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GH-16 Type β-1,3-Glucanase from *Lysobacter* sp. MK9-1 Enhances Antifungal Activity of GH-19 Type Chitinase, and Its Glucan-binding Domain Binds to Fungal Cell-wall

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Abstract: The GH-16 type β-1,3-glucanase (BgluC16MK) gene of *Lysobacter* sp. MK9-1 was cloned to study its antifungal activities. BgluC16MK displays amino acid sequence similarity with GluC from *L. enzymogenes* strain N4-7. BgluC16MK includes a signal sequence, a catalytic domain and carbohydrate-binding module family 6-type β-glucan binding domain (B-GBD). The expression of the BgluC16MK gene in *Escherichia coli* without the signal sequence resulted in antifungal activity at a dose of 0.6–0.8 nmol/disk. However, BgluC16MK displayed antifungal activity at a dose of 0.025 nmol/disk in combination with Chi19MK. Substrate-specific assay revealed that purified BgluC16MK hydrolyzed insoluble curdlan more readily than the soluble substrate. Furthermore, to explore the binding selectivity of B-GBD of BgluC-16MK, we constructed a fusion protein (B-GBD-GFP) using the B-GBD and green fluorescent protein. The activity of the fusion protein against various substrates indicates that B-GBD was selective for glucans with β-1,3-linkages. An additional study demonstrated the binding ability of B-GBD-GFP to the cell-wall of living fungi, such as *T. reesei* and *Aspergillus oryzae*. These findings suggest that BgluC16MK can be utilized to generate antifungal enzyme preparations and that the fusion protein B-GBD-GFP can be used to identify the fungal cell surface structure using β-glucans.

Key words: β-1,3-glucanase, glycoside hydrolase family 16, antifungal activity, fungal cell-wall, *Lysobacter*

INTRODUCTION

Several β-glucanases and chitinases have been studied as biological control candidates for pathogenic fungi, because β-glucan and chitin constitute the cell walls of most fungi, acting as skeletal polysaccharides.[1)2)]

β-1,3-Glucanases (EC 3.2.1.39) are classified into the families 5, 16, 17, 55, 64, 81, 128, 152, 157, and 158 of glycoside hydrolases (GHs) based on their amino acid sequences in the Carbohydrate Active enZymes (CAZy) database (http://www.cazy. org).[3)] As mentioned, β-1,3-glucan was found as the component of fungal cell-wall and also found in some of brown algae, which acts as a storage polysaccharide.[4)] β-1,3-glucanases hydrolyze internal β-1,3-glucosidic bonds in the glucans and release oligosaccharide. They are widely found in various organisms and have unique roles. In plants, β-1,3-glucanases are associated with defense against fungal pathogens, independently or in combination with chitinases and other antifungal proteins.[5)6)] Fungal β-1,3-glucanases hydrolyze their own cell-wall to facilitate extension, hyphal branching, sporulation, and budding. They can also hydrolyze extracellular glucans to assimilate hydrolysates.[7)8)] Most bacterial β-1,3-glucanases degrade extracellular glucans, and the hydrolysates are assimilated as an energy source.[9)10)]

The genus *Lysobacter* belongs to gram-negative Proteobacteria, and some species have been reported to exhibit lytic activity against fungi, oomycetes, algae, bacteria, and nematodes.[11)12)13)14)] Palumbo *et al.* reported that *Lysobacter enzymogenes* strain N4-7 produces three extracellular β-1,3-glucanases (GluA, GluB, and GluC), encoded by the genes *gluA*, *gluB*, and *gluC,* respectively, and classified as GH-16, GH-64, and GH-16 type enzymes, respectively.[14)] GluA and GluB only possess the catalytic domain, while

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Abbreviations: GH, glycoside hydrolase; B-GBD, β-glucan binding domain; GFP, green fluorenes protein; CBM, carbohydrate-binding module; BgluC16MK, GH-16 type β-1,3-glucanase from *Lysobacter* sp. MK9-1; Chi19MK, GH-19 type chitinase from *Lysobacter* sp. MK9-1; B-GBD-GFP, fusion protein of β-glucan binding domain and green fluorescent protein; IPTG, Isopropyl β-D-1-thiogalactopyranoside; KPB, Potassium phosphate buffer; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

GluC consists of an N-terminal catalytic domain and a C-terminal carbohydrate-binding module family 6 (CBM6) domain. Reports have shown that although deletion of these β-1,3-glucanase genes of *L. enzymogenes* strain C3 did not affect the hyphal growth inhibition activity against *Bipolaris sorokiniana* and *Pythium ultimum* on the plate, it significantly reduced the biological control activity against Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet.[15] These results suggested that β-1,3-glucanases of *L. enzymogenes* might exhibit antifungal activity and can be potential antifungal enzymes.

Previously, we cloned and expressed GH-19 type chitinase (Chi19MK) from *Lysobacter* sp. MK9-1.[16] Chi19MK inhibited hyphal extension in *Trichoderma reesei* and *Schizophyllum commune*. Although GH-18 type chitinases have been investigated in *Lysobacter*, Chi19MK is the first GH-19 type chitinase to be characterized and our finding suggested that Chi19MK might aid the antifungal activity of *Lysobacter*.

In this study, we used *Lysobacter* sp. MK9-1 to clone and express the GH-16 type β-1,3-glucanase (BgluC16MK) gene. We characterized BgluC16MK and performed a hyphal extension inhibition assay against *Trichoderma* to understand its role in antifungal action. Furthermore, to understand the binding mechanism of the β-glucan binding domain of BgluC16MK, we constructed a fusion protein (B-GBD-GFP) using the β-glucan binding domain and green fluorescent protein and studied substrate and fungal cell-wall binding activities. Our results revealed that BgluC16MK increased the antifungal activity of GH-19 type chitinase, and B-GBD-GFP was selectively bound to insoluble β-1,3-glucans and fungal cell walls.

MATERIALS AND METHODS

Materials. Laminarin and chitin powder were purchased from Nacalai Tesque, INC. (Kyoto, Japan). Zymosan A was supplied by Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Microcrystalline cellulose was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Pachyman, laminaribiose, and laminaritriose were purchased from Megazyme ltd. (Bray, Ireland). α-1,3-Glucan was prepared using a procedure described previously.[17] α-1,3-Glucanase Agl-KA from *Bacillus circulans* KA-304 was expressed in *E. coli* harboring pET-agl plasmid, according to previously described methods.[18] Other commercial reagents used were chemically pure grade.

Bacterial culture. *E. coli* DH 5α (TOYOBO CO., LTD., Osaka, Japan) was used as a host to construct recombinant plasmids and was cultured at 37 °C with shaking (100 rpm) in LB medium containing 100 μg/mL ampicillin. *E. coli* Rosetta-gami B (DE3) (Novagen Inc., Madison, WI, USA) was used for gene expression and the transformed cells were grown at 30 °C on a reciprocal shaker (100 strokes/min) in LB medium containing 100 μg/mL ampicillin, 10 μg/mL chloramphenicol, and 25 μg/mL kanamycin.

Cloning of the β-1,3-glucanase gene. Using *Lysobacter* sp. MK9-1 genomic DNA as a template, the β-1,3-glucanase gene was PCR amplified. The sense primer, BgcfullF1 (5′-CATCCGCGCAAAAGCCGGCGAAGTGTCGTGCGGTT-3′), and the antisense primer, R1bgcfull (5′-TGCGGACTGCGGCACAAAACGACGGTCTTGCAAAT-3′), were designed from upstream and downstream sequences of putative β-glucanase gene of gluc (AAN77505.1) from *L. enzymogenes* M497-1. The nucleotide sequence of the PCR product was determined.

Plasmid construction. The β-1,3-glucanase gene was PCR amplified using *Lysobacter* sp. MK9-1 genomic DNA as a template. The sense primer, BGlu16-for (5′-TTCGCCGGCGGCGCGCATATGCAAAACTGGCAGTT-3′ containing NdeI site, underlined), and the antisense primer, BGlu16-rev (5′-TGCGTTGCGCGGGCTCGAGTCAGATCTTGGTGATG-3′ XhoI site), were designed from gluc from *L. enzymogenes* M497-1. The sense primer was designed to replace Ala-22 with Met (initial codon). The PCR products were digested with NdeI and XhoI and ligated into pET22b (+) (Novagen Inc.). The plasmids expressing BgluC16MK were designated as pET-*bgluc16*.

The expression plasmids for B-GBD-GFP was constructed by amplifying the *b-gbd* gene from pET-*bgluc16* using the primer pair, CBDNdeF (5′-ACTATGTGCATATGTACAGCGGCGGCGGCAGCAAT-3′ NdeI site) and CBDBamR (5′-TATTAAGGATCCGCCTCCACCGCCACCTCCGATCTTGGTGATGCGTATCCAGTTGATGTT-3′ BamHI site). The amplified product was digested with NdeI and BamHI and ligated into pET-22b (+). The recombinant plasmid was designated as pET-*b-gbd*. The *ac-gfp* gene was amplified from pAcGFP1 Vector (Takara Bio Inc.) using the primer pair, GFP-BamH1-F (5′-TAGAGGATCCCGGGTACCGGTCGCCACCATGGTGA-3′ BamHI site) and GFP-Xho1-R (5′-AGTTGGAATTCTCGAGTCGCGGCCGCTCACTTGTA-3′ XhoI site). The product was treated with BamHI and XhoI and ligated into the pET-*b-gbd* plasmid to yield the recombinant plasmid, pET-*bgbd-gfp*.

Gene expression of recombinant enzyme. *E. coli* Rosetta-gami B (DE3) cells were transformed with pET-*bgluc16 and* pET-*bgbd-gfp*, inoculated into 2 L Sakaguchi flasks containing 1 L of LB medium with ampicillin, chloramphenicol, kanamycin, and tetracycline and incubated at 30 °C with shaking (100 rpm). After 5 h of incubation, isopropyl-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM and further incubated at 16 °C for 12 h. The cells were harvested by centrifugation (5,000 × G for 10 min) and suspended in 5 mL of 10 mM buffer. Potassium phosphate buffer (KPB) (pH 7.0) and Tris-HCl buffer (pH 8.0) were used for preparation of BgluC16MK and B-GBD-GFP, respectively. The cell suspensions were disrupted by sonication (4 °C, 10 min, 350–400 μA) on ice, and centrifuged (4 °C, 8,000 × G for 10 min) to remove cell debris. The resultant supernatant was dialyzed at 4 °C against the respective buffer overnight (cell-free extract).

Purification of BgluC16MK and B-GBD-GFP. BgluC16MK was purified from the cell-free extract using a TOYOPEARL DEAE-650M (Tosoh Corporation, Tokyo, Japan) column (2.5 × 8 cm). The extract was applied to the column equilibrated with 10 mM KPB (pH 7.0) and washed with the same buffer. The adsorbed proteins were eluted with 10 mM KPB containing 50 mM NaCl. The active fractions were concentrated with ammonium sulfate (70 % saturation) and stored at 4 °C for 2 h. After centrifugation at 8,000 × G for 15 min at 4 °C, the precipitate was dissolved in and dialyzed at 4 °C against the buffer. The resulting dialysate was mixed with ammonium sulfate at 20 % saturation, and applied onto a TOYOPEARL Butyl-650M (Tosoh Corporation) column (2.5 × 8 cm) equilibrated with 10 mM KPB (pH 7.0) containing 20 % ammonium sulfate. The column was washed with the same buffer, and then the enzyme was eluted in stepwise fashion with 10, 5, and 0 % ammonium sulfate in the buffer. BgluC16MK was eluted with the buffer without ammonium sulfate.

To purify B-GBD-GFP, the cell-free extract was applied to a TOYOPEARL DEAE-650M column equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and then washed with the same buffer. The fractions were eluted with 10 mM Tris-HCl buffer with 100 mM NaCl and dialyzed against 10 mM Tris-HCl buffer. Ammonium sulfate was added to the dialysate to reach 20 % saturation and this solution was applied to a TOYOPEARL Butyl-650M column. The column was washed with the buffer containing 20 % ammonium sulfate, and the fusion protein was eluted by the buffer containing 3 % ammonium sulfate. The fraction containing the fluorescent protein was dialyzed at 4 °C against 10 mM Tris-HCl buffer to obtain the purified protein.

β-1,3-Glucanases activity assay. β-1,3-Glucanases activity was assayed using laminarin as a substrate. A reaction mixture containing 1 % laminarin, 50 mM sodium acetate buffer (pH 5.0), and appropriate amounts of the enzyme was incubated at 30 °C. The reaction was stopped by immersing the mixture in boiling water for 5 min after allowing it to react for 3, 6, and 9 min. With the dinitrosalicylic acid reagent, the reducing sugar produced was measured as glucose,[19] and the initial velocity was calculated from the progress curve. One unit of the enzyme is defined as the amount of enzyme releasing 1 μmol of reducing sugar (as glucose) per min.

Antifungal activity assay. Antifungal activity was quantified as described previously.[20] *Trichoderma viride* was used as test strains. An agar disk (4 mm in diameter) containing mycelia was placed on a potato dextrose agar (PDA) plate containing 1.5 % agar, and each solution (5 μL) was overlaid onto the agar disks. The plate was incubated at 25 °C for 12 h and then photographed. We measured the area of the *T. viride* mycelial growth. The protein concentrations required for inhibiting the growth of the fungus by 50 % (IC_{50}) were determined by constructing dose-response curves (percentage of growth inhibition versus protein concentration).
Hyphal extension inhibition assays were performed as described previously.[16] *Trichoderma reesei* NBRC 4928 was used as the test strain. An agar disk (10 mm in diameter) containing mycelia was inoculated at the center of the plate, and paper disks were placed around the edge of the colony. The degree of inhibition of hyphal extension around the disks was observed.

Insoluble glucan binding assay. Glucan binding ability of B-GBD-GFP was analyzed according to the methods described previously.[17] We used zymosan A, curdlan, pachyman, chitin, chitosan, cellulose, and α-1,3-glucan as substrates. The reaction mixture contained 1 % substrates, 10 mM Tris-HCl buffer (pH 9.0), and 1 nmol/mL of B-GBD-GFP.

Fungal cell-wall binding assay. *T. reesei* NBRC 4928 and *Aspergillus oryzae* NBRC 100959 were used as test strains, and the assay was performed according to the methods described previously.[17] The reaction mixture, containing 10 mM Tris-HCl buffer (pH 8.0), the washed mycelia and B-GBD-GFP (2 nmol/mL), was incubated at 30 °C. The mycelia were washed several times with Tris-HCl buffer, and then visualized with an Olympus CKX53 fluorescent microscope.

Analytical methods. The protein concentration of purified BgluC16MK and B-GBD-GFP were measured using Lowry's method with bovine serum albumin as the standard.[21] Protein concentration was also estimated for BgluC16MK by measuring the absorbance at 280 nm with a molar absorption coefficient of 122,840 M^{-1} cm^{-1} based on its amino acid composition[22] and for of B-GBD-GFP, at 475 nm using a molar absorption coefficient of 32,500 M^{-1} cm^{-1} as derived from AcGFP1.

SDS-PAGE was performed using the Laemmli method.[23] PM1500 Excel Band All Blue Regular Range Plus Protein Marker, purchased from SMOBIO (Taiwan), was used as the molecular marker. Hydrolytic products were analyzed by thin-layer chromatography (TLC) by spotting them on a TLC Silica gel 60 (Merck, Darmstadt, Germany) and developing with a solvent composed of *n*-butanol: acetic acid: distilled water (2:1:1 v/v). The spots on the plate were stained by spraying with *p*-anisaldehyde regent (9.3 mL of *p*-anisaldehyde, 3.8 mL of acetic acid, 340 mL of ethanol, and 12.5 mL of sulfuric acid) and heated at 250 °C.

RESULTS

Expression and purification of BgluC16MK.

The *bgluc16 mk* gene was amplified by PCR from the *Lysobacter* sp. MK9-1 genomic DNA of *Lysobacter* sp. MK9-1. The primers were designed based on the putative β-1,3-glucanase gene of *L. enzymogenes* M497-1 *gluc* (BAV99893.1). This is because, the sequence of 16S rDNA gene of *Lysobacter* sp. MK9-1 was 99 % identical to those of *L. enzymogenes* M497-1, and the sequence of *chi19mk* (LC571610) of strain MK9-1 was also 98 % identical to that of putative GH-19 chitinase gene (LEN_2961) of *L. enzymogenes* M497-1.[16] The amplified fragment consisted of 1,152 nucleotides encoding a protein containing 383 amino acids (Fig. S1; see J. Appl. Glycosci. Web site). The gene and protein sequence data have been deposited in DDBJ under accession number LC672160. The encoded protein product exhibited high sequence similarity with GluC from *L. enzymogenes* M497-1, GluC (AAN77505.1) from *L. enzymogenes* N4-7, and GluC (AAT77162.1) from *L. enzymogenes* C3, with 99, 91, and 92 %, respectively (Fig. S2; see J. Appl. Glycosci. Web site), suggesting that amplified fragment encodes β-1,3-glucanase. The domain structure of BgluC16MK consists of N-terminal signal sequence, GH-16 type catalytic domain, and C-terminal carbohydrate-binding module family 6-type (CBM6) β-glucan binding domain (B-GBD) (Fig. 1).

Figure 2A shows multiple alignment of various GH-16 type catalytic domains with the catalytic domain of BgluC16MK, displaying the conserved EXDXXE motif. This result suggests that Glu-147 acts as the catalytic nucleophile, and Glu-152 acts as the general acid−base catalyst.[24] Figure 2B shows multiple alignment of CBM6s with the B-GBD of BgluC16MK, displaying sequence similarities with CBM6s of laminarinase ZgLamC from *Zobellia galacta-*

nivorans,[25)] endo-β-1,4-glucanase CelB from *Cellvibrio mixtus* ATCC 12120,[26)] and laminarinase BhGH81 from *Alkalihalobacillus halodurans* C-125 (66, 53, and 34 %, respectively).[27)]

To express BgluC16MK, *E. coli* Rosetta-gami B (DE3) were transformed with pET-*bgluc16* plasmid and significant β-1,3-glucanase activity was detected in the cell-free extract (3.4 units/mg). BgluC16MK was purified from the soluble fraction in two steps: anion exchange and hydrophobic column chromatography. BgluC16MK was purified 2.4-fold with an overall yield 17 %, and the final preparation was homogeneous on SDS-PAGE (Fig. 3). Purified BgluC16MK exhibited a specific activity of 8.3 units/mg (Table S1; see J. Appl. Glycosci. Web site).

Properties of BgluC16MK.

BgluC16MK exhibited optimal temperature at 40 °C (Fig. S3A; see J. Appl. Glycosci. Web site). The optimal pH of its was 5.0 (Fig. S3B; see J. Appl. Glycosci. Web site). BgluC16MK retained full activity after 10 min incubation at 30 °C and remained more than 50 % of maximum activity at 40 °C (Fig. S3C; see J. Appl. Glycosci. Web site). The enzyme was stable at pH 5.0 (Fig. S3D; see J. Appl. Glycosci. Web site).

Substrate specificity of BgluC16MK was measured using reaction mixtures containing 1 % polysaccharides.

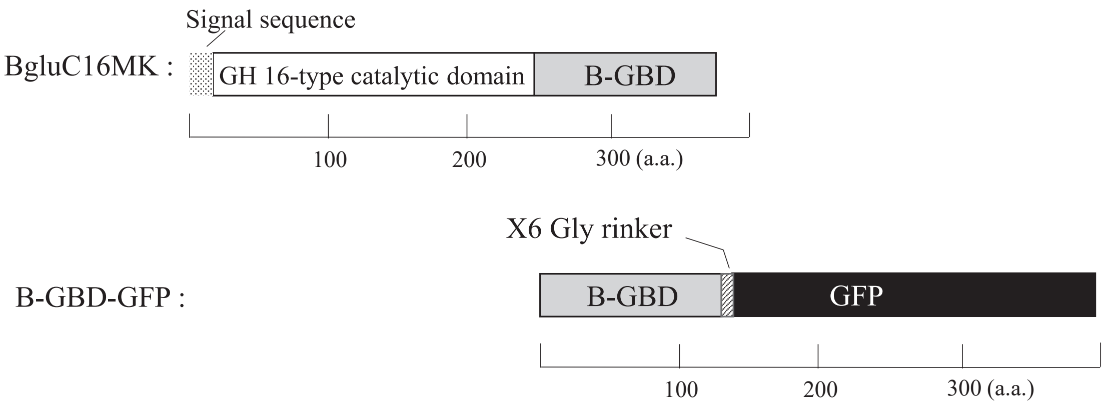

Fig. 1. Schematic structure of BgluC16MK and B-GBD-GFP.

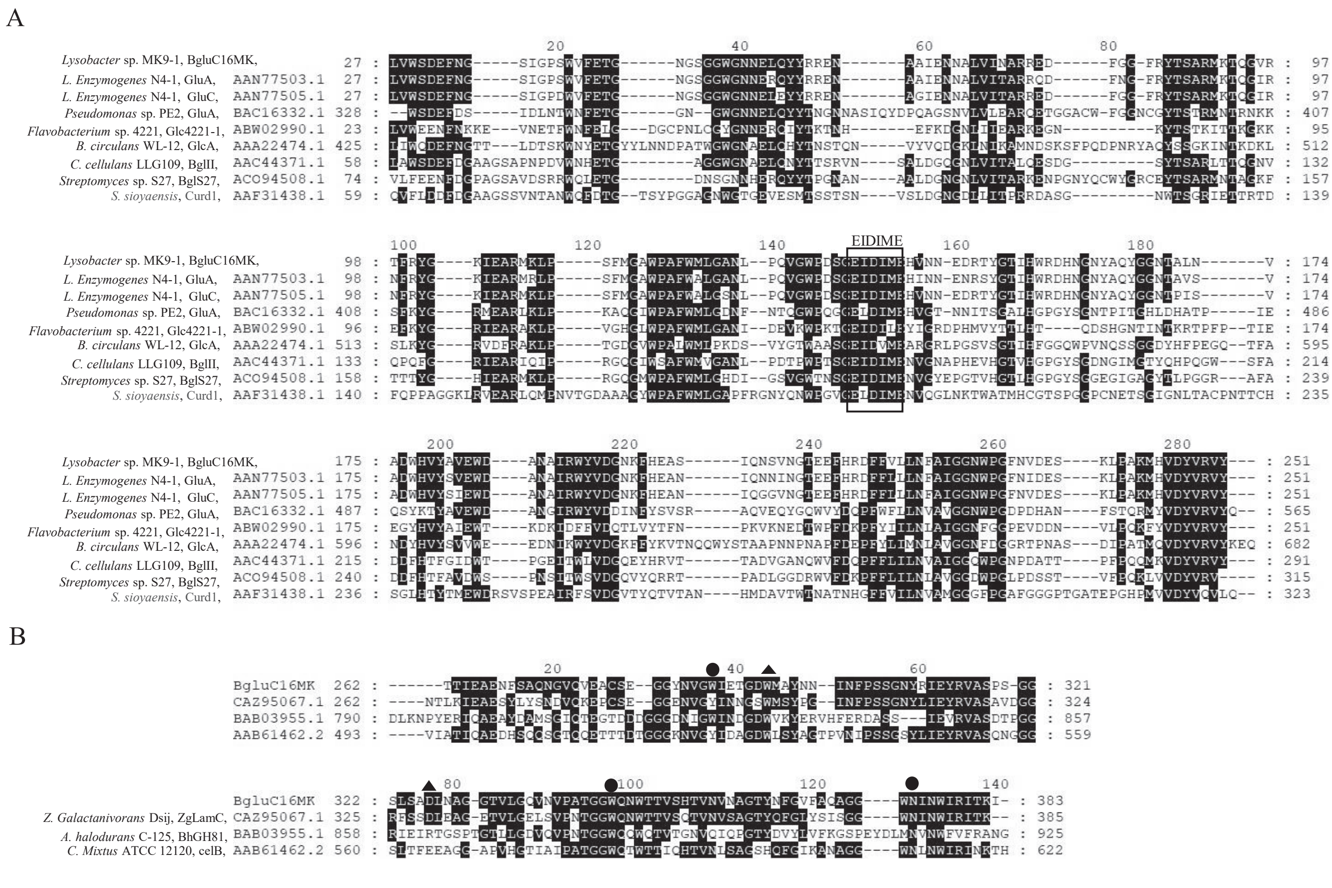

Fig. 2. Multiple alignments of amino acid sequences of the BgluC16MK catalytic domain with other GH-16 type β-1,3-glucanases (A), and the BgluC16MK glucan binding domain with other family 6 type carbohydrate-binding modules (B).

The amino acid sequences were aligned using Clustal W. The residue numbers of the first and last amino acids in each line are shown on the left and right sides. Completely identical amino acids are shaded in black. (A) The consensus EXDXXE motif sequence is enclosed in a box. (B) The substrate binding amino acids in the variable loop site are indicated by solid circles, and the residues in the concave site are indicated by solid triangles.

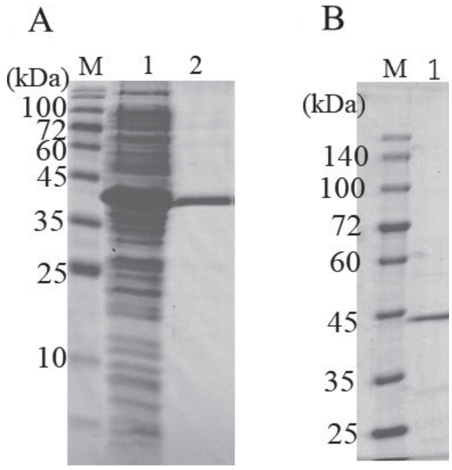

Fig. 3. SDS-PAGE analysis of BgluC16MK (A) and B-GBD-GFP (B).

The gel was stained with Coomassie Brilliant Blue R-250. (A) SDS-PAGE was performed using 15 % polyacrylamide gel. Lane M, Marker; lane 1, cell-free extract; lane 2, purified BgluC16MK. (B) SDS-PAGE was performed using 10 % gel. Lane M, Marker; lane 1, purified B-GBD-GFP.

BgluC16MK hydrolyzed laminarin (100 % relative activity), curdlan (249 %), pachyman (78 %), and zymosan A (59 %), while scarcely hydrolyzing microcrystalline cellulose (5 %). Reaction products from laminarin were analyzed by TLC (Fig. 4) and found to contain laminaritriose and oligosaccharides (longer than trisaccharide) during the reaction period (15–105 min). After 60 min of reaction, the amount of reducing sugars did not rise, suggesting that BgluC16MK had been inactivated. As a result, we tested the enzyme's stability at 30 °C in a 50 mM sodium acetate buffer (pH 5.0). Correspondingly, the enzyme's activity remained around 90 % after 120 min of treatment (Fig. S4; see J. Appl. Glycosci. Web site). The substrate, laminarin, remained at the origin in Fig. 4, indicating that it had not been entirely hydrolyzed. These data show that when a certain amount of oligosaccharides accumulates in the reaction mixture, the reaction will stop.

Antifungal activity.

Figiure 5 shows a dose-response curve of the antifungal activity of BgluC16MK against *T. viride*, yielding an IC_{50} value of 20.1 μM. Figure 6A shows a hyphal extension inhibition assay of BgluC16MK against *T. reesei*. The growth of *T. reesei* was weakly inhibited by BgluC16MK at concentrations of 0.6–0.8 nmol/disc. On the other hand, *Lysobacter* sp. MK9-1 Chi19MK produced clear inhibition zones at concentrations of 0.1 nmol/disc (Fig. 6B). To investigate the additional effects of BgluC16MK to Chi19MK, mixtures of BgluC16MK and Chi19MK were applied onto the disks, and the growth inhibition around the disks was observed. As shown in Fig. 6C, a mixture of 0.025 nmol each of BgluC16MK and Chi19MK produced clear inhibition zone, showing stronger growth inhibition than BgluC16MK and Chi19MK individually (compare Fig. 6C disk 3 with Fig. 6B disk 3).

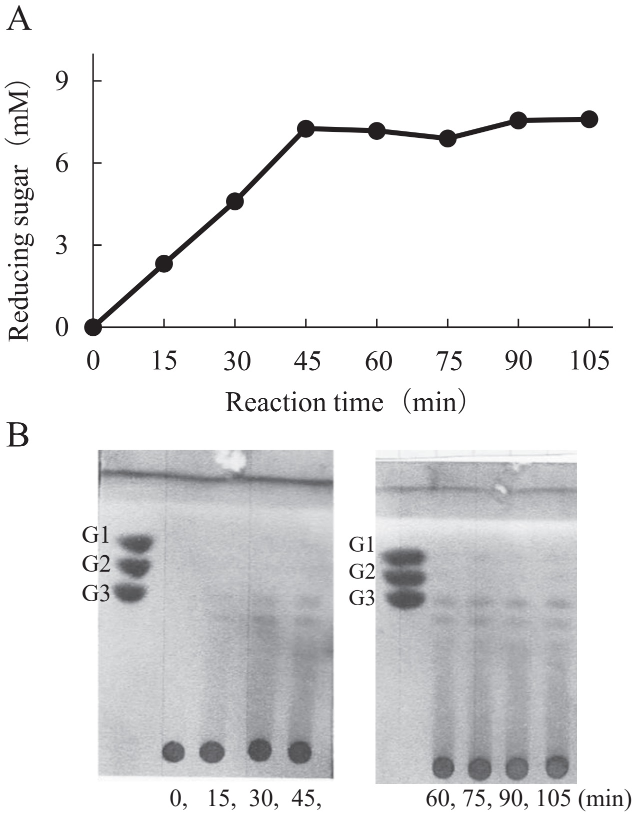

Fig. 4. Hydrolysis of laminarin by BgluC16MK (A), and TLC analysis of reaction products (B).

(A) The reaction mixture containing 50 mM acetate buffer (pH 5.0), 0.25 nmol/mL of BgluC16MK, and 1 % laminarin was incubated at 30 °C. (B) The reaction products from laminarin were analyzed by TLC. Glucose (G1), laminaribiose (G2), and laminaritriose (G3) were used as size markers.

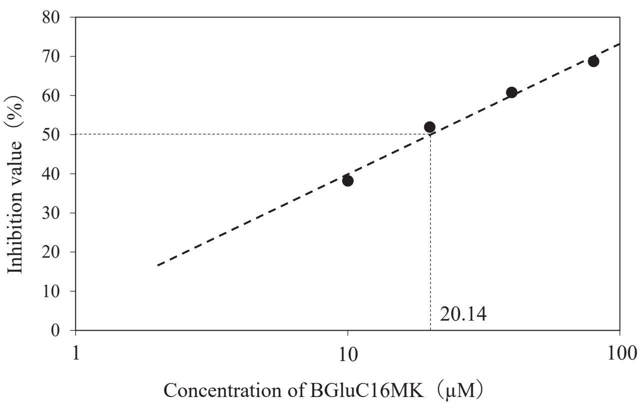

Fig. 5. Quantitative evaluation of the antifungal activity of BgluC16MK.

The agar disc (4 mm in diameter) containing *T. viride* mycelia was placed on a PDA plate, and then 5 μL of BgluC16MK was overlaid onto the agar disc. After incubating for 12 h at 25 °C, the area of mycelial regrowth was measured. The values are the averages of three independent experiments.

Insoluble glucan binding activity of β-glucan binding domain.

The binding specificity of N-terminal β-glucan binding domain of BgluC16MK was investigated with a fusion protein, B-GBD-GFP (Fig. 1). B-GBD-GFP was expressed in *E. coli* Rosetta-gami B (DE3), and purified by anion exchange and hydrophobic chromatography. The predicted molecular mass of B-GBD-GFP was approximately 42 kDa and the SDS-PAGE analysis results were consistent with this

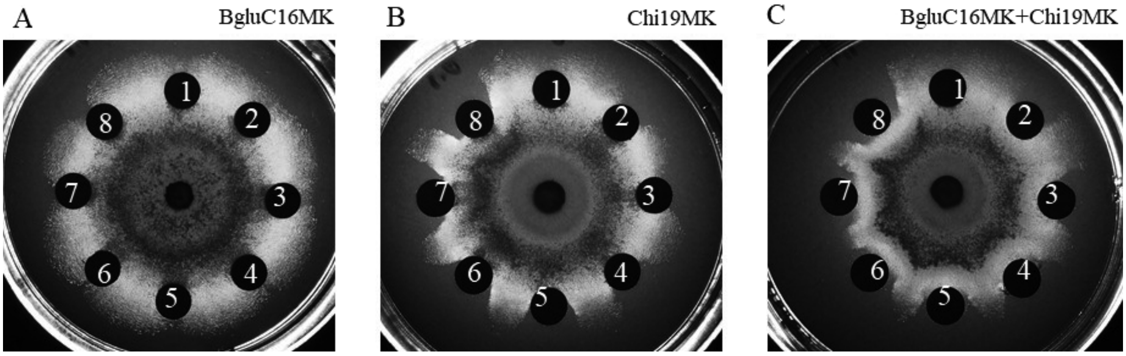

Fig. 6. Hyphal extension inhibition assay of BgluC16MK (A), Chi19MK Δ NTerm (B), and the mixture of BgluC16MK and Chi19MK Δ NTerm (C).

Trichoderma reesei was used as the test strain. The enzyme solution was added to the disks, and they were placed around the edge of the fungal colony. (A, B) Disc numbers 1−8: 0, 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 nmol of protein, respectively. (C) Each disc contained the same amounts of BgluC16MK and Chi19MK Δ NTerm. Disc numbers 1−8: 0, 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, and 0.4 nmol of each enzyme.

Table 1. Binding specificity of B-GBD-GFP.

Substrate	Glycosidic linkage-type	Binding efficiency (%)	
		AcGFP	BGBD-GFP
Zymosan A	β-1,3-	-	95.5 ± 0.1
Curdlan	β-1,3-	-	60.3 ± 5.4
Pachyman	β-1,3-		11.9 ± 0.3
Chitin	β-1,4-	-	5.2 ± 3.4
Chitosan	β-1,4-	-	5.2 ± 2.3
Cellulose	β-1,4-	-	-
α-1,3-glucan	α-1,3-	-	-

The amount of bound protein was estimated by subtracting the amount of free protein in the supernatant from the initial amount of protein. The binding efficiency is shown as percentage of the initial protein amount. The values are the means ± standard deviations of three independent experiments. '-' represents not detected.

value (Fig. 3). Table 1 shows that the binding specificity of B-GBD-GFP to zymosan A, curdlan, and pachyman was approximately 96, 60, and 12 %, respectively. B-GBD-GFP scarcely bound to chitin, chitosan, cellulose, and α-1,3-glucan, indicating that it specifically binds to glucans with β-1,3-linkages.

Binding assay of B-GBD-GFP against living fungal mycelium.

Figures 7A, B, C, and D shows that B-GBD-GFP binds to the cell-wall of *T. reesei* and *A. oryzae*, indicating that it might bind to living mycelium. In addition, B-GBD-GFP could bind to the entire mycelial surface of *T. reesei* and to some mycelial tips on *A. oryzae*. We assumed that the α-1,3-glucan accumulated on the cell surface of *A. oryzae* might have prevented the binding. Therefore, we performed the binding assay 2 h after treating the *A. oryzae* mycelium with α-1,3-glucanase. As shown in Figs. 7E and F, BGBD-GFP could bind to not only to the mycelial tips of *A. oryzae* but also to its overall mycelia.

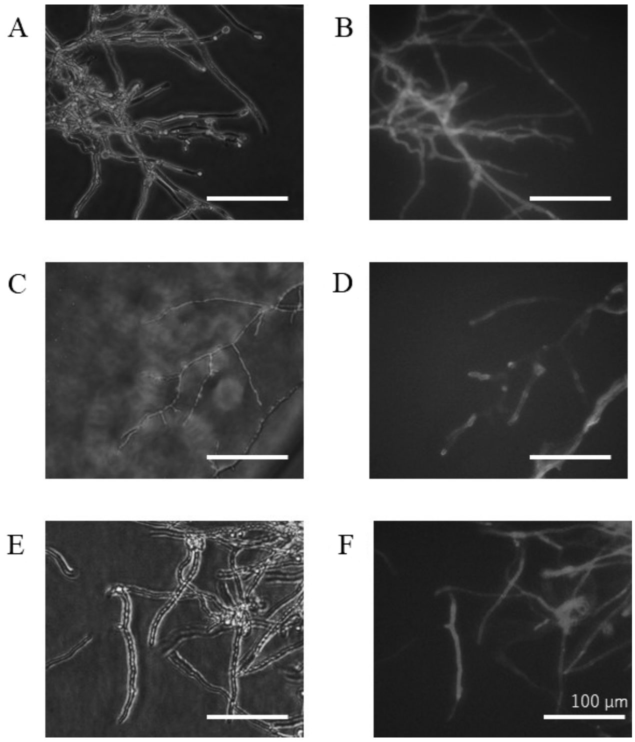

Fig. 7. Cell-wall binding assay of B-GBD-GFP against *Trichoderma reesei* (A, B) and *Aspergillus oryzae* (C, D, E, F).

A, B, C, D: The mycelia were incubated with B-GBD-GFP for 2 h; E, F: *A. oryzae* mycelia were treated with Agl-KA for 2 h and incubated with B-GBD-GFP for another 2 h. A, C, and E are light images. B, D, and F are fluorescence image.

DISCUSSION

In this study, we cloned the gene encoding GH-16 type β-1,3-glucanase (BgluC16MK) from *Lysobacter* sp. MK9-1 and expressed it in *E. coli*. BgluC16MK consists of N-terminal signal sequence, catalytic domain, and C-terminal CBM6 type B-GBD. It exhibits amino acid sequence similarity with β-1,3-glucanase GluC from *L. enzymogenes* C3, GluC from *L. enzymogenes* N4-7, and GluC from *L. enzymogenes* M497-1. Among the three enzymes, the enzymatic characteristics of *L. enzymogenes* N4-7 GluC were investigated.[14] BgluC16MK hydrolyzed laminarin, curdlan, pachyman, and zymosan A. The substrate specificity of BgluC16MK was similar to that of *L. enzymogenes* N4-7 GluC, although their relative activities on individual substrates were different. Furthermore, the optimum pH and temperature of BgluC16MK were almost same as those of *L. enzymogenes* N4-7 GluC. Thus, the differences between the enzymatic properties of *L. enzymogenes* N4-7 GluC and BgluC16MK were deemed negligible. As certain *Lysobacter* possess β-1,3-glucanase with high DNA sequence homology with GluC and BgluC16MK, we assumed that the functions and roles of these enzymes are similar.

BgluC16MK was more active against insoluble curdlan than soluble laminarin in the substrate specificity assay. The general comprehension that soluble substrates should be more readily hydrolyzed by the enzyme than insoluble substrates was supported by similar findings on enzymes, GH-16 type β-1,3-glucanase CcGluE of *Cellulosimicrobium cellulans* E4-5 and for GH-16 type enzyme Curd1 of *Streptomyces sioyaensis*.[28)29)] The spatial volume of the concave catalytic groove is regarded as limiting for CcGluE, and substrates having a β-1,6 side chain, such as laminarin, are difficult to accommodate. The C-terminal glucan binding domain of Curd1 contributes to the hydrolysis of the insoluble curdlan, and deletion of the glucan binding domain reduces activity against the insoluble substrate. The strong activity against curdlan is thought to be due to the B-GBD of BgluC16MK. As a result, we sought to build a mutant enzyme lacking the substrate-binding domain, but we were unable to do so because appropriate expression levels in the *E. coli* expression system could not be obtained. In this study, we focused on the involvement of BgluC16MK in *Lysobacter* sp. MK9-antifungal mechanism, but future research should focus on the enzyme's substrate specificity and steric structure to further understand its properties.

The antifungal activity of *L. enzymogenes* N4-7 GluC has not been investigated and therefore, we used BgluC16MK to perform the assay in this study. The quantitative evaluation of the antifungal activity of BgluC16MK against *T. viride* showed that the IC_{50} value was 20.1 μM (Fig. 5). Previously, we reported that the IC_{50} value of Chi19MK was 0.8 μM.[16)] These findings indicate that the antifungal activity of BgluC16MK is significantly weaker than that of Chi19MK. Contrastingly, hyphal extension inhibition assay showed that the addition of BgluC16MK to Chi19MK enhanced its antifungal activity (Fig. 6). This result suggests that BgluC16MK exerts its antifungal activity in combination with other lytic enzymes, such as Chi19MK. As mentioned in the introduction, Palumbo *et al.* reported that deletion of three β-1,3-glucanase genes of *L. enzymogenes* strain C3 significantly reduced the biological control activity of this strain against Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet.[15)] This might be due to the loss of the auxiliary role of β-1,3-glucanase, whose antifungal activity is similar function to that of BgluC16MK.

To understand the binding of BgluC16MK in order to use it as a fluorescence probe for fungal cell surface analysis, we also constructed a fusion protein, B-GBD-GFP, consisting of B-GBD from CBM6 and GFP. B-GBD-GFP bound to zymosan A, curdlan, and pachyman, but it scarcely bound to α-1,3-glucan and chitin (Table 1). Since chitin, β-glucan, and α-1,3-glucan are the main cell-wall components of many fungi, we assumed that B-GBD-GFP can selectively bind to β-1,3-glucan in the fungal cell-wall. The cell-wall binding assay showed that B-GBD-GFP bound to the cell-wall of *T. reesei* and *A. oryzae* (Fig. 7). In *A. oryzae*, B-GBD-GFP localized specifically to some mycelial tips. The mature cells of *A. oryzae* has accumulated α-1,3-glucan on the cell surface while the elongating mycelial tip contains less α-1,3-glucan and exposes chitin and β-glucan, which are cell-wall skeletal components. Therefore, the presence of α-1,3-glucan might interfere with the binding of B-GBD-GFP to mature mycelia. To mitigate this, we treated the *A. oryzae* mycelia with α-1,3-glucanase Agl-KA from *Bacillus circulans* KA-304 for 2 h and then performed the B-GBD binding assay. We observed that B-GBD bound to not only the elongating mycelial tip cell-wall but also the lateral cell-wall of mature mycelia, probably due to the degradation and removal of α-1,3-glucan from the cell-wall surface by α-1,3-glucanase. These findings suggest that B-GBD-GFP is a potential fluorescence probe candidate for the analysis of β-1,3-glucan in the fungal cell-wall.

We investigated the conformation and substrate binding sites of β-1,3-glucan binding CBM6s in ZgLamC from *Z. galactanivorans*, CelB from *C. mixtus* ATCC 12120, and BhGH81 from *A. halodurans* C-125. The CBM6s have two substrate binding clefts; the variable loop site and the concave site. Jam *et al.* reported substrate binding amino acid residues in CBM6 of ZgLamC and suggested that the residues, Y291, W348, and N377 at the variable loop site and W297 and D329 at the concave site contribute to substrate binding.[25)] B-GBD of BgluC16MK has a high amino acid sequence similarity with ZgLamC CBM6. Furthermore, B-GBD has conserved substrate binding amino acid residues at the concave site. W324 and N357 correspond to W348 and N377, and an aromatic amino acid, W268, corresponds to Y291 on ZgLamC CBM6 (Fig. 2B). Since the amino acid residues of CBM6s involved in binding to the fungal cell-wall have not been studied, our future goal is to investigate the contribution of the conserved amino acid residues in B-GBD of BgluC16MK in cell-wall binding.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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