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DISCOVERY OF A NOVEL CRYSTAL PROTEIN FROM PAKISTANI *BACILLUS THURINGIENSIS* STRAIN TOXIC TO *TRIBOLIUM CASTENEUM* HERBST (COLEOPTERA:TENEBRIONIDAE)

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Abstract

The biotoxicity analysis of crystal protein of some *Bacillus thuringiensis* strains has been carried out against the larvae of red flour beetle *Tribolium casteneum* Hebst. Seven isolates were found highly active against *T. casteneum*. The most toxic isolate SG31.11 has calculated LC_{50} value of 0.2 ug/mg of artificial diet. Presently, the active protein of isolate SG31.11 was sequenced and data showed that it resembles with a novel Cry3 protein.

Introduction

Tribolium castaneum, is a serious pest of stored grains throughout the world and also a genetic model for the Coleoptera. It not only affects the quantity but also the quality of stored grains. The quantitative estimation of the loss incurred by red flour beetle is difficult because this insect is found in flourmills, godowns and warehouses with other associated stored grain pest complex. To control the infestation of this insect, many synthetic pesticides have been used for several years. However, these pesticides produce several adverse effects, which include accumulation of lethal chemicals in food chain and environment, lack of selectivity towards beneficial insects and evolution of resistance. These factors have directed the attention of scientists from traditional chemical pesticides to biopesticides.

Microbial control of insect pest of crops using entomopathogens is an ecologically sound pest management strategy. Although insect viruses and fungal pathogens are used as microbial control agents, but *Bacillus thuringiensis* Berliner (Bt) appears to have the greatest potential for this purpose. This gram-positive, spore forming crystalliferous bacterium synthesizes a proteinaceous parasporal crystalline inclusion (δ-endotoxins) during the sporulation phase. These crystalline proteins are highly specific against different insect orders, and non-target organisms like parasitoids, predators and vertebrates are not affected by their use (Aronson *et al*., 1986: Whiteley & Schnepf., 1986). A promising variety of crystal proteins (Cry proteins) have been recognized in different Bt strains. Of these crystal proteins Cry3 are reported to be toxic against coleoptera. Our previous study presented initial efforts to assess the potential of Bt strains isolated from different environmental samples, as a biological control agent of *T. casteneum* (Malik & Riazuddin., 2000).

In the present study, the active protein of isolate SG31.11 was sequenced and data showed that it resembles with a novel Cry3 protein of *Bacillus thuringiensis* serovar japonensis strain Buibui, (toxic to larvae of the cupreous chafer, *Anomala cuprea* reported by Sato *et al*., (1994). Sequence of this gene is the same but our locally isolated Bt and target pest (*T. casteneum*) is different.

Materials and Methods

All the organic and inorganic chemicals used were from Sigma Chemical Company. Molecular weight protein markers, polyvinylidene difluoride (PVDF) membrane and Bradford protein assay reagents were from Bio-Rad Laboratories. *Tribolium castaneum* larvae were obtained from the insectory of the CEMB. All protein concentrations were measured by Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976). Cry3A clones were obtained from culture collection lab of CEMB.

Purification of insecticidal crystal proteins: Cry3A and SG31.11 proteins were purified by the procedure described by Lee *et al*., (1992). Purified proteins were solubilized in 50mM Na_2CO_3 pH 9.5, containing 10mM dithiothreitol treated with 5% trypsin at 37^oC for 4-hours and stored at 4^oC.

Biotoxicity assay: Biotoxicity assay was determined as reported by Malik & Riazuddin (2000). The most toxic isolate SG31.11 has calculated LC_{50} value of 0.2 ug/mg of artificial diet.

Protein purification and sequencing: Protein from *Bacillus thuringiensis* SG31.11 strain solubilized in alkaline buffer was resolved on non-denaturing protein gel. Protein band was excised from the gel to elute the protein. The eluted protein was transferred onto a PVDF membrane using semidry transblot apparatus, stained with Coomassie brilliant blue and used in amino acid sequencing by Edman degradation.

Results

Screening microbial collections to search for novel Bt proteins: Seven Bt were found toxic against *T. casteneum* during screening of Bt in the Pakistani environment to search for novel Bt proteins. Locally isolated Bt SG31.11 is highly toxic to the larvae in biotoxicity assays.

Amino acid sequencing: Purified protein sequenced by Edman degradation method of aminoacid sequencing.

Homology search: When the protein was sequenced by Edman degradation, yielded amino acid sequence which was searched for homology with other sequences in GenBank using Blastx, at http://www.ncbi.nlm.nih.gov/BLAST/.

There was significant sequence homology to a novel 130-kDa crystal protein antigen of *Bacillus thuringiensis* serovar japonensis strain Buibui in the database, under the Accession number U04366.

Amino acids sequence

MSPNNQNEYEIIDALSPTSVSDNSIRYPLANDQTNTLQNMNYKDYLKMTESTNA
ELSRNPGTFISAQDAVGTGIDIVSTIISGLGIPVLGEVFSILGSLIGLLWPSNNENVW
QIFMNRVEELIDQKILDSVRSRAIADLANSRIAVEYYQNALEDWRKNPHSTRSAA
LVKERFGNAEAILRTNMGSFSQTNYETPLLPTYAQAASLHLLVMRDVQIYGKEW
GYPQNDIDLFYKEQVSYTARYSDHCVQWYNAGLNKLRGTGAKQWVDYNRFRR

EMNVMVLDLVALFPNYDARIYPLETNAELTREIFTDPVGSYVTGQSSTLISWYDM
IPAALPSFSTLENLLRKPDFFTLLQEIRMYTSFRQNGTIEYYNYWGGQRLTLSYIY
GSSFNKYSGVLAGAEDIIPVGQNDIYRVVWTYIGRYTNSLLGVNPVTFYFSNNTQ
KTYSKPKQFAGGIKTIDSGEELTYENYQSYSHRVSYITSFEIKSTGGTVLGVVPIFG
WTHSSASRNNFIYATKISQIPINKASRTSGGAVWNFQEGLYNGGPVMKLSGSGSQ
VINLRVATDAKGASQRYRIRIRYASDRAGKFTISSRSPENPATYSASIAYTNTMST
NASLTYSTFAYAESGPINLGISGSSRTFDISITKEAGAANLYIDRIEFIPVNTLFEAEE
DLDVAKKAVNGLFTNEDALQTSVTDYQVNQAANLIECLSDELYPNEKRMLWDA
VKEAKRLVQARNLLQDTGFNRINGENGWTGSTGIEVVEGDVLFKDRSLRLTSAR
EIDTETYPTYLYQQIDESLLKPYTRYKLKGFIGSSQDLEIKLIRHRANQIVKNVPDN
LLPDVRPVNSCGGVDRCSEQQYVDANLALENNGENGNMSSDSHAFSFHIDTGEI
DLNENTGIWIVFKIPTTNGNATLGNLEFVEEGPLSGETLEWAQQQEQQWQDKMA
RKRAASEKTYYAAKQAIDRLFADYQDQKLNSGVEMSDLLAAQNLVQSIPYVYN
DALPEIPGMNYTSFTELTNRLQQAWNLYDLQNAIPNGDFRNGLSNWNATSDVN
VQQLSDTSVLVIPNWNSQVSQQFTVQPNYRYVLRVTARKEGVGDGYVIIRDGAN
QTETLTFNICDDDTGVLSTDQTSYITKTVEFTPSTEQVWIDMSETEGVFNIESVEL
VLEEE"

Nucleotide sequence

atgagtccaa ataatcaaaa tgagtatgaa attatagatg ctttatcacc cacttctgta tccgataatt ctattagata tcctttagca aacgatcaaa cgaacacatt acaaaacatg aattataaag attatctgaa aatgaccgaa tcaacaaatg ctgaattgtc tcgaaatccc gggacattta ttagtgcgca ggatgcggtt ggaactggaa ttgatattgt tagtactata ataagtggtt tagggattcc agtgcttggg gaagtcttct caattctggg ttcattaatt ggcttattgt ggccgtcaaa taatgaaaat gtatggcaaa tatttatgaa tcgagtggaa gagctaattg atcaaaaaat attagattct gtaagatcaa gagccattgc agatttagct aattctagaa tagctgtaga gtactatcaa aatgcacttg aagactggag aaaaaaccca cacagtacac gaagcgcagc acttgtaaag gaaagatttg gaaatgcaga agcaatttta cgtactaaca tgggttcatt ttctcaaacg aattatgaga ctccactctt acccacatat gcacaggccg cctctctgca tttgcttgta atgagggatg ttcaaattta cgggaaggaa tggggatatc ctcaaaatga tattgaccta ttttataaag aacaagtatc ttatacggct agatattccg atcattgcgt ccaatggtac aatgctggtt taaataaatt aagaggaacg ggtgctaagc aatgggtgga ttataatcgt ttccgaagag aaatgaatgt gatggtattg gatctagttg cattatttcc aaactacgat gcgcgtatat atccactgga aacaaatgca gaacttacaa gagaaatttt cacagatcct gttggaagtt acgtaactgg acaatcgagt acccttatat cttggtacga tatgattcca gcagctcttc cttcattttc aacgctcgag aacctactta gaaaacctga tttctttact ttgctgcaag aaattagaat gtatacaagt tttagacaaa acggtacgat tgaatattat aattattggg gaggacaaag gttaaccctt tcttatatct atggttcctc attcaataaa tatagtgggg ttcttgccgg tgctgaggat attattcctg tgggtcaaaa tgatatttac agagttgtat ggacttatat aggaaggtac acgaatagtc tgctaggagt aaatccagtt actttttact tcagtaataa tacacaaaaa acttattcga agccaaaaca attcgcgggt ggaataaaaa caattgattc cggcgaagaa ttaacttacg aaaattatca atcttatagt cacagggtaa gttacattac atcttttgaa ataaaaagta ccggtggtac agtattagga gtagttccta tatttggttg gacgcatagt agtgccagtc gcaataactt tatttacgca acaaaaatct cacaaatccc aatcaataaa gcaagtagaa ctagcggtgg agcggtttgg aatttccaag aaggtctata taatggagga cctgtaatga aattatctgg gtctggttcc caagtaataa acttaagggt cgcaacagat gcaaagggag caagtcaaag atatcgtatt agaatcagat atgcctctga tagagcgggt aaatttacga tatcttccag atctccagag aatcctgcaa cctattcagc ttctattgct tatacaaata ctatgtctac aaatgcttct ctaacgtata gtacttttgc atatgcagaa tctggcccta taaacttagg gatttcggga agttcaagga cttttgatat atctattaca aaagaagcag gtgctgctaa cctttatatt gatagaattg aatttattcc agttaatacg ttatttgaag cagaagaaga cctagatgtg gcaaagaaag ctgtgaatgg cttgtttacg aatgaaaaag atgccttaca gacaagtgta acggattatc aagtcaatca agcggcaaac ttaatagaat gcctatccga tgagttatac ccaaatgaaa aacgaatgtt atgggatgca gtgaaagagg cgaaacgact tgttcaggca cgtaacttac tccaagatac aggctttaat aggattaatg gagaaaacgg

atggacggga agtacgggaa tcgaggttgt ggaaggagat gttctgttta aagatcgttc gcttcgtttg acaagtgcga gagagattga tacagaaaca tatccaacgt atctctatca acaaatagat gaatcgcttt taaaaccata tacaagatat aaactaaaag gttttatagg aagtagtcaa gatttagaga ttaaattaat acgtcatcgg gcaaatcaaa tcgtcaaaaa tgtaccagat aatctcttgc cagatgtacg ccctgtcaat tcttgtggtg gagtcgatcg ctgcagtgaa caacagtatg tagacgcgaa tttagcactc gaaaacaatg gagaaaatgg aaatatgtct tctgattccc atgcattttc tttccatatt gatacgggtg aaatagattt gaatgaaaat acaggaattt ggatcgtatt taaaattccg acaacaaatg gaaacgcaac actaggaaat cttgaatttg tagaagaggg gccattgtca ggggaaacat tagaatgggc ccaacaacaa gaacaacaat ggcaagacaa aatggcaaga aaacgtgcag catcagaaaa aacatattat gcagcaaagc aagccattga tcgtttattc gcagattatc aagaccaaaa acttaattct ggtgtagaaa tgtcagattt gttggcagcc caaaaccttg tacagtccat tccttacgta tataatgatg cgttaccgga aatccctgga atgaactata cgagttttac agagttaaca aatagactcc aacaagcatg gaatttgtat gatcttcaaa acgctatacc aaatggagat tttcgaaatg gattaagtaa ttggaatgca acatcagatg taaatgtgca acaactaagc gatacatctg tccttgtcat tccaaactgg aattctcaag tgtcacaaca atttacagtt caaccgaatt atagatatgt gttacgtgtc acagcgagaa aagagggagt aggagacgga tatgtgatca tccgtgatgg tgcaaatcag acagaaacac tcacatttaa tatatgtgat gatgatacag gtgttttatc tactgatcaa actagctata tcacaaaaac agtggaattc actccatcta cagagcaagt ttggattgac atgagtgaga ccgaaggtgt attcaacata gaaagtgtag aactcgtgtt agaagaagag taa

Discussion

The use of Bt in controlling insect pests has increased over the past few decades. With the expansion of biotechnology in crop sciences use of Bt toxins is becoming a common practice (Sanchis & Lereclus, 1999). New variants of Bt with interesting toxicity spectra are also appearing. The search for Bt strains with novel toxicity, coupled with a more complete understanding of the toxins and their associated proteins, is paramount to current efforts to harness fully the potential of Bt technology. Shoaib *et al*., (2008) also revealed that *B. thuringiensis* has a great potential to prove as an alternative to methyl bromide for controlling coleopteran pests including *T. castaneum*. They concluded that there is a dire need to reach and test more and more isolates for tracing out the best to achieve maximum control of this pest.

During previous research, which was aimed at exploring the diversity of Bt in the Pakistani environment to search for novel Bt proteins, 7 Bt were found toxic against *T. casteneum.* The aim of the present study was to characterize Bt isolates toxic to coleopteran insect pests.

We report that locally isolated Bt SG31.11 is highly toxic to the larvae of *T. castaneum* and its protein sequence is identical with novel Cry3 crystal protein in the database, under the Accession number U04366. When we determined protein sequence, a report appeared by Sato *et al*., (1994) which showed proteins sequence similarity in Pakistani *Bacillus thuringiensis* strain SG31.11 and *Bacillus thuringiensis* serovar japonensis strain Buibui.

Sato *et al*., (1994) revealed that novel Cry3 protein of *Bacillus thuringiensis* serovar japonensis strain Buibui is toxic to larvae of the cupreous chafer (*Anomala cuprea*) which is a scarabaeid insect. However, there is homology between protein sequences of Pakistani *B. thuringiensis* strain SG31.11 and *B. thuringiensis* serovar japonensis strain Buibui, but our locally isolated *B. thuringiensis* and target pest (*T. casteneum*) is different. *T. casteneum* belongs to the family Tenebrionidae of the order Coleoptera.

The information obtained from these studies will be helpful in adopting strategies for controlling the insect pests of commercially important crops, better suited to Insect Resistance Management (IRM).

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