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ORIGINAL PAPER

Expression and characterization of *inhA* gene from *Bacillus thuringiensis* 8010

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Abstract InhA, a zinc metalloprotease secreted by *Bacillus thuringiensis*, specifically hydrolyzes antibacterial peptides produced by insect hosts. In this study, the *inhA* gene was cloned from *B. thuringiensis* 8010 using a pair of degenerate primers and the deduced 796 amino acid sequence showed a high degree of similarity with other InhA proteins in the *Bacillus cereus* group. The deduced amino acid sequence contained the zinc-binding motif (HEXXH), which is characteristic of the zinc-metalloprotease family. Additionally, the *inhA* gene was expressed in *Escherichia coli* BL21 (DE3). The expressed InhA protein was shown to be toxic to the third larvae of *Plutella xylostella*, contrary to preliminary study concerning the effect of InhA on *Bombyx mori*. This study provided insights into the potential of InhA for the biological control of certain lepidopteran insects.

Keywords *Bacillus thuringiensis* · Bioassay · Expression · *inhA* gene · Sequence analysis

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Introduction

Bacillus thuringiensis is a gram-positive, spore-forming bacterium capable of producing a number of toxins, including insecticidal endotoxins, exotoxins, haemolysins, enterotoxins, chitinase, and vegetative insecticidal proteins (VIPs), with toxicity to several insect orders, nematodes, mites and protozoa (Schnepf et al. 1998). Among these toxins, Immune Inhibitor A (commonly known as InhA), a zinc metalloprotease, is highly resistant to the humoral defense system of certain insects (Edlund et al. 1976). Previous evidence showed that the inhibition activity was mainly due to its selective degradation of antibacterial peptides attacins and cecropins in lepidopterans and dipterans (Dalhammar and Steiner 1984). As the major component of the exosporium, InhA was essential for spores of the *B. cereus* group to release from macrophages (Ramarao and Lereclus 2005). InhA also presented similarity with many zinc-containing proteases from pathogenic organisms, which caused necrotic or hemorrhagic tissue damage in the host by digesting important structural components (Lǒvgren et al. 1990; Ogierman et al. 1997; Miyoshi and Shinoda 2000).

InhA had a lethal effect when injected into *Trichoplusia ni* larvae (Lǒvgren et al. 1990). However, the *B. thuringiensis* 407 InhA-deficient mutant was slightly affected in its virulence when infecting *Bombyx mori* and *Galleria mellonella*, respectively (Fedhila et al. 2002). Up till now, attempts to evaluate the role of *inhA* have failed to obtain conclusive results with respect to a major role in virulence for this metalloprotease. To further understand the characteristics of *inhA* and its encoded protein, we cloned this gene from *B. thuringiensis* 8010 and analyzed its deduced amino acid sequence. The larvicidal activity of the InhA protein was examined as

well after expression of the corresponding gene in *Escherichia coli* BL21 (DE3).

Materials and methods

Bacterial strains, plasmids, and oligonucleotide primers

Bacillus thuringiensis subsp. *kurstaki* 8010 (serotype 3a3b) used in this study was collected by Guan (1997). *E. coli* strain JM109 and pMD18-T vector (TaKaRa, Shiga, Japan) were used for recombinant DNA cloning. *E. coli* BL21 (DE3) and pGEX-4T-3 vector (Pharmacia, Stockholm, Sweden) were used for the expression of InhA metalloprotease. One pair of degenerate primers was designed based on the consensus sequences of known *inhA* genes deposited in GenBank. Primer sequences are: inhAF (forward primer), 5′-CG**GGATCC**ATGARCAAGAAACCG-TTCAAAGT-3′ (the first two bases are protection bases, the boldface and underline show *Bam*HI site) and inhAR (reverse primer), 5′-CCG**CTCGAG**TTARCGATATAA-RCGAACAGCAC-3′ (the first three bases are protection bases, the boldface and underline show *Xho*I site). Throughout the experiments, bacteria were cultivated in Luria–Bertani (LB) liquid medium at 30 °C (37 °C for *E. coli*) with shaking at 230 rpm.

Gene amplification

Total DNA from *B. thuringiensis* 8010 was extracted as described by Kalman et al. (1995) and used as the template for PCR amplification. The full-length *inhA* gene was amplified with the primer pair inhAF/inhAR in 50 µl reaction volume containing 20 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 0.5 µM of each primer and 2.5 U *Taq* DNA polymerase. PCR was carried out for 30 cycles (at 94 °C for 1 min, 54 °C for 1.5 min, 72 °C for 3 min). The PCR product was analyzed on 1% agarose gel.

Construction of expression plasmid pGEXinhA

The PCR product was cloned into the pMD-18T vector to obtain the recombinant plasmid pMDinhA, and the selected clones were verified by DNA sequencing. The *Bam*HI/*Xho*I fragments were then recovered, cloned into the corresponding sites of the plasmid pGEX-4T-3 and transformed into competent cells of *E. coli* BL21 (DE3). One plasmid with the full-length *inhA* gene was obtained and designated pGEXinhA. All DNA manipulations including restriction digestion, ligation, agarose gel electrophoresis and transformation were carried out as described by Sambrook et al. (1989).

Sequence analysis

The nucleotide sequence of *inhA* and its deduced amino acid sequence were compared with the updated GenBank data by the BLAST search program. The theoretical molecular weight and pI value of deduced InhA were calculated by ExPASy. The prediction of the signal peptide was carried out by SignalP V2.0 software. Conserved domains of the deduced amino acid sequence were analyzed by Conserved Domain Search. Multiple-alignment of amino acid sequences was performed using DNAMAN V4.0 (Lynnon BioSoft, Vaudreuil, QC, Canada).

Expression of *inhA* gene in *E. coli*

Transformed *E. coli* BL21 (DE3) cells were grown at 37 °C in LB broth supplemented with 100 µg ampicillin ml^{-1} and induced by Isopropyl-*β*-D-thiogalactopyranoside (IPTG; 1 mM) when the culture reached an optical density at 600 nm of 0.6–0.8. Cells were harvested by centrifugation after overnight induction at 25 °C, resuspended in distilled water at a 50-fold dilution, and disrupted by ultrasonication. Samples were boiled (5 min) in the loading buffer, and the soluble mixtures were then analyzed by SDS-PAGE on a 7.5% (w/v) polyacrylamide separating gel.

Bioassay on *Plutella xylostella* larvae

The cultures of the transformant prepared as described above were used to test the toxicity of the expressed InhA against the laboratory-reared third larvae of *Plutella xylostella*. Bioassay and rearing were both conducted at 25 °C and 60% humidity with a photoperiod of 14 h of light and 10 h of dark. Toxicity assay was performed using leaf-dipped method. All materials tested were diluted 100 times with distilled water. Each treatment was repeated three times, with 30 larvae for a treatment. *E. coli* cells BL21 (DE3) [pGEX-4T-3] were added to the diet as the control. The mortality was scored after 72 h and the corrected mortality was also calculated.

Results

Sequence analysis of the *inhA* gene from *B. thuringiensis* 8010

The entire coding region of the *inhA* gene ~2.4 kb in length was produced by PCR using the primer pair inhAF/inhAR (Fig. 1). The PCR product was cloned and sequenced. Nucleotide sequence of this *inhA* gene (designated *inhA*-8010) has been deposited in GenBank (accession num-

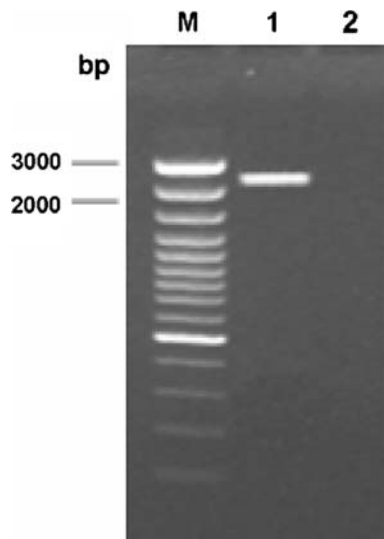

Fig. 1 Agarose gel electrophoresis of *inhA* gene amplified from *B. thuringiensis* 8010. Lane 1, PCR Product with inhAF/inhAR; Lane 2, Negative control; M, GeneRuler^{TM} 100 bp DNA Ladder Plus

ber AY945956). It consists of 2391 bases, encoding a protein of 796 residues with a calculated molecular weight of 86.5 kDa and a predicted pI value of 5.22. BLAST search showed that the deduced InhA-8010 had a similarity of 97.2% (774/796), 95.9% (763/796), and 95.5% (760/796) with that of *B. thuringiensis* 407 (AE287346), *B. cereus* ATCC 10987 (AE017268), *B. cereus* ZK (CP000001), respectively.

According to the SignalP site, the most likely cleavage site of InhA-8010 was between Ala-31 and Glu-32. The cleavage of the signal peptide generated an 83.3 kDa protein with a predicted pI value of 5.08. Conserved Domain Search revealed that the mature protein contained one large domain, Immune inhibitor A peptidase M6 (Pro-146 through Tyr-795), which consisted of 650 amino acids with a calculated molecular weight of 71.2 kDa (Fig. 2). Peptidase family M6 (Immune Inhibitor A family) displays the same HEXXHXXGXXD motif found in family M7 (*Streptomyces* extracellular small neutral proteases), in which the two histidines and the aspartate are zinc ligands and the glutamate is the catalytic residue. According to the recent classification system for zinc-dependent metallopeptidases, InhA belongs to the metzincins superfamily. Alignment of InhA-8010 with other InhA proteins demonstrated that they all lacked cysteine residues and contained the highly conserved zinc-binding motif (HEXXH) (Fig. 3), which is characteristic of the zinc-metalloprotease family (Jongeneel et al. 1989).

Expression of the *inhA* gene in *E. coli* BL21 (DE3) and its insecticidal activity

The *Bam*HI–*Xho*I fragment corresponding to the ORF of *inhA*-8010 was inserted into the expression vector pGEX-

MSKKPFKVLSSIALTAVLGLSFGAGTQSAYAETPVNKTATSPVDDHLIPEERLADALKKRG
VIDSKASETETKKAVEKYVENKKGENPGKEAANGDQLTKDASDFLKKVKDAKADTKEKLN
QPATGTPAATGPVKGGLNGKVPTSPAKQKDYNGEVRKDKVLVLLVEYADFKHNNIDKEPG
YMYSNDFNKEHYEKMLFGNEPFTLDDGSKIETFKQYYEEQSGGSYTVDGTVTKWLTVPG
KAADYGADAPGGGHDNKGPKGPRDLVKDALKAAVDSGIDLSEFDQFDQYDVNGDGNKN
QPDGLIDHLMIIHAGVGQEAGGGKLGDDAIWSHRWTVGPKPFPIEGTQAKVPYWGGKMA
AFDYTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGQGEPIEAWSIMSGGSWAGKIAGTT
PTSFSPQNKEFFQKTIGGNWANIVEVDYEKLNKGIGLATYLDQSVTKSARPGMIRVNLPDK
DVKTIEPAFGKQYYYSTKGDDLHTKMETPLFDLTNATSAKFDFKSLYEIEAGYDFLEVHAVT
EDGKQTLIERLGEKANSGNADSTNGKWIDKSYDLSQFKGKKVKLTFDYITDGGLALNGFAL
DNASLTVDGKVVFSDDAEGTPQLKLDGFVVSNGTEKKKHNYYVEWRNYAGADNALKFAR
GPVFNTGMVVWYADSAYTDNWVGVHPGHGFLGVVDSHPEAIVGTLNGKPTVKSSTRFQI
ADAAFSFDKTPAWKVVSPTRGTFTYDGLAGVPKFDDSKTYINQQIPDAGRILPKLGLKFEV
VGQADDNSAGAVRLYR

Fig. 2 The deduced amino acid sequence of *inhA*-8010. The signal peptide is *boxed*. The immune inhibitor A peptide M6 domain is *underlined*

Bt 8010_InhA	361	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGQGEPIEA
Bt 407_InhA	360	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGQGEPIEA
Bt 97-27_InhA	360	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGHGEPVQA
Bc 10987_InhA	361	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGQGEPIEA
Bc E33L_InhA	361	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGQGEPIEA
Ba Ames_InhA	360	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGHGEPVQA
Ba Sterne_InhA	360	YTIEPEDGAVGVFAHEYGHDLGLPDEYDTQYSGHGEPVQA

Fig. 3 Alignment of the zinc-binding domain of InhA-8010 with those of other InhA proteins. Sequences are from *B. thuringiensis* 8010 InhA (Bt 8010, this study), *B. thuringiensis* 407 InhA (Bt 407, accession number AAG00998), *B. thuringiensis* serovar *konkukian* 97-27 InhA (Bt 97-27, YP_035510), *B. cereus* ATCC 10987 InhA (Bc 10987, NP_977717), *B. cereus* E33L InhA (Bc E33L, YP_082776), *B. anthracis* Ames InhA (Ba Ames, NP_843763), and *B. anthracis* Sterne InhA (Ba Sterne, YP_027467). The conserved zinc-binding motif (HEXXH) is *shaded*

4T-3 between the *Bam*HI and *Xho*I sites. The resulting recombinant plasmid, designated pGEXinhA, was transferred to *E. coli* BL21 (DE3). Successful transformation was confirmed by both PCR amplification and nucleotide sequencing. The expression of the *inhA* gene was under the control of the tac promoter. Results of SDS-PAGE (Fig. 4) showed that the molecular weight of the expressed fusion protein (InhA plus additional 26 kDa GST carrier protein) was about 110 kDa, which corresponded with the ExPASy

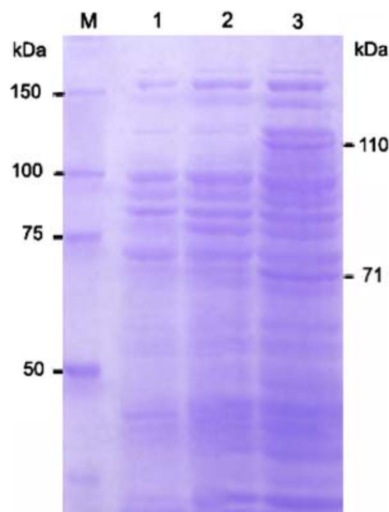

Fig. 4 SDS-PAGE analysis of the InhA produced in *E. coli* BL21 (DE3). Lane 1, *E. coli* BL21(DE3)/pGEX-4T-3 whole cell proteins after overnight induction with IPTG; lane 2, *E. coli* BL21(DE3)/pGEXinhA whole cell proteins without IPTG induction; lane 3, *E. coli* BL21(DE3)/pGEXinhA whole cell proteins after overnight induction with IPTG; M, Broad Range Protein Molecular Weight Markers (Promega, Madison, WI, USA)

calculation. Interestingly, another band of about 71 kDa, approximately the same size with the mature Immune Inhibitor A peptidase (71.2 kDa), was observed in *E. coli* BL21 (DE3) harboring pGEXinhA after IPTG induction (Fig. 4), suggestive of partial autocatalytic removal of the helper sequences from the original 796-amino-acid prepproprotein. This phenomenon was also mentioned in several other bacterial metalloproteases (Milton et al. 1992; Ogierman et al. 1997). The expressed InhA was demonstrated to be toxic against neonate *P. xylostella* larvae, with corrected mortality reaching 62.2% at 72 h after treatment (Table 1).

Table 1 Effect of the expressed InhA on *Plutella xylostella* larvae

Treatment[a]	Mortality (%)[b]
E. coli BL21 (DE3)/pGEXinhA	65.6
E. coli BL21 (DE3)/pGEX-4T-3 (IPTG induction)	8.9
E. coli BL21 (DE3)/pGEX-4T-3 (without IPTG induction)	7.8
E. coli BL21(DE3)	8.9
None	0

[a] *E. coli* BL21 (DE3)/pGEXinhA and *E. coli* BL21 (DE3)/pGEX-4T-3 refers to the recombinant *E. coli* BL21 (DE3) transferred with plasmid pGEXinhA and pGEX-4T-3, respectively. pGEXinhA harbors the *inhA* gene amplified from *B. thuringiensis* 8010

[b] Mortality as % of 90 larvae at 72 h after treatment

Discussion

We described the cloning, sequence analysis and expression of *inhA*, a gene encoding InhA metalloprotease, which mainly accounts for the high resistance of *B. thuringiensis* to humoral defense systems of insect hosts. The InhA from *B. thuringiensis* 8010 presented high similarity with other InhA metalloproteases, whose primary sequences have the unique canonical zinc-binding signature pattern present in various other metalloproteases. Besides, InhA-8010 lacked cysteine, a property shared with metalloproteases produced in other species of *Bacillus* (Dalhammar and Steiner 1984). Identification of putative signal peptide cleavage site between positions 31 and 32 of the deduced InhA amino acid sequence suggested that InhA is an exported protein, consistent with prior study with *B. thuringiensis* 407 (Grandvalet et al. 2001). The observation that the expressed 796-amino-acid InhA-8010 might be autocatalyzed into a shorter protein with a molecular weight of ~71 kDa is not unexpected, since several other bacterial zinc metalloproteases have been shown to be extracellular proteins which need signal and leader sequences to aid transport across the bacterial cell membranes (Milton et al. 1992).

Former study showed that the inactivation of *B. thuringiensis inhA* genes did not affect the ability of the bacteria to kill *B. mori* larvae via the intrahemocoelic route, hence the conclusion that InhA-like metalloproteases were not primary factors in intrahemocoelic infections (Fedhila et al. 2002). However, our study revealed that InhA-8010 did have toxicity effect on neonate *P. xylostella* larvae. PlcR is a pleiotropic regulator of virulence factors in *B. thuringiensis* and *B. cereus* (Agaisse et al. 1999; Økstad et al. 1999). It was reported that *inhA2*, highly homologous to *inhA*, was also regulated by PlcR (Fedhila et al. 2003). Possibly, the presence of one or several unidentified PlcR-regulated factors in *B. thuringiensis*, which compensated for the absence of InhA, might be the cause of reported inefficiency of InhA on *B. mori* larvae. Therefore, elucidation of the major role of InhA in pathogenesis was much complicated by the multifactorial characteristic of *B. thuringiensis* virulence.

This study provided some valuable insights into the virulence of InhA to lepidopteran insects. The toxicity of InhA-8010 against *P. xylostella* larvae demonstrated the potential of InhA for the biological control of certain insects. In previous study, it has been shown that the symptoms associated with the administration of InhA are typical of toxemia instead of bacterial septicemia as caused by insecticidal crystal proteins (ICPs), the major insecticidal endotoxins in *B. thuringiensis* (Lǒvgren et al. 1990). Although the precise role of InhA is still unclear, it is likely that the InhA metalloprotease interacts with a particularly important component of the host, leading to death (Fedhila

et al. 2002). Hence, InhA proteins are also likely to serve as supplements to ICP formulations, the persistent use of which has caused severe insect resistant problems worldwide.

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