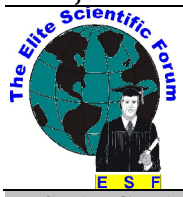
Pak. j. life soc. Sci. (2012), 10(2): 116-122 E-ISSN: 2221-7630;P-ISSN: 1727-4915



Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Heterologous expression and characterization of an antifungal chitinase Chi39 from *Bacillus thuringiensis* serovar *konkukian*

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ARTICLE INFO
Received: Sep 08, 2012
Accepted: Oct 21, 2012
Online: Oct 24, 2012

Keywords
Antifungal
Bacillus thuringiensis
Chitinase
Chitin degradation

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ABSTRACT

A chitinase gene from *Bacillus thuringiensis* serovar *konkukian* S4 was cloned, sequenced, and heterologously expressed in *Escherichia coli* M15. The recombinant enzyme (Chi39) was purified by Ni-NTA affinity column chromatography. The *chi39* gene was shown to contain a single open reading frame (ORF) with a capacity to encode a protein with a predicted molecular mass of 39 kDa and isoelectric point of 5.75. Comparison of Chi39 with other chitinases has shown this enzyme to contain a single N-terminal family 18 catalytic-domain. The turnover rate (K_{cat}) of the enzyme was determined (28.3±0.70 s^{-1}) using colloidal chitin as substrate. The purified enzyme was active at a broad range of pH (pH 4.5–8.0) and temperature (4-75 ^{o}C) with a peak activity at pH 5.0 and 60^{o}C. However, the enzyme activity was found to be stable up to 50 ^{o}C for longer incubation periods (36 h). Moreover, purified enzyme was shown to inhibit fungal spore germination and hyphal growth of pathogenic fungi *Fusarium oxysporum* and *Aspergillus niger*. The present study will lead us to develop biocontrol agent.

INTRODUCTION

Bacillus thuringiensis is an insecticidal bacterium whose activity is based on the effect of single or mixed Cry or Cyt proteins, acting additively or synergistically (Del Rinco´n-Castro et al., 1999). Along with the insecticidal proteins of *B. thuringiensis*, the chitinases of *B. thuringiensis* are also worth consideration due to their potential role as biological control agents for insects and plant-pathogenic fungi (Barboza-Corona et al., 2008; Driss et al., 2007; de la Vega et al., 2006). Several genera of bacteria, including *Serratia* (Mehmood et al., 2009; Suzuki et al., 2002; Watanabe et al., 1997), *Enterobacter* (Chernin et al., 1995), and *Aeromonas* (Mehmood et al., 2010a; Wang et al., 2003) produce high levels of chitinolytic enzymes. Generally, the expression level of the chitinases from *B. thuringiensis* is lower as compared to the chitinases from other sources, this is one of the reasons to clone, overexpress and characterize these chitinases. So far, several bacterial *chi* genes have been studied from *B. thuringiensis* (Mehmood et al., 2010; Barboza-Corona et al., 2008; Driss et al., 2007; Barboza-Corona et al., 2003; Thamthiankul et al., 2001; Chernin et al., 1995; Fuchs et al., 1986). But the insecticidal system of the *B. thuringiensis* needs to be improved due to several reasons such as; development of resistance against Cry-proteins, narrow range of activity, slower mode of action and the obligation of ingestion of Cry-proteins.

The peritrophic membrane of the insect midgut is reinforced with chitin fibers. If the peritrophic membrane is damaged or degraded, insecticidal activity of Cry-proteins is believed to be enhanced (Regev et al., 1996). Chitinases are the enzymes which may be exploited in this aspect. The role of chitinases in *B. thuringiensis* pathogenicity has been demonstrated previously (Ding et al., 2008; Thamthiankul et al., 2001; Sampson and Gooday 1998). Furthermore, chitinases from *B. thuringiensis* subsp. *kurstaki* HD-1(G) culture supernatants increased the insecticidal activity against *Plutella xylostella* (Wiwat et al., 2000). Quantitative synergistic effect of purified chitinases and Cry proteins has not been clearly demonstrated, possibly due to the low levels of expression of

chitinases in *B. thuringiensis*. The chitinases from bacteria have also been used in suppressing several fungal phytopathogens. The purified chitinases from *Serratia plymuthica* HRO-C48 and *Stenotrophomonas maltophilia* have shown inhibitory effect on the growth of *Botrytis cinerea* and *B. sorokiniana,* respectively (Frankowski et al., 2001; Zhang et al., 2000). It was elucidated that a single chitinase was responsible for the antifungal activity of *Stenotrophomonas maltophilia* 34S1 against a root infecting fungus *Magnaporthe poae* (Kobayashi et al., 2002). The purified chitinases from *Serratia proteamaculans* 18A1 and *Bacillus thuringiensis* subsp. *konkukian* showed inhibition to spore germination and hyphal extension of *Fusarium oxysporum* and *Aspergillus niger* (Mehmood et al., 2009).

Moreover, the human immune system can recognize and degrade chitin that is one of the important constituents of pathogenic fungal cell wall. Human chitinases and chitin binding proteins are becoming the point of interest for this reason. It is believed that the mechanisms of host chitinase responses may not only have applications in diagnostic assays as well as in the development of novel therapeutic approaches for patients that are at risk of fungal infections (Vega et al., 2012).

In the present study, we have characterized chitinase Chi39 from *Bacillus thuringiensis* serovar *konkukian.* We have shown that purified enzyme remains active at a broad range of temperature and pH, and has antifungal activity against pathogenic fungi such as *F. oxysporum* and *A. niger*.

MATERIALS AND METHODS

Microorganisms and plasmids

The *B. thuringiensis* serovar *konkukian* S4 was obtained from BIRCEN (Bio-Resource Centre) culture collection, NIBGE, Faisalabad, Pakistan. Strain was maintained in LB-medium (1.0 % Trypton, 0.5 % Yeast extract, 1 % NaCl) at 30 ^{o}C. The plasmid pQE-30 (Qiagen) was used as expression vector, and *E. coli* strain M15 was used as an expression host. Two fungal strains, *Aspergillus niger* and *Fusarium oxysporum*, were obtained from China General Microbiological Centre, Beijing, China. Fungal strains were grown on PDA (2 % Potato starch, 2 % Dextrose, and 1.5 % Agar) plates.

Cloning and sequencing of the chitinase gene

Genomic DNA was extracted from *B. thuringiensis* serovar *konkukian* S4 and purified as described by Li et al. (2007). This DNA was used as a template to amplify *chi39* gene by polymerase chain reaction (PCR). A pair of primers was designed according to the initial and terminal conserved regions of several chitinases of *B. thuringiensis* (Accession numbers). Primers ChiF39 (5'-CACGCATGCGCAAACAATTTAGGATCAAAACT A) and ChiR39 (5'-AGCCTGCAGTTATTTTGCAA GGGAATCGCAT-3') were used to amplify *chi39*, containing *Sph*I and *Pst*I restriction sites (underlined), respectively. The PCR was performed with *Pfu*-polymerase high fidelity enzyme (Tiangen) in Mastercycler gradient (Eppendorf) for 30 cycles of 94°C for 1 min, 55 °C for 1 min, 72 °C for 1 min in each cycle, followed by a 10 min final termination step at 72°C. The PCR product was purified using the QIAquick gel extraction kit (Qiagen). The amplicon was then digested with restriction enzymes and was ligated into pre-digested pQE-30 vector. The recombinant vector was transformed into chemical competent cells of *E. coli* M15 by heat shock method (Froger and James, 2007). The transformants were selected in Luria-Bertani (LB) agar supplemented with ampicillin (100 $\mu g.mL^{-1}$). The positive clones were screened by colony PCR. The recombinant plasmids were extracted by plasmid extraction kit (Qiagen) and selection was carried out by restriction analyses and sequencing (Sangon, Shanghai). The gene sequence was submitted to GenBank (GenBank data, GQ183830) and the recombinant plasmid was named as pQE-Chi39.

Expression and purification

The *E. coli* strain M15 harboring the pQE-Chi39 vector was grown at 37 ^{o}C. When the OD_{600} was reached to 0.8-1.0, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM. The culture was further incubated for 6 h. Cells were harvested by centrifugation and stored at -20 ^{o}C. Previously stored cells at -20 ^{o}C were transferred to ice and kept on ice for 30 min to thaw. The cells were resuspended in lysis buffer (50 mM Na-phosphate buffer pH 8.0, 300 mM NaCl, and 10 mM imidazole). Lysozyme was added at a final concentration of 1.0 mg mL^{-1} to facilitate the lysis. The cell lysis was performed mechanically using sonicator equipped with sonication microtip, using six 10 s bursts at 200–300 Watt, with a 10 s cooling period between each burst. After lysis, cell debris was removed by centrifugation at 20,000 x*g*. Total soluble proteins were applied to a Ni-NTA matrix column (Qiagen) and Chi39 was purified following the instruction of the manufacturer (Qiagen). Purified elutes were dialyzed against 25 mM Na-phosphate buffer (pH 8.0). After dialysis, purity of the Chi39 was further confirmed by SDS-PAGE (12.5 %) analysis and concentrations were measured by Bradford method (Bradford, 1976).

Enzyme activity and protein assays

The enzyme activity was measured using different subtypes of chitin. The colloidal chitin was prepared from crab shells according to the method of Shimahara and Takiguchi (Shimahara and Takiguchi 1988), unprocessed α-chitin from crab shell (Sigma), unprocessed β-chitin (prepared from octopus). The 50

pmol of Chi39 was incubated with 1.5 mg substrate (unprocessed α and β-chitin, colloidal chitin) in 600 µL 100 mM sodium phosphate buffer, pH 6.0, at 55 °C for 1 h. One unit of enzyme activity was defined as the amount of the enzyme required to release 1 µmol of reducing sugar in one min. The reducing sugars were measured according to DNS assay (Miller 1959). A range of substrate concentrations (colloidal chitin; 5-150 mg mL^{-1}) was used to determine the turnover rate (K_{cat}) of the enzyme. All the assays were performed in triplicates and the data were fitted into Michaelis-Menten equation by GraphPad Prism software (GraphPad Software Inc., San Diego, CA) and the turnover rate (K_{cat}) was calculated by the same software.

Effect of temperature, pH and metal ions on enzyme activity

Chitinase activity was assayed at different pH values (pH 2.5 to 9.0) using buffers, such as citrate-phosphate buffer (100 mM, pH, 2.5-7.0), sodium phosphate buffer (100 mM, pH, 7.0-8.5), and glycine-NaOH buffer (100 mM, pH, 8.5-10.0). The enzyme activity was also assayed at different temperatures ranging from 4-80 ºC at pH 5.0 in citrate phosphate buffer (100 mM). To determine thermostability, the purified enzyme was incubated at a range of temperatures (30-60 ºC) for different time intervals up to 36 h, prior to perform the enzyme activity assay. Moreover, the enzyme activity was determined in the presence of different metal ions at a range of ion concentrations (0-100 mM) under the standard assay conditions. Metal ions were provided as their chlorides or sulfates. All experiments were performed in triplicates, along with control and the data were adjusted to Gaussian model ($P< 0.05$) using Prism software.

Antifungal assays

The antifungal assays were performed as described previously (Mehmood et al., 2009; Ghosh 2006; Reyes-Ramírez et al., 2004). In spore germination inhibition assay, fungal spores were collected using sterile distilled water, containing 0.1 % (v/v) Tween 20, from PDA plates after 10 days of growth at 26 ^{o}C. Final concentrations of spores were adjusted to 1 x 10^6 by microscopy. Later, 100 µL of the spores were mixed with 20 µg of the purified enzyme and pre-incubated for 3 h. The mixtures were inoculated at the centre of the PDA plates and further incubated for 72 h. The 20 µg of BSA was mixed with 100 µL of spores in the same buffer and was considered as negative control. In fungal hyphal extension inhibition assay, the inocula were provided at the centre of the PDA plates surrounded by wells containing 20 µg of the enzyme per well. The plates containing equal amount of BSA protein instead of the Chi39 in the wells, were considered as negative control. Diameter of the fungal growth in control (only fungus) and experimental plates (fungus + purified Chi39) was measured and difference in growth on both plates gave us the inhibition. The percentage inhibition was calculated as described previously (Mehmood et al., 2009). All experiments were performed in triplicates and data were analyzed by GraphPad Prism software.

RESULTS AND DISCUSSION

The *B. thuringiensis* serovar *konkukian* S4 was found to be an efficient chitin utilizing strain on chitin agar plates. Furthermore, the strain has shown antifungal activity against the phytopathogenic fungi *F. oxysporum* (unpublished data). The *F. oxysporum* is resistant to common fungicides and causes wilt in more than 100 plant species (Mehmood et al., 2009). To demonstrate the role of chitinases in the antifungal activity of the S4, a chitinase gene (1,083 bp), encompassing the complete coding sequences of Chi39 was cloned and sequenced. The sequence analysis of *chi39* revealed a single open reading frame with a capacity to encode a protein containing 360 amino acid residues, with a deduced molecular mass of 39 kDa and an isoelectric point of 5.75. Comparison of deduced amino acid sequence with the chitinases of genus *Bacillus* has shown this to have an identity range of 94 and 98 %. The SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) program, indicated the presence of a putative signal peptide, recognized by Gram positive bacteria, predicting a cleavage site located between Ala-27 and Ala-28. The enzyme showed a single catalytic domain (Lys-34 through Asp-338) which belongs to family 18 of glycosyl hydrolases. A family 18 chitinase active site FDGIDIDLE was found to be present between Phe-137 through Glu-145, which is the most conserved among the family 18 chitinases. Comparison of Chi39 gene from *B. thuringiensis* serovar *konkukian* with reported chitinases indicated 98.6 % homology with chi36 from *B. cereus* (Wang et al., 2001) and 94.4 % homology with chitinase-A from *B. cereus* CH (Mabuchi and Araki 2001) which indicates the evolutionary closeness of the *B. thuringiensis* with *B. cereus* group.

The SDS-PAGE analysis of the purified protein has revealed the molecular mass of the purified protein close to 39 kDa (Fig. 1A), which corresponds well with the calculated molecular mass of 39, 432 Daltons. The enzyme was shown to degrade crystalline chitin (α and β-chitin) and colloidal chitin. The enzyme units were found highest for colloidal chitin (353 units) followed by unprocessed β-chitin (36 units) and unprocessed α-chitin (10 units) (Fig. 1B). The colloidal chitin was used as substrate to determine the rate of reaction (k_{cat} = 17.75 ± 0.93 s^{-1} and K_m = 17.75 ± 0.93 mg). The colloidal chitin is the pulverized form of the chitin due to the acid treatment during preparation that might be

the possible reason for higher activity units of the enzyme with colloidal chitin.

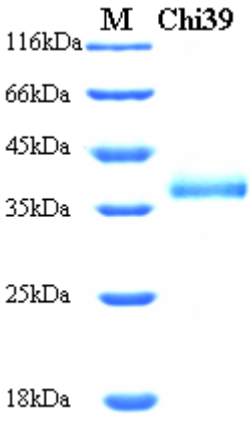
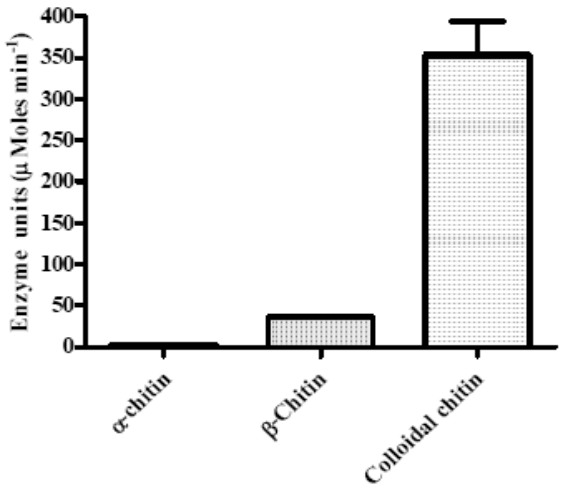


Fig. 1A: SDS-PAGE analysis of the purified Chi39
Single band on SDS-PAGE (12.5 %) shows the purified protein of almost 39 kDa in size, in comparison with marker (Fermentas).



1B: Polysaccharide degradation assay for Chi39
Enzyme assay was performed at 55°C, in 100 mM Na-phosphate buffer (pH = 6.0) using different polysaccharides (colloidal chitin, α-chitin, β-chitin) as substrate. All experiments were performed in triplicate along with control and blank.

The enzyme was active at a broad range of temperatures from 4 to 80 °C with peak activity at 60 °C (Fig. 2A). Although the enzyme was shown to retain 49 % of its activity at 75 °C, yet this activity was not stable at this temperature for longer incubation periods, as revealed by thermostability assay. However, the enzyme was quite stable up to 50 °C and could retain 80.0 % of its activity for the pre-incubation period of 36 h at 50 °C (Fig. 2B). Moreover, the enzyme was active at a range of pH (4.5-8.0), with the peak activity at pH 5.0 (Fig. 2C). Different metal ions were used to study their effect on the enzyme activity. The divalent ions Ca^{+2} (10 mM) and Cu^{+2} (10 mM) intended to increase the enzyme activity by 1.5 and 1.4 fold, respectively. Overall, mineral ions concentrations > 10 mM showed negative influence on the enzyme activity (Fig. 2D).
The purified Chi39 was shown to have antifungal potential against the pathogenic fungi *F. oxysporum* and *A. niger*. The enzyme inhibited spore germination by 90 % and 92 %, respectively. The hyphal extension was inhibited by 72 % and 82 %, respectively (Fig. 3). Hence, it was affirmed that production of Chi39 is one of the mechanisms used by the *B. thuringiensis* serovar *konkukian* to suppress the pathogenic fungi.

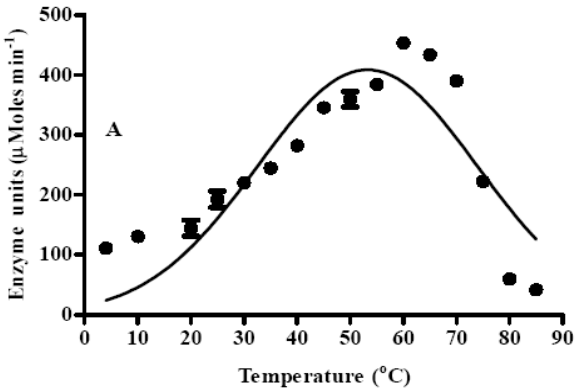
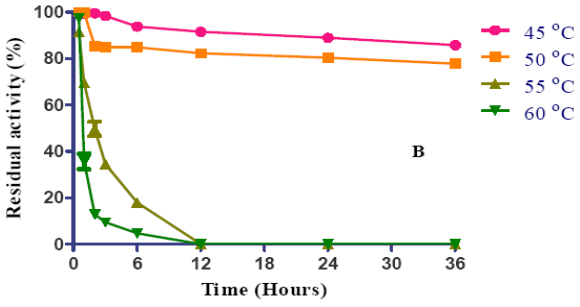
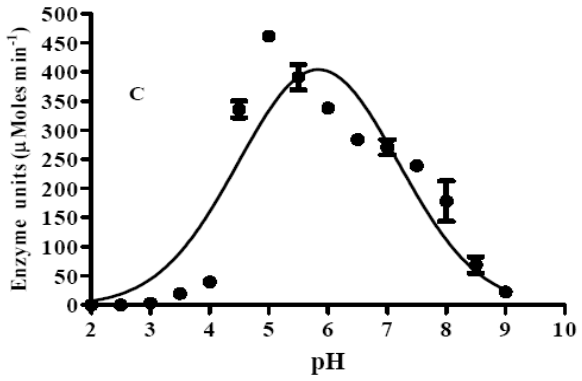


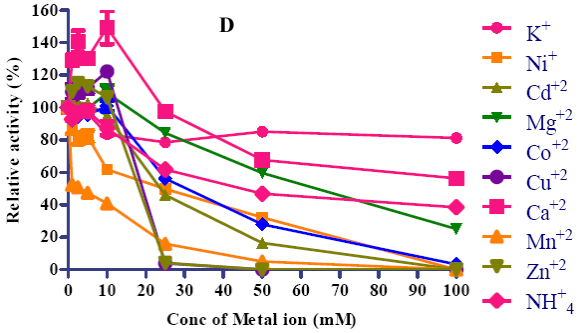
Fig. 2A: Effect of temperature on enzyme activity
Enzyme assays were performed at a range of temperature (4-75°C) under assay conditions using colloidal chitin as substrate.



2B: Thermostability of purified Chi39
Enzyme was pre-incubated at different temperatures (45-60°C) for 36 h. Control enzyme solution was stored at 4°C for comparison. Enzyme assays were performed under standard conditions. Bars indicate the average value of three independent experiments.



2C: Effect of pH on enzyme activity
Enzyme assays were performed at a range of pH (2.5-9.0) under assay conditions using colloidal chitin as substrate.



2D: Effect of metal ions on enzyme activity
Assays were performed in presence of different metal ions (0-100 mM) under assay conditions using colloidal chitin as substrate. All experiments were performed in triplicates along with control. Data were adjusted to Gaussian model (P>*0.05*) using Prism Software. Points indicate the average value of three independent experiments.

Conclusion and future perspectives

In conclusion, the enzyme could degrade crystalline chitin efficiently at a range of temperature and pH. The enzyme activity is quite stable up to 50 °C for 36 h incubation periods. These features make the enzyme suitable for the industrial, environmental and agricultural applications. Previously, the chitinase Chi74 (Mehmood et al., 2010b) and the chitin binding protein CBP50 (Mehmood et al., 2011) have shown antifungal against the phytopathogenic fungi such as *F. oxysporum* and *A. niger*. But, still further experimentation is required to reveal that whether these enzymes either have insecticidal activity or have synergistic effect with insecticidal system of the *B. thuringiensis*. Careful laboratory and field trials are required to demonstrate the role of Chi39 in insecticidal activity of Cry-proteins to improve the insecticidal system of the *B. thuringiensis*. Furthermore, co-expression of Chi39, Cry-proteins may also contribute to develop fungal resistance in economically important crop.

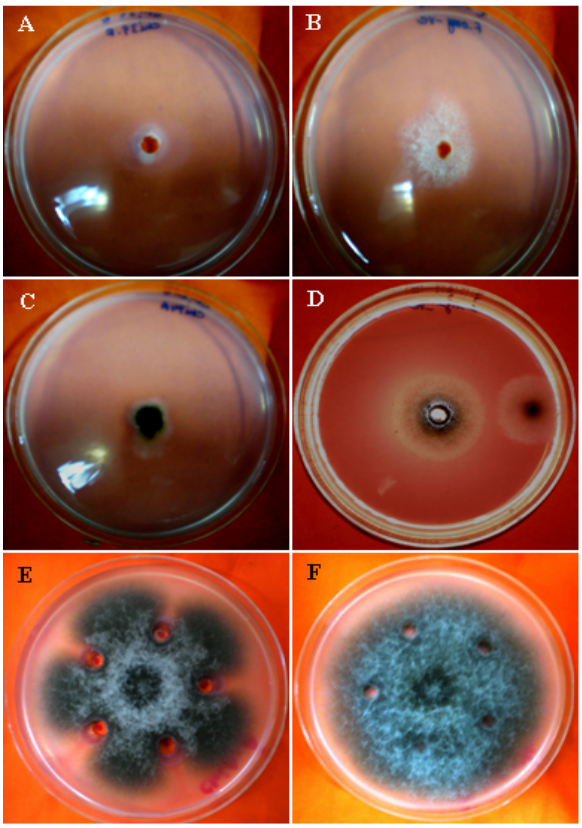


Fig 3: Antifungal assays
A & B: Spore germination inhibition of *F. oxysporum*, where B is negative control. C & D: Spore germination inhibition of *A. niger*, where D is negative control. E & F: Hyphal growth inhibition of *A. niger*, F is negative control. Diameter of the fungal growth in control and experimental plates was measured and difference in growth on control and experimental plates gave us the inhibition.

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