In silico and *in vitro* investigations on *cry4a* and *cry11a* toxins of *Bacillus thuringiensis var israelensis*

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Abstract

In the present study we attempted to correlate the structure and function of the cry11a (72 kDa) and cry4a (135 kDa) proteins of Bacillus thuringiensis var israelensis. Homology modeling and secondary structure predictions were done to locate most probable regions for finding helices or strands in these proteins. The JPRED (JPRED consensus secondary structure prediction server) secondary structure predictions were chosen for its ability to predict with high accuracies. The homology model predicted by CPH (CPH Homology Modelling server) modeler showed a distinct region of helices and sheets. The membrane spanning helices were predicted using TOPPRED (TOPPRED Topology prediction of membrane proteins server); these helices are known to play crucial role in cell lyses. The role of one such segment corresponding to amino acids 132-150 of cry4a protein, which had a large hydrophobic moment, was elucidated. The Circular Dichroism spectra of the peptide showed helical structure in methanol and β sheet structure in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The biological activity of this peptide was investigated. The peptide showed weak hemolytic activity in vitro. This may be due to the synthetic peptide used rather than the whole molecule in the native environment. The ability of the peptide and the alkali solubilized crystal proteins to perturb the synthetic membrane was investigated using carboxyflourescein trapped liposomes. The leakage caused by alkali solubilized extract was double than the leakage caused by synthetic peptide. In case of alkali solubilized extract, various osmoprotectants were seen to delay lytic activity. Thus it is clear that the cry proteins are highly active and lethal in their native state. Not only the membrane spanning segments but the whole molecule plays a crucial role in lysis.

Keywords*: Bacillus thuringiensis; Cry proteins; Homology modeling; Larvicidal activity; Amphipathic helices*

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INTRODUCTION

Mimicking the biological world in solving the problem provides reliable and effective solution. These biological molecules are specific in the mode of action when compared to synthetic chemical agents. Since its discovery in 1977, *B. thuringiensis var israelensis* has proved to be one of the most effective and potent biological pesticides (Becker and Margalit, 1993; de Barjac and Sutherland, 1990). Its discovery came at a time when resistance of mosquitoes and black flies to synthetic chemical pesticides was growing concern. Five *B. thuringiensis var israelensis cry* and *cyt* genes encode dipteran-active toxins: cry4A, cry4B, cry10A, cry11A, and cyt1A (cytolysin). These five genes are all located on a large plasmid of about 72 MDa that can be transferred to other *B. thuringiensis* strains by a conjugation-like process (Gonza´lez *et al*., 1982). Given the severe impact of mosquito and black fly borne human diseases, there is considerable interest in identifying additional dipteran-active toxins. *B. thuringiensis* is now the most widely used biologically produced pest control agent.

Synergistic interactions were demonstrated with all combinations of toxins, although the extent of this interaction was dependent on the combination. No combination, however, was as active as the native *B. thuringiensis* var. *israelensis* inclusion. There might be additional factors important for toxicity associated with the native crystal. It is also possible that native crystals might be ingested or solubilized more efficiently than those from the recombinant strains. Additionally, the presentation of all four toxins in a single crystal might be more efficient than a mixture of four inclusions (Suvarchala and Jamil, 2000). The effect of inactivating *cry11A* (Poncet *et al*., 1993) was

to halve the toxicity of the resulting strain to *A. aegypti* larvae. The structures of three crystal proteins-Cry3A (Li *et al*., 1991), Cry1Aa (Grochulski *et al*., 1995), and Cyt2A (Li *et al*., 1996) have been solved by X-ray crystallography.

Over 500 species of insects have become resistant to one or multiple synthetic chemical pesticide (Georghiou and Lagunes-Tejeda, 1991). In the past, it was assumed that the insects would not develop resistance to *B. thuringiensis* toxins since they have coevolved. However in 1980s a number of insect populations of several different levels of resistance to the *B. thuringiensis* cry proteins were obtained by laboratory selection experiments, using either laboratory adapted insects or insects collected from wild populations (Ferre *et al*., 1995; Tabashnik, 1994). Better understanding of the correlation between structure and function of the cry protein can lead to better pest management. The information that could locate the lytic segments of these toxic proteins is useful in understanding their mode of action.

The prediction of the secondary structure of proteins has been applied to many areas of protein chemistry. The experiments on proteins folding demonstrate that a protein could be denatured and refolded without loss of biological activity. This imply that the amino acid sequence contains all sufficient and necessary information to define a 3D structure of a protein. During the analysis, we needed a quick and effective way to build automatic alignments that would still give good predictions is needed such as JPred.

B. thuringiensis toxin interacting with and permeate cell membranes (Chang *et al*., 1993; Hofte and Whiteley, 1989). These properties are characteristic of naturally occurring membrane permeating short polypeptides (25-35 residues long) such as bee mellitin, shark repellant neurotoxin, paradoxin and antimicrobial peptides magainin, cecropin and dermaseptin. Some of these toxins are believed to exert their permeating activity via pore formation as is also hypothesized for *B. thuringiensis* toxin. A common feature for this family of toxins is their potential to form amphiphillic a helix structures. Several reviews have been published regarding the mechanism or the mode of action of the toxins (Gill *et al*., 1992; Knowles and Dow, 1993; Knowles, 1994; Schnepf, 1998)

The recognition of importance of amphipathic alpha helical conformation in lytic activity has lead to the design of synthetic peptides capable of forming amphipathic structures in general. It is accepted that a peptide of at least 20 amino acids is necessary to span the membrane to affect the formation of ion channels (Lear *et al*., 1988). The hydrophobic moment plot of *B. thuringiensis var israelensis* 135 kDa protein revealed certain putative surface seeking helices. In order to evaluate their role in inducing toxicity, a putative amphiphillic helix corresponding to 132-150 amino acid residues of the protein was synthesized and investigated in this study. This region corresponds to the $\alpha 3$ segment of the cry 3aγ endotoxin as revealed by x-ray crystallography.

MATERIALS AND METHODS

In silico studies

Homology modeling, secondary structure and transmembrane segment predictions: The two amino acid sequences for cry4a and cry11a were retrieved from SwissProt database (accessions P16480 and P21256, respectively) (Boeckmann *et al*., 2003). The homology models for the proteins were generated by submitting to the CPH modeler (Lund *et al*., 2002). The cry4ba and cry2aa proteins (PDB Id: 1W99 and 1I5P) were the templates on which the homology models of cry4a and cry11a were generated. The amino sequences of cry4a and cry11a were submitted to JPRED Server for secondary structure prediction (Cuff *et al*., 1998). The PFAM database was queried for the conserved domain recognition for the proteins (Bateman *et al*., 2002). The Transmembrane segments were identified and hydrophobicity values were calculated using TOPPRED for these proteins (Claros *et al*., 1994).

In vitro studies

Investigating the toxicity of the synthetic peptide: The segment of cry4a (135 kDa) protein corresponding to amino acids 132-150 ("TYISNANKILNRSFNVIST") was synthesized by Research Genetics Inc., USA., using solid phase method. The biological activity of this synthetic peptide was tested.

Mosquito larvicidal activity and the hemolytic assay: The larvicidal activity of the peptide was tested against 2-5 days old *Aedes aegypti* larvae by performing bioassays. About 10 larvae were used for each concentration of the synthetic peptide. The hemolytic activity of the protein was investigated as a function of time and concentration. The bioassays were performed in triplicates in microtitre plates. Controls contained only water. 5ml of fresh rat blood was centrifuged with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride to remove the buffy coat. Erythrocytes (0.5% v/v) were incubated at 37°C in the same buffer with different concentrations of peptide

(10-50 μg/ml) for 30 min. The suspension was centrifuged and absorbance if the supernatant was measured at 540 nm. The lysis obtained by treating with 1% Triton X-100 was taken as 100%.

Circular dichroism spectroscopy: Circular dichroism spectra of the synthetic peptide were recorded on a Jobin YVon Dicrograph V spectropolarimeter at 25°C in cells with path length of 0.1 cm. The concentration of peptide used was 0.038 mg/ml.

Membrane interaction of the peptide and the alkali solubilized activated crystal protein: The ability of the toxins to perturb the synthetic membrane was investigated using the peptide and alkali solubilized activated crystal proteins. The alkali solubilized crystal extract was prepared as follows; *Bacillus thuringiensis var israelensis* was grown on NYSM (nutrient yeast extract mineral salt medium) broth with 10% glycerol for 5 days on an orbital shaking incubator at 30°C. The sporulated culture was harvested by centrifugation at 6000 ×*g* for 10 min in a Sorvall centrifuge at 4°C and washed with distilled water. The cells consisted of spores and crystals which were separated by isopynic centrifugation at 25000 ×*g* in a Sorvall SS-34 rotor for 3 h in 50% w/v of sodium bromide. The crystals were buoyant on the top of the centrifuge tube, whereas spores and spore-crystal aggregates were at the bottom. The crystals were washed with double distilled water and solubilized in alkali (100 mm, pH 12) for 5 h at room temperature and then pH was lowered to 8 using 0.05 M Tris-Cl pH 8. The mixture was centrifuge to clarity and referred to alkali solubilized crystal extract. The guts were micro dissected , homogenized in a buffer containing 50 mm Sodium Carbonate (pH 10.5) and 10 mM DTT, centrifuged at 10000 ×*g* for 10 min, and the supernatant was stored in aliquots at -20°C. Alkali solubilized crystal extract was treated with these extracts for 30 min at 37°C (Drobniewski *et al*., 1989). To stimulate high pH of larval guts, trypsin was used for activation *in vitro* (1:20) at 25°C. The reaction was terminated by adding 2 μl of 100 mM phenyl methylsulfonyl fluoride.

Preparation of carboxyfluorescein loaded liposomes: A 100 mM solution of carboxyfluorescein was made in a buffer containing 5 mM HEPES and 120 mM sodium chloride an pH adjusted to pH 7.4 such that all the carboxyfluorescein (CF) just dissolved and the solution changed color from orange to maroon (Blumenthal *et al*., 1977; Weinstein *et al*., 1977). Phosphotidyl choline (4 mg/ml) was taken from a chloroform stock solution and dried to a thin film using Rotavaporator. The CF solution was added to the lipid film and vortexed. The lipid was completely suspended in the aqueous solution. This suspension of multilamellar vesicles was then sonicated for 30 min using sonifier. The initial turbid solution became clearer after 20 min of sonication. A gel filtration column of Sephadex G-75 was used to separate the liposomes from free carboxyfluorescein. The column was saturated with 5 mg of phosphotidyl choline to prevent non-specific binding of the lipid. The column was run with a buffer containing 120 mM sodium chloride and 5 mM HEPES (pH 7.4). The CF loaded liposomes eluted in the void volume and the free CF eluted much later. The CF vesicles were kept on ice and assayed for lipid concentration.

Quantification of phospholipids: Ferric thiocyanate (0.1 N) solution was prepared by mixing 27 gl^{-1} of ferric chloride hexahydrate and 30 gl^{-1} ammonium thiocyanate and 2 ml of this solution was mixed with 2 ml of lipid in chloroform. After mixing, the lower chloroform was transferred to another test tube, ensuring no ferric thiocyanate was transferred. The optical density at 488 was measured and plotted.

Carboxyfluorescein release assay: The fluorescence of the carboxyfluorescein is self quenched above 50 mM concentration. However, any agent causing the release of CF ensures its dilution in external buffer. This causes relief of this quenching and strong enhancement in fluorescence intensity. The release of CF from the encapsulated small unilamellar vesicles was measured using a Hitachi 650-10S spectrofluorimeter. The excited monochromator was set at 495 nm and the emission at 520 nm. The emission was continuously monitored after addition of solubilized crystal protein/peptide to the lipid vesicles. Enhancement of the fluorescence measures the permeabilisation of the vesicles. Complete release of the CF was obtained by the addition of Triton X-100 (0.1 % v/v).

The CF trapped liposomes were suspended in 1 ml of HEPES buffer; different concentrations of the peptide were added and measured continuously in spectrofluorimeter. The alkali solubilized activated protein was also treated similarly. For every set of the experiment, a positive control in terms of the value obtained after treating the liposomes with 0.1% Triton X-100 was used to adjust the sensitivity of the fluorimeter and to make quantitative comparisons between protein/peptide activities.

Osmotic protection experiment: Liposomes were suspended in a buffer (pH 7.4) containing 120 mM sodium chloride and 5 mM HEPES. The osmolarity was adjusted to 300 mOsm with D-mannitol, sucrose, raffinose, arabinose and PEG of molecular weight of 1540. Since the external medium will exert an osmotic pressure, various osmoprotectants were used to observe the effect of molecular weight on the variation in lysis time. Then the alkali solubilized crystal extract (5 μg) was added and lysis was determined as above after incubating for 30 min. The molecular diameters of the substances were taken as mannitol 7Å, sucrose 9Å, raffinose 110Å, arabinose 110Å and PEG 1540 240Å (Scherrer and Gerhardt, 1971)

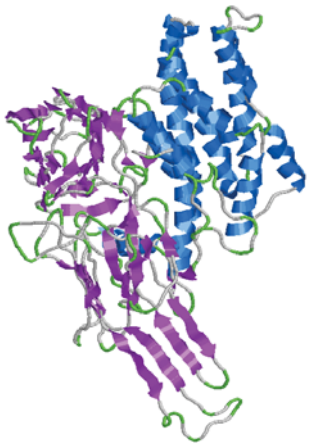

Figure 2. Homology modeling prediction of cry11a (72 kDa) protein.

RESULTS

The homology modeler returned incomplete structure for cry4a (135 kDa) protein (Fig. 1). The 3D prediction for the protein structure was for the two segments from 1 to 677 and 926 to 1055 amino acid residues. There is no prediction for the segment from 678 to 925 and 1056 to 1180 amino acids residues due to the unavailability of the suitable templates. The gap between the two predicted segments is of 248 amino acids in length. The structure contained segments with two amino acids making up helix which was not accurate prediction, but the helices were evident. The 3D structures for proteins with more than 1000 amino acids are rare in PDB and queries of this sequence length are not accepted by many online modelers like the automated Swiss modeler. The prediction of the cry11a (72 kDa) protein was complete but structurally inaccurate (Fig. 2). The returned prediction was 630 amino acids in length but the final 14 amino acids were not modeled. The surface seeking helices were evident, even though the structures were not technically correct the information. The returned secondary structure by the JPred server showed the most probable regions for finding a strand or a helix (Table 1). The prediction for cry4a protein showed two regions for helices and seven regions for the strands and for cry11a protein showed one region for helices and two for the strands. The secondary structures which were given low reliability scores were not recorded. In cry11a protein, the endotoxin N domain was from 32 to 235 amino acids and in cry4a protein was from 322 to 528 amino acids. But endotoxin M and endotoxin C domains were found exclusively in the 135 kDa cry protein. These domains contained amino acid residues from 70 to 317 and 538 to 678 (Table 2). The Transmembrane segments were found in both proteins. In cry4a protein there were 4 candidate segments, out of which only one was certain, whereas the other three were putative (Fig. 3 and Table 3). In cry11a protein (Fig. 4 and Table 3), 2 segments were found to be transmembrane with certainty.

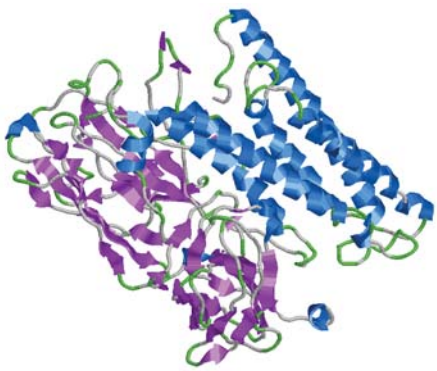

Figure 1. Homology modeling prediction of cry4a (135 kDa) protein.

Table 1. Protein regions showing most probable secondary structures prediction by *JPred*.

Proteins	Helices	Strands
CRY4A 135 KDa	1. From 110 to 309 2. From 1051 to 1155	1. From 0 to 110 2. From 323 to 455 3. From 500 to 672 4. From 680 to 752 5. From 768 to 857 6. From 1006 to 1045 7. From 1168 to 1178
CRY11A 72 KDa	1. From 70 to 257	1. From 317 to 409 2. From 445 to 639

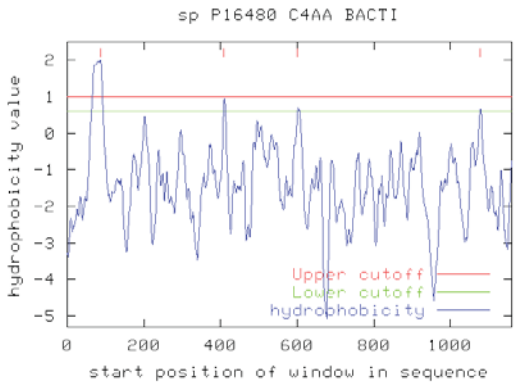

Figure 3. Transmembrane segment prediction cry4a (135 kDa) protein.

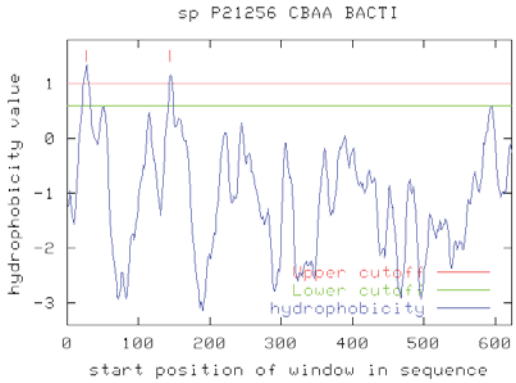

Figure 4. Transmembrane segment prediction cry11a (72 kDa) protein.

Toxicity of the synthetic peptide was tested against 2 to 5 day old larva *Aedes aegypti* larvae. The data shows that 1-3 day old are susceptible and LC_{50} value ranged 10 to 50 μg/ml. In contrast, the 5 day old larvae were totally resistant even at the concentration of 50 μg/ml (Table 4).The hemolysis of the peptide was investigated as a function of time and concentration. As shown in Figures 5 and 6 about 20% of red blood cells were lysed after 45 min at 50 μM concentration. The CD spectrum of the peptide showed helical structure in methanol (Fig. 7) and a β sheet structure in HEPES buffer (Fig. 8). These plots were interpreted by comparison with the standard plots of helices, sheets and coils (Kelly and Price, 2000). The effect of the peptide and the alkali solubilized activated protein on permeability was investigated by CF leakage experiments. The peptide released the encapsulated carboxyfluorescein dye from the liposomes at a concentration of 10 μm (Fig. 9). Further increasing the concentration caused less leakage. The release was very rapid initially followed by slow release over a period of 3 h. The release was around 20%. In case of alkali solubilized protein similar effect was observed (Fig. 10), but the protein caused rapid release at a concentration of 5 μg/ml. In the presence of osmoprotectants like mannitol, sucrose, raffinose and PEG 1540, the CF release was investigated for the alkali solubilized protein (Fig. 11). It was observed that depending on the molecular weight of the osmoprotectant, the lysis was delayed and 55% lysis was observed over a period of 30-40 min.

Table 2. Toxic domains.

CONSERVED ENDOTOXIN DOMAINS *(SOURCE: PFam)*	
Endotoxin-M	In 135kDa cry4a protein from 70 to 317 residues
Endotoxin-C	In 135kDa cry4a protein from 538 to 678 residues
Endotoxin-N	In 135kDa cry4a protein from 322 to 528 residues
	In 72kDa cry11a protein from 32 to 235 residues

Table 3. Transmembrane segments.

Transmembrane segments									
Cry4a					***Cry11a***				
Found: 4 segments;					Found: 2 segments;				
Helix	Begin	End	Score	Certainity	Helix	Begin	End	Score	Certainity
1	87	107	2.011	Certain	1	27	47	1.348	Certain
2	410	430	0.954	Putative	2	145	165	1.161	Certain
3	602	622	0.698	Putative					
4	1078	1098	0.640	Putative					

Table 4. Mosquito larvae bioassay with the peptide**.** Figures represent the range of peptide concentrations for which approximately 50% mortality was seen using a serial dilution probit analysis was not applied since the change in the toxicity from one dose to the next was quite dramatic .each experiment conducted in triplicates.

Species	Age(days)	Number of larvae	Number dead	Approx LC_{50} $\mu g/ml^{-1}$
A. aegypti	1	20	6	10-20
	2	20	2	10-30
	3	20	1	10-50
	4	20	-	10-50
	5	20	-	10-50

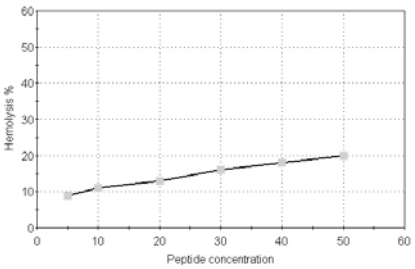

Figure 5. Hemolytic activity of the peptide - increasing concentration of the peptide.

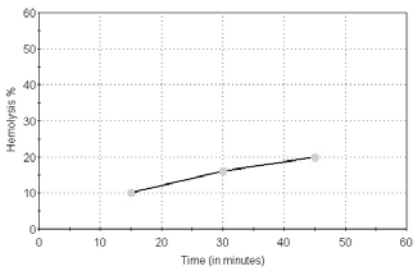

Figure 6. Hemolytic activity of the peptide-at different time periods and same concentration of the peptide (50 um).

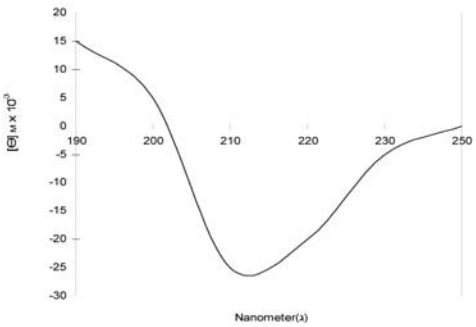

Figure 7. CD spectrum pf peptide in methanol. Concentration of the peptide = 0.038 mg/ml.

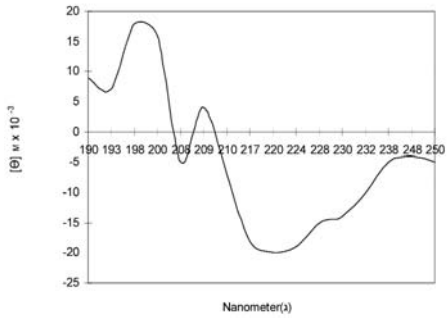

Figure 8. CD spectrum of peptide in 10 mM HEPES buffer (pH 7.4). Concentration of the peptide = 0.038 mg/ml.

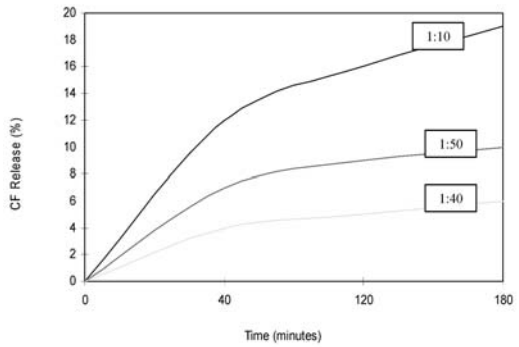

Figure 9. Release of encapsulated carboxyfluorescein from PC Vesicles in presence of peptide Liposomes containing trapped carboxyfluorescein were suspended in 1 ml of 5mM HEPES buffer (pH 7.4). Increase in fluorescence was monitored as a function of time and peptide concentration. Peptide lipid ratios are shown in figure. Triton X-100 was taken as 100%.

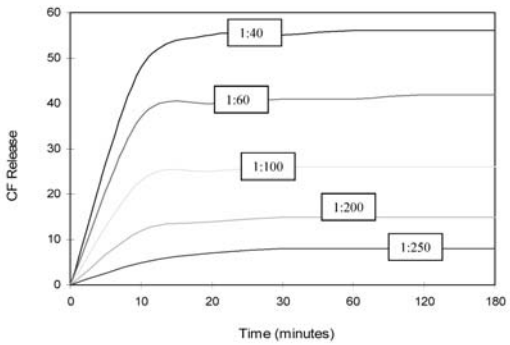

Figure 10. Release of encapsulated carboxyfluorescein from PC vesicles in presence of alkali solubilized extract. Increase in fluorescence was monitored as a function of time and protein concentration. Protein and lipid ratios are shown in the figure. Triton X-100 was taken as 100%.

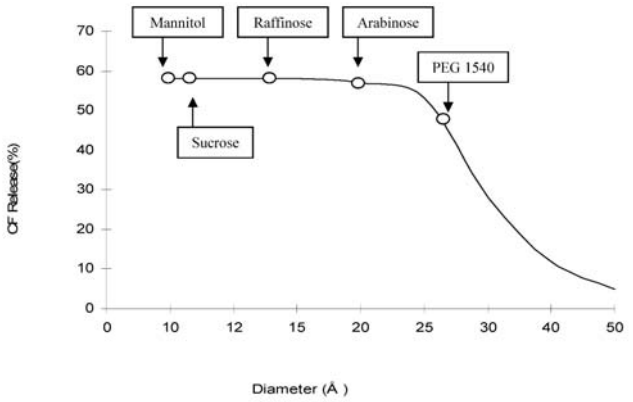

Figure 11. Alkali solubilized extract induced CF release in the presence of various osmoprotectants. Entrapped liposomes were suspended in 5 mM HEPES buffer and 300 mM protectant. Subsequently 5 μg of protein was added and lysis was determined after incubation for 30 min at 37°C.

DISCUSSION

The primary goal of protein engineering of *cry* proteins is to create better pesticides through rational design. A mutation (H168R) in helix a5 of Cry1Ac, domain I, caused a twofold increase in toxicity against *M. sexta* (Wu and Aronson, 1992). An increase in irreversible binding was correlated with the increase in toxicity for these mutants. For which a clear understanding of structure of the protein is essential.

The 3D structures and secondary structures were predicted for the cry11a and cry4a proteins. These proteins were seen containing toxin domains along with transmembrane segments. The consensus secondary structure prediction method of *JPRED* has accuracy of about 80%. Crystallographic methods cannot be replaced by these methods except for speed of getting structure prediction for a small sacrifice. The traditional Chou-Fasman and Garnier methods have accuracies of about 50-60%. Predicting the secondary structure for a protein sequence using these methods yields results which are less accurate, but when applied to a family of proteins rather than a single sequence, they prove much more accurate at identifying core secondary structure elements. The combination of sequence data with neural networks has lead to accuracies of above 70%. Though this seems a small percentage increase, these predictions are actually more useful for single sequences, since they tend to predict the core accurately. Moreover, the limit of 70-80% may be a function of secondary structure variation within homologous proteins.

JPred is a web server that takes a protein sequence or multiple alignment of protein sequences, and from these alignments secondary structure can be predicted by using a neural network called *JNet*. The prediction is the definition of each residue into alpha helix, beta sheet or random coil secondary structures. JPred was originally developed as a result of a study to compare various contemporary secondary structure prediction methods. The prediction by the homology model predicted helices in same region as the JPred prediction for both the proteins. The 3D structure of the proteins clearly shows the N-terminal bunch of helices which are membrane spanning. The proteins contained known domains, when searched for in the PFam database. These were toxin domains endotoxin N, endotoxin M and endotoxin C. The toxic domains are an important part of the proteins as they represent segment which has role in the toxicity of the protein. The endotoxin N domain was present in both the cry11a and cry4a proteins. But endotoxin M and endotoxin C domains were found exclusively in the cry4a protein (Table 2). The Transmembrane segments exhibit the secondary structure of amphipathic helices which span the cell membrane. These helices are known to play vital role in the toxic nature exhibited by the proteins (Suvarchala and Jamil, 2000). Such transmembrane segments were found in both proteins. (Figs 3, 4 and Table 3).

Toxicity of the synthetic peptide was tested against 2 to 5 days old *Aedes aegypti* larvae. The resistance of the 5 day old larvae, in contrast to the susceptibility of 1-3 day old larvae, might be due to degradation of the peptide by the proteases present in the larval midgut or the poor insertion of the peptide into membrane or the peptide unable to form functional ion channels. The hemolysis of the peptide was investigated as a function of time and concentration. The peptide showed very little hemolytic activity.

The CD spectrum was examined using Jobin YVon Dicrograph V spectropolarimeter. This was done in methanol and buffer (10 mM HEPES, pH 7.4) in order to compare the solubility of solute. The peptide showed helical structure in methanol (Fig. 7) and a β sheet structure in HEPES buffer (Fig. 8). This technique can be replaced by the computational secondary structure prediction programs since the results of the *in silico* technique are accurate enough. This was the case in our study.

CF release was monitored using phosphotidyl choline vesicles as a model system to prevent the contribution of phospholipids head group charges to binding process and preferential binding to the entire *B. thuringiensis* toxin (Thomas and Ellar, 1983). The

effect of the peptide and the alkali solubilized activated protein on permeability was investigate by CF leakage experiments. The peptide released the encapsulated carboxyfluorescein dye from the liposomes at a concentration of 10 μm and the release was very rapid initially followed by slow release over a period of 3 h. Sum of 20% of the dye was leaked out of the CF vesicles. In case of alkali solubilized protein similar effect was observed, but the protein caused rapid release at a concentration of 5 μg/ml. In the presence of osmoprotectants, the dye release was observed to be dependent on their molecular weight. It could be concluded that the peptide has less toxicity than the native protein. This is in corroboration with earlier results that there is a synergistic effect between the toxins. No single protein or the peptide is as toxic as the native crystal. Since the external medium will exert an osmotic pressure the toxin induced hole will not be enough for the dye to leak out of the cell. To investigate their effect on the leakage of the dye, various osmotic protectants were used. Large molecules were able to delay cytolysis.

In presence of the alkali solubilized crystal protein could adopt an amphipathic, alpha helical structures in which the axis of the helix lies parallel to the surface of the membrane. This process is initially driven by the electrostatic interactions between the protein and the phospholipids followed by the rotation of the helix about the axis to allow the hydrophobic interactions between the hydrophobic amino acids on the protein an the non polar head groups on the phospholipids as envisaged for the cecropin (Fink *et al*., 1989; Morriset *et al*., 1997).

The association of peptide or alkali solubilized protein with phosphotidyl choline vesicles result in efflux of carboxyfluorescein. The leakage of CF is due to appearance of pores in the membrane, thus at comparable peptide and lipid ratios more molecule of the proteins may bind to the vesicles and there might be hydrophobic interactions causing destabilization as envisaged for other cytolytic peptide (Suenaga *et al*., 1989). The whole *B. thuringiensis* toxin bind better to zwitterion phospholipids than to acidic ones (Thomas and Ellar, 1983). Addition of alkali solubilized extract caused a relatively rapid release followed by slow release phase. In the liposomes, which lack specific receptors, pore formation is dependent upon the relatively slow association of the toxin molecules by lateral diffusion. But in insect cells, the receptors in addition to recognizing and binding the toxins may promote toxin aggregation and thus accelerating the toxin formation. The hydrophobic groups may be brought together in close spatial proximity when protein binds receptor. It could be inferred that association between different toxin molecules to form the intramolecular aggregate and the specific assembly of the helices from several monomers to form a pore are essential steps in toxin action. For this reason probably the native crystal is more toxic than any single protein component. Several studies have been reported the synergistic effect of the toxin (Chang *et al*., 1993; Tabashnik, 1992; Wu and Chang, 1985).The low permeability of the *B. thuringiensis* peptide may be because of the disordered structures in the liposomes or it cannot self assemble. The interaction with the membrane may lead to some extent disruption of the membrane, causing small local leakage. The *cry* proteins generally form crystalline inclusions in the mother cell compartment. Depending on their protoxin composition, the crystals have various forms: bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A). This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. However, the crystals have to be solubilized rapidly and efficiently in the gut of insect larvae to become biologically active. The activation and toxicity of a crystal presumably depend on factors such as the secondary structure of the protoxin, the energy of the disulfide bonds, and other such structural characteristics.

In the present study, it was attempted to correlate the amino acid sequence of the protein to the function of toxicity. The structure-function correlation has shown the certainty of the involvement of the amphipathic helices in the toxic activity. Even though these membrane spanning helices are vital for the induction of toxicity, they cannot act in isolation. This is proven by the results from the experiments carried out on the synthetic putative membrane spanning peptide of the *cry4a* toxin of *Bacillus thuringiensis*.

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