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IMMUNOASSAY-BASED APPROACH FOR DETECTION OF NOVEL *BACILLUS THURINGIENSIS* δ-ENDOTOXINS, ENTOMOCIDAL TO COTTON APHIDS (*APHIS GOSSYPII*) AND WHITEFLIES (*BEMISIA TABACI*)

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Abstract

A new strategy, which is based on immunoassay, has been developed to screen locally isolated *Bacillus thuringiensis* (Bt). Delta endotoxins of three Bt strains (INS 2.13, HFZ 24.3 and GU 9.1) were prepared, electrophoresed on native PAGE and electroeluted the purified delta endotoxins from gels. Determined the toxicity of purified delta endotoxins against cotton aphids (*Aphis gossypii*) and whiteflies (*B. tabaci*). N-terminal sequences of the purified proteins (δ-endotoxins) of these three entomocidal Bt isolates showed no significant homology to protein sequences in the Gen bank (NCBI) protein database. The purified proteins having novelty in their sequences were used as antigens for the immunization of rabbits to raise polyclonal antibodies. The affinity purified antibodies were used in immunoassays to screen 170 Bt isolates for entomocidal activity and obtained promising results.

Introduction

Bacillus thuringiensis, is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. *B. thuringiensis* was initially characterized as an insect pathogen, and its insecticidal activity was attributed largely or completely (depending on the insect) to the parasporal crystals. This observation led to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera. There are more recent reports of *B. thuringiensis* isolates active against other insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against nematodes, mites, and protozoa (Feitlson, 1993). *B. thuringiensis* is already a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control. It is also a key source of genes for transgenic expression to provide pest resistance in plants. Interest in Bt proteins has increased during the last two decades because of their unique qualities which are unmatched by any conventional insecticide (Whalon & Wingerd, 2003).

Whiteflies (Homoptera: Aleyrodidae) are serious pests of many agricultural systems. They are the major carriers of cotton leaf curl disease (CLCuD) causing severe damage to the cotton crop in Pakistan (Briddon & Markham. 2000). The cotton aphids, *Aphis gossypii* Glov. (Homoptera: Aphididae) are significantly devastating pests of cotton in Pakistan (Mohyuddin, 1985). Both adults and nymphs imbibe liquid from host plant tissues by stylets (Pollard, 1973), this result in stunted growth, curled leaves and death of small plants. Heavy infestation on cotton later in the season causes shedding of curled leaves and pre mature opening of the bolls, thus reducing the yield and grade of lint. Furthermore, honeydew excreted by both adults and nymphs, is causative of black sooty mould, which makes cotton sticky and stained (Roy & Behvra, 1983). The importance of

Aphis gossypii Glover as a cotton pest is increasing throughout the cotton-producing regions of the world (Leclant & Deguine 1994). In central Africa, *A. gossypii* is the second most important economic cotton pest, following *Helicoverpa armigera* (Hübner). At least 60 host plants are known in Florida, and perhaps 700 host plants worldwide. The only control method available for the aphids is the foliar spray of insecticide. Resistance in cotton aphid (*Aphis gossypii*) is a major new threat to Australian cotton production (Herron *et al*., 2001).

The development of pest insect's resistance to chemical insecticide and general concern for environmental damage has shifted the trend towards biological control agents. Bt pesticidal action of protoxin proteins starts with solubilization. After solubilization, many protoxins must be processed by insect midgut proteases to become activated toxins. Activated Cry toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvillae of susceptible insects. Binding is a two-stage process involving reversible and irreversible steps. The latter steps may involve a tight binding between the toxin and receptor, insertion of the toxin into the apical membrane, or both. It has been generally assumed that irreversible binding is exclusively associated with membrane insertion (Hoffmann *et al*., 1988. Van Rie *et al*., 1989, 1990). Toxin insertion into the membrane, alters the electrochemical potential gradient across the midgut by generating pores (Wolfersberger, 1992. Knowles & Dow, 1993). This causes cell lysis and death of larvae.

Previously, we presented data, describing Bt δ-endotoxins activities toward *Aphis gossypii* Glov. (Homoptera: Aphididae) and found several toxic Bt isolates (Malik *et al*., 1998). This finding implies a potential control strategy for this important group of agricultural pest. The present study was done to find novel Bt against aphids, based on specific Immunoassays.

Materials and Methods

Aphis gossypii nymphs and whiteflies were obtained from the insectory of the Institute. Standard *Bacillus thuringiensis* subsp. *Kurstaki* HD73 type was purchased from ATCC. All protein concentrations were measured by Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976). 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.

Isolation and identification of *B. thuringiensis*: A large number of different samples from soil, animal dung, etc were collected from different ecological regions of Pakistan (Khan *et al*., 1995a, b) and Bt was separated by selective sodium acetate method (Martin & Travers, 1989). Isolated spore formers were checked for their spore and crystal production by a modified method (Schaeffer & Fulton, 1933). Crystal protein producers were maintained as sporulated cultures on T_3 slants (tryptone 3g, tryptose 2g, Yeast extract 1.5g, $MnCl_2$ $2H_2O$ 0.005g, 1M potassium phosphate buffer 2.5 ml, agar 15g, distilled water 1l, pH 6.8) and glycerol stocks.

***B. thuringiensis* toxin preparation:** Locally separated and selected Bt cultures were streaked on disposable plastic T_3 agar plates for synchronized growth of bacterium. Streaked plates were kept at 30°C until complete sporulation. The sporulated cultures were scraped off after 72 hrs. The spore/crystal mixture was used for toxin preparation.

The spore/crystal mixture was washed three times in chilled distilled water and pellet was resuspended in 5ml of alkalic buffer (50mM Sodium carbonate, 10mM dithiothreitol, pH 9.5) and incubated at 37ºC for 4-5 hrs with continuous shaking. After solubilization in alkalic buffer, protoxin was activated with 1:20 ratio of trypsin:protoxin for 2-3 hrs. Cry1Ac protein was prepared by using the same procedure from Standard *Bacillus thuringiensis* subsp. *Kurstaki* HD73 type.

Purification and Biotoxicity assays of delta endotoxins: Delta endotoxins were electrophoresed on native (without SDS) PAGE and bands of proteins were electroeluted to get the purified delta endotoxins. Biotoxicity assays were performed against cotton aphid (*Aphis gossypii*) nymphs and whiteflies by using the method described by Malik *et al*., (1998).

Amino acid sequencing: Purified proteins from three strains INS 2.13, HFZ24.8 and Gu 9.1 were electrophoresed on 10% SDS-PAGE (Laemmli, 1970). Then purified proteins were transferred to PVDF-membranes, stained with Coomassie brilliant blue, and used in N-terminal amino acid micro-sequencing by Edman degradation.

Homology search: The amino acid sequences were searched for homology with other sequences in GenBank using Blastx, at http://www.ncbi.nlm.nih.gov/BLAST/**.**

Production of antibodies: One milligram of purified proteins of INS 2.13, GU 9.1 and HD73 were used to immunize the rabbits according to the standard protocol. Antiserum separated and stored at -20ºC. Antibodies were further purified from one aliquot of antiserum by affinity purification. For affinity purification of antibodies, Immunoabsorbant technique (Rybicki *et al*., 1990) was used with slight modification.

Immunoblot analysis of *Bt* crystal proteins: Specific affinity of the purified crystal proteins was determined by dot blotting. After electrophoresis on 10% SDS-PAGE, *Bt* crystal proteins spotted on nitrocellulose membrane and incubated for one hour for blocking with 3% skimmed Milk. The dot blots were processed as Western blots as described by Towbin *et al*., (1979). Purified antiserums (polyclonal) against purified insecticidal crystal proteins (ICP) of INS 2.13, GU 9.1 and HD73 Bt isolates, used as primary antibody. Secondary antibody was AP-conjugated goat anti-rabbit IgG (H+L) purchased from Sigma Company.

Results

Biotoxicity assay against aphids: The isolates INS 2.13, HFZ24.8 and GU 9.1 exhibited the maximum LC_{50} values of 62ng/mL, 328ng/mL and 114ng/mL against aphids and 52.8ng/mL, 250 ng/mL, and 250 ng/mL respectively, against whiteflies.

Amino acid sequence comparison: When the purified insecticidal crystal proteins (ICP) of INS 2.13, HFZ24.8 and GU 9.1 Bt isolates (kDa), were sequenced by Edman degradation, following N-terminal amino acid sequences were obtained:

INS2.13	M/G P K T N V V E V L N K - V A N W N - L Y V F L
HFZ24.8	S T K T N V V E V L
Gu 9.1	(sequence not shown).

Interestingly, protein, isolated from HFZ24.8 had an eight amino acid identity with the newly identified sequence from INS 2.13 while GU9.1 has entirely different sequence from above mentioned sequences. It is on this basis that the newly identified proteins from INS 2.13 and GU 9.1 were chosen as immunogens.

When these sequences were used to BLAST search in the Gen Bank (NCBI) protein database, there were no significant sequence similarities to any of the protein in the database, which shows the uniqueness of these N-terminal sequences of proteins.

Antibody generation and immunore activity: Purified δ-endotoxins proteins (kDa) of novel entomocidal Bt (INS 2.13 and GU 9.1) and Cry1Ac were used as antigens to raise antibodies. The antibodies were used in dot blot experiments to investigate the antigenic relationship among δ-endotoxins of novel entomocidal Bt and local isolates. Total 170 Bt isolates were analysed, of these 49 with anti-INS 2.13, 11 with anti- GU 9.1 and 31with anti-Cry1Ac appeared to be more immuno-reactive (+++) while; 36 with anti-INS 2.13 and 19 with anti- GU 9.1 were immuno-reactive at moderate level (++) and 49 with anti-INS 2.13, 57 with anti- GU 9.1 and 32 with anti- Cry1Ac were less immuno-reactive (+) (table 1). Two isolates GRT44.5 and SL 43.3 gave good positive results with all three antibodies while HFZ 1.5 and CHT 6.10 were positive for both INS 2.13 and GU 9.1 antibodies. The results indicate diversity in protein profiles of these 4 isolates (GRT44.5, SL 43.3, HFZ 1.5, CHT 6.10).

Discussion

The use of Bt in controlling insect pests has increased over the past few decades. New variants of Bt with interesting toxicity spectra are also appearing. The Bt genes encoding the Cry1Ac, Cry2A and Cry3A toxins are now being used commercially in various crops around the world. Since the rediscovery of Bt in 1911, many Bt strains and their toxin genes have been discovered and modified to enhance toxicity against target insects (Sanchis *et al*., 1996). With only 297 known toxins to date, and with the frequent identification of new insecticidal proteins, the search for new Bt toxin genes has a great potential. New non-crystal forming proteins from Bt have been discovered which are either toxic to their target insects or synergistically enhance the efficacy of Cry proteins (Regev *et al*., 1996; Selvapandiyan, *et al.*, 2001). Likewise, several proteases of the native Bt cell are also being investigated (Fedhila & Lereclus, 2002; Barboza-Corona *et al*., 2003) for their insecticidal activity. 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.

It has also been reported that different Bt isolates can have a large variation in their biological activity against same species and a given isolate may be very active against one species and inactive against others (Jarrett & Burges, 1982). This amazing variability and complexity of host range in lepidopteran- active strains may be the result of multiple cry genes encoding the variety of different protoxins (Jarrett, 1985). Relative potency of cry preteins can be influenced with different factors, a) the origin of toxin (Bt isolate), b) the expression level of different multi-gene products, c) solubility and stability of toxins in the midguts of target larvae and d) the natural susceptibility of host to toxin (Jacquet, 1987; Aronson *et al*., 1994).

Table 1. *Bacillus thuringiensis* isolates: Their sources and immunoblot analysis.

Bt #	Bt isolates	Source	Locality	Immunoblot INS 2.13 antibody	Immunoblot GU 9.1 antibody	Immunoblot Cry1Ac antibody
1	MUL 10.2	Soil	Multan	+++	-	++
2	MUL 26.1	Cow dung	Multan	+++	+	++
3	MUL 4.4	Soil	Multan	+++	-	++
4	NW 4.12	Agriculture-land	Narowal	+++	-	++
5	KM 12.1	Wheat dust	Kashmir	+++	-	++
6	KM 16.5	Wheat dust	Kashmir	+++	-	++
7	KM 13.7	Beans	Kashmir	+++	-	++
8	KM 17.2	Wheat dust	Kashmir	+++	-	++
9	CL 10.5	Soil	Punjab	+++	+	+++
10	DN 4.8	Wheat dust	Deranawab	+++	+	+++
11	DN 3.1	Wheat dust	Deranawab	+++	+	-
12	DN 7.2	Wheat dust	Deranawab	+++	-	-
13	DN 4.7	Wheat dust	Deranawab	+++	+	++
14	RL 4.8	Railway-godown	Rawalpindi	+++	-	++
15	KSK-4	Soil	Kasoki	+++	-	++
16	FIC 26.7	Soil	Punjab	+++	++	+++
17	FIC25.7	Soil	Punjab	+++	-	+++
18	JR 7.24	Soil	Punjab	+++	+	+++
19	SG 31.10	Wheat Dust	Sargoda	+++	-	+++
20	GRT 39.1	Animal dung	Gujrat	+++	++	+++
21	GT 11.7	Forest soil	Punjab	+++	-	+++
22	3762	Soil	Punjab	+++	+	++
23	GRT 44.5	Animal dung	Gujrat	+++	+++	++
24	KRS 6.3	Sand	Punjab	+++	-	+++
25	RC 1.5	Soil	Punjab	+++	++	+++
26	ST 2.2	Dung & Soil	Punjab	+++	-	+++
27	ST 1.1	Buffalo dung	Punjab	+++	-	+++
28	ST 29.7	Dung & Soil	Punjab	+++	++	+++
29	ST 1.4	Dung & Soil	Punjab	+++	-	+++
30	JLP 14.5	Mud	Jalalpur	+++	+	+++
31	JLP 15.2	Mud	Jalalpur	+++	+	-
32	J 1.3	Soil	Jehlum	+++	+	++
33	SL 43.3	Soil	Sialkot	+++	+++	+++
34	CHN 8.1	Mud	Chiwinda	+++	-	+++
35	CHN 8.8	Mud	Chiwinda	+++	+	+++
36	MG 2.6	Soil	Muzafargur	+++	+	+++
37	LHR 14.4	Soil	Lahore	+++	-	++
38	LHR 12.1	Soil	Lahore	+++	-	++
39	LHR 26.11	Soil	Lahore	+++	+	-
40	LHR 11.5	Soil	Lahore	+++	-	+++
41	LHR 27.3	Soil	Lahore	+++	+	-
42	DK 2.5	Soil	Punjab	+++	+	+++
43	CN 2.1	Rice dust	Punjab	+++	+	-
44	DK 12.1	Rice dust	Punjab	+++	+	-
45	HFZ 3.1	Rice dust	Hafizabad	+++	-	-
46	HFZ 3.6	Soil	Hafizabad	+++	-	+++
47	HFZ 1.5	Soil	Hafizabad	+++	+++	-
48	CHT 6.10	Soil	Chishteean	+++	+++	-
49	3760	Soil	Punjab	++	+++	-
50	M 1.1	Soil	Punjab	+	+++	-
51	PI 6.1	Mud	Punjab	+	+++	-
52	KV 17.8	Mud	Punjab	++	+++	+++

53	RWK	Soil	Rawalakot	+	+++	+++
54	13.11	Soil	Punjab	-	+++	+++
55	FBD 1.2	Seed dust	Punjab	++	-	++
56	PH 11.7	Seed Dust	Punjab	+	-	-
57	PH 4.2	Soil	Punjab	+	+	++
58	LM 7.7	Wheat dust	Punjab	+	-	++
59	LM 13.5	Soil	Punjab	+	-	++
60	MUL 27.6	Goat dung	Multan	+	-	-
61	KM 7.6	Goat dung	Kashmir	+	++	-
62	KM 9.5	Goat dung	Kashmir	+	-	++
63	CL 4.1	Soil	Punjab	++	+	++
64	CL 6.6	Goat dung	Punjab	++	+	++
65	CL 4.4	Wheat dust	Punjab	++	++	++
66	CL 15.3	Wheat dust	Punjab	++	++	-
67	CL 3.1	Soil	Punjab	++	++	+++
68	DN 4.4	Soil	Deranawab	+	+	-
69	GT 12.3	Soil	Punjab	+	++	-
70	M2.3	Dead insect	Punjab	++	+++	-
71	SG 31.11	Soil	Sargoda	+	+	-
72	TB 2.1	Soil	Toba	+	+	-
73	INS 3.19	Soil	Lahore	++	+	-
74	QT 12.3	Dung & Soil	Punjab	+	-	-
75	D 1.1	Soil	Punjab	+	++	-
76	D 4.3	Soil	Punjab	+	-	-
77	ST 8 3375	Soil	Punjab	+	-	-
78	KAL 1.5	Soil	Punjab	++	-	+++
79	JR 6.3	Mud	Punjab	++	-	++
80	HN 1.1	Wheat dust	Punjab	+	++	-
81	D 3.11	Sheep dung	Punjab	+	+	-
82	MPK 7.4	Soil	Mir Pur	+	+	-
83	GU 29.2	Wheat dust	Gujranwala	+	+	-
84	Y 2	Wheat dust	Punjab	++	+	++
85	S 1.5	Seed dust	Punjab	+	-	-
86	PR 7.18	Seed dust	Peshawar	+	+	-
87	PR 10.3	Soil	Peshawar	++	-	-
88	LG 8.3	Soil	Punjab	+	-	-
89	LG 12.11	Rice dust	Punjab	-	+	-
90	GR 6	Soil	Punjab	+	++	-
91	GC 3.7	Soil	Punjab	+	+	+++
92	GC 14.3	Wheat dust	Punjab	+	-	++
93	GC 11.3	Soil	Punjab	++	++	++
94	GC 14.1	Mud	Punjab	++	+	++
95	BS 5.2	Donkey dung	Punjab	+++	-	-
96	FIC 25.10	Wheat dust	Punjab	+	-	-
97	RWK8.1	Wheat dust	Rawalakot	++	+	-
98	TA 9.3	Soil	Punjab	+	+	-
99	TA 13.9	Mud	Punjab	+	-	-
100	QT 11.2	Soil	Punjab	+	-	-
101	QT 7.4	Seed dust	Punjab	-	+	+++
102	JLP 15.2	Soil	Punjab	++	+	-
103	M 1.4	Soil	Punjab	+	+	-
104	LM 11.3	Dust	Punjab	+	+	-
105	KB 3.2	Animal dung	Punjab	++	+	-
106	PH 8.3	Mud	Punjab	++	++	-
107	JL 1.1	Wheat dust	Punjab	++	++	-
108	NM 7.14	Soil	Punjab	+	+	-

109	JLP 14.2	Soil	Jalalpur	++	+	++
110	HW 4.11	Soil	Punjab	++	-	-
111	M 2.4	Soil	Punjab	+	+	-
112	LHR 25.1	Soil	Lahore	++	+	-
113	LHR 10.7	Donkey dung	Lahore	+	-	-
114	BG 1.1	Mud	Punjab	+	+	-
115	BG 2.1	Soil	Punjab	+	-	-
116	BG 2.8	Soil	Punjab	++	-	-
117	RWK 15.5	Bird droping	Rawalakot	+	-	-
118	RA 2.12	Wheat dust	Punjab	-	+	-
119	SHD 38.7	Seed dust	Shahdara	-	+	-
120	SHD 42.1	Soil	Shahdara	+	+	-
121	PR 12.10	Wheat dust	Peshawar	+	+	-
122	BC 1408	Soil	Punjab	++	-	-
123	FBD 1.4	Soil	Punjab	++		-
124	T 3	Mud	Punjab	-	+	-
125	SR 10	Soil	Punjab	++	+	-
126	F 4.21	Soil	Punjab	++	-	++
127	KT 1.11	Soil	Punjab	++	+	-
128	KV 7.3	Rice dust	Punjab	++	+	-
129	H 2.3	Rice dust	Punjab	++	+	-
130	MR 19.1	Wheat dust	Punjab	++	-	++
131	HFZ 1.1	Donkey dung	Hafizabad	++	-	+++
132	HFZ 3.8	Rice dust	Hafizabad	+	+	-
133	HFZ 24.8	Rice dust	Hafizabad	+	+	-
134	HFZ 4.9	Soil	Hafizabad	+	-	-
135	HFZ 3.2	Soil	Hafizabad	+	-	-
136	HFZ 3.3	Soil	Hafizabad	+	++	-
137	CHT 27.13	Soil	Chishteean	++	-	-
138	CHT 17.12	Soil	Chishteean	++	++	-
139	CHT 37.8	Soil	Chishteean	+	+	-
140	KV 4.1	Maize field	Punjab	-	++	+++
141	GT 5.3	Soil	Punjab	+	++	-
142	KC 14.1	Soil	Punjab	-	+	-

In this study, immunoassay`s specificity was applied to screen novel Bt from local environment. In the present investigation, very promising results were obtained which appear to be important from the integrated pest management point of view. Further characterization of these proteins should give insight into the diversity of toxins effective against target pests.

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