Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite–humic acids–Al hydroxypolymers

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Abstract

The equilibrium adsorption and binding of the active toxin from *Bacillus thuringiensis* subsp. *kurstaki* on complexes of montmorillonite–humic acids–Al hydroxypolymers, as well as the biodegradation and the insecticidal activity of the bound toxin, were studied. Seventy percent of the total adsorption occurred within the first hour, and maximal adsorption occurred in 8 h. Adsorption of the toxin on a constant amount of the complexes increased as the amount of the toxin added increased, and equilibrium adsorption isotherms of the L-type were obtained. There was essentially no desorption of the toxin after extensive washing of the toxin–organomineral complexes with double distilled H2O and 1 M NaCl. The bound toxin was resistant to utilization by mixed microbial cultures from soil and to enzymatic degradation by Pronase E. Free and bound toxin were active against the larvae of *Manduca sexta*; the bound toxin retained the same activity after exposure to microbes or Pronase, whereas the toxicity of the free toxin decreased significantly. The results of these studies indicate that the release of transgenic plants and microorganisms expressing truncated genes that encode active insecticidal toxins from *B. thuringiensis* could result in the accumulation of these toxins in soil as a consequence of binding on surface-active soil particles. This persistence could pose a hazard to nontarget organisms, enhance the selection of toxin-resistant target species, and increase the control of target insect pests. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Bacillus thuringiensis*; Organomineral complexes; Insecticidal toxins; Binding; Insect bioassay; Biodegradation

1. Introduction

*Bacillus thuringiensis* is a gram-positive, spore-forming bacterium that forms parasporal crystal proteins during sporulation in the stationary phase of growth. These proteins, which are released with the endospore upon lysis of the sporangium, exhibit specific toxicities to insects, many of which are economically important crop pests. The primary site of action of the insecticidal toxins of various subspecies of *B. thuringiensis* is the brush border membranes of the midgut epithelium of susceptible larvae of Lepidoptera, Coleoptera, and Diptera (Bravo et al., 1992; Denolf et al., 1993). The nontoxic, parasporal, crystalline inclusions (protoxins) are solubilized after ingestion by sensitive larvae in the alkaline midgut (pH > 10) and proteolytically activated into toxins by specific proteases (Höfte and Whiteley, 1989). The active toxins interact with receptors on midgut epithelial cells, where the toxins form pores and destroy cells by colloidal osmotic lysis (Wolfersberger, 1990; Adang, 1991). Larvae of nontarget insects also contain receptors, but apparently in lower numbers (Höfte and Whiteley, 1989; Van Rie et al., 1990a), although in some cases, also in high numbers (Wolfersberger, 1990; Garcezynski et al., 1991).

Bioinsecticides, based on a mixture of cells, spores, and parasporal crystals of *B. thuringiensis*, have been used since the early 1960’s for the control of various insect species (Beegle and Yamamoto, 1992; Entwistle et al., 1993) and, more recently, against nematodes, mites, and protozoa (Feitelson, 1993; Feitelson et al., 1992). *B. thuringiensis* is a useful alternative or supplement to synthetic chemical pesticides in agriculture, forest management, and mosquito control. It is also the major source of genes for the expression of pest resistance in transgenic plants. Truncated forms of the genes that code for these toxins have been genetically engineered into plants that express the active toxins rather
than the inactive protoxins. Because a high gut pH and specific proteases are not necessary to activate the toxins, beneficial nontarget insects, as well as organisms at higher and lower trophic levels, could be harmed (Flexner et al., 1986; Goldberg and Tjaden, 1990; Addison, 1993; Johnson et al., 1995). Moreover, after commercially usable portions of transgenic plants have been harvested, the remainder of the plant biomass containing the toxins is usually incorporated into soil. The toxin expressed in transgenic corn is also released in pollen (Losey et al., 1999) and in root exudates (Saxena et al., 1999; Saxena and Stotzky, 2000).

These insecticidal proteins may be inactivated or removed from the soil environment by (i) consumption by insect larvae, (ii) degradation and mineralization by microorganisms, or (iii) sunlight. However, the toxins have been shown to bind rapidly on clay minerals (e.g., montmorillonite and kaolinite), humic acids from different soils, and the clay-size, but not the silt- and sand-size, fraction from soil. The structure of the toxins did not appear to be modified as a result of binding. The bound toxins retained, and in some cases had increased, insecticidal activity to target larvae (Tapp et al., 1994; Tapp and Stotzky, 1995b; Crecchio and Stotzky, 1998). The toxins persisted and retained insecticidal activity in nonsterile soils for at least 8 months (Tapp and Stotzky, 1995a, 1997, 1998; Palm et al., 1996; Koskella and Stotzky, 1997). Hence, the levels of active toxins in soil could be greater and be present longer than those introduced by conventional applications of commercial preparations of the protoxins. This could elicit and enhance the selection of toxin-resistant target organisms (Van Rie et al., 1990a,b; Tabashnik et al., 1990, 1994; Ferre et al., 1991; McGaughey and Whalon, 1992; Heckel, 1994; Tabashnik, 1994; Alstad and Andow, 1995), constitute a hazard to nontarget organisms (Addison, 1993; Charbonneau et al., 1994; Johnson et al., 1995), and increase the control of target organisms.

Although the persistence and insecticidal activity of the toxins bound either on clays or humic acids have been studied to some extent (Stotzky, 2000), the interactions of insecticidal toxins with organomineral complexes and the effect of such interactions on the persistence and bioactivity of the toxins have not been studied. Soil structure can be defined as an arrangement of mineral soil particles and organic compounds that form aggregates of different size and stability (Tisdall and Oades, 1982). Clay minerals in soil are partially or wholly covered with organic materials, among which humic substances resistant to biodegradation are the most important and prevalent. Monomers, polymers, and noncrystalline precipitation products of aluminum facilitate the adsorption of organic matter on clay surfaces (Violante and Huang, 1985). Organic compounds can also be present within the interlayers of expandable clay minerals (Stotzky, 1986; Theng et al., 1986).

To understand better the role of surface-active particles in the persistence of the insecticidal toxins in soil, organomineral complexes, which are presumably more representative of soil colloids than purified humic acids or clay minerals, and consisting of montmorillonite–humic acids–Al hydroxypolymers, were prepared. The adsorption and binding of the active toxin from B. thuringiensis subsp. kurstaki on the organomineral complexes, the insecticidal activity of the bound toxin to the larvae of the tobacco hornworm (Manduca sexta), and the resistance of the bound toxin to degradation by soil microorganisms and a commercial protease were investigated.

2. Materials and methods

2.1. Purification of the toxin from Bacillus thuringiensis subsp. kurstaki

Dipel technical powder (Abbott Laboratories) was washed twice with 1 M NaCl and twice with double distilled water (ddH2O) with vigorous shaking in a separatory funnel, the foam was discarded, and the suspension was centrifuged at 5900 g for 15 min. The pellet was extracted overnight with MOPS buffer (0.1 M 3-N-morpholinopropane–sulfonic acid, pH7.8) containing 1 M KSCN and 0.5 M dithiothreitol. After centrifugation at 5900 g for 15 min, the supernatant was dialyzed for 6–8 h, with hourly changes, against ddH2O. The toxin was precipitated with 17.5 g of (NH4)2SO4 100 ml⁻¹ of dialyze. After 2–3 h, the toxin was centrifuged at 16,000 g for 15 min, dialyzed against ddH2O, and lyophilized (Venkateswerlu and Stotzky, 1990; Tapp et al., 1994; Crecchio and Stotzky, 1998). The molecular mass and the purity of the preparations were assayed by SDS–PAGE and Western blot ELISA.

2.2. Preparation of organomineral complexes

The <0.2-μm fraction of a montmorillonite (Swy-1, Crook County, Wyoming) was separated by centrifugation (Jackson, 1979). The clay was saturated with Na (1 M NaCl), and the excess Cl⁻ was removed by repeated washings with ddH2O, followed by dialysis, until a negative test for Cl⁻ (AgNO₃) was obtained.

Humic acids were extracted from a forest soil with a mixture of 0.5 M NaOH and 0.1 M Na₂HPO₄ under a N₂ atmosphere for 24 h with vigorous shaking (10 l of solution kg⁻¹ of soil). The suspension was centrifuged at 6000 g for 20 min, the sediment was re-extracted overnight, and the pooled supernatants were precipitated with 12 M HCl (Schnitzer, 1978). The humic acids were centrifuged at 8000 g for 20 min, the pellet was re-extracted, re-precipitated, and treated with a mixture of 0.1 M HCl and 0.1 M HF (Piccolo, 1988), dialyzed against ddH₂O, and lyophilized.

Al(NO₃)₃ (0.3 mmol) was titrated (0.5 ml min⁻¹) with 0.25 M NaOH to pH5.0, and either 1 g of montmorillonite and 50 or 25 mg of humic acids were added (samples C1 and C2, respectively, in Table 1) or 25 mg of humic acids and, after 2 h, 1 g of montmorillonite was added (sample C3 in Table 1). The pH of the suspensions was raised to 7.0 with 0.25 M NaOH; the suspensions were diluted to 11 with
ddH₂O, shaken for 24 h, and centrifuged at 3000 g for 15 min; and the organomineral complexes were washed twice with ddH₂O, ultrasonically dispersed, and lyophilized.

2.3. Characterization of the organomineral complexes

Cation-exchange capacity (CEC) was determined by washing 100 mg of sample in 50 ml of 0.4 M BaCl₂ in the presence of triethanolamine buffer, pH 8.0. Barium was exchanged with magnesium (0.05 M MgSO₄ 7H₂O), and the amount of residual magnesium was determined by titration with 0.005 M EDTA (SISS, 1985). Organic carbon (OC) was determined with an EA 1108 CHN Analyzer (Fisons Instrument, Lucino di Rodano, Italy). Specific surface area (SSA) was determined by adsorption of ddH₂O at 20% relative humidity obtained with ammonium acetate, assuming that the weight of water required to form a monolayer on a square meter of surface is 2.78 £ 10⁻² g (Quirk, 1955). Powder X-ray diffractograms of oriented specimens were obtained with a Rigaku Geigerflex D/Max IIIC X-ray diffractometer using Fe-filtered Co–Kα radiation generated at 40 kV and 30 mA at a scanning speed of 5° 2θ min⁻¹. Samples were stored for 24 h at 20°C in a desiccator containing CaCl₂ before XRD analysis.

Some characteristics of the organomineral complexes are presented in Table 1.

2.4. Adsorption and binding of the toxin

The lyophilized toxin was dissolved in 0.1 M phosphate buffer (pH6.0), and any insoluble material was discarded after centrifugation at 16,000 g for 20 min. The protein concentration of solutions of the toxin was determined by the Lowry method (Lowry et al., 1951). The toxin (50–500 µg of protein) was added to suspensions of each of the organomineral complexes in ddH₂O (1–10 mg) and brought to a total volume of 1 ml with phosphate buffer (pH5–10). The mixtures were rotated at 40 rev min⁻¹ on a motorized wheel at 24 ± 2°C for 1–16 h. After adsorption, the suspensions were centrifuged at 16,000 g for 20 min and the toxin concentration in the supernatants determined by the Lowry method. The difference between the amount of the toxin added and the amount of toxin detected in the supernatant was used to calculate the amount of toxin adsorbed at equilibrium. No significant amounts of toxin adsorbed on the walls of the test tubes, as determined with control tubes not containing organomineral complexes. Amounts adsorbed vs equilibrium concentrations were plotted to obtain equilibrium adsorption isotherms (Crecchio and Stotzky, 1998).

After equilibrium adsorption, the organomineral–toxin complexes were washed five times with ddH₂O (5 min each washing by rotating the suspension on a motorized wheel) and once with 1 M NaCl (3 h). After centrifugation, the supernatants were assayed by (i) the Lowry method to determine the amount of the toxin bound, (ii) SDS–PAGE to determine whether contact with the organomineral
complexes modified the structure of the toxin, and (iii) insect bioassay for larvicidal activity (Crecchio and Stotzky, 1998).

2.5. Bioutilization of free and bound toxin

The utilization of the free and bound toxin as a source of carbon and energy was determined with a mixed microbial culture from soil that was either used immediately or grown in a protein-enriched medium before being used. Soil (100 g), freshly collected, was suspended in 500 ml of Davis Citrate Minimal Medium (DCMM; 7 g K2HPO4, 2 g KH2PO4, 40 mg sodium citrate, 100 mg MgSO4 7H2O, and 500 mg NH4NO3 in 1 l of ddH2O, pH7.2), centrifuged at 2000 g for 15 min to sediment the soil, the cells in the supernatant were sedimented at 27,000 g for 15 min, and the pellet of cells was resuspended in 5 ml of DCMM and immediately used. Alternatively, 10 g of freshly collected soil was shaken overnight at 28°C in 50 ml of DCMM containing 1 mg ml⁻¹ each of bovine serum albumin, pepsin, and toxin from B. thuringiensis subsp. kurstaki, and 5 ml was transferred to 45 ml of fresh medium and again shaken overnight at 28°C. This procedure was repeated three times. After centrifugation at 27,000 g for 15 min, the cells were washed and resuspended in DCMM to an optical density at 420 nm (OD420) of 1.0–2.0.

In both cases, the mixed microbial culture (diluted in 3.5 ml of DCMM to 0.1–0.2 OD420) containing either (i) 500 μg glucose ml⁻¹, (ii) 200 μg free toxin ml⁻¹, (iii) 10 mg organomineral complex ml⁻¹, (iv) 10 mg organomineral complex plus 500 μg glucose ml⁻¹, (v) 200 μg toxin bound on 10 mg organomineral complex ml⁻¹, or (vi) 200 μg free toxin plus 10 mg organomineral complex ml⁻¹ was rotated at 90 rev min⁻¹ at 28°C. Changes in OD420 were measured hourly for the first 5 h and then at longer intervals (Crecchio and Stotzky, 1998).

2.6. Insect bioassays

Insecticidal activity was determined with the larvae of the tobacco hornworm (Manduca sexta). Dilutions (100 μl) of the free toxin or equivalent amounts of the toxin bound on the organomineral complexes, before and after exposure to either (i) a mixed microbial culture grown for 4 days on the protein-enriched medium, (ii) Pronase E (Sigma) (1 mg ml⁻¹ phosphate buffer, pH7.8, 24 h at 25°C), or (iii) bound Pronase E (1 mg Pronase E bound on 4 mg organomineral complex ml⁻¹ of phosphate buffer, pH7.8, 24 h at 25°C), were pipetted onto 5 ml of solidified tobacco hornworm medium (Carolina Biological Co., Burlington, NC) in vials (3 cm diameter × 6 cm height) and allowed to dry. Four second-instar larvae of M. sexta hatched from eggs (Carolina Biological Co.) were placed into triplicate vials and incubated at 28 ± 2°C under a 40 W lamp for 7 days (Tapp and Stotzky, 1995b; Crecchio and Stotzky, 1998). Mortality responses were analyzed by the PC-POLO probit procedure (LeOra software, 1987) (Russell et al., 1977), and the lethal concentration to kill 50% of the larvae (LC50) and 95% confidence intervals (CI) were calculated. The relative potency (RP) of the bound toxin was determined: the LC50 of the free toxin was divided by the LC50 of the bound toxin of the same treatment. An RP of 1.0 indicates no difference in potency between free and bound toxin; an RP > 1.0 indicates a greater potency of the bound toxin; and an RP < 1.0 indicates a lower potency of the bound toxin.

2.7. Statistics

All experiments were conducted in triplicate. The data are presented as the means ± the standard error of the means (x ± SEM). When not indicated, the SEMs were within the dimensions of the symbols.

3. Results and discussion

3.1. Adsorption and binding of the toxin

Approximately 70% of the total adsorption of the toxin occurred within the first hour of contact. Maximal adsorption occurred in < 8 h, and longer contact times did not result in a decrease in the amounts of toxin adsorbed, indicating insignificant microbial growth in the suspensions and stability of the protein (Fig. 1).

Adsorption of the toxin on all complexes was greatest between pH 5 and 6 and generally decreased as the pH of the suspensions increased (Fig. 2). Values of pH lower than 5.0 were not studied, as the solubility of the toxin decreased and precipitation occurred. No significant difference among the complexes was observed when adsorption was highest whereas some differences, which were not consistent and the reasons for which are not known, were observed at pH values ≥ 7.0. The isoelectric point (pI) of the toxin is pH 5.5 (Bietlot et al., 1989). At pH values near the pI, a net neutral protein will encounter minimal repulsive forces, which

![Fig. 1. Effect of contact time on the adsorption of the toxin (100 μg) on the three organomineral complexes (10 mg) at pH7.0. Means ± SEM, which are indicated when not within the dimensions of the symbols. See text for details.](image-url)
results in maximal collisions with charged organomineral complexes and, hence, in increased adsorption.

Adsorption of the toxin on a constant amount of the organomineral complexes increased with an increase in the amount of toxin added (Fig. 3). The equilibrium adsorption isotherms were of the L-type (Giles et al., 1960), with none showing a plateau with the concentrations of toxin added, and there were no significant differences in maximal adsorption.

Almost no desorption (<2% of added toxin) was observed after five washings with ddH₂O (data not shown). No further desorption occurred after more stringent washing for 3 h with 1 M NaCl, confirming that essentially all the added toxin was bound on the organomineral complexes. This indicated unusually strong binding of a protein on soil particles: e.g., significant, although variable, amounts of the toxin and other proteins were desorbed from humic acids (Crecchio and Stotzky, 1998) and from montmorillonite and kaolinite homoionic to various monomeric cations of different valence or coated with polymeric oxyhydroxides of Fe (Fusi et al., 1989; Tapp et al., 1994; Vettori et al., 1999). Similar strong binding of DNA on these organomineral complexes has been observed (unpublished data). In these studies, organomineral complexes that were presumably more similar to natural soil particles than purified clays and humic acids alone were used. Further studies are necessary to understand which components of organomineral complexes and the type of interactions among them were responsible for such strong binding when compared with clays and humic acids alone. Despite the low amounts of toxin that were desorbed from the complexes, the structure and size of the toxin were not significantly modified as a result of their adsorption, as indicated by the same electrophoretic mobility (SDS–PAGE) and insecticidal activity of the desorbed toxin as of the free toxin that had not been in contact with the complexes (data not shown).

The adsorption and binding data indicated that, at least under the experimental conditions used, the amounts of humic acids added, the modality of preparation, and the differences in specific surface, cation-exchange capacity, and expansibility of the three complexes (Table 1) did not significantly affect adsorption. The high affinity of the toxin for the organomineral complexes, as indicated by the rapidity of adsorption, the broad range of pH values at which adsorption occurred, the lack of saturation in the equilibrium adsorption isotherms, and the small amount of toxin desorbed, was apparently more important than the differences among the complexes. In contrast, the adsorption and binding of the toxin on pure humic acids and clay minerals appeared to be more dependent on their
physicochemical characteristics (Tapp et al., 1994; Crecchio and Stotzky, 1998).

3.2. Bioutilization of free and bound toxin

Regardless of whether the mixed microbial cultures from soil were used immediately, to mimic the natural composition of the microbial community in soil, or after being grown in a protein-enriched medium, to enhance the selection of proteolytic microorganisms, the free toxin was readily utilized as a source of carbon and energy and supported the growth of the microbial cultures. In contrast, the bound toxin was resistant to utilization and did not support the growth of the microorganisms (Table 2 and Fig. 4). Controls, consisting of organomineral complexes alone or supplemented with glucose or free toxin, indicated that the organomineral complexes did not support microbial growth and were not toxic to microorganisms and that the lack of growth on the bound toxin was the result only of its low availability. The decrease in OD in the treatment with C1 and free toxin (Fig. 4) may have resulted from the binding of the toxin on the organomineral complexes during the growth period, as has also been observed with the toxin and humic substances alone (Crecchio and Stotzky, 1998). The bound toxin may have been protected more from microbial utilization by the inaccessibility of the terminal amino acids residues necessary for initiation of cleavage of the protein by peptidases than by intercalating the swelling 2:1-layer clay (Stotzky, 1986; Tapp et al., 1994).

3.3. Insect bioassays

Both the free and bound toxin was toxic to the larvae of M. sexta (Table 3). The LC50 values were lower, albeit within the same order of magnitude, for the bound than for the free toxin when not exposed to microbes or Pronase E. The higher insecticidal activity of the bound toxin may have been a result of the toxin being concentrated by binding on the complexes, and consequently, more toxin was ingested by the larvae than when the free toxin was uniformly spread over the surface of the food medium (Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998).

The toxin bound on organomineral complexes retained essentially the same insecticidal activity after exposure to microbes or Pronase E, whereas the LC50 of the free toxin increased significantly (i.e., less mortality), indicating that binding of the toxin on the complexes protected it from being inactivated (Table 3). Neither the organomineral complexes alone nor the microbes were toxic to the larvae, and bound Pronase retained a significant capacity for degrading free toxin when compared with the proteolytic activity of free Pronase (Table 3).

The ability of the bound toxin to kill larvae may have been a result of the higher affinity of receptors on the epithelium of the larval gut for the toxin than the lower affinity, because of nonspecific binding of the toxin for the organomineral complexes. The movement of the toxin from the organominerals to specific receptors was probably a result of the fact that the binding of proteins on soil colloids, including humic substances, is primarily by H-bonds, and

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<tr>
<th>Component</th>
<th>Composition of the incubation mixtures</th>
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<tr>
<td>Free toxin</td>
<td>– – – + + + – –</td>
</tr>
<tr>
<td>Glucose</td>
<td>+ – + – + – + +</td>
</tr>
<tr>
<td>Complex</td>
<td>– – + – – + – –</td>
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<tr>
<td>Bound toxin</td>
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* + = Component present; – = component absent. Each vertical column describes the composition of the mixture and whether or not the mixture supported growth of the culture.

![Fig. 4. Utilization of the toxin, free or bound on organomineral complex C1, by a mixed microbial culture from soil grown in a protein-enriched medium. Means ± SEM, which are indicated when not within the dimensions of the symbols. Similar results were obtained with organomineral complexes C2 and C3. See text for details.](image-url)
segments of bound proteins are constantly detaching and reattaching (Stotzky, 1986).

4. Conclusions

The results of the present study show that the toxin from B. thuringiensis subsp. kurstaki binds rapidly and strongly on complexes of montmorillonite–humic acids–Al hydroxypolymers. Although it has already been shown that the toxins from B. thuringiensis bind to organic (humic acids) and inorganic (clays) soil particles (Tapp et al., 1994; Tapp and Stotzky, 1995b; Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998), this is the first study in which organo-mineral complexes have been used. As organo-mineral complexes are presumably more representative of natural soils than either clays and humic substances alone, they can possibly substitute for whole soil to predict the fate of biomolecules (i.e., proteins, DNA) in the soil environment. In this way, many difficulties that can arise from the use of intact soil such as interference of dissolved organic matter with colorimetric assays, changes in soil structure as a result of sterilization by autoclaving, and inhomogeneous interactions of biomolecules with soil surfaces, can be minimized. Furthermore, by comparing, under laboratory conditions, adsorption and desorption processes, the contribution of different surface-active soil particles can be evaluated, as can the effects of differences in organic carbon content, cation-exchange capacity, specific external surface area, and expandibility of the clays on the adsorption process in terms of maximal amounts adsorbed, optimal pH, and mechanisms of adsorption. In the present study, differences in these characteristics between the three complexes did not appear to affect significantly the adsorption process.

As a result of binding, the toxin became resistant to degradation by soil microbes and by a commercial protease, but it retained insecticidal activity. The toxin, especially when expressed in transgenic biomass and subsequently released in an active form, may accumulate in soil in a form resistant to rapid inactivation (Saxena et al., 1999; Saxena and Stotzky, 2000; Stotzky, 2000). This may enhance the development of resistance to the toxin in target insects, pose a potential hazard to nontarget organisms, and increase the control of target insect pests. These results support the general concept that the risks, as well as the benefits, of releasing genetically modified organisms to the environment must be properly evaluated in studies in the laboratory as well as in the field.

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