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Potential Physiological Functions of Acceptor Products of Dextransucrase with Cellobiose as an Inhibitor of Mutansucrase and Fungal Cell Synthase

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A series of oligosaccharides (cellobio-oligosaccharides) ranging from degrees of polymer 3 to 6 were synthesized by *Leuconostoc mesenteroides* B-512 FMCM in the presence of cellobiose. The major oligosaccharides were the trisaccharides, α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glucopyranose and α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glucopyranose. These cellobio-oligosaccharides were inhibitory on mutansucrase, an enzyme that causes dental caries. They were also found to be effective antifungal agents against *Aspergillus terreus* acting by inhibiting β-(1→3)-glucan synthase, which is required for fungal cell wall formation.

KEYWORDS: Oligosaccharides; cellobiose; dextransucrase; *Aspergillus terreus*; dental plaque; antifungal agent

INTRODUCTION

Oligosaccharides have been widely used in food, feed, pharmaceutical, and cosmetic industries due to their beneficial effects for human and animals (*1*). Varoious oligosaccharides have been reported to modify numerous physiological functions, including immune-stimulating, anticariogenic, and prebiotic compounds as well as sweeteners, stabilizer, and bulking agents (*2*,*3*). Mostly the beneficial health effects originate from inhibition of enzymes involved in carbohydrate metabolism.

Dental caries is decay of the teeth that is mainly caused by oral streptococci such as *Streptococcus mutans* and *S. sobrinus*. Oral streptococci secrete mutansucrase, synthesizing water insoluble mutans with a highly branched structure, and a majority of α-(1→3)-glucosidic linkages from sucrose. Mutans enhance the attachment of bacteria to tooth surfaces enabling the development of dental caries. Some oligosaccharides such as isomaltosylfructoside (*3*), fructosylxyloside (*4*), maltosylsucrose (*5*), and glucooligosaccharides and fructooligosaccharides (*6*) have been reported to prevent the occurrence of dental caries (*2*).

Fungal infections in humans can cause fatalities at a high rate (50−90%). Opportunistic infectious fungal diseases have emerged as a major cause of morbidity and mortality in immunocompromised people (*7*, *8*). Recently, infection with *Aspergillus terreus* has emerged as a growing concern to the medical community because the infection is more aggressive and has a much higher mortality rate than infections caused by other *Aspergillus* species, such as *A. fumigates* and *A. flavus* (*9*,*10*). Besides medical problems, fungi have caused plant diseases with significant economic losses. Agricultural economic loss, caused from invasive plant pathogens by fungi, is estimated at $21 billion per year in United States, more than half of all plant diseases (*11*). Various fungal disease in the main crops have been reported including smut and rusts in wheat, boll-rot and root-rot in cotton, whip smut in sugar cane, grain and long smut in sorghum, green ear and smut in millets, and mildew in vegetables (*12*).

Fungal cell walls are composed of β-(1→6)-glucan, mannan, or mannoprotein in the outer layers and β-(*1*,*3*)-glucan and chitin in the inner layers. The β-(1→3)-glucan is a vital and unique structural polymer not found in other eukaryotes. Inhibition of β-(1→3)-glucan synthesis can show a high target specificity, reducing side effects to the host. Inhibition of β-(1→3)-glucan synthesis causes cytological and ultrastructural changes in fungi characterized by abnormal growth as pseudohyphae, swollen hyphae, thickened cell wall, or buds failing to separate from the mother cell (*13*,*14*). Moreover, cells become osmotically sensitive, and lysis occurs on the growing tips of budding cells. Echinocandins, pneumocandins, and papulacandins are known inhibitors of β-(1→3)-glucan synthase (BGS (*15*−*17*)). However, they each have drawbacks, such as limited effectiveness against some fungal species and insolubility (*15*−*17*).

In this work, we report on the synthesis and characteristics of cellobio-oligosaccharides (CBO) produced by *Leuconostoc mesenteroides* B-512 FMCM dextransucrase in the presence of cellobiose. The CBO showed inhibitory effects on mutansucrase and fungal cell synthase.

MATERIALS AND METHODS

Microorganism and Chemicals. *Leuconostoc mesenteroides* B-512 FMCM and a disclosing solution were gifted by Dr. Doman Kim of Chonnam National University in South Korea. The culture was maintained at 30 °C in LM medium [0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 2% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.001% (w/v) $MnSO_4 \cdot H_2O$, 0.013%

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(w/v) $CaCl_2 \cdot 2H_2O$] containing 2% glucose or 2% sucrose. It was maintained on glucose-LM medium containing 2% glucose and 1.5% agar at 4 °C and was transferred biweekly.

Bio-Gel P2 and Bradford protein assay kit were purchased from Bio-Rad Laboratories (Richmond, CA), Sirofluor was from Biosupplies (Parkville Victoria, Australia), and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Preparation of Acceptor Products (CBO). Dextransucrase was prepared from *L. mesenteroides* B-512 FMCM and used for transglycosylation of glucose between sucrose and cellobiose as previsously described (*18*). In brief, oligosaccharide (CBO) was synthesized from 300 mM of sucrose and 250 mM of cellobiose using 54 U dextransucrase in 500 mL of 20 mM citrate buffer (pH 5.2) with shaking at 150 rpm until the sucrose was depleted (*18*). The preparation was centrifuged at 6500 rpm for 45 min to remove insoluble polysaccharides. The soluble polysaccharide was precipitated with an equal volume of ethanol that was slowly added to the supernatant and the resulting solution stored in a refrigerator for 2 h. Any precipitate was eliminated by centrifugation at 6500 rpm for 45 min. The supernatant was analyzed by high performance anion exchange chromatography (HPAEC) using a Dionex Carbo-Pac PA 100 column (250 × 4 mm) by gradient elution using 1 M NaOH, water, and 480 mM sodium acetate at a constant flow rate of 0.5 mL/min (*18*). Oligosaccharide detection was carried out with an electrochemical detector (ED 40 (*18*)). The supernatant was concentrated 10-fold using a rotary evaporator and then freeze-dried prior to use. For structural analysis, CBO was loaded onto Bio-Gel P2 (fine) column (1.5 cm × 115 cm) and eluted with water. It was collected in 0.5−1.0 mL fractions. The purities of the individual fractions were analyzed using HPAEC. Those fractions with the same DP (degree of polymerization) were pooled and freeze-dried.

Structural Analysis. Mass spectrometry data of purified CBO were obtained from electrospray measurements. The solvent was ultrapure water at 7 μL/min and detection was performed in the positive mode. For the preparation of NMR samples, 50 mg of purified CBO was exchanged three to four times with 600 μL of pure D_2O and lyophilized twice, then dissolved in 600 μL of pure D_2O, and placed into 5 mm NMR tubes. NMR spectra were produced using a spectrometer, operating at 500 MHz for H and 125 MHz for ^{13}C at 25 °C. It was examined for the linkages between cellobiose and glucose from homonuclear correlation spectroscopy, total correlation spectroscopy, rotating frame overhause effect spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple quantum coherence spectroscopy spectra.

Inhibition of Mutansucrase. Oral bacteria were collected from human teeth and streaked onto a brain heart infusion (BHI) agar containing 4% sucrose. They were grown at 37 °C until visible colonies of oral *Streptococcus* species appeared. The colonies were grown in 1 L BHI at 37 °C with shaking at 150 rpm for 24−36 h to produce mutansucrase. After fermentation, the culture was harvested, centrifuged, and concentrated to 100 mL using a 30 K cutoff membrane filter. One unit of mutansucrase was defined as that amount of enzyme that catalyzes the formation of 1 μmol of fructose per min at 37 °C and pH 7.0 from 100 mM sucrose. The inhibition of CBO on the synthesis of water insoluble glucans by mutansucrase was determined as described by the modified method of Ryu et al. (*19*) and Seo et al. (*20*). Mutansucrase was incubated in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0) containing 1 M sucrose as a substrate and 50 mM of cellobiose or CBO as an inhibitor in glass vials at 37 °C for 48 h. The supernatants of individual reaction mixtures were discarded and insoluble glucans remained in the vial. The synthesized glucans were washed with a HEPES buffer and dissolved in 0.5 N NaOH for comparison of the amount of insoluble glucans. The absorbance of water insoluble glucans was measured at 595 nm (*5*).

Antifungal Effects. *A. terreus* (ATCC 20514) was maintained on potato dextrose agar (PDA) medium for 5 to 7 days at 28 °C. Conidia were collected with a cotton swab and suspended in 0.9% NaCl solution with 0.05% Tween 20. The heavy particles were allowed to settle for 2 h in cold solution. The viability was confirmed by plating serial dilutions onto PDA plates. For determination of the morphological changes, 2.5×10^4 conidia were inoculated into 2.9 mL of potato dextrose broth (PDB) with CBO and incubated for 10 d at 28 °C. The (1→3)-β-glucan synthase (BGS) was produced by a modified method of Kelly et al. (*21*). Approximately, 4.5×10^8 conidia were inoculated into 500 mL of YME medium containing 0.4% yeast extract, 1.0% malt extract, and 0.4% glucose and incubated at room temperature for 1 to 2 days with shaking at 150 rpm. The spherical mycelia grown on YME medium were harvested by centrifugation at 1500*g* for 10 min. Cells were washed extensively with water and then centrifuged at 1500*g* for 10 min. Cell breakage was performed using 20 cycles (1 min each) of vortexing with prechilled glass beads in chilled extraction buffer containing 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1 μg/mL of leupeptin, and 10 μM GTPγS at approximately 5 mL of buffer per cell. Cells were cooled for 5 min on ice between cycles. The homogenate was centrifuged at 1500*g* for 10 min to remove cell debris. After centrifugation at 23 000*g* for 10 min to remove mitochondrial membranes, the supernatant was ultracentrifuged at 100 000*g* for 1 h to recover microsomal membranes in a pellet. This pellet was resuspended in one-tenth the original volume of cold storage buffer containing 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM DTT, and 20% glycerol. Protein concentration of isolated β-(1→3)-glucan synthase was 2.5 mg/mL. All procedures for enzyme preparation were carried out at 4 °C. Protein concentration was determined by the method of Bradford using Bio-Rad reagent, and bovine serum albumin was used as standard. The activity of BGS was determined by the modification of a fluorescence method (*22*, *23*). The assay mixture (150 μL) contained 27 mM HEPES (pH 7.2), 7 μM GTP, 1.3 mM EDTA, 0.17% Brij 35, 2.2% glycerol, 0.7 mM UDP-Glc, and isolated BGS (0.83 μg/μL). For inhibition studies, 0.12−0.64% of CBO was added to the desired mixture. Reactions were started by the addition of BGS, incubated at 22 °C for 105 min and terminated by the addition of 10 μL of 6 N NaOH. Glucans were solublized in a water bath at 80 °C for 30 min followed by the addition of 20 μL of a 4:1 diluted Sirofluor. The mixtures were incubated for 50 min at 22 °C and measured with a fluorescence spectrophotometer (FluoroLog, Horiba Jobin Yvon) at an excitation wavelength of 390 nm and an emission wavelength of 455 nm. Standard curves were constructed using various concentrations of yeast glucan, dissolved in 300 μL of 1 N NaOH by heating 30 min at 80 °C, containing the same components as the reaction mixtures except for enzyme.

Scanning Electron Microsocopy. A study of any morphologic changes was conducted by scanning electron microscopy (SEM). Conidia (3.0×10^4) were inoculated in PDB and incubated at 28 °C. After 16 h incubation, CBO was added to a test tube and water into a second tube as a control. They were further incubated for 2 days at 28 °C. For SEM, cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4 °C for 2 h. After being washed with the buffer, specimens were postfixed for 2 h with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4 °C. Samples were dehydrated in graded acetone, freeze-dried in *t*-butyl alcohol, and the sputter coated with palladium−gold.

RESULTS AND DISCUSSION

Acceptor Products of Dextransucrase with Cellobiose (CBO). Transglycosylation reaction between 250 mM of cellobiose and 300 mM sucrose by 54 U *L. mesenteroides* B-512 FMCM dextransucrase synthesized a mixture containing 67% of two trisaccharides, 13% tetrasaccharide, 13% pentasaccharide, and 8% hexasaccharide (**Figure 1** and **Table 1**). Morales et al. (*24*) similarly reported that two trisaccharides were produced from *L. mesenteroides* B-512 F dextransucrase with a small oligosaccharide that was not identified (*24*).

The structures of the major transglycosylation products, A and B, were determined by ^1H and ^{13}C NMR spectrometry to determine the synthetic modes of cellobio-oligosaccharides. The NMR assignments indicated two forms of a trisaccharide. We denoted the smaller amount of a trisaccharide as a product A and the larger amount of a trisaccharide as a product B. When the integral of III-1 proton at 5.06 ppm was determined, the total integral of two III-1 proton at 5.33 and 4.96 ppm were 2.2 (**Figure 2**). Therefore, a trisaccharide having III H-1 at 5.06 ppm was determined as a product A and the other as a product B.

The new anomeric proton signals at 5.06 ppm (J = 3.5 Hz, doublet signal) was assigned, indicating that a glucosyl residue was connected to cellobiose with α-linkage (*25*). In a product A, the ^{13}C-chemical shift in cellobiose before and after the addition

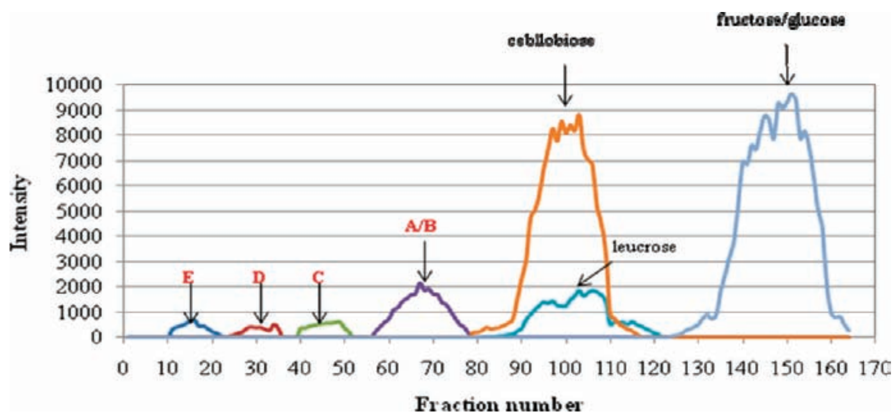

Figure 1. Bio-Gel P2 gel permeation column chromatogram of the *L. mesenteroides* B-512 FMCM dextransucrase reaction products. The column was 1.5 cm × 115 cm, eluted with water, and collected with 0.5 mL fractions.

Table 1. Concentration and Molecular Mass of Cellobio-Oligosaccharides

cellobio-oligosaccharides	concentration (g/l)	% in total CBO	molecular mass (g/mol)
A	1.5	14.0	504.1
B	5.5	52.7	504.1
C	1.4	12.9	660.0
D	1.4	13.3	828.3
E	0.9	8.1	990.3

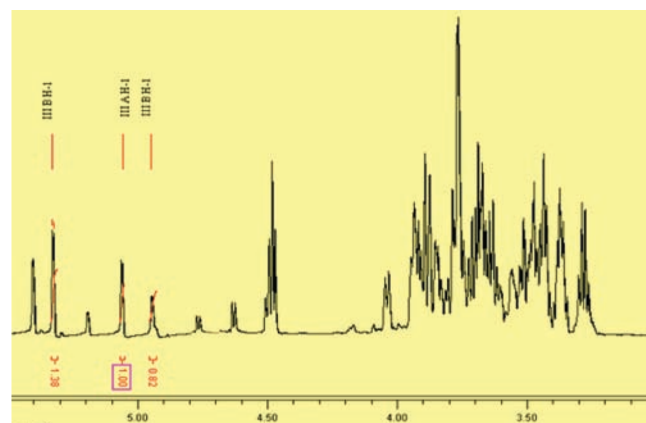

Figure 2. ^{1}H NMR spectrum of A [α-D-glucopyranosyl-(1→6)-cellobiose] and B [α-D-glucopyranosyl-(1→2)-cellobiose] in D_2O. Chemical shifts were measured at 500 MHz for ^{1}H NMR. Each of the residues of carbohydrates is designed by Roman numerals, started with I at the reducing-end residue.

of α-D-glucopyranose to cellobiose for C-6 was changed from 60.932 ppm to 66.354 ppm (**Table 2** and **Figure 3**). This chemical shift change is characteristic of the attachment of a D-glucopyranose unit to the original glucoside or aglycone (*25*−*27*). Except for this change for C-6, the spectra of product A gave no resonance changes. Therefore, the NMR result indicates that the D-glucopyranose unit was attached to the cellobiose ring by an α-(1→6) linkage. This cellobio-oligosaccharide structure was proposed in **Figure 4a**.

In product B, the ^{1}H chemical shifts of the new anomeric carbon (C-1) were 5.33 and 4.96 ppm with a coupling constant of 3.5 Hz, indicating that they were α-conformation (**Table 2** and **Figure 2** (*25*)). The corresponding ^{13}C chemical shifts appeared at 98.29 and 98.59 ppm (**Table 2** and **Figure 3**). According to Bock et al. (*28*), a ^{13}C chemical shift of C-1 in α-D-glucopyranose-(1→2)-β-D-glucopyranose was 98.6 ppm, which indicates an α-(1→2) linkage. Evidence for this linkage was

Table 2. ^{1}H NMR and ^{13}C NMR Chemical Shifts[a] for Product a and B Produced by the Reaction of Dextransucrase with Sucrose and Cellobiose (Units, ppm)

			Cellobiose (δ)	CBO-A[b] (δ_A)			CBO-B[c] (δ_B)		
			δ_C	δ_{AC}	δ_{AC}-δ_C	δ_H	δ_{BC}	δ_{BC}-δ_C	δ_H
I[d]	α-Glc	1[e]	92.20	92.33	0.13	5.19	89.69	−2.51	5.41
		2	71.60	71.83	0.24	3.56	70.32	−1.27	3.65
		3	71.71	72.28	0.58	3.94	72.24	0.53	3.94
		4	79.11	79.03	−0.07	3.67	79.06	−0.05	3.68
		5	70.48	70.39	−0.08	3.89	70.39	−0.08	3.89
		6	60.28	60.63	0.35	3.79	60.28	0.00	3.90
	β-Glc	1	96.12	96.53	0.41	4.78	96.31	0.18	4.63
		2	74.26	73.58	−0.68	3.38	73.74	−0.51	3.27
		3	74.66	73.46	−1.20	3.70	73.82	−0.84	3.49
		4	78.97	79.07	0.10	3.69	79.87	0.90	3.61
		5	75.16	73.58	−1.59	3.67	73.79	−1.37	3.47
		6	60.42	61.23	0.81	3.89	61.13	0.71	3.72
II	β-Glc	1	102.93	103.46	0.53	4.50	102.84	−0.09	4.48
		2	73.54	73.30	−0.24	3.30	76.73	3.20	3.30
		3	76.86	76.12	−0.74	3.49	76.12	−0.74	3.49
		4	69.82	70.11	0.28	3.37	69.72	−0.10	3.38
		5	76.33	75.32	−1.02	3.61	75.10	−1.24	3.71
		6	60.94	66.35	5.42	3.88	61.28	0.34	3.68
III	α-Glc	1		96.89		5.06	98.29/98.59		5.33/4.95
		2		71.74		3.53	71.96		3.53/3.55
		3		73.37		3.77	73.37		3.77
		4		69.82		3.43	69.82		3.43
		5		70.52		3.95	70.52		3.95
		6		61.31		3.78	61.31		3.78

[a] Chemical shifts were measured at 125 MHz for ^{13}C NMR and 500 MHz for ^{1}H NMR in D_2O at 25 °C with acetone as an internal standard. [b] α-D-glucopyranosyl-(1→6)-cellobiose. [c] α-D-glucopyranosyl-(1→2)-cellobiose. [d] Each of the residues of cellobio-oligosaccharide is designed by Roman numerals, started with I at the reducing-end residue. [e] The position of carbon and proton and the number starts from the anomeric carbon in a residue.

supported by a downfield ^{13}C shift for II C-2 of cellobiose from 73.54 to 76.73 (**Table 2** and **Figure 3**). These results identified this cellobio-oligosaccharide structure as α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (**Figure 4b**).

Our finding was agreed with Morales et al. (*24*) in the synthesis of two trisaccharides by *L. mesenteroides* B-512 F dextransucrase in the presence of cellobiose. Dextransucrase from *L. mesenterodies* is known primarily to transfer the D-glucose residue from sucrose to the nonreducing end 6-hydroxyl group of mono- and higher-saccharides in the presence of an acceptor molecule (*29*, *30*). Dextransucrase also synthesizes a different kind of oligosaccharides depending on the type of acceptor molecule. In the presence of β-glucosidic linkages in the acceptor molecule, the specificity of dextransucrase is changed to transfer the 2-OH

Figure 3. ^{13}C NMR spectra of (**a**) cellobiose and (**b**) α-D-glucopyranosyl-(1→6)-cellobiose and α-D-glucopyranosyl-(1→2)-cellobiose. Chemical shifts were measured at 125 MHz for ^{13}C NMR. Each of the residues of carbohydrates is designed by Roman numerals, started with I at the reducing-end residue.

(a) α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glucopyranose

(b) α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glucopyranose

Figure 4. Proposed chemical structures of (**a**) α-D-glucopyranosyl-(1→6)-cellobiose and (**b**) α-D-glucopyranosyl-(1→2)-cellobiose. Each of the residues of cellobio-oligosaccharides is designed by Roman numerals, started with I at the reducing-end residue.

group at the reducing end glucose rather than transfer 6-OH at the nonreducing end (*29−31*). It transfers the D-glucosyl residue to the nonreducing end OH of maltose or isomaltose in the presence of maltose or isomaltose, whereas it transfers D-glucose from sucrose to the reducing end, as well as the 6-OH groups of the nonreducing end in the presence of maltotriose and maltotetraose (*32*). When the acceptor molecule is lactose or raffinose, *L. mesenteroides* B-512 F dextransucrase transfers D-glucose from sucrose to the OH group at C-2 of the D-glucose residue (*29*, *30*).

Inhibition of Mutansucrase. The CBO inhibited the synthesis of water insoluble glucans in the presence of sucrose (**Figure 5**). In the presence of 50 mM CBO and 1 M sucrose, only 4% of insoluble glucans were produced compared to that produced in the absence of the CBO (**Figure 5a**). Addition of cellobiose (50 mM) did not affect insoluble glucan formation. The insoluble glucans adhered to an inner layer on a glass vial, as dental plaque does on teeth (**Figure 5b**). The quantity of insoluble glucans adhered to glass was less for the CBO mixture than the control or the cellobiose test. Mutansucrase from oral bacteria, *S. mutans* and *S. sobrinus,* synthesizes extracelluar water insoluble glucans in the presence of sucrose. The insoluble glucans become plaque on teeth and result in tooth decay. In addition, *S. mutans* and *S. sobrinus* synthesize intracellular polysaccharides as carbohydrate reserves, which can be converted to acids when dietary carbohydrates are available. Therefore, CBO has a potential to become an active ingredient in dental care products since it inhibits glucan formation. Inhibitory effects of oligosaccharides on mutansucrase have also been reported with oligosaccharides produced from *L. mesenteroides* B-512 FMCM (*20*), isomaltosylfructoside (*3*), fructosylxyloside (*4*), maltosylsucrose (*5*), and glucooligosaccharides and fructooligosaccharides (*6*). According to Seo et al. (*20*), oligosaccharides produced from a sucrose solution using B-512 FMCM inhibited growth of *S. sorbinus* but activated that of *Bifidobacterium* sp. in addition to inhibiting mutansucrase by oligosaccharides,

Antifungal Effects. Antifungal agents, the β-(1→3)-glucan synthase (BGS) inhibitors, have been validated as an effective treatment of fungal infections because they inhibit fungal cell wall synthesis, a target unique to these lower eukaryotes (*33*). The effect of CBO on β-(1→3)-glucan synthase, the essential enzyme that forms β-(1→3)-glucan fibrils from UDP-glucose, was evaluated. DP 3 oligosaccharides isolated from CBO were found to be strong inhibitory effect on BGS activity compared to CBO including DP 3 to DP 6 (**Figure 6**). It implies that inhibitory effects of CBO on BGS activity were caused by two trisaccharides among CBO. The addition of 0.64% DP 3-CBO in a reaction mixture containing 2 mM UDP-glucose inhibited BGS activity about 74%. The role of CBO on glucan synthase was evaluated by a kinetic study over a range of concentrations (0.05 to 8 mM) of UDP-glucose with CBO added at concentrations of 0, 0.16, and 0.24%. The reaction velocity was calculated, and a Lineweaver−Burk plot of 1/[substrate] and 1/velocity was plotted (**Figure 7**). The nonparallel lines, which converge at $x < 0$ and $y > 0$, are consistent with a mixed type of inhibition. There are no reports on oligosaccharides containing mixed β(1→4), α(1→2), and α(1→6) linkages and fungal glucan synthases. A cellotriose, comprising glucose linked with only β-(1→4), enhanced activity of glucan synthase isolated from *Euglena gracilis* (*34*). Differences between

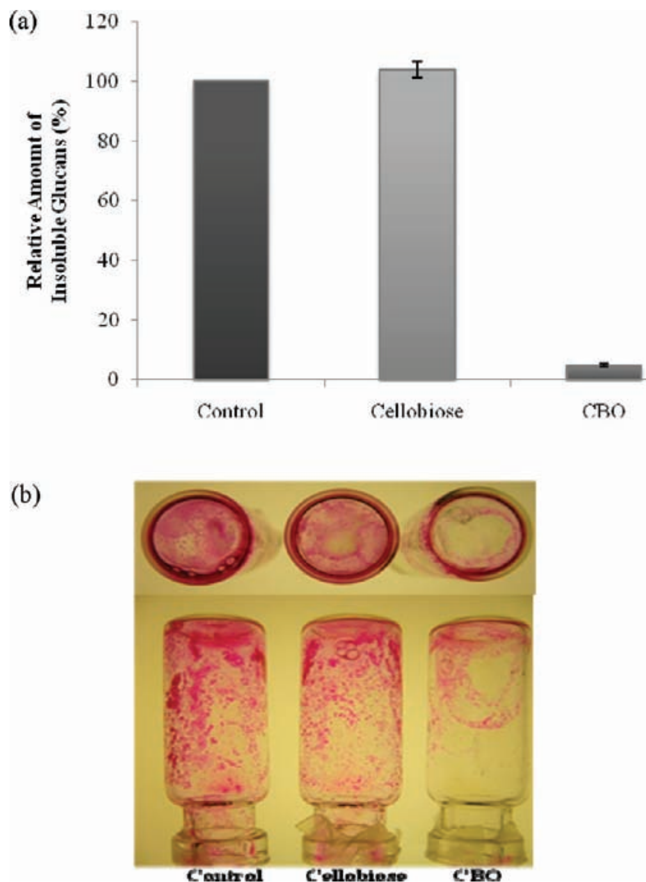

Figure 5. The effect of cellobiose or CBO on the formation of insoluble glucan by mutansucrase: (**a**) relative amount of insoluble glucan and (**b**) their adhesion on glass vial in the absence of additional sugar in the presence of 50 mM cellobiose or 50 mM CBO in 1 M sucrose.

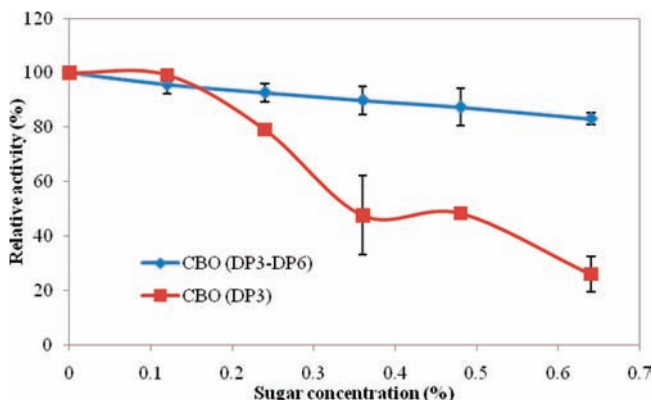

Figure 6. The inhibition effect of CBO or trisaccharides among CBO in β-(1→3)-glucan synthase acitivity from *A. terreus*. The BGS activity was determined using 0.7 mM UDP glucose as a substrate and 0.83 μg/μL 1, 3-β-D-glucan synthase as an enzyme in 27 mM HEPES (pH 7.2) containing 7 μM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol with the addition of 0−0.64% CBO at 22 °C for 105 min. Error bars show the standard deviation error of the mean.

our CBO and this cellotriose (*34*) demonstrate that the structural linkages are important in altering glucan synthase activity. Cellobiose has been reported to be a stimulator for glucan synthase production in sugar beet (*35*) and *Euglena gracilis* (*34*). However, cellobiose does not stimulate the glucan synthase of *S. cerevisiae* (*36*) or the germinating peanut, *Arachis hypogaea* (*37*). A simple sugar-based chemical, δ-gluconolactone, was an effective inhibitor of β-(1→3)-D-glucan synthase in the sugar beet (*35*) and in *S. cerevisiae* (*36*).

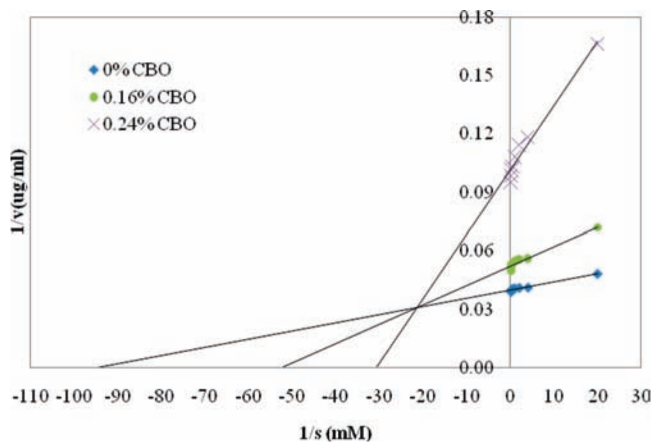

Figure 7. Lineweaver−Burk plot: inhibition of (1→3)-β-D-glucan synthase by CBO. The assay mixtures contained 27 mM HEPES (pH 7.2), 7 μM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol, varying concentration of UDP-glucose (0.05, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 μg/μL) also containing 0.83 μg/μL β−(1→3)-glucan synthase. The reaction mixtures were incubated for 105 min at 22 °C. BGS activity was also measured in 0−0.24% CBO.

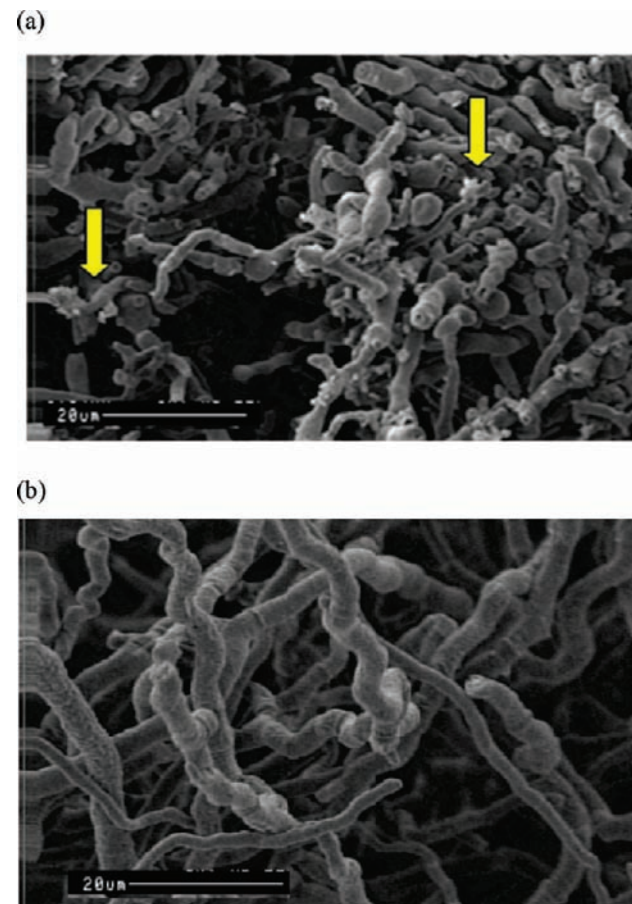

Figure 8. SEM images of *A. terreus* cells. (**a**) Control growth of cells (no cellobio-oligosaccharide); (**b**) growth of cells treated with cellobio-oligosaccharides. Arrows point at sporulation of the cells. Bars represent 20 μm at 1.34 k× magnification.

Most glucan synthase inhibitors induce profound morphological changes in fungal hyphae that correlate with inhibition of

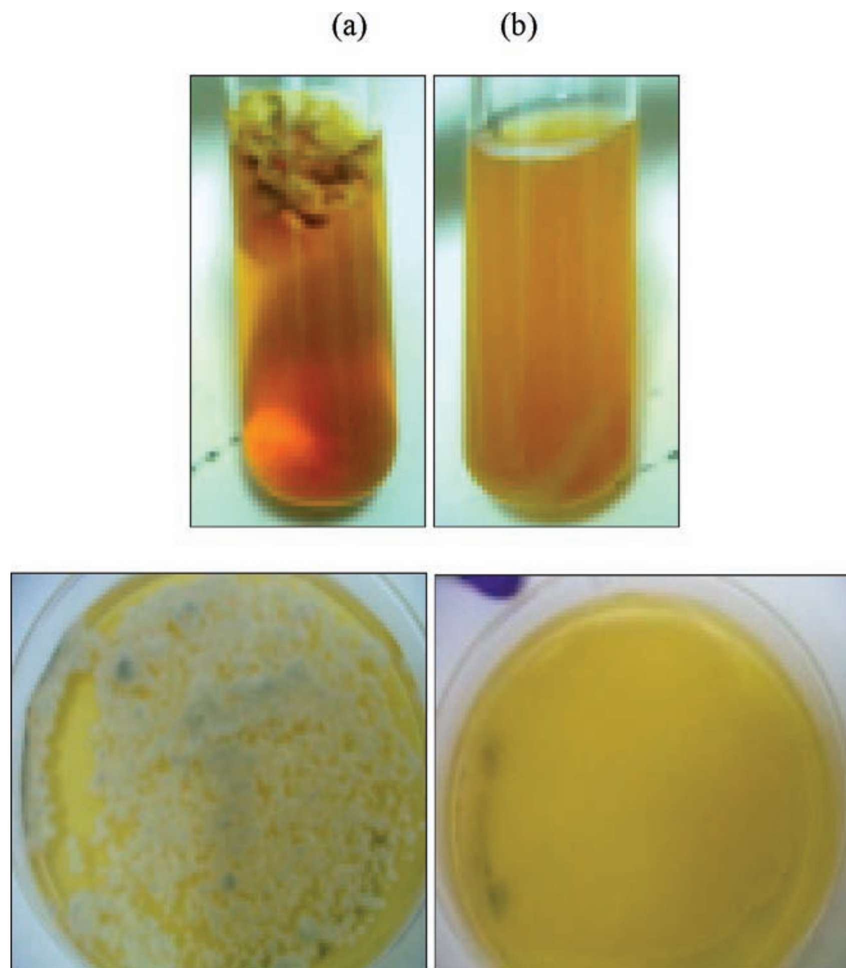

Figure 9. In vitro growth of *A. terreus* in PDB and PDA. (**a**) Control growth of cells; (**b**) growth of cells treated with cellobio-oligosaccharides. All tubes and Petri-dishes were photographed ten days after incubation at 30 °C.

glucan synthase (*13−15*). Observation of hyphal changes after addition of CBO was made using SEM. Hyphae of *A. terreus* showed distinct structural differences between control and CBO-treated cultures (**Figure 8**). The bud scar rings are found in several hyphae tips on the control but none on CBO treated *A. terreus*. The hyphae of CBO-treated *A. terreus* fail to bud, and the population does not increase. In addition, the average widths of hyphae were different between the two cultures when 20 hyphae were randomly selected and measured. The average width of twenty hyphae in CBO-treated *A. terreus* (3.4 μm) was 1.35 fold larger than the width in the control (2.5 μm). In the presence of CBO, the cells grew with swollen hyphae, indicating inhibition of glucan synthesis (*15*). Glucan synthesis inhibition will produce stunted, swollen hyphae, caused from a weakened cell wall that expands under high internal pressure (*33*). In the work of Kurtz et al. (*15*), pneumocandin-treated *A. fumigates* caused swelling and distension of the hyphae. Although the abundance of β-(1→3)-glucans in the cell walls formed during different stages of the *A. fumigatus* life cycle is not well characterized, the focus of new cell wall synthesis is the hyphae during vegetative growth (*38*, *39*). Inhibition of β-(1→3)-glucan synthesis has profound effects on cell wall structure in *A. fumigatus* (*15*). Inhibition of glucan synthesis results in structural changes, characterized as pseudohyphae, swollen hyphae, thickened cell wall, or buds failing to separate from mother cells (*13−15*). We explored the effect of CBO on *A. terreus* grown in PDB and PDA during extended incubation up to 10 d at 28 °C (**Figure 9**). When *A. terreus* was grown in PDB medium, it formed tangled hyphal masses on the surface in a tube. However, these masses were not observed when *A. terreus* was incubated with CBO in PDB medium. There was substantial growth in the untreated culture during the course of the experiment.

There is no comparable data that oligosaccharide alone works as an antifungal agent. Almost all proposed antifungal agents have complicated structures. Nevertheless, Kaur et al. (*40*) suggested that a small and simple sugar acid, D-gluconic acid from *Pseudomonas* strain AN5, may have antifungal activities against the take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Some researchers have reported that cellobiose-based lipids have fungicidal activities. Complex cellobiose-lipids of yeast fungi *Cryptococcus humicola* and *Pseudozyma fusiformata* (ustilagic acid B) inhibited the growth of a number of species that are important for medicine: *Candida. albicans*, *C. glabrata*, *C. viswanathii*, *F. neoformans*, *and Clavispora lusitaninae* (*41*, *42*). These lipids may stimulate the release of ATP from the test culture cells, indicating an increase in the permeability of plasma membrane, and resulting in cell death (*43*, *44*). Mimee et al. (*45*) isolated flocculosin, a low molecular weight cellobiose-lipid, from the yeastlike fungus *Pseudozyma flocculosa* to investigate antifungal activity. Flocculosin significantly inhibited the growth of *Candida lustitaniae*, *C. neoformans*, *Trichosporon asahii,* and *C. albicans*. Synergistic activity was also verified between flocculosin and amphotericin B, suggesting the potential for amphotericin B having a much lower MIC. Most isolated cellobiose-lipids have considerable efficacy as potential antifungal agents under the acidic condition (*42*, *45*). On the basis of our data, CBO has a

great potential to function as a new class of antifungal agent against fungi, which correlates with β-(1→3)-glucan synthase inhibition.

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