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**Study of the Basis for Toxicity and Specificity
of *Bacillus Thuringiensis* δ -Endotoxins**

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STUDY OF THE BASIS FOR TOXICITY AND SPECIFICITY OFBACILLUS THURINGIENSIS δ -ENDOTOXINSInvestigators' Names
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**STUDY OF THE BASIS FOR TOXICITY AND SPECIFICITY
OF *Bacillus thuringiensis* δ -ENDOTOXINS.**

Yechiel Shai, Arthur Aronson, Aviah Zilberstein and Bruch Sneh

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1. SUMMARY

The report contains three parts which summarize the three years' achievements of the three participating research groups; The Weizmann group, Tel-Aviv group and Purdue group. The first part describes the achievements obtained by Shai's group toward the elucidation of the mechanism of membrane insertion and the structural organization of the pores formed by the Cry3A and Cry1Ac *B. thuringiensis* δ -endotoxins. For that purpose Shai's group synthesized, fluorescently labeled and structurally and functionally characterized peptides corresponding to the seven helices that compose the pore-forming domain of Cry3A toxin, including mutant peptides and the hairpin $\alpha 4 \leftrightarrow \alpha 5$ of both Cry3A and Cry1Ac toxins composed of $\alpha 4$, $\alpha 5$ and the loop connecting $\alpha 4$ - $\alpha 5$. Among the synthesized peptides were three mutated $\alpha 4$ helices based on site directed mutagenesis done at Aronson's group that decreased or increased Cry1Ac toxicity. The results of these studies are consistent with a situation in which only helices $\alpha 4$ and $\alpha 5$ insert into the membrane as a helical hairpin in an antiparallel manner, while the other helices lie on the membrane surface like ribs of an umbrella (the "umbrella model"). In order to test this model Shai's group synthesized the helical hairpin $\alpha 4 \leftrightarrow \alpha 5$ of both Cry3A and Cry1Ac toxins, as well as,. Initial functional and structural studies showed direct correlation between the properties of the mutated helices and the mutated Cry1Ac. Based on Shai's findings that $\alpha 4$ is the second helix besides $\alpha 5$ that insert into the membrane, Aronson and colleagues performed extensive mutation on this helix in the Cry1Ac toxin, as well as in the loop connecting helices 4 and 5, and helix 3 (part two of the report). In addition, Aronson performed studies on the effect of mutations or type of insect which influence the oligomerization either the Cry1Ab or Cry1Ac toxins with vesicles prepared from BBMV. In the third part of the report Zilberstein's and Sneh's groups describe their studies on the three domains of Cry1C, Cry1E and cry1Ac and their interaction with the epithelial membrane of the larval midgut. In these studies they cloned all three domains and combinations of two domains, as well as cloning of the pore forming domain alone and studying its interaction with BBMV. In addition they investigated binding of Cry1E toxin and Cry1E domains to BBMV prepared from resistant (R) or sensitive larvae. Finally they initiated expression of the loop $\alpha 4 \leftrightarrow \alpha 5$ Cry3A in *E. coli* to be compared with the synthetic one done by Shai's group as a basis to develop a system to express all possible pairs for structural and functional studies by Shai's group (together with Y. Shai).

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OBJECTIVES OF THE ORIGINAL PROPOSAL

The objective of the proposed study is to have a better understanding of the mode of action and the basis for specificity of B.t. toxins. This will be achieved by using molecular biology and peptide synthesis approaches combined with functional and spectroscopic methods, to define the role of domain I in ion channel formation and the role of domains II and III in insect specificity. More specifically;

- (i) Introduction of single cys residues into each helix of domain I in the intact toxin and in synthetic helices, as well as, important loop between helices 2 and 3 (A92 and R93 residues in CryIAc).
- (ii) Use of fluorescent tag to determine location of each helix in lipid bilayer and in insect vesicle membranes.
- (iii). Determination of the localization of the helices using membranes that contain or that do not contain receptors to investigate the role of receptor in toxin location.
- (iv). To use probes to test if any of the helices interact or aggregate in the membrane
- (v). In different toxin molecules, differentially label helices known to insert to see if they interact or are in close proximity .
- (vi). If data with mutated toxins will correlate well with peptide data, it will be possible to determine the importance of helices interactions. It will be also possible also synthesize altered peptides to determine which aspects of sequence or structure is important.
- (vii). CryIAc point mutations, especially in helix 5, with low or no toxicity for cys-labeling will be also investigated to establish basis for toxicity loss.
- (viii) . The specificity region of the CryIC toxin will be defined by using larvae and membrane vesicles from a colony of *Spodoptera littoralis* sensitive to this toxin but resistant to the related CryIE toxin. Hybrid or mutant cryIE genes will be constructed and the products tested for their ability to overcome the resistance.

MODE OF COLLABORATION

The Weizmann and Tel-Aviv groups have an on-going collaboration in the following aspects: polypeptide labeling, development of methods to efficiently evaluate receptor – ligand binding and isolation of brush-border membrane vesicles. The Tel-Aviv Univ. group has focused the efforts towards expressing all three structural domains of Cry1C and Cry1E and combinations of domains in *E. coli* expression systems. With the aid of the Weizmann group, these Cry domains have been separated by FPLC and labeled with the fluorescent probes for receptor – ligand binding. In addition the Tel-Aviv groups are expressing one or more alpha helices by using *E. coli* expression vectors, in order to be used in functional studies by the Weizmann group. The same peptides are chemically synthesized in parallel in the Weizmann laboratory. At present the Tel-Aviv groups were able to express the $\alpha 4$ - $\alpha 5$ hairpin loop, which was further purified by the Weizmann group. However, at present the amount was insufficient to carry functional studies. Nevertheless, Shai group were able to make at present a synthetic $\alpha 4$ - $\alpha 5$ hairpin which showed membrane permeation activity. Since peptide synthesis of such long polypeptides is complicated, studies towards expression of the loop are still in progress by both the Tel-Aviv and the Purdue group in order to allow preparation of $\alpha 4$ - $\alpha 5$ hairpin mutants.

Prof. Aronson has helped the Tel-Aviv group in all aspects of introducing genes into *B. thuringiensis* and using *E. coli* – Bt shuttle vectors. They have also a continuous exchange of information and know-how with regard to domain binding and performance of ligand blotting. At the beginning of the study Prof. Aronson visited the Tel-Aviv and the Weizmann labs to form the basis of the mutual project. In September 1997 Yechiel Shai, and in June 1998, Aviah Zilberstein visited Aronson's group at Purdue University. The visit was focused on analyzing the data and planning further studies according to the already obtained results.

Studies on the role each helix of domain I of Cry1Ac has in the pore forming activity of the toxin were done in parallel by both the Weizmann and the Purdue groups. Whereas the Weizmann group focused on the properties of the isolated helices of the pore forming domain, the Purdue group did mutations in these helices, some of which were similar to those done in the isolated helices. Very importantly, the results of the mutational analysis and the synthetic peptide approach are in agreement one with each other. For example, Shai group studies showed that all the helices beside helix $\alpha 1$ bind membranes and that only helices $\alpha 4$ and $\alpha 5$ are inserted as a hairpin into the membrane to form the pore. Based on these studies Aronson group investigated: (1) The resistance of Cry1Ac toxin to digestion with proteaseK when bound to

vesicles prepared from the midguts of *Manduca sexta* larvae, and found that only primarily helix $\alpha 1$ was removed from the amino terminus in agreement with Shai's notion that this helix is exposed and not bound to membranes. (2) Based on Shai's group finding that $\alpha 4$ insert into the membrane and composes the pore, Aronson's group made ten different point mutations in helix $\alpha 4$ and found out that some of them reduced toxicity, some did not have an effect and some increased toxicity. Mutations in $\alpha 4$ were not done previously since this helix was not considered to be important for toxicity. Based on these mutations Shai's group synthesized $\alpha 4$ and three of its mutants, two which abolished toxin activity and one which increased toxicity. This was done to find out a direct correlation with Aronson's group mutations. As seen in the report functional studies revealed direct correlation between the properties of the synthetic $\alpha 4$ and its mutants and the effect of similar mutations in the intact toxin. These studies were presented as a shared abstract between the two groups in the Biophysical Meeting which was held in Baltimore on February, 1999. In addition the results of the synthetic peptides were summarized in a PNAS paper in which Aronson's group results were discussed in the Discussion section. In addition to these studies Aronson's group made several cysteine mutation in Cry1Ac toxin and send them to Shai's laboratory for fluorescent labeling and biophysical studies. Studies on these aspect were reported previously, and are still continued at present. We still do not have conclusive data from these studies, since the activity is very sensitive to the process of labeling and refolding.

Finally, the Tel-Aviv Univ. group have received *Bacillus thuringiensis* expression vectors that have been constructed by Aronson's group. These vectors are used to express either Cry1C or Cry1E in a Bt strain, which has been characterized by Aronson's group and shown to be devoid of any other cry genes

EVALUATION OF THE ACHIEVEMENTS TOGETHER WITH THEIR SCIENTIFIC AND/OR COMMERCIAL IMPACTS

The delta-endotoxins of *Bacillus thuringiensis* are considered as the most efficient and environmental friendly anti-insect toxins. Several transgenic crops expressing these proteins have been introduced as commercial crops in many places worldwide. In these transgenic fields many pests are differential rejected or killed without using chemical insecticides. However, due to the selective pressure exerted by the increasing number of transgenic fields, insect resistance to delta-endotoxin has gradually appeared. Identification of delta-endotoxin receptors and

unraveling the interaction mode of specific toxins with the epithelial membrane as has been achieved in our studies are of crucial importance to avoid the development of delta-endotoxin resistance in insect populations. Our research has dealt with various aspects of toxin – membrane interaction and the possibilities to develop or co-use other delta-endotoxin alternatives that would reduce the selectable pressure. We have done fine-mapping of the protein structural zones that specifically interact with the membrane which will allow to use their components for protein engineering that should lead to the production of a diverse array of delta-endotoxins that might be more effective and less involved in selection for insect resistance.

2. INTRODUCTION

Bacillus thuringiensis produces a wide variety of delta-endotoxins which are highly specific and very toxic for the larvae of at least three orders of insects. These toxins have been used successfully as spray applications for a number of crops and more recently, several of the genes have been incorporated into the genome of corn, cotton, potatoe, rice and other plant species. The initial field results are very promising so their potential as an alternative to chemical pesticides is substantial, given their effectiveness and specificity. A problem of major concern is the development of insect resistance that up to this time has been realized primarily in the laboratory. In order to cope with the occurrence of resistance, it would be important to understand the mode of action of these toxins. Our project was designed to elucidate the mode of action of a major class of these toxins and we have made considerable progress toward this goal.

The three dimensional structure of several of these toxins has been solved and despite very different specificities, the structures are almost superimposable. There are three domains and domain I which is comprised of a bundle of seven amphipathic helices is important for toxicity. One or more of these alpha helices inserts into the membrane to form ion channels. Either osmotic swelling of larval midgut cells or a more subtle perturbation of the ion flow across the cell membrane appears to account for the lethality.

3. PART I: Yechiel Shai- Weizmann Institute

STRUCTURE OF THE PORE FORMED BY Cry δ -endotoxin WITHIN PHOSPHOLIPID MEMBRANES.

The study was performed with two families of δ -endotoxins, the Cry3A (based on studies done at Tel-Aviv University) and Cry1Ac (based on studies done at Purdue University). Although there is a high homology between these two toxins, they share a significant difference in the composition of their amino acids, and therefore, it was interesting to compare the properties of their individual helices. The achievements obtained with these two families are summarized in the following paragraphs.

3. 1. The functional organization of the pore forming domain of Cry3A in phospholipid membranes (See enclosed paper: Gazit, E., La Rocca, P., Sansom, M.S. & Shai, Y. 1998 "The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* delta- endotoxin are consistent with an "umbrella-like" structure of the pore" *Proc. Natl. Acad. Sci. USA* 95, 12289-12294).

We have synthesized, fluorescently labeled and functionally and structurally characterized the seven helices that compose the toxin pore-forming domain of Cry3A of the *B. thuringiensis* δ - endotoxin. We determined the relative affinities of the seven helices towards membranes, their modes of membrane interaction, their structures within membranes, and their orientations relative to the membrane normal. In addition, we used resonance energy transfer measurements of all the possible combinatorial pairs of membrane bound helices to map the network of interactions between the helices in their membrane-bound state. The interaction of the helices with the bilayer membrane was also probed by a Monte Carlo simulation protocol to determine the lowest energy orientations. Our results are consistent with a situation in which only helices $\alpha 4$ and $\alpha 5$ insert into the membrane as a helical hairpin in an antiparallel manner, while the other helices lie on the membrane surface like ribs of an umbrella [the "umbrella model"(see Figure 7 in the enclosed PNAS paper)]. Our results also further support the suggestion that $\alpha 7$ may serve as a binding sensor to initiate the structural rearrangement of the pore-forming domain.

A very important contribution to our findings is the direct correlation between the experimental results and the computational studies. This is demonstrated for both the orientation of the helices in their membrane-bound state, and the energy of their binding to the membrane. For example a minimum energy configuration shown for $\alpha 6$ (Figure 6, panels A and B, in the enclosed PNAS paper) suggests a surface localization of $\alpha 6$, in agreement with surface localization found in the NBD fluorescence experiments and the ATR-FTIR studies (Tables 1 and 2 in the enclosed PNAS paper). It is significant that in these simulations neither $\alpha 4$ nor $\alpha 5$ inserts into the bilayer, despite the experimental evidence in favor of such insertion. We interpret this as indicating that in order to insert these two helices must self-associate to some extent, thus burying their hydrophilic sidechains from the core of the lipid bilayer. However, in the MC simulations helix $\alpha 7$ adopts a "semi-inserted" orientation. This is consistent with the overall

umbrella model of δ - endotoxin insertion, and suggests that $\alpha 7$ may adopt such an orientation independent of possible interactions with other helices. Regarding the energy of binding, a very interested finding is the direct correlation between the minimum value of the interaction energy ($EMIN$) calculated via Monte Carlo simulation and the experimentally determined free energy of peptide/bilayer interaction demonstrated for the first time for a membrane protein (Figure 6, panel C, in the enclosed PNAS paper). This figure shows that when this minimum energy was plotted against the experimental energies, the resultant linear correlation is good ($R = 0.93$).

3. 2. Structural and functional characterization of $\alpha 4$, $\alpha 5$ and $\alpha 4 \leftrightarrow \alpha 5$ hairpin of Cry1Ac toxin (see attached paper submitted for publication).

In order to investigate the generality of the “umbrella” model for the insertion of Cry δ -*endotoxins*, we investigated the pore region domain ($\alpha 4$ and $\alpha 5$) of Cry1Ac δ -*endotoxin*, the protein studied at Purdue University. Based on our findings with Cry3A toxin, described in the previous section, that $\alpha 4$ composes the loop that penetrates the membrane, the $\alpha 4$ helix of Cry1Ac was mutated by the Purdue group as described in the second part of the report. These mutations either did not affect the toxicity or alternatively, decreased or even increased toxicity. We selected three mutations, two of which decreased toxicity (neutral to charge mutation and hydrophobic to hydrophilic mutation), and the third one hydrophobic to hydrophobic mutation. The list of the peptides and their designations is given below (**X**- represent free amino group, or the fluorescent groups NBD, or rhodamine):

WT- $\alpha 4$: **X**-L R E E M R I Q F N D M N S A L T T A I P L F A

R8-WT- $\alpha 4$: **X**-L R E E M R I **R** F N D M N S A L T T A I P L F A

S7- $\alpha 4$: **X**-L R E E M R **S** Q F N D M N S A L T T A I P L F A

L9- $\alpha 4$: **X**-L R E E M R I Q **L** N D M N S A L T T A I P L F A

In addition we made the $\alpha 4$ -loop- $\alpha 5$ hairpin loop of both the Cry1Ac and Cry3A with the following sequences (bolded and underlined alanine residues represent isotopically labeled C^{13}

and N¹⁵ in $\alpha 4$ and $\alpha 5$, respectively). The isotopes were incorporated for future NMR and FTIR studies:

$\alpha 4 \leftrightarrow \alpha 5$ hairpin of Cry1Ac:

[-----Helix-4-----]---[---Loop---]-
X-ALREEMRIQFNDMNS ALT TA IPLFA-VQCYQVP-

[-----Helix-5-----]
--LLSVYVQA ANLHLSVLRDVSVFG

$\alpha 4$ -loop- $\alpha 5$ hairpin of Cry3A:

[-----Helix-4-----]---[---Loop---]-
X-HSQGRIRELFSQ AESHFRNSMPSFA-ISCYEVLL--

[-----Helix-5-----]
--LLSVYVQA ANLHLSVLRDVSVFG

The peptides were then structurally and functionally characterized as follows:

3. Ability to oligomerize in solution.
4. Ability to interact and insert into phospholipid membranes.
5. Ability to make pores in phospholipid vesicles.
6. Ability of the isolated helices to bind and interfere with the pore formed by the $\alpha 4$ -loop- $\alpha 5$ hairpin.
7. Ability of the peptides to self-associate or to coassemble within the membrane milieu.
8. The secondary structure of the peptides were measured in the membrane-bound state by using ATR-FTIR spectroscopy.

3.3 Summary of the results.

$\alpha 4$ and $\alpha 5$ are the two most hydrophobic helices that were implicated in channel formation by mutagenesis analysis in the full-length protein done at the Purdue University (see enclosed article by Kumar and Aronson, 1999 and Wu and Aronson, *J. Biol. Chem.*, 1992. 267, 2311-2317). Studies done at the Weizmann laboratory with the Cry3A toxin supported these

conclusions (Gazit and Shai, *Biochemistry*, 1993. 32, 3429-3436; Gazit and Shai, *Biochem. J.*, 1994. 304, 895-902; Gazit and Shai, *J. Biol. Chem.*, 1995. 270, 2571-2578).

Our studies on the ability of $\alpha 4$ and $\alpha 5$ to oligomerize in solution and in membranes revealed that only $\alpha 5$ is oligomer in both solution and membranes, whereas $\alpha 4$ and its mutants could self-associate only in the membrane. We also found that mutations in $\alpha 4$ did not alter their ability to coassemble with $\alpha 5$. Interestingly, these results correlate with mutations done in Purdue, since in the full-length Cry1Ac protein $\alpha 5$ oligomerization was found to be important for toxicity, as most active mutants at $\alpha 5$ have kept their oligomerization capability and inactive mutations lost it. In contrast, inactive mutations in $\alpha 4$ did not affect the protein's ability to form oligomers in the membrane.

We also found that the Cry1Ac segment of $\alpha 4$ -loop- $\alpha 5$ is much more active in membrane permeation than either one of the isolated helices by themselves, or a mixture of both of them. This provides strong evidence that both helices are needed to form the pore/channel and cause toxicity. It is also consistent with the Umbrella model we suggested (see enclosed PNAS paper), in which the $\alpha 4$ -loop- $\alpha 5$ hairpin, hidden in the hydrophobic core, is inserted into the membrane upon binding.

Further permeation analysis of the shorter helical segments suggests that $\alpha 4$ and $\alpha 5$ have distinctly different roles within the hairpin. Helix $\alpha 5$ is capable of K^+ diffusion potential dissipation and Calcein release (see Figure 4 in the enclosed manuscript). Namely, it can cause perforation of the membrane. Furthermore, $\alpha 5$ was previously shown to be important for oligomerization in the membrane (see enclosed article by Aronson et al., 1999). These facts imply that $\alpha 5$ plays a role in oligomerization of the channel and in pore formation. We found that helix $\alpha 4$ has also a capability to oligomerize within the membrane, both with $\alpha 5$ and with itself. On the other hand, $\alpha 4$ and its mutants were incapable of membrane permeation, except for a very mild ability of F9L $\alpha 4$. This indicates that $\alpha 4$ has no direct role in pore formation, but still it plays a role in oligomerization within the phospholipid bilayer.

What capacity does $\alpha 4$ have in channel/pore formation, if it is not directly involved in the pore? A very important observation in our study was that F9L $\alpha 4$, a peptide corresponding to an active mutation in the full-length protein (see enclosed article by Manoj Kumar & Aronson, 1999), was able to cause an immense decrease in $\alpha 4$ -loop- $\alpha 5$ membrane permeation (Figure 5 in the enclosed submitted article by Gerber & Shai). Whereas, the wild type $\alpha 4$, the inactive $\alpha 4$ mutants I7S and Q8R and also $\alpha 5$ could cause only limited inhibition of the $\alpha 4$ -loop- $\alpha 5$ activity.

Another observation is that wild type $\alpha 4$ and all $\alpha 4$ mutants show resonance energy transfer with $\alpha 5$, suggesting that the inhibition ability of $\alpha 4$ -loop- $\alpha 45$ by F9L is not due to the lack of oligomerization by the 'inactive mutants' (Figure 6 in the enclosed submitted article by Gerber & Shai). Contrarily, the binding isotherm curve of F9L $\alpha 4$ suggests positive cooperativity, a fact that may indicate that this specific mutation increases the role $\alpha 4$ takes in oligomerization within the membrane. Interestingly, this results are in agreement with the results obtained at Purdue University in which mutation in $\alpha 4$ of the intact toxin did not affect its oligomerization in the membrane.

4. PART II: Arthur Aronson - Purdue University

MUTATIONAL ANALYSIS INDICATES THAT HELICES 4 & 5 ARE IMPORTANT FOR TOXICITY (See Enclosed Paper: Kumar, M. A. S., and A. I. Aronson. 1999. Analysis of mutations in the pore-forming region essential for insecticidal activity of a *Bacillus thuringiensis* delta-endotoxin. *J. Bacteriol.* 181:6103-6107)

In previous work, we established that a number of mutations within helix alpha 5 but not within helices alpha 2 or 6 resulted in no or lower toxicity of the Cry1Ac1 toxin for three target insects. We have extended the mutagenic analysis to include helices alpha 3, alpha 4 and the loop connecting helices alpha 4 & 5. About 40% of the random mutations within a ten amino acid coding region of alpha 4 resulted in loss of toxicity (see reprint for exact changes). This value is comparable to that obtained for random mutations in helix alpha 5. Changes in helix alpha 3 were confined to a few residues in order to enhance the surface hydrophobicity of this helix. As discussed elsewhere in this report, Shai's lab had found that a synthetic peptide of helix alpha 3 bound poorly to artificial lipid bilayers (Gazit et al. *Proc. Natl. Acad. Sci. USA* 95, 12289-12294 (1998)). It was thought that increasing the hydrophobicity of this helix would enhance toxicity. Site directed mutations of four surface-exposed residues in this helix were made which should have increased the hydrophobicity but in no case was there a change in toxicity.

There were five random mutations within the loop connecting helices 4 & 5. None resulted in loss of toxicity despite several changes in size or charge of the amino acid residues. Assuming that helices 4 & 5 insert into the membrane (see results of Y. Shai), it is possible that

the loop could project into the cytoplasm of the larval midgut cell. If so, there could be interactions with cytoplasmic (or membrane) components which were important for toxicity. The limited mutagenic data suggest that such an interaction is not critical and as discussed below, there is evidence that little if any of the toxin projects through the membrane into the cytoplasm. Overall, the mutagenic data indicate that helices 4 & 5 but not 2, 3 or 6 are important for toxicity. As discussed below, the amino terminal part of the toxin comprised of helix 1 does not bind to the membrane so it is unlikely to be directly involved. A mutagenic study of helix 7 has not yet been done although its unique role in membrane insertion has been analyzed by Dr. Shai.

4.1. Active toxins or those with mutations in helix 4 aggregate within the membrane (see enclosed paper Aronson, A. I., C. Geng, and L. Wu. 1999. Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles. *Appl. Environ. Microbiol.* 65:2503-2507).

A prevalent model for toxin action is the formation of ion channels leading to osmotic swelling and larval death. Given our mutagenic results and the peptide studies of Dr. Shai discussed above, it appears that helices 4 & 5 are critical to the formation and function of this ion channel. This region of the toxin which forms a hairpin is the most hydrophobic part of the toxin and thus the most likely to insert into the membrane. It is not known, however, how many toxin monomers must aggregate to form a channel nor whether helices 4 & 5 have different functions in this process.

We devised an assay for measuring toxin aggregation in membrane vesicles prepared from larval midguts (BBMV). Initially we found that much of the toxin molecule (most studies done with the Cry1Ac1 toxin) bound to these vesicles was protected from digestion with protease K. The only susceptible part was helix alpha 1, the amino-terminal end of the toxin. Dr. Shai had demonstrated that a synthetic peptide of this helix was the only one of the seven in domain I which could not bind to synthetic phospholipid vesicles (Gazit et al. *Proc. Natl. Acad. Sci. USA* 95, 12289-12294 (1998)). This is one of several correlations between his peptide studies using synthetic phospholipid vesicles and our experiments with intact toxins and larval BBMV. It appears that much of the toxin is embedded in or on the surface of the BBMV. Recently, we preloaded BBMV with protease K and demonstrated that there was no digestion of Cry1Ac1

toxin which had bound to these vesicles. These results indicate that little if any of the bound toxin projects into the cytoplasm.

In order to examine the properties of the toxin bound to vesicles, we developed a protocol for extracting toxin antigen from BBMV under mild conditions, that hopefully preserved any reasonably stable aggregates that may have formed. We were able to extract from BBMV toxin antigen of about the size of trimers. There was some antigen of dimer size but very little monomer. A number of helix alpha 5 mutant toxins as well as other mutants in domain I which had lost toxicity did not form aggregates whereas mutant toxins which were still active, formed trimers. It should be noted that all of the toxins bound about equally well to the presumptive receptor in BBMV previously identified as aminopeptidase N. This binding was done in immunoblots so any differences in the rates of binding would not have been detected. It does appear, however, that the initial interaction with BBMV was unaltered by any of these mutations.

The one exception to a correlation between toxicity and aggregation in BBMV was a helix 5 mutation (H168R) which had somewhat higher toxicity but little if any aggregation in BBMV. There was a high concentration of bound monomers by this mutant toxin so binding appears to have been improved but aggregates, if they formed, were apparently less stable with the extraction conditions employed.

There was also a correlation between the extent of trimer formation and the susceptibility of the insect species serving as a source of BBMV. *Heliothis virescens* is more susceptible to the Cry1Ac1 toxin than is *Manduca sexta* and there was a higher concentration of aggregates extracted from BBMV prepared from the former than from the latter.

The size of the aggregates extracted from the BBMV was consistent with a toxin trimer although interaction of the toxin monomer with a BBMV component (most likely a protein) has not been excluded. One possibility was a toxin-aminopeptidase N (the presumptive toxin receptor) complex but there was no aminopeptidase N antigen in these toxin aggregates. This does not rule out the binding of a toxin monomer to another BBMV component(s).

Pure Cry1Ac1 toxin does contain a very small fraction of trimers. These toxin trimers may selectively insert into the BBMV and/or the interaction of the toxin with BBMV may enhance trimerization prior to or during insertion. No such trimers were found in purified preparations of a very closely related toxin, Cry1Ab1, nor in most of the mutant toxins examined. Trimerization in solution, therefore, may be helpful but is not essential for aggregation in BBMV. Their presence in the Cry1Ac1 toxin does indicate that the antigen

aggregates extracted from BBMV are likely to be such trimers. It is still not known at what stage in binding/insertion that the toxin aggregates nor the number of toxin molecules making up the ion channel. The presumptive trimers extracted in these experiments may reflect stable intermediates either in the formation of the channel or in its degradation.

Similar binding/ aggregation experiments were done with the non-toxic helix alpha 4 mutants. In this case, both the toxic and non-toxic mutant Cry1Ac1 toxins aggregated in the membrane to form apparent dimers and trimers. The rate of formation of such aggregates was not measured and may be slower for the inactive mutants. These mutant toxins did not form functional ion channels, however, as measured by light scattering. There was some permeability to potassium ion (i.e. a small decrease in swelling of the BBMV) but <5% of the rate found with the wild type toxin. Apparently toxins with an unaltered helix 5 can insert into the membrane but a functional ion channel cannot form without an unaltered helix 4. The implication of these results is that both helices are important for toxin function but they have distinct roles in this process.

5. PART III: Aviah Zilberstein and Baruch Sneh- Tel-Aviv University.

DOMAINS OF CRY1C, CRY1E AND OTHER CRY1A PROTEINS AND THEIR INTERACTION WITH THE EPITHELIAL MEMBRANE OF THE INSECT LARVAL GUT.

5.1 Abstract

Understanding the interaction module of the toxic part of Cry proteins with the epithelial membrane of the insect larval gut is of a great importance to develop strategies of preventing development of acquired resistance of target insects to the vastly used delta-endotoxins. During this study we have developed the approach of expressing single structural domains of Cry1 proteins and estimating their role in the specific interaction with the gut epithelial membrane of the insect larvae. Until now, strategies of studying the interaction of the full length toxic part of the Cry proteins have been developed, based on the concept that the correct folding of all three structural domains as a whole unit is required for the proper interaction with the membrane receptors.

Single Cry1C and Cry1E domains as well as combinations of consecutive domains were overexpressed in *E. coli* using T7-based expression systems. Binding assays of the purified

domains, performed in vitro with epithelial brush border membrane vesicles (BBMV), served as the tool for evaluating domain-membrane interactions.

Our study has pointed out the putative specific interaction of all three separate domains with the epithelial membrane and thereby complements the research performed by the other two partners of the project related to many aspects of domain I, such as specific interactions, orientation of components inside and on the membrane surface and structure- function relationships.

The results indicate that the main interacting domain is domain II and this interaction is as specific as the interaction with the membrane of the whole toxic part containing all three domains.

Experiments performed with membrane vesicles prepared from Cry1E-resistant and Cry1E-sensitive populations suggest the existence of two different receptors for Cry1E and Cry1C in the epithelial membrane of *Spodoptera littoralis* which is normally sensitive to both toxins.

The binding assays with these two types of membrane vesicles showed clearly the specificity of the interaction of all three domains, including domain I. Based on these findings we envisage a more complex specific interaction process of all three structural domains with the membrane in which the specific binding to certain receptor/s probably plays a key role but is not the sole interaction event which is specific.

5.2. Introduction

Studies of structure-function relationships related to the toxic parts of the Cry proteins have been started when the three dimensional structure of Cry3A and then Cry1Aa were determined (Li *et al.*, 1991; Grochulski *et al.*, 1995). Three different structural domains were clearly observed and different functional characteristics have gradually been attributed to each of them. While the first two parts of this study have been focused on the functional analysis of the helices comprising the N-terminal domain I of Cry1 proteins, the third part mainly deals with the receptor recognition features of the two other consecutive domains; domain II and domain III. Domain II, with its conserved beta sheet structure and intervening loops, exhibits relatively high diversity in amino acid sequence and therefore is considered as the highly variable domain. Intensive mutagenesis of domain II sequence in different *cry* genes has demonstrated the role of the intervening loops, protruding from the compact beta sheet structure, in toxicity and in dictating specificity of receptor recognition. However, with the time it has become evident that domain III with its beta-jelly roll structure (Li *et al.*, 1991) is also involved in receptor - toxin interaction (Burton *et al.*, 1999; De Maagd *et al.*, 1999; Lee *et al.*, 1999). Mutagenesis of

domain III as well as domain III swapping have shown that both specificity and toxicity of the Cry toxin could be changed. While a unique site, found only in Cry1Ac-domain III, has been shown to be specifically involved in N-acetyl galactosamine-mediated recognition of the aminopeptidase N receptor (Burton *et al.*, 1999; Lee *et al.*, 1999), no direct correlation between loss of binding capacity of this site and reduction in larval toxicity was observed. The maintenance of relatively high toxicity of Cry1Ac mutants devoid of domain III binding ability (Lee *et al.*, 1999) indirectly indicated that either another receptor might be simultaneously involved in binding of another Cry1Ac domain or another aminopeptidase N binding site exists in another domain of the toxin, as was shown by Masson *et al.*, 1995.

Two Cry1A receptors have been identified thus far. The aminopeptidase N (APN) putative receptor interacts with toxins of the Cry1A family. Purified *Manduca sexta* APN was shown to recognize two binding sites in Cry1Ac, whereas only a single binding site for APN was identified in Cry1Aa and Cry1Ab (Masson *et al.*, 1995). The latter two toxins lack the domain III binding site which interacts with N-acetyl galactosamine (Lee *et al.*, 1999).

Cadherin-like protein, was defined an epithelial receptor for as Cry1Aa. When its corresponding cDNA was isolated from *Bombyx mori* gut epithelium and expressed in Sf9 cells it conferred new sensitivity to Cry1Aa (Nagamatsu *et al.*, 1999). By using deletion mutants of the cadherin-like protein, the Cry1Aa binding site was confined to the ninth cadherin repeat and preceding transmembrane domain.

As yet, little is known about other receptors that might interact with other Cry1 proteins, such as Cry1C and Cry1E. The 40 kDa protein derived from *Spodoptera* gut membrane or Sf9 cells which was found to interact with Cry1C in ligand blots is the only evidence for a membrane protein that interacts with Cry1C.

The already defined interactions between Cry1 proteins and membrane proteins still need further clarification in order to differentiate between the direct interaction of the toxins with the primary receptors and possible accompanied interactions with adjacent proteins of the membrane surface. In addition, intermolecular Cry protein interactions are expected to occur as a part of the overall interactions of the toxin with the epithelial membrane surface. Aronson *et al.* (1999) found Cry1Ab and Cry1Ac oligomers bound to the surface of the gut membrane vesicles. The oligomerization was only formed when the helices alpha 4 and alpha 5 were properly inserted into the membrane. Mutations that cancel this specific insertion also affected toxin aggregation. These results have shown that domain I is involved in forming appropriate toxin-epithelial membrane interaction.

In this study the role of each of the structural domains has been investigated. Separate domains and combinations of two domains were expressed and their interactions with gut brush - border membrane vesicles (BBMV) were evaluated. The BBMVs were prepared from

Spodoptera littoralis larval population which was found to be fully sensitive to Cry1C and only partially sensitive to Cry1E toxin.

5.3 Results

5.3.1. Target insects

Larvae of *Spodoptera littoralis*, which have never been exposed to any Bt Cry toxin, were reared under lab conditions. This population of larvae revealed a consistent sensitivity to Cry1C and segregated in Cry1E sensitivity (Fig. 1). The Cry1E-insensitive individuals were defined, separated from the rest of Cry1E-very sensitive larvae and further propagated. Their offspring showed the same proportion of Cry1E insensitive and sensitive larvae. Consequently, it was impossible to enrich the larval population with Cry1E-resistant larvae. The Cry1E sensitivity did not correlate with known genetic segregation and therefore it was impossible to determine how many genes are involved in dictating Cry1E sensitivity or resistance.

This population was used for all toxicity bioassays and BBMV isolations (Fig. 1). Discrimination between Cry1E-sensitive and Cry1E-resistant larvae was performed by feeding the first instar larvae with artificial diet containing 20µg/ml Cry1E for 2 days and then both sensitive as well as resistant larvae were separately transferred to a new feeding medium devoid of Cry proteins.

5.3.2 Studying the binding capability of separate structural domains of Cry1C and Cry1E

(i) Cloning and expression of domains, purification of expressed proteins:

The role of different structural domains of Cry1C and Cry1E in the interaction with the epithelial receptors was estimated by measuring the binding capacity of each domain to BBMVs isolated from *S. littoralis* larval population mentioned in III.2.1. According to the 3D structure of Cry3a and Cry1Aa crystals (Li *et al.*, 1991; Grochulski *et al.*, 1995), separate structural domains and combinations of two domains were synthesized by PCR using the cloned toxic region of *cry1C* and *cry1E* genes as templates (Table 10 and Fig. 2). The amplified domains and domain combinations were cloned in the expression vectors pET11a or pET16b (Novagen) downstream to the T7-gene 10-promoter and *lac* operator. IPTG induced expression of domains was obtained in *E. coli* strain BL21(DE3) carrying a chromosomal copy of T7 RNA polymerase gene driven by *lac* promoter linked to *lac* operator (Fig. 3).

When the pET16b cloned Cry proteins or domains were expressed linked to the N-terminal 6 HIS tag, all the expressed proteins were insoluble and could not be separated by the metal chelation chromatography. In order to avoid such packaging of the pET11a products,

incubation of the BL21(DE3) cells carrying the cry plasmids was performed at 22°C and the synthesized proteins were dissolved at pH10 in the presence of DTT.

Analysis of Cry protein expression showed that all single domains and two domain combinations (I+II, II+III) could be overexpressed in the pET11-*E. coli* system and then partially solubilized, except of Cry1E domain II+III combination, which was always insoluble (Fig. 4).

Further purification of the full-length toxic part of Cry1C (65kDa) was achieved by two step column purification using at first Sephadex G25 and 50mM Na₂CO₃ elution buffer (pH 10) and then FPLC – MONO-Q column with the same buffer and 0-600mM NaCl gradient. Purification of Cry1C-domain II was also achieved by the same method with relatively low yield.

It was impossible to purify the 85kDa C-terminal truncated Cry1E and the rest of Cry1C and Cry1E domains by using the same approach. Therefore, the *E. coli* T7 S30 extract system for circular plasmids (Promega) was used to express purified labelled domains. The Cry domains were transcribed and then translated *in vitro* as ¹⁴C-labelled products by using ¹⁴C-glycine or/and leucine as precursors.

(ii). Interaction of Cry1C and Cry1E with membrane vesicles of the larval gut epithelium (BBMV).

Preliminary toxicity assays showed that *S. littoralis* larval population is homogeneously responding to Cry1C but only part of it is sensitive to Cry1E.

Binding assays with the 86kDa C-terminal truncated Cry1C or Cry1E, which were labelled with FITC, were performed *in vitro* with BBMVs prepared from *S. littoralis* Cry1E-sensitive or Cry1E-resistant larvae as well as from representatives of the same population that had never been exposed to any Cry protein.

Comparative binding assays showed that Cry1C equally interacts with both types of BBMVs (Fig. 5), while Cry1E revealed significantly higher interaction with BBMV isolated from the Cry1E-sensitive population (Fig. 6). These results indicate that Cry1C and Cry1E probably interact with different epithelial membrane receptors. Similar results of differential binding were obtained in Western blots used to analyze BBMV bound proteins resulting from binding assays performed *in vitro* (detailed below, section III.2.2.3).

(iii). Interaction of separate domains of Cry1C and Cry1E with BBMVs.

The hypothesis that the overall interaction of the Cry protein with the gut epithelial membrane involves more than a single Cry domain was verified by using the *E. coli* produced domains or combinations of two consecutive domains of Cry1C and Cry1E.

Two-step analysis of the interaction between domains of Cry1C and Cry1E and BBMV was performed, using the C-terminal truncated Cry1C or Cry1E containing all three domains as controls (86 kDa of Cry1C and Cry1E, or 65 kDa Cry1C). The binding reactions with BBMV comprised the first step (incubation of BBMV prepared from the gut epithelium of the fifth instar larvae with Cry protein/domain in 50mM PBS, pH 8, 0.1% BSA, for 30 min, at 25°C). After several washes (50mM PBS, pH 8), the BBMV attached proteins were analyzed on Western blots probed with anti-Cry1C polyclonal antibodies that also recognize Cry1E and all derived Cry1C and Cry1E domains. Figure 7 shows the binding of Cry1C and its three different domains to BBMV. All domains revealed certain interactions with the epithelial membrane, including domain I.

Cry1E domains were similarly examined in binding assays with BBMVs prepared either from Cry1E-resistant larvae or Cry1E-sensitive larvae. All domains revealed 80% reduction in binding to resistant BBMV. Similar reduced binding was observed for the 86kDa Cry1E, while the 86kDa Cry1C showed equal affinity to Cry1E-sensitive and Cry1E-resistant BBMVs (Fig.8). The equal interaction of Cry1C with the two types of BBMVs indicated that the Cry1E-resistant larvae were not defective in their general capacity to interact with Cry proteins. These data confirm that Cry1C and Cry1E interact with two different receptors. It also shows that the interactions of all domains are specific since all of them revealed equal reductions in binding affinity to the Cry1E-resistant membranes. The binding of domain I supports the findings of Aronson's group (Aronson *et al.*, 1999) that this domain is involved in the formation of Cry protein aggregates, upon interacting with the membrane, which is required for proper insecticidal activity.

Domain II of the Cry protein has always been considered as the one that dictates the specific interaction with the gut epithelial membrane (Li *et al.*, 1991). Thus, binding assays of FPLC-purified domain II of Cry1C (labelled with fluorescein) were performed in vitro with BBMV, to verify the kinetic parameters of the binding (Fig. 9). The binding constant (K_d), according to the calculated Scatchard plot (Fig. 9C), was 22.78mM and the linear Hill plot (Fig. 9B) showed that the N value is 1. This K_d value correlates well with the range of K_d values reported for the interaction of isolated Cry1A proteins with the isolated amino-peptidase-N putative receptor (Oltean *et al.*, 1999).

Competition assays performed between domain II and domain III of Cry1C (Fig 10) showed that the binding of domain III is strongly reduced by the presence of relatively low concentrations of domain II. However, binding of domain II was not interrupted by the presence of domain III, suggesting either that in addition to the main site of binding to domain II, there is additional site which interacts with domain III and also recognizes domain II, or the single domain II and III binding site has higher affinity for domain II.

Competition experiments with between Cry1C-domain II and Cry1E-domain II showed that the presence of an excess of Cry1E-domain II did not affect the binding of Cry1C-domain II. In addition, Cry1C-domain III binding is not interrupted by Cry1E-domain II (Fig. 11). These data support the concept that Cry1C and Cry1E interact with two different membrane receptors.

5.3.3 Exchange of domains

A general cloning vector (pET-based), containing domain I and III of Cry1C with a polylinker situated in between the two domains was constructed. It enables in-frame cloning of different Cry1-domain IIs, to evaluate changes in specificity dictated by domain II in the presence of structural constraints or features imposed by the physical linkage to the two adjacent domains. Cry1E-domain II was inserted between Cry1C domains I and II, yielding a chimeric protein of [Cry1C-domain I – Cry1E-domainII – Cry1C-domain III]. The fused protein was expressed in *E. coli*. and packed in inclusion bodies. It was impossible to dissolve the insoluble fraction in order to perform the binding experiments. Currently, we are trying to exchange the Cry1E-domain II with the similar domain of Cry1Ab which should be more soluble according to its primary structure.

5. 4. Conclusions

During this study we have developed the approach of expressing single structural domains of Cry1 proteins and estimating their role in the specific interaction with the gut epithelial membrane of the insect larvae. Until now, strategies of studying the interaction of the full length toxic part of the Cry proteins have been developed, based on the concept that the correct folding of all three structural domains as a whole unit is required for the proper interaction with the membrane receptors.

This study has pointed out the putative specific interaction of all three separate domains with the epithelial membrane and thereby complements the research performed by the other two partners of the project which shows the specific interactions, orientation inside and on the membrane surface, and correlation with the mode of action of domain I (Gazit *et al.*, 1998; Manoj-Kumar and Aronson, 1999).

The results indicate that the main interacting domain is domain II and this interaction is as specific as the interaction with the membrane of the whole toxic part containing all three domains.

Experiments performed with membrane vesicles prepared from Cry1E-resistant and Cry1E-sensitive populations suggest the existence of two different receptors for Cry1E and Cry1C in the epithelial membrane of *Spodoptera littoralis* which is normally sensitive to both toxins.

The binding assays with these two types of membrane vesicles show clearly the specificity of the interaction of all three domains, including domain I. Based on these findings we envisage a more complex interaction of all three structural domains with the membrane in which the specific binding to certain receptor/s plays a key role.

5.5. References

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Publications

Avisar, D., Keller, M., Sneh, B. and Zilberstein, A. Expression and specific interaction with membrane receptors of separate domains of the delta-endotoxins Cry1C and Cry1E. Abstract presented in the Meeting of Plant Genomics, Maagan, Israel, May 18-20, 1999.

Avisar, D., Keller, M., Gazit, E., Shai, Y., Dori, S., Zilberstein, A. and Sneh, B. Resistance of *Spodoptera littoralis* to Cry1E and evidence for the presence of different specific receptors for Cry1C and Cry1E in the midgut epithelium. Abstract presented in The 32nd Annual SIP Meeting, Irvine, California, USA, August 22-27, 1999

Avisar, D., Keller, M., and Sneh, B Gazit, E., Shai, Y. and Zilberstein, A. Specific and differential interaction of single structural domains of Cry1C and Cry1E with epithelial membrane vesicles, demonstrated by using sensitive and resistant populations of insect larvae. (in preparation).

Table 1:

Cry1C-I	756bp	252aa	28.80 kDa.
Cry1C-II	621bp	207aa	23.35 kDa.
Cry1C-III	525bp	175aa	19.15 kDa.
Cry1C-I+II	1377bp	459aa	52.14 kDa.
Cry1C-II+III	1146bp	382aa	42.48 kDa.
Cry1C	2268bp	756aa	86.00 kDa.
Cry1E	1890bp	630aa	65.00 kDa.
Cry1E-I	756bp	252aa	28.80 kDa.
Cry1E-II	612bp	204aa	22.86 kDa.
Cry1E-III	477bp	159aa	17.87 kDa.
Cry1E-I+II	1368bp	456aa	51.74 kDa.
Cry1E-II+III	1089bp	363aa	40.72 kDa.
Cry1E	2226bp	742aa	86.00 kDa.

Summary of Cry1C and Cry1E separate domains:

The plasmids containing the various cloned domains were transformed into the E. coli strain BL21. All of the domains and the full size toxins were expressed and the respective proteins were produced.

In addition, the full size Cry1C, Cry1E toxins and the Cry1C-65kDa protein, containing only the three domains shown to be the minimal size of the protein that could confer insect sensitivity, were expressed.

A.

B.

Figure 1:

A) Cry1E- Susceptible (left) and resistant (right) *Spodoptera littoralis* larvae.

B) Scanning electron microscope image of Brush Border Membrane Vesicles (BBMV), derived from isolated midgut of *S. littoralis* larvae.

10 20 30 40 50 60 70 80 90 100
cry1E 1: MEIVNNONOCVPYNCLNNEFENEILDIERNST.VATNIAIEISRLASA.TPIGGILLGLFPAIWGSSIGPSOWDLFLECIELLIDOKIEEFANNOAISRE 98
cry1Aa 1: MDRNPNINECIIPYNCLSNPEVVLGGERIETGYTPIDISLSLTOFLSEFVPGAGEVLGLVDIINGIFGFSOWDAFFVOIEOLINORIEEFANNOAISRE 100
cry1C 1: MEENNONOCIPYNCLSNPEVVLGGERISTGNCSSIDISLSVOLFVSNFVPGGGLVGLLDFVWGVGFSOWDAFFVOIEOLINERIEEFANNOAISRE 99

110 120 130 140 150 160 170 180 190 200
cry1E 99: EGISLYGIYEFREWEADPTNPALKEEMRTOFNDMNSILVTAIFLFSVONVUPFLDEVVCAANLHLVLDVVEFGQAWERTATTNRYNDLTRLE 198
cry1Aa 101: EGISLYGIYAESEFEWEADPTNPALKEEMRTOFNDMNSALTATPLAVQNYQVPLLSVYVQAANLHLVLDVSVFGQAWERTATTNRYNDLTRLE 200
cry1C 100: EGLGNNTIYVEAPKEWEEDPNPATRTRIDRFRILDGLLERDIPSRISGFEVPLLSVYVQAANLHLVLDVSVFGQAWERTATTNRYNDLTRLE 199

210 220 230 240 250 260 270 280 290 300
cry1E 199: PIYTDYAVRWYNTGLDRLPRTGGLRNWARENOFRRELTISVLDIISFFRNYDSILYPIPTSSQLTREVVYTDVINITDYRVGPSFE...NIENSAIRS 293
cry1Aa 201: GNYTDYAVRWYNTGLERV.WGPDSRDVRYNOFRRELTITVLDIVALPSNVDSFRYPRTVSQLTREIYTNFVLENFDGSPRGMAQ...RIEQN.IRC 293
cry1C 200: DEYADHCANTYNRGNNI.NPK.STYQDNITYNRRLRDLITVLDIAFFPNYDNFRYPIQPVGQLTREVVYTDVINITDYRVGPSFE...NIENSAIRS 297

310 320 330 340 350 360 370 380 390 400
cry1E 294: PHLMDELNNITIDTDLIRGVH..YVAGHRVT.SHEFGSSOVITTPGYGITANAEPRRTIADSTFGLNI.FVWTLANETFRRS..DMITPFLCINVVQAVG 388
cry1Aa 294: PHLMDELNNITIDTDLIRGVH..YVAGHRVT.SHEFGSSOVITTPGYGITANAEPRRTIADSTFGLNI.FVWTLANETFRRS..DMITPFLCINVVQAVG 388
cry1C 298: PHLMDELNNITIDTDLIRGVH..YVAGHRVT.SHEFGSSOVITTPGYGITANAEPRRTIADSTFGLNI.FVWTLANETFRRS..DMITPFLCINVVQAVG 390

410 420 430 440 450 460 470 480 490 500
cry1E 389: P...IOPNNAEVLKRSKSTVDSLENDLIDGENSL...VGYSHLSHVLIRKLNTHIT.OLTFVWTHSSATHTITHTFDITDQIDLYFGPFGGAT 480
cry1Aa 388: P...IOPNNAEVLKRSKSTVDSLENDLIDGENSL...VGYSHLSHVLIRKLNTHIT.OLTFVWTHSSATHTITHTFDITDQIDLYFGPFGGAT 480
cry1C 391: P...IOPNNAEVLKRSKSTVDSLENDLIDGENSL...VGYSHLSHVLIRKLNTHIT.OLTFVWTHSSATHTITHTFDITDQIDLYFGPFGGAT 480

510 520 530 540 550 560 570 580 590 600
cry1E 481: VLAQPGFTGQDILRNNGICEFUSTQUNINSPIQRYRLFRYASSRDARI...TVAIGGQIRVDNTELEKTEIGESLITORTFEYTNFENFFFRAN 573
cry1Aa 487: VVKQPGFTGQDILRNNGICEFUSTQUNINSPIQRYRLFRYASSRDARI...TVAIGGQIRVDNTELEKTEIGESLITORTFEYTNFENFFFRAN 579
cry1C 404: VITGPGFTGQDILRNNGICEFUSTQUNINSPIQRYRLFRYASSRDARVIVLTGAASTGVGGVSVNDELNTHTIDQNLGERTFVYTNFENFFFRAN 583

610 620 630 640 650 660 670 680 690 700
cry1E 574: PDIIRIAELP...IRCGELYIKIETILADATFEEDYDLERAQAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDDEFCLDEKRELSEKVKHAK 668
cry1Aa 580: PDIIRIAELP...IRCGELYIKIETILADATFEEDYDLERAQAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDDEFCLDEKRELSEKVKHAK 674
cry1C 584: PDIIRIAELP...IRCGELYIKIETILADATFEEDYDLERAQAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDDEFCLDEKRELSEKVKHAK 683

A.

B.

Figure 2:

Structural domains comprising the toxic part of d-endotoxins Cry1Aa, Cry1C and Cry1E.

A) Comparison of amino acid sequences of the three domains.

B) Three dimensional structure: Domain I - blue, Domain II - red and Domain III - green. (Grochulski *et al* 1995 and Li *et al* 1991).

Figure 3:

Cloning steps of *cry* domains in the expression vector pET11a. This figure represents an example of the cloning of Domain I of *cry1C*. A) PCR amplification of Domain I, using the planned primers. B) The amplified fraction with the sticky ends of A, was purified from the primer residues and was ligated to pUC57 with the sticky ends of T. C) The whole plasmid pUC57::*cry1C*-Domain I. D) Digestion with NdeI and BamHI yielded a fragment with the respective sticky ends. E) Cloning of domain I fragment in the NdeI and BamHI digested pET11a yielded the pET11a-*cry1C*-domainI plasmid.

Figure 4:

Over-expression of Cry1C, Cry1E and their separate structural domains in *E.coli*.

Verification by SDS-PAGE. Total proteins from B121(DE3)pET11a without any insert, similarly treated, served as a control.

● indicates the respective domain band in each lane.

Figure 5

Binding of fluorescein labelled Cry1C to BBMVs prepared from Cry1E-resistant-, Cry1E-sensitive larvae and non-Cry selected larvae.

BBMV (10µg) prepared from the fifth instar larvae of the mentioned sub-populations were incubated with increasing concentration of Cry1C for 30 min at room temperature.

Following three washes, the fluorescence of the bound Cry1C was measured (excitation at 475nm, emission at 520nm). Each point represents two replicates.

Figure 6

Binding of fluorescein labelled Cry1E to BBMVs isolated from Cry1E-sensitive- and Cry1E-resistant larvae. Binding assays performed as detailed in Fig. 5.

Figure 7:

Binding of Cry1C and its domains to BBMV isolated from *Spodoptera littoralis* larvae. Binding reactions were performed *in vitro* by incubating BBMV (50mg) with *E. coli* produced Cry1C (250nM) or Cry1C domains (250nM) for 5min at room temperature in 500ml 50mM PBS, pH8. BBMV - bound proteins were identified by Western analysis. Total soluble protein extracted from *E. coli* that carries pET11a served as control (depicted as *E.coli* + BBMV or *E.coli*) and did not bind to BBMV (lanes 1 and 17). (●) indicates the respective domain band in each lane.

Figure 8:

Binding of Cry1E and its domains 1E-I, 1E-II, and 1E-I+II to BBMVs isolated from resistant (R) or susceptible (S) *Spodoptera littoralis* larvae.

Binding reactions were carried out as described in Fig. 5. (An additional 25 kDa protein was identified by the anti-Cry1C antibodies in the R- BBMVs).

* Quantitative estimation of the binding was calculated as arbitrary units, quantified by the NIH Image 1.62b7 computer program following scanning of specific bands. Binding value of each toxin fractions to the S-BBMV was considered as 1.

● indicates the respective domain band in each lane.

Figure 9:

Binding assay of fluorescein labeled Cry1C-domain-II.

A) Binding to BBMV. Non specific binding is verified in the presence of 1000 nM unlabeled Cry1C-domain-II. Specific binding = Total - Non specific.

B) Hill plot: $n=1$.

C) Scatchard plot: $K_d=22.78 \text{ nM}$.

A.

B.

C.

Figure 10:

Competition between Cry1C domain-II and domain-III on binding to *S.littoralis* BBMV.

A) BBMV (25mg) were incubated with a constant concentration of domain-III (260nM) and increasing concentrations of domain-II (lane1-0nM, 2-43nM, 3-86nM, 4-172nM, 5-344nM). Binding reactions were carried out as described in Fig. 5.

B) Quantitative evaluation of domain-II and III binding deduced from panel A: The gradual decrease in domain-III (blue) binding (1-750ng, 2-500ng, 3-300ng 4-250ng, 5-185ng) correlated with the increase in domain-II concentrations (red). Quantification performed by the NIH Image 1.62b7 program.

C) Binding of domain-II and domain-III to BBMV. Lane1: Incubation of BBMV with domain-II (43nM) alone resulted in binding of ca. 250ng domain-II. Lane2: Incubation with a mixture of 43nM domain-II and 10mM domain-III resulted in binding of about 250ng domain-II (100%) + 400ng domain-III (40%). Lane3: Incubation with domain-III (10mM) alone resulted in binding of ca. 1000ng domain-III.

The presence of domain-II reduced the binding of domain-III (lane2).

Figure 11:

No Competition between Cry1E domain-II and Cry1C domain-II or Cry1C domain-III.

A) BBMV (25mg) were incubated with a constant concentration of Cry1C-II (43nM) and increasing concentrations of Cry1E-II (lane1-0nM, 2-43nM, 3-86nM, 4-172nM, 5-344nM). Binding reactions were carried out as described in Fig. 5.

B) Quantitative evaluation of the domain binding shown in panel A:

There is no effect of increasing concentrations of Cry1E-II on Cry1C-II binding.

C) Binding of Cry1E-II and Cry1C-III to BBMV. Incubation of BBMV with 2500nM (lane1) and 10mM (lane 5) cry1C-III resulted in binding of 200ng and 1000ng cry1C-III, respectively. Incubation with 43nM (lane2) and 172nM (lane6) cry1E-II resulted in binding of 250ng and 1200ng cry1E-II respectively. Incubation with a mixture of 10mM Cry1C-III and 43nM Cry1E-II (lane3) or a mixture of 2500nM Cry1C-III and 172nM Cry1E-II (lane4) had no effect on binding of each of these domains.

The presence of Cry1E-II does not interfere with the binding of Cry1C-III to BBMV.

1

1
1
1