Galactosylation at Side Chains of Branched Cyclodextrins by Various β-Galactosidases

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The galactosyl transfer reaction to branched cyclodextrins (CDs) was investigated using lactose as a donor substrate and branched CDs as acceptors by various β-galactosidases. *Bacillus circulans* β-galactosidase synthesized galactosyl transfer products to branched CDs, of which the galactose residues were linked at side chains of branched CDs, not directly at CD rings. *Aspergillus oryzae* and *Penicillium multicolor* β-galactosidases also produced derivatives galactosylated at side chains of branched CDs. The structures of main transgalactosylation products of branched CDs by these β-galactosidases seem to be different from those by *B. circulans* β-galactosidase, judging from the retention times on high performance liquid chromatography.

Cyclodextrins (CDs) are the cyclic, non-reducing oligosaccharides produced by the action of cyclomaltooligosaccharides produced by the action of cyclomaltooligosaccharide glucanotransferase (EC 2.4.1.19, CGTase) on α-glucans. Among those CDs, αCD, βCD, and γCD which are respectively composed of 6, 7, and 8 D-glucopyranosyl residues linked by α-1,4-linkages, are well known and used for various purposes. Recently, to improve the solubility of these CDs, branched CDs have been synthesized. The branched CDs that have been prepared have one or more side chains consisting of an α-D-glucose or an α-maltooligosaccharide linked at C-6 of CDs. Among them, glucosyl-CDs (G1-CDs) were produced by the action of CGTase on amylopectin, following digestion by glucoamylase. Maltosyl-CDs (G2-CDs) were formed by condensation reaction of maltose and CDs with debranching enzymes (pullulanase or isomaltase), and by the transfer action of debranching enzymes from *M. aruginosa* to CDs. All these CDs are all homogeneous oligosaccharides composed of only glucose. These products have the ability to form inclusion compounds with various kinds of inorganic and organic compounds, and consequently have become widely used for stabilizing labile materials, emulsifying oils, masking odors, increasing solubility, and changing viscous or oily compounds into powders.

In this work, to develop new applications different from conventional ones, we tried to synthesize heterogeneous branched CDs. Recently, we found that β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) transferred a galactosyl residue to the side chains of branched CDs. This study deals with the production of novel heterogeneous branched CDs by transgalactosylation with β-galactosidases.

Materials and Methods

**Materials**

G1-αCD, G1-βCD, G2-αCD, and G2-βCD were supplied from the Ensuiko Sugar Refining Co., Ltd. β-Galactosidase preparations from *Aspergillus oryzae*, *Bacillus circulans*, *Penicillium multicolor*, *Rhizopus oryzae*, and *K. lactis* were supplied from Amano Pharmaceutical Co., Ltd., Daiwa Kasei K.K., K-I Chemical Industry Co., Ltd., Novo Nordisk A/S, and Godo Shuise Co., Ltd., respectively. β-Galactosidase from *Escherichia coli* was purchased from Sigma Chemical Co. A crystalline glucoamylase (25 units/mg) from *Rhizopus niveus* was prepared in our institute.

**Assay of β-galactosidase activity.** Fifty µl of an enzyme solution was incubated with 400 µl of 5 mm p-nitrophenyl β-galactoside in 25 mm buffer (optimum pH of each enzyme) at 40°C for 10 min. The reaction was stopped by adding 0.5 ml of 0.2 m Na2CO3, and spectrophotometrically measured the release of p-nitrophenol from the substrate. One unit of the enzyme activity is defined as the amount releasing 1 µmol of p-nitrophenol per min.

**High-performance liquid chromatography.** The analysis of the reaction products by high-performance liquid chromatography (HPLC) was done under the following conditions: column, TSK-Gel Amide-80 (4.6 × 250 mm); solvent system, 60% acetonitrile; flow rate, 1 ml/min; column temperature, 60°C; detector, Shodex SE-11 refractometer. Conditions for preparative HPLC were as follows: column, YMC-Pack SH-345-5 AQ (20 × 500 mm × 2); solvent system, 5% or 8% methanol; flow rate, 7 ml/min.

**Calculation of the yield of transfer products.** The yield of transgalactosylation products to branched CDs was defined as the amount of transfer product (mol)/(amount of branched CDs used (mol) × 100 (%)).

**Fast atom bombardment mass spectrometry (FAB-MS).** FAB-MS was done with a JEOL JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluorooctylphosphazene (Ultra Mark), and glycerol was used as the matrix. Galactosylated derivatives of branched CDs were analyzed in the negative-ion mode.

**Results and Discussion**

Transgalactosylation of branched CDs by β-galactosidases from various origins

β-Galactosidases from *B. circulans*, *A. oryzae*, *P. multicolor*, *K. fragilis*, *K. lactis*, and *E. coli* (1 unit) were separately incubated with a mixture of lactose (20 mg) as a donor and branched CD (G1-α, G1-β, G2-α, or G2-βCD) (40 mg) as an acceptor in a total volume of 100 µl of 50 mm

Abbreviations: CD, cyclodextrin; αCD, α-cyclodextrin; βCD, β-cyclodextrin; G1-CD, glucosyl-cyclodextrin; G2-CD, maltosyl-cyclodextrin; CGTase, cyclomaltooligosaccharide glucanotransferase.
buffer (pH 6.0 (B. circulans and K. lactis), pH 4.5 (Asp. oryzae and P. multicolor), pH 6.5 (K. fragilis), and pH 7.3 (E. coli)) at 40°C for 60 min. Two-μl samples of the reaction mixtures were removed for analysis by HPLC.

β-Galactosidases from K. fragilis and K. lactis catalyzed the hydrolytic reaction of lactose but did not transgalactosylation. E. coli β-galactosidase catalyzed transgalactosylation only to lactose but did not to branched CDs. In the case of B. circulans β-galactosidase, the products, which seem to be transgalactosylation derivatives of branched CDs (G1-α1 and G1-α2 from G1-αCD, G1-β1 and G1-β2 from G1-βCD, G2-α1 and G2-α2 from G2-αCD, and G2-β1 from G2-βCD), were detected other than lactose, branched CDs, hydrolyzates of lactose (glucose and galactose),

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**Fig. 1.** High-Performance Liquid Chromatograms of Reaction Products of β-Galactosidases from Bacillus circulans and Penicillium multicolor on the Mixture of Lactose as a Donor and G1-α, G1-β, G2-α, or G2-β as Acceptors.

A1, A2, A3, and A4: reaction products of B. circulans β-galactosidase on G1-α, G1-β, G2-α, and G2-βCD, respectively. B1, B2, B3, and B4: reaction products of P. multicolor β-galactosidase on G1-α, G1-β, G2-α, and G2-βCD, respectively.

**Table I.** Transfer Products to G1-α, G1-β, G2-α, and G2-βCD by Various β-Galactosidases

<table>
<thead>
<tr>
<th>Yield (%) a</th>
<th>G1-αCD</th>
<th>G1-βCD</th>
<th>G2-αCD</th>
<th>G2-βCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1-α1</td>
<td>G1-α2</td>
<td>G1-α3</td>
<td>G1-β1</td>
</tr>
<tr>
<td>B. circulans</td>
<td>22</td>
<td>2.5</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td>Asp. oryzae</td>
<td>—</td>
<td>—</td>
<td>5.5</td>
<td>—</td>
</tr>
<tr>
<td>P. multicolor</td>
<td>—</td>
<td>—</td>
<td>6.7</td>
<td>—</td>
</tr>
</tbody>
</table>

a [Amount of transfer product (m)/Amount of branched CDs used (m)] × 100.
and transgalactosylation products of lactose (Fig. 1. A1, A2, A3, and A4). Asp. oryzae and P. multicolor β-galactosidases produced also products which seem to be transgalactosylation derivatives of branched CDs (G1-α1 and G1-α3 from G1-α2CD, G1-β1 and G1-β3 from G1-βCD, G2-α1 and G2-α3 from G2-α2CD, and G2-β1 and G2-β3 from G2-βCD) (Fig. 1. B1, B2, B3, and B4), like the reaction by B. circulans β-galactosidase, though the amount of transfer products, especially G1-α1, G1-β1, G2-α1, and G2-β1, were small. Also in the reaction mixtures of B. circulans β-galactosidase, products which seem to be G1-α2, G1-β3, and G2-α3, judging from the retention times, are detected between the peaks of G1-α1 and G1-α2, G1-β1 and G1-β2, and G2-α1 and G2-α2, respectively. The peak eluted after G2-β1 (Fig. 1. A4) and G2-β3 (Fig. 1. B4) is a impurity in G2-βCD preparation. Therefore, it is not clear that the transfer product (G2-β2) corresponding to G2-α2 (Fig. 1. A3) is produced or not. The yield of main transgalactosylation derivatives of branched CDs was summarized in Table I.

Effects of concentration of G1-βCD and lactose on the formation of G1-β1

The amount of transfer product is usually affected by the concentrations of donor and acceptor substrates. Therefore, the effects of substrate concentrations on the formation of transfer products were investigated, using for an example G1-β1.

To investigate the effects of G1-βCD concentration on G1-β1 formation, the B. circulans β-galactosidase (8 units/ml) was incubated with 0.3 M lactose in the presence of various concentrations of G1-βCD at 40°C. After 15, 30, 45, and 60 min, the amounts of G1-β1 produced in the reaction mixtures were measured by HPLC. As shown in Table II, the yields of G1-β1 were not almost affected by G1-βCD concentration, though the amounts of G1-β1 increased with increment of G1-βCD concentration.

For the purpose of investigation of the effects of lactose concentration on G1-β1 formation, B. circulans β-galactosidase (8 units/ml) was incubated with various concentrations of lactose in the presence of 0.2 M G1-βCD at 40°C. The amounts of G1-β1 produced in the reaction mixtures were measured by HPLC (Table III). The yield of G1-β1 increased with increase of lactose concentration, as the efficiency of transgalactosylation was enhanced. The maximum yield of G1-β1 (25%) was obtained at the lactose concentration of 0.8 M, practically the usable maximum concentration, at 120 min. The yield of G1-β1 decreased to 24% after 190 min because of the hydrolysis of the G1-β1 produced.

Isolation of transgalactosylation products from G1-βCD and G2-αCD

B. circulans and P. multicolor β-galactosidases (50 units) were separately incubated with a mixture of lactose (5 g) and branched CD (G1-β or G2-αCD) (2.5 g) in a total volume of 11 ml of 50 mm buffer (pH 6.0 for B. circulans and pH 4.5 for P. multicolor) at 40°C for 4 hr. The reaction mixtures were heated at 100°C for 15 min to inactivate the enzyme and centrifuged to remove the insoluble materials. The yields of G1-β1, G1-β2, G2-α1, and G2-α2 (by B. circulans β-galactosidase) and G1-β3 and G2-α3 (by P. multicolor β-galactosidase) were 18.4%, 3.9%, 21.1%, 6.3%, 5.4%, and 3.7%, respectively. Each transfer product

Table II. Effects of G1-βCD Concentration on the Production of Transfer Product (G1-β1) to G1-βCD from Lactose by B. circulans β-Galactosidase

<table>
<thead>
<tr>
<th>Yield (%)</th>
<th>0.1 M</th>
<th>0.15 M</th>
<th>0.2 M</th>
<th>0.3 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>17%</td>
<td>16%</td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>30 min</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>45 min</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>60 min</td>
<td>12</td>
<td>14</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

Lactose concentration, 0.3 M.

* [Amount of transfer product (m)/Amount of branched CDs used (m)] × 100.

Table III. Effects of Lactose Concentration on the Production of Transfer Product (G1-β1) to G1-βCD from Lactose by B. circulans β-Galactosidase

<table>
<thead>
<tr>
<th>Yield (%)</th>
<th>0.05 M</th>
<th>0.1 M</th>
<th>0.2 M</th>
<th>0.3 M</th>
<th>0.4 M</th>
<th>0.5 M</th>
<th>0.8 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>2.9%</td>
<td>4.3%</td>
<td>13%</td>
<td>15%</td>
<td>20%</td>
<td>20%</td>
<td>15%</td>
</tr>
<tr>
<td>60 min</td>
<td>3.4</td>
<td>12</td>
<td>13</td>
<td>17</td>
<td>21</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>120 min</td>
<td>2.9</td>
<td>4.9</td>
<td>11</td>
<td>16</td>
<td>12</td>
<td>23</td>
<td>25</td>
</tr>
</tbody>
</table>

G1-βCD concentration, 0.2 M.

* [Amount of transfer product (m)/Amount of branched CDs used (m)] × 100.

Fig. 2. High-Performance Liquid Chromatogram of Hydrolyzates by B. circulans β-Galactosidase of G1-β1.
Branched CDs Galactosylated by β-Galactosidases

was isolated by preparative HPLC.

Structural elucidation of transgalactosylation products

The saccharides G1-β1 and G1-β2 were completely hydrolyzed by B. circulans β-galactosidase to G1-βCD and galactose (these were identified by comparing the retention times of HPLC with those of authentic samples) in the molar ratio of 1:1 and 1:2, respectively (Fig. 2). The molar ratio was calculated with the calibration curves of authentic galactose and G1-βCD. The saccharides G2-α1 and G2-α2 were also completely hydrolyzed to G2-αCD and galactose in the molar ratio of 1:1 and 1:2, respectively. And the saccharides G1-β3 and G2-α3 were completely hydrolyzed by P. multicolor β-galactosidase to G1-βCD and galactose, and G2-αCD and galactose in the molar ratio of 1:1, respectively. These results indicated that G1-β1, G1-β2, G1-β3, G2-α1, G2-α2, and G2-α3 must be a mono-β-α-galactosyl-G1-βCD, di-β-α-galactosyl-G1-βCD, mono-β-α-galactosyl-G1-βCD, mono-β-α-galactosyl-G2-αCD, di-β-α-galactosyl-G2-αCD, and mono-β-α-galactosyl-G2-αCD, respectively. It is judged from their different retention times that the structures of G1-β3 and G2-α3 (main transgalactosylation derivatives of branched CDs produced by A. oryzae and P. multicolor β-galactosidases) would be different from G1-β1 and G1-β2 (those by B. circulans β-galactosidase), respectively.

When αCD or βCD was used as an acceptor, B. circulans and P. multicolor β-galactosidases did not produce the transgalactosylation derivatives of CDs (data not shown). Furthermore, G2-α1, G2-α2, and G2-α3 were not hydrolyzed by glucoamylase, though the parent G2-αCD was hydrolyzed to G1-αCD and glucose by glucoamylase. These results suggest that the galactosyl residues in G1-β1, G1-β2, and G1-β3 are linked at the side chain of G1-βCD by β-linkage, and those in G2-α1, G2-α2, and G2-α3 to the non-reducing end glucosyl unit at the side chain of G2-αCD by β-linkage, not directly at CD rings.

The FAB-MS spectra of these compounds in the negative mode were consistent with their structures. Figure 3 shows spectra of G1-β1, G1-β2, and G2-α2, for example. The [M – H]− peak is clearly observed at m/z 1457 in spectrum of G1-β1, and at m/z 1619 in each spectrum of G1-β2 and G2-α2. Other than these molecular ion peaks, two to four fragment ion peaks can be detected in each of these spectra. They must be formed through one cleavage of the side chain (primary fragments) and therefore, suggest the length of side chain. Namely the length of side chains of G1-β1, G1-β2, and G2-α2 are DP 2, 3, and 4, respectively. In each spectrum of G2-α1 and G2-α3, the [M – H]− peak and primary fragments were observed at m/z 1457, 1295, 1133, and 971, and the spectrum of G1-β3 was similar to the spectrum of G1-β1.

The further structural analyses and the measurements of physical properties of G1-β1, G1-β2, G1-β3, G2-α1, G2-α2, and G2-α3 will be presented in our next paper.

References