions

Total DNA extraction from *Bt* strains was performed as described by Delecluse et al, 1991. *Pfu* DNA polymerase was used at each step of PCR amplification of domain coding regions, single strand amplification and ssOE-PCR to maintain high fidelity. Amplification of *crylAc* domain I-II coding region (1.4 kb) and *crylEa* domain III coding region (2.2 kb) was performed using total DNA of *Btk* HD73 and plasmid pTZ19R-crylEa respectively. Amplification of *crylAc* domain I (0.7 kb) and *crylAb* domain II-III (2.9 kb) coding regions was done using total DNA of *Btk* HD73 and *Btk* HD1.
respectively. PCR systems and programs are mentioned in Materials and Methods section 6.3.2.

Table 5A.2: Characteristics of primer pairs used in present study

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Ta &amp; extension time</th>
<th>Domain coding region amplified</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) cry1Ac Full F:</td>
<td>CGGGATCCATGGATAAACATCCGAAMAT</td>
<td>55°C, 1 min</td>
<td>Domain I cry1Ac</td>
</tr>
<tr>
<td>cry1Ac I R: GGGGAATTCGATAATCCTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) cry1Ab II-III F: GGGGATTCGATCCGGCAGCTAT</td>
<td>60°C, 3 min</td>
<td>Domain II-III cry1Ab</td>
<td>2960</td>
</tr>
<tr>
<td>cry1Ab Full R: TGTCGACTCTGACACCATACATTATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) cry1Ac Full F:</td>
<td>CGGGATCCATGGATAAACATCCGAAMAT</td>
<td>53°C, 1 min &amp; 30 sec</td>
<td>Domain I-II cry1Ac</td>
</tr>
<tr>
<td>cry1Ac I-II R: GCACACGATCGATCCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) cry1Ea III F: TTGGACACATCACAGTG</td>
<td>57°C, 2 min</td>
<td>Domain III cry1Ea</td>
<td>2184</td>
</tr>
<tr>
<td>cry1Ea III Full R: TGTCGACTATTCCCTCCATAAGAAGTA</td>
<td>&amp; 20 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) cry1A/Ea deg II-III F: TAATCAATACGAAATCGGTGGTA</td>
<td>49°C, 3 min</td>
<td>Domain II-III cry1</td>
<td>~2.8 kb</td>
</tr>
<tr>
<td>C32: TATCGGTTTCTGCGAAGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ta: Annealing temperature

5A.2.3 Single strand amplification of domain coding regions

In traditional OE-PCR final amplification of chimera is done using outer flanking primers, in the method described here single strand amplification of the respective domain coding regions was done by using only one flanking primer and the corresponding double strand amplification product as template DNA (Fig. 5A.1). PCR conditions for single strand amplification were same as for its double strand amplification of each domain coding region except use of a single primer and double strand amplification product as.
template DNA. PCR systems and programs are mentioned in Materials and Methods section 6.3.2. Single strand amplification of cry1Ac domain I coding region was done using only forward primer cry1Ac Full F (0.2 μM final concentration) with ~3 ng of its double strand amplification product. For single strand amplification of cry1Ab domain II-III coding region, primer cry1Ab Full R with ~6 ng of its double strand amplification product was used. Similarly for single strand amplification of cry1Ac domain I-II and cry1Ea domain III coding regions, primers cry1Ac Full F and cry1Ea III R were used respectively with ~5 ng of their double strand amplification product as template DNA. Electrophoresis of both single strand and double strand amplification product was performed on a 1% agarose gel to check purity and concentration.

5A.2.4 Generation of hybrid cry genes by single strand OE-PCR (ssOE-PCR)

Single strands generated as described above, were then used as templates in ssOE-PCR to perform recombination by an extension from free 3' ends after annealing (Fig. 5A.3). Equimolar concentration of single strand product of respective domain coding regions was used in ssOE-PCR with the following PCR program; first denaturation step at 94°C for 1 min, 15 cycles of denaturation step at 94°C for 30 seconds, annealing step at 50°C for 45 seconds and extension step at 72°C followed by final extension at 72°C for 10 min. The extension time in 15 cycles of PCR program was 3 min for recombination of cry1Ac and cry1Ab genes and 2 min 30 seconds for that of cry1Ac and cry1Ea genes. PCR systems and programs are mentioned in Materials and Methods sections 6.3.3 and 6.3.4.

5A.2.5 Expression of hybrid Cry toxins

The ssOE-PCR products of hybrid genes cry1AcAcEa and cry1AcAbAb were given Taq DNA polymerase treatment at 72°C for 10 min and cloned using InstTA clone PCR cloning kit, Fermentas, as per the manufacturer's
instructions. Hybrid genes were excised from pTZ57R vector backbone by restriction digestion with *Bam* HI and *Sal* I. Excised inserts were ligated to *Bam* HI and *Sal* I digested pET30a vector and transformed to *E. coli* DH5α, followed by transformation to *E. coli* BL21 (DE3). Methods for ligation, plasmid extraction and transformation are described in chapter 6. Expression of hybrid toxins was performed by induction of transformants using 0.5 mM IPTG and incubation at 30°C overnight.

5A.2.6 Purification of hybrid and native toxins

The overnight grown culture (100 ml) of *E. coli* BL21 transformants was centrifuged at 10,000 rpm for 10 min. Pellet was resuspended in 50 mM Tris buffer pH 8.0. Cells were lysed by lysozyme treatment (1 mg/ml final concentration and incubation for 30 min at 37 °C) and sonication treatment (at 35 amplitude for 3 min) followed by centrifugation at 10,000 rpm for 10 min and three times wash with buffer (0.1 M Tris, EDTA 1 mM, pH8.0, phenyl methyl sulphonyl fluoride 1 mM). Pellets were solubilized in 40 ml of 0.1 M Na₂CO₃ (pH 10.5) by incubating at 42 °C for four hours. Undissolved pellet was removed by centrifugation at 10,000 rpm for 5 min. Supernatant (30 ml) was mixed by equal volume of 2X native buffer (100 mM NaH₂PO₄, 600 mM NaCl, 20 mM imidazole) and pH was adjusted to 8.0 using NaOH. Mixture was passed through Ni-NTA sepharose column (5 ml, Qiagen) at linear flow rate of 1 ml/min to allow binding of 6X His tagged hybrid Cry proteins and flow through was discarded. Wash buffer volume of 30 ml (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) was passed through column for three times to wash unbound proteins. Elution of the hybrid Cry proteins was carried out by passing 10 ml volume of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) and collecting last 5 ml. Protein concentration of purified toxins was determined by Bradford method using bovine serum albumin as standard. Native Cry1Ac proteins were...
purified from *E. coli* ECE 53 strain harboring pKK223-cry1Ac constructs as described by Ge et al, 1990.

5A.2.7 Insect bioassay

Insect bioassay of native Cry1Ac and hybrid toxins Cry1AcAbAb and Cry1AcAcEa was performed with laboratory reared 3rd instar larvae of *H. armigera*. Four different concentrations with two fold increment from 0.125 to 1 µg/ml were used in triplicates for the assay. Twenty larvae were used for each replicate. An aliquot of 2.5 ml from Cry protein suspensions with 0.1% Triton X100 was spread homogenously on five cm² cabbage leaf piece and dried under laminar air flow. The leaf piece was further cut into 1 cm² leaf discs and single piece was fed to each larva kept individually. Insect bioassay with native Cry1Ac protein was performed in similar manner to compare the toxicity of hybrid toxins. Mortality was recorded on 7th day of treatment. Probit analysis was performed using EPA Probit Program version 1.5 to derive LC₅₀ values.

5A.2.8 Comparison of conventional OE-PCR with ssOE-PCR

Conventional OE-PCR was performed by directly mixing individual PCR amplification products of cry1Ac domain I and cry1Ab domain II-III coding regions. PCR system and program is mentioned in Materials and Methods section 6.3.5. The ssOE-PCR was performed for same genes as described earlier. Recombination products were loaded onto 1% agarose gel and electrophoresis was carried out to observe efficiency of recombination.

5A.2.9 PCR amplification using degenerate primer

Nucleotide sequences of cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca and cry1Ea genes were aligned using clustal W and degenerate primer cry1A/Ea deg II-III F was designed from the homologous region in block 2. Domain II-III coding regions of cry1Ac, cry1Ab, cry1Aa, and cry1Ea genes were amplified using plasmids containing respective gene as template and primers cry1A/Ea
deg II-III F and CJ2 (Table 5A.2). Plasmid constructs with various cryI genes were obtained from Bacillus Genetic Stock Center.

5A.3 Results

5A.3.1 Double strand and single strand amplification of domain coding regions

Expected amplification product size of 1450 bp and 2184 bp was obtained for cryIAc domain I-II and cryIEa domain III coding regions respectively (Fig. 5A.2A, lanes 1 & 3). Amplification products for single strands of respective domain coding regions were observed to migrate at the same position to its corresponding double strand products (Fig. 5A.2A, lanes 2 & 4). Similarly cryIAc domain I coding region (798 bp) and cryIAb domain II-III coding region (2960 bp) were amplified (Fig. 5A.2B, lanes 2 & 4). Single strand products of the respective domain coding regions also migrated at the same position to its corresponding double strand products (Fig. 5A.2B, lanes 1 & 3).

5A.3.2 ssOE-PCR for recombination of cryIAc and cryIEa genes

A short overlapping sequence of 18 bp with three mismatches was utilized to recombine domain coding regions of cryIAc and cryIEa genes. The reverse primer for amplification of cryIAc domain I-II coding region and forward primer for cryIEa domain III coding region were designed from the same 18 bp homologous sequences (Fig. 5A.4A) from block 3 so as to generate overlapping ends of 18 bp in amplification products (Fig. 5A.3A). By ssOE-PCR mediated recombination of cryIAc domain I-II coding region (1450 bp) with cryIEa domain III coding region (2184 bp) yielded an expected hybrid gene cryIAcAcEa of size 3616 bp (Fig. 5A.5A, lane 1).

5A.3.3 ssOE-PCR for recombination of cryIAc and cryIAb genes

In this case, extended overlapping sequence of 233 bp with 17 mismatches (Fig. 5A.4B) was utilized to exchange domain coding regions of cryIAc and cryIAb genes. Reverse primer for amplification of cryIAc domain I coding
region was designed from homologous site in domain II coding regions as shown in Fig. 5A.3B. Similarly forward primer for cry1Ab domain II-III coding region was designed from homologous site in domain I coding region so as to generate an overlap of 233 bp in amplification products (Fig. 5A.3B). Recombination of cry1Ac domain I coding region (798 bp) with cry1Ab domain II-III coding region (2960 bp) yielded an expected hybrid gene cry1AcAbAb of size 3.5 kb (Fig. 5A.5B, lane 1). Sequencing of hybrid cry1AcAbAb gene confirmed recombination of the two cry1 genes (GenBank Accession no. FJ536844).

5A.3.4 Expression and toxicity of hybrid Cry toxins

Hybrid genes were ligated with TA vector pTZ57R. Cloned fragments were released from the vector backbone by digestion with restriction enzymes. Expected bands of ~3 kb for vector backbone, 3.6 for cry1AcAcEa and 3.5 kb for cry1AcAbAb were observed (Fig. 5A.6). Released fragments were ligated with pET-30a expression vector, cloned into E. coli DH5α strain followed by plasmid extraction and transformation into E. coli BL21 strain for expression of hybrid proteins. Confirmation of the pET and hybrid gene constructs into E. coli DH5α clones and E. coli BL21 transformants was done by amplification with primers for domain coding regions (Fig. 5A.7). Confirmation for cry1AcAcEa and cry1AcAbAb genes was done by primers for amplification of cry1Ea domain III and cry1Ab domain II-III coding regions respectively. Expected bands of 2.1 kb and 2.9 kb were observed for cry1AcAcEa and cry1AcAbAb GENES respectively. Hybrid toxin Cry1AcAcEa showed a band of ~135 kDa protoxin when expressed in E. coli BL21 strain (Fig. 5A.8, lane 1). Toxicity of the hybrid toxin to H. armigera was observed to be 3.6 times lower than parental Cry1Ac toxin (Table 5A.3). A band of protoxin of size ~130 kDa was observed in SDS-PAGE analysis, upon expression of hybrid toxin Cry1AcAbAb into E. coli BL21 strain (Fig. 5A.8,
lane 2). However, LD_{50} toxicity of the toxin was observed to be 2.4 times lower than the parental toxin Cry1Ac (Table 5A.3).

Figure 5A.2: PCR amplification of double and single strands of domain coding regions for OE-PCR. (A) Lanes 1 & 2: dsDNA and ssDNA PCR product of cry1Ac domain I-II coding region respectively, Lanes 3 & 4: dsDNA and ssDNA PCR product of cry1Ea domain III coding region respectively (B) Lanes 1 & 2: ssDNA and dsDNA PCR product of cry1Ac domain I coding region respectively, Lanes 3 & 4: ssDNA and dsDNA PCR product of cry1Ab domain II-III coding region respectively, Lane M: DNA ladder

Figure 5A.3A: Schematic representation of primer positions, domain coding regions and strategy used for generation of cryl gene hybrids using cry1Ac and cry1Ea genes
Chapter 5A: Construction of hybrid Cry toxins & ssOE-PCR

Figure 5A.3B: Schematic representation of primer positions, domain coding regions and strategy used for generation of cry1 gene hybrids using cryIAc and cryIAb genes

\[
\text{cryIAc} \quad \text{TTGGGACACATCACAGTGC} \quad 18 \\
\text{cryIAb} \quad \text{TTGGGATACATCGTAGTGC} \quad 18
\]

\[\text{(A)}\]

\[
\begin{align*}
\text{cryIAc} & \quad \text{TGGGAGTTTCACTGACCAGCGCATTAAAATGCTTTTATGATTATAGTTATATTCATAGCCTGCTAT} \\
\text{cryIAb} & \quad \text{TGGGAAATTTCACTGACCAGCGCATTAAAATGCTTTTATGATTATATTCATAGCCTGCTAT}
\end{align*}
\]

\[
\begin{align*}
\text{cryIAc} & \quad \text{AACTTAGAAGATTTGACGCGACTATCAATAGTCGCTTATAATGATXGTTGCTTATTGGC} \\
\text{cryIAb} & \quad \text{TGGGGATTTGATGCCGCGACTATCAATAGTCGCTTATAATGATXGTTGCTTATTGGC}
\end{align*}
\]

\[
\begin{align*}
\text{cryIAc} & \quad \text{GATTCTAGAGATTGAGAGTGATGTTAATCTCAATTTAAGAGAAGAAGCTAACTTTACTATTAGATT} \\
\text{cryIAb} & \quad \text{GATTCTAGAGATTGAGAGTGATGTTAATCTCAATTTAAGAGAAGAAGCTAACTTTACTATTAGATT}
\end{align*}
\]

\[
\begin{align*}
\text{cryIAc} & \quad \text{GATATCGTTGCTCTATTCTCAAATTATGATAGTCGAAGGTATCCAATTCGAAC} \\
\text{cryIAb} & \quad \text{GATATCGTXTCTCTTXCTAGAAATTACGAXTCTAGATTATATCCAAXTCCAAC}
\end{align*}
\]

\[\text{(B)}\]

Figure 5A.4: Sequence alignments (A) 18 bp overlap sequences for recombination of cryIAc domain I-II coding region with cryIEa domain III coding region in first approach (B) 233 bp overlap sequences for recombination of cryIAc domain I coding region with cryIAb domain II-III coding region in second approach.
Figure 5A.5: Agarose gel electrophoresis of single strand OE-PCR product (A) Lane 1: *cry1Ac*-cry1Ea ssOE-PCR product, Lane M: DNA ladder (B) Lane 1: *cry1Ac*-cry1Ab ssOE-PCR product, Lane M: DNA ladder

Figure 5A.6: Release of insert from pTZ57R vector backbone by digestion with *Bam* HI and *Sal* I, lane M: mix DNA ladder, lane 1: pTZ-*cry1AcAcEa* digest, lane 2: pTZ-*cry1AcAbAb* digest
Figure 5A.7: Confirmation of clones and transformants by PCR amplification with primers for domain coding regions. Lane ML: mix DNA ladder, lane 1: colony lysate of *E. coli* DH5α harboring pET-cry1AcAcEa construct as template, lanes 2 & 3: colony lysate of *E. coli* BL21 harboring pET-cry1AcAcEa construct as template, lane 4: colony lysate of *E. coli* DH5α harboring pET-cry1AcAbAb construct as template, lanes 5 & 6: colony lysate of *E. coli* BL21 harboring pET-cry1AcAbAb construct as template.

Figure 5A.8: SDS-PAGE analysis of purified His tagged hybrid toxins through Ni-NTA column. Lane M: Protein molecular weight marker, lane 1: Cry1AcAcEa proteins, lane 2: Cry1AcAbAb proteins.
Table 5A.3: LC₅₀ values of parental and hybrid toxins against *H. armigera*

<table>
<thead>
<tr>
<th>Toxins</th>
<th>LC₅₀ µg/ml</th>
<th>95% Confidence limits</th>
<th>Slope ± (SE)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Cry1Ac</td>
<td>0.368</td>
<td>0.268 - 0.504</td>
<td>1.37 (0.26)</td>
<td>1.008</td>
</tr>
<tr>
<td>Cry1AcAcEa</td>
<td>1.354</td>
<td>0.968 - 2.831</td>
<td>1.99 (0.47)</td>
<td>0.570</td>
</tr>
<tr>
<td>Cry1AcAbAb</td>
<td>0.906</td>
<td>0.614 - 0.949</td>
<td>1.22 (0.27)</td>
<td>0.147</td>
</tr>
</tbody>
</table>

X²: Chi square values for heterogeneity was less than tabular values (p < 0.05).

5A.3.5 Single strand Overlap extension PCR (ssOE-PCR)

The basic OE-PCR technique was devised for generation of site directed mutagenesis by Ho et al, 1989. It has also applications in gene recombination [Horton et al, 1989] and gene synthesis [Stemmer et al, 1995]. OE-PCR basically involves the use of two flanking and two overlapping primers as depicted in Chapter 1. Two separate amplifications using individual pairs of primers are done. The PCR products are then mixed, where they anneal through their overlapping region and extended to full length gene through their free 3'. The product obtained is then PCR amplified for cloning. However it has been modified over time by many investigators to improve the method [Urban et al, 1997, Wurch et al, 1998, Mehta et al, 1999, An et al, 2005, Peng et al, 2006, Xiao et al, 2007].

However there are a few drawbacks still associated with the method which need to be addressed.

1) Low efficiency of recombination [Mehta et al, 1998; Andag et al, 2001]
2) Smear and or no amplification of full length hybrid genes [Gao et al, 2003; Xiong et al, 2004].

Similar problems were faced during recombination of cry genes in present study which could be due to larger size of fragments. A modification was done
in the method to increase the efficiency of recombination and allow easy cloning of the recombinant gene. The above drawbacks can be taken care of by simply getting rid of those single strands which after annealing cannot be extended due to presence of 5' ends as shown in figure 5A.9 marked as (×).

![Figure 5A.9: Schematic representation of overlap extension PCR](image)

A simple strategy was used to tackle the problem. Single strand amplification was done using double stranded amplification products as template. Here the primer used was the one that amplifies the strand which after annealing has free 3' end that can be extended further as shown in figure 5A.9 marked as (√) above.

### 5A.3.6 Comparison of conventional and ssOE-PCR

The ssOE-PCR was performed using single strands of domain I and domain II-III coding regions. Conventional method of OE-PCR was performed using double stranded PCR amplification product of domain I and domain II-III coding regions. Electrophoresis of conventional OE-PCR products and ssOE-PCR products was done to observe efficacy of the methods. Products of conventional OE-PCR showed a band of recombinant gene of 3.5 kb size along with two more bands of size ~2.9 kb and ~800bp (Fig. 5A.10 lane 1). These bands might represent unused single strands of domain II-III and domain I coding regions respectively. Products of ssOE-PCR showed an intense band of
3.5 kb which represents recombinant (hybrid) gene. Single strands of domain II-III coding region have been completely utilized, while very few single strands of domain I coding region have remain unutilized as represented by a very faint band of size 800 bp. As expected, 100% recombination was achieved with ssOE-PCR but not with the conventional method.

![Figure 5A.10: Agarose gel electrophoresis of conventional and ssOE-PCR](image)

Figure 5A.10: Agarose gel electrophoresis of conventional and ssOE-PCR

5A.3.7 Amplification of domain coding regions using degenerate primers

In order to ascertain the exchange of domain coding regions among multiple cry genes for construction of hybrid cry $l$ gene library, a degenerate primer cry1A/Ea deg II-III F was designed from block 2 (Fig. 5A.11a). Successful amplification of size ~2.7 kb for domain II-III coding region of cry1Ac, cry1Ab, cry1Aa, cry1Ba, cry1Ca, cry1Ea genes with the degenerate primer was obtained (Fig. 5A.12). Similarly sufficient homology required to design other degenerate primers was also located within block 3 by multiple sequence alignment among cry1la and other cry genes (Fig. 5A.11b).
Figure 5A.11: Sequence alignments for primer design (A) degenerate primer cry1A/Ea deg II-III F at conserved block 2 and (B) degenerate primer design at conserved block 3. Note: Dark shaded region represents sequences for degenerate primer design.

Figure 5A.12: PCR amplification with degenerate primer cry1A/Ea deg II-III F using plasmid constructs of various cry1 genes.
5A.4 Discussion

A majority of the Cry toxins consist of five conserved blocks [Hofte and Whiteley 1989]. Block 2 was observed to be located at the boundary region between domain I and domain II coding regions, while block 3 at that of domain II and domain III [Knight et al, 2004]. In present investigation, the conserved blocks were found to contain sufficient nucleotide sequence homology for designing primers to be used for recombination of domain coding regions. OE-PCR being a well known approach for site directed mutagenesis and gene recombination, an OE-PCR based strategy for domain swapping of Cry toxins was designed using conserved block 2 and 3. Also exchange of domains from the boundary region would have less impact on structure and function compared to exchange started within domain coding regions.

The overlap sequences selected from conserved block 2 and 3 for OE-PCR showed presence of mismatches during alignment. Thus two approaches were used in order to determine an easier approach for exchange of larger sizes of cry1Ab and cry1Ea domain coding regions. Although ~2.1 kb sequence of cry genes encoding three domains is sufficient for the activity, study of full length cry genes was preferred in order to test the efficiency of two approaches. The first approach was tried with 18 bp overlap sequence including three mismatches for recombination of cry1Ea domain III coding region of size 2184 bp. Since increase in length of overlap region improves heteroduplex formation [Meza et al, 1996], 233 bp overlap region (17 mismatches) was considered in second approach for recombination of cry1Ab domain II-III coding region of size 2960 bp. Though both approaches were successful for recombination and cloning, second approach could be preferred more comparatively. Concurrent to results by Meza et al, 1996 it yielded more assembled fragments thereby making easy for cloning. In addition it also demonstrated the possibility of recombining larger size of 2.9 kb fragments.
Nevertheless both approaches can be used successfully depending on the length of overlap sequence available for recombination.

Replacement of Cry1Ac domain III with that of Cry1Ea resulted in 2.4 folds low toxicity to *H. armigera* during present study. Thus indicating domain III of Cry1Ac plays an important role directly or indirectly in pore formation. Role of domain III in increasing toxicity of hybrid toxins was elucidated by [de Maagd et al, 1996a, Karlova et al, 2005]. In contradiction, Sakai et al, 2007 observed domain III not crucial for replacement in Cry1C toxins. Hence it can be concluded that domain III has diverse effects in different Cry toxins.

Cry1Ab toxins are shown to be one of the most toxic proteins to *H. armigera* [Liao et al, 2002]. Estela et al, 2004 showed different receptor sites for Cry1Ab and Cry1Ac toxins in *H. armigera* besides some common sites. Considering these facts in mind the domain I of Cry1Ab was replaced with that of Cry1Ac. However lower activity of the hybrid Cry1AcAbAb toxins compared to parental toxin was observed in present study. Similarly Rang et al, 2001 observed lower toxicity of four hybrid toxins containing domain I from other toxins. Thus it indicates pore forming ability of domain I could be difficult to improve upon exchange with other toxins.

Overall low toxicity of hybrid toxins along with the parental toxin Cry1Ac could be attributed to variations observed in susceptibility of *H. armigera* populations from different states of India [Gujar et al, 2007]. Nevertheless, domain swapping should be performed with many other Cry toxins in order to achieve desired results [de Maagd et al, 2000; Karlova et al, 2005; Rang et al, 1999].

The basic OE-PCR technique for generation of site specific mutations or hybrid genes has been modified over time since its discovery. The main reasons were low efficiency of recombination [Mehta et al, 1998], smear and or no amplification of full length hybrid genes [Gao et al, 2003]. Similar problems were faced while generating hybrid *cry* genes of *Bacillus*...
The difficulty was due to larger size of the DNA fragments to be assembled. To overcome this problem, the number of cycles for assembly PCR was increased to maximize the chimeric gene product. However, generation of smear and unused strands leads to unsatisfactory amplification of chimeric gene. Thus further modification was done by employing single strands of DNA fragments for assembly PCR.

The ssOE-PCR method was observed to result in 100% recombination of two amplification products compared to traditional method. The major advantage of using the ssOE-PCR method was that the efficiency of recombination increased to 100%. Second advantage was easier cloning of the recombinant product. Since the ssOE-PCR method produces higher yields of the recombinant product, it can be eluted from the gel and cloned directly. Thus amplification of the recombinant product as in traditional method is not required in ssOE-PCR. The third advantage was the efficiency of recombining large size of DNA fragment up to 3 kb.

Degenerate primers designed from homologous regions of conserved block 2 and 3 would greatly reduce the task of designing and using many specific primers for each cry gene. Rather a single primer would serve the purpose of domain swapping among many cry genes and construction of library of hybrid cry genes. In case of recombination between distantly related cry genes where homologous region cannot be found, the gene splicing method (SOEing) of OE-PCR [Horton et al, 1989] or Sticky PCR [Yamabhai 2009] can be used. Henceforth, OE-PCR can be useful for domain swapping among any cry genes.

Since the methods used till date are random or cumbersome, there is a need for an appropriate method. OE-PCR strategy with the ease of recombination and no constraint of the choice for cry genes would be the method of choice for generation of hybrid Cry toxins for future use.
Chapter 5B

In silico structural analysis of Cry toxins
Chapter 5B: In silico structural analysis of Cry toxins

5B. 1 Introduction

Protein structure analysis is important in order to understand the structure-function relationship, molecular mechanism of particular domains and interaction with other proteins, ligands or receptors. With the advancement in bioinformatics, large numbers of computational tools are available to perform in silico structural analysis of proteins. Swiss Institute of Bioinformatics server named Expasy (http://expasy.org/tools/) and European Bioinformatics Institute server (http://www.ebi.ac.uk/Tools/structural.html) mainly provides tools for these purposes.

Homology modeling is one of the computational tools to predict the three dimensional (3D) structure of a protein using a template structure of other protein which has been experimentally determined. It is the most reliable and accurate method for structure prediction. It is useful for functional characterization of proteins. Structure based homology searches can be used to assign protein families. Location of active sites, receptor/ligand binding sites, domains etc. is also possible with the predicted structure. Interaction studies with other proteins, ligands or drug molecules can also be performed. The predicted structure can serve as basis for mutagenesis experiments for improvement in activity or change in specificity etc.

Similarly structural alignment (superimposition) is another tool to compare structure of two or multiple proteins. It can help to identify conformational and topological differences among similar proteins. Structural variations in active sites or receptor/ligand binding sites can be detected.

On the other hand, protein dossier tool is useful for analysis of various physicochemical properties of protein sequence [Neshich et al, 2004]. Each amino acid in the sequence can be annotated for various parameters through graphical view.
Chapter 5B: In silico structural analysis of Cry toxins

Structure of various Cry1Aa, Cry2Aa, Cry3Bb, Cry4Aa proteins have been determined experimentally [Grochulski et al, 1995, Morse et al, 2001, Cody 2001, Boonserm et al, 2005]. Prediction of theoretical 3D structure of other Cry toxins such as Cry5Aa and Cry10Aa have been done using these structures as templates [Xin-Min et al, 2009, Mahalakshmi et al, 2010]. In this study we have predicted theoretical 3D structures of Cry1Ab and Cry1Ac proteins. Structural alignment of these predicted structures was performed with Cry1Aa and native protein structures and analyzed.

5B.2 Materials and Methods

5B.2.1 Structure prediction
The structure of Cry1Ab and Cry1Ac protein was predicted by homology modeling using Cry1Aa (1CIY) as template. Amino acid sequence of Cry1Ab (M13898) and Cry1Ac (M11068) proteins was submitted to the Swiss Model server (http://swissmodel.expasy.org/workspace) for structure prediction. PDB file obtained from the server was viewed with Discovery studio visualizer 3.0 (http://accelrys.com/products/discovery-studio).

5B.2.2 Structural alignment
Multiple protein structural alignment was performed by submission of PDB files of Cry1Aa and Cry1Ab proteins to online structural alignment program Protein 3D fit (http://protein3dfit.tu-bs.de/cgi-bin/3daligner.py). PDB file for structural alignment was viewed with Discovery studio visualizer 3.0 to analyze the difference in structure of the two proteins. Similarly PDB files from Cry3Ba and Cry3Bb amino acid sequences (X17123 & M89794) were generated and structural alignment was performed. Accordingly structural alignment of Cry1Ac & Cry1Ea and Cry1Ac & Cry1Ab was performed.

5B.2.3 Structural analysis
For structural analysis of proteins, Protein dossier program was used from Blue Star Sting web server (http://sms.cbi.cnptia.embrapa.br/SMS). Amino
acids were mapped and evaluated according to: conservation, solvent accessibility, side-chain volume, effect on amino-acid interactions, protein electrostatics, and physicochemical properties [Neshich et al, 2004].

5B.3 Results:

5B.3.1 Three dimensional structures of Cry toxins

From the figure 5B.1, it can be observed that the toxins have almost similar three dimensional (3D) structures. They have 95% amino acid sequence similarity, which is also evident from the structure resemblance of the toxins. Mutational studies have shown that amino acids comprising the loop regions are important for specificity [Schnepf et al, 1998]. The importance of these amino acids is also confirmed by the fact that these loop regions are present at the surface, thus making their interaction with receptor more favorable.

Figure 5B.1: Three dimensional structures of Cry1Aa and Cry1Ab. Note: Coiled Ribbon: α helices, Straight ribbon: β pleated sheets, Gray line: loops
5B.3.2 Structural alignment of Cry1Aa and Cry1Ab

The amino acid sequence alignment shows that there is more than 95% homology whereas only 75% homology is shared in domain II and domain III among Cry1Ac and Cry1Ab genes. The difference in the sequence in domain II-III is not as a contiguous stretch but mostly spread throughout the primary sequence (Fig. 5B.2). However this difference is not apparent in the tertiary structure of domain II and III. The tertiary structure did not show major differences except in the loop regions. In the figure 5B.3 showing structural alignment of the two toxins, domain I exhibited exact superimposition and similar was the case with the β-sheets of domain II and domain III. However the loop region in domain II differed markedly as highlighted with gray (round) portion. Amino acids at these positions (337-341, I I L G S in Cry1Aa and R P F N I in Cry1Ab) were also different in both toxins.

5B.3.3 Structural alignment of Cry3 proteins

Similarly Cry3Ba and Cry3Ba proteins showed exact superimposition except the loop regions (Fig. 5B.4). The round highlighted portion shows difference in the structure in domain II and square highlighted portion in domain III respectively. In order to analyze the structure function parameters the protein dossier tool was used. The amino acids showing difference in domain II were valine, tryptophan and proline position (409-411) and in domain III the positions were 516-528 & 571-578. These amino acids showed highest solvent accessibility, side chain volume and interface contacts when analyzed by protein dossier (Fig. 5B.5).
Chapter 5B: In silico structural analysis of Cry toxins

Figure 5B.2: Primary sequence alignment of domain II in Cry1Aa and Cry1Ab proteins. Dark areas indicate exact match and light areas indicate mismatch.

Figure 5B.2 Structural alignment of Cry1Aa and Cry1Ab proteins
Figure 5B.4: Structural alignment of Cry3Aa and Cry3Bb proteins
**5B.3.4 Structural alignment for hybrid toxins**

As described in chapter 5A, hybrid toxin Cry1AcAcEa had domain I & II from Cry1Ac and domain III from cry1Ea and hybrid Cry1AcAbAb had domain I from Cry1Ac and domain II & III from Cry1Ab. In order to observe differences between the 3D structures of hybrid and native Cry toxins, the 3D structures were predicted and aligned. Structure of Cry1Ea was predicted as for Cry1Ab. Five major structural differences of Cry1Ea compared to Cry1Ac were observed as pointed by arrows in figure 5B.6a. Topological differences in the loops of domain II were observed as shown by arrows 1 and 2 (Fig. 5B.6a). Position of β-sheet was changed and a loop in domain II was absent as shown by arrow 3. Similarly difference in structure of β-sheet and loop of domain III was observed as shown by arrow 4 and arrow 5 respectively. Accordingly differences were observed in Cry1Ab compared to Cry1Ac (Fig. 5B.6b). Difference in loop structure and presence of an extra β-sheet was observed in domain II as shown by arrows 1 and 2 respectively. Also structure of β-sheet in domain III was found different as shown by arrow 3.

![Structural alignment of Cry1Ac with Cry1Ea proteins](image)

Figure 5B.6a: Structural alignment of Cry1Ac with Cry1Ea proteins
5B.4 Discussion

The molecular mechanism of Cry toxins for insect toxicity has remained controversial as described in chapter 1. A complete understanding of Cry toxin structure can help better understand the mechanism. Towards this effort, X-ray crystallography for structure determination has been performed for several Cry proteins. The \textit{in silico} methods of structure prediction have been developed to construct a theoretical model with higher accuracy. As a result theoretical three dimensional structures for Cry1Ab21 [Kasyap et al, 2011], Cry2Ab10 [Lin et al, 2008], Cry5Aa [Xin-Min et al, 2009], Cry5Ba [Xia et al, 2008] and Cry11B [Gutierrez et al, 2001] have been constructed by homology modeling using the known structures as templates. Structure of \textit{S. littoralis} receptor protein aminopeptidase N (APN) was also predicted by homology modeling [Alejandro 2008]. Lin et al, 2008 performed molecular docking studies with theoretical 3D structure of Cry2Ab10 and predicted
receptor binding pocket for APN. These structures can be useful to provide information regarding structural similarities and difference compared to other toxins. At the same time docking studies could provide useful information for receptor binding sites.

Amino acid sequence similarity was reflected in structures of Cry1Aa and Cry1Ab proteins (Fig. 5B.1). Structural alignment of the two toxins revealed difference only in loop regions of domain II. Similarly Kashyap et al, 2011 observed difference in these loop regions in comparison to Cry1Aa and Cry1Ac proteins. Mutational studies reviewed by Schnepf et al, 1998 also supported the role of domain II loops in specificity determination. Domain II is made of three antiparallel β-sheets, oriented parallel to the α-helices of domain I. Apex of domain II is formed by three surface exposed loops of variable length and the tips of these hairpins are comprised of residues 310 to 313, 367 to 379 and 438 to 456 from sheets 1, 2 and 3 respectively [Grochulski et al, 1995]. These surface exposed loops located in the hypervariable blocks of domain II of the δ-endotoxins are identified as specificity determining regions. Also, this region has difference in the amino acid sequence in different toxins as shown in sequence alignment file (Fig. 5B.2). Thus, the highlighted region of loop can be predicted to be responsible for changed specificity of the two toxins.

Cry3Ba (CryIIIb) and Cry3Bb (CryIIIb2) exhibited 94% nucleotide sequence similarity but displayed different toxicity to Colorado potato beetle (CPB) and southern corn root worm [Donovan et al, 1992]. Cry3Bb protein was toxic to both insects while Cry3Ba only to CPB. The loop regions of domain II and III were found topologically different during structural alignment of these proteins indicating role of these loops in specificity determination and toxicity. Structure function analysis by Protein dossier also supported the role of these loops in interaction with receptors of insects. The highest solvent
accessibility, side chain volume and interface contacts by the amino acids in these loop regions indicated their possible role in receptor interaction.

Similarly several differences observed during structural alignment of Cry1Ac with Cry1Ab and Cry1Ea proteins revealed possible role of loop regions and β-sheets in domain II and III for specificity. Cry1Ab protein structure showed more differences compared to that of Cry1Ea which could be due to the reason that Cry1Ab is more closely related to cry1Ac than Cry1Ea comparatively. These differences could possibly be involved in lowering the toxicity of hybrid toxins towards *H. armigera* as described in chapter 5A. The loop regions in domain II of both Cry1Ea and Cry1Ab proteins were topologically different than its counterpart in Cry1Ac protein. As a result the binding of the toxins could be affected which lead to lower toxicity.

Besides structure prediction and structural alignments, *in silico* structural analysis can be useful to predict role of various structural elements of proteins, their interaction with receptor, ligand or drug molecules, inter or intra chain/domain amino acid interactions. Mutational studies can be planned to achieve desirable change in structure or function of a protein. At the same time it can help to develop strategy for domain swapping among similar proteins.