Enzyme and Microbial Technology 46 (2010) 252–256

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Purification and partial characterization of a 36-kDa chitinase from *Bacillus thuringiensis* subsp. *colmeri*, and its biocontrol potential

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ARTICLE INFO

Article history:
Received 6 August 2009
Received in revised form 1 October 2009
Accepted 14 October 2009

Keywords:
Bacillus thuringiensis
ChiA
Dimmer
Characterization
Antagonism
Synergism

ABSTRACT

Chitinase A (ChiA) produced by *Bacillus thuringiensis* subsp. *colmeri* 15A3 (*Bt.* 15A3) was expressed in *Escherichia coli* XL-Blue. The ChiA was purified using Sephadex G-200 and its molecular mass was estimated to be 36 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Detection of chitinase activity on SDS-PAGE after protein renaturation indicated that the molecular mass of the protein band expressing chitinase activity was approximately 72 kDa. This suggests that the dimeric form of ChiA is the enzymatically active form when glycol chitin is used as a substrate. ChiA has optimal activity at 50 °C and retains most of its activity between 20 and 60 °C. The optimum pH for ChiA activity is pH 5.0, and the enzyme is active between pH 4.0 and 8.0. The enzyme activity was significantly inhibited by Ag^+ and Zn^{2+}. ChiA significantly inhibited the spore germination of four species of fungi. The median inhibitory concentrations (IC_{50}) of ChiA on the spore germination of *Penicillium glaucum* and *Sclerotinia fuckelian* were 11.27 and 10.57 μg/ml, respectively. In surface contamination bioassays, the crude ChiA protein (12.6 mU) reduced the LC_{50} (50% lethal concentration) of the crystal protein of *Bt.* 15A3 against the larvae of *Spodoptera exigua* and *Helicoverpa armigera*.

1. Introduction

Chitin, a homopolymer of β-1,4-linked *N*-acetyl glucosamine (GlcNAc), is the second most abundant polysaccharide found in nature. It is a main structural component of the fungal cell wall and insect exoskeletons [1,2]. The linear polymer can be hydrolyzed by bases, acids, or enzymes such as lysozyme and chitinases. Chitinases (EC3.2.1.14) are produced by a large group of chitin-degrading organisms, including fungi, bacteria, insects, plants, and animals. In yeasts such as *Saccharomyces cerevisiae*, chitinase plays a morphological role in promoting efficient cell separation [3]. Plants express chitinases as a defense against chitin-containing pathogens that have chitin as a structural constituent. Bacterial chitinases degrade chitin primarily for use as carbon and energy sources [4].

Chitinase-producing microorganisms have been reported as biocontrol agents for different types of fungi. Driss et al. [5] observed that the culture supernatant of *Bacillus thuringiensis* (*Bt.*) strain BUPM255 has high antifungal activity against *Aspergillus niger*. The chitinolytic bacterium *Bacillus cereus* 28-9 produces a chitinase (ChiCW) that is found to be effective against the fungal pathogen *Botrytis elliptica* [6]. de la Vega et al. [7] reported that a purified 66-kDa chitinase from *Bt.* subsp. *aizawai* exhibits lytic activity against the cell walls of six phytopathogenic fungi and inhibited the mycelial growth of *Fusarium* sp. and *Sclerotium rolfsii*. Further, the chitinases from two fungal strains can degrade the cell walls of *Fusarium solani* and also inhibit microconidial germination in this species [8].

B. thuringiensis is a gram-positive, endospore forming, soil bacterium that is used worldwide for pest control in agriculture and for the control of various disease-related insect vectors. Several studies have demonstrated that co-expression of heterologous chitinase genes in *B. thuringiensis* can enhance the insecticidal activity of this bacterium [9–13]. The involvement of endogenous chitinases during *Bt.* subsp. *aizawai* infection of *Spodoptera littoralis* larvae in the presence of allosamidin, a specific chitinase inhibitor, was demonstrated to increase the LC_{50} value of the toxin [14].

We have reported that *Bt.* subsp. *colmeri* 15A3 (*Bt.* 15A3) constitutively expresses two chitinases, namely, ChiB and ChiA, with molecular masses of 70 and 36 kDa, respectively [15], and subsequently described the biochemical characterization and biocontrol effects of ChiB expressed in *Escherichia coli* [16].

Although three chitinases with a molecular weight of 36 kDa have been identified in *Bacillus* spp. [17–21], none of these enzymes have been biochemically well characterized, and little is known of the insecticidal activity of ChiA. Consequently, the potential applications of ChiA remain unexplored.

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doi:10.1016/j.enzmictec.2009.10.007

In this study, we report the purification and biochemical characterization of ChiA isolated from *Bt.* 15A3 expressed in *E. coli*. For the first time, we show that the active form of ChiA is dimeric. We also examine the antifungal activity of ChiA *in vivo* against four species of fungi and assess the synergistic effects of ChiA in combination with *Bt.* 15A3 crystal protein on larvicidal activity against *Spodoptera exigua* and *Helicoverpa armigera*.

2. Materials and methods

2.1. Strains and culture conditions

E. coli XL-Blue carrying the plasmid pUCm-*chiA* (the construction of which is described in our previous paper [15]) was grown at 37 °C in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin in order to induce protein expression. The phytopathogenic fungal species *Rhizoctonia solani*, *Physalospora piricola*, *Penicillium chrysogenum*, and *Botrytis cinerea* were kindly provided by Professor Fang Liu, of the Department of Microbiology, Nankai University. These fungi were maintained on potato dextrose agar (PDA) slants and stored at 4 °C.

2.2. Chemicals

All chemicals were of analytical grade for biochemical use and used as described by the respective manufacturers. Glycol chitin and Calcofluor white M2R were obtained from Sigma (Steinheim, Germany). Sephadex G-200 was obtained from Pharmacia.

2.3. Colloidal chitin preparation

Colloidal chitin was prepared according to the method of Sandhya et al. [22] with some modifications: 10 g of chitin flakes were added into 40 ml of acetone and ground gently for 10 min. Then 400 ml of concentrated HCl was added in the solution with agitating gently for 3 h on a magnetic stirrer. This solution was stored at 4 °C for 24 h and then filtered to 2 l of 50% ethanol with constant mixing. After centrifugation at 10,000 rpm for 20 min at 4 °C, the precipitate was formed and washed in distilled water repeatedly until the pH reached to 5.5. The supernatant was discarded and 1 l of distilled water was added to form 1% colloidal chitin, then the colloidal chitin was kept in refrigerator for future use.

2.4. Purification of ChiA

An overnight culture of *E. coli* XL-Blue carrying the pUCm-*chiA* construct was added to fresh LB medium supplemented with ampicillin and cultured at 30 °C for 72 h to induce the expression of ChiA. Culture supernatant was collected by centrifugation at 8000 × *g* for 20 min. Proteins in the culture supernatant were precipitated with 70% $(NH_4)_2SO_4$ at 4 °C. The precipitated protein was recovered by centrifugation (12,000 × *g*, 20 min, and 4 °C), dissolved in a small amount of 50 mmol/l Tris–HCl buffer (pH 6.0), and dialyzed overnight against the same buffer. The dialysate was then loaded onto a Sephadex G-200 column (1.5 cm × 25 cm) pre-equilibrated with 50 mmol/l Tris–HCl buffer (pH 6.0). After washing the column with the same buffer, the enzyme was fractionated in the 50 mmol/l Tris–HCl buffer at a flow rate of 6 ml/h. Fractions of 4 ml were collected and analyzed for chitinase activity.

2.5. Protein concentration determination

The protein concentration was measured using the method described by Bradford [23], with bovine serum albumin as a standard.

2.6. Chitinase activity assay

Chitinase activity was measured using colloidal chitin as a substrate following the method of Joshi et al. [24]. The amount of reduced sugar released was determined by recording the absorbance at 565 nm. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmol of *N*-acetyl glucosamine (GlcNAc) per hour under the above conditions.

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

SDS-PAGE was performed according to the method of Laemmli [25]. Two types of molecular weight protein markers (Takara and Transgen) were used as molecular standards.

In order to identify the activity of the enzyme, zymography was conducted according to the method of Trudel and Asselin [26], and Sugai et al. [27] with minor modifications. The enzyme was subjected to SDS-PAGE on a separating gel containing 0.01% glycol chitin. After electrophoresis, proteins in the gel was renatured by incubating the gel overnight at 37 °C in 100 mmol/l sodium acetate buffer (pH 5.0) containing 1% Triton X-100. Subsequently, the gel was stained for 1 h at room temperature with 0.01% (w/v) Calcofluor white M2R in a staining solution (50 mM Tris–HCl, pH 8.9) and then incubated at room temperature in distilled water. Bands with chitinase activity were visualized under a UV transilluminator, and the molecular weight was estimated by comparison with the molecular standards in the gel.

2.8. Effect of temperature and pH on chitinase activity and enzyme stability

For determination of the optimal temperature for chitinase activity, the enzyme was incubated in 50 mmol/l Tris–HCl buffer at pH 6.0 for 1 h at temperatures ranging from 20 to 100 °C, using colloidal chitin as a substrate. In order to determinate the thermostability of the chitinase, the enzyme was preincubated in reaction buffer at pH 6.0 for 1 h at temperatures ranging from 20 to 100 °C without substrate. Thereafter, colloidal chitin was added as a substrate for determination of the relative residual enzyme activity under the standard conditions described above.

The pH optimum was investigated by incubating the enzyme at 4 °C in reaction buffers with pH values between pH 4.0 and 10.0 for 1 h, using colloidal chitin as a substrate. For the determination of pH stability, the enzyme was incubated in reaction buffers with pHs ranging from 4.0 to 10.0 at 4 °C for 1 h without substrate, and the relative residual enzyme activity was measured as described above using colloidal chitin as a substrate. All measurements were repeated three times.

2.9. Effect of various chemicals on chitinase activity

The effects of various chemicals on enzyme activity were investigated by adding different metals and other reagents to the reaction mixture at 10 mmol/l, preincubating the mixture for 60 min at 4 °C, and then measuring the relative activity (compared with the enzyme solution lacking any added chemical) by using colloidal chitin as a substrate. The following 10 mmol/l solutions were used in the assay: Mn^{2+} ($MnCl_2$), Cr^{3+} ($CrCl_3$), Li^+ (LiCl), Na^+ (NaCl), Cu^{2+} ($CuSO_4$), Mg^{2+} ($MgCl_2$), Fe^{3+} ($FeCl_3$), Ca^{2+} ($CaCl_2$), Zn^{2+} ($ZnSO_4$), Ag^+ (AgCl), EDTA, and SDS.

2.10. Antifungal activity assay

Four species of fungi were used in this assay: *R. solani*, *P. piricola*, *P. chrysogenum*, and *B. cinerea*. The concentration of the spore suspensions was adjusted to 10^6 spores ml^{-1}. Stainless steel cylinders of uniform size (8 mm × 6 mm × 10 mm) were placed on the surface of PDA plates, and then 100 μl of 210 mU crude chitinase and 100 μl spore suspension were added to the cylinder. For the control treatment, an equal amount of sterile water mixed with 100 μl spore suspension was added to another cylinder. The plates were incubated at 30 °C for 72 h, and then inhibition of spore germination in the cylinders was judged by visual inspection.

In order to evaluate the inhibitory activity of ChiA against fungal spore germination, a spore germination inhibition assay was employed. The concentration of the spore suspensions was adjusted to 10^5 spores ml^{-1}. The spore suspensions were added to PDA medium on glass slides, with and without ChiA, then cultured at 28 °C for 48 h. The inhibition of spore germination was examined under a microscope at ×200 magnification for 10 different fields of view. All experiments were conducted three times and the median inhibitory concentrations (IC_{50}) of ChiA on *P. glaucum* and *S. fuckelian* spore germination were calculated according to the method of Tan et al. [28].

2.11. Bioassays for insecticidal activity

The enhancing effects of ChiA on the insecticidal activity of *Bt.* 15A3 crystal protein on the larvae of *S. exigua* and *H. armigera* were studied by a surface contamination bioassay, using an artificial diet in 24-well trays. First, 10–15 mg spore-crystal complex of strain *Bt.* 15A3 was diluted to give a range of doses. Each dilution mixed with artificial diet was added to the 24-well trays. Crude chitinase (20 μl, 12.6 mU) was soaked into each cube of artificial diet. For the control treatment, bioassay buffer instead of crystal protein was added to the artificial diet. Dilutions were tested against first instar larvae by incubating for 3 d at 28 °C. The median lethal concentration (LC_{50}) for the larvae of *S. exigua* and *H. armigera* was calculated by probit analysis (SPSS for Windows software). The LC_{50} values and 95% fiducial limits were determined from three independent assays, and each assay was performed in triplicate. The values were calculated based on the data from all repeats.

3. Results

3.1. Enzyme production and purification

The protein in supernatant from *E. coli* XL-Blue (pUCm-*chiA*) cultures grown in LB medium was purified by ammonium sulfate precipitation and Sephadex G-200 chromatography. The enzyme activity and protein concentration of the fractions were determined. The molecular mass of the major band of purified ChiA was estimated to be 36 kDa by SDS-PAGE (Fig. 1A). This molecular mass corresponds well with the estimated molecular mass of 36.3 kDa for 360 amino acids. The molecular mass of the protein exhibiting

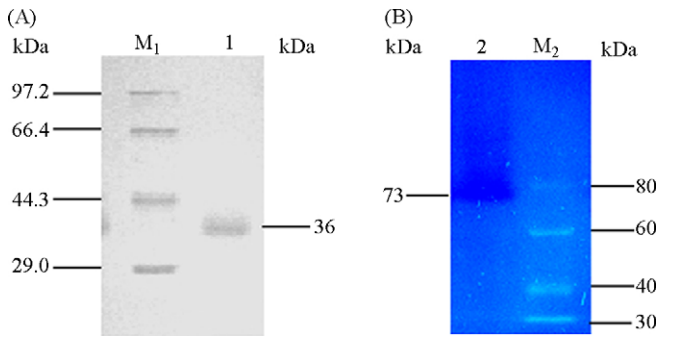

Fig. 1. SDS-PAGE and zymogram analysis of ChiA purified from a culture of *E. coli* XL-Blue (pUCm-*chiA*). (A) SDS-PAGE of the gel filtration-purified active fractions, gel stained with Coomassie brilliant blue G-250. (B) Chitinase activity detected after SDS-PAGE in a gel containing 0.01% glycol chitin. The dark zone represents the hydrolysis of glycol chitin in the gel. Lanes M_1 and M_2, molecular weight standards. Lanes 1 and 2, gel filtration chromatography of ChiA.

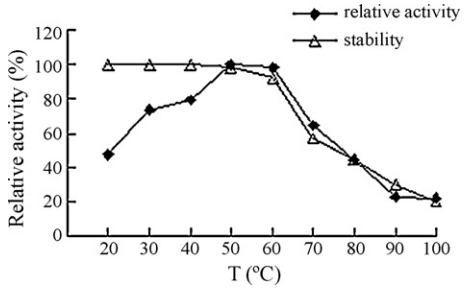

Fig. 2. Effect of temperature on the relative activity and stability of ChiA.

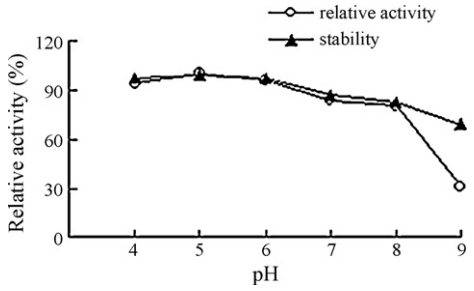

Fig. 3. Effects of pH on the relative activity and stability of ChiA.

enzyme activity, determined by an in-gel activity assay with glycol chitin as a substrate, was approximately 72 kDa, which is twice the mass of monomeric ChiA (Fig. 1B). These results indicate that ChiA could be active against glycol chitin as a dimer.

3.2. Characterization of chitinase

The optimal temperature for ChiA enzyme activity was 50 °C, although activity at 60 °C was almost the same (Fig. 2). The enzyme was found to be very stable from 20 to 60 °C and retained 45% of its activity at 80 °C (Fig. 2).

The optimal pH for ChiA activity was pH 5.0. The enzyme appeared to have broad pH optima between pH 4.0 and 8.0 with at least 90% activity (Fig. 3). Between pH 4.0 and 8.0, the enzymatic activity is as high as 100%. The overall optimal conditions for ChiA activity were 50 °C and pH 5.0.

The effects of metal ions and other reagents on enzyme activity were examined by preincubating the crude enzyme with chemicals in 50 mmol/l sodium phosphate buffer (pH 5.0) for 1 h at 4 °C, and then measuring the residual enzyme activity. The results are presented in Table 1. These results represent the averages of three independent experiments. The data indicate that there was a slight increase in enzyme activity in the presence of Cu^{2+}, Fe^{3+}, Na^+, Mg^{2+}, and Ca^{2+}, whereas the enzyme activity was markedly inhibited by Zn^{2+} and Ag^+. In contrast, enzyme activity was not sensitive to SDS or the metal-chelating agent EDTA, indicating that divalent cations are not required for enzyme activity.

Table 1
Effects of metal ions, EDTA, and SDS on the relative activity of ChiA.[a].

Substances (10 mmol/l)	Relative activity (%)
None	100
Cu^{2+}	115 ± 0.29
Mg^{2+}	107 ± 0.24
Na^+	106.2 ± 0.31
Fe^{3+}	106 ± 2.64
Ca^{2+}	103 ± 1.63
Li^+	98.7 ± 0.72
Mn^{2+}	92.4 ± 0.25
Cr^{3+}	87.7 ± 0.15
Ag^+	43.8 ± 0.18
Zn^{2+}	36.2 ± 0.31
EDTA	106.9 ± 0.12
SDS	90.9 ± 0.37

[a] Mean ± SD; $n = 3$.

Table 2
IC_{50} of ChiA on the spore germination of *P. glaucum* and *S. fuckelian*.

Fungus species	*R*	IC_{50} (μg/ml)	95% fiducial limits (μg/ml)
Penicillium glaucum	0.92	11.27	11.26–11.28
Sclerotinia fuckelian	0.97	10.57	10.55–10.58

3.3. Antifungal activity of chitinase

Germination of the spores of four fungal species was significantly inhibited by up to nearly 100% after incubation with ChiA at 30 °C for 72 h (Fig. 4).

It is clear that ChiA almost completely inhibits the spore germination of *R. solani* and *B. cinerea*, although it is less effective in inhibiting spore germination in *P. chrysogenum* and *P. piricola*.

The median inhibitory concentrations (IC_{50}) of ChiA on the spore germination of *P. glaucum* and *S. fuckelian* were calculated to be 11.27 and 10.57 μg/ml, respectively (Table 2).

3.4. Insecticidal activity

A comparison of 3-d insecticidal bioassays revealed that the LC_{50} for *Bt.* 15A3 crystal protein towards *S. exigua* and *H. armigera* was 19.42 and 12.82 μg/ml, respectively. When crystal protein was added in combination with ChiA, the LC_{50} was decreased by 18% and 19.8%, respectively (Table 3).

4. Discussion

Three previous studies have used this zymogram technology to detect the activity of 36-kDa chitinase [18,19,21]. It should be noted, however, that in only two of these studies was the molecular mass of the band with enzyme activity found to be exactly 36 kDa, and that these studies used different substrates in the gel: carboxylmethyl Remazol Brilliant Violet chitin (CM-chitin-RBV) [18], and 4-methylumbelliferyl diacetylchitobiose [4-MU-$(GlcNAc)_2$] [20]. In the present study, glycol chitin was used as the substrate, and we revealed that the molecular mass of the enzymatic band was 72 kDa, which is twice the molecular weight of the purified ChiA determined by SDS-PAGE (Fig. 1A and B). These results reveal that ChiA may form a dimer in order to facilitate the hydrolysis of glycol

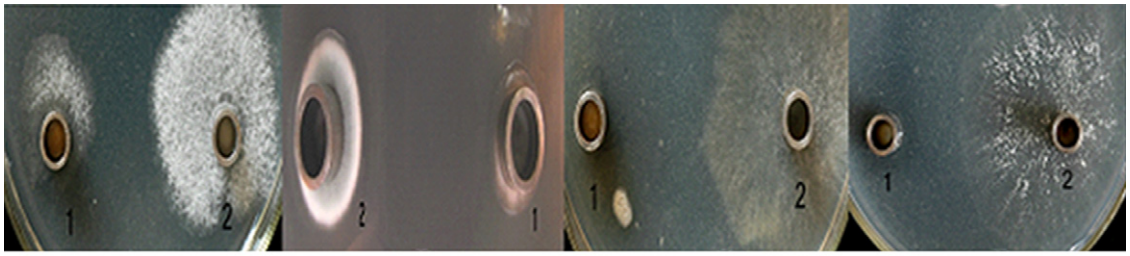

Fig. 4. The antimicrobial action of ChiA on the spores of four species of fungi. (A) *Physalospora piricola*; (B) *Penicillium chrysogenum*; (C) *Rhizoctonia solani*; (D) *Botrytis cinerea* (1) ChiA + spores; (2) control.

Table 3
Insecticidal activity of ChiA and crystal protein of *Bt.* 15A3 against *S. exigua* and *H. armigera*.

Insect species	Samples	LC_{50} (μg/ml)	95% fiducial limits (μg/ml)
Spodoptera exigua	*Bt.* 15A3 crystal protein	19.42	17.16–21.72
	Bt. 15A3 crystal protein + ChiA	15.93	13.08–18.19
Helicoverpa armigera	*Bt.* 15A3 crystal protein	12.82	10.47–16.11
	Bt. 15A3 crystal protein + ChiA	10.28	7.84–12.5

chitin. There are a number of studies that have demonstrated dimer formation in enzymes [29–31]. We can deduce from these results that with different substrates in the gel, ChiA may form different conformations to hydrolyze the different substrates. Using other substrates to determine the molecular mass of the active band and investigating whether different substrates effect the formation of ChiA will constitute the next stage of our work. This is the first study to suggest that the 36-kDa ChiA may need to dimerize in order to exert its enzyme activity on glycol chitin.

The 36-kDa ChiA contains a conserved region of family 18 chitinases. ChiA consists of a catalytic domain, but lacks a fibronectin domain and a chitin-binding domain, the latter of which is involved in binding chitin [16]. We accordingly deduce that the ChiA of *Bt.* 15A3 has a unique mechanism for hydrolyzing chitin and its analogues. In the present study, we found that ChiA exhibits no enzyme activity in its monomeric form.

The optimum pH for the ChiA of *Bt.* 15A3 is approximately 5.0, whereas that for the ChiA of *B. cereus* is 6.0 [18], and that for the Chi36 of *Bt.* HD-1 is 6.5 [17]. In the present study, ChiA was demonstrated to exhibit broad pH and temperature optima. Several chitinases have broad pH optima, including the chitinase of *B. cereus* (pH 4.0–7.0) [18] and an exochitinase from *Bt.* subsp. *aizawai* (pH 5–8) [7]. Compared with the previously reported chitinases, however, the ChiA of *Bt.* 15A3 has a broader pH range, pH 4.0–8.0.

The optimum temperature for ChiA activity is 50 °C and the enzyme retains 45% of its activity at 80 °C. The thermostability of the ChiA of *Bt.* 15A3 is also higher than that reported for two other 36-kDa chitinases.

The partial characterizations of ChiA and ChiB, which are both from *Bt.* 15A3 and expressed in *E. coli*, would appear to be almost the same. The optimal temperature for ChiB activity is 60 °C and retains nearly 80% of its activity at 50 °C. The optimal pH for ChiB is also 5.0, and ChiB is also stable between the pH 4.0 and 8.0.

The effects of metal ions and other reagents upon ChiA are diverse. There have been no previous reports regarding the effects of metal ions and other reagents upon two other 36-kDa chitinases. This paper is therefore the first to report the effects of metal ions and other reagents upon a 36-kDa chitinase.

In spite of lacking a chitin-binding domain, the ChiA of *Bt.* 15A3 displays a high activity against colloidal chitin. This indicates that the chitin-binding domain is not a requirement for the hydrolysis of chitin. Similarly, the 36-kDa chitinase of *B. cereus*, which lacks a chitin-binding domain, is active on a polymeric substrate [18]. Further, the Chi36 enzyme of *Bt.* HD-1, which is an exochitinase, is active on the trisaccharide MU-$(GlcNAc)_3$ [17].

The biocontrol potentials of ChiA and ChiB would appear to be different. The inhibitory effect of ChiA on fungal spore germination and its synergistic activity are lower than those of ChiB. The IC_{50} of ChiB on *P. glaucum* and *S. fuckelian* was 4.12 and 4.02 μg/ml, respectively, therefore lower than that of ChiA on spore germination in these two fungal species. This difference could be due to the lack of a chitin-binding domain in the former. A number of studies have suggested that when the chitin-binding domain of chitinases is deleted, the antifungal effect of these chitinases is almost completely abolished [32,33]. In our recent study, when we deleted the whole chitin-binding domain of ChiB, we found that the chitinase loses its ability to inhibit spore germinations in *P. piricola* and *P. chrysogenum*, whereas it is still able to significantly inhibit spore germination in *R. solani* and *B. cinerea* (unpublished results).

Only one previous study has analyzed the insecticidal effect of Chi36 from *Bt.* HD-1 in combination with the Vip (vegetative insecticidal protein) against *S. litura* neonate larvae [17]. In the present study, using insecticidal bioassays, we demonstrated that ChiA can decrease the LC_{50} of *Bt.* 15A3 crystal protein against the larvae of *S. exigua* and *H. armigera*.

Acknowledgement

This research is supported by grants from the National Natural Science Foundation of China (No. 30971957 and No. 30600015).

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