

Protein Engineering of *Bacillus thuringiensis* Toxins

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Insect-resistant crops have been one of the major successes of applying plant genetic engineering to agriculture. Transgenic crops are part of global agriculture has led to considerable reduction in pesticide usage and lower production costs. The source of the insecticidal toxins produced in commercial transgenic plants is the soil bacterium *Bacillus thuringiensis* (*Bt*). *Bt* strains show differing specificities of insecticidal activity toward pests and constitute a large reservoir of genes encoding insecticidal proteins, which are accumulated in the crystalline inclusion bodies produced by the bacterium on sporulation (Cry proteins, Cyt proteins) or expressed during bacterial growth (Vip proteins). However, the future of *Bt* crops is threatened by the possible evolution of insect resistance. Owing to the enormous selective pressure imposed by widespread use of Cry proteins in agriculture worldwide, the development of better Cry toxins with broader insecticidal spectrum is of ever increasing importance. An emerging strategy is the use of protein engineering approaches to counter the adaptive behavior of insects based on a molecular understanding of the toxin and its interaction with proteins in the insect gut. The ultimate goal of protein engineering of the insecticidal Cry proteins is to be able to design any Cry toxin to possess toxic activity against any insect. A more immediate goal is to introduce a specific activity into a toxin that does not possess it.

Protein engineering studies are based on methods for introducing mutations in the genes that encode proteins of interest and for producing the proteins in large amount in bacteria (*E. coli*) for further analysis. In the last decade numerous modifications were created in δ -endotoxins using various protein engineering techniques such as a) single or multiple amino acid change in variable and conserved regions through site directed mutagenesis; b) restriction fragment exchange between closely related cry toxins or with other bacterial toxins; and c) exchange of domains

between toxins through PCR mediated cloning or *in vivo* recombination in Rec A+ *E. coli* strains. The effects of mutations or modifications in delta endotoxins have been studied on various aspects like, 1) pore forming capacity in artificial membranes and measuring ion channel conductance using voltage patch clamping, light scattering and light quenching techniques; 2) binding of toxins to BBMV isolated from susceptible larval midguts using labeled toxins; 3) stability of proteins using proteases like trypsin and larval midgut juice; 4) *in vivo* toxicity analysis-larval bioassay; 5) lysis of cells *in vitro* in insect cell lines lawn assay; 6) prediction of secondary structural changes using CD spectrometry. Mutagenesis of Cry toxins has been used extensively in studying the mechanism of action of these proteins (Bravo *et al.*, 2007) and has been exploited to produce novel recombinant toxins.

Structure and sequence-based engineering

Properties of proteins are determined by their three dimensional (3D) structure, with the precise configuration of specific amino acid residues contributing to the functional site(s) within the protein. In structure-based protein engineering, appropriate sites are selected for mutation based on an examination of the 3D structure of the protein and the mutants characterized for desired activity. Similarly, knowledge-based protein engineering of the family of δ -endotoxins from *Bacillus thuringiensis* has resulted in the development of novel toxins with enhanced insecticidal activity and specificity. In addition to the “high dose/refuge strategy” recommended by the U.S. Environmental Protection Agency (www.epa.gov/pesticides/biopesticides/white_bt.pdf), an emerging strategy is the use of protein engineering approaches to counter the adaptive behavior of insects based on a molecular understanding of the toxin and its interaction with proteins in the insect gut.

Mutagenesis of three-domain Cry toxins

Mutations were carried out to: (1) increase hydrophobicity of the protein in regions in domain I containing sheets of bound water molecules and in loop regions; (2) increase the mobility of the channel-forming helices in domain I by disrupting hydrogen-bond formation; (3) increase the mobility and flexibility of loop regions in domain I; (4) alter potential ion-pair interactions and metal-binding sites; and (5) reduce or eliminate binding to carbohydrates in the insect gut by mutation of a loop region between domains I and II.

Domain exchange in three-domain Cry toxins

✓ **Mechanisms of evolution**

Phylogenetic studies suggested that the members of this family evolved from a common origin and that the high diversity of these proteins might be generated by sequence divergence and by homologous recombination that leads to domain III and carboxy-terminal extension interchange among different toxins. It was suggested that the Cry9Aa toxin evolved independently from other Cry9 toxins but obtained the same C-terminal extension, probably by a recombination. There are multiple clear examples of domain III swapping, particularly among Cry1 toxins. Particularly interesting are the toxins with dual (lepidopteran and coleopteran) insect-order specificity, Cry1I and Cry1B toxins, with domains I and II that share high similarity with coleopteran specific toxins, whereas their domains III are more closely related to those of lepidopteran-active toxins. Moreover, interchange of domain III sequences confirmed the hypothesis that domain III shuffling can be a mechanism for generating new specificities in nature.

✓ **Domain swapping as a putative mechanism for evolution of new specificities**

Although it seems that domains I and II have coevolved, experimentation shows that this is not necessarily owing to structural constraints because domain I swapping between different toxins can yield biologically active toxins. Such experiments show that exchange of domain I can affect the size of the membrane pores formed and can negatively affect toxicity against an insect. Although domains I and II, and in some cases domain III as well, seem to have co-evolved towards certain specificities, there are also strong indications that part of the variation in toxin structure and specificity is a result of domain III swapping by homologous recombination, especially among Cry1 toxins. The structural similarity of all members of the family of three-domain *Bt* toxins and the separate roles of the domains in the processes of receptor binding and channel formation, suggested that combining domains from different proteins could generate active toxins with novel specificities.

Advantages of Protein Engineering

- Protein engineering also helps in understanding the structure-function relationships of Cry toxin proteins and also in creating mutated proteins with an enhanced insecticidal activity.
- Site directed mutagenesis and domain exchange studies demonstrated that the chimeric toxins could be produced so as to increase the toxin activity, spectrum of activity or direct resistance towards a particular type of insects (de Maagd *et al.*, 2001). These new insecticidal toxins may provide new weapons for the fight against insect infestation.
- The reason identified for resistance development is loss of receptor binding and cross resistance is observed when δ -endotoxins with high homology are used. Therefore use of δ -

endotoxins which bind to different receptors in combination might delay the resistance and cross resistance development in insects. These toxins could be used in resistant management as alternatives for the toxins already in use to which insects may become resistant by losing receptor

Conclusion

About 40% of the currently identified Bt toxins are not active on insects, due to various reasons like low solubility in the insect gut environment, lack of binding to Brush Border Membrane Vesicles in the larval midgut, presence of protease cleavage sites. Knowledge of δ -endotoxins can be utilized to make these inactive toxins active by protein engineering. Domain swapping experiments suggests that domain swapping between two *cry* genes with limited stretches of homology and subsequent screening for the production of soluble protoxin can be a powerful tool to modify toxicity and to increase available repertoire of active toxins against pest insects. Evolution of resistance to more than one toxin is associated with the alteration of a common binding site in several insect species. Thus, knowledge of which toxins share binding sites can help in choosing appropriate sets of toxins for delaying resistance.

References

1. Bravo, A., Gill, S.S. and Soberon, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **49**:423-435.
2. 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.