



The chitinase C gene *PsChiC* from *Pseudomonas* sp. and its synergistic effects on larvicidal activity

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Abstract

Pseudomonas sp. strain TXG6-1, a chitinolytic gram-negative bacterium, was isolated from a vegetable field in Taixing city, Jiangsu Province, China. In this study, a *Pseudomonas* chitinase C gene (*PsChiC*) was isolated from the chromosomal DNA of this bacterium using a pair of specific primers. The *PsChiC* gene consisted of an open reading frame of 1443 nucleotides and encoded 480 amino acid residues with a calculated molecular mass of 51.66 kDa. The deduced *PsChiC* amino acid sequence lacked a signal sequence and consisted of a glycoside hydrolase family 18 catalytic domain responsible for chitinase activity, a fibronectin type III-like domain (FLD) and a C-terminal chitin-binding domain (ChBD). The amino acid sequence of *PsChiC* showed high sequence homology (> 95%) with chitinase C from *Serratia marcescens*. SDS-PAGE showed that the molecular mass of chitinase *PsChiC* was 52 kDa. Chitinase assays revealed that the chitobiosidase and endochitinase activities of *PsChiC* were 51.6- and 84.1-fold higher than those of pET30a, respectively. Although *PsChiC* showed little insecticidal activity towards *Spodoptera litura* larvae, an insecticidal assay indicated that *PsChiC* increased the insecticidal toxicity of SpltNPV by 1.78-fold at 192 h and hastened death. These results suggest that *PsChiC* from *Pseudomonas* sp. could be useful in improving the pathogenicity of baculoviruses.

Keywords: catalytic domain, chitin-binding domain, fibronectin type-like domain, gene cloning, insecticidal activity.

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Introduction

Chitin, an insoluble polymer of N-acetyl-D-glucosamine (GlcNAc), is an important component of insect exoskeletons, crustacean shells and fungal cell walls. Chitin is the second most abundant component of biomass in nature after cellulose and can be degraded to GlcNAc monomers by chitinase. Chitinases are found in a wide range of organisms, including bacteria, fungi, plants, insects, crustaceans and some vertebrates. Chitinases, including endochitinase, exochitinase, β -N-acetylglucosaminidase and chitobiase, play important physiological and ecological roles in chitin metabolism (Flach *et al.*, 1992; Felse and Panda, 1999). Many microbial species produce chitinase and digest chitin as a carbon and energy source. Bacteria in particular can produce high levels of chitinolytic enzymes. Based on their amino acid sequence similarities, chitinases are grouped into families 18 and 19 of glycoside hydrolases (Henrissat and Bairoch, 1993). Family 19 chitinases include classes I, II and IV of plant chiti-

nases (Collinge *et al.*, 1993; Levorson and Chlan, 1997; Gomez *et al.*, 2002) and family 18 chitinases include most of the chitinases from bacteria, fungi, insects, animals and plants (class III and V chitinases).

Spodoptera litura multicapsid nucleopolyhedrovirus (SpltNPV) is highly specific and infects only a single host, the cotton leaf worm (*Spodoptera litura*). This insect is an economically important polyphagous pest in China, India and Japan, where it causes considerable economic loss to many vegetable and field crops. SpltNPV has been successfully applied in large scale as a commercial biological insecticide against the cotton leaf worm in China (Pang *et al.*, 2001). However, like other biopesticides, SpltNPV has several potential limitations for broad commercial use, such as slow speed of kill (ranging from five days to more than two weeks), short field stability, very narrow host specificity, susceptibility to UV light, short shelf-life and high production costs (Inceoglu *et al.*, 2006; Mills and Kean, 2010). Although there is considerable information on bacterial chitinase genes, there have been no reports on the chitinase genes from *Pseudomonas* species. In this study, we isolated a bacterium with chitinase activity from a vegetable field and identified this strain as a *Pseudomonas* species. To fur-

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ther characterize the chitinase genes from this bacterium, we initially cloned the chitinase C gene and analyzed the resulting sequence. Subsequently, the *Pseudomonas* chitinase C gene (*PsChiC*) was expressed in *Escherichia coli* BL21(DE3) using a 6xHis-fusion protein expression system and the synergistic effects of *PsChiC* in combination with SpltNPV on larvicidal activity against *S. litura* were evaluated.

Material and Methods

Isolation of chitinase-producing microorganisms

To isolate chitinase-producing microorganisms, soil was collected from a vegetable field in Taixing city, Jiangsu Province, China, crushed and suspended in sterile water with shaking for 2 h. After centrifugation at 8000 x g for 10 min, the supernatant was diluted and spread on synthetic medium containing 1.4% agar. Analysis of the 16S rDNA sequence, as well as biochemical and physiological characterization of the isolated strain were done by the TAKARA Company, China.

Strains, plasmids and media

Pseudomonas sp. TXG6-1 was grown in synthetic medium containing colloidal chitin (5 g/L), MgSO₄•7H₂O (0.3 g/L), KH₂PO₄ (0.3 g/L) and K₂HPO₄ (0.3 g/L) at 30 °C. Colloidal chitin was prepared from crude chitin (Sigma, USA) by the method of Roberts and Selitrennikoff (1988), with a few modifications. *Escherichia coli* DH5α and *E. coli* BL21(DE3) for gene cloning, were grown in LB medium at 37 °C. The vectors pGEM T-easy (Promega, USA) and pET30a+ were used for cloning and expression of the chitinase gene, respectively.

PCR cloning and DNA sequencing

Chromosomal DNA of *Pseudomonas* sp. TXG6-1 was isolated using an EZNA bacterial DNA kit (Omega Bio-Tek, USA). The 16S rRNA gene sequence of TXG6-1 was amplified with two conserved bacterial 16S rDNA PCR primers (27F and 1495R) and sequenced using an ABI PRISM 3100 Genetic Analyzer system (Applied Biosystems, Inc., USA). The 16S rRNA gene sequence was determined and compared to those of related taxa retrieved from the *Pseudo*MLSA Database (Bennasar *et al.*, 2010), GenBank and EMBL databases.

Based on the sequence of the bacterial chitinase *ChiC* gene, the following pair of primers was designed for PCR cloning: P1: 5'-AGC TGA TAT TGC CGG CGA G-3' and P2: 5'-AAA TAG TTG TTT GCT TAG GTG CGG-3'. The PCR amplification conditions for each cycle were: 95 °C (1 min), 55 °C (1.5 min) and 72 °C (1 min) for a total of 30 cycles. The corresponding gene from TXG6-1 was then inserted into the pGEM T-easy vector and sequenced by the dideoxy chain-termination method in an ABI 3730 automatic sequencer. The nucleotide sequences have been de-

posited in the GenBank nucleotide sequence database under accession number GU724605 for *PsChiC*.

Using the identified gene fragment encoding the chitinase as template, PCR was done with primers P3: 5'-GAC CAT ATG GCA CCA ATA ACA TTA TT-3' and P4: 5'-GAC CCA TGG GGC GAT GAG CTG CCA CAG-3'. The PCR product was cleaved with *NdeI* and *NcoI*, and then ligated into *NdeI-NcoI* digested pET-30a+ to construct the recombinant plasmid pET-*PsChiC*.

Bioinformatics analysis of *PsChiC*

Analysis of the DNA sequences was done using the DNAMAN software package (version 4.0, Lynnon Corporation, Pointe-Claire, QC, Canada) (Huang and Zhang 2004). Homology searches were done using the BLAST algorithms against various databases in GenBank. Protein domain predictions, including the determination of the glycosyl hydrolase family 18 domains, were obtained from the SMART 7 - protein domain annotation resource (Letunic *et al.*, 2012) and from the SBASE Domain Prediction System. The theoretical pI/MW were computed using the ExPASy Bioinformatics Resources Portal (Atrimo *et al.*, 2012).

Expression and purification of *PsChiC*

The plasmid pET-*PsChiC* was transformed in *E. coli* BL21 (DE3) competent cells using the CaCl₂ heat shock method (Sambrook and Russell, 2001). The clones were streaked onto Luria-Bertani (LB) agar plates containing kanamycin (50 µg/mL) and the plates then incubated at 37 °C for 16 h. A single clone was selected and used to inoculate 10 mL of LB broth containing kanamycin (50 µg/mL) followed by incubation at 37 °C with vigorous agitation in a shaking incubator. Approximately 5 mL of overnight culture was used to inoculate 100 mL of LB medium containing kanamycin (50 µg/mL) in a 500 mL fermenter and the culture was grown at 30 °C with vigorous agitation. When cells reached an optical density of 0.6 at 600 nm, isopropyl-β-D-thiogalactoside (IPTG; 0.8 mM) was added to induce chitinase gene expression. After an appropriate period of induction (Fan *et al.*, 2007), the cells were harvested by centrifugation (3,000 x g, 15 min, 4 °C), ruptured on ice in an Ultrasonic Cell Disruptor and either used immediately or stored at -20 °C. After centrifugation (10,000 x g, 20 min) to remove cells, the total cell extract (crude chitinase) containing the intracellular chitinase was collected for measurement of chitinase activity or subjected to His-Tag affinity purification.

Chitinase assay

Pseudomonas sp. TXG6-1 was grown in 100 mL of basal medium containing 0.1% K₂HPO₄ and 0.05% MgSO₄•7H₂O (pH 7.0), and supplemented with 0.5-2% (w/v) of colloidal chitin as the sole carbon/nitrogen (C/N) source. The bacteria were cultured aerobically in a 250 mL Erlenmeyer flask at 30 °C for three days on a rotary shaker

(150 rpm). After centrifugation (3,000 x g, 4 °C, 15 min), the supernatants were collected for measurement of chitinase activity.

Chitinase activity was measured using a chitinase assay kit (Sigma-Aldrich), according to the manufacturer's instructions. The exochitinase (N-acetyl- β -glucosaminidase), chitobiosidase and endochitinase activities in the harvested culture medium were determined using p-NP-GlcNAc, p-NP-(GlcNAc)₂ and p-NP-(GlcNAc)₃ as substrates, respectively. The release of *p*-nitrophenol was monitored at 405 nm. A molar extinction coefficient for *p*-nitrophenol of $\epsilon = 17,700 \text{ M}^{-1}\text{cm}^{-1}$ was used to calculate the product concentration. Protein concentrations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Insecticidal bioassay

The synergistic effects of *PsChiC* on the insecticidal activity of *Spodoptera litura* nucleopolyhedrovirus (SpltNPV) on second-instar larvae of *S. litura* were studied using a surface contamination bioassay in 24-well trays. SpltNPV (1×10^7 particles) mixed with an artificial diet was added to the 24-well trays. Crude chitinase (20 μL , 50 mU) was soaked into each cube of artificial diet. For the control treatment, water instead of SpltNPV was added to the artificial diet. Three replicates were done for each treatment and each replicate contained 30 larvae. The plates were incubated at 26 ± 1 °C, 85% humidity and a 12 h light/dark photoperiod. The confidence intervals and other regression parameters were determined using the SPSS program.

Results

Identification of *Pseudomonas* TXG6-1

For morphological and biochemical classification, the microscopic characteristics of the *Pseudomonas* strain were analyzed with a Nikon H-600L system (Nikon, Japan). The sequences of TXG6-1 16S rRNA (1400 bp) were provisionally identified and classified based on BLASTN analysis against several 16S rRNA gene databases (*Pseudo*MLSA, GenBank and EMBL). A homology search indicated that *Pseudomonas* strain TXG6-1 had > 99% sequence identity with *Pseudomonas putida* ZX-PKU-004 (GenBank accession number DQ912807). Figure 1 shows a Neighbor-Joining phylogenetic tree inferred from the sequences of the isolate and related organisms and clearly indicates that the isolate belongs to the genus *Pseudomonas*.

Bioinformatic comparison of *PsChiC*

The open reading frame of *PsChiC* had A+T = 40.4% and G+C = 59.6%. *PsChiC* consisted of 480 amino acids, with a predicted molecular mass of 51.66 kDa and pI of 5.36. Amino acid sequence analysis revealed that *PsChiC* shared high sequence homology with several *Serratia* and

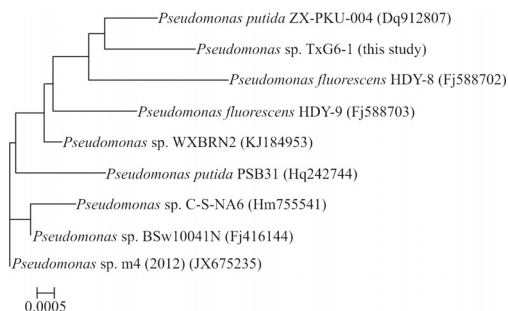


Figure 1 - Rooted neighbor-joining distance matrix tree derived from the 16S rRNA gene sequences of TXG6-1 and related bacterial. Accession numbers for the 16S rRNA gene sequences used are given in parentheses after the species and strain names. The scale bar represents a 0.05% nucleotide substitution rate based on Kimura's two-parameter model.

Pseudomonas chitinases, such as *Serratia marcescens* chitinase C1 (99.17%, accession no. BAA76623; 98.54%, CAF74787; 98.13%, ABI79318), *Pseudomonas aeruginosa* PA7 chitinase (66.25%; ABR83459) and *Pseudomonas fluorescens* Pf-5 chitinase (65.83%; AAY91366). Bacterial family 18 chitinases can be divided into three subfamilies (A, B and C) based on the amino acid sequence similarity of their catalytic domains (Suzuki *et al.*, 1999). The major structural difference deduced from comparison of the amino acid sequences of subfamilies A and B is that chitinases in subfamily A have an insertion domain between the seventh and eighth β -strands of the $(\alpha+\beta)_8$ -barrel catalytic domain that is absent in subfamily B chitinases. *PsChiC* has the catalytic domain of subfamily B instead of subfamily A. Prediction of the functional domains of the protein revealed a glycoside hydrolase family 18 catalytic domain, a fibronectin type III-like domain (FLD) and a chitin-binding domain (ChBD).

The *PsChiC* N-terminal region (residues 25-305) showed similarity to the catalytic domains of chitinases belonging to glycosyl hydrolase family 18 (Henrissat and Bairoch, 1996; Henrissat, 1999). The region exhibited extensive similarity to the catalytic domains of several bacterial chitinases, such as *Serratia marcescens* chitinase C1 (97.51%) and *Vibrio harveyi* chitinase A (60.14%) (Figure 2). In this region, the deduced amino acid sequence from residues 134 to 141 (DGLDIDLE) was homologous to the active site motif of enzymes in glycosyl hydrolase family 18 ([DN]-G-[VFILM]-[DN]-[LFIMV]-[DN]-X-E) that form the fourth β -strand in the $(\beta/\alpha)_8$ -barrel of their catalytic domain (Henrissat and Bairoch, 1993; Aronson Jr *et al.*, 2006). Furthermore, amino acid residues D-137, D-139 and E141 of *PsChiC* (corresponding to D-200, D-202 and E204 of *Bacillus circulans* chitinase ChiA) were well conserved and may play an essential role in chitinase activity (Watanabe *et al.*, 1993).

As shown in Figure 3, the C-terminal region (residues 433 to 479) exhibited significant similarity not only to the ChBDs of various bacterial chitinases, but also to the C-ter-

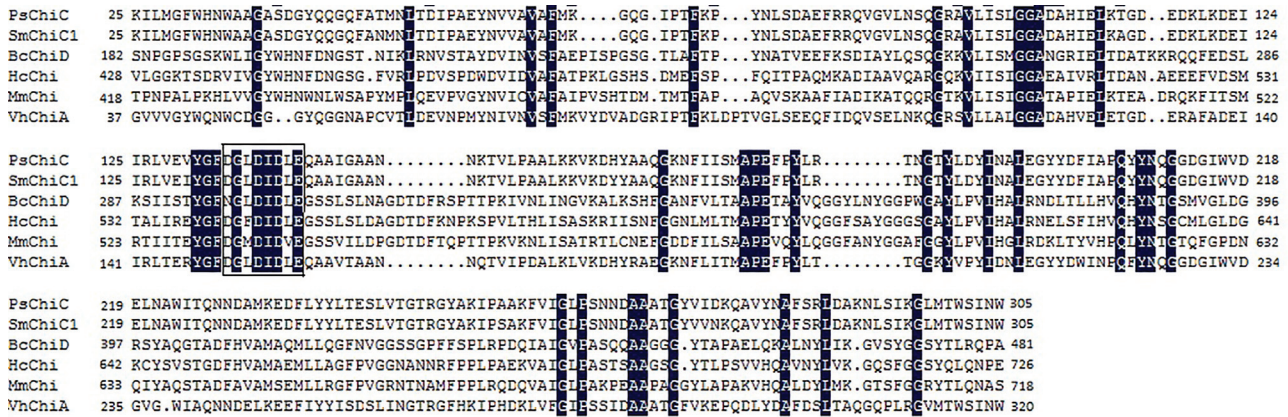


Figure 2 - Amino acid sequence alignment of the catalytic regions of *PsChiC* and several bacterial chitinases. *PsChiC* - *Pseudomonas* sp. TXG6-1 chitinase C (this study), *VhChiA* - *Vibrio harveyi* chitinase A (AAC46383), *SmChiC1* - *Serratia marcescens* chitinase C1 (CAF74787), *BcChiD* - *Bacillus circulans* chitinase D (P27050), *HcChi* - *Hahella chejuensis* KCTC 2396 chitinase (YP_432187) and *MmChi* - *Microscilla marina* ATCC 23134 chitinase (ZP_01688190). Black shading indicates amino acid residues that are identical. The active site motif is boxed.

minal regions of *Streptomyces griseus* serine protease (Sidhu *et al.*, 1994), and *Vibrio alginolyticus* deacetylase (Ohishi *et al.*, 2000). In the ChBD region of many chitinases, aromatic amino acids (W and Y) are highly conserved and may play a crucial role during binding to the pyranosyl rings of N-acetylglucosamine residues in chitin (Morimoto *et al.*, 1997).

The middle region (residues 338 to 411) showed similarity to the fibronectin type III-like domain (Fn3) sequences found in the R-1 and R-2 regions of *Bacillus circulans* chitinase A1 (32.20% and 30.77% identity, respectively; Watanabe *et al.*, 1990), and Chi74FLD1 and Chi74FLD2 of *B. thuringiensis* (16.22% and 24.32%

identity, respectively; Barboza-Corona *et al.*, 2003) (Figure 4). Fibronectin is a multifunctional extracellular matrix and plasma protein and plays a significant role in cell adhesion. Fibronectin type III-like domain (FLD) has been found in chitinase, cellulases, α -amylase and poly(3-hydroxybutyrate) (PHB) depolymerase (Little *et al.*, 1994). Among bacterial enzymes capable of degrading insoluble substrates, the Fn3 domains are most frequently found in chitinases. The widespread occurrence of this domain in chitinases may have special relevance for the degradation of insoluble and crystalline polysaccharide chitin (Suzuki *et al.*, 1999).

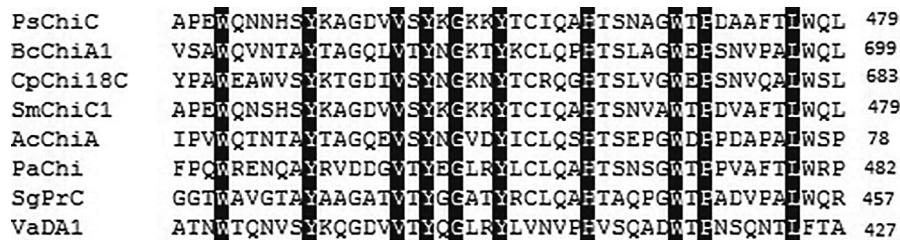


Figure 3 - Amino acid sequence alignment of the chitin-binding domains of *PsChiC* with those of other bacterial enzymes (protease, deacetylase and chitinases). *PsChiC* - *Pseudomonas* sp. TXG6-1 chitinase C (this study), *BcChiA1* - *Bacillus circulans* chitinase A1 (AAA81528), *CpChi18C* - *Clostridium paraputrificum* chitinase 18C (BAD29950), *SmChiC1* - *Serratia marcescens* GEI chitinase C1 (ACX42073), *AcChiA* - *Acidobacterium capsulatum* ATCC 51196 chitinase A (ACO33699), *PaChi* - *Paeruginosa* UCBPP-PA14 chitinase (ABJ11480), *SgPrC* - *Streptomyces griseus* serine protease (AAA26813), and *VaDA1* - *Vibrio alginolyticus* deacetylase DA1 (CAC29092). Black shading indicates identical amino acid residues.

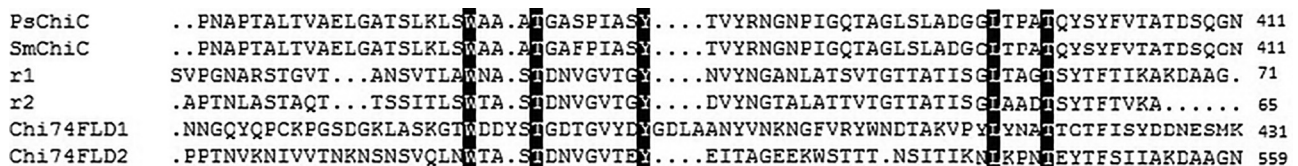


Figure 4 - Alignment of the Fn3-like domain of *PsChiC* with those of other bacterial enzymes. The sequences of *PsChiC* Fn3-like are aligned with those of *Serratia marcescens* chitinase C1 (CAF74787), *Bacillus circulans* chitinase A1 (r1 and r2, AAA81528) and *Bacillus thuringiensis* serovar kenaya ChiA74 (Chi74FLD1 and Chi74FLD2, AAL17867). Black shading indicates identical amino acid residues.

Expression and purification of *PsChiC*

The *PsChiC* gene was subcloned into pET30a to construct the recombinant expression vector pETPsChiC. The identity of pETPsChiC was identified by restriction enzyme digestion and DNA sequencing. The protein in supernatant from *E. coli* BL21 (DE3) cultures grown in LB medium was purified through a His-Tag affinity resin designed for the recombinant chitinase. The enzyme activity and protein concentration of the fractions were determined. The molecular mass of the major band of purified *PsChiC* was estimated to be 52 kDa by SDS-PAGE (Figure 5). This molecular mass agreed well with the calculated molecular mass of 51.66 kDa based on the 480 amino acid sequence. Expression of the fusion protein was observed 1 h after induction, with maximal expression occurring at 6 h.

Chitinase assay

Chitinolytic activities were quantified with a liquid assay using p -NP-(GlcNAc)_n (n = 1-3) as substrates. The chitinase expressed in *E. coli* BL21(DE3)/ pETPsChiC showed hydrolytic activity towards the chitin tetrameric derivative (4-MU-(GlcNAc)₃) and trimeric derivative [(4-MU-(GlcNAc)₂], with practically no activity against the dimeric derivative (4-MU-GlcNAc) (Table 1). This finding indicated that *PsChiC* had both chitobiosidase and endochitinase activities, but no exochitinase activity.

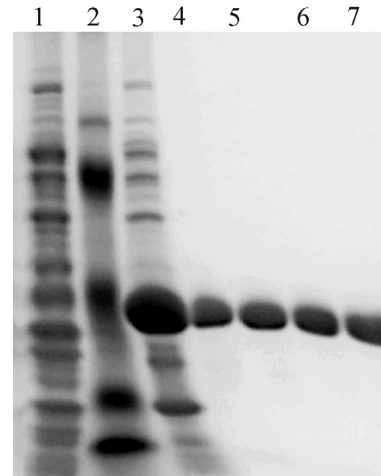


Figure 5 - SDS-PAGE of recombinant *PsChiC*. The gel was stained with Coomassie brilliant blue R-250. Lane 1 - induced BL21(DE3)/pET30a, lane 2 - molecular mass markers (105, 71, 50, 35 and 25 kDa), lane 3 - BL21(DE3)/pETPsChiC induced for 4 h, lanes 4-7 - purified *PsChiC*.

Insecticidal activity

Although crude *PsChiC* had little activity against *S. litura* larvae, co-culture with SpltNPV enhanced the insecticidal activity of the latter (Table 2). When crude *PsChiC* was mixed with 1×10^7 SpltNPV, some *S. litura* larvae began to die after 72 h while larvae fed an artificial diet of SpltNPV alone began to die at 96 h. The mortality rates were 91.1%, 51.1% and 17.8% at 192 h for *S. litura* larvae

Table 1 - Chitinolytic activities of *Pseudomonas* chitinase C (*PsChiC*) expressed in *E. coli*.

| Sample | Protein | Chitinolytic activity (units/mg of protein) | | |
|--|---------------|---|---------------------------|--------------------------|
| | | Endochitinase* | Chitobiosidase | Exochitinase |
| <i>E. coli</i> without vector | Secreted | 0.00 ± 0.00 ^d | 0.05 ± 0.01 ^c | 0.01 ± 0.01 ^b |
| <i>E. coli</i> with pET- <i>PsChiC</i> | Secreted | 63.02 ± 0.11 ^b | 21.33 ± 0.07 ^b | 0.01 ± 0.01 ^b |
| <i>E. coli</i> with pET- <i>PsChiC</i> | Intracellular | 34.42 ± 0.32 ^c | 10.47 ± 0.04 ^b | 0.02 ± 0.01 ^b |
| <i>Pseudomonas</i> sp. TXG6-1 | Secreted | 15.26 ± 0.94 ^a | 8.55 ± 0.66 ^a | 1.73 ± 0.13 ^a |

The data are expressed as the mean ± SD. Values with the same letter were not significantly different as determined by Tukey's multiple range test ($p < 0.05$). *The substrates used for the enzymatic assays were: p -nitrophenyl N,N'-diacetyl- β -D-chitobioside (chitobiosidase activity), p -nitrophenyl N-acetyl- β -D-glucosaminide (exochitinase activity) and 4-nitrophenyl β -D-N,N',N''-triacylchitotriose (endochitinase activity). In all cases, formation of the reaction product (p -nitrophenol), which ionizes in basic pH, was monitored colorimetrically at 405 nm.

Table 2 - Synergistic effects of *Pseudomonas* chitinase C gene (*PsChiC*) on the insecticidal activity of SpltNPV.

| Treatment (h)* | 72 | 96 | 120 | 144 | 168 | 192 |
|-------------------------|-----------------------|------------------------|-------------------------|-------------------------|-----------------------|-----------------------|
| SpltNPV | 0 ^{ij**} | 2.22 ^{Hij} | 10 ^{FGghi} | 13.33 ^{DEFfgh} | 20 ^{De} | 51.11 ^{Bc} |
| SpltNPV + <i>PsChiC</i> | 4.44 ^{GHIij} | 12.22 ^{EFfgh} | 15.56 ^{DEFfgh} | 27.78 ^{Cd} | 57.58 ^{Bb} | 91.11 ^{Aa} |
| <i>PsChiC</i> | 0 ^{ij} | 0 ^{ij} | 0 ^{ij} | 4.44 ^{GHIj} | 8.89 ^{FGHhi} | 17.78 ^{DEef} |
| CK | 0 ^{ij} | 0 ^{ij} | 0 ^{ij} | 0 ^{ij} | 2.22 ^{Hij} | 2.22 ^{Hij} |

All data analyses were done using SPSS software. *Three replicates were done for each treatment and each replicate contained 30 larvae. **Numbers indicate mortality rate (%), uppercase letters indicate $p < 0.01$ and lowercase letters indicate $p < 0.05$. SpltNPV - *Spodoptera litura* multicapsid nucleopolyhedrovirus.

grown on artificial diet supplemented with 1×10^7 SpltNPV + 50 mU crude chitinase, 1×10^7 SpltNPV, and 50 mU crude chitinase, respectively. *PsChiC* alone was also insecticidal to *S. litura* larvae.

Discussion

The low yield of chitinase-producing strains is one of the major problems in the study and application of chitinases. The production of recombinant proteins in active form is therefore of great importance for studying the applications of these enzymes. Expression systems based on *E. coli* have been commonly used to express heterologous proteins because this bacterium is well characterized with regard to its molecular genetics, physiology and availability of different expression systems (Balbás, 2001). In this study, PCR primers specific for the *chiC* gene were used to amplify a chitinase gene fragment and to confirm the presence of the *PsChiC* gene in *Pseudomonas* strain TXG6-1. The chitinase *PsChiC* gene was transformed in an expression vector pET30a to construct the recombinant expression plasmid, pETPsChiC. The fusion protein PsChiC-His was successfully expressed in *E. coli* cells. The ability to produce this chitinase in *E. coli* will facilitate large-scale production and subsequent structural and functional studies of this protein.

Chitinases from similar or closely related species exhibit high sequence similarity (Henrissat, 1991). Based on the 16S rDNA sequence analysis, strain TXG6-1 belongs to the genus *Pseudomonas*, and comparison of the amino acid sequence of *PsChiC* from strain TXG6-1 with the *chiC* sequences of other bacterial chitinase indicated that *PsChiC* had a high level of homology (77.1-99.2%) with the corresponding enzymes from some gram-negative bacteria, including *Serratia marcescens*, *S. proteamaculans*, *S. odorifera*, *P. aeruginosa* and *P. fluorescens*. This finding suggests that the *PsChiC* gene of strain TXG6-1 and the *chiC* genes of these gram-negative bacteria were recently derived from the same ancestral chitinase gene.

The effective control of pest insect populations is an essential prerequisite for producing food and commodities for man and domestic animals. Chemical insecticides are highly effective in controlling pest insect populations. However, the use of chemical pesticides has led to several problems, including environmental contamination, the development of resistance to insecticides, the elimination of beneficial or non-target insects, and adverse effects on human health, including cancer and several immune system disorders (Andersen *et al.*, 2012; Baldwin *et al.*, 2013). The emergence of pesticide-resistant insect populations has also caused major outbreaks of secondary pests (Devine and Furlong, 2007). Thus, the search for safer agents and methods of plant protection has intensified in recent years.

The use of biological insecticides, alone or in combination with other agents and methods, in an integrated pest management system has gained momentum during the last

30 years (Ashour *et al.*, 2007). Nucleopolyhedroviruses (NPVs) (family Baculoviridae) infect the larvae of many important lepidopteran pests and several of these viruses have been developed as commercial biopesticides (Moscardi, 1999). However, NPVs are of limited usefulness since their narrow spectrum of activity enables them to kill only certain insect species. NPVs are also not very resistant in the environment and require precise application practices since many of these pathogens are specific to young insect larval stages or are sensitive to radiation. The most important limitation is that the killing rate of NPVs is lower than that of chemical insecticides. Consequently, there is a need for agents to improve the speed of killing by NPVs so as to expand the use of NPVs. Several studies have demonstrated the potential use of chitinase for controlling insect pests because of the enzymes disrupting effect on the peritrophic membrane. In lepidopteran larvae, the chitinases (from AcMNPV and SpliMNPV-K1, respectively) act by disrupting the peritrophic membrane and increasing its permeability (Rao *et al.*, 2004; Wang *et al.*, 2013). As shown here, *PsChiC* alone had limited insecticidal activity against *S. litura* larvae but markedly enhanced the activity of SpltNPV against these larvae. This finding suggests that *PsChiC* could be useful in enhancing the pathogenicity of baculoviruses.

Acknowledgments

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Internet Resources

- SMART 7 - protein domain annotation resource, <http://smart.embl-heidelberg.de/> (accessed on May 5, 2014).
- SBASE Domain Prediction System, <http://hydra.icgeb.trieste.it/sbase/> (accessed on May 5, 2014).
- ExpASy Bioinformatics Resources Portal, http://www.expasy.org/cgi-bin/pi_tool.html server (accessed on May 5, 2014).

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