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ORIGINAL ARTICLE

Degradation of the insecticidal toxin produced by *Bacillus thuringiensis* var. *kurstaki* by extracellular proteases produced by *Chrysosporium* sp.

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bioassays, Cry1Ac toxin, degradation, insecticidal activity, proteases.

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Abstract

Aims: Some Cry proteins produced by the soil bacterium *Bacillus thuringiensis* (*Bt*) or by transgenic *Bt* plants persist in agricultural soils for an extended period of time, which may pose a hazard for nontarget soil organisms. The aims of our study were to screen for soil fungi capable of degrading the Cry1Ac toxin and to identify the mechanisms that lead to the inactivation of this protein.

Methods and Results: Of the eight fungal strains screened, only one, *Chrysosporium* sp., was found to produce extracellular proteases capable of degrading the 66-kDa Cry1Ac at the *N*-terminal end of amino acid 125 (alanine). The proteolytic products of the Cry1Ac toxin did not exhibit any insecticidal activity against *Helicoverpa armigera*, in contrast to its high toxicity exhibited in the native form.

Conclusions: Proteases elaborated by the *Chrysosporium* sp. degrade the Cry1Ac toxin in a way that it looses its insecticidal activity against *H. armigera*.

Significance and Impact of the Study: *Chrysosporium* sp., a specific soil microorganism capable of producing proteases that degrade the Cry1Ac toxin into inactive products under controlled conditions is being reported for the first time. Application of this observation needs to be further tested in field conditions.

Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive spore-forming bacterium that forms crystalline protein inclusions during growth. These inclusions consist of proteins with fairly specific insecticidal activities (Aronson *et al.* 1986; Whiteley and Schnepf 1986) against the larvae of many insect species of Lepidoptera, Coleoptera, and Diptera (Beegle and Yamamoto 1992). Some strains of *Bt* are also active against other insect orders, such as Hymenoptera, Homoptera, Orthoptera, and Mallophaga, as well as against some nematodes, mites, and protozoa (Feitelson *et al.* 1992; Feitelson 1993). Among the *Bt* endotoxins, that are active against Lepidoptera, the major component is a 130–140-kDa nontoxic protoxin that is converted to a 60–70-kDa active toxin in the larval midgut by the action of protease(s) (Tojo and Aizawa 1983). Exogenous proteases, such as trypsin, chymotrypsin, and papain, also produce an active toxin from the *Bt* protoxin (Johnston *et al.* 1995). Even proteases of *Bt* itself produced during sporulation convert the protoxin into toxin (Suresh and Venkateswerlu 1998b; Rukmini *et al.* 2000).

Modified, truncated versions of *cry* genes from *Bt* strains have been introduced into the genome of various crops, which enable them to produce Cry proteins throughout their life cycle. Cry proteins produced by the transgenic plants are released into the soil through root exudates of *Bt* corn (Cry1Ab protein), *Bt* potato (Cry3A protein), *Bt* cotton (Cry1Ac and Cry2Ab proteins), and *Bt* rice (Cry1Ab protein) but not of *Bt* canola, and *Bt* tobacco (Cry1Ac protein) (Saxena *et al.* 2002; Stotzky 2004; Knox *et al.* 2007). Dead plant material during plant

growth, and crop residue left on the field after harvest are other sources of *Bt* toxin input into the soil environment (Zwahlen *et al.* 2003; Baumgarte and Tebbe 2005). Therefore, nontarget organisms associated with the soil ecosystem might be exposed to *Bt* toxins from these various sources during an extended period of time and might be indirectly or directly affected by them (Wandeler *et al.* 2002; Baumgarte and Tebbe 2005; Castaldini *et al.* 2005; Harwood *et al.* 2005; Pont and Nentwig 2005; Zwahlen and Andow 2005; Harwood and Obrycki 2006).

The insecticidal toxins (CryI and CryIII) can remain active in soil for at least 234 days, as they bind on clay particles and humic acids (Venkateswerlu and Stotzky 1992; Tapp *et al.* 1994; Tapp and Stotzky 1995, 1998; Crecchio and Stotzky 1998, 2001). Transgenic plant *Btk* toxin persists for several weeks or months in soil (Palm *et al.* 1996). Therefore, it is important to identify plant residue-colonizing micro-organisms that can degrade active *Bt* toxins released into the environment in exudates of transgenic *Bt* plants and their biomass.

In our study, we focus on the degradation of the Cry1Ac from the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 strain by soil fungi. This strain contains a single crystal protein gene *Cry1Ac* that produces the Cry1Ac, which is toxic to lepidopteran species (LC_{50} value of 1·8 μg ml^{-1} diet for *Helicoverpa armigera*). The *cry1Ac* gene has been expressed in several crop plants, of which *Bt* cotton and *Bt* corn have been released for cultivation in several countries (Sharma *et al.* 2004). Therefore, it is important to understand the fate of *Bt* toxins in the soil, and to identify enzymes that are effective in degrading *Bt* toxins and minimize the adverse effects of these toxins and the degraded products on nontarget soil organisms.

Therefore the present studies were undertaken to: (i) screen a few selected soil fungi for their ability to degrade the Cry1Ac toxin under controlled conditions; (ii) evaluate the toxicity of the Cry1Ac toxin degraded by proteases to the lepidopteran insect, *H. armigera* and (iii) sequence the degraded Cry1Ac product to find out the site of cleavage by the protease. In this communication, we report, for the first time, a protease system secreted by a soil micro-organism, *Chrysosporium* sp., with the ability to degrade the Cry1Ac toxic protein into an innocuous product.

Materials and methods

Culture conditions

Bacillus thuringiensis subsp. *kurstaki* HD-73 (BGSC # 4D4) was obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH, USA). The bacterium was cultured in a liquid medium (1% tryptose, 0·3% beef extract, 0·5% NaCl, 5 mmol l^{-1} $MgSO_4$, and 20 μmol l^{-1} $MnCl_2$; pH 7·2) for 5 days at 28°C and was shaken continuously (150 rev min^{-1}; LEAD-Orbital shaker, Bangalore, India).

Isolation of the Cry1Ac toxin

The sporulated culture was harvested by centrifugation at 4800 ***g*** for 20 min in a Remi C-23 centrifuge (Mumbai, India) at 4°C. The biomass was washed thrice with 1 mol l^{-1} NaCl, followed by several washes with distilled water, and the biomass was lyophilized (FTS Systems, Dura-Dry μP, New York, USA). The material was stored at −20°C until further use.

The Cry1Ac toxin was isolated from the biomass according to the method described by Venkateswerlu and Stotzky (1990). The lyophilized material (2 g) was suspended in 15 ml of 0·1 mol l^{-1} MOPS buffer (3-*N*-morpholino propane sulfonic acid), pH 7·8, containing 1 mol l^{-1} KSCN (potassium thiocyanate) and 0·5 mol l^{-1} dithiothreitol. The suspension was gently agitated overnight in a shaking water bath (Julabo-SW21; Labortechnik GmbH, Seelbach, Germany) at 40°C, the contents centrifuged (10 000 ***g*** for 30 min at 4°C; rotor # 12159; Sigma 3k 30, Osterode am Harz, Germany), and the supernatant obtained after centrifugation was dialysed extensively against distilled water at 4°C. The precipitated protein after dialysis was recovered by centrifugation (12 000 ***g*** for 30 min at 4°C), lyophilized and stored at −20°C.

Screening of fungal cultures for proteases acting on the Cry1Ac toxin

For screening the soil fungal proteases, the soil fungi, *Neurospora crassa* (FGSC 4200 'a'), *Aspergillus flavus* (MTCC 873), *Aspergillus fumigatus* (MTCC 4330), *Aspergillus niger* (MTCC 4285), *Trichoderma viridae* (MTCC 4329), *Mucor racemosus* (OUFH 187), *Paecilomyces variotii* (OUFH 197) (all obtained from the Department of Botany, Osmania University, Hyderabad, India) and *Chrysosporium* sp. (isolated by Prof G. Venkateswerlu, Department of Biochemistry, Osmania University, Hyderabad and identified with the help of the International Mycological Institute, UK), were chosen for the study.

The fungal cultures were grown in Sabourauds medium, pH 5·6 (1% peptone and 4% dextrose), in five 250-ml conical flasks containing 50 ml of the medium at 28°C (150 rev min^{-1}; LEAD-Orbital shaker, Bangalore, India) for 5 days. *Chrysosporium* sp. was cultured in modified Sabourauds medium containing 1·5% chitin at 28°C

(150 rev min^{-1}; LEAD-Orbital shaker) for 7 days. Three replicates (batches) of each fungal strain were used for studying the effect of proteases isolated from these strains on the Cry1Ac toxin.

In addition three bacterial strains (*Bacillus subtilis*, *Bacillus megalotherium*, and *Agrobacterium* sp.) were also screened.

Isolation of extracellular proteases from fungal strains

The individual culture fluids, after growth, were filtered through a Büchner funnel, and the resulting filtrate was left overnight at 4°C after saturation with $(NH_4)_2SO_4$ (ammonium sulfate, 80%). The precipitated protein was recovered by centrifugation (12 000 ***g*** for 20 min at 4°C; Sigma 3k 30, rotor # 12159), suspended in distilled water, and dialysed extensively against the distilled water at 4°C.

Quantification of proteases from fungal strains

In order to quantify the protease activity of the extracellular proteases isolated from eight fungal strains, an azocasein hydrolysis was carried out as described by Prestidge *et al.* (1971). The reaction mixture (1 ml) containing 20 μg of protein (extracellular proteases) and 3 mg of azocasein in 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, was incubated in a water bath (Julabo-SW21; Labortechnik GmbH) for 1 h at 30°C. The reaction was stopped with 2 ml of ice-cold 7% perchloric acid, and the protein precipitated at room temperature was removed by centrifugation (4800 ***g*** for 15 min; Remi C-23, Mumbai, India). Then 0·3 ml of 10 N NaOH was added to the supernatant, and the intensity of the colour developed was measured at 440 nm (Elico SL-150, UV-Visible Spectrophotometer, Hyderabad, Andhra Pradesh, India). One unit of protease activity was equivalent to the amount of protein that produced an increase of 1·0 unit of absorbance at 440 nm h^{-1} under the experimental conditions. Protease activity was expressed as units mg^{-1} protein.

Protein quantification

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

In vitro hydrolysis of the Cry1Ac toxin with extracellular proteases from fungal strains

In this assay, the reaction mixture, containing 100 μg of *Bt* toxin in 100 μl of 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, was incubated with 0·2 units of the extracellular proteases from different fungal species at 30°C for 1 h. After being incubated for 1 h, an equal volume of Laemmli sample buffer was added, and boiled immediately at 100°C for 5 min to denature the protein. The size of the samples then analysed using SDS-PAGE or immunoblot (see next for details). As the proteases only from the *Chrysosporium* sp. degraded the Cry1Ac, further studies were carried on the proteolysis of the Cry1Ac, using these proteases at 15, 30, 45, and 60 min, of incubations. The samples were then analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Identification of proteases

As only the *Chrysosporium* sp. could degrade the Cry1Ac toxin, further efforts were made to identify the kind of proteases, produced by this organism (no other organism tested could degrade the Cry1Ac toxin). Specific protease inhibitors were used to identify the kind of proteases produced by *Chrysosporium* sp., these were metalloprotease inhibitors (EDTA, EGTA, and 1,10-phenanthroline), serine protease inhibitors (PMSF [phenylmethylsulfonyl fluoride], leupeptin, aprotinin, chymostatin, TLCK [*N-p*-tosyl-L-lysine chloromethyl ketone], and TPCK [*N-p*-tosyl-L-phenylalanine chloromethyl ketone]), and cysteine protease inhibitors [E-64, *trans*-Epoxysuccinyl-L-leucyl-amido {4-guanidino} butane], iodoacetamide and mercuric chloride). Before adding the substrate azocasein, the *Chrysosporium* proteases were incubated with specific inhibitor for 15 min. The residual activity that remained after incubation with the inhibitor was determined by the standard azocasein assay. The protease inhibitors were obtained from Roche, Mannheim, Germany.

The effects of protease inhibitors, PMSF, chymostatin, and EDTA, on proteolysis of the Cry1Ac toxin by the proteases from *Chrysosporium* were also evaluated. The proteases from *Chrysosporium* were incubated with the inhibitors for 15 min before the addition of the Cry1Ac toxin, and then maintained at 30°C for 1 h. The products were then analysed using SDS-PAGE and immunoblot, as described next.

Electrophoresis and immunoblot

SDS-PAGE was performed according to the method of Laemmli (1970) using 10% gels and stained with Coomassie Brilliant Blue. For immunoblot, proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane by the method of Towbin *et al.* (1979). Membranes were blocked for 1 h in phosphate-buffered saline (PBS) + 5% fat-free milk powder + 0·1% Tween-20, and then incubated with rabbit anti-serum against the Cry1Ac

toxin (1 : 1000 in blocking buffer) for 1 h. Membranes were washed with PBS + 0·1% Tween-20 (PBS-T, 3 × 5 min), and then incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) (1 : 1000 in PBS-T) for 1 h. Membranes were washed with PBS-T (3 × 5 min) and then developed with NBT-BCIP (p-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate.

N-terminal amino acid sequencing

N-terminal amino acid sequencing of the proteolytic product (a 57-kDa fragment) of the Cry1Ac toxin by *Chrysosporium* proteases was carried out to identify the site of protease cleavage in the *Bt* toxin. The Cry1Ac toxin treated with proteases from *Chrysosporium* separated on 10% SDS-PAGE were electroblotted onto polyvinylidene difluoride membrane (Towbin *et al.* 1979). The blot was stained with Coomassie Brilliant Blue R-250 (0·25% in 45% methanol and 10% acetic acid), washed (2 × 30 s) in destainer I (7% methanol and 10% acetic acid), and then rinsed in destainer II (50% ethanol) until the background was completely clear. The band corresponding to the 57-kDa fragment (cleavage product of the Cry1Ac toxin) was cut and analysed for *N*-terminal amino acid sequencing on Applied Biosystems Precise 492 dual-column instrument (Foster City, CA, USA).

Bioassays

Bioassays were conducted to evaluate the biological activity of the proteolytic products of the Cry1Ac toxin generated by *Chrysosporium* proteases against the neonate larvae of *H. armigera*. The larvae of *H. armigera* were reared at 27 ± 2°C on artificial diet (300 g of chickpea flour, 4·7 g of ascorbic acid, 3 g of sorbic acid, 5 g of methoxy-4-hydroxy benzoate, 11·5 g of aureomycin, 10 ml of vitamin mix, 48 g of yeast, and 17·3 g agar l^{-1}) (Armes *et al.* 1992).

The *H. armigera* were reared on artificial diet treated with *Chrysosporium* proteases alone (8 μg 100 ml^{-1}), Cry1Ac toxin alone (50 μg 100 ml^{-1}), Cry1Ac toxin (50 μg) treated with *Chrysosporium* proteases (8 μg) at 30°C for 1 h, and the untreated control diet. There were three replicates (10 larvae in each replicate) in completely randomized design. Larval weights were recorded at day 5 after initiating the experiment.

Statistical analysis

Data from the bioassay tests were analysed with ANOVA, using GenStat software version 8.0 (VSNi, UK) to determine the significance of the differences.

Results

Effect of different fungal proteases on the Cry1Ac toxin

Although the $(NH_4)_2SO_4$ (ammonium sulfate) fractionated culture filtrates of *N. crassa, A. flavus, A. fumigatus*, *A. niger*, *T. viridae*, *M. racemosus*, and *P. variotii* showed protease activity by azocasein assay (6·8, 17·3, 5·2, 8·3, 10·0, 3·8, and 2·2 units mg^{-1} protein respectively), none of these proteases resulted in proteolysis of the Cry1Ac toxin (data not shown). Azocasein assay of the culture filtrates of *Chrysosporium* sp. showed 12 units of protease activity mg^{-1} of protein. Eighty per cent of $(NH_4)_2SO_4$ fractionation precipitates and accounts for 69·7% of the total protease activity in the *Chrysosporium* culture filtrate. In contrast to the other fungal strains, proteases obtained from *Chrysosporium* sp. exhibited proteolytic activity towards Cry1Ac toxin. The protein band corresponding to the 66-kDa CrylAc toxin disappeared with a concomitant appearance of a new protein band with an apparent molecular mass (M_r) of 57 kDa (Fig. 1, lane 3).

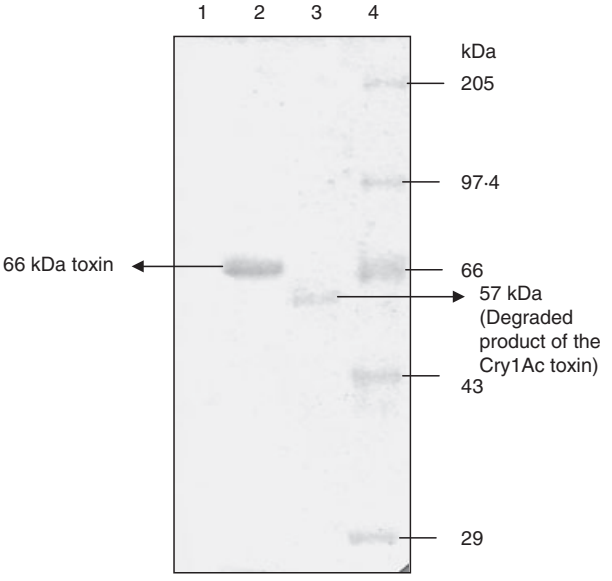

Figure 1 Degradation of the Cry1Ac toxin by *Chrysosporium* proteases, as determined by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, *Chrysosporium* proteases (3 μg); lane 2, Cry1Ac toxin (20 μg); lane 3, Cry1Ac toxin incubated with *Chrysosporium* proteases; the reaction mixture (50 μl) containing 50 μg of the Cry1Ac and 8 μg of *Chrysosporium* proteases in 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, were incubated for 1 h at 30°C; a volume of 20 μl of the reaction mixture was loaded (3 μg of *Chrysosporium* proteases and 20 μg of Cry1Ac toxin equivalent); lane 4, standard protein markers.

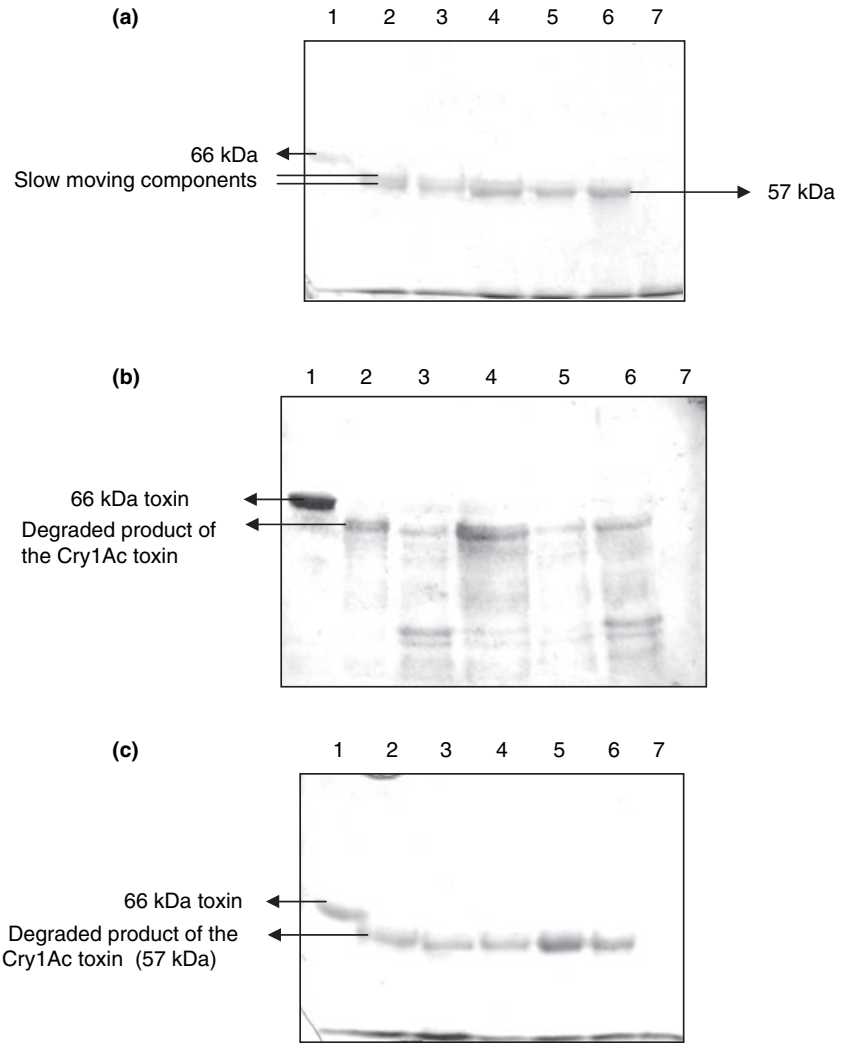

Figure 2 Influence of incubation time on the degradation of the Cry1Ac toxin by *Chrysosporium* proteases. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue. Lane 1, Cry1Ac toxin (20 μg); lanes 2–6, Cry1Ac toxin incubated with *Chrysosporium* proteases for 15, 30, 45, 60, and 120 min, respectively; the reaction mixture (50 μl) containing 50 μg of the Cry1Ac and 8 μg of *Chrysosporium* proteases in 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, were incubated for different time periods at 30°C; a volume of 20 μl of the reaction mixture was loaded; lane 7, *Chrysosporium* proteases (3 μg). (b) Immunoblot developed by using antibodies specific to the Cry1Ac toxin. Lane 1, Cry1Ac toxin (20 μg); lanes 2–6, Cry1Ac toxin incubated with *Chrysosporium* proteases for 15, 30, 45, 60, and 120 min, respectively; the reaction mixture (50 μl) containing 50 μg of the Cry1Ac and 8 μg of *Chrysosporium* proteases in 0·1 mol l^{-1} of Tris-HCl buffer, pH 8·0, were incubated for different time periods at 30°C; a volume of 20 μl of the reaction mixture was loaded; lane 7, *Chrysosporium* proteases (3 μg). (c) SDS-PAGE stained with Coomassie brilliant blue. Lane 1, Cry1Ac toxin (20 μg); lanes 2–6, Cry1Ac toxin incubated with *Chrysosporium* proteases for 1, 3, 6, 9, and 12 h, respectively; the reaction mixture (50 μl) containing 50 μg of the Cry1Ac and 8 μg of *Chrysosporium* proteases in 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, were incubated for different time periods at 30°C; a volume of 20 μL of the reaction mixture was loaded; lane 7, *Chrysosporium* proteases (3 μg).

Influence of incubation time on the proteolysis of the Cry1Ac toxin by proteases from *Chrysosporium* sp.

After 15 min of incubation of the Cry1Ac toxin with *Chrysosporium* proteases, two slow-moving components (Fig. 2a, lane 2) in addition to the 57-kDa protein were observed; this disappeared as the intensity of the 57-kDa protein band increased with the duration of incubation. However, in addition to the 57-kDa protein, two fast-moving components of low M_r were observed by immunoblotting (Fig. 2b) to 120 min. The results of the Cry1Ac toxin incubated with *Chrysosporium* proteases for longer time periods (1, 3, 6, 9, and 12 h) and analysed using SDS-PAGE are shown in Fig. 2c. The gel pattern

was found to be similar at all prolonged time intervals, no further proteolysis of 57-kDa fragment of the Cry1Ac toxin was observed during further incubation also (Fig. 2c).

Effect of protease inhibitors on the activity of proteases from *Chrysosporium*

Inhibition of *Chrysosporium* protease activity by PMSF indicated the presence of serine-catalysed protease activity. No inhibition was observed with EDTA, EGTA, and 1,10-phenanthroline, suggesting that the protease is not a metalloprotease. Iodoacetamide and E-64 showed no inhibition, suggesting the absence of thiol protease. The alkylating reagent, TLCK, a specific inhibitor of trypsin-like proteases had no effect on protease activity of *Chrysosporium* sp. However, chymotrypsin-like serine protease inhibitors, such as TPCK, and chymostatin, showed 100% inhibition, indicating the presence of chymotrypsin-like protease activity by the *Chrysosporium* proteases. $HgCl_2$ (mercuric chloride) also decreased the proteolytic activity to 4%, possibly acting as a denaturant of the proteases (Table 1).

The gel pattern showed a 57-kDa protein band when the Cry1Ac toxin was incubated with *Chrysosporium* proteases alone or with *Chrysosporium* proteases and EDTA (Fig. 3a, lanes 4 and 5, respectively) indicating that EDTA had no influence on protease activity. However, the 66-kDa protein band corresponding to the Cry1Ac toxin was observed when incubated with PMSF and chymostatin (Fig. 3a, lanes 2 and 3), which suggested the inhibitory effect of PMSF and chymostatin on protease activity. This observation was also substantiated by immunoblotting of the proteolytic products (Fig. 3b).

Biological activity of degraded products against *Helicoverpa armigera*

There were no significant differences in the weight of *H. armigera* larvae reared on artificial diet treated individually with *Chrysosporium* proteases alone or pretreated Cry1Ac toxin with C*hrysosporium* proteases, and those treated with untreated artificial diet, indicating that *Chrysosporium* proteases degraded the Cry1Ac toxin resulting in a total loss of insecticidal activity to *H. armigera* (Fig. 4). However, the larvae reared on diet treated with the Cry1Ac toxin alone, showed significantly reduced body mass by 75%.

N-terminal amino acid sequencing

Amino terminal analysis of the 57-kDa fragment produced through degradation of the Cry1Ac toxin by *Chrysosporium* proteases yielded a sequence ^{125}ALREE, which was located in deduced amino acid sequence of crystal protein of *Btk* HD-73 corresponding to the position 125,

Table 1 Influence of protease inhibitors on the protease activity of *Chrysosporium* proteases*

Protease inhibitor	Specificity of the inhibitors	Effective concentration of inhibitor	Protease activity† (% of control)
Control	–	–	100
EDTA	Metalloproteases	20 mmol l^{-1}	100 ± 1·42
EGTA	Metalloproteases	20 mmol l^{-1}	100 ± 1·07
1,10 Phenanthroline	Metalloproteases	20 mmol l^{-1}	100 ± 2·3
PMSF	Ser and some Cys proteases	5 mmol l^{-1}	35 ± 2·0
Leupeptin	Ser and Cys proteases	5 μmol l^{-1}	93 ± 0·54
Aprotinin	Ser proteases	3 μmol l^{-1}	100 ± 0·8
Chymostatin	Chymotrypsin-like Ser proteases	10 μmol l^{-1}	NA
TPCK	Chymotrypsin-like Ser proteases	2 μmol l^{-1}	NA
TLCK	Trypsin-like Ser proteases	2 μmol l^{-1}	100 ± 2·0
E-64	Cys proteases	2 μmol l^{-1}	100 ± 1·45
Iodoacetamide	Cys proteases	20 mmol l^{-1}	100 ± 0·87
$HgCl_2$	Denaturant	5 mmol l^{-1}	4 ± 0·57

**Chrysosporium* proteases were incubated with the inhibitor for 15 min before adding azocasein. The reaction was conducted for 1 h at 30°C. The activity was determined by azocasein assay.

†Activities are expressed as a percentage of the activity in control samples without the inhibitor. The data were mean ± SE of three independent experiments.

NA, no detectable activity; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-p-Tosyl-L-lysine chloromethyl ketone; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone; E-64, *trans*-Epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; $HgCl_2$, mercuric chloride.

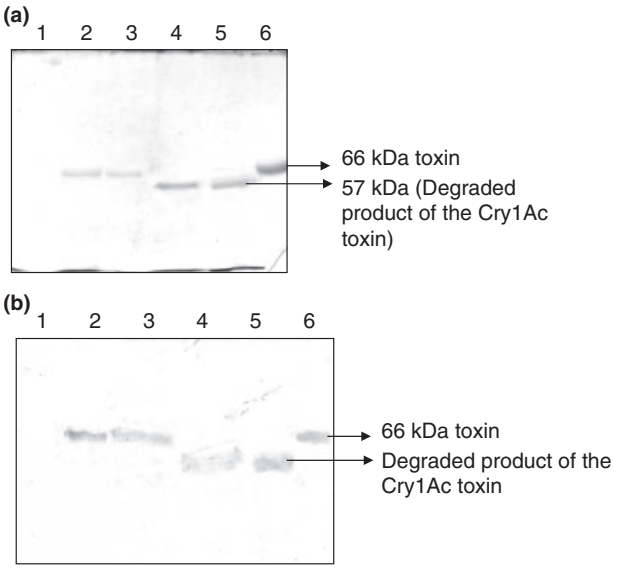

Figure 3 Effect of protease inhibitors on the degradation of the Cry1Ac toxin by *Chrysosporium* proteases. The *Chrysosporium* proteases were incubated with the respective inhibitor for 15 min before the addition of the Cry1Ac toxin and then maintained at 30°C for 1 h before the analysis of degraded products. The reaction mixture (50 μl) containing 50 μg of the Cry1Ac and 8 μg of *Chrysosporium* proteases (either preincubated with the inhibitor or untreated) in 0·1 mol l^{-1} Tris-HCl buffer, pH 8·0, were incubated for 1 h at 30°C. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue. Lane 1, *Chrysosporium* proteases (3 μg); lanes 2–4, Cry1Ac toxin incubated with *Chrysosporium* proteases incubated with phenylmethylsulfonyl fluoride (PMSF), chymostatin, and EDTA, respectively (a volume of 20 μl of the reaction mixture was loaded); lane 5, Cry1Ac toxin incubated with *Chrysosporium* proteases (a volume of 20 μl of the reaction mixture was loaded); lane 6, Cry1Ac toxin (20 μg). (b) Immunoblot developed by using antibodies specific to the Cry1Ac toxin. Lane 1, *Chrysosporium* proteases (3 μg); lanes 2–4, Cry1Ac toxin incubated with *Chrysosporium* proteases incubated with PMSF, chymostatin, and EDTA, respectively (a volume of 20 μl of the reaction mixture was loaded); lane 5, Cry1Ac toxin incubated with *Chrysosporium* proteases (a volume of 20 μl of the reaction mixture was loaded); lane 6, Cry1Ac toxin (20 μg).

which is an alanine residue (Table 2). *Chrysosporium* proteases cleaved the Cry1Ac toxin at the *C*-terminal end of proline, which is at the 124th position, leading to the removal of 96 (from 29 to124) amino acids from the *N*-terminal end of the active 66-kDa toxin, which corresponds to a difference of 9 kDa in molecular weight between the 66-kDa active toxin and the 57-kDa proteolytic fragment of Cry1Ac by *Chrysosporium* proteases.

Discussion

Of the eight fungi evaluated, only proteases from the culture filtrate of *Chrysosporium* sp. degraded the Cry1Ac toxin. Although *A. flavus* exhibited highest protease activity (17 units mg^{-1} protein as compared with *Chrysosporium* 12 units mg^{-1} protein), did not degrade the Cry1Ac toxin. In addition, three bacterial strains were screened. Although these bacteria did elaborate proteases, however, they were incapable of degrading the Cry1Ac toxin. We did not include this data, as we had used only three strains of the bacteria. This is the first report documenting the involvement of a specific soil micro-organism in the degradation of a biologically active Cry1Ac toxin. Incubation of active toxin with *Chrysosporium* proteases for different time intervals resulted in the degradation of the toxin into products with lower M_r. *Chrysosporium* is a keratinophylic filamentous fungus commonly isolated from soil, plant material, and dung. *Chrysosporium* sp. being a natural soil isolate has a better potential for testing its efficacy in field conditions.

The inhibitory studies using azocasein as a substrate indicated that chymostatin and TPCK completely inhibited the protease activity of *Chrysosporium*, whereas PMSF exhibited only a partial inhibition (65%). Both PMSF and chymostatin inhibited the proteolysis of the 66-kDa Cry1Ac toxin by *Chrysosporium* proteases. Experimental results of the present study indicated that chymotrypsin-like serine proteases have a major role in the degradation and inactivation of the Cry1Ac toxin. The bioassay indicated that the proteolytic products derived from the Cry1Ac toxin by *Chrysosporium* proteases did not retain insecticidal activity against *H. armigera* larvae, in contrast to the parent Cry1Ac toxin. The differences in weights of larvae reared on untreated diet and diets with *Chrysosporium* proteases were not statistically significant, indicating that *Chrysosporium* proteases did not have any adverse effects on the growth of *H. armigera* larvae.

These findings are complementary to previous studies in which transgenic plants with a combination of *Bt* toxin genes and protease inhibitor genes were produced to protect and enhance the insecticidal activity of *Bt* toxin (MacIntosh *et al.* 1990). Trypsin and chymotrypsin inhibitors enhanced the insecticidal activity of *Bt* toxins, both in the diet and in transgenic tobacco plants (MacIntosh *et al.* 1990; Zhu *et al.* 2007). Indrasith *et al.* (1991) observed that immobilized chymotrypsin exhibited more intense degradation and resulted in lower insecticidal activity of *Btk* HD-1 and *Btk* HD-73 toxins. Pang and Gringorten (1998) suggested the enhancement of insecticidal activity of *Btk* HD-1 by protease inhibitors as a result of reduced toxin degradation. Shao *et al.* (1998) observed some degradation of active toxin with chymotrypsin.

Activation of *Bt* protoxins is a prerequisite for toxicity, and insufficient processing or overdigestion of the toxin may render it inactive (Lightwood *et al.* 2000). Lightwood *et al.* (2000) demonstrated that the difference in toxin

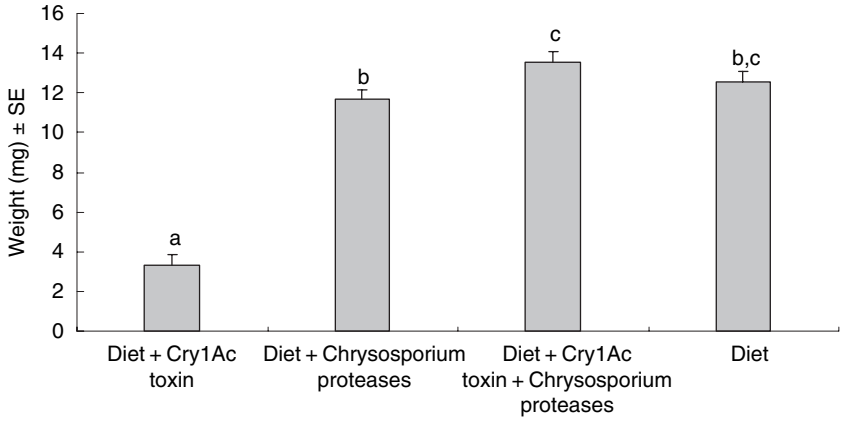

Figure 4 The effect of the degradation of Cry1Ac toxin by *Chrysosporium* proteases against neonate larva of *Helicoverpa armigera*. Each treatment contained 30 neonates and were weighed after 5 days. Error bars represent SE. Bars with same letters were not statistically significant ($P < 0{\cdot}05$). Treatments include artificial diet mixed with: (i) Cry1Ac toxin (50 μg 100 ml^{-1} diet), (ii) *Chrysosporium* proteases (8 μg 100 ml^{-1} diet), (iii) Cry1Ac toxin (50 μg) pretreated with *Chrysosporium* proteases (8 μg) and (iv) artificial diet alone.

Table 2 *N*-terminal amino acid sequence of the Cry1Ac toxin treated with *Chrysosporium* proteases

Sample	Cleavage site	*N*-terminal sequence
*Btk*HD-73 protoxin treated with trypsin*	28–29	^{29}IETGYTIPIDIS
*Btk*HD-1 Dipel toxin treated with endogenous proteases†	29–30	^{30}ETGYTIPIDIS
*Btk*HD-73 toxin treated with *Chrysosporium* protease	124–125	^{125}ALREE

*Adang *et al.* (1985).
†Suresh Kumar and Venkateswerlu (1998a).

potency to larvae of susceptible *Pieris brassicae* (large white butterfly) and nonsusceptible *Mamestra brassicae* (cabbage moth) was related to the differences in proteolysis of the Cry1Ac protein by the proteases within these two species. The lack of toxicity of *Bt* subsp. *kurstaki* HD-1 to another lepidopteran species, *Spodoptera litura* (Tobacco cutworm) is due to complete degradation of toxin by the gut proteases (Inagaki *et al.* 1992). Similarly, Keller *et al.* (1996) observed that increased degradation of the CrylC toxin in the less susceptible larvae of *Spodoptera littoralis* (Egyptian cotton leafworm) was the major reason for its reduced toxicity to this insect. Ogiwara *et al.* (1992) studied the proteolytic cleavage sites of the *Bt* subsp. *kurstaki* HD-1 and HD-73 endotoxins, and found that the proteolysis of the Cry toxins depended on the proteases in the insect gut and that the differences in proteolysis are reasons for the differences in toxicity of the *Bt* toxins towards different insect species.

Amino terminal analysis of the toxin fragment produced by *Chrysosporium* proteases indicated that the proteolysis site corresponds to alanine at position 125, which is present in the α4 of domain I, as predicted from the three-dimensional structure of the Cry1Aa protein (Grochulski *et al.* 1995). Domain I exists as a helical bundle with the hydrophobic faces of the amphipathic helices surrounding a hydrophobic central helix. Proteolytic nicking within domain I imparts more flexibility to the molecule. Previous studies have shown that domain I is involved in the insertion of the protein into the membrane and pore formation, and that mutations in domain I reduced the irreversible binding of the toxin to the brush border membrane (Chen *et al.* 1995). The *N*-terminal region between 28 and 37 amino acids has been shown to be essential to have toxic effects by deletion studies with *Bacillus thuringiensis* subsp. *berliner*, closely related to lepidopteran-specific bacterium, *Btk* HD-73. Further, the shortest reported toxic fragment was found to be localized between codons 29 and 607 for Cry1Ab (Höfte *et al.* 1986). Additionally, removal of four codons from the 3′ end or eight codons from the 5′ end completely abolished the toxic activity of the gene product (Wabiko *et al.* 1986). Similar observations were also made for proteins expressed by the *cry1Aa* (Schnepf and Whiteley 1985) and *cry1Ac* (Adang *et al.* 1985) genes. *Chrysosporium* proteases apparently cleaves the *Bt* toxin between the amino acids, the 124th *C*-terminal proline and the 125th *N*-terminal alanine, deleting a 96-amino acid fragment from the *N*-terminal end, which resulted in the loss of insecticidal activity.

The toxic domain of the activated Cry toxin consists of several conserved hydrophobic regions (Gill *et al.* 1992), and site-directed mutagenesis in this region causes a decrease in toxicity, indicating the importance of this hydrophobic region in toxicity (Wu and Aronson 1992). It is also known that activated toxin can be folded in such a conformation that the hydrophobic regions are internalized in the molecule, which precludes further processing by proteases. Chymotrypsin hydrolyses the peptide bonds of amino acids with hydrophobic lateral chains. The inhibitory studies revealed that chymotrypsin-like proteases are responsible for the degradation of the Cry1Ac toxin, demonstrating that proteases are involved in

hydrolysing and altering the hydrophobic regions of the toxin, which seem to be important for its toxicity against *H. armigera*.

In conclusion, the outcome of the present study indicated that proteases elaborated by the *Chrysosporium* sp. degraded the 66-kDa Cry1Ac toxin, thus leading to the loss of biological activity of the Cry1Ac toxin against *H. armigera*, but possibly also other species. Although the effect of microbes present in soil on the *Bt* toxins (Cry1 and Cry3 proteins) have been known for a long time (Koskella and Stotzky 1997), this is the first time that the action of a protease secreted by a specific soil microorganism has degraded the Cry1Ac toxin and is the possible mechanism that led to the inactivation of this protein has been reported. Detailed further studies using this isolate of *Chrysosporium* sp. needs to be carried out under field conditions to correlate its potential application in reducing the nontarget effects of the *Bt* toxins in soil. Further it is also pertinent to note that under natural conditions, how this isolate of *Chrysosporium* sp. elaborates the proteases that can subsequently degrade the Cry1Ac toxin in relation to the microenvironment that exists around the transgenic plant are yet to be tested.

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