Molecular Analysis of Genes Involved in Chitin Degradation from the Chitinolytic Bacterium *Bacillus Velezensis*

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Abstract

Bacillus velezensis RB.IBE29 is a potent biocontrol agent with high chitinase activity isolated from the rhizosphere of black pepper cultivated in the Central Highlands, Vietnam. Genome sequences revealed that this species possesses some GH18 chitinases and AA10 protein(s); however, these enzymes have not been experimentally characterized. In this work, three genes were identified from the genomic DNA of this bacterium and cloned in *Escherichia coli*. Sequence analysis exhibited that the ORF of *chiA* consists of 1,203 bp and encodes deduced 45.46 kDa-chitinase A of 400 aa. The domain structure of chitinase A is composed of a CBM 50 domain at the N-terminus and a catalytic domain at the C-terminus. The ORF of *chiB* includes 1,263 bp and encodes deduced 47.59 kDa-chitinase B of 420 aa. Chitinase B consists of two CBM50 domains at the N-terminus and a catalytic domain at the C-terminus. The ORF of *lpmo10* is 621 bp and encodes a deduced 22.44 kDa-AA10 protein, BvLPMO10 of 206 aa. BvLPMO10 contains a signal peptide and an AA10 catalytic domain. Chitinases A and B were grouped into subfamily A of family 18 chitinases. Amino acid sequences in their catalytic domains lack aromatic residues (Trp, Phe, Tyr) probably involved in processivity and substrate binding compared with well-known bacterial GH18 chitinases. *chiB* was successfully expressed in *E. coli*. Purified rBvChiB degraded insoluble chitin and was responsible for inhibition of fungal spore-germination and egg hatching of plant-parasitic nematode. This is the first report describing the analysis of the chitinase system from *B. velezensis*.

Introduction

Chitin, poly-β-1,4-N-acetylglucosamine (GlcNAc), is presumed to be the most abundant biopolymer in the aquatic biosphere, with an annual production of 10^{11} tons (Yu et al. 1993). Chitin is widely distributed in nature, e.g., as a constituent of insect exoskeletons, crustacean shells, fungal cell walls, and nematode eggshells and pharynx (Synowiecki and Al-Khateeb 2003). Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of chitin. These enzymes are found in both chitin-containing and non-chitin-containing organisms, such as bacteria, fungi, insects, plants, and animals. All most chitinases are classified into two different families of glycoside hydrolases (GH), families 18 and 19, based on the amino acid sequences in their catalytic domains (Henrissat 1991; Henrissat and Bairoch 1993). The catalytic domains of GH18 chitinases have $(\beta/\alpha)_8$ barrel folds (Perrakis et al. 1994; van Aalten et al. 2000), whereas those of GH19 chitinases have high α-helical content (Hart et al. 1995; Hahn et al. 2000). Bacterial family 18 chitinases can be further classified into three subfamilies A, B, and C. Subfamily A chitinases have an extra domain with a small $\alpha + \beta$ domain inserted into the seventh and eighth $(\alpha/\beta)_8$ barrel at the catalytic domain, while subfamilies B and C have no such domain (Suzuki et al. 1999). In addition to GH18 and GH19 chitinases, chitinases belonging to GH23 and GH48 are rarely found in some organisms. For instance, a family 23 chitinase was found in the moderately thermophilic bacterium, *Ralstonia* sp. A-471 (Ueda et al. 2009; Arimori et al. 2013), whereas a family 48 chitinase was found in the leaf beetle, *Gastrophysa atrocyanea* (Fujita et al. 2006).

Numerous studies have demonstrated that bacterial chitinases display an important role in inhibiting hyphal growth of phytopathogenic fungi (Ohno et al. 1996; Watanabe et al. 1999; Tsujibo et al. 2000;

Kawase et al. 2006; Huang et al. 2012a,b; Pentekhina et al. 2020). In addition, it was reported that chitinolytic bacteria normally produce plant growth-promoting traits, extracellular enzymes, antifungal compounds other than chitinases to control phytopathogenic fungi and promote plant growth (Gu et al. 2017; Tran et al. 2018; Trinh et al. 2019). On the other hand, chitin is the main component of nematode eggshells and the pharynx. Therefore, any disturbance in the synthesis or hydrolysis of chitin could lead to nematode embryonic lethal, laying defective eggs, and/or moulting failure. Hence, the critical components in the chitin metabolic process are targets for the development of anti-nematode agents (Chen and Peng 2019). Bacterial chitinases have been shown to have activity against egg hatching of nematodes (Jung et al. 2002; Lee et al. 2014, 2015). Consequently, chitinase-producing bacteria could be used for crop production as biological agents to reduce the use of chemical agents in controlling fungal phytopathogens (Kurze et al. 2001; Kobayashi et al. 2002; Bhattacharya et al. 2007) and plant-parasitic nematodes (Lee et al. 2015; Chen and Peng 2019).

Chitinolytic bacteria usually produce chitinases and/or auxiliary activities family 10 (AA10) proteins to degrade insoluble chitin efficiently for their carbon and nitrogen sources. AA10 proteins are enzymes that were previously classified into carbohydrate-binding modules in family 33 and have been reclassified into the auxiliary activities family 10 of lytic polysaccharide monooxygenases, according to the CAZy database (Levasseur et al. 2013). Bacterial AA10 proteins were reported to boost the activity of chitinases (Vaaje-Kolstad et al. 2005a, 2010). In addition, combinations of chitinases and AA10 proteins have been demonstrated to have enhanced hydrolytic chitin-degrading activity in comparison with individual chitinases (Gutiérrez-Román et al. 2014; Pentekhina et al. 2020).

The species *Bacillus velezensis* is widely distributed in the soil environment. It has been reported that *B. velezensis* formed biofilms to promote biocontrol ability (Krober et al. 2016) and produced secondary metabolites against the growth of plant pathogenic fungi, including fengycin (Koumoutsi et al. 2004), bacillomycin D (Gu et al. 2017), bacillibactin C (Chen et al. 2007), di□cidin (Wu et al. 2015), bacilysin (Wu et al. 2014), and amylocyclicin (Scholz et al. 2014). In addition, this species produced plant-growth-promoting traits, such as cytokinin (Arkhipova et al. 2007). Based on the evidence, *B. velezensis* has been widely applied for agricultural cultivation as biocontrol and plant-growth-promoting agents (Cai et al. 2017; Lim et al. 2017). On the other hand, *B. velezensis* could be a potent chitin degrader due to the presence of family 18 chitinases and AA10 protein according to the CAZy database (http://www.cazy.org/bB.html). However, such chitinases and protein are revealed by genome sequence analysis only, and no experimental studies thus far have described this chitinase system and its utilization in the hydrolysis of chitin. Moreover, it is well known that degradation and utilization of insoluble chitin by chitinolytic bacteria typically consists of two principal steps: (1) bacteria secrete families 18 and 19 chitinases to cleave chitin polymers into oligomers, and dimers $(GlcNAc)_2$ are the major products, and then (2) the dimers are cleaved into monomers (GlcNAc) by the action of family 20 glycoside hydrolase (Beier and Bertilsson 2013). Genome sequences in the CAZy database show that *B. velezensis* possesses GH18 chitinases and AA10 protein only, posing the question of how this species converts chitin into GlcNAc for its carbon source utilization. In addition, the primary structures of two

chitinases of this species contain carbohydrate-binding module family 50 (CBM50) domains. It was reported that the CBM50 domain in plant chitinases and fungal proteins are involved in substrate binding and antifungal activity (Seidl-Seiboth et al. 2013; Inamine et al. 2015), but the role of such domains in bacterial chitinases have not been revealed. Hence, the chitinase system from this species appears unique among those that have already been characterized and may possess antifungal activity.

We previously isolated a chitinolytic strain, RB.IBE29 (formerly DS29), from the rhizosphere of black pepper cultivated in the Central Highlands of Vietnam and identified it as *B. velezensis*. Strain RB.IBE29 showed high chitinase and antifungal activities against *Phytophthora*, the main cause of black pepper wilt disease in the region, as examined *in vitro* and the greenhouse condition (Trinh et al. 2019). Our field study showed that a combination of strain RB.IBE29 and other chitinolytic bacteria exhibited the most significant effect against *Phytophthora* and *Fusarium* in the soil and roots of black pepper. This combination also increased the chlorophyll a and b contents of black pepper, indicating that using indigenous bacteria, including *B. velezensis*, is a good solution for sustainable and green cultivation of black pepper in the Central Highlands of Vietnam (Nguyen et al. 2021). Considering the information on chitinases and AA10 protein genes revealed by genome sequencing and experimental studies on biocontrol and the plant-growth-promoting ability of *B. velezensis*, our next step was to identify and characterize chitinase molecules from *B. velezensis* RB.IBE29 by applying gene cloning and expression, then clarifying their bio-properties and synergistic effects for sustainable and green crop production.

In order to analyze the chitinase system of this bacterium, in this initial work, we present (1) isolation, cloning, and sequencing analysis of two chitinase and one AA10 protein genes of strain RB.IBE29, (2) analysis of primary structures of the deduced enzymes, (3) expression of *chiB* in *E. coli* BL21-CodonPlus (DE3)-RIPL cells, and examination of purified recombinant enzyme concerning chitinase, antifungal, and anti-nematode activities.

Materials And Methods

Bacterial strains, plasmids, and culture conditions

B. velezensis RB.IBE29 (Trinh et al. 2019) was used as a source of chromosomal DNA for chitinase and AA10 protein gene cloning. *E. coli* DH5α and *E. coli* BL21-CodonPlus (DE3)-RIPL were used as host microorganisms for gene cloning and protein expression. Fungal spores of *Fusarium* sp. were used for the spore-germination test. pUC19 and pColdII vectors were used for gene cloning and protein expression, respectively. Luria-Bertani (LB) medium was used for routine cultures and protein production.

Gene isolation

To identify genes related to the chitinase system in strain RB.IBE29, we based on sequences of genes coding for chitinases and AA10 protein in *B. velezensis* CAU B946 (HE617159.1) available in the CAZy databases. On the basis of the sequence of each gene and surrounding regions of the gene in *B. velezensis* CAU B946, primers were designed to identify genes related to chitinases and AA10 protein in

strain RB.IBE29 by polymerase chain reaction (PCR). A schema for gene identification is shown in Fig. S1A. PCR reactions were conducted using primers (Table S1), the genomic DNA of *B. velezensis* RB.IBE29, and *Taq* DNA polymerase (Bioline, USA) according to the manufacturer's instructions. The reaction mixtures were incubated in a C1000 thermal cycler (Bio-Rad, USA) and amplified products were then analyzed by electrophoresis on agarose (1.5%, w/v) gels.

Gene cloning and sequencing analysis

A fragment (1,765 base pairs [bp]) containing the *chiA* gene, including 304 bp upstream and 169 bp downstream of the *chiA* open reading frame (ORF), was amplified using the genomic DNA of *B. velezensis* RB.IBE29 as the template, the primers chiA-f and chiA-r, and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, USA), following the manufacturer's instructions.

Similarly, a fragment (1,929 bp) containing the *chiB* gene, including 513 bp upstream and 174 bp downstream of the *chiB* ORF, was amplified using the genomic DNA of strain RB.IBE29, primers chiB-f and chiB-r, and Phusion High-Fidelity DNA polymerase. A fragment (1,139 bp) containing *lpmo10* gene, including 264 bp upstream and 255 bp downstream of the *lpmo10* ORF, was amplified from the genomic DNA of strain RB.IBE29 using primers (lpmo10-f and lpmo10-r) and Phusion High-Fidelity DNA polymerase. The amplified fragments were then individually ligated into the plasmid pUC19 (Thermo Fisher Scientific, USA) previously treated with *Sma*I (New England Biolabs, USA) using a DNA ligation kit (Mighty mix, Takara, Japan) to generate recombinant plasmids, pUC-*chiA*, pUC-*chiB*, and pUC-*lpmo10*, respectively. Primers used in this work are listed in Table S1.

Finally, the recombinant plasmids were individually transformed into *E. coli* DH5α by heat shock. Transformants were grown at 37°C on LB agar plates containing ampicillin (100 μg/mL), X-Gal (0.04 mg/mL), and Isopropyl *β*-D-thiogalactopyranoside (0.1 mM) and then selected on the basis of the blue/white selection assay.

Recombinant plasmids from the positive colonies examined by colony-PCR were isolated and purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). The purified recombinant plasmids were sequenced at the 1st BASE Company (Selangor, Malaysia) using the Sanger method.

ORFs of the genes were predicted using the ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Putative promoters were identified using the CNNProm program (Umarov and Solovyev 2017).

Primary structure analysis of deduced proteins

Signal peptide sequences were deduced using SignalP (http://www.cbs.dtu.dk/services/SignalP). Domain structures and their functions were examined using Pfam (http://pfam.sanger.ac.uk) and SMART (http://smart.embl-heidelberg.de/), respectively. The molecular weight of deduced proteins was computed using the Compute pI/Mw tool (https://web.expasy.org/compute_pi/). Finally, the BLASTp program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to examine homology among the deduced domains.

Phylogenetic analysis

Phylogenetic trees based on amino acids in the catalytic domains of deduced chitinases were produced using MEGA version 6.0 software (Tamura et al. 2013) after multiple data alignments by Clustal W (Larkin et al. 2007) implemented in MEGA software. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987), and evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965). Finally, a bootstrap analysis (1000 replications) was performed to evaluate the topology of the resulting tree.

Construction of the expression plasmid

The gene *chiB*, which encodes a unique two CBM50-domain-containing chitinase among three genes, was chosen for expression in *E. coli* as the first step. The ORF (full length) of *chiB* was amplified by PCR using primers (Table S1), the purified pUC-*chiB*, and Phusion High-Fidelity DNA polymerase. The amplified fragment was then purified using a Wizard SV Gel and Clean-Up Kit (Promega Co., USA) and digested with FastDigest *Bam*HI and *Hind*III (Thermo Fisher Scientific, USA), respectively. The digested fragment was inserted into the *Bam*HI-*Hind*III site of pColdII (Takara, Japan) using the DNA ligation kit to generate a recombinant vector, pCold-*chiB*. Finally, pCold-*chiB* was transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent, USA) by heat shock for expression of chitinase B. The insert from positive colonies was confirmed by sequencing.

Expression, extraction, and purification of recombinant chitinase B

E. coli BL21-CodonPlus (DE3)-RIPL harboring pCold-*chiB* was cultivated aerobically in 1.0 L of LB medium containing ampicillin (100 μg/mL) at 33 ℃ with shaking (150 rpm). When the OD_{600nm} reached 0.4–0.6, the culture was incubated at 15 ℃ for 30 min. After that, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture (0.5 mM, final), and the culture was grown continuously at 15 ℃ for 24 h with shaking. Cells (about 8.0 g) were harvested by centrifugation (4,600 rpm, 4 ^{o}C, 20 min), suspended in 80 mL of 20 mM sodium phosphate buffer (pH 7.4) (0.5 M NaCl, 40 mM imidazole), and disrupted by sonication (model Q700, Qsonica, USA) with an amplitude of 40%, 15 seconds ON and 45 seconds OFF, and a total ON time of 12 min. Cell debris was removed by centrifugation (13,000 rpm, 4 ^{o}C, 10 min), and recombinant chitinase B in the supernatant was then purified by loading onto the HisTrap FF column (Cytiva, Germany) previously equilibrated with binding buffer (20 mM phosphate buffer, 0.5 M NaCl, 40 mM imidazole, pH 7.4). Unbound and weakly bound proteins were washed with a 15-column volume of the binding buffer at a 1.0 mL/min flow rate. His-tagged proteins were then eluted with 5 column volumes of elution buffer (20 mM phosphate buffer, 0.5 M NaCl, 500 mM imidazole, pH 7.4). After the molecular sizes of the proteins in the eluted samples were verified by the electrophoresis on 11.5% SDS–PAGE gels, fractions containing the target protein were collected and dialyzed in 20 mM phosphate buffer (pH 6.0) at 4 ℃ overnight. The dialyzed proteins were stored at −80 ℃ in the same buffer containing 10% glycerol for further examination.

SDS–PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was conducted as previously described (Laemmli 1970) with minor modifications (Tran et al. 2018).

Measurement of chitinase activity and protein concentration

The chitinase activity assay was conducted in a 600-µL reaction mixture containing shrimp shell colloidal chitin, shrimp shell powdered chitin, shrimp shell chitin flakes, and squid chitin flakes (0.1% each), and 5 µg of purified recombinant chitinase B in 20 mM phosphate buffer (pH 6.0). The mixture was incubated for 20 min at 37°C. Measurement of chitinase activity was performed using a modified version of the Schales' procedure (Imoto and Yagishita 1971) with GlcNAc as the standard. Protein concentration was measured using the Bradford method (Bradford 1976) with bovine serum albumin as the standard. Shrimp shell colloidal chitin was prepared as described previously (Berger and Reynolds 1958).

Spore-germination inhibition assay

Fungal spores were collected as described previously (Mehmood et al. 2009), with some modifications. First, plant pathogenic fungus was grown on PDA (potato dextrose agar) plates at 28°C for 7 days and then suspended in 5 mL of autoclaved peptone (0.1%) water. Next, the suspension was filtered through sterile paper tissues. Finally, the density of spores was counted using a CX31 upright microscope (Olympus, Japan) and a Neubauer improved hemocytometer counting chamber (Marienfeld, Germany).

Antifungal activity was evaluated by inhibiting spore-germination of *Fusarium* sp., as described previously (Mehmood et al. 2009; Seidl-Seiboth 2013), with some modifications. Fifty microliters containing spores (approximately 4,000 spores) and 20 µg of purified recombinant chitinase B were incubated in a 200-mL Eppendorf tube at 28 ^{o}C for 15 h. Subsequently, the spores were observed using a CX31 upright microscope at ×400 magnification. On the other hand, after 15 h of incubation, the solution was also inoculated at the center of the PDA plate and incubated for a further 48 h at 28°C. The solution containing fungal spores and 20 mM phosphate buffer (pH 6.0) only was used as the control. After incubation of inoculated plates, the diameter of fungal growth was measured in control and treated plates, and antifungal activity was calculated by the formula:

$$\% \text{ Inhibition } = \frac{D - d}{D} \times 100$$

Where: D is the diameter of fungal growth in the control (mm)

d is the diameter of fungal growth in the treatment (mm)

Inhibition of nematode egg hatching

Preparation of root-knot nematode eggs: Eggs of *Meiloidogyne* spp. were isolated from roots of black pepper trees as previously described (Khan et al. 2008) with some modifications (Nguyen et al. 2019).

Estimation of the egg hatching was conducted as described by Lee and Kim (2015) with minor modifications. Briefly, a solution (150 µL) containing eggs (about 40–50 eggs) and 20 µg of purified recombinant chitinase B was incubated in a 200-mL Eppendorf tube at 26 ^{o}C in the dark for three days. For the control, 20 mM phosphate buffer (pH 6.0) was applied instead of purified enzyme. After incubation, the eggs were observed and hatched juveniles from the eggs were counted using the CX31 upright microscope at ×100 magnification.

Results

Identification of chitinase and AA10 protein genes

In the CAZy databases, the genome sequence of *B. velezensis* CAU B946 (HE617159.1) possesses *yaaH* and *ydhD* encoding two putative GH18 chitinases, *yucG* encoding a putative AA10 protein, respectively. Based on the sequence of each gene and surrounding regions of the gene in *B. velezensis* CAU B946 (HE617159.1), primers were designed to identify chitinase and AA10 protein genes in the genomic DNA of *B. velezensis* RB.IBE29. Our result showed that two chitinase genes (*chiA* and *chiB*, respectively) and one AA10 protein gene (*lpmo10*) were successfully identified in the genomic DNA of the strain RB.IBE29 by PCR (Fig. S1B).

Nucleotide sequence, domain structure, and subfamily of chitinase A and B

Each chromosomal DNA fragment containing each gene coding for chitinase or AA10 protein was amplified by PCR using primers (Table S1). The PCR products were inserted into pUC19 previously digested with *Sma*I, following which the nucleotide sequences of the genes were determined and analyzed. The ORF of *chiA* consists of 1,203 base pairs in length and encodes a deduced protein of 400 amino acids (aa) with a predicted molecular mass of 45.46 kDa. The presumed ribosome binding site, AGGAGG, was found upstream of the initial codon. The possible promoter sequences, TTGATA for the –35 region and TATATT for the –10 region with 19-bp spacing between them, were found upstream of the start codon. A 7-bp inverted repeat, which may serve as a transcription termination signal, was observed downstream of the stop codon of *chiA* (Fig. S2).

The primary structure of the deduced protein was analyzed by using the SMART and Pfam programs. The result showed that the deduced protein lacks a signal sequence, and contains a carbohydrate-binding module family 50 (45 aa, residues 21–65) and a catalytic domain of family 18 chitinases (308 aa, residues 73–380) (Fig. 1). Therefore, the deduced protein was named chitinase A (BvChiA). The catalytic domain of BvChiA showed 100% identity to that of an uncharacterized chitinase (AXY36285.1) of *B. velezensis* CAU B946 (Blom et al. 2012), followed by well-characterized chitinases, including 55.7% identity with chitinase (Q9K3E4.1) from *B. cereus* (Chen et al. 2000) and chitinase (WP_000614841) from

B. anthracis (Bateman et al. 2000), followed by 25.8% identity to chitinase A1 (AAA81528) of *B. circulans* WL-12 (Watanabe et al. 1990), 23.9% and 22.6% identities to chitinase A (BAA31567) and chitinase B (BAA31568), the two chitinases of *S. marcescens* 2170 (Watanabe et al. 1997). In addition, a glutamate residue, which was reported to be essential for hydrolysis of family 18 chitinases acting as a proton donor, was found in the catalytic domain of BvChiA (corresponding to Glu-187). Other residues, which might be necessary for saccharide binding (corresponding to Trp-375) and catalytic reaction (corresponding to Asp-185 and Tyr-252), are conserved in the catalytic domain of BvChiA (Watanabe et al. 1993; Itoh et al. 2014) (Fig. S3).

The ORF of *chiB* includes 1,263 bp and encodes a deduced protein of 420 aa. The deduced protein has a molecular mass of 47.59 kDa. A sequence located upstream of the start codon, AGGAGG, was predicted to be a sequence for the ribosome binding site. The possible promoter sequences were identified upstream of the start codon, TTGAGT for the –35 region and TATAAT for the –10 region. A 6-bp inverted repeat sequence, which may serve as a transcription termination signal, was observed downstream of the stop codon of *chiB* (Fig. S4).

The primary structure of the deduced enzyme lacks a signal peptide, and contains two CBM 50 domains (the first domain, 44 aa, residues 3–46; and the second domain, 45 aa, residues 49–93) and a catalytic domain of family 18 chitinases (311 aa, residues 93–403) (Fig. 1). The deduced protein was named chitinase B (BvChiB). The catalytic domain of BvChiB showed 100% identity to that of an uncharacterized chitinase (AXY37439.1) of *B. velezensis* CAU B946 (Blom et al. 2012), followed by characterized chitinases, including 32.3% identity to chitinase (WP_000614841) from *B. anthracis* (Bateman et al. 2000), 31.9% identity to chitinase (Q9K3E4.1) from *B. cereus* (Chen et al. 2000), 22.9% identity to chitinase A1 (AAA81528) of *B. circulans* WL-12 (Watanabe et al. 1990), and 25.4% and 22.1% identities to chitinase A (BAA31567) and chitinase B (BAA31568), respectively, the two chitinases of *S. marcescens* 2170 (Watanabe et al. 1997). Furthermore, a glutamate residue that is essential for the hydrolysis of family 18 chitinases, was found in the catalytic domain of BvChiB (corresponding to Glu-212). In addition, residues that were revealed to be important for saccharide binding (corresponding to Trp-398) and catalytic reaction (corresponding to Asp-211 and Tyr-275) are also conserved in the catalytic domain of BvChiB (Watanabe et al. 1993; Itoh et al. 2014) (Fig. S3). These analyses indicate that BvChiA and BvChiB are members of bacterial family 18 chitinases.

Bacterial GH18 chitinases can be further divided into three subfamilies, A, B, and C. Subfamily A has an extra domain with a small $\alpha+\beta$ domain inserted into the core TIM (triosephosphate isomerase)-barrel fold, while subfamilies B and C have no such domain (Suzuki et al. 1999). Hence, they display different chitin-degradation properties. To classify the subfamily of BvChiA and BvChiB from *B. velezensis* RB.IBE29, amino acids in the catalytic domain of these enzymes and those of other bacterial GH18 chitinases were aligned using the Clustal W program, and phylogenetic analysis was then performed from this alignment using the neighbor-joining method. The result showed that BvChiA and BvChiB are grouped into subfamily A (Fig. 2). In addition, the small $\alpha+\beta$ domain inserted between the seventh and eighth β-strands of (β/α)8 was identified in the catalytic domain sequences of BvChiA and BvChiB (Fig.

S3). These analyses suggest that BvChiA and BvChiB belong to subfamily A of bacterial GH18 chitinases.

Nucleotide sequence and domain structure of BvLPMO10

The ORF of *lpmo10* consists of 621 bp encoding a deduced protein of 206 aa with a predicted molecular mass of 22.44 kDa. A Shine-Dalgarno sequence, AGGAGG, was identified upstream of the start codon of *lpmo10*. The possible promoter sequences, TTGAAG for the –35 region and TTTTAT for the –10 region with 18-bp spacing between them, were identified upstream of the initial codon of *lpmo10*. A 9-bp inverted repeat sequence, which may serve as a transcription termination signal, was found downstream of the stop codon of *lpmo10* (Fig. S5).

The primary structure of the deduced protein contains a signal peptide (19 aa, residues 1–19) and an AA10 catalytic domain (175 aa, residues 28–202) (Fig. 1). Therefore, the deduced protein was named BvLPMO10. The calculated molecular mass of BvLPMO10 without the signal peptide is 20.67 kDa.

Amino acids of the catalytic domain of BvLPMO10 exhibited 100% identity to those of an uncharacterized AA10 protein (AXY37869.1) of *B. velezensis* CAU B946 (Blom et al. 2012), 49.15% to *Sm*LPMO10A (formerly CBP21, AAU88202) (Vaaje-Kolstad et al. 2005b) and to *Sm*CBP21 (formerly CBP21 (BAA31569) (Suzuki et al. 1998), 44.9% to *Sp*CBP50 (ABV43333) (Purushotham et al. 2012). In addition, two coordinating histidine residues that are important for the copper active site of AA10 proteins (Chaplin et al. 2016; Forsberg et al. 2016) were well conserved in the catalytic domain of BvLPMO10 (corresponding to His-28 and His-125 of BvLPMO10). Moreover, four out of the six surface-located polar residues of *Sm*LPMO10A and *Sp*CBP50 that are important in the chitin-binding (Vaaje-Kolstad et al. 2005b; Purushotham et al. 2012) were also found in the catalytic domain of BvLPMO10 (corresponding to Glu-68, His-125, Asp-191, and Asn-194). Tyr-54 and Glu-55 in *Sm*LPMO10A were replaced by Asp-62 and Asn-63 in the BvLPMO10A (Fig. S6). These analyses suggest that BvLPMO10 from *B. velezensis* RB.IBE29 is a member of the auxiliary activities family 10 proteins.

Expression and purification of recombinant chitinase B

The gene *chiB*, which encodes the two CBM50 domain-containing chitinase, was successfully expressed in *E. coli* BL21-CodonPlus (DE3)-RIPL cells and the resulting recombinant protein (rBvChiB) was purified. The molecular size of rBvChiB was estimated to be 51 kDa by SDS–PAGE. This estimation was in good agreement with the calculated molecular mass of the protein based on its amino acid sequence (BvChiB, 47.59 kDa + polyhistidine tag, 3.18 kDa = 50.77 kDa) (Fig. 3).

Hydrolytic activity of purified recombinant chitinase B

As shown in Fig. 4, purified rBvChiB had the highest hydrolytic activity against colloidal chitin. The activity was seven times higher than that of shrimp shell powdered chitin and shrimp shell chitin flakes used as the substrates, respectively. Furthermore, among the insoluble forms of chitin, rBvChiB exhibited

approximately five fold higher chitinase activity against β-chitin (squid chitin flakes) than that of α-chitin (shrimp shell powdered chitin and shrimp shell chitin flakes).

Effect of purified recombinant chitinase B on fungal spore-germination

Black pepper cultivation in the Central Highland of Vietnam faces several root diseases, such as the slow decline disease, the primary cause of *Fusarium* spp. (Nguyet et al. 2021). To evaluate antifungal activity by purified enzyme, purified rBvChiB was incubated with spores of fungal *Fusarium* sp., we found that purified rBvChiB had a significant activity against spore-germination of *Fusarium* when 20 µg of the enzyme was applied for the test. rBvChiB affected the tips of fungal spores compared with the control (Fig. 5A-B), resulting in suppression of spore-germination by 54.5% as examined using a PDA plate (Fig. 5C). These results indicate that chitinase B possesses an inhibiting activity against spore germination of phytopathogenic fungi.

Effect of purified recombinant chitinase B on hatching of nematode eggs

Root-knot nematodes (genus *Meloidogyne*) have been recognized as a major pathogen of pepper worldwide (Djian-Caporalino et al. 2007; Lee and Kim 2015). Black pepper cultivation in the Central Highlands, Vietnam, also faces other problems caused by the primary agent of root-knot nematode, *Meloidogyne* spp. (Tran et al. 2019). To estimate anti-nematode activity by chitinase B, the eggs of *Meloidogyne* spp. were incubated with 20 µg of purified rBvChiB. We found that the egg hatching in the presence of rBvChiB was significantly decreased compared to that of the control (Fig. 6A-B). For example, on day 3 of incubation, the egg hatching in the treated sample was 3.45%, while the untreated sample was 100%. In addition, our observation found that all second-stage juveniles (J2) hatched from eggs in the treatment were dead after 4 days of incubation; however, those in control were healthy (Fig. 6A). This result implies that chitinase B of *B. velezensis* RB.IBE 29 possesses anti-nematode activity.

Discussion

In this study, to identify genes related to chitinolytic enzymes from the genomic DNA of *B. velezensis* RB.IBE29, we based on sequences of genes encoding chitinases and AA10 protein in *B. velezensis* CAU B946 (HE617159.1) available in the CAZy databases because of the 16S rDNA sequence of strain RB.IBE29 was 100% identical to that of *B. velezensis* CAU B946. In the CAZy database, strain CAU B946 possesses two family 18 chitinases and one AA10 protein. Moreover, the primary structures of these chitinases contain CBM50 domain(s), which was just characterized in plant chitinase and fungal protein (Seidl-Seiboth et al. 2013; Inamine et al. 2015). Additionally, the species *B. velezensis* was reported to be a strong chitinase-producing bacterium, biocontrol and plant-growth promoting agent, and has been widely used for agricultural cultivation (Lim et al. 2017; Cai et al. 2017). Our strain, RB.IBE29 also

possesses such properties as examined *in vitro*, greenhouse, and field conditions (Trinh et al. 2019; Nguyen et al. 2021). To our knowledge, experimental characterization of chitinases and AA10 protein from *B. velezensis* has not been reported thus far. Hence, strain RB.IBE29 was a good candidate to continuously explore its chitinase system concerning chitinase, antifungal, and anti-nematode activities for crop production.

The CBM50 (previously LysM) domain is a family of carbohydrate-binding modules. This domain contains 44–65 amino acids in length and binds to *N*-acetylglucosamine-containing glycans, such as peptidoglycan, chitin, and its derivatives (Buist et al. 2008). This domain is found in many enzymes involved in cell wall degradation and other proteins associated with bacterial cell walls (Onaga and Taira 2008). Furthermore, it was reported that the CBM50 domain possesses antifungal activity against various phytopathogenic fungi (Seidl-Seiboth et al. 2013; Inamine et al. 2015; Takashima et al. 2020). Hence, the CBM50 domains from BvChiA and BvChiB of *B. velezensis* RB.IBE29 (Fig. 1) probably have such activity. However, in this study, the amino acid sequences of the CBM50 domains of BvChiA and BvChiB shared less than 36.4% identity to each other and less than 37% identity with those of characterized CBM50-containing plant chitinases and CBM50-containing fungal proteins (Onaga and Taira 2008; Seidl-Seiboth et al. 2013; Inamine et al. 2015). Therefore, BvChiA and BvChiB may show different chitinase and antifungal activity properties. To our knowledge, the role of CBM50 domains from bacterial chitinases has not been reported thus far. Consequently, it is necessary to characterize the CBM50 domains of BvChiA and BvChiB concerning their chitin-binding, chitinase, antifungal and anti-nematode activities in further studies. Moreover, to date, reported bacterial chitinases usually possess CBM5, fibronectin type III, CBM12, and CBM73 domains in their primary structures (Ohno et al. 1996; Suzuki et al. 1999; Huang et al. 2012a; Pentekhina et al. 2020). Hence, chitinases from *B. velezensis* RB.IBE29 seem to be a novelty compared to the known bacterial chitinases.

BvChiA and BvChiB belong to subfamily A of bacterial GH18 chitinases based on phylogenetic analysis using the amino acids in their catalytic domains (Fig. 2). Various chitinases of subfamily A are processive chitinases and possess high hydrolytic activity toward crystalline chitin, such as ChiA and ChiB from *S. marcescens* 2170 (Uchiyama et al. 2001; Hult et al. 2005). Hence, it is hoped that BvChiA and BvChiB of our bacterium, *B. velezensis* RB.IBE29 are processive chitinases. Moreover, BvLPMO10 can act on the surface of crystalline chitin. It can introduce breaks in the chitin chain and generate oxidized chain ends, thus promoting further chitin degradation by BvChiA and BvChiB. In this study, purified rBvChiB has significant inhibitory activity against spore germination of *Fusarium* (Fig. 5) and egg hatching of root-knot nematode, *Meloidogyne* (Fig. 6). Hence, the synergistic action of three enzymes of this bacterium probably increases the efficiency of hydrolysis of chitin, antifungal and anti-nematode activities. Further studies are necessary to examine the synergistic action of these enzymes in detail.

Amino acid sequences in the catalytic domain of BvChiA and BvChiB share a low level of sequence identity (32%). Therefore, these chitinases may possess different chitin-binding and enzymatic properties. Sequence analysis revealed that aromatic residues (tryptophan, phenylalanine, and tyrosine), which are important for guiding a chitin chain into the catalytic cleft during the crystalline chitin hydrolysis and

substrate binding of ChiA and ChiB from *S. marcescens* 2170 (Uchiyama et al. 2001; Katouno et al. 2004), and ChiA1 from *B. circulans* WL-12 (Watanabe et al. 2003), are lacking in the catalytic domain of BvChiA and BvChiB (Fig. S3). This analysis implies that the lack of these aromatic residues of BvChiA and BvChiB may affect the chitinase, antifungal, and anti-nematode activities of the enzymes. On the other hand, the result of hydrolytic activity against crystalline chitin by purified rBvChiB (Fig. 4) revealed that the hydrolytic activity of the enzyme was approximately 62% and 25% less than that of ChiA and ChiB, respectively, from *S. marcescens* 2170 (Suzuki et al. 2002), and 30% less than that of chitinase A from *Aeromonas salmonicida* SWSY-1.411 (Pentekhina et al. 2020). Therefore, most probably, the main reason for its low activity on the crystalline substrates could be attributed to the lack of these exposed aromatic residues in the catalytic domain of BvChiB.

The gene *chiB* was successfully expressed in *E. coli* and purified. A test of biocontrol ability revealed that purified rBvChiB (the family 18 chitinase) had significant activity against spore-germination of *Fusarium* (Fig. 5) and inhibitory effect on egg hatching of root-knot nematode, *Meloidogyne* (Fig. 6). To date, it is clear that chitin is the main component of the fungal cell wall and a component of eggshells of various nematodes, including plant-parasitic nematodes *Meloidogyne* spp. (Fanelli et al. 2005). Previous studies have estimated that the eggshells of *Meloidogyne* contain about 30% chitin (Clarke et al. 1967; Bird and Mcclure 1976). Therefore, the inhibition of spore-germination and egg hatching by purified rBvChiB could be attributed to its digestion of chitin in the walls of fungal spores and eggshells of nematode. In addition, it was reported that chitin has also occurred in the pharynx of nematodes other than in the eggshells (Zhang et al. 2005). Our microscope observation found that all J2s hatched from eggs in the treated sample were dead after 4 days of incubation, but those in control were healthy (Fig. 6A). Hence, the main reason for this result could be caused by the degradation of chitin in the pharynx of J2s. On the other hand, it was previously revealed that among the bacterial chitinases, family 19 chitinases had been shown as primary enzymes involved in the antifungal activity (Ohno et al. 1996; Watanabe et al. 1999; Tsujibo et al. 2000; Kawase et al. 2006; Huang et al. 2012a; Pentekhina et al. 2020). Since purified rBvChiB showed significant antifungal and anti-nematode activities by our *in vitro* test, it is implied that the role and potent application of bacterial GH18 chitinases within the CBM50-domain-containing chitinases in biocontrol of plant pathogenic fungi and root-knot nematodes could be expanded.

In conclusion, three genes encoding two family 18 chitinase and an AA10 protein were identified from the genomic DNA of *B. velezensis* RB.IBE29 and then analyzed. Analyses of primary structures of enzymes and examination of purified rBvChiB indicate that the chitinase system of this bacterium is a possible novel system and probably possesses interesting bio-properties of chitin-degradation, antifungal and anti-nematode activities. Strain RB.IBE29 and rBvChiB could be further developed to control plant pathogenic fungi and plant-parasitic nematodes under a biocontrol agent alternative chemical pesticide for sustainable crop production. Currently, characterization of rBvChiA, rBvChiB, and rBvLPMO10 and their synergistic effects on the hydrolysis of insoluble chitin and ability against phytopathogenic fungi and plant-parasitic nematodes are underway. This is the first description of sequence analysis of the chitinase system from *B. velezensis*.

Abbreviations

GlcNAc, *N*-acetyl-D-glucosamine, GH, glycoside hydrolases, AA10, auxiliary activities family 10, CBM50, carbohydrate-binding module family 50, LB medium, Luria-Bertani medium, PCR, polymerase chain reaction, bp, base pair, ORF, open reading frame, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, aa, amino acid.

Declarations

AUTHOR CONTRIBUTIONS

D.M. Tran conceived the study, designed the experiments, analyzed, and wrote the manuscript. D.M. Tran, T.U. Huynh, T.H. Nguyen, T.O. Do, Q.V. Nguyen, and A.D. Nguyen performed experiments. A.D. Nguyen involved in the interpretation of the data and critical reading of the draft. All authors reviewed and approved the final manuscript.

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CONFLICT OF INTERESTS

The authors reported no potential conflict of interest.

DATA AVAILABILITY

Sequences in this study have been deposited in the DNA Data Bank of Japan database under accession numbers LC649802–LC649804.

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Figures

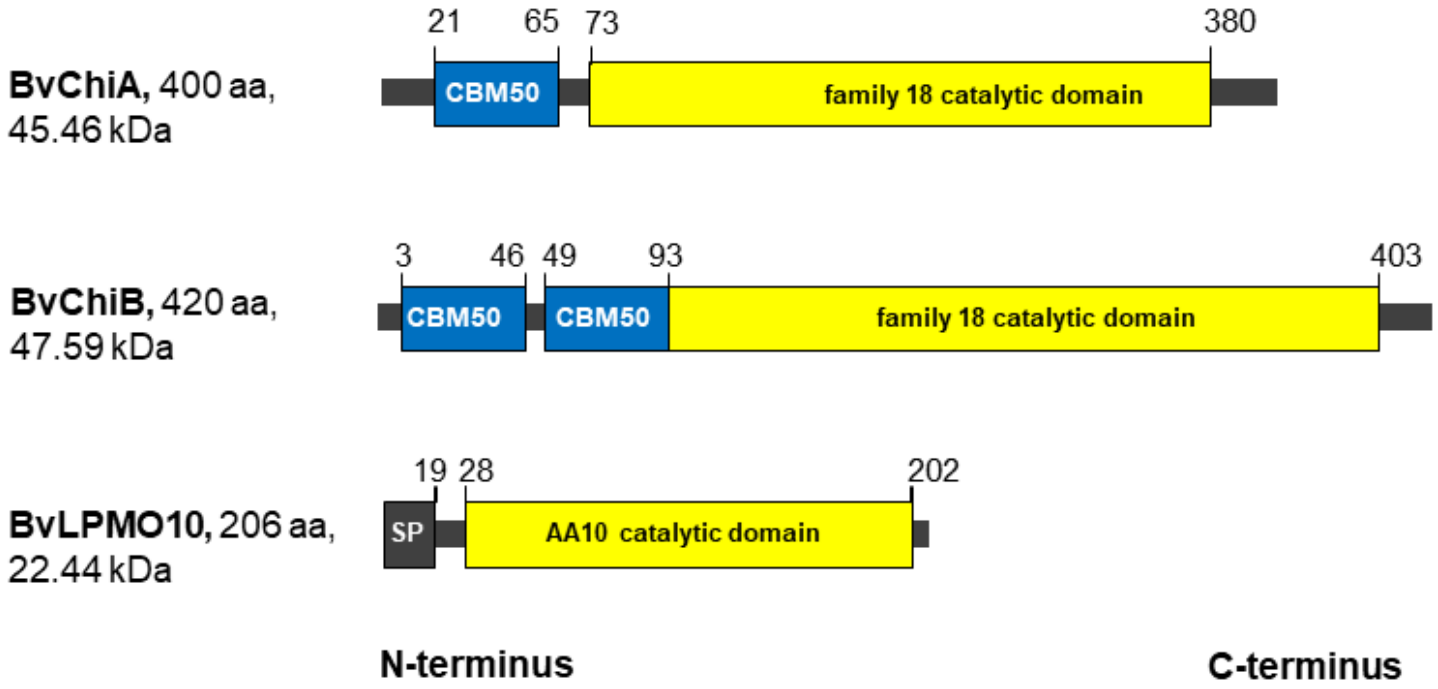

Fig. 1

Figure 1

Domain structures of three proteins. Numerals in the domain structures of proteins indicate the number of amino acids starting from the start codon of the ORFs. SP, signal peptide, CBM50, carbohydrate-binding module family 50.

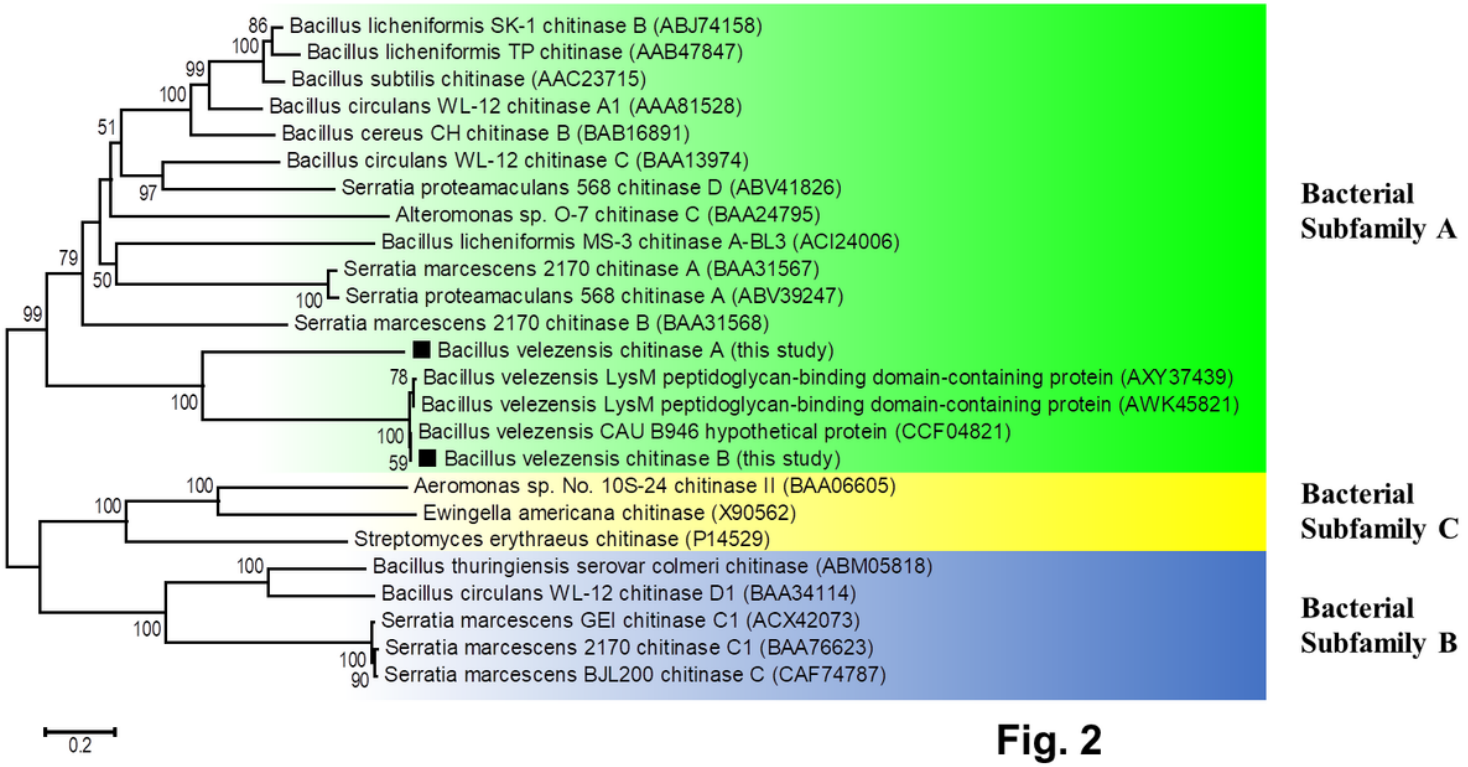

Figure 2

Phylogenetic relationships among family 18 chitinases. Amino acids of the catalytic domain of BvChiA and BvChiB (filled rectangle) and those of other bacterial GH18 chitinases were used for the analysis. Phylogenetic trees were produced using the MEGA 6.0 after multiple alignments of data by the Clustal W. The tree was constructed using the neighbor-joining method, and evolutionary distances were computed using the Poisson correction method. Numbers at the branches indicate bootstrap confidence percentages (%) based on 1000 resampled data sets, only bootstrap confidence percentages > 50% are shown. Bar, 0.2 substitutions per amino acid position.

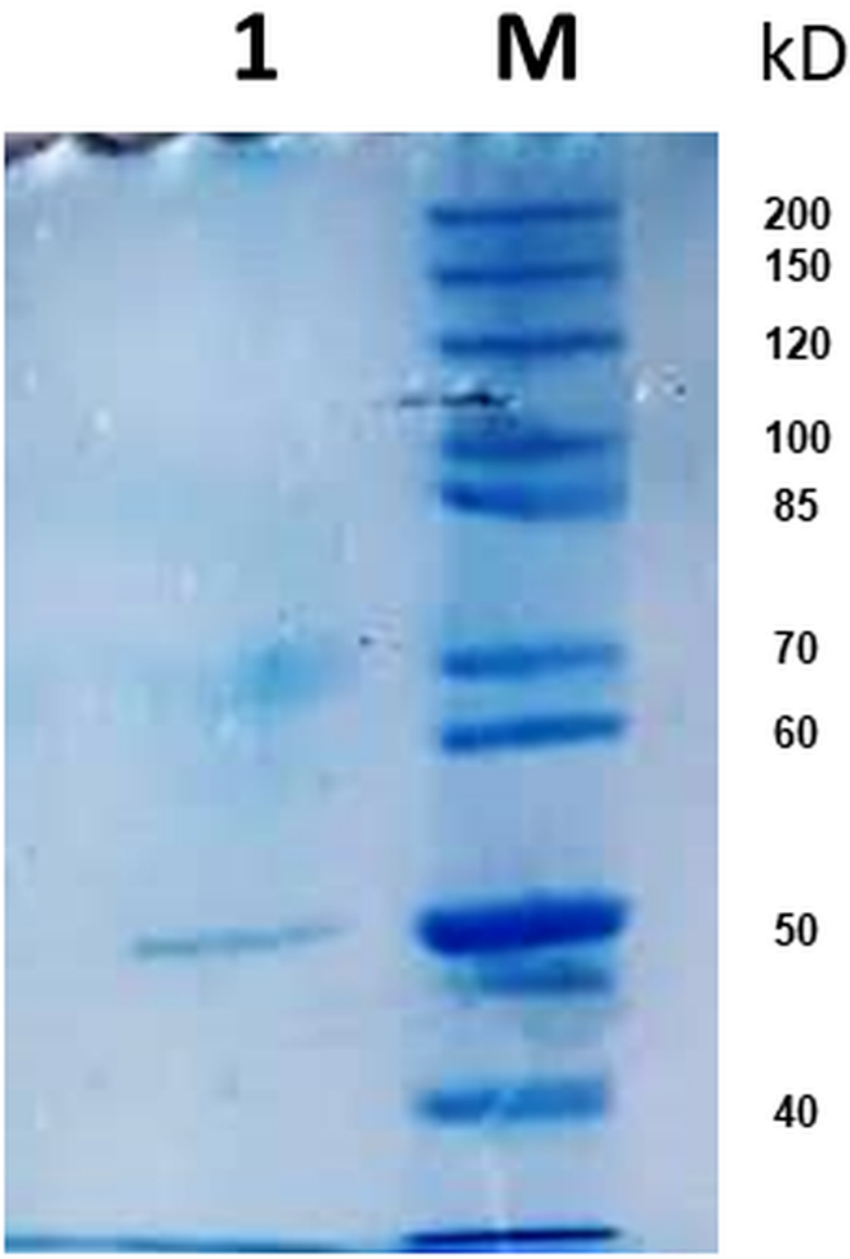

Fig. 3

Figure 3

SDS–PAGE analysis of purified rBvChiB. Lane M, size markers in kDa, lane 1, purified rBvChiB (2 µg). The SDS–PAGE analysis was done on an 11.5% polyacrylamide gel.

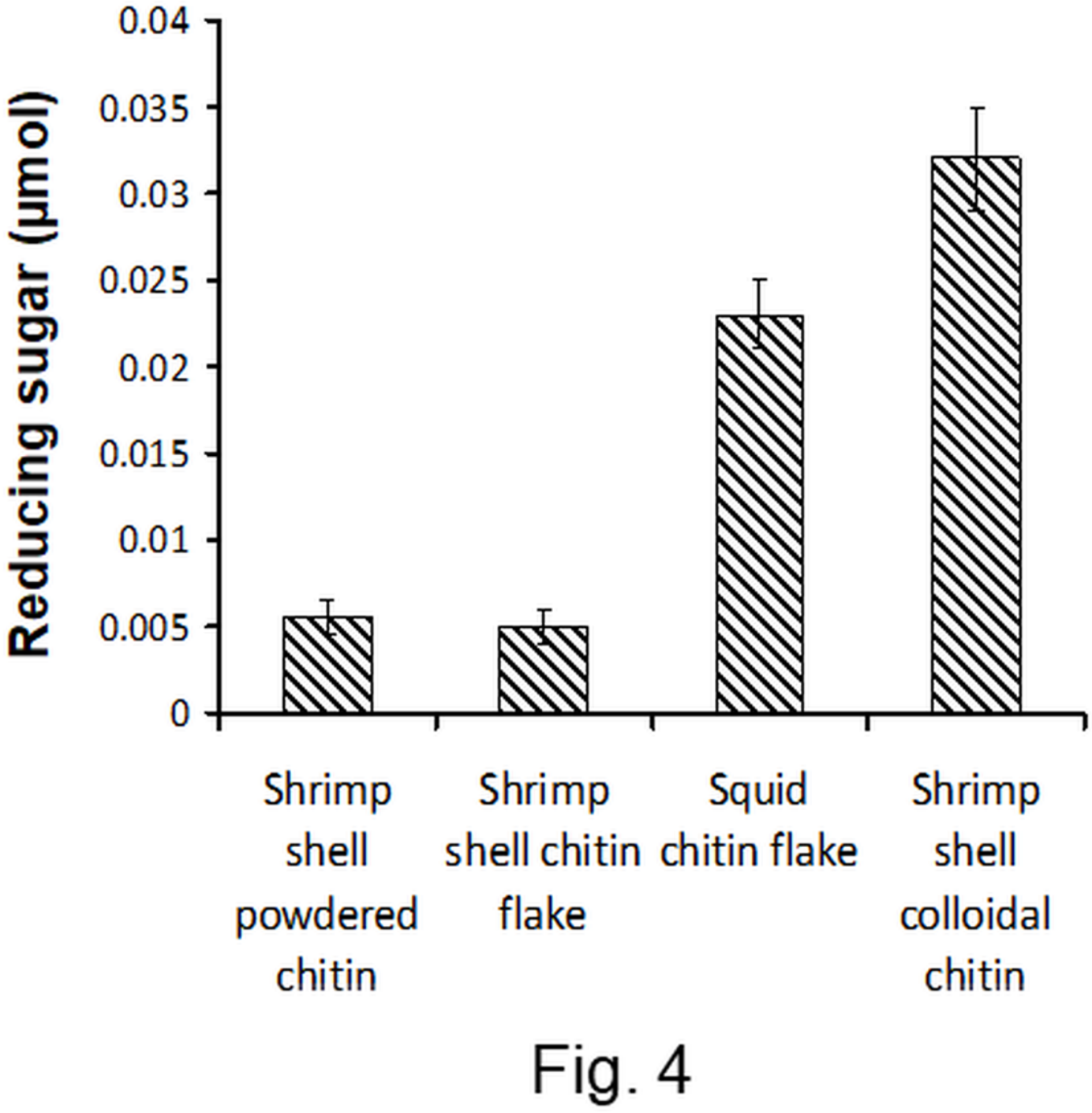

Figure 4

Hydrolytic activity of purified rBvChiB against chitinous substrates. The hydrolysis of chitin by purified rBvChiB was measured using the modified version of Schales' procedure and GlcNAc as the standard.

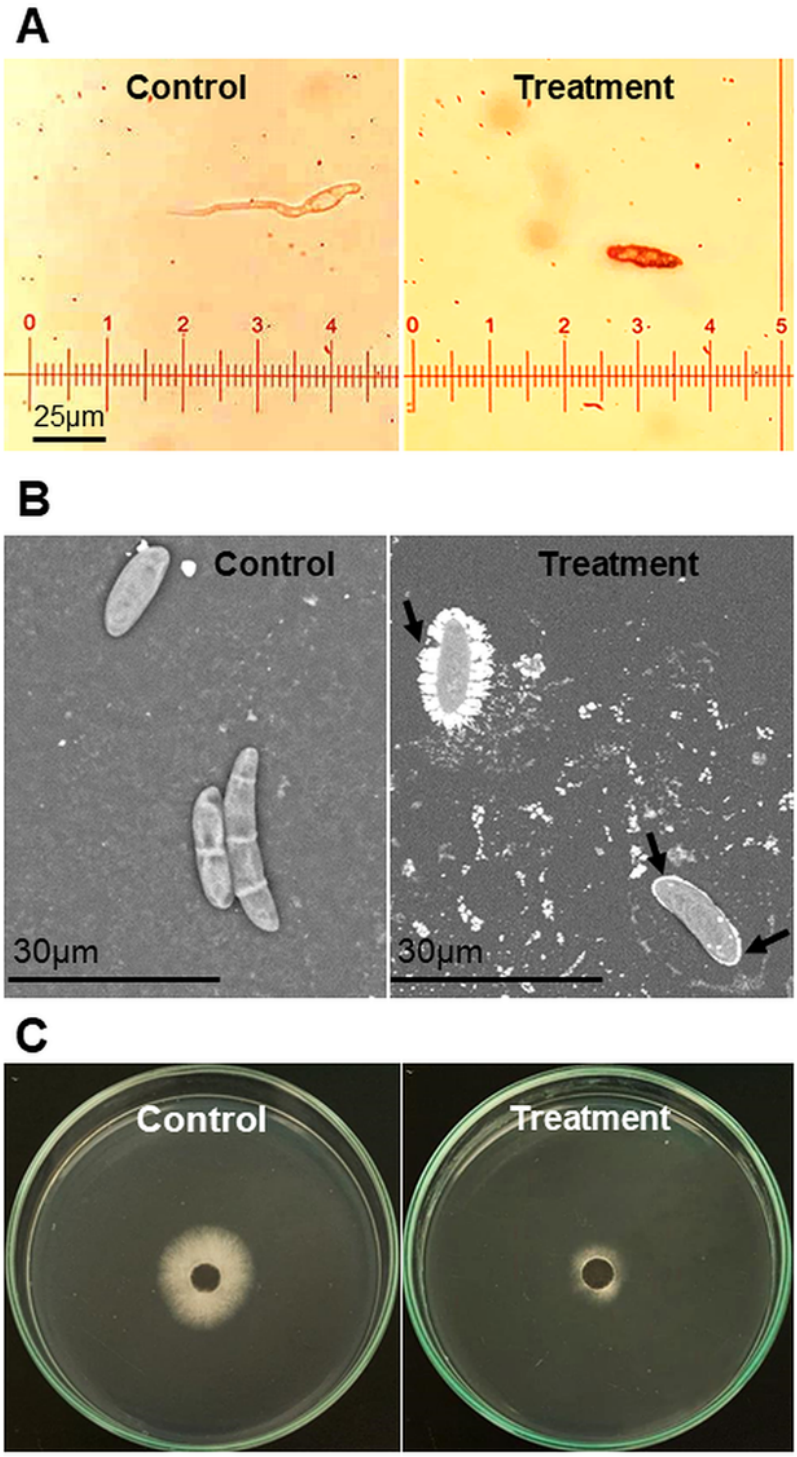

Fig. 5

Figure 5

Inhibition of spore-germination by purified rBvChiB. (A), spores observed using a microscope at 400×, (B), spores observed using the scanning electron microscope (SEM) (Phenom ProX, Thermo Fisher Scientific, USA), (C), inhibition of spore-germination after incubating treated spores on PDA plates. A solution (50 µL) containing spores (approximately 4,000 spores) and 20 µg of purified rBvChiB were set in a 200-mL Eppendorf tube and incubated at 28 oC for 15 h. Subsequently, the treated spores were observed using a

CX31 upright microscope at 400× magnification. On the other hand, the treated spores were inoculated at the center of the PDA plate and incubated for a further 48 h at 28 °C. In control, spores were treated with 20 mM phosphate buffer (pH 6.0). Arrows indicate the possible destruction of the wall of spores.

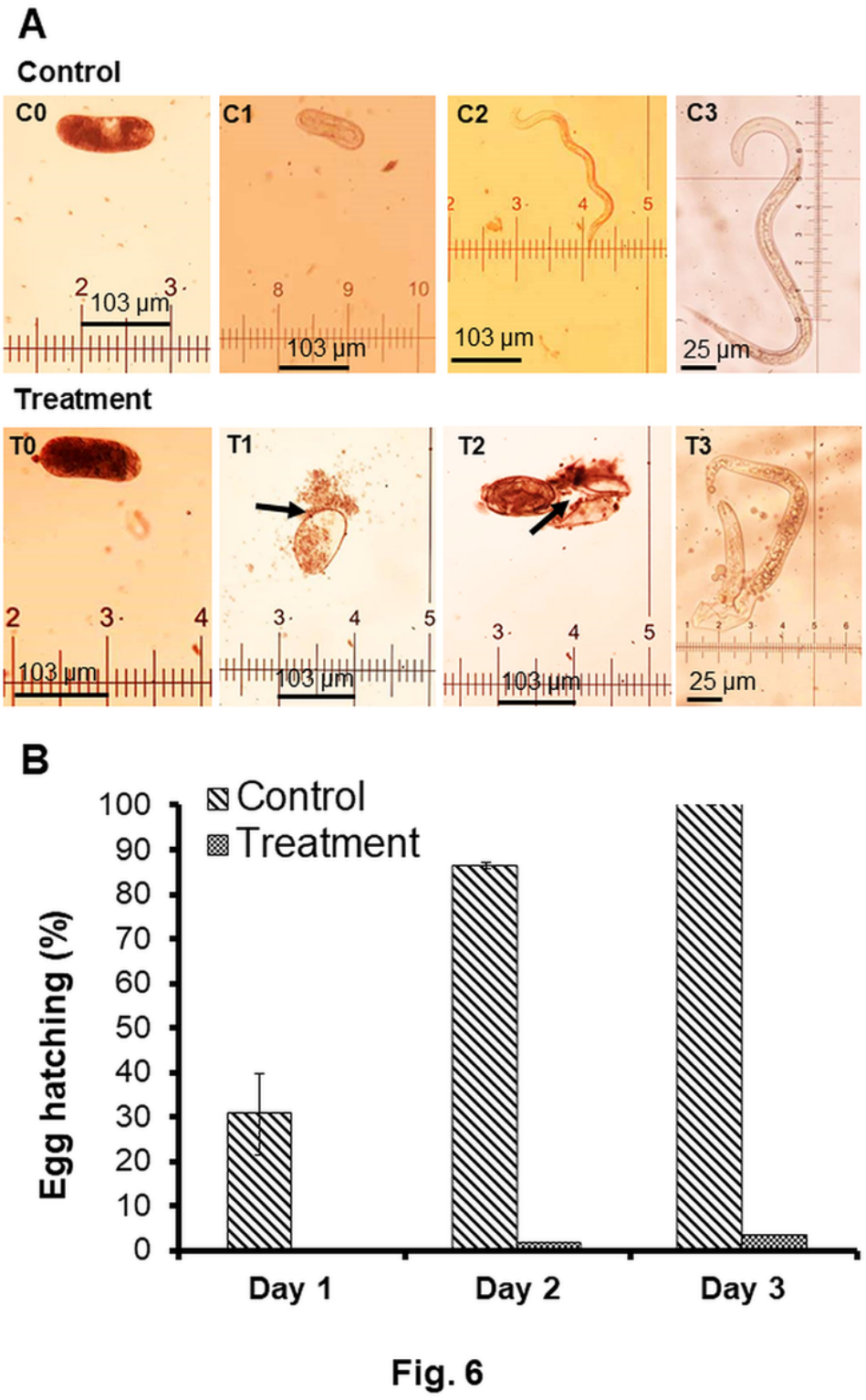

Figure 6

Inhibition of nematode egg hatching by purified rBvChiB. (A), eggs were observed using a microscope at 100× magnification. Panel Control, the egg before incubation (C0), eggs after incubation, and appearance

of the first stage of the juvenile (C1) and J2 (C2), and healthy J2 (C3). Panel treatment, the egg before treatment (T0), the egg after one day (T1) and two days (T2) of treatment, and died J2 (C3), (B), %inhibition of nematode egg hatching by purified rBvChiB. A solution (150 µL) containing Meloidogyne eggs (40–50 eggs) and 20 µg of purified rBvChiB were incubated in a 200-mL Eppendorf tube at 26 oC in the dark. In control, eggs were treated with 20 mM phosphate buffer (pH 6.0). Arrows indicate the possible destruction of the eggshell.

Supplementary Files

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