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Enzymatic synthesis of dimaltosyl-β-cyclodextrin via a transglycosylation reaction using TreX, a *Sulfolobus solfataricus* P2 debranching enzyme

Hee-Kwon Kang [a], Hyunju Cha [a], Tae-Joo Yang [a], Jong-Tae Park [a], Seungjae Lee [a], Young-Wan Kim [b], Joong-Hyuck Auh [c], Yasuyo Okada [d], Jung-Wan Kim [e], Jaeho Cha [f], Chung Ho Kim [g], Kwan-Hwa Park [a,*]

[a] *Center for Agricultural Biomaterials, Department of Food Science and Biotechnology, College of Agriculture and Life Sciences, Seoul National University, San 56-1, Shillim-dong, Kwanak-gu, Seoul 151-921, Republic of Korea*
[b] *Department of Food and Biotechnology, Korea University, Jochiwon, Chungnam 339-700, Republic of Korea*
[c] *Department of Food Science & Technology, Chungang University, Anseong 456-756, Republic of Korea*
[d] *School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya 663-8179, Japan*
[e] *Department of Biology, University of Incheon, Incheon 402-749, Republic of Korea*
[f] *Department of Biological Science, Pusan National University, Busan 609-735, Republic of Korea*
[g] *Department of Food and Nutrition, Seowon University, Cheongju 361-742, Republic of Korea*

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Abstract

Di-*O*-α-maltosyl-β-cyclodextrin ($(G2)_2$-β-CD) was synthesized from 6-*O*-α-maltosyl-β-cyclodextrin (G2-β-CD) via a transglycosylation reaction catalyzed by TreX, a debranching enzyme from *Sulfolobus solfataricus* P2. TreX showed no activity toward glucosyl-β-CD, but a transfer product (**1**) was detected when the enzyme was incubated with maltosyl-β-CD, indicating specificity for a branched glucosyl chain bigger than DP2. Analysis of the structure of the transfer product (**1**) using MALDI-TOF/MS and isoamylase or glucoamylase treatment revealed it to be dimaltosyl-β-CD, suggesting that TreX transferred the maltosyl residue of a G2-β-CD to another molecule of G2-β-CD by forming an α-1,6-glucosidic linkage. When [^{14}C]-maltose and maltosyl-β-CD were reacted with the enzyme, the radiogram showed no labeled dimaltosyl-β-CD; no condensation product between the two substrates was detected, indicating that the synthesis of dimaltosyl-β-CD occurred exclusively via transglycosylation of an α-1,6-glucosidic linkage. Based on the HPLC elution profile, the transfer product (**1**) was identified to be isomers of $6^1,6^3$- and $6^1,6^4$-dimaltosyl-β-CD. Inhibition studies with β-CD on the transglycosylation activity revealed that β-CD was a mixed-type inhibitor, with a K_i value of 55.6 μmol/mL. Thus, dimaltosyl-β-CD can be more efficiently synthesized by a transglycosylation reaction with TreX in the absence of β-CD. Our findings suggest that the high yield of $(G2)_2$-β-CD from G2-β-CD was based on both the transglycosylation action mode and elimination of the inhibitory effect of β-CD.

Keywords: *Sulfolobus solfataricus* P2 glycogen-debranching-like enzyme (TreX); α-(1,6)-Transglycosylation activity; Dimaltosyl-β-cyclodextrin ($(G2)_2$-β-CD)

Derivatives of cyclodextrins prepared by chemical and enzymatic modification have been widely studied for improved bioavailability, solubility, and stability in aqueous solutions [1]. Branched cyclodextrins, that is, cyclodextrin (CD) bound with various malto-oligosaccharides by α-(1,6)-glucosidic linkages, are one class of these derivatives. Because of their high solubility compared to the parent CDs, synthesis of branched CDs has been pursued by many researchers [2–5]. In addition, branched CDs show low hemolytic activity. For the synthesis of branched CDs, the action of cyclodextrin glucanotransferase on amylopectin and the reverse condensation reaction with debranching

* Corresponding author. Fax: +82 2 873 5095.
E-mail address: parkkh@plaza.snu.ac.kr (K.-H. Park).

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enzymes such as pullulanase or isoamylase, have been used [6–9]. Abe et al. [7] reported the synthesis of doubly branched β-CD via the reverse action of *Pseudomonas* isoamylase on maltose and β-CD. Di-*O*-α-maltosyl-β-CD ($(G2)_2$-β-CD) was produced as a minor product (yield < 5%) in addition to maltosyl-β-CD (G2-β-CD). By condensation reactions with various branching enzymes, including pullulanase from *Aerobacter aerogenes*, isoamylase from *Pseudomonas amyloderamosa*, and pullulanase from *Bacillus acidopullulyticus*, a small amount of $(G2)_2$-β-CD (0.2 mmol) was formed from 40 mM maltose and 90 mM β-CD [9]; high concentrations of substrates were used to produce the branched cyclodextrins.

The transfer action of debranching enzymes has also been used to produce maltosyl-CDs using α-maltosyl fluoride [9]. In this process, the maltosyl-CD was formed in high yield (~40%), but the doubly branched product was not produced.

We previously cloned the debranching enzyme gene from the trehalose biosynthesis gene cluster of *Sulfolobus solfataricus* P2 and characterized the properties of the enzyme (TreX) [10]. The enzyme was present in dimeric or tetrameric form; higher oligomerization to a tetramer resulted in enhanced catalytic activity. An organic solvent (DMSO) induced oligomerization of TreX to a tetramer [11]. Although TreX shares high homology with several isoamylases and pullulanase having only α-1,6-hydrolyzing activity, it also showed disproportionation activity, as seen in GDEs from yeast and rabbit. The synthesis of doubly branched CDs has been investigated via the reverse action (condensation) of various debranching enzymes. However, the synthesis of doubly branched CDs by a transglycosylation reaction of the enzyme has not previously been reported.

In this study, we describe an efficient synthesis of $(G2)_2$-β-CD using the transglycosylation activity of TreX. We also discuss the transglycosylation activity and the inhibitory effect of β-CD on the transglycosylation activity of TreX.

Experimental procedures

Chemicals and enzymes. 6-*O*-α-Glucosyl-β-CD (G1-β-CD) and 6-*O*-α-maltosyl-β-CD were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and β-CD was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucoamylase from *Sulfolobus tokodaii* was purified through Ni-NTA affinity chromatography and heat treatment [12]. The purity was above 90%. Isoamylase was purchased from Hayashibara Biochemical Laboratories, Inc. (Fujisaki, Japan).

Purification of TreX. Escherichia coli MC1061 [F^-, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, Δ*lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] was used as a host for the expression of the GDE from *S. solfataricus* P2 (TreX). The *E. coli* transformants were cultured in Luria–Bertani (LB) medium [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl], containing ampicillin (100 μg/mL), at 37 °C. Six-His-tagged TreX was efficiently purified using a Ni-NTA column (1 × 4 cm, Ni-NTA superflow; Qiagen, Hilden, Germany) as described previously [13]. The purity of the enzyme was confirmed by SDS–PAGE analysis.

Transglycosylation reaction. G2-β-CD (2.5%, w/v) and G1-β-CD in 50 mM sodium acetate buffer (pH 5.5) were reacted, catalyzed by TreX (0.1 mg/mg substrate). The reactions were carried out at 70 °C. The products were analyzed by thin layer chromatography (TLC). Reaction mixtures were spotted on a silica gel plate (K5F TLC plate; Whatman, Maidstone, UK) and placed in a TLC chamber containing a solvent mixture of ethanol:butanol:water [5:5:3 (v/v/v)] and developed twice at room temperature. The plate was dried thoroughly and developed by dipping rapidly into a methanol solution containing 3 g of *N*-(1-naphthyl)-ethylene-diamine and 50 mL of concentrated H_2SO_4/L. The plate was dried and then placed in an oven at 110 °C for 10 min. For the labeled maltose reaction, [^{14}C]-maltose (10%, w/v) was reacted with G2-β-CD (1% w/v) in the presence of the enzyme at 70 °C. The procedures for the TLC analysis were the same as above except for the visualization. After it was developed, the TLC plate was placed on an imaging plate for 12 h and the radioactivity in each spot was measured using an image analyzer (Fuji Film BAS2500; Fuji Photo, Tokyo, Japan).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Mass spectra of $(G2)_2$-β-CD and diglucosyl-β-CD ($(G1)_2$-β-CD) were collected with a MALDI-TOF mass spectrometer (Voyager-DE; Perceptive Biosystems, Framingham, MA, USA); 2,5-dihydroxybenzoic acid (2,5-DHB) was the matrix for carbohydrates, and the final concentration of the matrix was 0.1–5 pmol/μL. For the calibration analysis, 1% (w/v) bovine serum albumin (Sigma Chemical Co.) solution was prepared. Purified $(G2)_2$-β-CD or $(G1)_2$-β-CD (1 μL; 0.1–0.001 mg/μL) and 1 mL of the prepared matrix were mixed and dropped on a sample plate and air-dried until crystallization occurred. The sample plate was then loaded into a Voyager biospectrometer. The molecular weight of the sample was analyzed under appropriate conditions (grid voltage: 87; grid wire voltage: 0.3; delayed extraction: 300 ns; laser: 2000).

Recycling-preparative high-performance liquid chromatography. For recycling-preparative HPLC analysis, we used a JAIGEL W-251 column (2 × 50 cm; JAI, Tokyo, Japan) and an RI detector RI-50 (JAI). Distilled water was used to elute the sample at a flow rate of 2.0 mL/min.

HPLC analysis. A 600E HPLC system (Waters, Milford, MA, USA) with Hypercarb (100 × 4.6 mm i.d.; Shandon Scientific, Astmoor, UK) was used. The analysis was performed at 60 °C using a gradient solvent system consisting of acetonitrile and water. The peaks were identified with

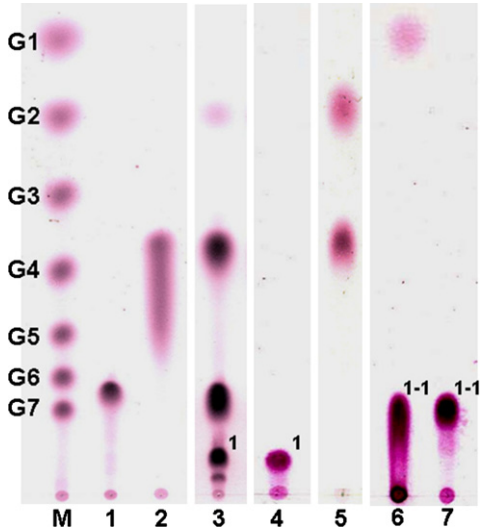

Fig. 1. Thin-layer chromatography of reaction products and compound **1** treated with isoamylase and glucoamylase. Lane Std represents maltooligosaccharides (G1–G7); lane 1, maltosyl-β-CD; lane 2, β-CD; lane 3, maltosyl-β-CD treated with TreX; lane 4, purified compound 1; lane 5, compound **1** treated with isoamylase; lane 6, compound **1** treated with glucoamylase; lane 7, purified compound **1-1**. Spot **1** represents the transfer product.

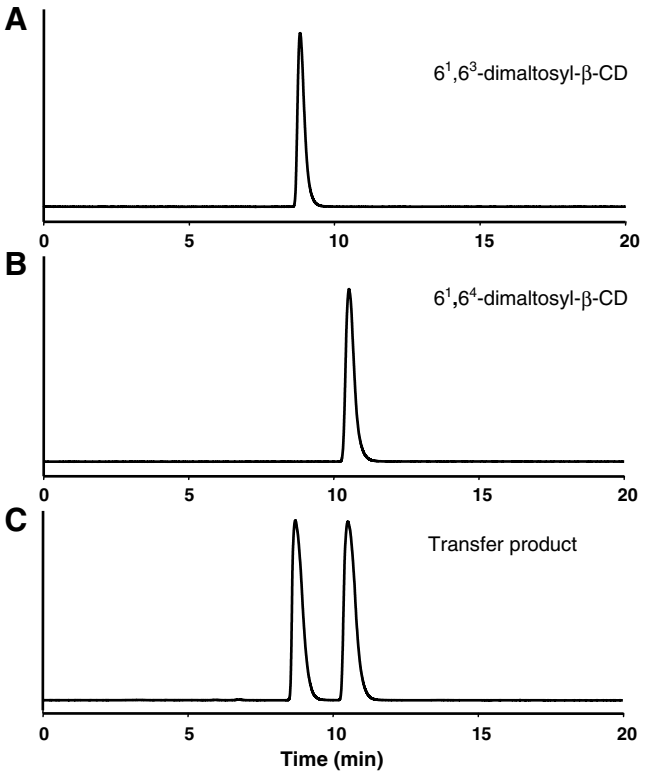

Fig. 2. HPLC analysis of $6^1,6^3$-$(G2)_2$-β-CD (A), $6^1,6^4$-$(G2)_2$-β-CD (B), and transfer product **1** (C).

a JEOL LC mate mass spectrometer (JEOL, Tokyo, Japan) by positive ion mode ESI-LC/MS $6^1,6^2$-, $6^1,6^3$-, and $6^1,6^4$-dimaltosyl-β-CD were prepared as described in previously [14].

Determination of kinetic parameters for the production of $(G2)_2$-β-CD by TreX. The reaction was carried out with substrate concentrations ranging from 0.75 to 2 mM for G2-β-CD in 50 mM sodium acetate buffer (pH 5.5) at 75 °C. Each aliquot was boiled for 10 min to stop the reaction and was stored at 4 °C until HPLC analysis. The changes in dimaltosyl-β-CD and β-CD content were measured by HPLC. The amounts of dimaltosyl-β-CD and β-CD were calculated from a standard curve. The effect of β-CD on dimaltosyl-β-CD production from β-CD was analyzed at substrate concentrations of 1–2 mM of G2-β-CD and 250–400 μmol/mL of β-CD.

Results and discussion

Production and identification of the branched cyclodextrins

To make the branched cyclodextrins, TreX was used to catalyze the reaction between 10% (w/v) maltose (G2) and 2% (w/v) β-CD in 50 mM sodium acetate buffer (pH 5.5) at 70 °C. The reactants were analyzed using thin-layer chromatography (TLC) over a time course (data not shown). G2-β-CD was first produced after 24 h and then increased. After a 72-h reaction, an unknown product appeared; the amount of this unknown compound increased with time.

The reaction product in which the unknown compound reached maximum yield was analyzed by TLC (Fig. 1, lane 3) and the unknown compound (**1**) was further analyzed after purification by recycling-preparative HPLC (Fig. 1, lane 4). The molecular weight of **1**, determined using MALDI-TOF/MS analysis, was 1805.5 $[M+Na]^+$, indicating that the product consisted of 11 glucose units (data not shown). The structure may be either maltoteraosyl-β-CD

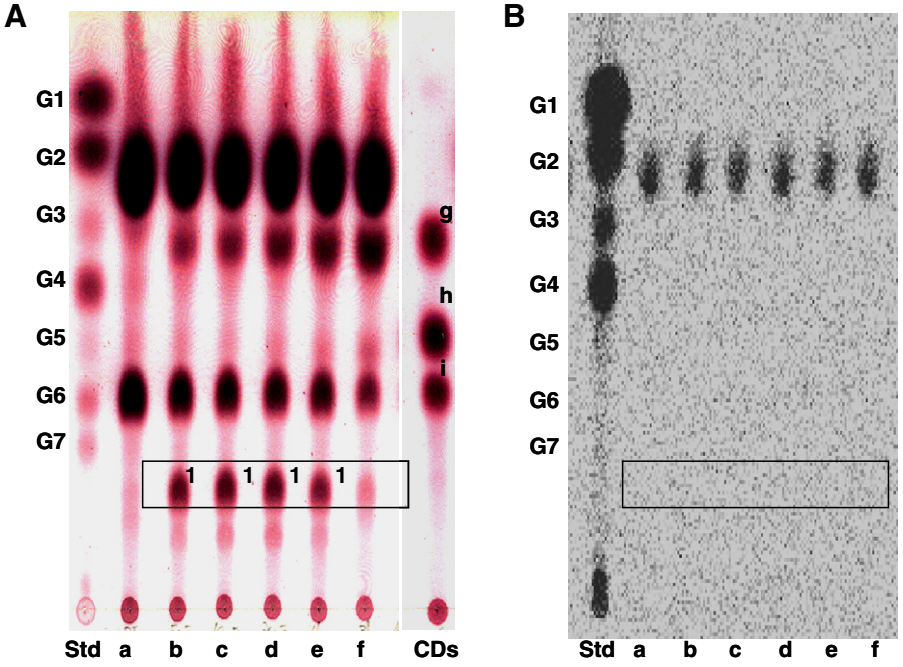

Fig. 3. Time course analysis of maltosyl-β-CD catalysis by TreX. Lane Std was spotted with labeled (^{14}C) standard malto-oligosaccharides (G1*–G7*), lane CDs, branched cyclodextrins. Lanes a–f represent 5, 10, 30, 60, and 180 min, respectively. Spot 1 represents the transfer product; spot g, β-CD; spot h, G1-β-CD; spot i, G2-β-CD. The chromatography was visualized using the naphtol-H_2SO_4 method (A) and autoradiography (B).

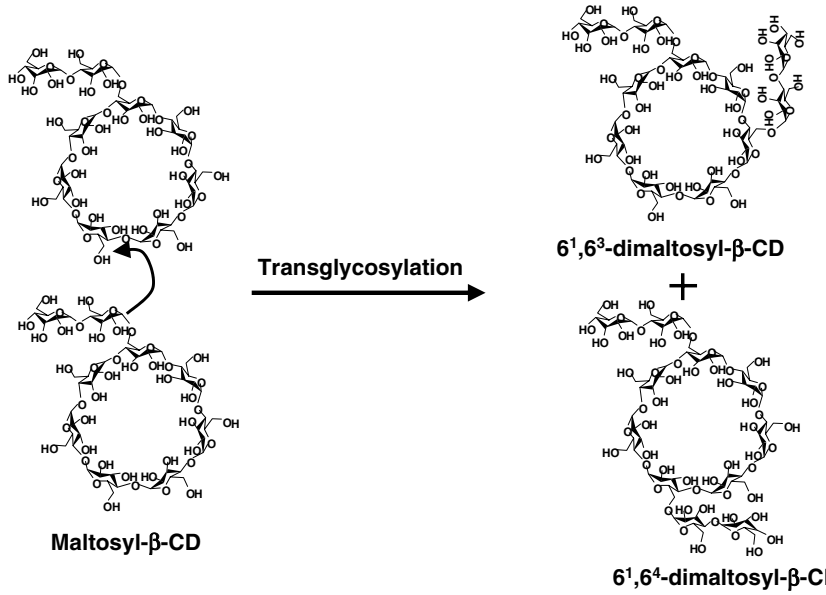

Fig. 4. Proposed mechanism of $(G2)_2$-β-CD production by TreX. $(G2)_2$-β-CD was produced from G2-β-CD via transglycosylation of TreX.

(G4-β-CD) or dimaltosyl-β-CD [$(G2)_2$-β-CD], via α-1,4- or α-1,6-transglycosylation of maltose to maltosyl-β-CD.

To examine the structure of purified transfer product, it was treated with isoamylase, which hydrolyzes only α-1,6-glycosidic linkages (Fig. 1, lane 5). If the transfer product was generated by forming an α-1,4-glycosidic linkage to the maltosyl moiety of G2-β-CD, maltotetraose (G4) and β-CD would be produced from the transfer product by isoamylase treatment. However, the products (Fig. 1, lane 5) were maltose and β-CD, not G4 and β-CD, indicating that the maltosyl moiety of the donor molecule was transferred to β-CD via formation of an α-1,6-glycosidic linkage.

To analyze the $(G2)_2$-β-CD structure further, the purified transfer product was hydrolyzed completely by glucoamylase for 10 h (Fig. 1, lane 6). If a maltosyl moiety was transferred to the β-CD moiety of G2-β-CD, glucose and $(G1)_2$-β-CD would be produced from $(G2)_2$-β-CD after the glucoamylase reaction. If a maltosyl moiety was transferred to the maltose moiety of maltosyl-β-CD, glucose and maltotriosyl-β-CD would appear by glucoamylase treatment. TLC analysis (Fig. 1, lane 6) showed that glucoamylase treatment resulted in the production of glucose and an unknown compound (compound **1-1**) from compound **1**. After removing glucose by recycling-preparative HPLC (Fig. 1, lane 7), compound **1-1** was subjected to MALDI-TOF/MS. The molecular weight of compound **1-1** was 1481.6 $[M+Na]^+$, indicating that it consisted of nine glucose units, corresponding to the molecular weight of $(G1)_2$-β-CD (data not shown). Thus, compound **1-1** consisted of β-CD with two glucose molecules linked by an α-1,6-glycosidic linkage. Consequently, compound **1** was $(G2)_2$-β-CD. Compound **1** could be a mixture of $(G2)_2$-β-CD isomers, as reported by Koizumi et al. [14]. To identify the position of the maltosyl moiety of G2-β-CD, we performed HPLC analysis. As shown in Fig. 2, in a comparison of the chromatographic behavior of known samples, compound **1** was a mixture of $6^1,6^3$- and $6^1,6^4$-dimaltosyl-β-CD at a ratio of 1:1.

Mechanism of dimaltosyl-β-CD production

To produce $(G2)_2$-β-CD from G2 and β-CD, we suspected that a transglycosylation or condensation reaction was involved in the reaction mechanism. To clarify the mechanism of $(G2)_2$-β-CD production, labeled G2* (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used as the substrate. When labeled G2* and β-CD were reacted with TreX, we could not determine whether the resulting $(G2)_2$-β-CD came from a transglycosylation or condensation process. Thus, labeled G2* and G2-β-CD substrates were reacted, catalyzed by TreX. If a condensation reaction was catalyzed by TreX, labeled G2* would be attached to G2-β-CD and the resulting $(G2^*)_2$-β-CD. From the TLC analysis (Fig. 3), compound **1** was detected in the dipping solution, but not in the radiogram.

Table 1
Summary of the reactions for the synthesis of $(G2)_2$-β-CD with TreX

	Condensation		Transglycosylation
Substrates	G2 and β-CD (17.6 μmol/mL)[a]		G2-β-CD (6.8 μmol/mL)
Reaction times	100 h		15 min
Products	G2-β-CD (3.6 μmol/mL)	$(G2)_2$-β-CD (0.3 μmol/mL)	$(G2)_2$-β-CD (1.4 μmol/mL)
Yield[b] (%)	20.5%	1.7%	20.6%

G2-β-CD, 6-*O*-α-maltosyl-β-CD; $(G2)_2$-β-CD, di-*O*-α-maltosyl-β-CD.

[a] Concentration of β-CD.

[b] Yield (%) = (μmol of product)/(μmol of initial substrate) × 100; yield of G2-β-CD in condensation reaction = 3.6/17.6 × 100; yield of $(G2)_2$-β-CD in condensation reaction = 0.3/17.6 × 100; yield of $(G2)_2$-β-CD in transglycosylation reaction = 1.4/6.8 × 100.

Table 2
Kinetic parameters for β-CD inhibition of the transglycosylation activity of TreX

Substrate	Type of inhibition	K_m (μM)	k_{cat} (s^{-1})	K_i (μM)	K_i' (μM)
Maltosyl-β-cyclodextrin	Mixed type	1591.5 ± 241.5	$11.3 \times 10^{-3} \pm 2.6 \times 10^{-3}$	55.6 ± 9.8	130.2 ± 24.1

This indicated that production of $(G2)_2$-β-CD involved only transglycosylation by TreX. Based on these results, we propose a transglycosylation mechanism of TreX for $(G2)_2$-β-CD production from G2-β-CD (Fig. 4). TreX released G2 from G2-β-CD via its α-1,6-hydrolyzing activity and transferred G2 to another molecule of G2-β-CD via its α-1,6-transferring activity.

In the previous report, for synthesis of G2- or G3-β-CD *Pseudomonas* isoamylase was used to catalyze the reaction between high concentrations of maltose or maltotriose (about 50%) and β-CD (about 10%) at 45 °C for 48 h [6]. Using pullulanase, heterobranched CDs were produced with high concentrations of galactosyl-maltose and β-CD [5]. These products came from the reverse reaction of the debranching enzymes, isoamylase and pullulanase, which have α-1,6-glycosyl hydrolase activity.

The yield of $(G2)_2$-β-CD

The reaction producing G2-β-CD, catalyzed by TreX, was monitored for 120 min using HPLC (data not shown). Then, 1.714 mM of G2-β-CD was incubated with 0.6 U of TreX in 50 mM sodium acetate buffer (pH 5.5) at 75 °C. In the hydrolyzate, β-CD from G2-β-CD increased continuously, while G2-β-CD decreased. Compound **1**, $(G2)_2$-β-CD, increased rapidly during the first 40 min of the reaction and was maintained for the next 20 min. After 60 min of reaction, compound **1** slowly decreased. The yield of compound **1** via transglycosylation or condensation (reverse reaction) catalyzed by TreX was calculated (Table 1). $(G2)_2$-β-CD produced through condensation was 1.7% and that produced through transglycosylation reaction was 20.6%.

When isoamylase and pullulanase were used to produce branched cyclodextrins via reverse reaction [5,6], high concentrations of substrates and long reaction times were needed. In addition, most reaction temperatures were below 60 °C and thus β-CD was added into the reaction solution as a powder or in liquefied form because of the low solubility of β-CD. Only very low productivity or even no detection has been reported regarding dibranched CDs [5,6].

Inhibition of β-CD on transglycosylation activity of TreX

The type of inhibition and inhibitor constant, K_i, of β-CD were determined for the transglycosylation activity of TreX. The Lineweaver–Burk and Dixon plots indicated that the inhibition of β-CD followed mixed-type competitive inhibition. The K_i value, 55.6 μmol (Table 2) is quite low, indicating that β-CD significantly inhibited the transglycosylation reaction of TreX. Iwamoto et al. [15,16] showed that pullulanase from *Klebsiella pneumoniae* was competitively inhibited strongly by β-CD; β-CD inhibition was analyzed by determining a decrease in the end hydrolysis endproduct. In contrast, we obtained kinetic parameters for the transglycosylation activity by measuring the decrease in substrate concentration.

We prepared the debranching enzyme (TreX) from *Sulfolobus*, which grows at above 75 °C, because the optimum temperature of TreX is 75 °C and it has high thermal stability. We first found that $(G2)_2$-β-CD can be efficiently formed from G2-β-CD via the α-1,6-transglycosylation activity of TreX. The yield by transglycosylation was about 12 times higher than that through condensation. Compared to condensation, lower substrate concentrations and shorter reaction times were required for the transglycosylation reaction. Additionally, the synthesis of $(G2)_2$-β-CD can be carried out without inhibition by β-CD, resulting in higher yields. Based on our results, the transglycosylation mechanism, compared with condensation, is more effective for the production of $(G2)_2$-β-CD.

Acknowledgments

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