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ORIGINAL ARTICLE

Isolation, characterization and toxicity of native *Bacillus thuringiensis* isolates from different hosts and habitats in Iran

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Abstract

Bacillus thuringiensis is a Gram-positive, aerobic, facultative anaerobic and endospore-forming bacterium. Different strains of this species have the ability to produce parasporal crystalline inclusions which are toxic to larvae of different insect orders and other invertebrates and cause rapid death of the host. To determine the importance of this species in microbial control, we collected native strains and studied their virulence on the diamondback moth, *Plutella xylostella*. More than 148 samples were collected from Alborz, Guilan and Mazandaran Provinces. Experimental samples, including soil samples from forests, fruit gardens, agricultural fields, diseased and dead larvae, were transferred to a laboratory in sterile plastic containers. For evaluating *B. thuringiensis* isolates virulence, a cabbage leaf dip method with 10^6 cell · ml^{-1} concentration of various Bt isolates was applied to diamondback moths. Larval mortality was recorded 72 h after treatment. Based on bioassay results, all isolates were classified into three high, medium and low virulence groups. Protein level characterization based on the SDS-PAGE gel analysis showed that two isolates from a high virulence group have proteins of high molecular masses of 121 and 109 kDa. Results revealed that there is a positive correlation between protein masses and virulence of isolates. In addition, this research introduced nine strains that are highly toxic to *P. xylostella* and would be valuable as insecticidal agents for controlling lepidopteran pests.

Key words: *Bacillus thuringiensis*, characterization, isolation, *Plutella xylostella*, SDS-PAGE, virulence

Introduction

Bacillus thuringiensis is a Gram-positive, aerobic, facultative anaerobic, endospore-forming bacterium and is distinguished from other *Bacillus* spp. by its ability to produce indigenous crystals during sporulation. Crystal proteins from many *B. thuringiensis* strains are toxic to lepidopteran pests (Zhong *et al.* 2000). These crystals are predominantly composed of one or more proteins (Cry and Cyt toxins), also called δ-endotoxins (Bravo *et al.* 2007), which is specific primarily at the level of insect orders, particularly Lepidoptera, Coleoptera and Diptera and cause gut paralysis in some insect orders, resulting in an inability to feed, starvation and death (Faknath 1999). Cry proteins are parasporal inclusion (Crystal) from *B. thuringiensis* that exhibit experimentally verifiable toxic effects on a target organism and Cyt proteins are parasporal inclusion from *B. thuringiensis* that exhibit hemolytic (Cytolitic) activity (Bravo *et al.* 2007). In Lepidoptera specificity is partly due to an alkaline midgut environment that is needed to solubilize the protoxin into active toxin (Broderick *et al.* 2006). Protoxins are activated by midgut proteases and bind to specific receptors on the intestinal epithelium by forming pores. Toxins then enter gut cells, leading to cell lysis which provides spores access to a more

favorable environment of hemocoel. Spores germinate, reproduce and cause septicemia and death (Schnepf *et al.* 1998). *Bacillus thuringiensis* is ubiquitous in natural environments and is readily isolated from soil, warehouses, the leaf surfaces of broad leaf trees, conifers, grasses and insect habitats (Zhong *et al.* 2000).

Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera, Plutellidae), is a serious and important pest of cruciferous plants worldwide (Talekar and Shelton 1993; Sarfraz *et al.* 2006; Li *et al.* 2016). *Plutella xylostella* occurs annually throughout different regions of Iran wherever cruciferous crops are grown and can cause substantial crop losses during outbreak years (Keyhanian *et al.* 2005). In Indonesia this pest has been reported as a primary factor in limiting the production of cabbage in many areas (Ahmad 1999). In this study, the virulence of *B. thuringiensis* isolates was evaluated on *P. xylostella* larvae. The method of controlling this pest has been to spray with chemical pesticides, a strategy that also kills natural enemies and can lead to the rapid development of insecticide resistance in pest species. Therefore, any effort to find new microbial pesticides to control this pest, e.g. ones based on the bacterium *B. thuringiensis*, is highly necessary and desirable.

Isolating native *B. thuringiensis* strains from different habitats, evaluating the insecticidal activity of the strains, characterizing the protein level diversity and finally, determining the correlation between protein masses and virulence were the main objectives of this study.

Materials and Methods

Sample collection

A total of 148 samples were collected from environmentally diverse sources in Alborz, Guilan and Mazandaran provinces in Iran. Experimental samples, including soil samples from forests, fruit gardens, agricultural fields, beach sands, urban and rural areas, diseased and dead larvae, were transferred to a laboratory in sterile plastic containers. Soil samples were collected at a depth of 5 to 15 cm under the surface, away from direct sunlight (Ohba and Aizawa 1986b).

Isolation from soil samples

Bt strains were isolated according to the sodium acetate selective method of Travers *et al.* (1987). Approximately 1 g of soil samples were suspended in 10 ml nutrient broth medium containing 0.25 M and 0.35 M sodium acetate and left for microbial growth at 37°C for 4 h. Every 10 min the containers were shaken well. Heat treatment was then applied for 7 min at 80°C to kill vegetative cells. Using this method, the germination of *B. thuringiensis* spores was inhibited by sodium acetate, thus the germinated spores and other non-spore forming bacteria were eliminated by heat treatment. Next, samples were plated on nutrient agar, incubated at 30°C for 4 days and then examined for colony morphology and the presence of parasporal crystals by phase contrast microscopy. All crystal forming colonies were sub-cultured and maintained for further investigation and stored in sterile liquid nutrient broth containing 50% glycerol at –20°C.

Isolation from dead larvae

Insect cadaver samples were surface sterilized with 0.2% sodium hypochlorite solution, 70% ethanol and sterile distilled water. The dissected body contents were placed on nutrient agar. The plates were incubated at 30°C for 4 days, and processed as mentioned in the previous section.

Insect toxicity assay

Mortality data were generated by a cabbage leaf dip bioassay on diamondback moth second instar larvae. An experimental population of *P. xylostella* was established from cabbage farms in Alborz Province. Cabbage leaf disks (50 mm diameter) were dipped for 10 sec in a 10^6 cell · ml^{-1} concentration of various Bt isolates, allowed to air dry and inoculated with 20 larvae per leaf. Mortality was determined after 72 h but after 24 h of feeding on contaminated leaves, the treated cabbage disks were replaced with clean ones. Sterile distilled water was used for negative control. Larvae that failed to respond to gentle prodding were considered as dead. Bioassay was carried out on 68 native Bt isolates and Biolep commercial Bt formulation from Fanavary Zisti Tabiatgara company (Karaj, Iran) in three replicates. Also three reference strains, Bt subsp. *kurstaki*, Bt subsp. *israelensis* and Bt subsp. *tenebrionis,* which were kindly provided by dr. Zihni Demirbag from Karadeniz Technical University (Trabzon, Turkey) were used in this study. According to bioassay results, all isolates were classified into three, high, medium and low, virulence groups.

Growth curve analysis

Bacterial growth curves were traced for six Bt isolates. Two isolates from each group of virulence were selected. The designated isolates were inoculated in nutrient broth medium. The growth curve, based on absorbance at 540 nm at an interval of 24 to 120 h, was generated. During this period, the cells were observed by phase contrast microscopy to identify the sporulation phase.

Crystal morphology

Parasporal inclusions were examined before cell lysis by phase contrast microscopy at 1,000× magnification. Smears of bacterial strains were stained with coomassie brilliant blue solution and observed by phase contrast microscopy with oil emersion. From the three virulence groups, 18 isolates were chosen and inoculated in nutrient broth medium and left for 120 h to sporulate at 200 rpm. When sporulation was completed and crystals and spores were released (as checked by phase contrast microscopy), the colony material was removed from the medium by centrifugation at 6,000×g for 15 min, and transferred to sterile microtubes containing 1 ml 0.5 M NaCl. Re-suspended samples were centrifuged at 17,000×g for 12 min at 4°C. The discarded supernatant and pellets were re-suspended in 1 ml 0.5 M NaCl. The suspension was centrifuged again as above. The washing procedure was performed twice. The final pellet containing crystals was collected for microscopic observation.

Protein extraction

From each group of virulence, six Bt isolates and three standard Bt isolates were designated for protein extraction by using the Lowry method (Lowry 1951). After complete autolysis, 1.5 ml of distilled water containing lysed cells were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 2 ml of sterile distilled water. The protein quantification was determined by taking an absorbance reading at 630 nm.

Protein electrophoresis

The protein contents of 21 Bt strains were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis, as described by Leamlli (1970), using 5% and 12.5% acrylamide resolving and separating gels.

Results

Isolation and morphological characteristics

Soil samples were the most abundant source of *B. thuringiensis*. Of the 148 analyzed samples, 155 native strains showing typical colonies of the *Bacillus* genus were found. All isolates were rod shaped indicating their vegetative stage. Based on the capacity of producing parasporal crystal inclusions, Bt isolates were detected. Phase contrast microscopic observation showed both the endospores and parasporal bodies clearly. Most cells were lysed and released the spore and crystals after 120 h. On the basis of microscopic observation, parasporal crystals produced by *B. thuringiensis* isolates, were classified into four morphological groups; bipyramid crystals were mainly in high virulent groups, cube and polyhedron (triangular) crystals were identified in medium virulent groups and round crystals were the most abundant in low virulent groups. Crystal morphology of Bt can give information about target insect spectra (Maeda *et al.* 2000). Therefore, in order to determine the crystal pathogenicity and virulence, the crystal morphology by phase contrast microscopy should be examined.

Insect toxicity assay

The bioassay results indicated that nine Bt isolates were highly toxic against second instar larvae of *P. xylostella*. The percentages of mortality of Bt isolates in the high virulent groups are shown in Table 1. Among nine isolates of Bt subjected to the high virulent groups, only three isolates of Bt-IE, Bt-IP and Bt-U3a induced mortality above 95% against second instar larvae of *P. xylostella*. The Bt-IE and Bt-IP isolates produced 100% mortality. The percentages of mortality were: 100, 100, 78.4, 97.4, 84.9, 88.9, 77.8, 84.5 and 91.2 with isolates of Bt-IP, Bt-IE, Bt-AR4, Bt-U3a, Bt-AP, Bt-MCh, Bt-MWa, Bt-Sarv1 and Bt-DC, respectively. Bioassay results showed that the commercial and reference isolate (biolep) induced 100% mortality against second instar larvae of *P. xylostella*.

Bacterial growth curve

The bacterial growth curve was plotted for six Bt isolates at an interval of 24 to 120 h to determine their life cycles. During the period of 24, 48, 72, 96 and 120 h, the bacterial cells were observed under a phase contrast microscope to identify the sporulation phase. Based on the results, two isolates of the high virulent groups, Bt-IP and Bt-IE were found to sporulate at 72 h after inoculation (Fig. 1). Bt-Bua isolate from the medium virulent group sporulated at 72 h while Bt-DP isolate from the same group sporulated at 68 h (Fig. 2). Two isolates of the low virulent groups, Bt-BaR and Bt-MW, sporulated 64 and 92 h after inoculation, respectively (Fig. 3).

Protein extraction

Quantitative comparison of whole cellular proteins (mg · ml^{-1}) of Bt isolates using spectrophotometry showed that the amounts of proteins differed and ranged from 3.39 to 6.01 mg · ml^{-1} in high virulent groups, from 0.49 to 4.33 mg · ml^{-1} in medium virulent groups and from 1.84 to 3.78 mg · ml^{-1} in low virulent groups.

Table 1. The percentages of mortality of diamondback moth (*Plutella xylostella*) second instar larvae caused by *Bacillus thuringiensis* isolates in the high virulent group

Isolate	Sample source	Crystal morphology	% Mortality±SE
Bt-IP	*Plodia interpunctella* larvae	bipyramid	100±00.0
Bt-IE	*Ectomyelois ceratonia* larvae	bipyramid	100±00.0
Bt-AR4	agricultural field (rice)	bipyramid	78.4±04.4
Bt-U3a	forest	bipyramid	97.4±07.5
Bt-AP	agricultural field (pepper)	bipyramid	84.9±12.5
Bt-MCh	fruit garden (cherry)	bipyramid	88.9±02.2
Bt-MWa	agricultural field (wheat)	bipyramid	77.8±08.9
Bt-Sarv1	forest	bipyramid	84.5±03.8
Bt-DC	agricultural field (cabbage)	bipyramid	91.2±04.4
Bt-biolep	formulation Bt subsp. *kurstaki*	bipyramid	100±00.0

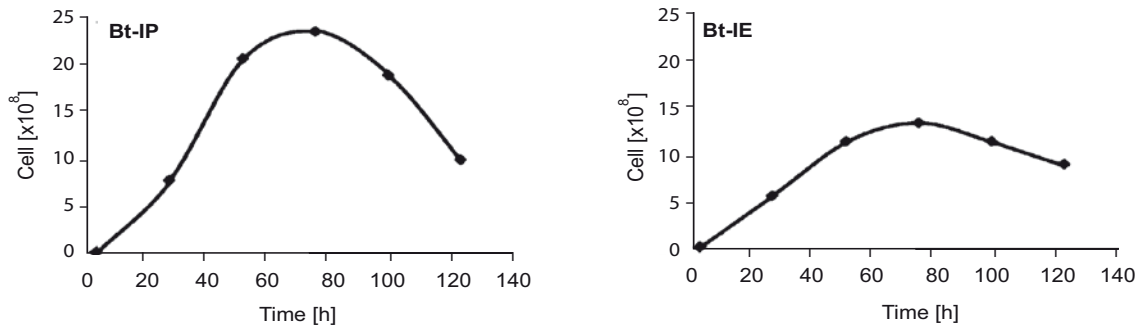

Fig. 1. Growth curves of two *Bacillus thuringiensis* isolates from the high virulent group: Bt-IP and Bt-IE

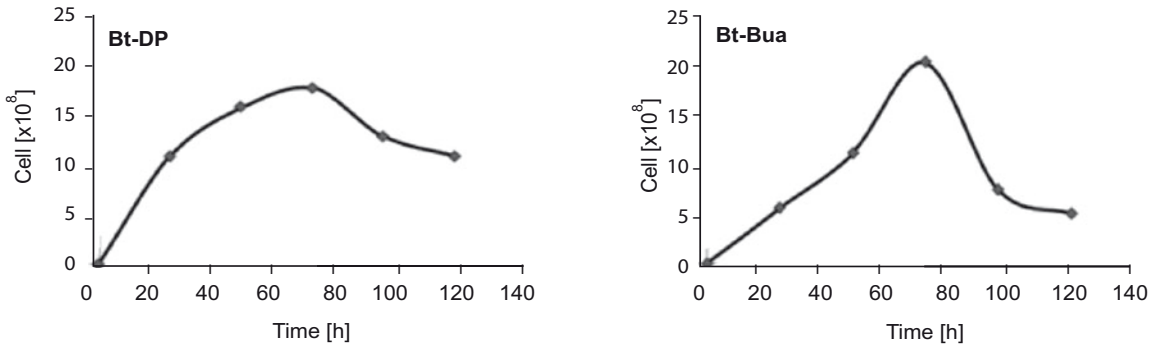

Fig. 2. Growth curves of two *Bacillus thuringiensis* isolates from the medium virulent group: Bt-DP and Bt-Bua

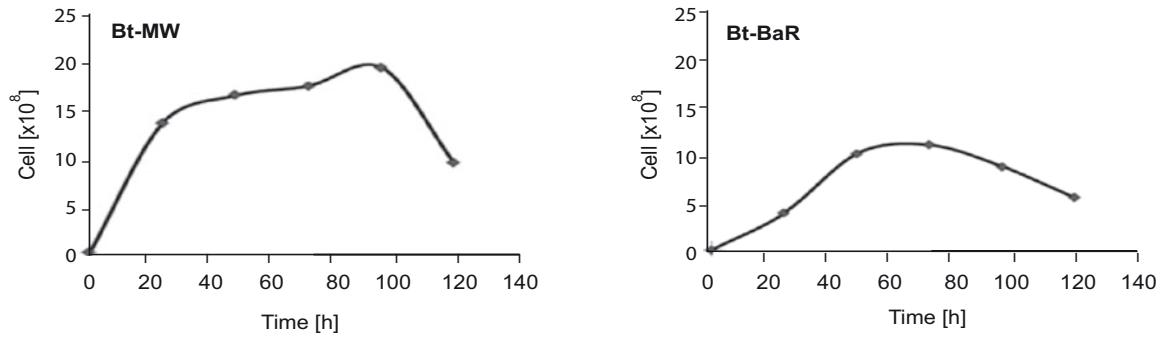

Fig. 3. Growth curves of two *Bacillus thuringiensis* isolates from the low virulent group: Bt-MW and Bt-BaR

Protein electrophoresis

The whole cellular proteins of 21 *B. thuringiensis* strains as well as three reference strains of this species, *B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *tenebrionis*, were analyzed by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). A great diversity of protein patterns, including both the number and size of protein bands, was found among 21 tested Bt isolates. All the isolates show distinct and clear bands so the existence of Cry proteins can be confirmed (Fig. 4). On the basis of molecular weight, the protein profiles of Bt strains were in the range of 134.8 kDa to 12.3 kDa. The high virulent group exhibited the most protein bands from a maximum molecular mass of 134.8 kDa to minimum of 12.1 kDa. Moreover, SDS-PAGE of the high virulent group of isolates showed a protein composition resembling that of the lepidopteran toxic Bt consisting of major polypeptides in the range of 130 to 140 kDa. In the medium virulent group the number and size of protein bands differed from 24 kDa to 16.1 kDa and in the low virulent group the mass of proteins ranged from 112.2 kDa to 12.1 kDa.

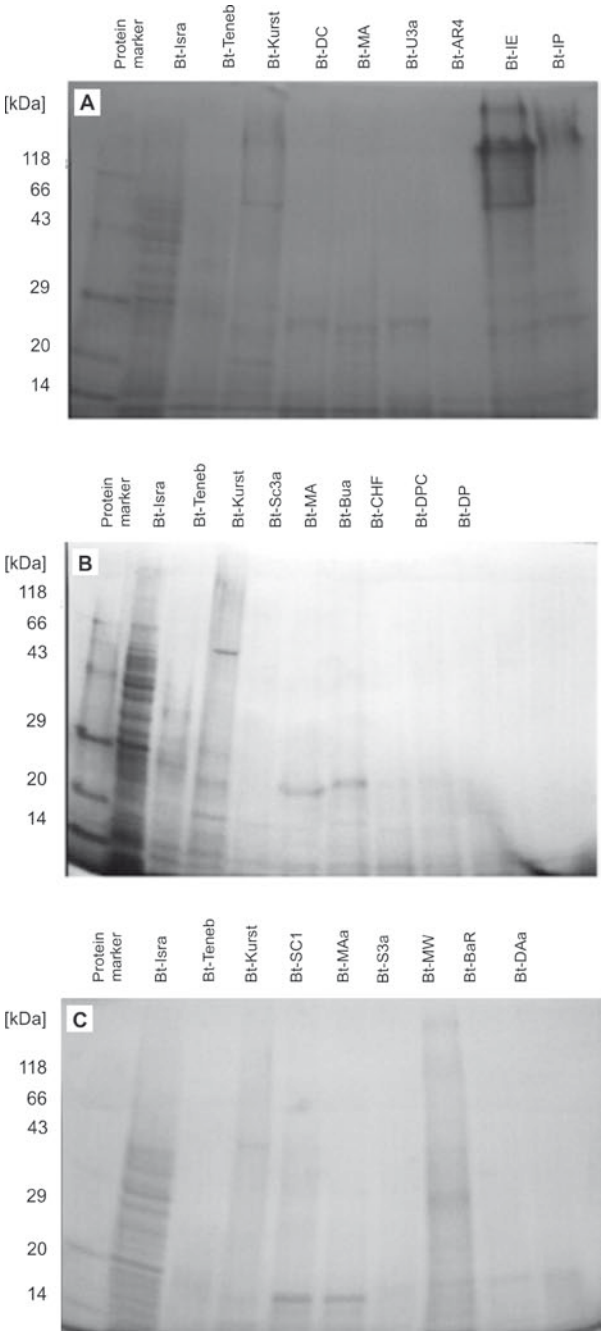

Fig. 4. Protein profiles of whole cellular proteins of *Bacillus thuringiensis* strains: Bt-Isra, Bt-Teneb and Bt-Kurst are the reference strains. A – high, B – medium, C – low virulent group

Discussion

In the present study, native strains of *B. thuringiensis* were isolated from soil samples and insect cadavers. A total of 155 *B. thuringiensis* isolates were confirmed according to Travers *et al.* (1987), bacterial shape, gram staining, the catalase test and the presence of spores and crystals. All isolates were rod shaped, Gram-positive, catalase positive and endospores and parasporal bodies were clearly observed inside all bacterium during the sporulation phase. The autolysis phase of bacterial cells occurred 68 to 92 h after inoculation. This shows a similar sporulation time as Renganathan *et al.* (2011) and the standard Bt strain which is approximately 92 h (Bulla *et al.* 1980). The bioassay results revealed that Bt isolates differed in their insecticidal activity against second instar larvae of *P. xylostella*. The slight difference of toxicity between strains could be due to the spore crystal ratio, different Cry protein content and activity of trypsin (Oppert *et al.* 2010). The results of the bioassay of 68 isolates led to the introduction of nine isolates that are highly toxic to *P. xylostella* larvae causing 100% to 78.3% mortality and will be valuable as insecticidal agents. These nine strains originated from different sources: insects, agricultural fields, forests and fruit gardens (Table 1).

In this study the differences among Bt isolates determined according to parasporal crystals morphology, spectrophotometry to reveal protein concentration and SDS-PAGE to specify the presence of proteins in spores and crystal mixtures. Bt strains produce parasporal inclusions with different morphologies, sizes and numbers. On the basis of microscopic observations, parasporal crystals were classified into four morphological classes: bipyramid, cubical, polyhedron (triangular) and round crystals. Diverse crystal inclusion morphologies were identified among 376 isolates that Vidal-Quist *et al.* (2009) studied: bipyramid, round, adhered to spore, small and irregular. It has been shown that some distinct morphologies are apparent; bipyramidal crystals related to Cry1 proteins, cuboidal inclusions related to Cry2 proteins, flat and square

crystals related to Cry3 proteins. Crystal morphology of Bt can provide valuable information on target insect spectra (Hernstand *et al.* 1986; Ohba and Aizawai 1986a; Asokan *et al.* 2013). In this study, bipyramid crystals were observed in isolates with the highest toxicity against second instar larvae of *P. xylostella*. Quantitative protein analysis indicated that Bt isolates differed in their amount of protein and it is noteworthy that there is a correlation between the amount of protein and the insecticidal activity of isolates. These results are similar to Schnepf *et al.* (1998) who reported that the entomopathogenic properties of Bt strains are attributed largely to the parasporal crystals, which contain one or more insecticidal protoxins produced during bacterial sporulation. Also, Maeda *et al.* (2000) believed that there is a relationship between toxic activity and the crystal shape of Bt strains. The total protein extracts of the 21 isolated strains revealed protein bands ranging from 134.8 kDa to 12.3 kDa, suggesting variable biological activity and specificity for different insect pests. The 130–140 kDa proteins are the usual bands produced by lepidopteran-active strains of Bt producing bipyramidal crystals comprised of Cry1 proteins (van Frankenhuyzen 2009). Bt-IP and Bt-IE isolates displayed similar results. Hongyu *et al.* (2000b) found five protein profiles through characterization of Bt strains from Chinese stored products. Quezada-Moraga *et al.* (2004) reported 65 different protein profiles among 109 native Bt strains. Hernandez-Fernandez *et al.* (2010) introduced 18 different profiles based on the presence of four crystal forms. There is a probable relationship between molecular weight and Cry proteins (Aronson *et al.* 1986; Crickmore *et al.* 2010). Federici *et al.* (2006) reported that the Bt strains are recognized as types of crystal proteins. Cry proteins are generally either 60–80 kDa or 130–150 kDa. Cyt proteins are approximately 28 kDa. The most common pattern of protein profiles in Swamy *et al.* (2013) isolates was composed of proteins with molecular weights between 60–135 kDa. The Lip strain that El Khoury *et al.* (2014) studied, produced bipyramidal and cubic crystalline inclusions suggesting the presence of Cry1A (135 kDa) and Cry2A (70 kDa) proteins. SDS-PAGE separation of crystal proteins also showed two significant bands with weights of 135 and 65 kDa. Morphology and genetic composition of Bt is highly variable among different types of soils and places. Bt composition seems to be influenced by several factors including soil humidity, organic matter, temperature, structure and pH, micro/macro-nutrients, richness and local insect distribution (Uribe 2004).

Further investigation is needed to characterize the *B. thuringiensis* isolates from other habitats such as stored products. Hongyu *et al.* (2000a) and Bernhard *et al.* (1997) reported that Bt is more abundant in the environments of stored products than in soil, dust and leaf surfaces, so that new isolates of Bt could be identified and used as an effective insect pathogen against pests of different orders of insects. Observed SDS-PAGE profiles with corresponding gene profiles via PCR should be compared.

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