Production of Turanose by Cyclomaltodextrin Glucanotransferase from *Bacillus stearothermophilus*

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Abstract: Cyclomaltodextrin glucanotransferase (EC 2.4.1.19, abbreviated as CGTase) from *Bacillus stearothermophilus* produced a series of transfer products from a mixture of cyclomaltohexaose and D-fructose. After digestion with glucoamylase, the resultant product contained turanose and trehalulose in addition to D-glucose and D-fructose. These results showed that CGTase transferred the glucose residue to the C3- and C1-hydroxy groups of fructopyranose. The optimum conditions on the turanose production from α -CD and D-fructose using CGTase were examined. The reaction mixture containing 30% of α -CD and 30% of D-fructose and 300 U/g of α -CD of CGTase from *B. stearothermophilus* was incubated at 60°C and pH 5.5 for 24 h and then treated with glucoamylase. Turanose was produced in a yield of about 45%.

Key words: turanose, trehalulose, cyclomaltodextrin glucanotransferase, cyclomaltodextrin, transglycosylation

Cyclomaltodextrin glucanotransferase (EC 2.4.1.19, CGTase) catalyzes not only the cyclization reaction forming cyclodextrins from starch but also the transglycosylation reaction in the presence of suitable acceptors.¹⁾ D-Glucose and the oligosaccharides containing D-glucose at the non-reducing side of the oligosaccharide such as maltose and sucrose are good acceptors. The transglycosylation reaction occurs at the C4-hydroxyl group of D-glucose or glucosyl residue and forms α -glucose- and α -maltooligosaccharide-linked transfer products. D-Xylose and L-sorbose, which have a D-glucose-like conformation in their pyranose rings, are effective acceptors. Although D-xylose gives the transfer products linked to the C4-hydroxyl group,²⁾ the transglycosylation to L-sorbose occurs at the C3-hydroxyl group.²⁾ When a large amount of CGTase was used, the transglycosylation reaction was observed in various saccharides and compounds. The transglycosylation to L-ascorbic acid occurred preferentially at the C2-hydroxyl group.³⁾ D-Galactose gave the transfer products for C1-, C3- and C2-(C4) hydroxyl groups in the ratio of 26 : 10 : 1, respectively.⁴⁾ The transfer products for lactose were glucosyl lactoside (α -D-glucosyl O- β -D-galactosyl-(1 $\rightarrow$ 4)- β -D-glucoside) as a main product and 3^{Gal}-O-, 4^{Gal}-O- and 2^{Gal}-O- α -D-glucosyl-lactose as minor products.^{5,6)} Thiamin⁷⁾ and catechin⁸⁾ also functioned as acceptors of the CGTase reaction. Transglycosylations to fructose by CGTases from *Bacillus stearothermophilus*, alkalophilic *Bacillus* sp. and *Brevibacterium* sp. has been reported already.^{9–11)} The structure of transfer product, however, was not determined hitherto.

In this study, we report on the structure of the transfer product for D-fructose and determine the linkage site of D-

fructose. Optimum conditions for transglycosylation products are also examined.

MATERIALS AND METHODS

Saccharides. Maltose, neotrehalose, maltotriose, maltotetraose, maltopentaose, and cyclomaltohexaose (α -CD), a commercially available reagent, were produced at our laboratory. D-Glucose, D-fructose, trehalulose, turanose, and soluble starch were purchased from Wako Junyaku Kogyo Co. (Tokyo, Japan). Dextrin (Pine dex #1) was purchased from Matsutani Chemical Industry Co., Ltd. (Itami, Japan).

Enzymes. CGTases from *B. stearothermophilus* and *B. circulans* were prepared in our laboratory.¹²⁾ The enzyme from *B. macerans* from *Aspergillus niger* was obtained from Amano Pharmaceutical Co. (Nagoya, Japan). Glucoamylase from *Rhizopus niveus* was purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

Assay of enzyme activity.

CGTase activity. The activity of CGTase was measured by the method of Shiosaka and Bunya.¹³⁾ The reaction mixture containing 0.2 mL of enzyme solution and 5 mL of 0.3% soluble starch in 20 mM acetate buffer (pH 5.5) and 1 mM CaCl₂ was incubated at 40°C for 10 min. The reaction was stopped by adding 0.5 mL of the reaction mixture to 15 mL of 0.02 N sulfuric acid, and 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 activity. The activity of glucoamylase was measured according to the method reported previously.¹⁴⁾ 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

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 Abbreviations: CGTase, cyclomaltodextrin glucanotransferase (EC 2.4.1.19); α -CD, Cyclomaltohexose.

min. The reaction was stopped by adding 1.0 mL of the reaction mixture to 2.0 mL of Somogyi reagent. The amount of reducing sugar produced was measured by the Somogyi-Nelson method.¹⁵⁾ One unit of glucoamylase activity was defined as the amount of the enzyme that produces a level of reducing sugar equivalent to 1.0 μ mol 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 μ m, Katayama Chemical Co., Osaka, Japan) and by deionization using a micro acilyzer G0 (Asahi Chemical Co., Tokyo, Japan). HPLC was done using a Tosoh HPLC system (Tosoh, Tokyo, Japan) composed of a CCPM pump, an AS8010 auto-sampler, and an RI-8012 refractive index flow analyzer. Sugars were separated on a YMC PA-03 (4.6 $\times$ 250 mm) using a 70 : 30 mixture of acetonitrile and water as the mobile phase, at a flow rate of 0.5 mL per min. The yield of transfer products showed on sugar composition.

Gas liquid chromatography (GLC). After trimethylsilylation, the sample was analyzed by GLC (GC-14B, Shimadzu Seisakusho, Kyoto, Japan) in an OV-1701 capillary column (25 $\times$ 0.25 mm i.d., GL science, Tokyo, Japan) at 140–180°C (2°C/min).

Instrumental analyses. A liquid chromatograph mass spectrometer (LC-MS) used a model LCQ advantage ion-trap mass analyzer (Thermo Quest, Tokyo, Japan). H-NMR and ¹³C-NMR was done in D₂O using a model JNM-AL300 spectrometer (JEOL, Tokyo, Japan) at 300 and 75.45 MHz, respectively.

RESULTS

Formation of saccharide A and saccharide B.

Transglycosylation to fructose by CGTase was examined using α -CD as the glycosyl donor. A reaction mixture (100 g) containing 25% of D-fructose, 25% of α -CD, and 5000 U of CGTase from *B. stearothermophilus* in 50 mM acetate buffer (pH 5.5) was incubated at 60°C for 24 h. Figure 1(a) shows an HPLC chromatogram of the resultant reaction mixture was analyzed using an YMC PA-03 column. A series of transfer products were formed in addition to D-fructose and D-glucose.

To hydrolyze the α -1,4-glucan chains of the transfer products, 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 U of glucoamylase, and then incubated at 40°C for 24 h. Figure 1(b) shows an HPLC chromatogram of the hydrolyzate. The HPLC profile was rather simple and showed only four components present, which were D-fructose, D-glucose, saccharide A and saccharide B, of which the contents were 22.2, 19.6, 44.0 and 9.8%, respectively. The contents of saccharide A and saccharide B were increased by the glucoamylase-treatment, meaning that the transfer products linked to the α -1,4-glucan chain coexisted with saccharide A and B.

Isolation of saccharide A.

Saccharide A was isolated from the reaction mixture after glucoamylase digestion using a preparative ODS col-

Fig. 1. HPLC profiles of the reaction mixture.

(a) α -CD and fructose-derived products after glycosyl transfer catalyzed by CGTase. Glc, glucose; Fru, fructose; A, saccharide A; B, saccharide B; C, saccharide C; D, saccharide D; E, saccharide E; F, saccharide F. (b) Hydrolyzate catalyzed by glucoamylase. Glc, glucose; Fru, fructose; A, saccharide A; B, saccharide B. (c) Purified saccharide A.

umn (4.6 $\times$ 250 mm, YMC-Pack ODS-AQ-303). When the solution of saccharide A was concentrated up to 75% and methanol was added up to 75% of the final concentration, crystals of saccharide A were formed. The purified saccharide A was obtained 2.8 g (Fig. 1(c)). Saccharide A was identified by HPLC and GLC (data not show) with authentic turanose. Spectrograms of H-NMR and ¹³C-NMR of saccharide A completely agreed with those of turanose (data not show). We, therefore, concluded that saccharide A is turanose (α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-fructose).

Saccharide B.

LC-MS analysis gave an ion (M+Na)⁺ at MS m/z 365 for saccharide B, meaning that saccharide B is a disaccharide consisting one molecule of D-glucose and one molecule of D-fructose. As compared with various forms of α -D-glucosyl-D-fructose, the retention time of saccharide B by HPLC agreed with that of trehalulose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-fructose). GLC analysis also showed the same retention time for saccharide B as that for trehalulose. Saccharide B was not purified, but it is clear that this saccharide is trehalulose.

Thus, CGTase catalyzes the transglycosylation reaction to D-fructose, and produces turanose and trehalulose in a ratio of 9 : 2. The transfer sites of D-fructose were the C3- and C1-hydroxyl groups.

Effects of CGTase concentrations on the production of turanose.

The effects of a range of concentrations of CGTase

(20–1000 U/g of α -CD) on transglycosylation were examined. The CGTase reactions were done using 25% of D-fructose and 25% α -CD at pH 5.5 and 60°C, for 24 h. The reaction mixture was treated with glucoamylase as described above. The resultant saccharide contents are listed in Table 1. The turanose content reached 44.3% with 300 U/g- α -CD of the enzyme concentration, and seemed to decrease slightly with excess amounts of the enzyme (400 and 1000 U/g- α -CD). On the other hand, the content of trehalulose and other transfer products were produced by the excess amounts of the enzyme, especially 1000 U/g- α -CD. LC-MS analysis showed that these products were a di-saccharide, probably neotrehalose, and tri-saccharide, but the structures of the trisaccharide were not determined in this study.

Comparison with other CGTases.

In addition to the CGTase from *B. stearothermophilus* (an intermediate type between α - and β -CD-forming enzyme), the enzymes from *B. macerans* (α -CD-forming enzyme) and *B. circulans* (β -CD-forming enzyme) were tested for catalysis of transglycosylation to fructose. As shown in Table 2, both CGTases also produced turanose and trehalulose, but the yields of turanose and trehalulose were low compared to that from *B. stearothermophilus* CGTase when the enzyme concentration used was 200 U/g- α -CD. The enzymes from *B. stearothermophilus* and *B. circulans* produced turanose and trehalulose in the ratio of about 4 : 1, but the enzyme from *B. macerans* gave a ratio of about 2 : 1, suggesting that the enzyme from *B.*

macerans tends to transfer glucosyl residues to the C1-hydroxy group of D-fructose in comparison with the *B. stearothermophilus* and *B. circulans* enzymes.

Glycosyl donor.

We tested various saccharides as donor substrates for transglycosylation to D-fructose. D-glucose, maltose, maltotriose, maltotetraose, maltopentaose, dextrin (DE 5%), soluble starch and α -CD were used as the donor substrates. The CGTase reactions (200 U/g- α -CD) were done using 25% of D-fructose and 25% of donor at pH 5.5 and 60°C, for 24 h. The reaction mixtures were treated with glucoamylase as described above. Figure 2 shows the yields of turanose from the donor substrates. Among the substrates tested in this study, α -CD was the best substrate yielding 43.7% of turanose. Next to α -CD, soluble starch and dextrin produced 34.2 and 31.5% of turanose, respectively. Linear maltooligosaccharides gave turanose according to the number of glucose residues of maltooligosaccharides, but the yields of turanose were less than 30%.

Effects of substrate concentrations on the production of turanose.

The effects of concentrations of substrate on transglycosylation were tested. The CGTase reactions (200 U/g- α -CD) were done using 8.5–32.5% of D-fructose and 8.5–32.5% of α -CD at pH 5.5 and 60°C, for 24 h. The reaction mixtures were treated with glucoamylase as described above. Figure 3 shows the relationship of the total sub-

Table 1. Effects of CGTase concentrations on the production of turanose.

CGTase (U/g)	Sugar composition (%)				
	Glucose	Fructose	Turanose	Trehalulose	Others
20	33.3	38.2	24.2	4.3	0.0
50	27.0	34.9	31.3	6.1	0.5
100	24.1	31.9	35.1	7.7	1.2
200	21.3	26.6	41.2	8.7	2.2
300	20.0	23.6	44.3	9.3	2.8
400	21.2	23.2	43.1	9.4	3.1
1000	19.7	20.6	41.5	11.9	6.3

The reaction mixture (5 g), containing 1.25 g of α -CD and 1.25 g of fructose, and from 20 to 1000 U of CGTase from *B. stearothermophilus*, was incubated at 60°C and pH 5.5 for 24 h. The yields of glucoamylase-resistant molecules, glucose, fructose, turanose, trehalulose and others (unknown saccharides) were measured by HPLC.

Fig. 2. Effects of substrate degree on the production of turanose.

G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose Dextrin, pine-dex #1; S.S, soluble starch; α -CD, cyclomaltotetraose.

Table 2. Production of turanose by other CGTases.

Origin	Sugar composition (%)				
	Glucose	Fructose	Turanose	Trehalulose	Others
<i>Bacillus stearothermophilus</i>	26.6	21.3	41.2	8.7	2.2
<i>Bacillus circulans</i>	41.2	38.1	17.1	3.3	0.3
<i>Bacillus macerans</i>	42.5	41.1	10.5	4.9	1.0

The reaction mixture (5 g), containing 1.25 g of α -CD and 1.25 g of fructose, and 200 U of CGTase from *B. stearothermophilus*, *B. macerans* and *B. circulans* was incubated at 60°C and pH 5.5 for 24 h. The yields of glucoamylase-resistant molecules, glucose, fructose, turanose, trehalulose and others (unknown saccharides) were measured by HPLC.

strate concentration and yield of turanose. The yields of turanose slightly increased as the substrate concentrations increased. The highest concentration (65%) gave 43.7% of turanose.

Effects of the ratios of fructose to α -CD.

We investigated the formation of turanose using different substrate ratios of D-fructose to α -CD (Table 3). Turanose yield reached 44.0% when the concentration of D-fructose (25%) was equal to that of α -CD (25%). The yield of trehalulose was a maximum (9.9%) in the α -CD-rich substrate; α -CD : D-fructose = 33.3 : 16.7. Other transfer products increased as the ratio of D-fructose decreased. A main component of other transfer products was probably neotrehalose.

DISCUSSION

It is known that CGTase catalyzes the transferring of α -1,4-glucan chains to the C4-hydroxyl groups of D-glucose. From analysis of the derivatives of D-glucose, Kitahata *et al.* reported that the configuration of the C2-, C3-, C4-hydroxyl groups of D-glucose was the key structure for the transglycosylation of CGTase.¹⁾ We studied

Fig. 3. Effects of substrate concentrations on the production of turanose.

Table 3. Effects of donor/acceptor ratios on the production of turanose.

α -CD (%)	Fructose (%)	Sugar composition (%)				
		Glucose	Fructose	Turanose	Trehalulose	Others
40	10.0	51.3	3.0	21.8	7.5	16.4
37.5	12.5	44.8	4.1	27.1	9.2	14.8
33.3	16.7	37.5	7.6	35.4	9.9	9.6
25.0	25.0	22.2	19.6	44.0	9.8	4.4
16.7	33.3	13.4	39.5	38.0	7.2	1.9
12.5	37.5	10.1	52.5	31.4	5.8	0.2
10.0	40.0	9.3	59.0	26.2	4.7	0.8

The reaction mixture (5 g), containing 3.0 g of α -CD and fructose, and from 200 U/g- α -CD of CGTase from *B. stearothermophilus* was incubated at 60°C and pH 5.5 for 24 h. The yields of glucoamylase-resistant molecules, glucose, fructose, turanose, trehalulose and others (unknown saccharides) were measured by HPLC.

glycosyltransfering to D-fructose by CGTase using α -CD as the glycosyl donor. The glycosylated sites of D-fructose were determined as the C3- and C1-hydroxyl groups of D-fructose. The main product was turanose, the glucose residue of which was linked with the C3-hydroxyl group of D-fructopyranose. When the configuration of D-fructopyranose is compared with D-glucopyranose (Fig. 4), the C5-, C4- and C3-hydroxyl groups of D-fructopyranose correspond to the C2-, C3- and C4-hydroxyl groups, respectively, of D-glucopyranose. It is reasonable that the C3-hydroxyl group of D-fructopyranose, which corresponds to the C4-hydroxyl group of D-glucopyranose, is the acceptor site of the transglycosylation of CGTase. Although all of these hydroxyl groups of D-fructopyranose are equatorial, the C5-hydroxyl group of D-fructopyranose is axial and the C3- and C4-hydroxyl groups are equatorial. The difference in conformation of the C5-hydroxyl group between D-glucopyranose and D-fructopyranose may affect the transglycosylation. D-Mannopyranose has the D-fructopyranose-like conformation in which C2-, C3- and C4-hydroxyl groups are axial, equatorial and equatorial, respectively.

Mori *et al.* observed that CGTase from *Brevibacterium* catalyzed the transglycosylation to the C4-hydroxyl group of D-mannopyranose.¹¹⁾ These results suggest that the third hydroxyl group may act as the linkage site for the transglycosylation of CGTase when the successive three hydroxyls of the pyranose ring (C5, C4 and C3 in the case of D-fructopyranose) form the axial-equatorial-equatorial conformation. It is known that α -glucosyltransferase from *Pro-*

Fig. 4. Conformation of glucopyranose and fructopyranose.

(a) glucopyranose, (b) fructopyranose.

taminobacter rubrum transfers a glucosyl residue to the C1- and C6-hydroxyl groups of D-fructose to produce trehalulose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-fructose) and palatinose (α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-fructose).¹⁶⁾ Our observation showed that CGTase produced trehalulose but no palatinose. D-Fructose has two primary hydroxy groups (C1 and C6) in structure of D-fructofuranose. However, D-fructopyranose has only one primary hydroxyl group (C1) in the structure. CGTase possesses the ability to transfer the D-glucose residue to the primary hydroxyl group of alcohol such as ethanol. Probably, CGTase utilizes D-fructopyranose, but no D-fructofuranose, as the acceptor for the transglycosylation, and transfers the glucosyl residue to the C1-hydroxyl group of D-fructopyranose in addition to the C3-hydroxyl group of the ring.

We determined the optimum condition for the turanose production from α -CD and D-fructose by CGTase. The reaction mixture consisting of 30% of α -CD, 30% of D-fructose, and 300 U/g- α -CD was incubated at 60°C and pH 5.5 for 24 h and then treated by glucoamylase. Turanose was produced in a yield of 45% under the conditions. However, α -CD is an expensive saccharide and unsuitable for industrial production; therefore we selected starch as the glycosyl donor for the reaction. After our trial using starch and D-fructose, we demonstrated the large-scale production of turanose from starch and D-fructose by CGTase. The sweetness of turanose is half that of sucrose. Turanose inhibits the activity of α -glucosidase.¹⁷⁾ Turanose may be anticipated as a new sweetener for foods, cosmetics and medicines.

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Bacillus stearothermophilus 由来の

シクロマルトデキストリングルカノ

トランスフェラーゼによるツラノースの製造

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Bacillus stearothermophilus 由来の CGTase (EC 2.4.1.19) を用いて、フラクトースへの糖転移および糖転移物の生成条件について検討した。*Bacillus stearothermophilus* 由来の CGTase を α -CD とフラクトースの混合水溶液に作用させたところ、一連の糖転移物が生成した。この CGTase 反応液をグルコアミラーゼで分解したところ、主たる糖転移物としてツラノースとトレハロースの生成が認められ、CGTase はフラクトピラノースの 3 位と 1 位水酸基にグルコシル転移することがわかった。そこで、主たる糖転移物のツラノースの最適な生成条件を検討した。その結果、30% α -CD と 30% フラクトースの混合水溶液に CGTase を 300 U/g- α -CD, 60°C, pH 5.5 で 24 時間作用させ、その後グルコアミラーゼ処理することによりツラノースは約 44% 生成した。