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In-silico Analysis of Loop Residues of the Receptor Binding Domain of *Bacillus thuringiensis* Cry2Ac11 Toxin

Faiza Saleem,[1] Quratulain Amjad,[1] Afshan Kaleem,[1,*] Roheena Abdullah[1], Mehwish Iqtedar[1], Shagufta Naz[1] and Abdul Rauf Shakoori[2,*]

[1]*Department of Biotechnology, Lahore College for Women University, Lahore*

[2]*School of Biological Sciences, University of the Punjab, Lahore*

ABSTRACT

Genetic engineering of *Bacillus thuringiensis* Cry proteins has resulted in the synthesis of various novel toxin proteins with enhanced insecticidal activity and broader specificity spectrum. Toxin-receptor interaction is known to be the most crucial and rate limiting step for toxin activity and specificity determination but still very less information is available regarding the receptors of Cry2 proteins and the mechanism of receptor-ligand interactions of BT toxins. In the present work, detailed *in-silico* studies of the binding epitopes of *Bacillus thuringiensis* Cry2Ac11 toxin are done. To get the knowledge about the binding characteristics of each single residue in the receptor binding region, various bioinformatics tools have been employed and protein modeling, sequence and structural homology analysis, alanine scanning and protein docking studies were done. Also the binding interactions of individual residues with different lepidopteran and dipteran receptors have been predicted. The collective knowledge of Cry toxin interactions with its potential receptors will lead to a more critical understanding of the structural basis for receptor binding and specificity determination. This study will serve as a starting point for designing mutagenesis strategies aimed to improve the insecticidal activity of Cry proteins.

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Authors' Contribution

FS and ARS conceived and designed the study. QA and AK developed, analyzed and verified the 3D structure. RA, MI and SN analyzed the data. FS, AK, SN and ARS wrote the article.

Key words

Azomite, GMT, body composition, growth performance, specific growth rate, feed conversion rate, lipase.

INTRODUCTION

A major cause of the loss of agricultural crops is the destruction caused by insect pests. In a country like Pakistan, where agriculture supports our economy, such loss is not bearable. Chemical pesticides may however play an important role in eradicating these insect pests and boosting agricultural production, but their haphazard use can also lead to harmful consequences eventually affecting the human health. Bio-pesticides provide quite safer alternative to their chemically synthetic counterparts. For increasing percentage yield and pest control *Bacillus thuringiensis*, a rod shaped gram-positive bacterium, has been used as substitute or supplement to chemical pesticides (Seifinejad *et al.*, 2008; Wu *et al.*, 2007). The insecticidal properties of *Bt* toxins were discovered in the early 20th century (Seifinejad *et al.*, 2008) and since then it has been widely used either in form of bio-pesticides or transgenic plants.

Crystalline (Cry) protein is produced during stationary phase of *Bt* at the end of sporulation, which is accumulated as inclusion bodies in the mother cell compartment constituting up to 25% of total dry weight of the sporulated cells (Lima *et al.*, 2008). On ingestion, these proteins solubilize and on activation cause the formation of pores in the apical membrane of midgut cells. As a result of which the insect dies (Soberón *et al.*, 2012). Hundreds of Cry toxins have been discovered and are classified into several groups based on their amino acid homology. Five subgroups of Cry2A family are known, Cry2Aa, Cry2Ab, Cry2Ac, Cry2Ad and Cry2Ae (Lima *et al.*, 2008; Zhang *et al.*, 2007). Cry2Ab along with Cry2Ac, Cry2Ad, Cry2Ae, and Cry2Af are known to be toxic to only lepidopteran pests (Zheng *et al.*, 2010); Whereas Cry2Aa exhibits broader toxicity against Diptera (mosquitoes and flies) and Lepidoptera (butterflies and moths). Five tertiary structures of Cry proteins have been determined through X-ray crystallography, namely Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, and Cry4Ba. All these reported structures suggested that the active toxins are actually globular molecules consisting of three domains that are conserved. This property necessitated elucidation of three-dimensional structures of the rest of the reported Cry family members.

Several reports have demonstrated that Domain II is responsible for receptor recognition and specificity determination among all three domains of Cry proteins. Mutagenesis data also revealed the possible roles of loops of Domain II in receptor binding (Likitvivatanavong *et al.*, 2009; Fernández *et al.*, 2005; Cantón *et al.*, 2011). Two ways of binding of Cry protein are reported. In first,

* Corresponding author: arshaksbs@yahoo.com, afshankaleem@yahoo.dk

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to induce toxicity the sequential binding takes place with multiple receptors, while according to the second, binding to the cadherin stimulates an intracellular reactions cascade leading to the toxicity in cell. Frequently mentioned Cry toxin receptors include cadherin-like proteins, GPI anchored APN and ALP (alkaline phosphatase) and also a glycoconjugate (270 kDa) (Likitvivatanavong *et al.*, 2009; Schnepf *et al.*, 1998). A number of studies regarding the mutational analysis of the binding epitope have been done previously and the effect of these mutations has been analyzed on receptor binding and toxicity. For example in the case of Cry3Aa the replacement of N353 and D354 of loop 1 with alanine resulted in loss of the receptor binding and toxicity. The replacement of W357 of loop 1 in binding domain of Cry19Aa with alanine resulted in the loss of toxicity against mosquitoes. The mutations Y410A, W416A, and D418A of the loop 2 in binding domain of Cry19Aa resulted in reduced toxicity against *Culex, Aedes aegypti*. The alanine scanning of Cry4Aa mutants showed comparable toxicity to wildtypes (Boonserm *et al.*, 2006). Unlike these reports the mutational analysis in present study indicated no marked difference in insect specificity and toxicity as compare to wild type and the toxicity profiles were quite similar to wild type.

In the present study binding interaction of Cry2Ac11 has been examined with its putative receptors. Moreover, the binding epitope of Cry2Ac11 has been analyzed in detail by generating different mutations and observing their effect on receptor binding and toxicity.

MATERIALS AND METHODS

Sequence retrieval and sequence alignment

Sequences of Cry2Ac11 (strain HD29 ID AM689531) was retrieved from NCBI (www.ncbi.nlm.gov). The Sequence of Cry2Aa (accession number UniProtKB: P0A377) was also retrieved from NCBI. Both the sequences were multiply aligned using ClustalW2 with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Amino acid similarities and differences in both specificity determining regions and binding regions were observed. Cry2Ac11 was also aligned with other Cry2Ac type proteins Cry2Ac7 (AM292031) Cry2Ac8 (AM421903), Cry2Ac9 (AM421904) and Cry2Ac12 (AM689532) and their similarities and differences were analyzed.

Construction of mutants

Several mutants were designed by replacing different amino acids from loop 1 and 2 of Domain II. The mutants were generated by replacing amino acids with the ones having similar chemical properties or having entirely different properties, to predict their role in receptor binding and toxicity. The details about the mutants are given in Table I.

Table I.- Mutants of loop 1 and 2 of Domain II of Cry2Ac11.

Sr. No.	Mutations	Amino acid sequence
1.	**L**324**R**	FPNIGG**R**PG
2.	**G**322**P**	FPNI**P**GLPG
3.	**I**321**S**	FPN**S**GGLPG
4.	**I**321**S, L**324**T**	FPN**S**GG**T**PG
5.	**P**319**A**	F**A**NIGGLPG
6.	**P**319**A, P**325**A**	F**A**NIGGL**A**G
7.	**A**314**I,R**315**F**	IFTTFPNIG
8.	**T**332**A,H**334**L**	STTTQALL
9.	**V**385**K**	REG**K**ATST
10.	**A**386**K**	REGV**K**TST
11.	**A**386**E**	REGV**E**TST
12.	**G**384**D**,**A**386**N**	RE**D**V**N**TST
13.	**V**385**S**	REG**S**ATST
14.	**T**387**D**	REGVA**D**ST
15.	**T**387**A**	REGVA**A**ST
16.	**V**385**A,T**387**A**	REG**A**A**A**ST
17.	**A**386**P**	REGV**P**TST

Structure prediction of Cry2Ac11 and mutants

Sequences of Cry2Ac11 and its potential receptors including *Helicoverpa armigera* aminopeptidase N (APN) (Accession: AAN75694.1) and Cadherin (Accession: AFQ60152.1) and *Aedes aegypti* ALP (Accession: ACV04847.1) were retrieved from NCBI (www.ncbi.nlm.gov) and were used to predict the 3D structures using Phyre2 software (www.sbg.bio.ic.ac.uk/phyre2) and RaptorX (http://raptorx.uchicago.edu/) by using default settings.

3D structures of mutants of receptor binding loops (loop1 and loop 2) of Domain II of Cry2Ac11 were also predicted using Phyre2 software.

Structural alignment

The 3D structure of Cry2Aa reported using X-ray crystallography was used as template for homology modeling and was aligned with the predicted structure of Cry2Ac11.the structural alignment was done using pyMol software and RaptorX. The differences in the binding loops and specificity determining regions of both proteins were analyzed.

Quality analysis of predicted protein structures

For the quality analysis and structure validation of all protein structure PROCHECK and Rampage was used and Raswin and pyMol were used for the visualization of 3D structures.

Prediction of receptor-ligand interactions by protein–protein docking of Cry2Ac11 mutants and receptors

The 3D structures containing mutated loop residues of Cry2Ac11 were used to generate the protein complex comprising the receptor binding loops and receptors. The rigid-body docking program (ZDOCK) employing a fast Fourier transform (FFT) algorithm and ClusPro, were used to generate the receptor ligand interactions. The top 10 predicted complexes were selected. Complexes showing most stable interactions with the domain II of the Cry protein and low binding score (the first model) were selected for further analysis and their bonding interactions were examined.

Domain-II loop 1 and loop 2 residues were selected as the interacting residues with receptor in the ZDOCK program. The Vasker Lab program was also used to search the receptor-ligand interactions of proteins with their potential receptors.

Analysis of docking by PDBsum

All docking predictions were further analyzed on PDBsum generate (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html). The interaction profiles of all proteins and their receptors were analyzed. The binding strengths of all receptor-ligand complexes were predicted by explicating the number and types of bonds between proteins and receptors.

Analysis of binding interactions by pyMOL and VMD1.9.1

All receptor-ligand complexes were further visualized, analyzed and labeled on pyMOL Rasmol and VMD1.9.1 softwares. The bond lengths were also labeled using these softwares.

Table II.- Alanine scanning of loop 2 of Domain II.

Sr.No	Mutations	Amino acid sequence
1.	**T**380**A**	**A**DREGVATST
2.	**D**381**A**	T**A**REGVATST
3.	**R**382**A**	TD**A**EGVATST
4.	**E**383**A**	TDR**A**GVATST
5.	**G**384**A**	TDRE**A**VATST
6.	**S**388**A**	TDREGVAT**A**T
7.	**T**389**A**	TDREGVATS**A**

*The mutated amino acids are indicated in red.

Alanine scanning of loop 2 residues

For further analysis of the role of individual residues in receptor binding alanine scanning was done (Table II). The interaction profile of these loop residues was also predicted by using docking programs and then all docking predictions were analyzed using above mentioned software's. These binding predictions of alanine substituted epitopes were than compared to the binding profiles of each original residue with potential receptors.

RESULTS AND DISCUSSION

Homology modeling

Sequence of Cry2Ac11 was aligned with Cry2Aa to analyze the similarities as well as substitutions. (Fig. 1) Cry2Aa showed 98% sequence similarity with Cry2Ac11. The alignment of specificity determining regions of both proteins also showed conserved amino acids with very few substitutions (Fig. 2). The sequence of Cry2Aa was retrieved from PDBsum database and was used as a template for structure prediction of Cry2Ac11 and mutants. For homology modeling the predicted structure of Cry2Ac11 was aligned with the reported structure of Cry2Aa (Fig. 3). Despite few differences in amino acid sequences, the 3D structures of various Cry toxins showed similarities. So to compare the sequence similarities of Cry2Aa with Cry2Ac11, their structures were also aligned which showed only few points of differences with more than 90% sequence similarity.

Fig. 1. Sequence alignment of Cry2Aa and Cry2Ac11 by Clustal W Omega. A: Alignment of complete sequence of Cry2Ac11 and Cry2Aa. B: Alignment of Specificity determining region of Cry2Aa with Cry2Ac11. Dipteran specificity region is from amino acid 307-340 while Lepidopteron specificity region is from 341-412. The substitutions are highlighted.

Dipteran specificity region comprises 307-340 amino acid while lepidopteran specificity region is from residues 341-412. The alignment of these D and L blocks of Cry2Aa with Cry2Ac showed that in these proteins 11 amino acids that are different in D-block while 20 amino acids differ in lepidopteran specificity determining region.

3D structures of all mutants and receptor protein were predicted using Phyre2 software and RaptorX. The predicted structures showed that all mutant proteins contain similar supra secondary structures having three distinct domains containing alpha helices and beta sheets (Fig. 2).

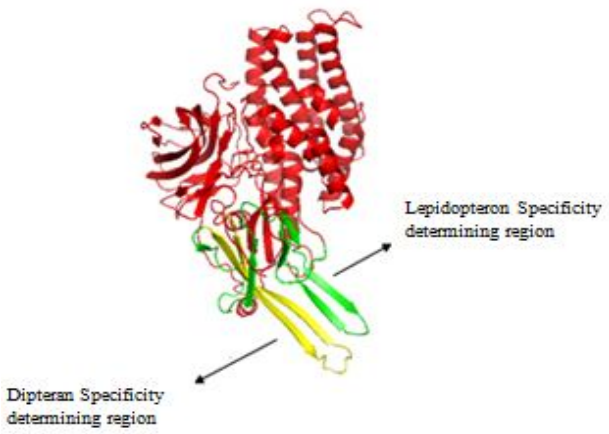

Fig. 2. Specificity determining regions. The specificity determining region for Dipterans is labelled in yellow colour, whereas the specificity determining region for Lepidopterans is shown in green colour. Loop 1 is included in D-block while loop2 is included in L-Block.

Fig. 3. Structural superposition of Cry2Ac11 with Cry2Aa using pyMol. Cry2Ac11 is shown in red color whereas Cry2Aa is in cyan.

Quality analysis

All the predicted protein structures were passed through quality analysis and structural validation by Ramachandran plot assessment. The Ramachandran plot for Cry2Ac11 is shown in Figure 4A.

For Cry2Ac11, the evaluation of residues showed 98% residues in favored region, 4.9% residues in allowed region and 13 residues in outlier region. These regions are differentiated on basis of torsion angles and favored rotation of phi and psi angles. As the induced mutations can potentially affect the protein conformation and structural fidelity, so all predicted mutated protein structures were also passed through structural validation and quality check. All the subtituted residues came into allowed region except one. In mutant pPFS2Ac11L2-9 the replaced proline at position 386 fell in outlier region (Fig. 4B). Moreover, the numbers of residues in favored region and in allowed region were different as result of each mutation. The same was true for the residues in outlier region indicating that each mutation has somewhat effect on protein conformation, although the effect was not that much to reflect any alteration in protein 3D structure.

Protein docking

To examine the mode of binding, binding stability and interaction profiles of ligand and receptor proteins, all mutant proteins were subjected to docking with *Helicoverpa armigera* Cadherin and APN receptors and *Aedes aegypti* ALP by clusPro(2.0) software. These complexes were further reranked by employing the ZDOCK and RDOCK programs.

Docking results (Table III) showed different interaction profiles of all mutants with their potential receptors. From the ten displayed models the first model was selected for further analysis. Binding interactions and bonding statistics were analyzed by PDBsum generate. The interface statistics of each protein including all mutants and Cry2Ac11 were analyzed for all three receptors including *Helicoverpa armigera* APN and Cadherin and *Aedes aegypti* ALP one by one. Interaction profile of Cry2Ac11 with ALP showed 2 salt bridges, 21 hydrogen bonds and 224 other interactions including non-bonded contacts predicting it as a weak interaction. Interaction profile of Cry2Ac11 with APN showed 18 hydrogen bonds and 220 non-bonded contacts predicting the interaction not to be very strong. As for the interaction profile of Cry2Ac11 with cadherin 3 salt bridges, 36 hydrogen bonds and 511 non-bonded contacts were observed predicting it to be a quite strong binding interaction. To check the effect of mutations on binding interactions with potential Cry2Ac11 receptors, the docking predictions for mutants were also further analyzed. All mutants showed different interaction profiles with lepidopteran and dipteran receptors when analyzed on PDBsum generate. With ALP (*Aedes aegypti*) the loop 1 mutants G322P and P319A formed 1 salt bridge each with ALP. Among loop 2 mutants, the mutant G384D/A386N and T387D showed 1 salt bridge formation each with ALP. Although the predicted binding

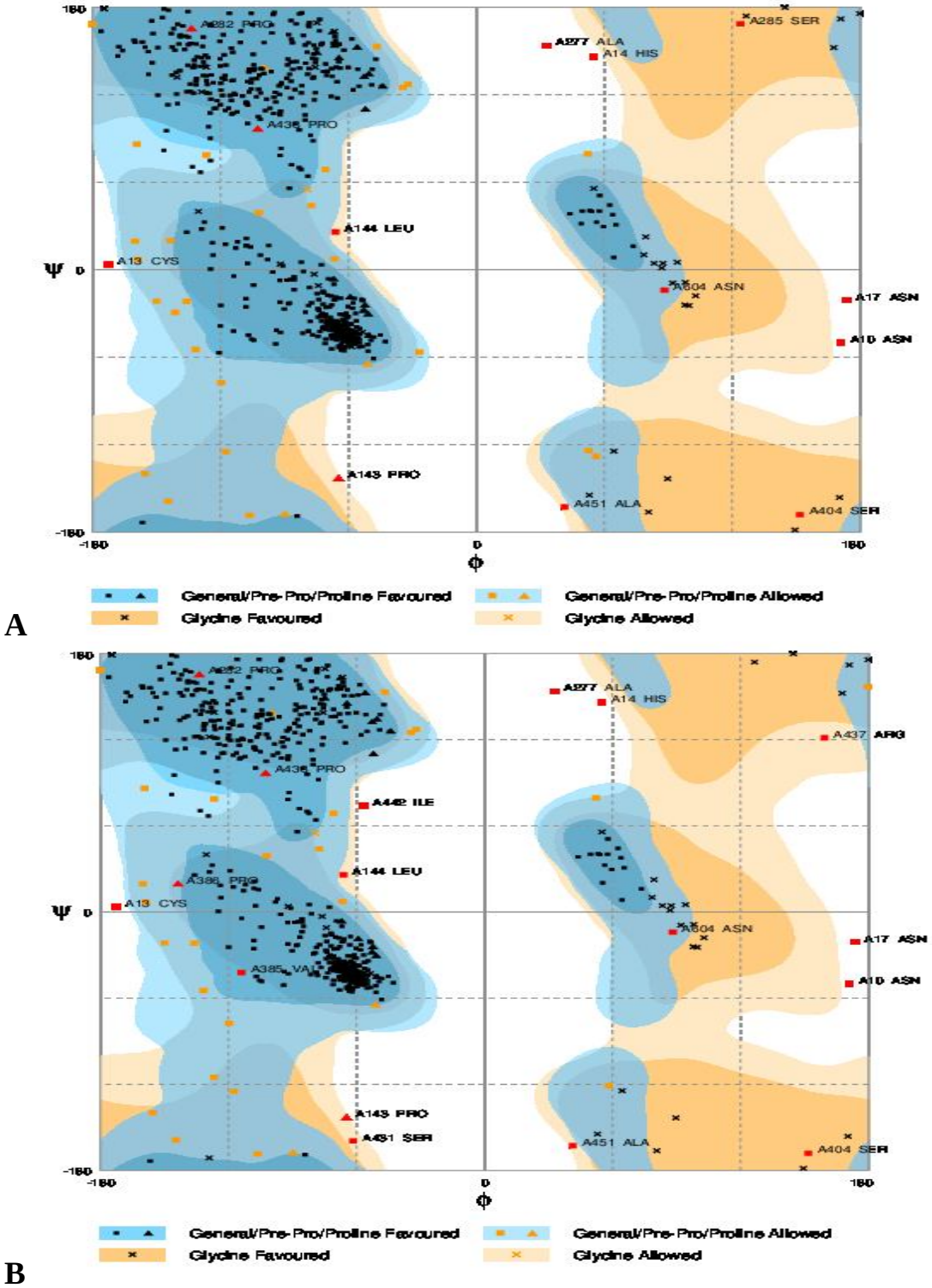

Fig. 4. Quality analysis of Cry2AC11 (A) pPFS-2Ac11L2-9 (B) by Ramachandran plot.

Table III.- Interface statistics: Binding interactions between Cry2Ac11 mutants and ALP.

Sr. No	Mutants	Mutations	Mutants and ALP			Mutant and APN			Mutant an d CDH		
			Salt bridges	Hydrogen bonds	Other interactions	Salt bridges	Hydrogen bonds	Other interactions	Salt bridges	Hydrogen bonds	Other interactions
1.	2Ac11L1-1	**L324R**	-	29	314	-	18	210	-	31	262
2.	2Ac11L1-2	**G322P**	1	26	269	-	20	238	3	18	237
3.	2Ac11L1-3	**I321S**	-	23	278	-	15	177	-	30	285
4.	2Ac11L1-4	**I321S, L324T**	-	16	221	-	21	251	-	29	302
5.	2Ac11L1-5	**P319A**	1	26	348	-	19	209	2	17	205
6.	2Ac11L1-6	**P319A, P325A**	-	16	221	-	17	224	-	33	368
7.	2Ac11L1-7	**A314I, R315F**	-	29	273	1	19	243		30	279
8.	2Ac11L1-8	**T332A, H334L**	-	28	305	2	20	251	3	19	210
9.	2Ac11L2-1	**V385K**	-	21	202	-	17	245	-	27	306
10.	2Ac11L2-2	**A386K**	-	17	191	-	18	212	-	26	277
11.	2Ac11L2-3	**A386E**	-	12	161	-	13	183	-	9	213
12.	2Ac11L2-4	**G384D**, A386N	1	15	248	-	15	199	-	11	210
13.	2Ac11L2-5	**V385S**	-	18	217	-	19	230	-	31	320
14.	2Ac11L2-6	**T387D**	1	15	263	-	23	247	2	25	253
15.	2Ac11L2-7	**T387A**	-	17	216	-	23	210	1	27	316
16.	2Ac11L2-8	**V385A, T387A**	-	16	238	-	20	227	-	20	227
17.	2Ac11L2-9	**A386P**	1	30	333	-	19	219	-	20	307

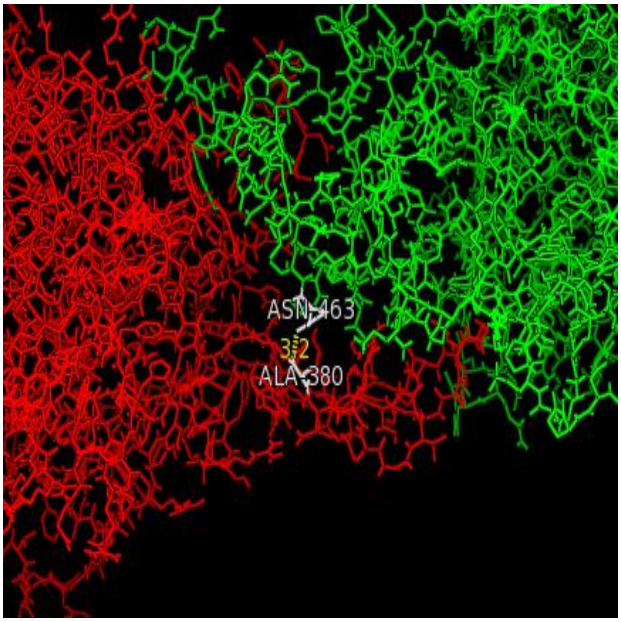

Fig 5. Hydrogen bond formation between Ala 380 of Cry2Ac11 alanine substitution mutant and Asn 463 of *Aedes aegypti* ALP. Bond length between the two interacting residues is also shown.

interactions of all loop mutants is not that strong with ALP but still to some extend it can be predicted that these mutants would show some toxicity against *Aedes aegypti in-vitro* but this toxicity is less in all loop mutants as compared to Cry2Ac11(showing 2 salt bridges). With APN *(Helicoverpa armigera)* loop 1 mutant with proline replaced with alanine showed one salt bridge and mutant withT332A and H334L showed two salt bridges whereas Cry2Ac11 showed no salt bridge formation with APN. Loop 2 mutants also showed no salt bridge formation in their predicted interactions profile. With cadherin *(Helicoverpa armigera)* none of loop 1 and 2 mutants showed binding strength greater to Cry2Ac11. But still the binding profiles showed stronger interactions as compare to that of APN and ALP. 3 salt bridges each were shown by loop 1 mutants having mutation G322P and the other with two mutations T332A and H334L. Loop 1 mutant having mutation P319A showed two salt bridges. Among loop 2 mutants the mutant having mutation T387D showed 2 salt bridges with cadherin and T387A showed 1 salt bridge. The remaining loop 1 and loop 2 mutants showed no salt bridge. Although the number of hydrogen bonds predicted was quite large for all mutants but none of them showed number of hydrogen bonds and non-bonded interactions greater than that of Cry2Ac11. So it can be predicted that all the mutants may show toxicity against *Helicoverpa armigera in-vitro* but

Table IV.- Predicted effect of alanine replacing single loop 2 residue on the overall binding interactions of protein with its potential receptors.

Sr. No.	Mutation	Predicted interactions with ALP *(Aedes aegypti)*			Predicted interactions with APN (*Helicoverpa armigera)*			Predicted interactions with CADH *(Helicoverpa armigera)*		
		Salt bridges	H-Bonds	Other contacts	Salt bridges	H-Bonds	Other contacts	Salt bridges	H-Bonds	Other contacts
1.	**T**380**A**	-	24	205	-	13	175	-	11	215
2.	**D**381**A**	11	13	172	-	20	235	-	22	308
3.	**R**382**A**	11	25	249	-	19	193	1	23	288
4.	**E**383**A**	-	8	367	-	20	263	-	5	256
5.	**G**384**A**	-	18	213	-	20	228	-	33	329
6.	**S**388**A**	-	13	203	-	21	232	-	25	262
7.	**T**389**A**	-	17	210	-	18	168	-	28	316

the toxicity would not be greater than Cry2Ac11. If we compare the toxicity predictions for loop 1 and loop 2 mutants against *Helicoverpa armigera* loop 1 mutants showed relatively greater number of hydrogen bonds whereas the number of non-bonded contacts were same. As the loop 2 residues are included in lepidopteran specificity determining region (Widner and Whitely, 1990) so it can be predicted that the mutations may somehow affect insect specificity towards lepidopteran receptors as well.

Alanine scanning

To check the role of an individual amino acids in receptor binding, alanine scanning was done. All residues in the binding epitope were replaced by alanine one by one as mentioned in Table IV. The potential role in receptor binding and toxicity was predicted by comparing the binding interactions of an amino acid and receptor protein to the interactions of alanine with receptor. The binding of individual loop 2 residues was also compared to interaction of alanine substituted in its place. With ALP, T380 did not show any bonding interaction but when it was replaced with alanine, the alanine formed a hydrogen bond with asparagine in ALP (N463). The bond length was 3.17 (Fig. 5).

Bacillus thuringiensis is a potential candidate for pest control due to its ability to produce insecticidal proteins but very few data has been generated about the receptor ligand interactions of Cry proteins.

The binding interactions of loop 1 and 2 mutants of domain II were predicted *in-silico*. All mutants displayed different binding profiles with all three receptors *in-silico*. When results were analyzed, most of the mutants showed interaction profiles comparable to those of Cry2Ac11 so to predict the role of individual amino acids towards receptor binding, alanine scanning of loop 2 residues was done. All alanine substitution mutants also showed only slight changes in interaction profiles. The slight conformational changes in protein caused by mutations were also not that much to affect protein folding. These results clearly indicate that there are multiple interaction sites in the binding epitope of Cry2Ac11 protein and the substitution of one or two amino acids cannot impart any remarkable change in toxicity profiles of protein.

Considering the findings of Liang and Dean (1994) and Widner and Whitely (1990) the peptide stretch including residues 307-412 is the specificity determining region. As the mutations done were present in this area so there was little possibility that the insect specificity and host range of Cry2Ac11 may also get affected due to the induced mutation.

To conclude, the *in-silico* analysis indicate that mutations of one or two amino acids in loop 2 of Domain II cannot impart any remarkable difference in insect specificity and toxicity spectrum for both lepidopteran and dipteran insect species as compare to wildtype

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