Khurshid *et al.*
Egyptian Journal of Biological Pest Control (2023) 33:86
https://doi.org/10.1186/s41938-023-00731-x

Egyptian Journal of Biological Pest Control

RESEARCH

Open Access

Site-directed mutagenesis in Cry proteins of *Bacillus thuringiensis* to demonstrate the role of domain II and domain III in toxicity enhancement toward *Spodoptera litura*

Huma Khurshid[1], Hafsa Zaheer[1], Fakhar-un-Nisa Yunus[1*], Farkhanda Manzoor[1], Aasma Abdul Latif[1] and Farzana Rashid[1]

Abstract

Background *Bacillus thuringiensis* (*Bt*) is a gram-positive bacterium responsible for the production of a wide variety of insecticidal Cry, Cyt, and Vip proteins with distinct insect specificities. The bioinsecticides derived from *Bt* Cry proteins account for > 95% of the microbial biopesticide market to combat a variety of pest species belonging to the order Lepidoptera (including *Spodoptera* spp.), Coleoptera, Diptera, etc. Cry proteins are engineered by using different molecular techniques to control the development of multiple insecticide resistance problems in major insect pests using bio-toxicity assays.

Main body It is common knowledge that the Cry proteins domain II and III are involved in pore formation or interaction between several insect larval receptors and the membranes of epithelial cells. In the present research, the PCR site-directed mutagenesis technique was used to introduce a total of four mutations into the *cry* genes (*cry*1 and *cry*2) near key regions of active proteins. The diet overlay bioassay was used to test the efficacy of expressed mutant Cry proteins against *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), one of the most damaging bollworms to cotton, causing severe output losses.

Results Two amino acid replacements in the receptor binding domain of Cry1Ac toxins (S573, L588) showed decrease in activity for Cry1Ac mutants. However, amino acid substitutions in the receptor binding/pore formation domain of Cry2Aa (T325, S445) were to some extent proved more toxic than wild-type recombinant Cry2Aa protein, with an increase in mortality percentage from 3.33 to 6.66% after 24 to 72 h of treatment, respectively, against 2nd instar larvae of *S. litura*.

Conclusion A comparison of activity demonstrated that larvae of *S. litura* were more susceptible to Cry2Aa toxins than those of Cry1Ac toxins after being treated with *Bt* toxins. The LC_{50} values of Cry2Aa mutants were slightly reduced for *S. litura* larvae than those of Cry1Ac mutants.

Keywords *Bacillus thuringiensis*, Overlap extension PCR, Site-directed mutagenesis, *Spodoptera litura*

Background

The decline in the production of cotton in tropical and subtropical areas has been recorded due to many reasons such as the high cost of input, biotic or abiotic factors, and the extremely harmful invasion of insect pests (Trebicki et al. 2017). Fifteen out of 31 species of genus

*Correspondence:
Fakhar-un-Nisa Yunus
fakhar_yunus@yahoo.com
[1] Department of Zoology, Lahore College for Women University, 54000-Jail Road, Lahore, Pakistan

Spodoptera among Noctuidae were identified to be agricultural pests (Pogue 2002). Due to their widespread distribution in tropical and subtropical regions, *Spodoptera* species including *S. littoralis*, *S. frugiperda*, *S. exigua*, and *S. litura* cause significant economical yield losses of several crops. *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), also referred to as cotton leafworm, armyworm, tobacco cutworm, or common cutworm, is one of the most destructive sporadic and polyphagous pests distributed worldwide (Fand et al. 2015). It infests more than 180 plant species including 112 field crops and vegetables from 44 families such as cotton, rice, maize, soybean, groundnut, celery, sweet potato, cabbage, etc. (Pazhanisamy et al. 2019). On average, its larvae can cause 26–100% damage to major cash crops resulting in huge yield loss if not controlled (Tuan et al. 2014). Over the past few decades, frequent applications of different pest control methods and excessive use of pesticides have caused the development of resistance in insect pests (Shad et al. 2012). The cotton crop in Pakistan was frequently attacked by *S. litura* in the mid-1990s and again in 2003 in the cotton belt of South Punjab caused farmers to use insecticides indiscriminately, which failed several management strategies to control *S. litura* (Saleem et al. 2008). Resistance to insecticides was suspected to be the primary factor contributing to the ineffectiveness of pest control efforts (Abbas et al. 2012). Unfortunately, significant selection pressure has also resulted in insect resistance due to the world-scale cultivation of *Bt* crops and the simultaneous usage of *Bt* spray products (Jakka et al. 2015). According to the mode of action, the toxin core of *Bt* crystals is solubilized and proteolytically activated in the gastrointestinal juice of the midgut of susceptible insects after ingestion. The binding of *Bt* Cry toxins to their respective receptors in the insect midgut is the essential stage that leads to membrane insertion and pore formation, to eventually cause osmotic stress-induced cell death via pore formation and signal transduction pathway (Adang et al. 2014).

Site-directed mutagenesis is a molecular technique for altering a protein's amino acid sequence in order to assess its function in a molecule. A mutant primer is often used to insert the altered nucleotide, and DNA polymerase extends the primer in vitro (Saiki et al. 1988). *B. thuringiensis* produces 3D-Cry toxins, which have undergone substantial mutations in an effort to better understand the intricate process underlying their ability to kill vulnerable insects. The mutant protein is expressed and evaluated for activity after the in vitro generated DNA (the mutant DNA) is counter-selected from the wild-type DNA. When an amino acid involved in a protein's mechanism of action is altered, the protein's function is affected or ultimately abolished, however this method might potentially be utilized to create novel proteins with enhanced properties (Vilchez 2020). Cry1A and Cry2A toxins, widely used in *Bt* transgenic crops, can specifically bind to insect midgut and exert their insecticidal effects (Yuan et al. 2017). In this work, site-directed mutagenesis technique by overlapping PCR method was used to introduce the variants in *cry*1Ac domain II and *cry*2Aa domain III to understand their function in activity against *S. litura*. Furthermore, this strategy is highly efficient, simple, cost effective and fast to introduce specific mutations into template DNA sequence.

Methods

All DNA markers, Lambda HindIII DNA, 100 bp and10 kb ladder were purchased from the New England Biolabs (NEB) and Fermentas, Protein high molecular weight marker from Santa Cruz Biotechnology, Inc. (USA). Macrogen, Inc. Seoul Republic of Korea synthesized all the primers. KOD Plus mutagenesis kit was purchased from Toyobo, Beijing, China, whereas, cloning vector pGEM-TEasyT7 and *Escherichia coli* BL21pLysS purchased from Promega. *B. thuringiensis* strains HD-73 and HD-1 were procured from Bacillus Genetic Stock Center (BGSC).

Design and construction of *cry*1Ac and *cry*2Aa recombinant strains

C*ry*1Ac and *cry*2Aa complete genes were amplified from wild type strains of HD-73 (4D4) strain of *B. thuringiensis* subsp. *kurstaki* (*btk*) and HD-1 (4D6) strain, respectively. Designed primers were used for overlap extension PCR cloning contained flanking overhang region of pGEM cloning vector (Table 1). Master Mix prepared by mixing 1 µl DNA (50 ng/µl), 5 µl 10xBuffer, 5 µl dNTPs, 1.5 µl (2 µm) solution of each forward and reverse primers, 2 µl $MgSO_4$, 33 µl H_2O and 1 µl KOD plus in the final volume of 50 µl. Reaction mix divided into two tubes (50 µl per tube). The reaction was initiated with denaturation and preheating at 95 °C for 2 min, 95 °C for 30 s, followed by 30 cycles of 1 min/kb at 68 °C extension phase and a final extension of 10 min at 72 °C. After PCR, the product was analyzed by 1% agarose gel electrophoresis and gel purified by gel purification mini kit (FavorPrep™). PCR products ligated for insert and vector in 1:1 ratio. After the PCR, DpnI restriction enzyme (2 µl) added to the PCR tube and incubated for 1h at 37 °C to degrade the original parental plasmid and transformed in *E. coli* host system.

Site-directed mutagenesis through overlap extension PCR

Four mutants were designed by amino acids in domain II and III in order to create a number of distinct mutants to predict their function in toxicity. All mutations were selected near to key regions involved in receptor binding and toxicity. Site-directed mutagenesis was performed

Table 1 Specifically designed chimeric primers for overlap extension PCR of *cry*1Ac and *cry*2Aa genes

Primer name	Sequence 5′-3′
*cry*1Ac-pGEM-1F	GACCCAGGGTGTTGGGTACCGAGCTCGAATTCGCCC
*cry*1Ac-pGEM-2R	AGGATGTTTTTGTCGGGGATCCTCTAGAATCGAATTCCCGCGGCCG
*cry*1Ac-Ser-1F	CCGCGACTATCAATGCGCGTTATAATG
*cry*1Ac-Ser-2R	CATTATAACGCGCATTGATAGTCGCGG
*cry*1Ac-Leu-1F	CGTTATAATGATGCGACTAGGCTTATTGGC
*cry*1Ac-Leu-2R	GCCAATAAGCCTAGTCGCATCATTATAACG
*cry*1Ac insert-vect-1F	GGGCGAATTCGAGCTCGGTACCCAACACCCTGGGTC
*cry*1Ac-insert-vect-2R	CGGCCGCGGGAATTCGATTCTAGAGGATCCCCGACAAAAACATCCT
*cry*2Aa-pGEM-1F	CAATACATTATTCATATAAAATTCCTCCTTAATCGAATTCCCGCGGCCG
*cry*2Aa-pGEM-2R	CTTCCACCACTTTATTAAGGTTTGAGTGAATCACTAGTGAATTCGCG
*cry*2Aa-Thr-1F	CTTAATACAGATGCGCTTGCTCGTG
*cry*2Aa-Thr-2R	CACGAGCAAGCGCATCTGTATTAAG
*cry*2Aa-Ser-1F	CAATAACTTCTGCGGTTAATACAATGC
*cry*2Aa-Ser-2R	GCATTGTATTAACCGCAGAAGTTATTG
*cry*2A-insert-vect-1F	CGGCCGCGGGAATTCGATTAAGGAGGAATTTTATATGAATAATGTATTG
*cry*2A-insert-vect-2R	CGCGAATTCACTAGTGATTCACTCAAACCTTAATAAAGTGGTGGAAG
*cry*2A-seq'P	GCTGCTGAAATACCCCATTCATCTGC
*cry*1Ac-seq'P	CGGGAACAGAGCAACGATATCTAATACAG

through overlap extension PCR by using the KOD plus mutagenesis kit (Toyobo). 3D structures of mutants were predicted with Phyre2, and 3D structures were visualized by pyMol, while PROCHECK was employed for protein structure quality analysis and validation. The recombinant plasmids of *cry*1Ac and *cry*2Aa were spared for site-directed mutagenesis where two amino acids leucine and serine were replaced with alanine (Alanine scanning) in *cry*1Ac domain II and amino acids serine and threonine in *cry*2Aa domain III. For amplification of four mutant gene inserts and vectors, the PCR program began with pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 15 s, and annealing at 45 °C, 48 °C, 56 °C, 50 °C, and 65 °C for 30 s at a rate of 60 s/kb lasting for 30 cycles. The elongation phase was terminated at 72 °C for 5 min. The products of first PCR ligated to the vector using the previous conditions. The amplified products after treated with DpnI transformed into *E. coli* strain BL21pLysS host system.

Protein expression in *cry*1Ac and *cry*2Aa mutants

Initially, protein from different mutants of *cry*1Ac and *cry*2Aa and optimized basic parameters of proteins expression were prepared, solubility of the crystal proteins in alkaline buffer and stability to trypsin to characterize the mutant library. Clones that showed the enhanced expression, solubilized in alkaline buffer, were assayed against 2nd instar insect larvae. Initially, a discriminatory concentration was used just to detect large differences in activity. Biotoxicity assays were performed after preliminary characterization involved combining mutant forms of one toxin (Cry1Ac and Cry2Aa) with non-mutant forms of the other in order to detect change in toxicity.

Biotoxicity assays

Biotoxicity assays were performed after the optimization of different protein expression parameters against *S. litura*. Seed culture of cotton leaf worms (*S. litura*) was collected from infested cotton fields, and larvae were reared on artificial diet [pinto beans flour 17%, semolina 5%, Ravi yeast 2%, vitamin C 0.3%, sorbic acid 0.1%, formaldehyde 0.15%, parahydroxy benzoic acid 0.2%, agar 1.28%, distilled water 75%/L]. Five different protein concentrations were used for biotoxicity assays ranged from 50 to 250 µg/gm. Each assay was performed in triplicate, and the second instar larvae were used for the biotoxicity assay. For a negative control, the same quantity of buffer was added to the diet and incubated at 28 ± 2 °C, 60 ± 5% relative humidity. Mortality was monitored for 24 to 72 h, and bioassays data were statistically analyzed using the Quantal Computer Program.

Statistical analysis

The statistical analysis was performed through SPSS, Quantal Program, and probit analysis to find out the mortality rate and LC_{50} values of mutants and non-mutant clones.

Results

Toxicity of mutants

The *cry*1Ac (4.1 kb) and *cry*2Aa (1.9 kb) genes were amplified through overlap extension PCR and cloned into the cloning vector pGEM through insert mega primer contains the flanking region of vector (Fig. 1). These two clones were spared for site-directed mutagenesis where in *cry*1Ac two codons serine (AGT) and leucine (TTA) and two amino acids in *cry*2Aa serine (TCG) and threonine (ACC) were replaced with alanine (GCG). For site-directed mutagenesis, designed primers were used specifically for both genes contain the mutagenic sites for both amino acids (Table 1), which produced mutants of *cry*1-Ser573A, *cry*1-Leu588A in domain II and *cry*2-Thr325A, *cry*2-Ser445A in domain III of 7.1kb and 4.9 kb mutants, respectively. All of the mutants were successfully expressed in *E. coli* where four mutants of *cry*1Ac showed the replacement of alanine with serine and leucine. In *cry*2Aa, five mutants showed replacement of threonine and serine with alanine (Fig. 2).

Protein expression in *Bt* Cry1Ac and Cry2Aa mutants

Expression of recombinant mutants proteins (Cry1-Leu588A, Cry1-Ser573A, Cry2-Ser445A, Cry2-Thr325A) in *E. coli* verified the site-directed mutagenesis effectiveness by comparing with non-mutant Cry1Ac and Cry2Aa proteins (Fig. 1) showed 130 kDa and 65 kDa bands, respectively, indicating that all mutants were successfully expressed in *E. coli*. All constructs expressed mutant proteins suggesting that changes did not likely affect the overall structure of the protoxin.

Determination of insecticidal activity

Biotoxicity assays were performed by selective mutants against 2nd instar of *S. litura* that revealed the toxicity effect of mutants and non-mutants Cry proteins after 72 h. The insecticidal activity of Cry1Ac and its mutant suppressed with 0.99 toxicity indexes; however, Cry2Aa and its mutants showed slight increase in insecticidal activity with toxicity index ~ 1.01 against *S. litura* (Table 2).

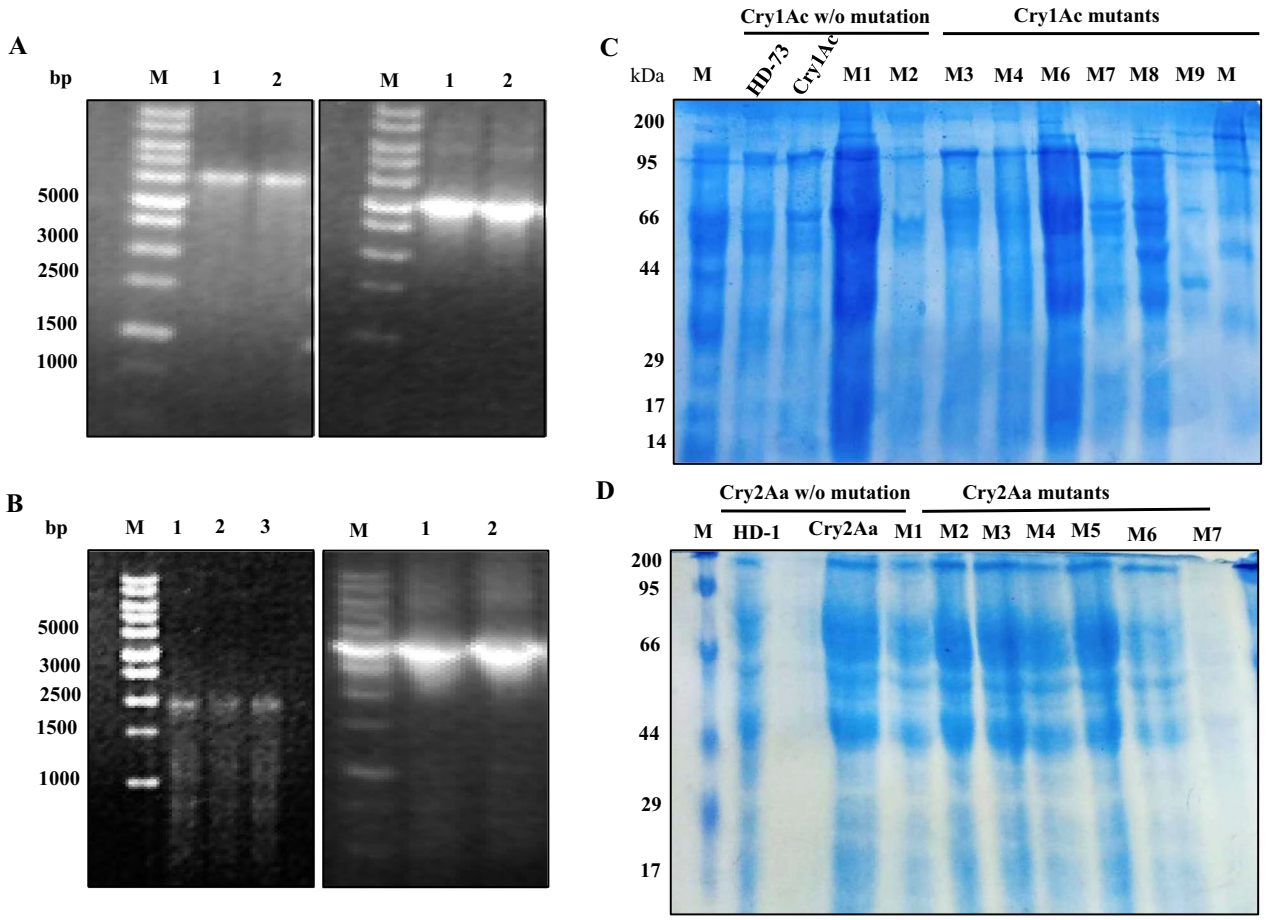

Fig. 1 Site-directed mutagenesis PCR for *cry*1Ac and *cry*2Aa full-length genes through KOD Plus mutagenesis kit **A** Amplification of *cry*1Ac 4.1 kb gene and amplified vector (pGEM) 3 kb **B** Amplification of *cry2Aa* 1.9 kb gene and amplified vector (pGEM) 3 kb **C** SDS-PAGE analysis for Cry1Ac non-mutant and mutant proteins expressed in *Escherichia coli.* **D** SDS-PAGE analysis for Cry2Aa non-mutant and mutant proteins

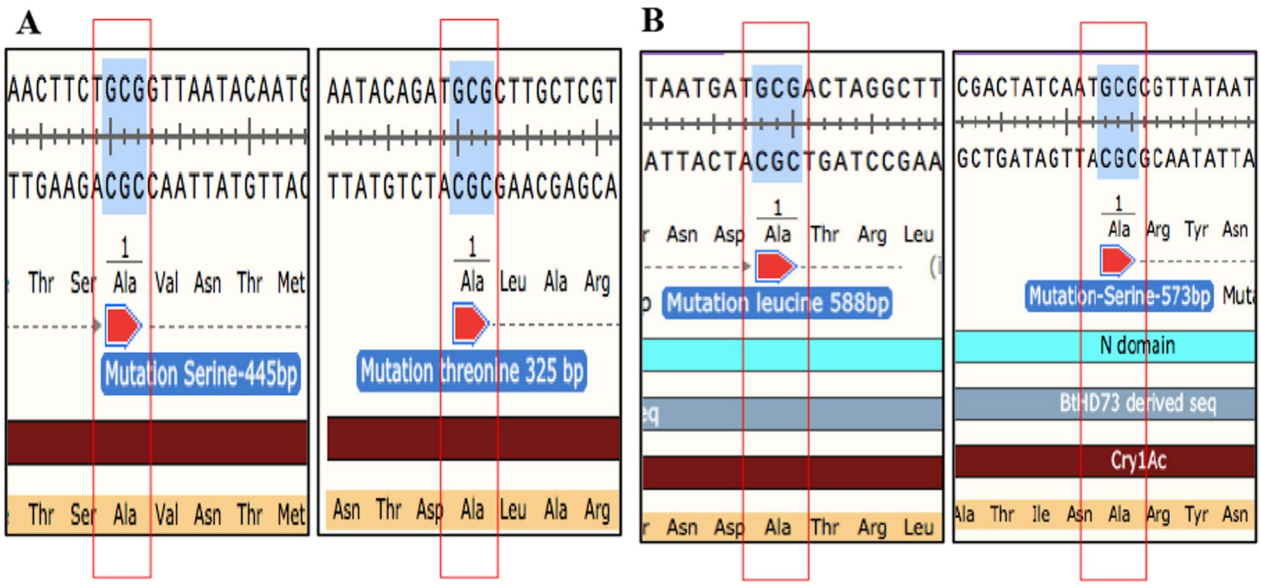

Fig. 2 Site-specific mutagenesis of *cry*1Ac and *cry*2Aa genes through alanine scanning. **A–B** Comparison of mutants and non-mutants clones of *cry*1Ac and *cry*2Aa genes, respectively. Amino acids substituted with alanine are highlighted in red

Table 2 Biotoxicity assays of Cry1Ac and Cry2Aa mutant proteins against 2nd instar larvae of *Spodoptera litura*

Treatments (µg/g)	LC_{50} values[a]	95% Confidence limits		Slope ± SE	X^2
		LL	UL		
Cry1Ac/pGEM	248.120	178.089	607.361	1.608 ± .476	0.553
Cry1-L588A (M1)	249.842	185.649	514.620	1.856 ± .506	1.257
Cry1-L588A (M2)	249.294	193.585	429.468	2.269 ± .556	1.09
Cry1-L588A (M3)	249.539	191.650	443.347	2.152 ± .534	0.675
Cry*1*-S573A (M1)	248.528	180.710	568.758	1.692 ± .486	0.553
Cry*1*-S573A (M2)	248.485	183.132	533.895	1.785 ± .499	1.741
Cry*1*-S573A (M3)	248.669	189.444	456.132	2.065 ± .525	0.439
Cry2Aa/pGEM	169.195	121.185	302.855	1.450 ± .442	0.320
Cry2-Thr325A (M1)	168.763	126.397	265.461	1.672 ± .452	0.157
Cry2-Thr325A (M2)	168.720	121.243	298.467	1.464 ± .442	0.359
Cry2-Thr325A (M3)	168.055	133.038	295.122	1.646 ± .453	0.565
Cry2-S445A (M1)	167.581	130.924	237.658	1.974 ± .466	0.408
Cry2-S445A (M2)	167.232	126.704	254.069	1.746 ± .454	0.581
Cry2-S445A (M3)	167.628	135.007	223.489	2.280 ± .490	1.023

LC_{50}: Lethal concentration at 50% mortality; [a]µg of purified protein per gram diet; LL-UL: lower and upper limits of the LC_{50} at $p = 0.05$; χ^2: Chi-square value with four degrees of freedom

Mutants of Cry1Ac proteins with low toxicity contained serine and leucine amino acids replacements. These residues in domain II may have some function in the insecticidal mechanism of Cry1Ac where LC_{50} values of Cry1-Leu588A mutants were 249.294, 249.539, and 249.842 µg/g for serine and leucine amino acids, respectively. The LC_{50} values of Cry1-Ser573A mutants (248.528, 248.485, and 248.669 µg/g) were observed slightly less than Cry1-Leu588A mutants (249.294, 249.539, and 249.842 µg/g) for the same three contained mutants Cry proteins, respectively. On the basis of mortality percentage after given intervals, Cry1-Ser573A and Cry1-Leu588A mutants showed 46.66% mortality rate that means non-significant effect of mutation observed in these mutants compared to the recombinant Cry1Ac toxin ($LC_{50} = 248.120$ µg/g) (Fig. 3). In case of Cry2Aa, results showed that Cry2-Ser445A toxin mutants exhibited lower LC_{50} values 167.232, 167.581 and 167.628 µg/g, followed by Cry2-Thr325A mutants ($LC_{50} = 168.763$, 168.720 and 168.055 µg/g) for insect mortality ranging from 13.33, 43.33 and 66.66 percent at an interval of 24, 48 and 72 h, respectively, compared to the recombinant Cry2Aa toxin, where LC_{50} value was 169.195 µg/g. It was found that Cry2-Ser445A mutants showed enhanced mortality of 66.66% that is 6.66% more than recombinant Cry2Aa and Cry2-Thr325A which caused 63.33% mortality that is 3.33% more than recombinant Cry2Aa without mutation (Fig. 4).

Predicted impact of mutations on protein structure and toxicity

Cry1Ac mutants of Cry1Ac were located in the loop connecting β19-β20 of domain III. A decrease in insecticidal efficacy observed when S573A was altered, suggesting that it occupied a pivotal place in the molecule's tertiary

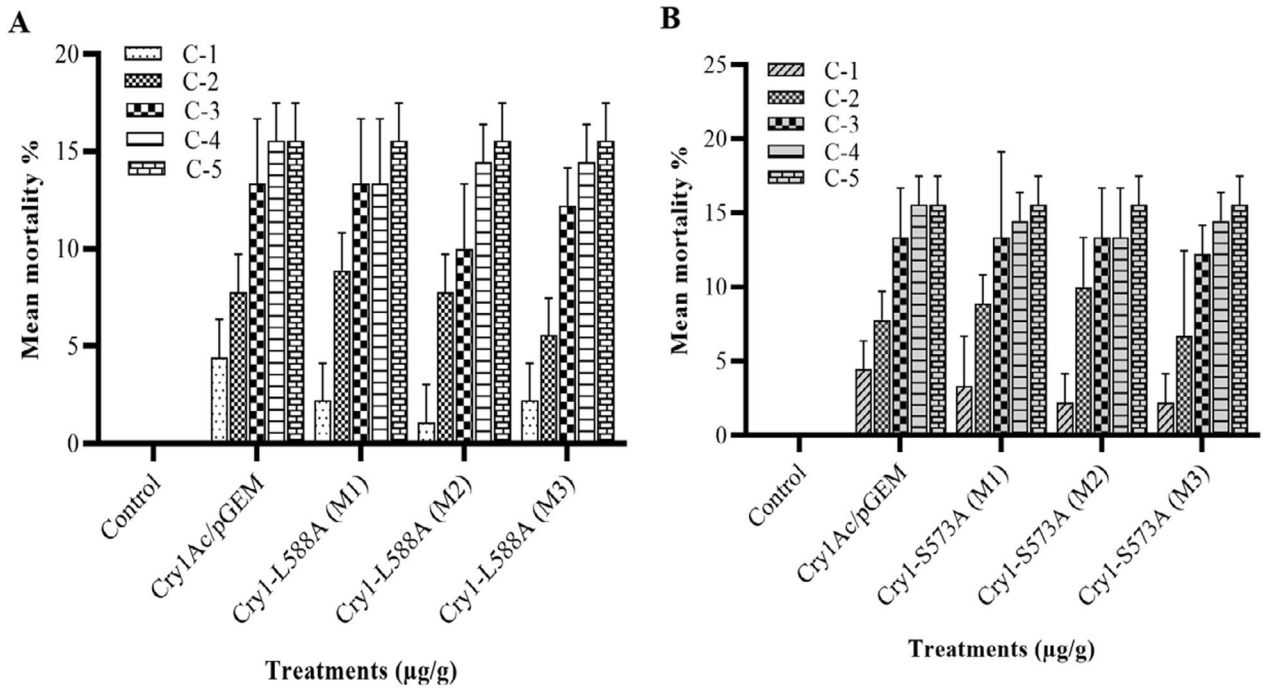

Fig. 3 Graphical representation of mean mortality percentage of biotoxicity assay for various concentrations of Cry proteins with and without mutations **A**–**B** Comparison of mean mortality percentage of Cry1Ac mutants with original

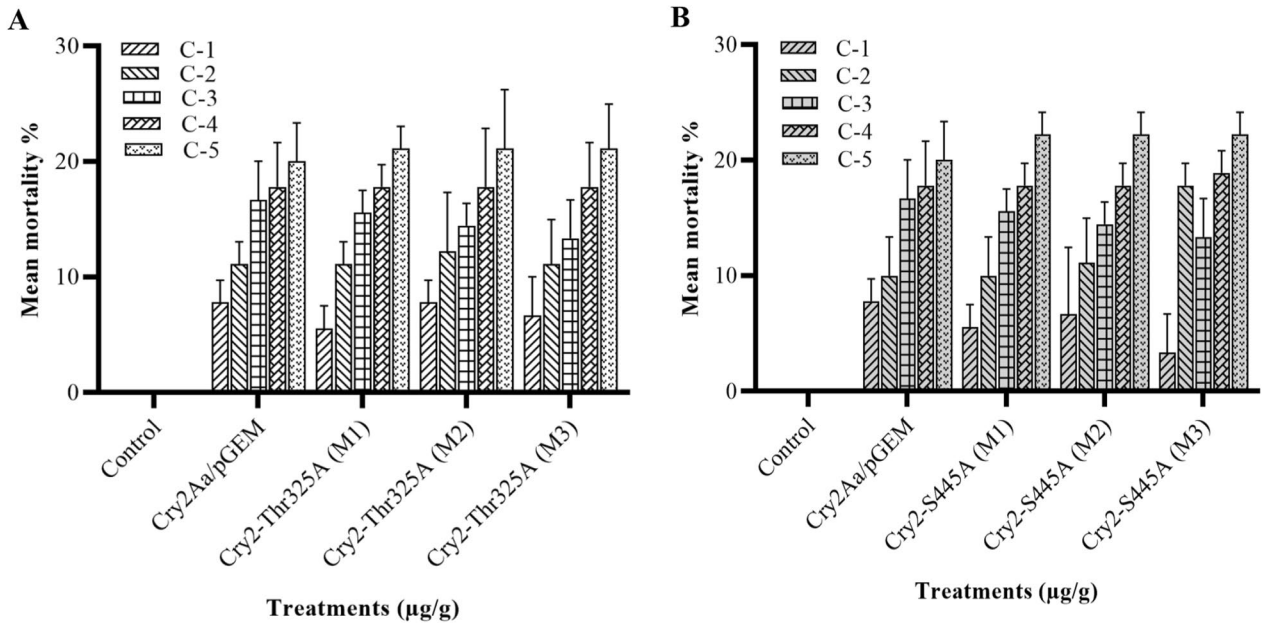

Fig. 4 Graphical representation of mean mortality percentage for biotoxicity assay with different protein concentrations with and without mutations. **A**–**B** Comparison of mean mortality percentage of Cry2Aa mutants with non-mutated protein

structure. L588A of Cry1Ac markedly reduced insecticidal action may have resulted from the aberrant conformational change by the loss of charge at this location, which disrupted the typical operation of the toxin's core region. The fact that Cry1Ac toxin was more effective at killing *S. litura* than L588A suggests that this amino acid

position is critical to the protein's core active area. T325A of Cry2Aa located close to the β17 region of domain II involved in structure stability of the toxin. The S445A of Cry2Aa was located in the middle of β18-β19 of domain II. The mutant's effect on insecticidal activity against *S. litura* was a result of the steric hindrance being reduced after S445 was replaced by alanine. However, additional tests, such as ligand binding, must be performed to verify this.

Discussion

To understand the function of these sites in insect toxicity, the behavior of mutants with parent toxin compared to gain insight into the importance of these areas in determining its toxicity. In domain II of Cry1Ac the polar residue serine and non-polar/hydrophobic residue leucine and in domain III of Cry2Aa the two polar residues serine and threonine were replaced with non-polar/hydrophobic alanine residue. According to the findings of phylogenetic research, domain II is made up of three β-sheets, features loops at the apexes of β-hairpin extensions. Several locations in the three surface-exposed loops play a significant part in the process of receptor binding and its specificity. Domain III is composed of two anti-parallel β-sheets sandwiched together, capable of performing functions related to receptor binding as well as insect specificity (Herrero et al. 2016).

The study focused on the use of overlap PCR, in the creation of a modified Cry1Ac and Cry2Aa of *B. thuringiensis* toxin with altered toxicity. PCR-directed recombination is faster way to clone gene inserts and vectors than traditional ligation-dependent cloning. PCR product of two strands of insert used as mega primers with the template vector, which already has the flanking region to match with overlap primers of insert by KOD Plus mutagenesis kit (Toyobo, China). Alanine mutagenesis facilitated the investigation of systematic residue replacements in a protein sequence without affecting the secondary structure as alanine has the shortest side chain of all the amino acids. Substituting a residue for non-polar alanine assure the most frequent dihedral angle that connects the side chain to the amino acid backbone without extreme steric or electrostatic effect (Morrison and Weiss 2001). Dean et al. (1996) used site-directed mutagenesis to introduce amino acid changes into Cry1Ab toxins (Y153A, Y153D, and Y153R) concluded that negatively charged residue is less suitable for membrane insertion in Cry1A toxins than non-polar residues like alanine and positively charged residues like arginine.

All mutations (Cry1Ac-Leu588A, Cry1Ac-Ser573A, Cry2Aa-Ser445A, and Cry2Aa-Thr325A) expressed protoxins at a level comparable to that of wild type Cry1Ac and Cry2Aa and as stable as parent toxin of the natural type. The activity of Cry1Ac was found slightly suppressed (TI = 0.99) against *S. litura* when a single residue at Ser573 and Leu588 in domain II replaced with alanine. This indicated that the functional determination of the insecticidal activity of Cry1Ac occurs in domain II, but not in a single amino acid residue. Cry toxins efficacy and target insect spectrum are both heavily dependent on their ability to connect to their specific receptor. Cry toxins have a limited insecticidal spectrum, implying that this family of toxins has a specific receptor in susceptible insects. So far, Cry proteins and a few receptors specific to *S. litura* have been discovered. The low Cry1Ac toxicity to *S. litura* in the present bioassays agrees with previous reports (Liu et al. 2018). In order to further understand the role of domain II in determining the insecticidal action, additional mutants need to be constructed in which different residues in domain II require to be substituted with alanine.

Cry2Aa mutants in domain III slightly increased the toxicity level (TI = ~ 1.01) against *S. litura*. The behavior of inebriated *S. litura* larvae revealed a considerable distinction between the modes of action of CrylAc and Cry2Aa. The Cry2Aa-fed larvae regurgitated faster than the Cry1Ac-fed larvae, despite eating on the toxins for the same period of time. The insects fed Cry2Aa did not recover from the toxicity as quickly as those administered Cry1Ac. It revealed that Cry2Aa was either a toxin with a quicker onset of action or that the delay in regurgitation increased its toxicity. Previously, mutants (S509A, V513A, and N514A) were created by alanine scanning in domain III of Cry1Ab resulted in 9.5, 12.7, and 51 fold increase in toxicity against *S. frugiperda* (Gomez et al. 2018). *Bt* Cry2A proteins have a distinct structure and insecticidal mechanism from the commonly used Cry-1 proteins, making them excellent candidates for the control of insect resistance (Kumar et al. 2004). Cry2A toxins have different receptor binding epitopes (Morse et al. 2001), low degree of sequence homology with Cry1 toxins, and do not share binding sites with Cry1A toxins in BBMV derived from lepidopteran larvae (Jurat-Fuentes et al. 2002). The alterations in the basic structure of *cry*1Ac and *cry*2Aa can lead to a shift in insect toxicity without entirely diminish its insecticidal action (McNeil and Dean 2011). However, several residues in different locations of domain II and/or domain III may be needed for complete first contact to the receptor's broad surface. Thus, domain III mutations alone or in tiny blocks may not have caused toxicity loss. Furthermore, binding experiments will be conducted to determine saturation binding, and concentrations of BBMV will be chosen for subsequent competition experiments.

Conclusion

Spodoptera litura is emerging as a threat to the cotton crop. There is a possibility that the process of developing pesticide resistance in insect pests can be delayed or perhaps avoided entirely by appropriately incorporating *Bt* into the integrated pest management system. In the present study, a feasible technique was demonstrated for creating and screening mutant cry genes from *B. thuringiensis* with insecticidal activity. Based on probit analysis results against the devastating crop pest *S. litura*, our data support the observation that amino acid residues in Cry2Aa are susceptible to modification, making this potential agent for remodeling of 3D-Cry toxin against *S. litura;* however, further investigation needs to identify receptor molecules and binding epitopes would be essential for the development of insect resistance management strategies.

Abbreviations

Cry	Crystal protein
cry	Crystal protein gene
Bt	*Bacillus thuringiensis*
PCR	Polymerase chain reaction

Acknowledgements

The authors wish to thank our project collaborator Dr. Neil Crickmore, University of Sussex, BN1 9QH, United Kingdom, his support and guidance.

Author contributions

FY designed the work. HK did the experiments and analyzed the data. HZ, FM, AL, and FR were involved in the MS preparation and revised the paper. All authors read and approved the final manuscript.

Funding

The work is part of the first author's PhD dissertation, which was supported and facilitated by the National Research Program of Universities, Higher Education Commission, Pakistan (3528).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This is to state that this work is author's PhD dissertation work, so I give permission for the publication.

Competing interests

The authors declare that they have no competing interests.

Received: 5 June 2023 Accepted: 22 August 2023
Published online: 30 August 2023

References

Abbas N, Shad SA, Razaq M (2012) Fitness cost, cross resistance and realized heritability of resistance to imidacloprid in *Spodoptera litura* (Lepidoptera: Noctuidae). Pestic Biochem Physiol 103(3):181–188. https://doi.org/10.1016/j.pestbp.2012.05.001

Adang MJ, Crickmore N, Jurat-Fuentes JL (2014) Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: Advances in insect physiology, pp 39–87 https://doi.org/10.1016/B978-0-12-800197-4.00002-6

Dean DH, Rajamohan F, Lee MK, Wu SJ, Chen XJ, Alcantara E, Hussain SR (1996) Probing the mechanism of action of *Bacillus thuringiensis* insecticidal proteins by site-directed mutagenesis a minireview. Gene 179:111–117. https://doi.org/10.1016/S0378-1119(96)00442-8

Fand BB, Sul NT, Bal SK, Minhas PS (2015) Temperature impacts the development and survival of common cutworm (*Spodoptera litura*): simulation and visualization of potential population growth in India under warmer temperatures through life cycle modelling and spatial mapping. PLoS One 10(4):e0124682. https://doi.org/10.1371/journal.pone.0124682

Gomez I, Ocelotl J, Sanchez J, Lima C, Martins E, Rosales-Juarez A, Soberon M (2018) Enhancement of *Bacillus thuringiensis* Cry1Ab and Cry1Fa toxicity to *Spodoptera frugiperda* by domain III mutations indicates there are two limiting steps in toxicity as defined by receptor binding and protein stability. Appl Environ Microbiol 84(20):e01393-e1418. https://doi.org/10.1128/AEM.01393-18

Herrero S, Bel Y, Hernandez-Martinez P, Ferre J (2016) Susceptibility, mechanisms of response and resistance to *Bacillus thuringiensis* toxins in *Spodoptera* spp. Curr Opinion Insect Sci 15:89–96. https://doi.org/10.1016/j.cois.2016.04.006

Jakka S, Ferre J, Jurat-Fuentes JL, Soberon M, Gao Y, Bravo A (2015) Cry toxin binding site models and their use in strategies to delay resistance evolution. In: Soberon M, Gao Y, Bravo A (eds) *Bt* Resistance: Characterization and Strategies for GM Crops Producing *Bacillus thuringiensis* Toxins, (Centre for Agriculture and Biosciences International, Oxfordshire, UK) 24:138–149. https://doi.org/10.1079/9781780644370.0138

Jurat-Fuentes JL, Gould FL, Adang MJ (2002) Altered glycosylation of 63 and 68 kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. Appl Environ Microbiol 68:5711–5717. https://doi.org/10.1128/AEM.68.11.5711-5717.2002

Kumar S, Udayasuriyan V, Sangeetha P, Bharathi M (2004) Analysis of Cry2A proteins encoded by genes cloned from indigenous isolates of *Bacillus thuringiensis* for toxicity against *Helicoverpa armigera*. Current Science pp 566–570

Liu L, Chen Z, Yang Y, Xiao Y, Liu C, Ma Y, Liu K (2018) A single amino acid polymorphism in ABCC2 loop 1 is responsible for differential toxicity of *Bacillus thuringiensis* Cry1Ac toxin in different *Spodoptera* (Noctuidae) species. Insect Biochem Mol Biol 100:59–65. https://doi.org/10.1016/j.ibmb.2018.06.004

McNeil BC, Dean DH (2011) *Bacillus thuringiensis* Cry2Ab is active on *Anopheles* mosquitoes: single D block exchanges reveal critical residues involved in activity. FEMS Microbiol Lett 325:16–21. https://doi.org/10.1111/j.1574-6968.2011.02403.x

Morrison KL, Weiss GA (2001) Combinatorial alanine-scanning. Curr Opin Chem Biol 5:302–307. https://doi.org/10.1016/S1367-5931(00)00206-4

Morse RJ, Yamamoto T, Stroud RM (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9:409–417. https://doi.org/10.1016/S0969-2126(01)00601-3

Pazhanisamy M, Senthilkumar M, Sathyaseelan V (2019) Seasonal incidence of leaf eating caterpillar, *Spodoptera litura* (Fabricius) in groundnut ecosystem during kharif season. Plant Archives 19(2):3351–3354

Pogue MG (2002) A world revision of the genus *Spodoptera* Guenee (Lepidoptera:Noctuidae). Mem Am Entomol Soc 43:1–202

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491. https://doi.org/10.1126/science.2448875

Saleem MA, Ahmad M, Ahmad M, Aslam M, Sayyed AH (2008) Resistance to selected organochlorin, organophosphate, carbamate and pyrethroid, in *Spodoptera litura* (Lepidoptera: Noctuidae) from Pakistan. J Econ Entomol 101(5):1667–1675

Shad SA, Sayyed AH, Fazal S, Saleem MA, Zaka SM, Ali M (2012) Field evolved resistance to carbamates, organophosphates, pyrethroids, and new

chemistry insecticides in *Spodoptera litura* Fab. (Lepidoptera: Noctuidae). J Pest Sci 85:153–162. https://doi.org/10.1007/s10340-011-0404-z
Trebicki P, Dader B, Vassiliadis S, Fereres A (2017) Insect–plant–pathogen interactions as shaped by future climate: effects on biology, distribution, and implications for agriculture. Insect Sci 24(6):975–989. https://doi.org/10.1111/1744-7917.12531
Tuan SJ, Li NJ, Yeh CC, Tang LC, Chi H (2014) Effects of green manure cover crops on *Spodoptera litura* (Lepidoptera: Noctuidae) populations. J Econ Entomol 107:897–905. https://doi.org/10.1603/EC13435
Vilchez S (2020) Making 3D-cry toxin mutants: Much more than a tool of understanding toxins mechanism of action. Toxins 12(9):600. https://doi.org/10.3390/toxins12090600
Yuan X, Zhao M, Wei J, Zhang W, Wang B, Khaing MM, Liang G (2017) New insight on the role of alkaline phosphatase 2 from *Spodoptera exigua* (Hubner) in the action mechanism of *Bt* toxin Cry2Aa. J Insect Physiol 98:101–107. https://doi.org/10.1016/j.jinsphys.2016.12.004

Publisher's Note