IDENTIFICATION AND SEQUENCE ANALYSIS OF A FAMILY 18 CHITINASE-ENCODING-GENE *(chiB)* FROM A CHITINOLYTIC BACTERIUM ISOLATED FROM THE CENTRAL HIGHLAND REGION

NHẬN DIỆN VÀ PHÂN TÍCH TRÌNH TỰ GENE CHITINASE HỌ 18 (*chiB*) Ở VI KHUẨN PHÂN LẬP TẠI TÂY NGUYÊN

Bich Thuy Vu[1], Thi Anh Do[1], Thi Huyen Nguyen[1], To Uyen Huynh[1], Tu Oanh Do[1], Iuliia Pentekhina[2], Anh Dzung Nguyen[1], Dinh Minh Tran[1*]

[1]Tay Nguyen University

[2]School of Economics and Management, Far Eastern Federal University, Russia

*Corresponding author: tmdinh@tnu.edu.vn

(Received: October 19, 2020; Accepted: December 21, 2020)

Abstract - Chitinolytic bacteria and their chitinases have attracted great attention due to potential applications in various fields, including medicine, food processing, agriculture. To develop a novel type of biocontrol agents alternative chemical agents for phytopathogenic controlling, we focus on bacteria possessed high chitinase activity. In this study, a chitinase gene (*chiB*) from *Bacillus velezensis* RB.IBE29 was identified, cloned, and analyzed. The ORF of *chiB* consists of 1,263 base pairs and encodes a deduced protein (*Bv*ChiB) of 420 amino acids with a predicted molecular mass of 47.59 kDa. The primary structure analysis of *Bv*ChiB revealed that the deduced enzyme is composed of two carbohydrate-binding module family 50 domains at the N-terminus and a catalytic domain at the C-terminus. *Bv*ChiB was grouped into subfamily A of bacterial GH18 chitinases based on phylogenetic analysis. Analyses based on the primary and three-dimensional structures showed that differences of important residues were observed between *Bv*ChiB and well-known chitinases reported. These analyses imply that *Bv*ChiB possibly possesses an interesting role in the degradation of insoluble chitin. This is the first report describing sequence analyses of the chitinase gene from the bacterium. We are conducting the expression, purification, and characterization of *Bv*ChiB concerning chitinase and antifungal activities.

Key words - chitinase; *chiB* gene; sequence analysis; CBM50

Tóm tắt -. Vi khuẩn sở hữu chitinases có tiềm năng ứng dụng lớn trong các lĩnh vực như y học, thực phẩm và nông nghiệp... Để phát triển tác nhân sinh học mới thay thế thuốc hóa học trong kiểm soát nấm bệnh hại cây trồng, chúng tôi tập trung nghiên cứu vi khuẩn sinh chitinase. Trong nghiên cứu này, một gene mã hóa chitinase (chiB) ở Bacillus velezensis RB.IBE29 đã được nhận diện, tạo dòng và phân tích. ORF của chiB gồm 1263 bp, mã hóa protein (BvChiB) dài 420 aa với khối lượng 47,59 kDa. Phân tích cấu trúc bậc một của BvChiB cho thấy enzyme gồm hai domain bám CBM50 ở đầu N và một domain xúc tác ở đầu C. Phân tích cây phân loại kết luận BvChiB thuộc vào họ phụ A của chitinase họ 18. Các phân tích dựa vào cấu trúc bậc một và bậc ba cho biết có sự khác biệt về các phân tử quan trọng giữa BvChiB và các chitinase đã được báo cáo. Các phân tích này chỉ ra rằng BvChiB có thể sở hữu vai trò mới trong trong việc phân hủy chitin. Đây là nghiên cứu đầu tiên về trình tự gene chitinase ở vi khuẩn này. Các nghiên cứu về biểu hiện, tinh sạch và vai trò của BvChiB liên quan tới phân hủy chitin và kháng nấm bệnh đang được thực hiện.

Từ khóa - enzyme chitinase; gen chiB; phân tích trình tự; CBM50

1. Introduction

Chitin is an insoluble linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc). Chitin is the most abundant biopolymer in the aquatic biosphere with annual production estimated to be 10^{11} tons [1] and is widely distributed in nature such as constituent of insect exoskeletons, shells of crustaceans, and cell walls of fungi [2]. Chitinases (EC 3.2.1.14) are enzymes that degrade chitin. These enzymes are found in both chitin-containing organisms and nonchitin-containing organisms such as bacteria, fungi, insects, plants, and animals. To date, most chitinases are classified into two different families of glycoside hydrolases (GH), families 18 and 19, based on their amino acid sequences in the catalytic domain [3].

Various chitinase genes have been cloned, analyzed, and characterized in detail from a variety of bacteria such as *Serratia marcescens* 2170 [4, 5, 6], *Bacillus circulans* WL-12 [7, 8], *Chitiniphilus shinanonensis* SAY3 [9, 10], *Paenibacillus* sp. FPU-7 [11], *Alteromona*s sp. O-7 [12]. A large number of studies have been demonstrated that bacterial chitinases display an important role in inhibiting hyphal growth of phytopathogenic fungi, among them, GH19 chitinases have been shown as primary enzymes involved in such activity [9, 13]. Therefore, chitinase-producing bacteria could be widely applied for crop production as biocontrol agents of fungal phytopathogens alternative chemical agents [14]. Chitinolytic bacteria normally produce several chitinases and/or auxiliary activities family 10 (AA10) proteins to efficiently degrade insoluble chitin for their carbon and nitrogen sources. AA10 proteins are enzymes that were previously classified into carbohydrate-binding modules (CBM) in family 33 and have been reclassified into the auxiliary activities family 10 of lytic polysaccharide monooxygenases.

Recently, we isolated and identified a promising bacterium, *Bacillus velezensis* RB.IBE29 (previously *B. velezensis* RB.DS29), from the rhizosphere of the Central Highlands of Vietnam, where black pepper is cultivated. The bacterium showed high chitinase and antifungal activities against *Phytophthora* sp. which is the main cause of black pepper wilt disease in the Central Highlands [15]. In the CAZy database (http://www.cazy.org/b.html), *Bacillus velezensis* is shown to be possessed several GH18 chitinases and AA10 proteins. However, to the best of our

knowledge, no studies on chitinases and AA10 proteins from the bacterium concerning chitinase and antifungal activities have been reported so far.

In this report, we describe identification, cloning, and sequencing analyses of the *chiB* gene encoding a family 18 chitinase from the genomic DNA of *B. velezensis* RB.IBE29.

2. Materials and methods

2.1. Bacterial strain, plasmid, and culture medium

B. velezensis RB.IBE29 [previously *B. velezensis* RB.DS29, 15] was used as the source of chromosomal DNA for gene identification. *Escherichia coli* DH5α was used as the host for gene cloning. pUC19 was used as the vector for gene cloning. Luria-Bertani (LB) medium was used for routine cultures.

2.2. Gene identification

To identify *chiB* in the genomic DNA of strain RB.IBE29, we based on sequences of genes encoding chitinases in *B. velezensis* species available in the CAZy databases (http://www.cazy.org/b.html) to design primers for polymerase chain reaction (PCR). PCR-reactions were conducted using primers (GH18-2f: 5'-CCGCTATATTGCTTGCATGAG-3' and GH18-2r: 5'-AGCCTCGTTGATATACTGCTC-3'), genomic DNA of strain RB.IBE29, and Mytaq DNA polymerase (Bioline, USA) according to the manufacturer's instructions. The reaction mixtures were incubated in a C1000 thermal cycler (Bio-Rad, USA) and the amplified products were then analyzed by electrophoresis on agarose gel (1%, w/v).

2.3. Gene cloning and sequencing analysis

A fragment containing the *chiB* gene, including 543 bp upstream of and 124 bp downstream of the *chiB* ORF, was amplified using the genomic DNA, primers GH18-2f and GH18-2r, and Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific Inc., USA). The amplified fragment was then ligated into the plasmid pUC19 previously treated with *Sma*I (New England Biolabs, USA) by using a DNA ligation kit (Mighty mix, Takara Bio Inc., Shiga, Japan) to generate the recombinant plasmid pUC-*chiB*. Finally, the recombinant plasmid pUC-*chiB* was 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 based on the blue/white selection assay [16].

The recombinant plasmid from the positive colonies examined by colony-PCR was isolated and purified using an AccuPrep Plasmid Miniprep Kit (Bioneer Co., Republic of Korea) and sent to the First base Company (Malaysia) for sequencing.

Nucleotide sequences obtained by the sequencing were analyzed using Blastn on NCBI (https://blast.ncbi.nlm.nih.gov/Blast). The ORF was predicted using the ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

2.4. Analysis of the primary structure of chitinase B

The signal peptide of the amino acid sequence was deduced using the SignalP (http://www.cbs.dtu.dk/services/SignalP). Domain structure and its function were examined by the Pfam (http://pfam.sanger.ac.uk) and the SMART (http://smart.embl-heidelberg.de/), respectively. The molecular weight of the deduced protein was computed using the Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The BLASTp program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to examine the homology of deduced domains.

2.5. Phylogenetic analysis

A phylogenetic tree using amino acids in the catalytic domain of deduced protein was produced using the MEGA version 6.0 software after multiple alignments of data by the Clustal W implemented in the MEGA software. The tree was constructed using the neighbor-joining method [17] and evolutionary distances were computed using the Poisson correction method [18]. A bootstrap analysis (1000 replications) was carried out to evaluate the topology of the resulting tree.

2.6. Three-dimensional structure analysis

The predicted structure model of *Bv*ChiB was constructed using the SWISS-MODEL program (https://swissmodel.expasy.org). The position of aromatic residues of *Bv*ChiB and figures was analyzed and prepared using Chimera 1.13.1 program [19].

3. Results

3.1. Identification and nucleotide sequence of the chiB gene

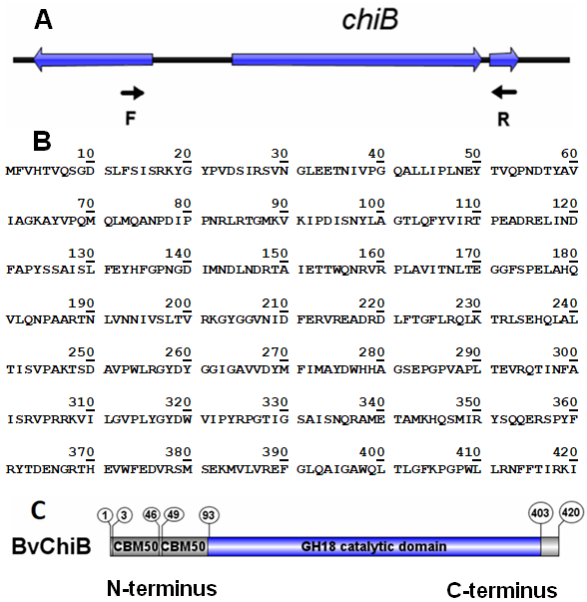

Figure 1. *Schematic presentation of chiB identification, amino acid sequence and domain structure of chitinase B. A, Schematic presentation of chiB identification; B, amino acid sequence; C, domain structure of chitinase B; F, forward primer; R, reverse primer*

Based on information of the *B. velezensis* genome sequences available in the CAZy database to designed primers and orientation for *chiB* identification by PCR from our isolates (Figure 1A, we successfully amplified the target gene. We then cloned *chiB* in *E. coli* DH5α, screened, and sequenced the positive transformation. Sequence analyses showed that the ORF of *chiB* consists

of 1,263 base pairs (bp) in length and encodes a deduced protein of 420 amino acids (aa) (Figure 1B).

3.2. Domain structure of chitinase B

The primary structure of the deduced protein was analyzed by using the SMART and Pfam programs. The result showed that the deduced protein contains two carbohydrate-binding module family 50 (CBM50) domains at the N-terminus (the first domain, 44 aa, residues 3–46 and the second domain, 44 aa, residues 49–103) and a catalytic domain (311 aa, residues 102–403) at the C-terminus of the deduced protein (Figure 1C). The deduced protein was named *Bv*ChiB. The calculated molecular mass of *Bv*ChiB is 47.59 kDa and the pI (isoelectric point) of the protein is 8.53.

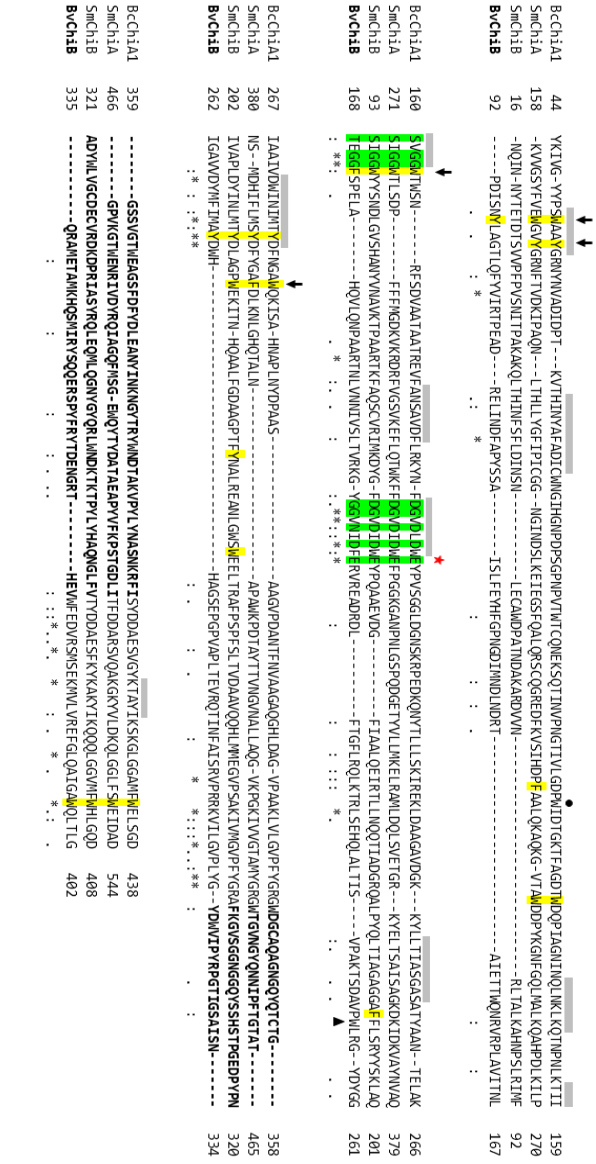

Figure 2. *Amino acid sequence alignment*

Amino acid sequence in the catalytic domain of B. velezensis BvChiB is aligned with those of other GH18 bacterial chitinases. BcChiA1, chitinase A1 from B. circulans WL-12; SmChiA, SmChiB are corresponding to chitinase A, chitinase B from S. marcescens 2170; BvChiB is chitinase B from B. velezensis (this study). The two conserved motifs in family 18 chitinases are shown with green backgrounds; a red star indicates the glutamate residue. Conserved aromatic residues that are important for processivity are underlined by the arrow. All other important conserved aromatic residues are shown with yellow backgrounds. Aromatic residue corresponded to W122 in BcChiA1 is underlined by a circle. A small α+β domain inserted between the seventh and eighth β-strands of (β/α)8 is bolded. The aromatic residue of BvChiB is underlined by a solid triangle.

The catalytic domain of *Bv*ChiB showed 99% identity to that of an uncharacterized chitinase (QDF55640), followed by characterized chitinases such as 22.8% identity to *Bc*ChiA1 (AAA81528) from *B. circulans* WL-12, 22.1% identity to *Sm*ChiA (BAA31567) from *S. marcescens* 2170. These chitinases were reported to be family 18 chitinases. In addition, a glutamate residue that was reported to be essential for hydrolysis of family 18 chitinases acting as a proton donor was found in the catalytic domain of *Bv*ChiB. Furthermore, the conserved regions in GH18 chitinases, SxGG and DxxDxDxE, were found in the catalytic domain of *Bv*ChiB (Figure 2). These conserved regions were reported to be involved in the substrate-binding and active sites [20, 21]. The small *α+β* domain inserted between the seventh and eighth β-strands of (β/α)8 was identified in the sequence of *Bv*ChiB (Figure 2). Taken together, these analyses indicate that *Bv*ChiB is a member of the bacterial family 18 chitinases.

3.3. The subfamily of chitinase B

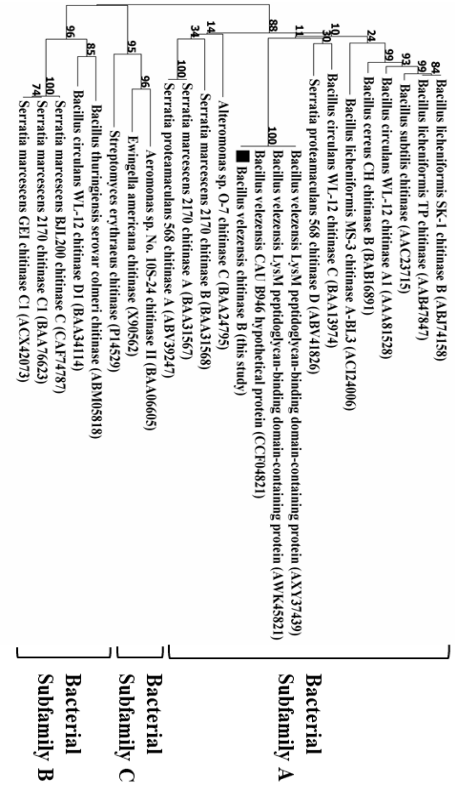

Figure 3. *Phylogenetic relationships among family 18 chitinases*

The amino acid of the catalytic domain of BvChiB of B. velezensis analyzed in this study ***(filled rectangle)*** *and other bacterial GH18 chitinases were compared*

Bacterial GH18 chitinases can be further divided into three subfamilies, A, B, and C. Subfamily A has an extra domain with a small *α+β* domain inserted into the core TIM (triosephosphate isomerase)-barrel fold, while

subfamilies B and C have no such domain [5]. Hence, they display different properties on chitin-degradation. To classify the subfamily of *Bv*ChiB, amino acids in the catalytic domain of *Bv*ChiB and those of the other family 18 chitinases were aligned by using the Clustal W program, and phylogenetic analysis was then performed from this alignment using the neighbor-joining method. The result showed that *Bv*ChiB has a close genetic relationship to *Bacillus* chitinases and is grouped into subfamily A (Figure 3). Moreover, the small $\alpha+\beta$ domain inserted between the seventh and eighth β-strands of (β/α)8 was identified in the sequence of *Bv*ChiB (Figure 2), suggesting that *Bv*ChiB belongs to subfamily A of family 18 chitinases.

3.4. Three-dimensional structure of chitinase B

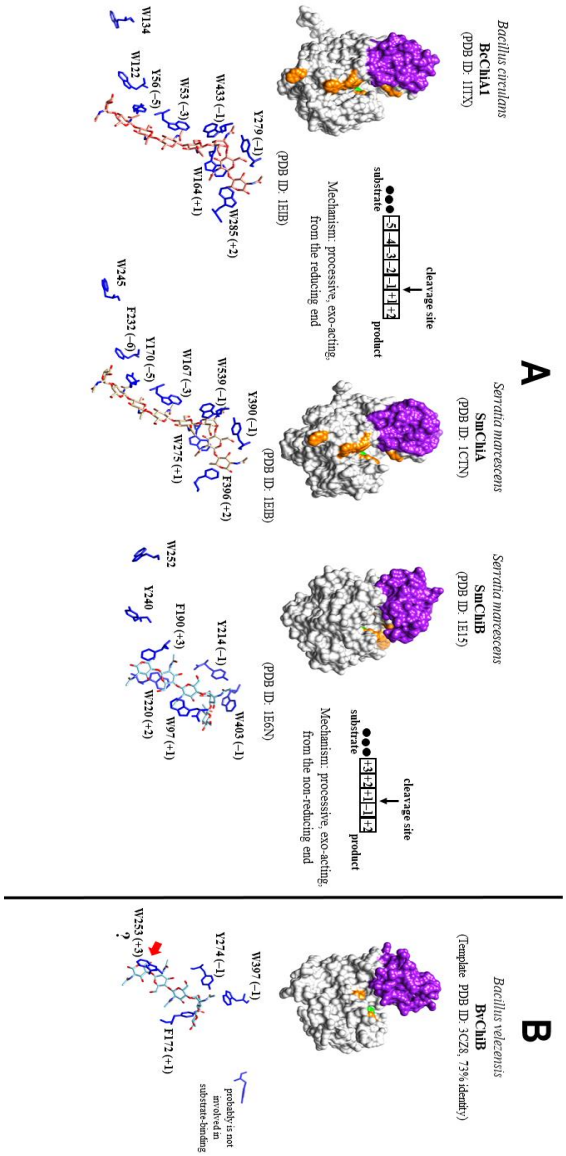

Figure 4. *Predicted structure models and position of aromatic residues of BvChiB of B. velezensis in comparison with the known structure of SmChiA and SmChiB of S. marcescens, and BcChiA1 of B. circulans*

Panel A, X-ray crystal structure and aromatic residue involved in the hydrolysis of chitin of the catalytic domain of chitinases of S. marcescens, SmChiA (PDB ID: 1CTN; 1EIB); SmChiB, PDB ID: 1E15; 1E6N), and B. circulans, BcChiA1 (PDB ID: 1ITX; 1EIB). Panel B, predicted structure and aromatic residues of the catalytic domains of BvChiB of B. velezensis (PDB ID 3CZ8 corresponds to putative sporulation-specific glycosylase ydhD from B. subtilis). An $\alpha+\beta$ domain is shown in purple color. The important aromatic residues for catalysis and/or binding are colored by orange. The glutamate residue for catalysis is shown with green color

Analysis of the predicted 3-D structure models of GH18 chitinases in comparison with known processive chitinases *Sm*ChiA and *Sm*ChiB of *S. marcescens* 2170 [26] and *Bc*ChiA1 of *B. circulans* WL-12 [27] suggests that *Bv*ChiB is closer to *Sm*ChiA and *Bc*ChiA1 and therefore, it has been proposed that *Bv*ChiB has an open active cleft as *Sm*ChiA and *Bc*ChiA1 (Figure 4). A comparison of the predicted subsite structure of *Bv*ChiB with the well-characterized GH18 chitinases [26, 27] suggests that there are some differences in aromatic residues between *Bv*ChiB, *Sm*ChiA, *Sm*ChiB, and *Bc*ChiA1. Aromatic residues lack in the catalytic domain of *Bv*ChiB (Figure 4).

4. Discussion

CBM50 which probably binds chitin was originally identified as a component of bacterial lysins. This domain is found in many enzymes involved in cell wall degradation and is also present in other proteins that are associated with bacterial cell walls. Basal level resistance by plants against certain pathogens also appears to involve the recognition of chitin oligosaccharides and related compounds [28]. Inamine has reported that CBM50 from chitinase-A of a horsetail *(Equisetum arvense)* involved in antifungal activity of the chitinase [29]. In this study, *Bv*ChiB of *B. velezensis* contains two CBM50 (Figure 1C); therefore, this chitinase is hopeful to be possessed antifungal activity against plant pathogenic fungi. To the best of our knowledge, no studies on chitinases from the bacterium concerning chitinase and antifungal activities have been reported. Hence, it is necessary to characterize *Bv*ChiB and its CBM50 in the next studies with respect to chitinase and antifungal activities.

The primary and 3-D structure analyses of *Bv*ChiB indicate that *Bv*ChiB belongs to the subfamily A of bacterial GH18 chitinases and contains a small $\alpha+\beta$ domain inserted between the seventh and eighth β-strands of (β/α)8 in the catalytic domain sequence of the enzyme, implying that the enzyme can be a processive chitinase. These processive chitinases degrade the crystalline chitin with high efficiency caused by a processive mode of action [23]. On the other hand, *Bv*ChiB has a small deletion in the amino acid sequence of the catalytic domain compared with those of *Bc*ChiA1 from *B. circulans* WL-12 and *Sm*ChiA from *S. marcescens* 2170 (Figure 2). This deletion leads to a lack of a tryptophan residue in the catalytic domain of *Bv*ChiB that corresponds to Trp-134 of *Bc*ChiA1 from *B. circulans* WL-12 and Trp-245 of *Sm*ChiA from *S. marcescens* 2170. Moreover, the catalytic domain of *Bv*ChiB lacks exposed aromatic residues compared to those of *Bc*ChiA1 from *B. circulans* WL-12 and *Sm*ChiA from *S. marcescens* 2170 which contributed to guiding a chitin chain into the catalytic cleft during the crystalline chitin hydrolysis or in the chitin-binding of these chitinases [23, 30] (Figure 4). These analyses imply that the lacking of

these exposed aromatic residues in the catalytic domain of *Bc*ChiA1 may affect the chitinase activity of the enzyme. Consequently, it is necessary to characterize *Bc*ChiA1 as well as its domains in detail in further study.

In conclusion, a gene *(chiB)* encoding a GH18 chitinase was identified from the genomic DNA of *B. velezensis* RB.IBE29 and then analyzed. Primary and 3-D structure analyses of the enzyme indicate that the deduced chitinase from the bacterium probably plays an interesting role in the hydrolysis of insoluble chitin and/or inhibition of the hyphal growth of fungi. This is the first description of the sequence analysis of chitinase from the bacterium so far. Currently, studies on expression, purification, and characterization of the enzyme concerning chitinase and antifungal activities are underway.

Acknowledgments: This work was supported by the Ministry of Education and Training under the grant number B2020-TTN-04.

REFERENCES

[1] Yu, C., Bassler, B.L., Roseman, S., 1993. Chemotaxis of the marine bacterium *Vibrio furnissii* to sugars. a potential mechanism for initiating the chitin catabolic cascade. *J Biol Chem.* 268, 9405–9409.

[2] Synowiecki, J., Al-Khateeb, N.A., 2003. Production, properties, and some new applications of chitin and its derivatives. *Crit Rev Food Sci Nutr.* 43(2); 145–1471.

[3] Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J*. 280, 309–316.

[4] Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S., Regue, M., 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J Bacteriol*. 179, 7111–7117.

[5] Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., Watanabe, T., 1999. The third chitinase gene (*chiC*) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. *Biochem J*. 343, 587–596.

[6] Suzuki, K., Sugawara, N., Suzuki, M., Uchiyama, T., Katouno, F., Nikaidou, N., Watanabe, T., 2002. Chitinases A, B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. *Biosci Biotechnol Biochem.* 66, 1075–1083.

[7] Watanabe, T., Oyanagi, W., Suzuki, K., Tanaka, H., 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J Bacteriol.* 172, 4017–4022.

[8] Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K., Tanaka, H., 1992. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *J. Bacteriol.* 174, 408–414.

[9] Huang, L., Garbulewska, E., Sato, K., Kato, Y., Nogawa, M., Taguchi, G., Shimosaka, M., 2012a. Isolation of genes coding for chitin-degrading enzymes in the novel chitinolytic bacterium, *Chitiniphilus shinanonensis*, and characterization of a gene coding for a family 19 chitinase. *J Biosci Bioeng.* 113, 293–299.

[10] Huang, L., Shizume, A., Nogawa, M., Taguchi, G., Shimosaka, M., 2012b. Heterologous expression and functional characterization of a novel chitinase from the chitinolytic bacterium *Chitiniphilus shinanonensis*. *Biosci Biotechnol Biochem.*76, 517–522.

[11] Itoh, T., Hibi, T., Fujii, Y., Sugimoto, I., Fujiwara, A., Suzuki, F., Iwasaki, Y., Kim, J.K., Taketo, A., Kimoto, H., 2013. Cooperative degradation of chitin by extracellular and cell surface-expressed chitinases from *Paenibacillus* sp. strain FPU-7. Appl Environ Microbiol. 79, 7482–7490.

[12] Tsujibo, H., Orikoshi, H., Baba, N., Miyahara, M., Miyamoto, K., Yasuda, M., Inamori, Y., 2002. Identification and characterization of the gene cluster involved in chitin degradation in a marine bacterium, *Alteromonas* sp. strain O-7. *Appl Environ Microbiol.* 68, 263–270.

[13] Ohno, T., Armand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M., Watanabe, T., 1996. A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. *J. Bacteriol.* 178, 5065–5070.

[14] Bhattacharya, D., Nagpure, A., Gupta, R.K., 2007. Bacterial chitinase: properties and potential. *Crit Rev Biotechnol*. 27, 21–28.

[15] Trinh, T.H.T., Wang, S.L., Nguyen, V.B., Tran, M.D., Doan, C.T., Vo, T.P.K., Huynh, Q.V., Nguyen, A.D., 2019. A potent antifungal rhizobacteria *Bacillus velezensis isolated from black pepper. Research on Chemical Intermediates.* 45:5309–5323.

[16] Green, M.R., Sambrook, J., 2012. Molecular coling: a laboratory manual, 4th edition, vol. 1, pp. 210–212. New York (USA): Cold Spring Harbor Laboratory Press.

[17] Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol. 4*, 406–425.

[18] Zuckerkandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in proteins. Edited in Evolving Genes and Proteins by Bryson, V. and Vogel, H. J., 97–166. Academic Press, New York.

[19] Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera - a visualization system for exploratory research and analysis. *J Comput Chem*. 25:1605-1612.

[20] van Aalten, D.M., Synstad, B., Brurberg, M.B., Hough, E., Riise, B.W., Eijsink, V.G., Wierenga, R.K., 2000. Structure of a two-domain chitotriosidase from *Serratia marcescens* at 1.9-Å resolution. *Proc Natl Acad Sci USA*. 97, 5842–5847.

[21] Tsujibo, H., Kubota, T., Yamamoto, M., Miyamoto, K., Inamori, Y., 2003. Characterization of chitinase genes from an alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131. *Appl Environ Microbiol*. 69, 894–900.

[22] Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M., Tanaka, H., 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol Chem.* 268, 18567–18572.

[23] Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., Watanabe, T., 2001. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. *J. Biol Chem*. 276, 41343–41349.

[24] Katouno, F., Taguchi, M., Sakurai, K., Uchiyama, T., Nikaidou, N., Nonaka, T., Sugiyama, J., Watanabe, T., 2004. Importance of exposed aromatic residues in chitinase B from *Serratia marcescens* 2170 for crystalline chitin hydrolysis. *J. Biochem*. 136:163-168.

[25] Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, H., Nikaidou, N., Kezuka, Y., Nonaka, T., Sugiyama, J., 2003. Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. *Biochem J*. 376:237-244.

[26] Igarashi, K., Uchihashi, T., Uchiyama, T., Sugimoto, H., Wada, M., Suzuki, K., Sakuda, S., Ando, T., Watanabe, T., Samejima, M., 2014. Two-way traffic of glycoside hydrolase family 18 processive chitinases on crystalline chitin. Nat Commun. 5:3975.

[27] Imai T, Watanabe T, Yui T, Sugiyama, J., 2002. Directional degradation of β-chitin by chitinase A1 revealed by a novel reducing end labelling technique. *FEBS Lett.* 510:201–205.

[28] Ohnuma, T., Onaga, S., Murata K., Taira T., Etsuko K., 2008. LysM domains from Pteris ryukyuensis chitinase-A: a stability study and characterization of the chitin-binding site. *J. Biol Chem.* 283:5178–5187.

[29] Inamine, S., Onaga, S., Ohnuma, T., Fukamizo, T., Taira, R., 2015. Purification, cDNA cloning, and characterization of LysM-containing plant chitinase from horsetail (*Equisetum arvense*). *Biosci Biotechnol Biochem.* 79:1296–1304.

[30] Watanabe, T., Ishibashi, A., Ariga, Y., Hashimoto, M., Nikaidou, N., Sugiyama, J., Matsumoto, T., Nonaka, T., 2001. Trp122 and Trp134 on the surface of the catalytic domain are essential for crystalline chitin hydrolysis by *Bacillus circulans* chitinase A1. *FEBS Lett.* 494, 74–78.