Characterization of new oligosaccharide converted from cellobiose by novel strain of *Bacillus subtilis*

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Abstract Six bacterial strains isolated from various Korean fermented foods were cultured in cellobiose-containing medium to investigate their potential for producing new kind of oligosaccharides. After bacterial culture in a liquid medium, each culture medium was concentrated and analyzed. TLC analysis revealed that only one strain (*Bacillus subtilis* SS-76) produced new spot on the TLC plate, indicating that it could convert cellobiose into a new oligosaccharide. Following purification of the culture supernatant of *B. subtilis* SS-76, the fractions containing the oligosaccharide produced were pooled, and the concentrated fraction was analyzed for its chemical and structural characteristics. By using various analytical techniques such as sugar composition analysis, glycosidic linkage analysis, and molecular weight determination, the new oligosaccharide was identified as glucotriose connected with (1 → 4) and (1 → 3) glycosidic linkages. In addition, the result of specific enzyme catalysis suggested that the new glucotriose might contain only β -configurations in their anomeric configurations.

Keywords *Bacillus subtilis* · Cellobiose · Oligosaccharide · Structural characterization

Introduction

According to the International Union of Pure and Applied Chemistry (IUPAC), oligosaccharides are defined as low-molecular-mass sugars that typically contain between 3 and 10 residues. In the food and nutraceutical industries, oligosaccharides have gained attention for their great potential in improving the quality of many food products, such as modifying the flavor and improving the physicochemical characteristics of the food [1, 2]. In particular, non-digestible oligosaccharides are known to be prebiotics that stimulate the growth of beneficial bacteria in the colon. The industrial production of oligosaccharides has typically depended on the degradation of polysaccharides, or on synthesis from monosaccharide and disaccharide substrates [3]. In particular, the simpler enzymatic synthesis of oligosaccharides has been increasingly favored over the cumbersome chemical synthesis method that generally relies on multiple steps [4]. In enzymatic synthesis, oligosaccharide-synthesizing enzymes, specifically glycosidases and glycosyltransferases, have generally been used for catalyzing the transglycosylation of ubiquitous substrates [5–10]. Transglycosylation is a general term for the process that transfers a glycosidic residue from one sugar to another.

Cellobiose, one of the substrates for oligosaccharide synthesis, is a reducing-sugar disaccharide consisting of two β -glucose molecules connected by β -(1 → 4) linkage [7, 11]. Although cellobiose is used as a raw material in the production of bioenergy such as bioethanol, it is an expensive material owing to its high production cost. However, given the growing number of reports on improving the production of cellobiose [12, 13], the production costs are predicted to become more affordable, thereby accelerating the industrial production of this

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important disaccharide for its bioconversion into high-value substances. In particular, studies on the conversion of cellobiose into functional materials such as novel oligosaccharides are under the spotlight [14].

In a recent study, 6 bacterial strains (viz., SS-02, SS-21, SS-27, SS-46, SS-65, and SS-76) were isolated from various Korean fermented foods and their potential for producing oligosaccharides was investigated [15]. Among these six strains, only one strain (SS-76; isolated from traditionally fermented soybean paste) produced an uncommon oligosaccharide from maltose as a sole carbon source. Based on scanning electron microscopy and 16S rDNA sequence analysis results, the rod-shaped bacterial strain SS-76 had the closest relatedness to *Bacillus subtilis*. The novel oligosaccharide revealed on the TLC plate was purified and finally identified as a trisaccharide panose [α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose] through diverse chemical and structural analyses [15]. Based on the previous study, this study aimed to identify whether this strain could produce novel oligosaccharides by transglycosylation in a cellobiose-containing medium.

Materials and methods

Chemical and biological reagents

Glucose, cellobiose, and cellotriose were all obtained from Sigma-Aldrich (St. Louis, MO, USA). The basal medium without a carbon source consisted of 0.5% peptone, 0.1% yeast extract, 0.1% potassium phosphate dibasic, 0.06% sodium phosphate monobasic dihydrate, and 0.05% magnesium sulfate dehydrate, with the pH adjusted to 6.8. All culture materials were obtained from BD Diagnostics (Franklin Lakes, NJ, USA).

Isolation of bacterial strains from various fermented foods

According to the isolation method of bacterial strains reported by Bae et al. [16] and Seo and Shin [17], diverse bacterial strains were isolated from various Korean fermented food samples, such as soybean paste, soy sauce, and heavily salted fermented shrimp, to find novel strain that could produce new oligosaccharide. Briefly, from those fermented foods, individual strains were purely separated by diluting fermented food samples with saline solution, plating and culturing the solution on nutrient agar, and finally transferring morphologically distinguishable colonies onto fresh nutrient agar. A total of 116 strains were discriminated separately and stored (in nutrient broth

with 20% glycerol) as frozen stock cultures at -70°C until use.

Cultivation of bacterial strains for estimation of oligosaccharide-producing activity

All bacterial strains were cultured individually in nutrient broth medium for 1 day, and their potential to produce novel oligosaccharides was determined by analyzing the culture supernatant by TLC (Kieselgel 60 plate, Merck, Darmstadt, Germany), as described by Seo and Shin [17]. Briefly, harvested and washed cells were suspended with 100 mM acetate buffer (pH 5.5) containing 1% cellobiose, and incubated at 30°C for 4 h. When the culture supernatant was spotted and developed with eluent (BtOH:pyridine:water, 6:4:1) on the TLC plate, 6 bacterial strains (viz., SS-02, SS-21, SS-27, SS-46, SS-65, and SS-76) were found to produce oligosaccharides. These 6 strains were further individually cultured in basal medium containing 2% cellobiose as a sole carbon source in a shaking incubator (Lab Companion, Seoul, Korea) at 30°C for 1 day, and the culture supernatant was again analyzed by TLC to determine the production of new oligosaccharides.

Fractionation and purification of new oligosaccharide converted from cellobiose

To purify the oligosaccharide, the oligosaccharide-producing strain was repeatedly cultured in the same medium at 30°C for 3 days, and the culture supernatants were pooled and concentrated using a vacuum rotary evaporator (Eyela; Tokyo Rikakikai Co., Tokyo, Japan). The concentrated culture supernatant was then fractionated using a HPLC device equipped with a Prevail Carbohydrate ES column (250 mm $\times$ 4.6 mm, 5 μm ; Grace Davison Discovery Sciences, Deerfield, IL, USA). A 70% acetonitrile solution was used as the eluent at a flow rate of 0.5 mL/min, and the eluates were sequentially collected in test tubes using a fraction collector (1200 Series; Eyela, Tokyo, Japan). The eluates in all tubes were monitored by TLC to verify the oligosaccharide production.

Analytical methods

The total carbohydrate and protein contents were respectively measured by the phenol-sulfuric acid method [18], using glucose as a reference, and the Bradford method [19] using protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as a reference. The monosaccharide composition of the oligosaccharide was analyzed by a modified alditol acetate method [20], using a gas chromatography (GC) apparatus (6000 Series; Young-Lin Co., Anyang, Korea) equipped with an

SP-2380 capillary column (0.2 $\mu\text{m} \times 0.25 \text{ mm} \times 30 \text{ m}$; Supelco, Bellefonte, PA, USA) and flame ionization detector (FID). The temperature program for the analysis was the same as that used by Kim et al. [21], and the molar ratio of monosaccharides was calculated from the peak areas and response factors.

Determination of glycosidic linkage type by methylation analysis

In order to verify the glycosidic linkage information of the new oligosaccharide, methylation analysis, followed by hydrolysis, reduction, and acetylation, was performed according to the methods described by Hakomori [22], Waeghe et al. [23], and Kim et al. [21], using the above-described GC system equipped with the SP-2380 capillary column and FID. The analysis was performed at 60 °C for 1 min, followed by incremental increases in temperature of 60 °C $\rightarrow$ 150 °C (30 °C/min), 150 °C $\rightarrow$ 180 °C (1 °C/min), 180 °C $\rightarrow$ 231 °C (1.5 °C/min), and 231 °C $\rightarrow$ 250 °C (30 °C/min), and finally 250 °C for 10 min. The PMAAs were identified by their fragment ions and relative retention times, and their molar ratios were estimated from the peak areas and response factors.

Mass spectrometry and nuclear magnetic resonance spectrometry

MALDI-TOF/MS and ESI-LC-MS/MS were used to determine the precise molecular mass of the novel oligosaccharide. The MALDI-TOF mass spectra were obtained on a 4700 Proteomics Analyzer/Voyager DE STR workstation (Applied Biosystems, Framingham, MA, USA) following the conditions described by Seo and Shin [17]. The matrix for oligosaccharide analysis was 0.1 M 2,5-dihydroxyacetophenone in 50% aqueous acetonitrile. The ESI-LC-MS/MS spectra were obtained on an Agilent 1200 HPLC system (Palo Alto, CA, USA) interfaced with a triple quadrupole tandem MS (Agilent) fitted with an ESI source. The mass spectrometer was operated in the positive-ion mode, the ESI voltage was set at 5 kV, and the gas temperature was 350 °C.

Specific enzyme treatment for identification of the anomeric configuration of new oligosaccharide

In order to identify the anomeric configuration of new oligosaccharide produced from cellobiose, glucoamylase from *Aspergillus niger* (EC 3.2.1.3) and β -glucosidase from almond (EC 3.2.1.21) were purchased from Sigma. Enzyme reaction of each enzyme was performed according to the manufacturer's recommendations.

Results and discussion

Screening and identification of an oligosaccharide-producing bacterial strain

Based on our previous study [15], we hypothesized that another saccharide with β -linkage (i.e., cellobiose) could be converted into different type of oligosaccharides. Therefore, in the present study, the same 6 bacterial strains were re-examined to consider this possibility. The strains were incubated in basal medium containing 2% cellobiose and the culture supernatants were analyzed on thin layer chromatography (TLC) plates. As shown in Fig. 1, relative to the TLC spots corresponding to cellotriose and cellobiose, all six strains showed a spot corresponding to cellobiose. Interestingly, only one strain (lane 7, strain SS-76) showed two additional spots, one of them presumably corresponding to an unknown oligosaccharide and the other to a monosaccharide (Fig. 1). This demonstrated that only strain SS-76 was likely to convert cellobiose into another oligosaccharide and to degrade cellobiose into an

Fig. 1 TLC analysis of culture supernatants from 6 bacterial strains cultured in basal medium containing 2% cellobiose for 3 days. The culture supernatants were spotted onto a TLC plate and developed twice with a solvent containing butanol:pyridine:water (6:4:1). Sugar spots were detected by spraying the plates with 20% sulfuric acid in methanol, followed by heating the plates at 120 °C for 10 min. Lane 1, cellobiose and cellotriose standards; lane 2, culture supernatant of strain SS-02; lane 3, culture supernatant of strain SS-21; lane 4, culture supernatant of strain SS-27; lane 5, culture supernatant of strain SS-46; lane 6, culture supernatant of strain SS-65; lane 7, culture supernatant of strain SS-76

Fig. 2 (A) HPLC elution profile of the culture supernatant of *Bacillus subtilis* SS-76 cultured in cellobiose-containing medium. (B) TLC analysis of the fractions obtained by preparative HPLC. Lanes 1–14, fractions collected by a fraction collector with retention times ranging from 27 to 33 min on the HPLC chromatogram

additional monosaccharide (possibly glucose). This also suggested that strain SS-76, which was identified as a *B. subtilis* (>99% similarity) strain in the preliminary research, has great potential for producing another oligosaccharide as well as panose [15]. To our best knowledge, there have been no other similar reports, with the exception of our preliminary paper, on the production of oligosaccharides using *Bacillus subtilis*.

Purification of the new oligosaccharide produced by *B. subtilis* SS-76

For successful separation of the novel oligosaccharide, the cultured medium of strain SS-76 was fractionated by

preparative HPLC system. As shown in Fig. 2(A), the supernatant produced peaks “A” and “B” at retention times 28.0383 min and 31.0350 min, respectively, in addition to the peak identified as glucose and cellobiose. Subsequently, eluates that had passed through the detector were sequentially collected using a fraction collector, whereupon a total of 80 fractions (250 μ L per tube) were obtained and were further screened using TLC. As shown in Fig. 2(B), a total of 14 fractions corresponding to retention times ranging from 27 to 33 min on HPLC spectrum were analyzed by TLC. Relative to the glucose, cellobiose, and cellotriose standards (data not shown), the fractions spotted seemed to be oligosaccharides composed of more than three sugar residues. To obtain a pure

Table 1 Chemical composition and glycosidic linkages of the oligosaccharide fraction A generated by *Bacillus subtilis* SS-76

Chemical composition	(%)
Carbohydrate	99.3
Protein	n.d. ^a
Monosaccharide composition	(Mol%) ^b
Glucose	99.7
Other sugars	Trace
Linkage composition ^c	(Mol%)
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylhexitol (T-Glcp)	35.7
1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methylhexitol (3-linked Glcp)	30.4
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methylhexitol (4-linked Glcp)	33.9

The monosaccharide composition was analyzed using the alditol acetate method, and the linkage information was determined by methylation analysis

^aNot detected

^bMol% calculated from the detected neutral sugar

^cGlcp, glucopyranoside; T, non-reducing terminal

oligosaccharide fraction, the spots labeled as “fraction A” (corresponding to peak “A” in the HPLC analysis) were collected from lanes 2 to 7, and the oligosaccharide labeled as “fraction B” (corresponding to peak “B” in the HPLC analysis) was collected from lanes 9 to 14. In this study, former peak (fraction A) was chosen for further structural elucidation. Of course, latter peak (fraction B) should be further identified to understand the reaction condition, such as substrate variety, of strain SS-76, and it will be done in further study. Meanwhile, our previous study showed that strain SS-76 could also produce two peaks in the HPLC analysis when another disaccharide maltose was supplied as a sole carbon source and a latter peak was identified isomaltooligosaccharide panose.

Monosaccharide composition and linkage analysis of the novel oligosaccharide fraction

As shown in Table 1, the oligosaccharide fraction A produced by *B. subtilis* SS-76 contained 99.0% of neutral saccharides without any proteins, indicating that it was purely isolated. Analysis of the monosaccharide composition using the alditol acetate method indicated that fraction A consisted only of one sugar unit, glucose, demonstrating that the fraction was definitely not cellobiose but another oligosaccharide. To clarify the fine structure of this fraction A oligosaccharide, methylation analysis was carried out. The term methylation analysis usually encompasses the full steps of derivation of the carbohydrate for methylation analysis, followed by hydrolysis, reduction, and acetylation, resulting in partially methylated alditol acetates (PMAAs) from the carbohydrate. Although the methylation analysis was first applied to the structural analysis of

carbohydrates over a century ago, it is still known as the most powerful method for structural analysis of carbohydrates as well as an essential step for determining the position of the glycosidic linkages in carbohydrates [24, 25]. The methylation analysis showed that fraction A was composed of 3 types of glucosyl residues in similar proportions; that is, a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol corresponding to non-reducing terminal glucopyranose, a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol corresponding to (1 → 3)-glucopyranose, and a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol corresponding to (1 → 4)-glucopyranose, in molar ratios of 35.7, 30.4, and 33.9%, respectively (Table 1). Interestingly, produced oligosaccharide has not only (1 → 4) glycosidic linkage, but also (1 → 3) glycosidic linkage. This result strongly suggests the possibility that transglycosylation reaction by *B. subtilis* SS-76 can lead to make oligosaccharide with new type of linkages. To date, different kind of methods for production of β-(1 → 3)/(1 → 4)-glucotriose was introduced in some literatures. Takeo and Suzuki [26] reported a method related to synthesis of glucotriose and glucotetraose connected with β-(1 → 3) and/or β-(1 → 4) by organic synthesis using silver trifluoromethanesulfonate-promoted Koenigs-Knorr type condensation. Whereas, Hughes et al. [27] reported in vitro fermentation of oat and barley β-glucans using human faecal bacteria for production of β-glucan oligosaccharide (average molecular mass of 137, 172 and 137 kDa from barley, 230 and 150 kDa from oat). Consequently, component sugar and methylation analyses identified the structure of oligosaccharide fraction A as a glucotriose connected with (1 → 3) and (1 → 4) glycosidic linkages.

Fig. 3 Spectra obtained by (A) MALDI-TOF/MS and (B) ESI-LC-MS/MS analyses of the oligosaccharide fraction A produced by *Bacillus subtilis* SS-76. The major pseudomolecular ion obtained by MALDI-TOF/MS at m/z 527 seems to correspond to 3 glucosyl residues with one sodium adduct; the ions obtained by ESI-LC-MS/

MS at m/z 527, 365, and 203 are separated by 162 mass units, suggesting the presence of a hexotriose. The fragment ions observed in the ESI-LC-MS/MS spectrum were named according to the nomenclature suggested by Domon and Costello (30)

Determination of the molecular mass and degree of polymerization using spectrometric analyses

In order to determine its precise molecular mass, oligosaccharide fraction A was analyzed using mass spectrometric technologies. As shown in Fig. 3(A), two Matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry (MALDI-TOF/MS) signals (m/z 527 and 543) were detected. The pseudomolecular ion at m/z 527 corresponds to three hexosyl residues with one sodium

adduct ($[3 \text{ hexose} + \text{Na}]^+$), whereas the ion at m/z 543 corresponds to those with one potassium adduct ($[3 \text{ hexose} + \text{K}]^+$). MALDI-TOF/MS analysis is suitable for detecting molecular masses ranging from 500 to 4000 Da. In general, the limitation of MALDI-TOF/MS is use of the matrix, which interferences with the detection of small compounds below 400 Da, including monosaccharides and disaccharides [28]. Therefore, the degree of polymerization (degradation products of m/z 527) was confirmed by electrospray ionization-based liquid chromatography-mass

Fig. 4 Specific enzyme catalysis to identify the anomeric configuration of oligosaccharide fraction A produced by *Bacillus subtilis* SS-76. Enzyme digests were spotted onto a TLC plate, and developed twice with a solvent containing butanol:pyridine:water (6:4:1). Saccharide spots were detected by spraying the plate with 20% sulfuric acid in methanol, followed by heating the plate at 120 °C for 10 min. Lane 1, mixture of certificated standard references including glucose (G1), cellobiose (G2), cellotriose (G3), cellotetrose (G4), and cellopento (G5); lane 2, certificated standard references of glucose (G1) and maltose (G2); lane 3, culture supernatant of *B. subtilis* SS-76 cultured with cellobiose-containing medium; lane 4, oligosaccharide fraction A purified from lane 3; lane 5, product obtained after glucoamylase digestion of lane 4, lane 6, product obtained after β -glucosidase digestion of lane 4

Fig. 5 Putative structure of novel oligosaccharide produced by *Bacillus subtilis* SS-76. The oligosaccharide indicated an β -(1,3)-glucosylcellobiose [β -D-glucopyranosyl-(1 $\rightarrow$ 3)- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose]

spectrometry/mass spectrometry (ESI-LC-MS/MS) analysis [Fig. 3(B)], where the peaks observed at m/z 527, 365, and 203 differed by 162 mass units, suggesting the presence of a hexotriose. In addition, recorded tandem MS spectra are known to contain two types of fragment ions, reflecting glycosidic cleavage and cross-ring cleavage, respectively, in which the carbon-to-carbon or carbon-to-oxygen linkage is broken within the monosaccharide ring [29–31]. As shown in Fig. 3(B), the ions at m/z 466 and 305 resulted from the loss of $C_2H_4O_2$ (–60 Da) from the parent ions at m/z 527 and 365, respectively, indicating cross-ring cleavage of the hexotriose unit. This indicated that both sugar units possessed (1 $\rightarrow$ 3) and/or (1 $\rightarrow$ 4) linkages, rather than (1 $\rightarrow$ 2) and (1 $\rightarrow$ 6) linkages. Given the monosaccharide association and linkage compositions, the oligosaccharide fraction A should be a glucotriose, being connected seriatim by (1 $\rightarrow$ 4) and (1 $\rightarrow$ 3) linkages from the reducing-end residue.

Identification of anomeric determination of the oligosaccharide using specific enzyme hydrolysis

Finally, anomeric configuration of the produced oligosaccharide was identified using specific enzyme catalysis. Of the two kind of glucosidases, glucoamylase (1,4- α -D-glucan glucohydrolase) catalyzes the hydrolysis of α -1,4 and α -1,6 glycosidic linkages to release β -D-glucose from the non-reducing ends, whereas β -glucosidase is known that it involved in the hydrolysis of β -1,3 and β -1,4 glycosidic linkages connecting carbohydrate residues in β -D-glycosides. In other word, glucoamylase acts on carbohydrates connected with α -glycosidic linkages, whereas β -glucosidase works on carbohydrates linked with β -linkages. Figure 4 showed that the purified fraction (A, lane 4) was hydrolyzed into monosaccharide-like molecule not by glucoamylase (lane 5) but with β -glucosidase (lane 6). The result suggests that oligosaccharide fraction A should be composed of only β glycosidic linkages. Meanwhile, it is well known that some β -glucosidases exhibit transglycosylation activity (via acceptor reactions) as well as cellulolytic activity, forming oligosaccharides with 3–6 degrees of polymerization [7, 11]. Ever since the reaction was first established by Barker et al. [32], a number of studies have reported oligosaccharide production by transglycosylation using cellobiose as the substrate. The resulting oligosaccharides are theoretically able to possess diverse linkages of β -(1 $\rightarrow$ 2), β -(1 $\rightarrow$ 3), β -(1 $\rightarrow$ 4), and β -(1 $\rightarrow$ 6) types. However, interestingly, experimental results have shown that oligosaccharides produced enzymatically via transglycosylation predominantly have the β -(1 $\rightarrow$ 6) linkage (6,11,32). Especially, Fujimoto et al. [6] proposed that this was due to the free energy of the β -(1 $\rightarrow$ 6) linkage in

glycosylation being much smaller than that of the other linkages. From this perspective, our result seems to suggest new possibility of transglycosylation reaction using novel bacterial strain. Based on above results, the transglycosylation mechanism of *B. subtilis* SS-76 using cellobiose may be speculated as follows: The cellobiose-degrading enzyme from the strain cleaves the (1 → 4) linkage of cellobiose to form two glucose units, and the glucosyl residue is subsequently specifically transferred to the C(O)3 of the non-reducing end of another cellobiose molecule, yielding one molecule of the trisaccharide. In order to elucidate this possibility clearly, the enzyme involving transglycosylation in the *B. subtilis* SS-76 should be purified and examined closely, and it might be elucidated in our near future works.

Our findings strongly speculated that cellobiose acts as a glycosyl acceptor, transferring a glucosyl residue to another cellobiose unit to form the trisaccharide. To the best of our knowledge, this is the first report on the production of a novel glucotriose possessing β-(1 → 3) and β-(1 → 4) linkages via bacterial fermentation in a cellobiose-containing medium. Consequently, a possible structure of the analyzed oligosaccharide can be suggested as shown in Fig. 5.

Meanwhile, further studies are required to improve the quality of the present work, as follows: First, NMR spectroscopic data must be presented to prove exact structure of the confirmed oligosaccharide, and the study might be done in our near future work. Second, in order for industrial application of the present work, studies on the specific enzyme associated with the oligosaccharide synthesis in *B. subtilis* SS-76, i.e., identification of transglycosylation-related enzyme and investigation of optimal conditions for the enzyme reaction, should be conducted. Finally, estimation of functional or biological activities of the new oligosaccharide is must be done to prove its utility value.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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