Acting as skeletal polysaccharides.1)2) β-glucan and chitin constitute the cell walls of most fungi, and biological control candidates for pathogenic fungi, because Several sequences in the Carbohydrate Active enZymes (CAZy) database (http://www.cazy.org).3) As mentioned, β-glucosidase hydrolases (GHs) based on their amino acid families 5, 16, 17, 55, 64, 81, 128, 152, 157, and 158 of Lysobacter sp. MK9-1; Chi19MK, GH-19 type chitinase from Lysobacter sp. MK9-1; BgluC16MK, GH-16 type β-glucan binding domain; GFP, green fluorescent protein; CBM, carbohydrate-binding domain; 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.

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;1)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...
GluC consists of an N-terminal catalytic domain and a C-terminal carbohydrate-binding module family 6 (CBM6) domain. Reports have shown that 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 Schizopilum 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, laminarinbiose, 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’-CATCGCGCCGAAAGCCCGCGGAAGTGTGGTGCTGCGGT-3’), and the antisense primer, R1bgcfull (5’-TGGCGGACTCAGAAACGACGGTCTTGCAAT-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’-TTGGGGCCGCG CGCGCATATGCAAACACTCGAGCT-3’ containing NdeI site, underlined), and the antisense primer, BGLu16-rev (5’-TGGCGTTGC GGCGGTCCAGATCTTGTTGATG-3’ Xhol 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 Xhol 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, CBDBNdeF (5’-ACTATAGTGCAATGTCAGC GCGCGGCAGCAAT-3’ NdeI site) and CBDBBamR (5’-TATAGAAGATCCGCCCTCACCGCCACCTCGGAT CTGGTGATCGTGATCCGTTGATG-3’ BamHI site). The amplified product was digested with NdeI and BamHI and ligated into pET22b (+). 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’-TAGAGGATCCCGGTACCGGAT CGCCACCATGTGA-3’ BamHI site) and GFP-Xho1-R (5’-AATTGGAATTCTCGAGTCGCGGCCGCTCAA-3’ Xho1 site). The product was treated with BamHI and Xhol and ligated into the pET-b-gbd plasmid to yield the recombinant plasmid, pET-hb-gbd-fp.

**Gene expression of recombinant enzyme.** E. coli Rosetta-gami B (DE3) cells were transformed with pET-bgluc16 and pET-hb-gbd-fp, 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 (pH 7.0) and washed with the same buffer. The resulting dialysate was mixed with
ammonium sulfate at 20% saturation, and applied onto a TOYOPEARL Toyobeads O-Sephrose 650M (Tosoh Corporation) column (2.5 x 8 cm) equilibrated with 10 mM Tris-HCl pH 8.0. 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 Toyobeads O-Sephrose 650M column equilibrated with 10 mM Tris-HCl 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 Toyobeads O-Sephrose 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, and the initial velocity was calculated from the progress curve. One unit of the enzyme releasing 1 μmol of reducing sugar (as glucose) per min.

Antifungal activity assay. Antifungal activity was quantified as described previously. 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% (IC50) were determined by constructing dose-response curves (percentage of growth inhibition versus protein concentration).

Hyphal extension inhibition assays were performed as described previously. Trichoderma reesi 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. 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. reesi NBRC 4928 and Aspergillus oryzae NBRC 100959 were used as test strains, and the assay was performed according to the methods described previously. 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 was measured using Lowry’s method with bovine serum albumin as the standard. 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 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. PM1500 Excel Band All Blue Regular 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 reagent (9.3 mM 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-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. 10 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 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 type 6 (CBM6) β-glucan binding domain (B-GBD) (Fig. 1).

Figure 2 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. Figure 2B shows multiple alignment of CBM6s with the B-GBD of BgluC16MK, displaying sequence similarities with CBM6s of laminarinase ZgLamC from Zobellia galacta-


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.
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.**

Figure 5 shows a dose-response curve of the antifungal activity of BgluC16MK against *T. viride*, yielding an IC₅₀ 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).

**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
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.

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. 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

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

| Substrate    | Glycosidic linkage-type | AcGFP Binding efficiency (%) | BGBD-GFP Binding efficiency (%) |
|--------------|-------------------------|-------------------------------|---------------------------------|
| 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.

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.

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.

Figures 6A, 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.
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 sioyaeensis.2930 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 IC50 value was 20.1 µM (Fig. 5). Previously, we reported that the IC50 value of Chi19MK was 0.8 µM.40 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.45 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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