Transglycosylation of Glycosyl Residues to Cyclic Tetrasaccharide by Bacillus stearothermophilus Cyclomaltodextrin Glucanotransferase Using Cyclomaltodextrin as the Glycosyl Donor

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Cyclomaltodextrin glucanotransferase (EC 2.4.1.19, abbreviated as CGTase) derived from Bacillus stearothermophilus produced a series of transfer products from a mixture of cyclomaltohexaose and cyclic tetrasaccharide (cyclo(→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→), CTS). Of the transfer products, only two components, saccharides A and D, remained and accumulated after digestion with glucoamylase. The total combined yield of the saccharides reached 63.4% of total sugars, and enzymatic and instrumental analyses revealed the structures of both saccharides. Saccharide A was identified as 4-mono-α-D-glucosyl-CTS, (→6)-[α-D-GlcP-(1→4)]-α-D-GlcP-(1→3)-α-D-GlcP-(1→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→), and saccharide D was 4,4-di-O-α-glucosyl-CTS, (→6)-[α-D-GlcP-(1→4)]-α-D-GlcP-(1→3)-α-D-GlcP-(1→6)-[α-D-GlcP-(1→4)]-α-D-GlcP-(1→3)-α-D-GlcP-(1→). These structures led us to conclude that the glycosyltransfer catalyzed by CGTase was specific to the 4-OH of the 6-linked glucopyranosyl residues in CTS.

Key words: cyclic tetrasaccharide; cyclomaltodextrin glucanotransferase; cyclomaltodextrin; transglycosylation

Previously, Côté et al. reported that a cyclic tetrasaccharide, cyclo(→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→3)-α-D-GlcP-(1→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→) (abbreviated as CTS), was found to be a degradation product derived from alternan by its alternanase.1-3) Alternan is a polysaccharide consisting mainly of alternating 1,6-α-D-glucopyranosyl and 1,3-α-D-glucopyranosyl residues, which is synthesized from sucrose by the alternansucrase from Leuconostoc mesenteroides.4) Recently, we isolated a bacterial strain, Bacillus globisporus C11, from soil. The latter strain produced two enzymes, α-1,6-glucosyltransferase (6GT) and α-1,3-isomaltosyltransferase (IMT), that are involved in the synthesis of CTS from maltooligosaccharide.5) We succeeded in producing CTS in high yields from starch using both transferases.6) Cyclomaltodextrins (CD) are cyclic glucans composed of six or more glucose residues joined by α-1,4 linkages. For the purpose of expanding the properties of CD and discovering new functions, several glycosylated derivatives of CD were synthesized by enzymatic reactions.7,8) For example, maltosyl β-CD produced by isoamylase showed about centuple solubility when compared to the original β-CD.9) In the case of CTS, derivatives having glucosyl or isomaltoxy residues were isolated from the reaction mixtures catalyzed by alternanase or 6GT/IMT.10) In addition, a galactosyl derivative was reportedly synthesized with α-galactosidase.10) Cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) is one of the enzymes useful for transglycosylation of CD to produce various new saccharides. It is known that CGTase catalyzes the transferring to the 4-OH group of a free glucose or a glucose residue of saccharides.11) Because CTS has four 4-OH groups in its structure, it is expected that CGTase transfers glucosyl or glycosyl residues to these groups in CTS. In this paper, we describe glycosyltransferring to CTS catalyzed by CGTase using cyclomaltohexose as the glycosyl donor, and discuss the glycosylated OH groups of CTS.

Materials and Methods

Saccharides. CTS (99.9%-purity) and 4-O-α-glucosyl CTS were prepared in our laboratories.6) Cyclomaltohexaose (α-CD, 99%-purity) is a commercially available reagent produced at our laboratories.

Enzymes. CGTases from B. stearothermophilus and B. circulans were prepared in our laboratories.21) The enzyme from B. macerans and α-glucosidase from Aspergillus niger were obtained from Amano Pharmaceutical Co. (Nagoya, Japan). Glucoamylase

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Abbreviations: CTS, cyclic tetrasaccharide; CGTase, cyclomaltodextrin glucanotransferase (EC 2.4.1.19); α-CD, Cyclomaltohexose
Transglycosylation to CTS by CGTase

1095

from *Rhizopus niveus* and β-amylase from sweet potato were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and Sigma-Aldrich Co. (St. Louis, Mo), respectively.

**Assay of enzyme activity**

**CGTase activity.** The activity of CGTase was measured by the method of Shiosaka and Bunya. The reaction mixture containing 0.2 ml of enzyme solution and 4.0 ml of 0.2 M acetate buffer (pH 5.5) and 1 mM CaCl₂ was incubated at 40°C for 10 min. The reaction stopped by adding 0.5 ml of the reaction mixture to 15 ml of 0.02 M sulfuric acid, then 0.2 ml of 0.1 N iodine solution was added. The color of the solution was measured at 660 nm. One unit of CGTase activity was defined as the amount of the enzyme that catalyzed a 10% decrease of the absorbance per min under these conditions.

**Glucoamylase and β-amylase activity.** The activities of glucoamylase and β-amylase were measured according to methods reported previously. The reaction mixture containing 0.2 ml of enzyme solution and 5.0 ml of 1.0% soluble starch in 20 mM acetate buffer (pH 5.5) was incubated at 40°C for 10 min. The reaction was stopped by adding 1.0 ml of the reaction mixture to 2.0 ml of Somogy reagent. The amount of reducing sugar produced was measured by the Somogy-Nelson method. One unit of glucoamylase or β-amylase activity was defined as the amount of the enzyme that produces a level of reducing sugar equivalent to 1.0 μmol of glucose per min under these conditions.

**α-Glucosidase activity.** A reaction mixture containing 0.2 ml of enzyme solution and 4.0 ml of 0.2% maltose in 50 mM acetate buffer (pH 5.5) was incubated at 40°C for 20 min. The reaction was stopped by boiling for 10 min and the amount of glucose produced in the reaction mixture was measured by the glucose-oxidase method. One unit of α-glucosidase activity was defined as the amount of the enzyme producing 2.0 μmol of glucose per min under these conditions.

**High-pressure liquid chromatography (HPLC).** Samples were first treated by filtration using a filter kit, KC prep dura (0.45 μm, Katayama Chemical Co., Osaka, Japan) and by deionization using a micro acilyzer G0 (Asahi Chemical Co., Tokyo, Japan). HPLC was done using a Tosho HPLC system (Tokyo, Japan) composed of a CCPM pump, an AS8020 auto-sampler, and an RI-8020 refractive index flow analyzer. Two types of HPLC columns were used for sugar analysis. Mono- to oligo-saccharides were first separated on a YMC-Pack ODS-AQ-303 (4.6×250 mm, TMC Co., Kyoto, Japan) using water as the mobile phase at a flow rate of 0.5 ml per min. The components, including high-molecular weight saccharides, were then analyzed using an MCl-gel CK04SS column (10×200 mm, Mitsubishi Chemical Co., Tokyo, Japan). The yield of transfer products showed on sugar composition.

**Methylation analysis.** One hundred micrograms of saccharide A was methylated by the method of Hakomori. After extraction with chloroform, a methylated sample was hydrolyzed with 90% acetic acid containing 0.5 N H₂SO₄ at 80°C for 6 h. The methylated monosaccharides were reduced with sodium borohydride and then acetylated with acetic anhydride at 100°C for 4 h. The resulting partially methylated alditol acetates were analyzed by GLC (Shimadzu GC-14B) in an OV-1701 capillary column (25×0.25 mm i.d.) at 140–180°C (2°C/min).

**Instrumental analyses.** A liquid chromatograph mass spectrometer (LC-MS) used a model LCQ advantage iontrap mass analyzer (Thermo Quest, Tokyo, Japan). 13C-NMR spectroscopy measurement was done in D₂O using a model JNM-AL300 spectrometer (JEOL, Tokyo) at 75.45 MHz.

**Results**

**Transglycosylation to CTS by CGTase**

Transglycosylation to CTS by CGTase was examined using α-CD as the glycosyl donor. A reaction mixture (50 g) containing 20% CTS, 20% α-CD, and 100 units of CGTase from *B. stearothermophilus* in 50 mM acetate buffer (pH 5.5) was incubated at 50°C for 24 h. After the CGTase-catalyzed reaction, α-CD was completely consumed, but a small amount of CTS (2.8%) remained as unglycosylated CTS. Figure 1(a) shows an HPLC chromatogram of the resultant reaction mixture analyzed using an ODS-AQ-303 column. Many peaks other than that of CTS were detected as products. In addition to these products, high-molecular weight products were observed by further HPLC analysis using an MCl-gel CK04SS column (Data not shown).

**Digestion by β-amylase and glucoamylase**

To characterize the reaction products, we tested the resultant mixture for digestibility by β-amylase or glucoamylase. One gram of the mixture was added to 3 g of 50 mM acetate buffer (pH 5.5) containing 20 units of β-amylase, and was then incubated at 40°C for 24 h. Several of the original reaction products, including the high-molecular weight ones, were degraded by β-amylolysis, but nine of the products (namely saccharides A to I) remained, as shown in Fig. 1(b). The amounts of these β-limited saccharides, except A and D, increased after β-amylolysis. There was a
negligible difference between the amounts of CTS before and after the β-amylolysis. LC-MS analysis gave ions (M + Na)+ at MS m/z 833 for A, 995 for B and D, 1157 for C and E, 1319 for F and G, 1481 for H, and 1643 for I, corresponding to the ions derived from CTSs linked to one of the six glucosyl residues.

Digestion by glucoamylase was done as follows; the CGTase-reaction mixture (45 g) was added to 135 g of 50 mM acetate buffer (pH 4.5) containing 900 units of glucoamylase, and was then incubated at 40°C for 24 h. Figure 1(c) shows an HPLC chromatogram of the hydrolyzate. The HPLC profile was rather simple and showed only four components present, which were glucose, CTS, saccharide A, and saccharide D, of which the contents were 35.7, 7.3, 34.5, and 22.2%, respectively. The content of CTS was negligibly increased by the glucoamylase treatment similarly to that induced by β-amylolysis. On the other hand, the contents of both saccharides A and D were significantly increased by the treatment. It was considered that CGTase-reaction products other than saccharides A and D were degraded to glucose and either saccharide A or D. Consequently both saccharides accumulated to high levels after the glucoamylase treatment.

Isolation of saccharides A and D

The saccharides A and D were isolated from the reaction mixture after glucoamylase digestion using a preparative ODS column (4.6×250 mm, YMC-Pack ODS-AQ-303). When each saccharide was concentrated up to 75% and stored at room temperature, crystals of both saccharides were formed. Figure 3 shows a photograph of the colorless, pillar-shaped crystals of saccharide D. The purified saccharides A and D were obtained 5.0 g and 2.9 g, respectively.

Saccharide A

When the purified saccharide A (5 mg/ml) was incubated with 5 units/ml of α-glucosidase at 50°C for 24 h and pH 5.0, glucose and CTS were produced at a molar ratio of 1:1, indicating that saccharide A is a mono-glucosyl CTS. This agrees with the results of the mass analysis described above. 1H- and 13C-NMR analyses showed that the chemical shifts of saccharide A were identical to those of 4-α-D-glucosyl CTS, cyclo { [α-D-GlcP-(1→4)]-α-D-GlcP-(1→3)-[α-D-GlcP-(1→6)]-α-D-GlcP-(1→3)-α-D-GlcP-(1→)}. We, therefore, concluded that saccharide A was 4-α-D-glucosyl CTS (abbreviated as MGC), the structure of which is illustrated in Fig. 2(a).

Saccharide D

Saccharide D was hydrolyzed to glucose and CTS at a molar ratio of 2:1 by the α-glucosidase digestion. This means that saccharide D is a di-glucosyl CTS, which is also in agreement with the result of mass analysis mentioned above. In the initial stages of
the hydrolysis, α-glucosidase produced MGC from saccharide D, implying that MGC is a partial hydrolyzate of saccharide D, i.e. saccharide D contains MGC in its molecular structure. The methylation of saccharide D gave 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl glucitol, and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl glucitol at a molar ratio of 1:0.7:0.7. These results led us to the conclusion that a possible structure of saccharide D is cyclo{[→6]-[α-ᴅ-Glcp-(1→4)]-\(\alpha\)-ᴅ-Glcp-(1→3)-α-ᴅ-Glcp-(1→6)-[α-ᴅ-Glcp-(1→4)]-\(\alpha\)-ᴅ-Glcp-(1→3)-α-ᴅ-Glcp-(1→)} (Fig. 2(b)). The \(^{13}\)C-NMR spectrum of the saccharide produced only 18 signals, in agreement with the symmetric molecule deduced from the projected structure. The \(^{13}\)C-NMR data of 4,4′-di-α-glucosyl CTS (abbreviated as DGC), together with those of CTS and MGC, are listed in Table 1. The C-1 chemical shift (102 ppm) of the branched glucose residues proved the anomer of C-1 to be in the alpha configuration.

On the basis of the structures of MGC and DGC, we concluded that CGTase transferred glycosyl residues to one or both 4-OH groups of the 6-linked glucose residues of CTS specifically.

Effects of CGTase concentrations on the formation of MGC and DGC

The effects of a range of concentrations of CGTase (3 to 1000 units per one gram of α-CD) on transglycosylation were examined. The CGTase reactions were done using 20% CTS and 20% α-CD at pH 5.5 and 50°C, for 24 h. The reaction mixtures were treated with glucoamylase as described earlier. The resultant saccharide contents, together with the molar percentages of MGC, DGC, and unglycosylated CTS, are listed in Table 2. Among the tested concentrations of CGTase, a rather low concentration (3 units/g-CD) gave the highest yield (37.7%) of MGC. The molar percentages of MGC and DGC relative to total CTS concentration were 61.2% and 10.8%, respectively. The MGC yield decreased and remained at approximately 28% as the CGTase concentration increased. On the other hand, the DGC yield reached 29.1% at 30 units/g-CD of CGTase, but decreased significantly after addition of higher concentrations of CGTase. The concentration of 30 units/g-CD gave the maximum total yield (56.6%) of the glycosylated CTSs.

Transglycosylation to CTS by other CGTases

In addition to the CGTase from \(B.\) stearothermophilus, enzymes isolated from \(B.\) macerans and \(B.\) circulans were tested for catalysis of transglycosylation to CTS. As shown in Table 3, both of these CGTases produced MGC and DGC. The yields were
zyme. *B. circulans* 

**Table 3.** Transglycosylation to CTS by Other CGTases

<table>
<thead>
<tr>
<th>Origin</th>
<th>CGTase (units/g)</th>
<th>Sugar composition (%)</th>
<th>Sugar composition (% mole)</th>
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<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>CTS</td>
</tr>
<tr>
<td><strong>Bacillus macerans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>3</td>
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<td>30</td>
<td>35.9</td>
<td>8.3</td>
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<td>7.1</td>
<td>26.7</td>
</tr>
<tr>
<td>1000</td>
<td>26.2</td>
<td>9.2</td>
<td>25.6</td>
</tr>
<tr>
<td><strong>Bacillus circulans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23.1</td>
<td>34.2</td>
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<tr>
<td>1000</td>
<td>34.0</td>
<td>8.0</td>
<td>28.5</td>
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</table>

The reaction mixture (5 g), containing 1.0 g of α-CD and CTS, and from 3 to 1000 units of CGTase from *B. macerans* and *B. circulans* was incubated at 50°C and pH 5.5 for 24 h. The yields of glucoamylase-resistant molecules, glucose, CTS, MGC (glucosyl-CTS), DGC (diglucosyl-CTS), and others (unknown saccharides) were measured by HPLC.

**Table 4.** Effects of Donor/Acceptor Ratios on the Transglycosylation to CTS as Catalyzed CGTase

<table>
<thead>
<tr>
<th>CTS (%): α-CD (%)</th>
<th>Sugar composition (%)</th>
<th>Sugar composition (% mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>CTS</td>
</tr>
<tr>
<td>10</td>
<td>8.0</td>
<td>32.0</td>
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<td>13.3</td>
<td>26.7</td>
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<td>20.0</td>
<td>20.0</td>
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<td>13.3</td>
<td>67.2</td>
</tr>
<tr>
<td>32.0</td>
<td>8.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

The reaction mixture (5 g), containing 2.0 g of α-CD and CTS, and from 30 units/g-α-CD of CGTase from *B. steaetherophilus* was incubated at 50°C and pH 5.5 for 24 h. The yields of glucoamylase-resistant molecules, glucose, CTS, MGC (glucosyl-CTS), DGC (diglucosyl-CTS), and others (unknown saccharides) were measured by HPLC.

nearly equal to those produced by *B. steaetherophilus* CGTase when the enzyme concentrations used were 3-fold, namely, 100 units/g-CD for *B. macerans* and *B. circulans* and 30 units/g-CD for *B. steaetherophilus*, respectively (see Table 2). Lower concentrations of *B. macerans* CGTase, excepting 3 units/g-CD, gave high yields of MGC similarly to the *B. steaetherophilus* enzyme, but the combined MGC and DGC yields were a few percent lower than those produced by the *B. steaetherophilus* enzyme. *B. circulans* enzyme yielded both glycosylated CTSs at almost the same rates of 27–29%, except for concentrations of 3 to 10 units/g-CD which caused unreacted α-CD to remain.

**Effects of the ratios of CTS to α-CD on the formation of the glycosylated CTSs**

We investigated the formation of glycosylated CTSs using different substrate ratios of CTS (the reaction acceptor) to α-CD (the glycosyl donor). CGTase from *B. steaetherophilus* (30 units/g-CD) catalyzed the reaction. The total substrate concentrations were 40%, at pH 5.5, and 50°C for 24 h, and then the reaction mixture was treated with glucoamylase. The yields of MGC and DGC, and together with their molar percentages are listed in Table 4. The MGC yield reached up to 42.9% under acceptor-rich conditions, when the concentrations of CTS and α-CD were 32% and 8%, respectively. The molar percentage (43.6%) of MGC was about 4.5-fold higher than that of DGC (9.7%). On the other hand, conditions favoring the donor-rich substrate (8% CTS and 32% α-CD) gave the highest molar percentage (67.0%) of DGC, although the maximum yield of DGC (29.8%) was obtained with a ratio of 20% CTS to 20% α-CD. The total yield of the glycosylated CTSs reached a maximum of 63.4% (41.4% MGC plus 22.0% DGC) when the substrate ratios were 26.7% CTS and 13.3% α-CD.

**Discussion**

It is known that CGTase catalyzes the transferring of α-1,4-glucan chains to the hydroxyl groups of glucose-containing molecules, such as maltose and sucrose. We studied glycosyltransferring to CTS as catalyzed by CGTase using α-CD as the glycosyl donor. The resultant reaction mixture seemed to contain glycosylated CTSs of various chain-lengths (Fig. 1). Actually, after the mixture had been treated with glucoamylase, only two products, MGC and DGC remained, indicating that the glycosylated transfer products other than MGC and DGC were hydrolyzed by the enzyme. CTS has four 4-OH groups with the potential to be glycosylated by CGTase; two are the 3-linked glucosyl residues of...
CTS and the others are the 6-linked residues. Our structural analyses of the two products showed that MGC is a mono-branched CTS with one α-glucosyl residue linked one of the 4-OH of the 6-linked glucosyl residues of CTS (see Fig. 2(a)), and that DGC was a di-branched product with two α-glucosyl residues linked to both 4-OHs (Fig. 2(b)). On the basis of the molecular mass data and the specificity of α-amylase, the α-limited products were considered to be the mono- or di-branched derivatives with α-glucosyl, α-maltosyl or α-maltotriosyl branches linked to the 4-OH of the 6-linked glucosyl residues of CTS, similarly to MGC and DGC. These results mean that CGTase transferred glycosyl chains to the 4-OH groups of the 6-linked glucosyl residues of CTS but not to the 4-OH groups of the 3-linked residues. Previously, Kitahata et al. reported that the structure of an effective acceptor in the CGTase reaction was of the pyranose type with the same configuration as glucopyranose, namely with free 2-, 3-, and 4-OH groups. The 6-linked glucosyl residues of CTS have free 2-, 3-, and 4-OH groups facing away from the CTS molecule. The 4-OH groups of the residues are, therefore, considered to be the acceptor sites for the CGTase glycosylation reaction. In contrast, regarding the 3-linked glucosyl residues of CTS, the 3-OH groups are not free and the 4-OH groups are half-buried within the CTS molecule by the presence of the 6-C carbons and the 2-OH groups. These structural restraints may inhibit the transferring of glycosyl residues to the 4-OH groups of the 3-linked residues.

It was reported that CGTase from B. stea-rothermophilus shows a higher transferring-activity than similar enzymes from other sources. As shown in Tables 2 and 3, when compared with B. macerans and B. circulans enzymes, a lower concentration (30 units/g-CD) of B. stea-rothermophilus enzyme gave a maximum total yield (56.6%) of the glycosylated products (MGC plus DGC). All of these enzymes yielded MGC at high rates when the enzyme concentrations were low (3 or 10 units/g-CD), and DGC increased in quantity as the enzyme concentrations increased, implying that the transglycosylation of CTS occurs preferentially at either of the two 4-OH groups of the 6-linked residues and then both 4-OHs are glycosylated. It is notable that an excess, (300 units/g-CD) of B. stea-rothermophilus enzyme decreased the yield of DGC and increased the amount of free CTS. Because the enzyme has weak hydrolytic activity, the branched chain of the glycosylated products might be cleaved by the activity. Excess concentration also yielded small amounts of products other than MGC and DGC. One of the minor products was neotrehalose, α-glucopyranosyl β-glucopyranoside (data not shown), which had been reported as a transfer product of CGTase. The other products have not been sufficiently analyzed as to whether they are CTS derivatives or not in our current study.

Previously, we reported that MGC was isolated from a crude CTS preparation produced from starch using 6GT and IMT. However, DGC was not detected in the latter crude preparation. MGC is also synthesized to a yield of about 17% by the transglycosylation action of 6GT, but DGC is not (unpublished data). Compared with 6GT, CGTase is an effective enzyme for glycosyltransfering to CTS, and efficiently produces various branched products. Chemical branching catalyzed by the enzyme has been observed in the transglycosylation to trehalose and neotrehalose. CTS is a cyclic saccharide with a small cavity in the central part of the molecule, a structure similar to that of cyclodextrines. It has been speculated that CTS and its derivatives, including MGC and DGC, may physically interact with various substances via the cavity, such as binding. In addition to glucosyl residues, galactosyl or N-acetyl glucosaminyl residues are also attached to CTS by the enzymatic action of β-galactosidase or lysozyme. These enzymatic reactions and their implications will be reported on elsewhere.

Acknowledgments

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