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Research Article

Cloning of the *Bacillus thuringiensis* serovar *sotto* chitinase (*Schi*) gene and characterization of its protein

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Abstract

Chitinase plays a positive role in the pathogenicity of *Bacillus thuringiensis* to insect pests. We used touchdown PCR to clone the chitinase (*Schi*) gene from *Bacillus thuringiensis* serovar *sotto* (*Bt sotto*) chromosomal DNA. Our DNA sequencing analysis revealed that the *Bt sotto Schi* gene consists of an open reading frame (ORF) of 2067 nucleotides with codes for the chitinase precursor. We also found that the putative promoter consensus sequences (the -35 and -10 regions) of the *Bt soto Schi gene* are identical to those of the *chiA71* gene from *Bt Pakistani*, the *chiA74* gene from *Bt kenyae* and the *ichi* gene from *Bt israelensis*. The Schi chitinase precursor is 688 amino acids long with an estimated molecular mass of 75.75 kDa and a theoretical isoelectric point of 5.74, and contains four domains, which are, in sequence, a signal peptide, an N-terminal catalytic domain, a fibronectin type III like domain and a C-terminal chitin-binding domain. Sequence comparison and the evolutionary relationship of the *Bt sotto* Schi chitinase to other chitinase and chitinase-like proteins are also discussed.

Key words: Bacillus thuringiensis serovar *sotto*, chitinase, touchdown PCR, gene cloning, characterization analysis.

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Introduction

Chitin, a (1,4)-β-linked homopolysaccharide made up of *N*-acetylglucosamine residues, is absent from vertebrates and plants but is commonly found in the exoskeletons of insects and crustaceans as well as in fungi and some algae. Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin, and is found in a wide variety of organisms including bacteria, fungi, invertebrates, plants and animals (Goody, 1990), but the roles of chitinases in different organisms are diverse. Bacteria produce chitinases to digest chitin for use as a carbon and energy sources, while fungi produce this enzyme to modify the important cell wall component chitin and invertebrates require chitinases for the partial degradation of old exoskeletons. In plants, however, chitinases are part of the plants defense mechanisms against fungal pathogens.

Some chitinolytic bacteria have been shown to be potential agents for the biological control of both phytopathogenic fungi and insect pests (Chernin *et al.*, 1997; Sampson and Gooday, 1998). One of the most important insecticidal microbes used in biological control is *Bacillus thuringiensis* (*Bt*), a gram-positive, rod-shaped, spore-forming bacterium that forms insecticidal protein(s) during the stationary phase of its growth cycle. Insecticidal proteins, mainly Cry (crystal) proteins, play the leading role in controlling of insect pests. It has been reported that chitinases are widely distributed in *Bt* strains and that some of the chitinase-producing strains can enhance the insecticidal activity of *Bt* (Liu *et al*., 2002), although, as far as we know, only a few chitinase genes have yet been cloned (Thamthiankul *et al*., 2001; Barboza-Corona *et al*., 2003; Zhong *et al*., 2003; Lin and Guan, 2004) and furthermore, the synergistic effect of purified *Bt* chitinase and Cry proteins has still not been quantitatively demonstrated (Barboza-Corona *et al*., 2003).

It is known that *B. thuringiensis* serovar *sotto* (*Bt sotto*) has strong larvicidal activity against lepidopteran pests because of Cry proteins (Zhong *et al*., 2004). We cloned the *cry1Aa13* gene from *Bt sotto* plasmid and expressed it in *Escherichia coli*, but expression of the *cry1Aa13* gene product was low (Zhong *et al*., 2004). In order to further determine the synergistic action between the *Bt sotto* Cry1Aa13 protein and *Bt sotto* Schi chitinase, it is necessary to clone and sequence the Schi chitinase. This re-

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port deals with the cloning, sequencing and partial characterization of *Bt sotto Schi* gene.

Materials and Methods

Bacterial strains, plasmids and culture condition

We obtained *Bt sotto* from Dr. Dai LY (Chinese Academy of Forestry, Beijing) and grew cultures at 30 °C in nutrient yeast extract salts medium (NYSM; consisting of (gL^{-1}) nutrient broth, 8; $CaCl_2.2H_2O$, 0.103; $MnCl_2.4H_2O$, 0.01; $MgCl_2.6H_2O$, 0.203) for chromosomal DNA extraction. The plasmid T-easy vector (Promega) was used as a cloning vector and *Escherichia coli* strain DH5α as the transformation host, this strain being grown at 37 °C in Luria-Bertani (LB) medium (containing (gL^{-1}) tryptone, 10; NaCl, 10; yeast extract, 5) and transformants in LB supplemented with 50 μg/mL of ampicillin.

Primer design and oligonucleotide synthesis

Oligonucleotide primers P1 (5'-GGG CCC TTT CCT CCC ATA CCA-3') and P2 (5'-GGG CCC CGA AAG CCT TTC CTA-3') were synthesized using β-cyanoethyl phosphoramidite chemistry and an Expedite Nucleic Acid Synthesis System (workstation) in TaKaRa Dalian Corporation (Dalian, China).

DNA manipulation and cloning of the *Bt sotto Schi* gene

Touchdown PCR avoids the need for complicated optimization of the annealing temperature of the primers by using a 0.5-1 °C sequential decrease from a high annealing temperature in the first PCR cycle to a "touchdown" annealing temperature which is then used for several more cycles (Don *et al.*, 1991). Touchdown PCR was carried out in a TGradient Thermocycler (Biometra) using the following program: 94 °C for 3 min, 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1.5 min, followed by 24 cycles at decreasing annealing temperatures in decrements of 0.5 °C per cycle, then 10 cycles of 1 min at 94 °C, 1 min at 52 °C, 1.5 min at 72 °C, and final extension at 72 °C for 10 min. The reaction mixture contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer, 1U *Taq* plus DNA polymerase (TaKaRa) and 30 ng of *Bt sotto* total genomic DNA, extracted according to Zhong *et al.* (2003), as template. Small-scale plasmid isolation, transformation, restriction enzyme digestion and agarose gel electrophoresis were performed by standard methods, *i.e.* 1% (w/v) agarose gel electrophoreses and purification using the Wizard SV Gel and PCR Clean-Up System (Promega), the resulting PCR product being ligated to the T-easy vector which was used to transform *Escherichia coli* DH5α competent cells with the *Schi* gene. The positive recombinant clone was screened and used for nucleotide sequencing.

Nucleotide sequence analysis

The *Bt sotto Schi* gene nucleotide sequence was determined at least once in each strand by the dideoxy chain termination method using an ABI 377 sequencer. DNA and deduced amino acid sequences were compared with those of other chitinase genes from GenBank, using both the BLAST (http://ncbi.nlm.nih.gov/BLAST/) and CLUSTAL W multiple sequence alignment tool.

Results

Cloning and nucleotide sequencing of the *Bt sotto Schi* chitinase gene

We successfully cloned the *Bt sotto Schi* chitinase gene using touchdown PCR with primers P1 and P2. The *Bt sotto Schi* gene structural and flanking region sequence was deposited in the GenBank database under accession number AY129671. The manuscript has *Schi* gene region contains an open reading frame (ORF) with a base composition of 33.9% adenine and 28.4% thymine (A + T = 62.3%) and 19.7% guanine 18.0% cytosine (G + C = 37.7%) consisting of 2067 nucleotides encoding 688 amino acid residues with a deduced molecular weight of 75.75 Da and a theoretical isoelectric point of 5.74. The codon usage is shown in Table 1, from which it can be seen that because of the relatively high A + T content there is a preference for A and T at the third base position in the codons, the A:G ratio being 3.67:1 and the T:C ratio 3.08:1.

The putative promoter consensus region, spanning nucleotide positions 59 to 64 (TTGAGA, -35) and 79 to 84 (TTAATA, -10) of the *Schi* gene are identical to those of the *chiA71* gene from *B. thuringiensis* serovar *Pakistani* (Thamthiankul *et al*., 2001), the *chiA74* gene from *B. thuringiensis* serovar *kenyae* (Barboza-Corona *et al*., 2003), and the *ichi* gene from *B. thuringiensis* serovar *israelensis* (Zhong *et al*., 2003). The putative promoters also show obvious homology to the -35 (TTGACA) and -10 (TATAAT) consensus sequence for an *E. coli* promoter and *Bacillus subtilis* σ^A. Two stem-loop structures (one at 25 to 41 bp and the other at 69 to 94 bp from the TAG stop codon) of the putative transcriptional terminator were found at the 3' end, these structures which causing the polymerase to pause and subsequently cease transcription, similar results having been reported by us for the *Bt ichi* sequences (Zhong *et al*., 2003).

Amino acid sequence analysis of the Schi

The predicted 46 N-terminal amino acids of the Schi chitinase precursor exhibited a typical feature of the signal peptide characteristic of Gram-positive bacteria. There was a positively charged hydrophilic N-terminal segment ending in lysine with a net charge of +3, followed by a hydrophobic amino acid sequence. Cleavage of the signal sequence of the Schi chitinase precursor occurred at between A-46 and D-47. Interesting, the signal peptide may

Table 1 - Amino acid codons for the *Bacillus thuringiensis* serovar *sotto* (*Bt sotto*) *Schi* gene.

Amino acid*	Codon	Nr	Amino acid*	Codon	Nr
Alanine (A)	GCG	3	Glutamine (Q)	CAG	0
	GCA	15		CAA	17
	GCT	19	Glycine (G)	GGG	5
	GCC	7		GGA	19
Arginine (R)	AGG	1		GGT	25
	AGA	1		GGC	14
	CGG	0	Histidine (H)	CAT	5
	CGA	2		CAC	4
	CGT	6	Isoleucine (I)	ATA	5
	CGC	3		ATT	26
Asparagine (N)	AAT	46		ATC	1
	AAC	11	Leucine (L)	TTG	3
Aspartic acid (D)	GAT	34		TTA	19
	GAC	9		CTG	1
Cysteine (C)	TGT	4		CTA	11
	TGC	4		CTT	12
Glutamic acid (E)	GAG	3		CTC	6
	GAA	27	Lysine (K)	AAA	47

Amino acid*	Codon	Nr	Amino acid*	Codon	Nr
Lysine (K)	AAG	5	Threonine (T)	ACG	10
Methionine (M)	ATG	7		ACA	27
Phenylalanine (F)	TTT	17		ACT	19
	TTC	7		ACC	6
Proline (P)	CCG	1	Tryptophan (W)	TGG	20
	CCA	13	Tyrosine (Y)	TAT	21
	CCT	22		TAC	12
	CCC	0	Valine (V)	GTG	3
Serine (S)	AGT	13		GTA	14
	AGC	7		GTT	15
	TCG	4		GTC	4
	TCA	9	End (.)	TGA	0
	TCT	15		TAG	1
	TCC	2		TAA	0

*Amino acid or punctuation one-letter code in parenthesis.
Nr: Number of residues.

also be recognized by gram-negative bacterial and eukaryotic organisms according to the SignalP server (http://www.cbs.dtu.dk/services/SignalP/). We estimated the mature Schi chitinase molecular weight to be 70.50 kDa and the isoelectric point to be 5.55. Computer analysis of the deduced mature Schi chitinase amino acid sequence revealed that it consists of three discrete domains, an N-terminal catalytic domain, a fibronectin type III-like domain (FLD) and a chitin-binding domain (ChBD).

The *Bt* Schi chitinase N-terminal region (residue 151 to 255) showed similarity to catalytic domains of chitinases belonging to glycosyl hydrolase family 18. In particular,

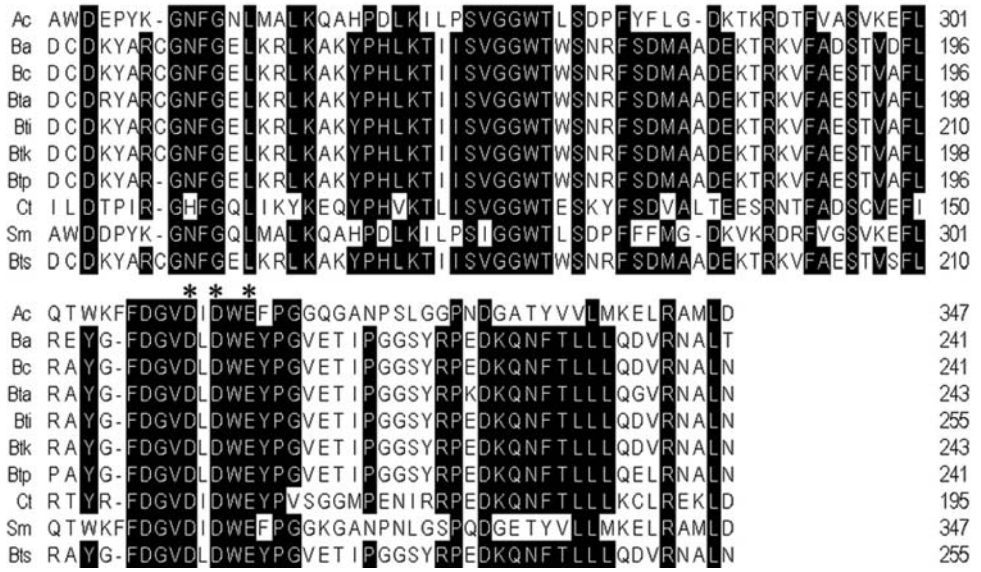

Figure 1 - Alignment of the *Bacillus thuringiensis* (*Bt*) serovar *sotto* (*Bt sotto*) Schi catalytic domain with those of other chitinases (Chi). Sequences are from *Aeromonas caviae* chitinase (*Ac*), *Bacillus anthracis* chitinase B (*Ba*), *Bacillus cereus* chitinase B (*Bc*), *Bt alesti* ChiA74-HD16 (*Bta*), *Bt israelensis* Ichi chitinase (*Bti*), *Bt kenyae* ChiA74 (*Btk*), *Bt pakistani* ChiA71 (*Btp*), *Clostridium thermocellum* (*Ct*) and *Serratia marcescens* ChiA (*Sm*). Putative proton donor amino acid residues are marked with an asterisk. Dashes indicated gaps inserted to improve alignment. Numbers refer to the amino acid residue at the end of the respective lines.

the N-terminal region showed high sequence similarity to the following chitinases (Chi); *Bt pakistani* ChiA71 (95.2% similarity; Thamthiankul *et al.*, 2001); *Bt kenyae* ChiA74 (99%; Barboza-Corona *et al.*, 2003); *Bt alesti* ChiA74-HD16 (96.2%; Lin and Guan, 2004); *Bt israelensis* Ichi chitinase (99%; Zhong *et al.*, 2003); *Bacillus cereus* chitinase B (99%; Mabuchi and Araki, 2001); and *Bacillus anthracis* chitinase B (96.2%; Read *et al.*, 2003) (Figure 1). Further analysis of the *Bt* Schi chitinase showed that there was some degree of similarity between the catalytic domain of this chitinase and that of other bacterial chitinases, such as the chitinases from *Aeromonas caviae* (39.8% similarity) and *Clostridium thermocellum* (57.7%) as well as *Serratia marcescens* chitinase A (40.8%) (Figure 1). In this region, the deduced amino acid sequence from residue 215 to 223 (FDGVDLDWE; q.v. Table 1 for amino acid key) was homologous to the active site motif of enzymes in glycosyl hydrolase family 18 ([FILMVY]-[DN]-G-[VFILM]-[DN]-[LFIMV]-[DN]-X-E; q.v. Table 1 for amino acid key). Furthermore, amino acid residues D-219, D-221 and E223 of the Schi chitinase (corresponding to D-200, D-202 and E204 of *Bacillus circulans* chitinase ChiA) were well conserved and may pay an essential role in chitinase activity.

The *Bt* Schi chitinase middle region (residues 502 to 575) showed similarity to the fibronectin type III-like domain (FLD) sequences found in the R-1 and R-2 regions of *Bacillus circulans* chitinase A1 (44% and 48.8% similarity respectively; Watanabe *et al*., 1990) and also to the FLD1 and FLD2 regions of *Bt* Chi74 chitinase (13.1% and 94% respectively; Barboza-Corona *et al*., 2003) as well as to regions in the *Bt* ChiA71 chitinase (86.9%; Thamthiankul *et al*., 2001) and the *Bt* Ichi chitinase (95.2%; Zhong *et al*., 2003). Interesting, the middle region of the *Bt* Schi chitinase were identity to the FLD of *Bt alesti* chitinase ChiA74-HD16 (Lin and Guan, 2004) (Figure 2).

The *Bt* Schi chitinase C-terminal region (residues 600 to 641) showed sequence homology to the chitin-binding domain found in the following chitinases: Chi71 (residues 454 to 495; Thamthiankul *et al*., 2001); Chi74 (residues 588 to 629; Barboza-Corona *et al*., 2003); *Bt* Ichi (residues 600 to 641; Zhong *et al*., 2003); ChiA74-HD16 (residues 588 to 629; Lin and Guan, 2004); Ac1 and Ac2 of *Aeromonas caviae* ChiA (residues 770 to 813 and 817 to 859 respectively; Sitrit *et al*., 1995); *Alteromonas* sp. strain O-7 Chi85 (residues 776 to 818; Tsujibo *et al*., 1993) and *Vibrio harveyi* ChiA (residues 512 to 557; Svitil and Kirchman, 1998) (Figure 3).

Discussion

According to our catalytic domain analysis, the *Bt* chitinase *Schi* gene we cloned and sequenced during this study seems to be the *chiA* gene encoding the chitinase precursor. The phenomenon of possible promoter showed homology with *B. subtilis* σ^A indicated that the expression of Schi take place during the vegetative (*i.e.* exponential) growth phase.

Figure 2 - Alignment of the fibrinectin type III-like domain (FLD). The sequences of the FLD domain of the *Bacillus thuringiensis* (*Bt*) serovar *sotto* (*Bt sotto*) chitinase (Chi) Schi (*Bts*) are aligned with those of *Bacillus circulans* chitinase A1 (R-1 and R-2), *Bt alesti* ChiA74-HD16 (*Bta*), *Bt israelensis* Ichi chitinase (*Bti*), *Bt kenyae* ChiA74 (*Btk*1 and *Btk*2) and *Bt pakistani* ChiA71 (*Btp*). Aromatic amino acid residues typical for FLD regions are indicated by an asterisk.

Figure 3 - Composition of the C-terminal domain of *Bacillus thuringiensis* (*Bt*) serovar *sotto* (*Bt sotto*) chitinase (Chi) Schi with chitin-binding domain (ChBD) of other chitinase. Sequences are from *Aeromonas caviae* ChiA (*Ac1* and *Ac2*), *Alteromonas* sp. strain O-7 Chi85 (*Al*), *Bt alesti* ChiA74-HD16 (*Bta*), *Bt israelensis* Ichi (*Bti*), *Bt kenyae* ChiA74 (*Btk*), *Bt pakistani* ChiA71 (*Btp*) and *Vibrio harveyi* ChiA (*Vb*). Highly conserved aromatic amino acids are marked with an asterisk.

The signal sequence of the *Bt sotto* Schi chitinase precursor contains an extremely long N-terminal hydrophilic segment, with the signal peptide cleavage in the Schi precursor occurring between amino acid residues A-46 and D-47. The signal peptide is the same length as that of the *Bt* ichi chitinase but longer than that of the chiA71 and chiA74 chitinases.

The high homology (97% identity) between the *Bt sotto* Schi chitinase and the *B. cereus* strain CH chiB chitinase (Mabuchi and Araki, 2001) supports the view that even though *B. thuringiensis* and *B. cereus* belong to different species they are closely related evolutionary.

The mature *Bt sotto* Schi chitinase seems to be composed of a glycosyl-hydrolase family 18 catalytic domain, a fibronectin type III-like domain and a chitin-binding domain. Chitinases are acid-base catalysts and from sequence comparison with *B. circulans* chitinase A1 (Watanabe *et al.*, 1994), it seems that the catalytic residues acting as proton donors are the amino acid residues D-219, D-221 and E-223. Fibronectin is a multifunctional extracellular matrix and plasma protein that plays a significant role in cell adhesion. Fibronectin type III-like domains (FLD) have been found in chitinase, cellulases, α-amylase, and poly-3-hydroxybutyrate (PHB) depolymerase. In the chitin-binding domain (ChBD) of many chitinase, the aromatic amino acids tryptophan (W) and tyrosine (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). In *Bt sotto* Schi chitinase we found that tryptophan and tyrosine residues (*e.g.* W-603, Y-607, W-624 and W-638) were well conserved. It is also known that Schi chitinases show homology with the cellulose-binding domain of some cellulases and some researchers consider that chitinases and these cellulases have common substrate-binding mechanisms (Morimoto *et al*., 1997). The work presented in this paper has partially elucidated the role of each domain but the structure of the *Bt sotto* Schi chitinase is still not fully understood because of the limitations inherent in attempting to characterization of enzyme from amino acid sequences.

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