

A NOVEL DNA SEQUENCE OF *BACILLUS THURINGIENSIS* δ -ENDOTOXIN RECEPTOR IN *HELICOVERPA ARMIGERA*

KAUSAR MALIK AND SHEIKH RIAZUDDIN

National Center of Excellence in Molecular Biology,
University of the Punjab 87-West Canal Bank Road,
Thokar Niaz Baig Lahore-53700, Pakistan

Abstract

The pesticidal bacterium *Bacillus thuringiensis* has been the subject of intensive research. *Bacillus thuringiensis* (Bt) crystal proteins are effective in controlling agriculturally and biomedically harmful insects. However, the mechanism of Bt protein pesticidal action is not well understood. It is assumed that the pesticidal protein has affinity for specific receptors in the midgut of the susceptible larvae and binds irreversibly to create holes in the gut leading to eventual death of the target larvae. The study is endeavored to characterization of Bt delta endotoxin receptor in agronomically important pest, American bollworm (*Helicoverpa armigera*). Presence of a novel Protein is reported in the extract of the larval midgut membrane of *Helicoverpa armigera* as putative receptor for Bt Cry1A delta-endotoxins. The gene sequence has novelty because it has no significant homology to already existing sequences of Bt receptor.

Introduction

Bacillus thuringiensis (Bt) is a valuable source of insecticidal proteins for use in conventional sprayable formulations and in transgenic crops. It is the most promising alternative to synthetic insecticides. However, evolution of resistance in insect populations is a serious threat to this technology. Several important crops have been engineered to express toxins of *Bacillus thuringiensis* (Bt) for insect control. In principle, the mechanism of insect resistance to Bt could be located at each of the various steps (solubilization, proteolytic processing, passage through the peritrophic membrane, receptor binding, membrane insertion, pore formation, and osmotic lysis of midgut cells) in the mode of action of Bt Cry proteins. Conclusively, three different biochemical mechanisms of resistance to Bt have been observed so far, proteolytic processing of protoxin, improved repair of damaged midgut cells and modification of a Cry protein-binding site.

Binding site modification is thought to be the major mechanism of resistance to Cry1A toxins in *Pectinophora interpunctella* (Van Rie *et al.*, 1990), *H. virescens* (Lee, *et al.*, 1995), and the diamondback moth (Sayyed *et al.*, 2000). This has also been proposed to be responsible for Cry1F resistance in the diamondback moth (Ballester *et al.*, 1999). Central to learning to curb resistance to Bt is understanding the mechanism by which an insect resists the toxins.

Aminopeptidase N has been reported to be a *Bacillus thuringiensis* (Bt) Cry1A toxin-binding protein in several lepidopteran insects. It is believed that insect resistance build up emanates from mutational changes in the receptor protein (Aronson, and Shai, 2001).

The cotton bollworm *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) is one of the most serious insect pests in many cotton-producing countries, including Australia, India, China and Pakistan. The larva causes substantial economical losses to

legume, fibre, cereal oilseed and vegetable crops. This pest has proven to be difficult to control by conventional means, mainly due to the development of pesticide resistance (Estebanez *et al.*, 2001; Krathi *et al.*, 2001). The main crops affected are cotton, chickpea, maize, wheat, sorghum, sunflowers, tomatoes, potato and variety of vegetables and fruits.

Toxicity of insecticidal endotoxins produced by *Bacillus thuringiensis* correlates with the presence of specific proteins in the midgut of susceptible larvae. So characterization of the novel Bt receptor present in the midgut of the American bollworm *Helicoverpa armigera*, was done and cDNA sequencing of Bt receptor gene was performed. The present study relates to a novel DNA sequence of the gene of Bt receptor protein in the brush border membrane vesicles of *Helicoverpa armigera*.

Materials and Methods

Purification of insecticidal crystal proteins, preparation of brush border membrane vesicles, purification of the receptor protein, analysis of its affinity for various *Bt* crystal proteins and associated aminopeptidase, alkaline phosphatase activities, N-terminal amino acid sequencing, characterization of receptor protein by cloning and expression of the receptor protein, were done according to the procedures described in Malik *et al.*, 2006.

Sequencing of Cry1A-receptor gene: Partial sequencing of the confirmed clone of receptor gene, by automated sequencing system (ABI) was performed.

Automated DNA sequencing system (ABI 3100) from applied Biosystems was used along with ABI PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing Kit according to manufacturer's instructions. This method is based on dye terminator chemistry, in which each of the four dideoxynucleotides is labeled with a different fluorochrome (Prober *et al.*, 1987; Lee *et al.*, 1992). The ABI 3100 can simultaneously detect fluorescence at four different wavelengths, set to coincide with the emission of four different fluorescent dyes. The reaction mixture was run in a single capillary so that color of each band passing the detector represents the DNA sequences.

Homology studies of sequenced nucleotides: Homology studies of the nucleotide sequence of clone with known nucleotide sequences present in gene data bank was done through standard nucleotide-nucleotide and protein (swiss prot) BLAST (Basic Local Alignment Search Tool) software (Altschul *et al.*, 1997) available at NCBI web site.

www.ncbi.nlm.nih.gov/home/BLAST <<http://www.ncbi.nlm.nih.gov/home/BLAST>>

Results

Nucleotide sequence: Partial nucleotide sequence of cDNA from *Helicoverpa armigera*, midgut's BBMVs is given below:

GATTCATATGGCACCCGCCCGGTCACCCAGCCCCAGCACGCAGCCCTGGGAG
CATGTGAATGCCATCCAGGAGGCCCGGCGGCTCCTGAACCTGAGTAGAGACA
CTGCTGCTGAGATGAATGAAACAGTAGAAGTGATATCAGAAATGTTTGACCT
CCAGGAGCCGACTTGCTACAGACCCGGCCTGGAGCTGTACAAGCAGGGCCT
GCGGGGCAGCCTACCAAGCTCAAGGGCCCCTTGACCATGATGGCCAGCCAC

TACAAGCAGCACTGCCCTCCAACCCCGGAAACTTCCTGTGCAACCCAGATTA
 TCACCTTTGAAAGTTTCAAAGAGAACCTGAAGGACTTCCTGCTTGTCATCCCC
 TTTGACTGCTGGGAGCCAGTCCAGGAGTGAGGATCCAATCACTAGTGAATTC
 GCGGCCGCCTGCAGGTGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA
 TAGCTTGAGTATTCTATAGTGTACCTAAATAGCTTGGCGTAATCATGGTGCAT
 AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACATTTCCACACAACATACGA
 GCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCA
 CATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGC
 CAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTG
 GCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTTCGGCTG
 CGGCGA

Homology studies: Homology studies of the nucleotide sequences of clone with known nucleotide sequences present in gene data bank was done. The results showed no significant homology with existing sequences so this is a novel Bt receptor gene.

Discussion

Toxicity of insecticidal endotoxins produced by *Bacillus thuringiensis* correlates with the presence of specific proteins in the midgut of susceptible larvae. In susceptible lepidopteran insects, aminopeptidase N and cadherin-like proteins are the putative receptors for *Bacillus thuringiensis* (Bt) toxins.

The CryIA toxin-binding proteins that have been characterized are primarily leucine aminopeptidases (Denolf *et al.*, 1997; Gill *et al.*, 1995; Knight *et al.*, 1995; Yaoi *et al.*, 1999), cadherinlike proteins (Nagamatsu *et al.*, 1998; Vadlamudi *et al.*, 1995), and, in one study, biotin-containing proteins (Du *et al.*, 1996). Aminopeptidase cDNAs have been cloned from *H. virescens* (Gill *et al.*, 1995; Luo *et al.*, 1997a; Jurat *et al.*, 2001; Oltean *et al.*, 1999), *Manduca sexta* (Denolf *et al.*, 1997; Knight *et al.*, 1995), *Plutella xylostella* (Denolf *et al.*, 1997; Luo *et al.*, 1997b; Nakanishi *et al.*, 1999; Chang, *et al.*, 1999), *Bombyx mori* (Yaoi *et al.*, 1999), *L. dispar* (Valaitis *et al.*, 1995; Lee and Dean, 1996; Garner *et al.*, 1999) *Helicoverpa punctigera* (Emmerling *et al.*, 2001) *Helicoverpa armigera* (Rajagopal *et al.*, 2003) and *Plodia interpunctella*, while cadherinlike CryIA-binding proteins have been cloned from *M. sexta* (Vadlamudi *et al.*, 1995) and *B. mori* (Nagamatsu *et al.*, 1998).

Homology studies of the DNA sequence of this invention with known nucleotide sequences present in gene data bank was done. The results showed no significant homology with existing sequences of Bt receptor gene.

The purpose of present study was molecular characterization of Bt receptor protein in the brush border membrane vesicles of *Helicoverpa armigera*. The results of this study and a comparison between susceptible and resistant insects receptor gene sequences will help in the elucidation of mode of action of Bt pesticidal protein, specificity and insect resistance build up.

References

- Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.*, 25: 3389-3402.

- Aronson, A.I and Y. Shai. 2001. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique feature of their mode of action. Mini Review. *FEMS Microbiol. Letters*, 195: 1-8.
- Ballester, V., F. Granero, B.E. Tabashnik, T. Malvar and J. Ferré. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). *Appl. Environ. Microbiol.*, 65: 1413-1419.
- Chang, W.X.Z., L.J. Gahan, B.E. Tabashnik and D.G. Heckel. 1999. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. *Insect Mol. Biol.*, 8: 171-177.
- Denolf, P., K. Hendricks, J. Van Demme, S. Jensens, M. Peferoen, D. Degheele and J. Van Rie. 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase-N enzyme related to *Bacillus thuringiensis* toxin-binding proteins. *Eur. J. Biochem.*, 24: 8748-761.
- Du, C. and K.W. Nickerson. 1996. The *Bacillus thuringiensis* insecticidal toxin binds biotin-containing proteins. *Appl. Environ. Microbiol.*, 62: 2932-2939.
- Emmerling, M., D. Chandler and M. Sandeman. 2001. Molecular cloning of three cDNAs encoding aminopeptidases from the midgut of *Helicoverpa punctigera*, the Australian native budworm source. *Insect Biochem. Mol. Biol.*, 31(9): 899-907.
- Estebanez-Perpina, E., A. Bayes, J. Vendrell, M.A. Jongsma, D.P. Bown, J.A. Gatehouse, R. Huber, W. Bode, F.X. Aviles and D. Reverter. 2001. Crystal structure of a novel mid-gut procarboxypeptidase from the cotton pest *Helicoverpa armigera*. *J. Mol. Biol.*, 313: 629-638.
- Garner, K.J., S. Hiremath, K. Lehtoma and A.P. Valaitis. 1999. Cloning and complete sequence characterization of two gypsy moth aminopeptidase-N cDNAs, including the receptor for *Bacillus thuringiensis* Cry1Ac toxin. *Insect Biochem. Mol. Biol.*, 29: 527-535.
- Gill, S.S., E.A. Cowles and V. Francis. 1995. Identification, isolation and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding protein from the midgut of lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.*, 270: 27277-27282.
- Jurat-Fuentes, J.L., D.H. Dean and M.J. Adang. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa delta-endotoxin binding to a novel 110-kDa aminopeptidase in *Heliothis virescens* is not N-acetylgalactosamine mediated. *Insect Biochem. Mol. Biol.*, 31: 909-918.
- Knight, P.J.K., B.H. Knowles and D.J. Ellar. 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis*. Cry1Ac toxin. *J. Biol. Chem.*, 270: 17765-17770.
- Krathi, K.R., D.R. Jadhav, R.R. Wanjari, S.S. Ali and D. Russel. 2001. Pyrethroid resistance and mechanism of resistance in field strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.*, 94: 253-263.
- Lee, M.K. and D.H. Dean. 1996. Inconsistencies in determining *Bacillus thuringiensis* toxin binding sites relationships by comparing competition assays with ligand blotting. *Biochemical and Biophysical Research Communications*, 220: 575-580.
- Lee, M.K., F. Rajamohan, F. Gould and D.H. Dean. 1995. Resistance to *Bacillus thuringiensis* Cry1A delta-endotoxins in a laboratory selected *Heliothis virescens* strain is related to receptor alteration. *Appl. Environ. Microbiol.*, 61: 3836-3842.
- Lee, M.K., R.E. Milne, A.Z. Ge and D.H. Dean. 1992. Location of *Bombyx mori* receptor binding region on a *Bacillus thuringiensis* δ -endotoxin. *J. Biol. Chem.*, 267: 3115-3121.
- Luo, K., B.E. Tabashnik and M.J. Adang. 1997b. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant *Plutella xylostella*. *Appl. Environ. Microbiol.*, 63: 1024-1027.
- Luo, K., S. Sangadala, L. Masson, A. Mazza, R. Brousseau and M.J. Adang. 1997a. The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A delta-endotoxin binding and pore formation. *Insect Biochem. Mol. Biol.*, 27: 735-743.
- Malik, K., A.S. Riazuddin and S. Riazuddin. 2006. Identification, Purification, Cloning and expression of receptor for *Bacillus thuringiensis* Cry1A delta-endotoxins in the brush border membranes of the *Helicoverpa armigera* (Lepidoptera: Nuctoidae). *Pak. J. Bot.*, 38(3): 767-778.

- Nagamatsu, Y., S. Toda, T. Koike, Y. Miyoshi, S. Shigematsu and M. Kogure. 1998. Cloning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal Cry1Aa toxin. *Biosci. Biotechnol. Biochem.*, 62: 727-734.
- Nakanishi, K., N. Shimada, T. Kadotani and R. Sato. 1999. *Bacillus thuringiensis* insecticidal Cry1Aa toxin binds to a highly conserved region of aminopeptidase N in the host insect leading to its evolutionary success; *Biochim. Biophys. Acta*, 1432: 57-63.
- Oltean, D.I., A.K. Pullikuth, H.K. Lee and S.S. Gill. 1999. Partial purification and characterization of Bt. Cry1A toxin. Receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. *Appl. Environ. Microbiol.*, 65: 4760-4766.
- Prober, J.M., G.L. Trainor, R.J. Dam, F.W. Bobbs, C.W. Robertson, R.J. Zagursky, M.A. Jensen and K.A. Baumeister. 1987. A system for rapid DNA sequencing with fluorescent chain terminating dideoxynucleotides. *Science*, 238: 336-341.
- Rajagopal, R., N. Agrawal, A. Selvapandian, S. Sivakumar, S. Ahmad and R.K. Bhatnagar. 2003. Recombinantly expressed isoenzymic aminopeptidases from *Helicoverpa armigera* (American cotton bollworm) midgut display differential interaction with closely related *Bacillus thuringiensis* insecticidal proteins. *Biochem. J.*, 370: 971-978.
- Sayed, A.H., R. Howard, S. Herrero, J. Ferré and D.J. Wright. 2000. Genetic and biochemical approach for characterisation of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth. *Appl. Environ. Microbiol.*, 66: 1509-1516.
- Tabashnik, B.E., N. Finson, F.R. Groeters, W.J. Moar, M.W. Johnson, K. Luo and M.J. Adang. 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. *Proceedings of the National Academy of Sciences of the USA.*, 91: 4120-4124.
- Vadlamudi, R.K., E.T. Weber and L.A. Jr. Bulla. 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem.*, 270: 5490-5494.
- Valaitis, A.P., M.K. Lee, F. Rajamohan and D.H. Dean. 1995. Brush border membrane aminopeptidase-N in the midgut of the gypsy moth serves as the receptor for the Cry1Ac δ -endotoxin of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol.*, 25: 1143-1151.
- Van Rie, J., McGaughey, W.H., D.E. Johnson, B.D. Barnett and H. Van Mellaert. 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science*, 247: 72-74.
- Yaoi, K., K. Nakanishi, T. Kadotani, M. Imamura, N. Koizumi, H. Iwahana, R. Sato. 1999. cDNA cloning and expression of *Bacillus thuringiensis* Cry1Aa toxin binding 120 kDa aminopeptidase N from *Bombyx mori*. *Biochim. Biophys. Acta*, 1444: 131-137.

(Received for publication 5 January 2008)