Acceptor Specificity of Cyclodextrin Glycosyltransferase from Bacillus stea
termophilus and Synthesis of α-D-Glucosyl O-β-D-galactosyl-(1→4)-β-D-glucoside

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Bacillus stea
termophilus CGTase had a wider acceptor specificity than Bacillus maceran
t CGTase did and produced large amounts of transfer products of various acceptors such as D-galactose, D-mannose, D-fructose, D- and L-arabinose, D- and L-fucose, D-ra
hmannose, D-glucosamine, and lactose, which were inefficient acceptors for B. maceran
t CGTase. The main component of the smallest transfer products of lactose was assumed to be α-D-glucosyl O-β-D-galactosyl-(1→4)-β-D-glucoside.

Cyclomaltodextrin glycosyltransferase (CGTase) converts starch to cyclodextrin (CD) by intramolecular transglycosylation. In the presence of suitable acceptors such as D-glucose, the enzyme also catalyzes intermolecular transglycosylation, where glycosyl residues are transferred to the acceptors. To date, at least 9 bacteria have been found to produce CGTases. The specificities in the intramolecular transglycosylation of the CGTases have been well-studied, and it is reported that the ratios of α, β, and γCDs produced from starch differed depending on the source of CGTases. With respect to the intermolecular transglycosylation, the acceptor specificities of CGTases from Bacillus megaterium and B. macerans have been investigated using various kinds of monosaccharides and sugar alcohols. It was concluded that the requirement for an acceptor of these CGTases was the pyranose structure with the same configurations of the free C2-, C3-, and C4-hydroxyl groups as D-glucose. Therefore D-galactose, D-fructose, D-mannose, D-glucosamine, and D-ribose were inefficient acceptors for the CGTases. However, the acceptor specificity of the enzymes from other origins have been poorly understood.

B. stea
termophilus CGTase has been reported to transfer glycosyl residues to the hydroxyl groups of L-ascorbic acid and trimethylpropane, and B. obhensis CGTase also to the hydroxyl group of inositol. These reports indicated that the acceptor specificity in intermolecular transglycosylation depended on the origin, and B. stea
termophilus CGTase had wider acceptor specificity than B. megaterium and B. macerans CGTases did, and may be useful in the synthesis of various hetero

glicosaccharides.

This paper deals with the acceptor specificity of CGTase from B. stea
termophilus, comparing with those of B. macerans and B. circu
tans CGTases. This paper also describes synthesis of the transfer products of lactose by B. stea
termophilus CGTase.

Materials and Methods

Materials. Crude CGTase preparations from B. circulans and B. stea
termophilus were supplied by Hayashibara Biochemical Laboratos, Inc. Crude CGTase preparation from B. macerans was supplied by Amano Pharmaceutical Co., Ltd. The purified preparations of the CGTases were obtained as described previously. β-Galactosidase from Escherichia coli was purchased from Sigma Chemical Company. A crystalline glucoamylase from Rhizopus niveus was prepared by the method described before. α, β, and γCDs were supplied from Ensuiho Sugar Refining Co., Ltd. D-Glucose, D-galactose, D-ribose, D-mannose, D-glucosamine, D- and L-arabinose, D-fructose, D-rhamnose, D- and L-fucose, lactose, trehalose, and isomaltose were purchased from Wako Pure Chemical Industries. Neotrehalose was synthesized by the procedure of Helferich and Weis. The peracetylated disaccharide fraction was separated from the reaction product by column chromatography on a Lobar prepacked column, LiChroprep Si 60, size C (E Merck, Darmstadt, F.R.G) with benzene-acetone (7:1, v/v) and was deacetylated. Two isomeric trehaloses, neotrehalose and isotrehalose, were separated by HPLC on an Asahipak NH2P-50 (250 × 10 mm i.d.) (Asahi Kasei, Tokyo, Japan) with acetonitrile-water (75:25, v/v) and characterized by 1H-NMR. Kojibiose and nigerose were gifts.

Enzyme assay. The CGTase activity was assayed using soluble starch as a substrate by measuring the decrease in iodine-staining power, as described previously. Glucoamylase activity was assayed using soluble starch as a substrate. β-Galactosidase activity was measured with lactose as a substrate. One unit of the enzyme activity was defined as the amount liberating 1 μmol of glucose per minute.

Analytical methods. The reducing power was measured by Somogyi and Nelson’s method. Fast atom bombardment mass spectrometry (FAB-MS) was done with a JEOL JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy equivalent to 6 keV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkyl-phosphazene (Ultra Mark), and glyceral was used as the matrix.

Thin layer chromatography (TLC). TLC was done on plates of Kiesel
gel 60 TLC plate (Merck Co., Ltd.) with a solvent system of ethyl acetate-acetic acid–water (3:1:1, v/v). The sugars on the TLC plate were made visible by heating at 110–120°C after they were sprayed with 20% sulfuric acid–methanol solution.

High performance liquid chromatography (HPLC). HPLC analyses were done with a Hitachi L-6000 pump and a Hitachi 655A-30 RI monitor, and a Jasco 880-PU pump and a Showa Denko SE-61 RI monitor. The columns used were an Asahipak NH2P-50 (250 × 6.4 mm i.d.) and Hyper

carb (100 × 4.7 mm i.d.) (Shandon Scientific, Cheshire, England). HPLC
analyses at constant temperature were done with a Uniflow column oven CD-1093C. A Hitachi 833A data processor and a Shimadzu Chromatopac C-R3A digital integrator were used to calculate peak areas. Preparative HPLC was done on an Asahipak NH2P-50 column (250 x 10 mm i.d.) with acetonitrile-water (65:35, v/v) at ambient temperature and a flow rate of 2 ml/min.

Results

Acceptor specificities of CGTases

The acceptor specificities of CGTases from B. circulans and B. steaerothermophilus were investigated using various kinds of monosaccharides, which were reported to be inefficient acceptors for B. megaterium and B. macerans CGTases. d-Glucose, an efficient acceptor, was used as a reference saccharide. The rate of transglycosylation was indirectly measured by the decrease of the iodine-staining of starch, on the basis of the fact that the rate of starch degradation followed by the iodine-staining power increased with the rate of transglycosylation in the presence of an acceptor. The formation of transfer products of acceptors was also confirmed by TLC and HPLC analyses.

The CGTases from B. circulans, B. steaerothermophilus and B. macerans (0.8 units) were incubated with soluble starch and acceptor (each 15 mg) in 0.6 ml of 50 mM pH 5.6 acetate buffer at 40°C. After 20 min, fifty μl of the reaction mixture was removed and added to 1 ml of 10 mM I₂-KI solution containing 0.1 N HCl solution. The results are shown in Table I. The initial rates of starch degradation in the presence of acceptors increased as follows except when d-ribose was used as an acceptor; B. steaerothermophilus > B. circulans > B. macerans. In the case of B. macerans CGTase, the acceleration of starch degradation more than 10% was observed only in the presence of d-glucose, d-arabinose, and l-fucose. d-Glucose was the most effective acceptor for B. steaerothermophilus CGTase, followed by d-arabinose, l-arabinose, l-fucose, d-galactose, d-mannose, d-fructose, l-rhamnose, d-fucose, d-glucosamine, and d-ribose.

To confirm the transfer products, soluble starch (10 mg) and acceptors (10 mg) in 100 μl of 50 mM pH 5.6 acetate buffer were incubated with a higher activity of CGTases (10 μl, 5 units) at 40°C. After 24 hr, half μl and 2 μl of the samples were taken for TLC and HPLC analyses, respectively. Figure 1 shows typical thin-layer chromatograms. The three CGTases formed transfer products of d-galactose, d-ribose, d-fructose, d-mannose, d-arabinose, and l-fucose. On the other hand, B. steaerothermophilus and B. circulans CGTases gave the transfer products of l-arabinose, d-fucose, l-rhamnose, and d-glucosamine, but B. macerans CGTase did not. In HPLC analysis, α, β, and γCD which were synthesized by intramolecular transglycosylation were also detected, other than intermolecular transfer products of acceptors. The amounts of transfer products of acceptors increased in the following order of enzyme origins; B. steaerothermophilus > B. circulans > B. macerans.

Transfer reaction in the presence of lactose

The possibility of transglycosylation to lactose (4-O-β-d-galactosyl-d-glucose) and its transfer position by CGTases were investigated. Three CGTases (0.5 units/mg starch) were incubated with a mixture of soluble starch (10%) and lactose (10%) at 50°C (for B. steaerothermophilus) and at 40°C (for B. circulans and B. macerans). After 24 hr and 48 hr, two μl of the reaction mixtures were removed to analyze the products by HPLC on an Asahipak NH2P-50 column (Fig. 2). B. steaerothermophilus CGTase produced much more transfer products (Lac-1, Lac-2, Lac-3) than those from B. circulans and B. macerans CGTases.

Isolation of the transfer products of lactose

The reaction mixture (3 ml) containing 300 mg of soluble starch, 300 mg of lactose, and B. steaerothermophilus CGTase (150 units) was incubated at 50°C for 24 hr, and then heated.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>B. steaerothermophilus</th>
<th>B. circulans</th>
<th>B. macerans</th>
</tr>
</thead>
<tbody>
<tr>
<td>No acceptor</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>680</td>
<td>590</td>
<td>363</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>126</td>
<td>114</td>
<td>105</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>111</td>
<td>123</td>
<td>105</td>
</tr>
<tr>
<td>d-Manose</td>
<td>126</td>
<td>114</td>
<td>105</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>125</td>
<td>110</td>
<td>105</td>
</tr>
<tr>
<td>d-Glucosamine</td>
<td>113</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>d-Arabinose</td>
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<tr>
<td>l-Arabinose</td>
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<td>106</td>
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<tr>
<td>d-Fucose</td>
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</tr>
<tr>
<td>l-Fucose</td>
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<td>110</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>125</td>
<td>110</td>
<td>109</td>
</tr>
</tbody>
</table>

a Relative rates of starch degradation in the presence of acceptor to that in its absence.
at 100°C for 10 min to stop the enzyme reaction. After addition of 10 mL of ethanol to the reaction mixture, the resulting precipitate was removed by centrifugation. The supernatant solution was evaporated to 1.5 mL. The saccharide corresponding to the peaks designated as Lac-1, Lac-2, and Lac-3 in Fig. 2 were isolated by preparative HPLC and lyophilized (yields: Lac-1, 100 mg; Lac-2, 72 mg; Lac-3, 41 mg).

**Structure elucidation of the transfer products**

The isolated saccharide Lac-1 was analyzed by HPLC. The saccharide, Lac-1, showed two shoulders by HPLC analysis on an Asahipak NH2P-50 column (Fig. 3). And by HPLC analysis on a graphitized carbon column, Hypercarb, the Lac-1 was separated into several peaks. The peaks 1 and 1', peaks 2 and 2' (shoulder), and peaks 3 and 3' were those of α- and β-anomers of the same saccharides, respectively (Fig. A). These double peaks became single peaks upon the addition of 1 mM alkali to the eluent (Fig. 4B). The saccharide 4 corresponding to main peak 4, however, had no anomers. The saccharide 4 was isolated by a graphitized carbon column, and the reducing power and molecular weight were measured by the Somogyi–Nelson method and FAB-MS analysis, respectively. It had no reducing power, and its FAB-mass spectrum in the negative mode showed the [M – H]⁻ peak at m/z 503. These results indicate that the saccharide 4 is a non-reducing trisaccharide. Based on the above results, the saccharide 4 was elucidated to be either α-D-glucopyranosyl 3-β-D-galactopyranosyl-(1→4)-α-D-glucopyranoside or α-D-glucopyranosyl 3-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside. The proportion of the saccharide 4 in Lac-1 was calculated as 63–64% by HPLC analysis with the eluent containing 1 mM alkali (Fig. 4B). The Lac-1 (3 mg) was hydrolyzed by E. coli β-galactosidase (2 units) at 40°C. After 30 min and 120 min, the reaction product was analyzed by HPLC (Fig. 5). About 70% of Lac-1 was hydrolyzed to galactose and a disaccharide. About 30% of Lac-1 remained unhydrolyzed even after 120 min of incubation. The elution profile of the main disaccharide on a Hypercarb column suggested that it was neotrehalose, and not trehalose (Fig. 6A). Consequently, the saccharide 4 was assumed as α-D-glucopyranosyl 3-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside. A minor reducing disaccharide in the hydrolyzates of Lac-1 was identified as kojibiose (Fig. 6B).

The isolated saccharides, Lac-2 and Lac-3, were completely hydrolyzed to glucose and Lac-1 by glucoamylase. Based on the result together with the action pattern of CGTases, Lac-2 and Lac-3 were considered to be...
Discussion

The acceptor specificity of *B. stearothermophilus* CGTase was investigated, comparing it with those of *B. circulans* and *B. macerans* CGTases. CGTase catalyzes intermolecular transglycosylation in the presence of suitable acceptors and produces maltooligosylated acceptors. But in the absence of an acceptor or in the presence of a poor acceptor CGTase catalyzes mainly intramolecular transglycosylation and produces cyclodextrins. Saccharides that do not have the pyranose structure with the same configurations as D-glucopyranose in C2-, C3-, and C4-hydroxyl groups are reported to be poor acceptors for *B. macerans* CGTase. Although the rate of starch degradation at the initial stage of reaction by *B. macerans* CGTase did not increase more than 20% in the presence of D-galactose, D-ribose, D-mannose, D-arabinose, D-fructose, and L-fucose which were poor acceptors, transfer products of those acceptors were observed on TLC and HPLC analysis in the final stage of reaction. Main products, however, were β and γCD and the amounts of transfer products of those acceptors were smaller than those of *B. stearothermophilus* CGTase (data not shown). When CGTase was incubated with starch in the presence of poor acceptors such as D-arabinose and D-galactose, the CGTase produced mainly CDs in an early stage of reaction. But CGTase is a transferase which scarcely catalyzes hydrolysis reactions. Therefore when high activity of CGTase was incubated with starch in the presence of poor acceptors for a long time, the CDs produced in the initial stage of reaction may act further as donor substrates in the intermolecular transglycosylation to the acceptor, and transfer products were gradually accumulated. In the presence of ineffective acceptors such as L-rhamnose, *B. macerans* CGTase produced mostly CDs. A small amount of glucose and maltooligosaccharides were also produced by hydrolytic action of starch or CDs but no intermolecular transfer products of the acceptor.

*B. stearothermophilus* CGTase transferred the glycosyl moieties of starch to lactose as an acceptor and produced several kinds of transfer products. The main component of the smallest transfer product (Lac-1), saccharide 4, was assumed to be α-D-glucosyl O-β-D-galactosyl-(1→4)-β-D-glucoside. And also one of the minor components was assumed to be 2-O-α-D-glucosyl-lactose. These results
are compatible with the reports of Kobayashi et al.\textsuperscript{17,18}\textsuperscript{18}. When \textit{B. macerans} CGTase was incubated with a high concentration of maltose or a mixture of maltose and 2\textalpha,CD, neotrehalose (\textalpha-\textbeta-glucosyl \textbeta-D-glucoside) and kojibiose were produced. Considering the results of the HPLC analysis of hydrolyzates of Lac-1 by \textbeta-galactosidase, the minor components, non-hydrolyzed by \textbeta-galactosidase, seemed to be a mixture of at least three kinds of trisaccharides, which were synthesized by transferring the glucosyl residues in starch to the galactosyl moiety in lactose. The elution profile of the hydrolyzates on a graphitized carbon column also supported this result (data not shown). Our previous studies\textsuperscript{19} showed that \textit{B. megaterium} CGTase produced four kinds of transfer products of \textbeta-galactoside, though the yield of transfer products was very small compared with those of \textbeta-glucose, and the transglycosylation occurred at C1-, C3-, and (C2- and C4-) hydroxyl groups in the proportion of 26:10:1. Therefore the other minor components in Lac-1 were considered to be a mixture of 3\textgamma\textbeta-O-, 4\textgamma\textbeta-O-, and 2\textgamma\textbeta-O-\textalpha-D-glucosyl-lactose. The structures of these minor saccharides will be further studied.

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