Synthesis of Branched Cycloisomaltooligosaccharides by the Action of Cyclodextrin Glucanotransferase

Tetsuya OGUMA,* Toshiko KUROKAWA, Tadahiro NAGATA,1 Mayumi O-KAMEYAMA,1 Satoshi KITAO,2 Kouichiro TOBE2 and Mikihiko KOBAYASHI1

Noda Institute for Scientific Research (399 Noda, Noda, Chiba 278, Japan)
1 National Food Research Institute, Ministry of Agriculture, Forestry, and Fisheries (2-1-2, Kannondai, Tsukuba, Ibaraki 305, Japan) 2 Research and Development Division, Kikkoman Corporation (399, Noda, Noda, Chiba 278, Japan)

Two kinds of branched cycloisomaltooligosaccharides, P1 and P2, were synthesized from cycloisomaltoheptaose and maltose by the action of cyclodextrin glucanotransferase. P1 was hydrolyzed into glucose and cycloisomaltoheptaose with the same molar ratio by the action of glucoamylase. P2 was hydrolyzed into glucose and cycloisomaltoheptaose with a molar ratio of two to one. P2 was also hydrolyzed into maltose and cycloisomaltoheptaose by acid degradation. Their molecular weight were determined by mass spectra analysis to be 1296 and 1458, respectively. Carbon and proton magnetic resonance analysis suggested that they contained $\alpha$-1,4-glucosidic linkage. From these results, they were identified as 4-O-$\alpha$-glucosyl-cycloisomaltoheptaose and 4-O-$\alpha$-maltosyl-cycloisomaltoheptaose. They had higher resistance to hydrolytic action by endo-dextranase than the parent cycloisomaltoheptaose.

Cyclodextrans (cycloisomaltooligosaccharides; CIs) are cyclic oligosaccharides composed of 7, 8 or 9 glucose residues linked by $\alpha$-1,6 linkages.1,2 CIs are synthesized from dextran by the action of CI glucanotransferase (CITase) from Bacillus circulans T-3040.3 Since they have larger cavities in the center of their rings than that of cyclodextrin, they are expected to be a novel dominant candidate for making inclusion complexes with larger compounds.1,4 Moreover, they are also expected to be anticarcinogenic reagents because of their prominent inhibition in the action of glucan synthetase from Leuconostoc mesenteroides and Streptococcus mutans.5,6 To develop another novel function of CIs, we attempted to synthesize modified CIs by enzymatic methods. Recently, we found that branched CIs were synthesized by the action of cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) on CI and maltose. This paper is concerning with the method of synthesis and the determination of structures of branched CIs.

MATERIALS AND METHODS

Enzymes. Endo-dextranase (EC 3.2.1.11) from Penicillium sp. and $\beta$-amylase (EC 3.2.1.2) were purchased from Sigma Chemical Co.; CGTase from Bacillus sp., glucoamylase (EC 3.2.1.3) from Rhizopus sp., and glucodextranase (EC 3.2.1.70) from Arthrobacter globiformis I42 were purchased from Amano Pharmaceuticals, Toyobo Co., Ltd., and Seikagaku Co., respectively.

Carbohydrates. Dextran 40 was purchased from Meito Sangyo Co., Ltd. CIs were prepared from dextran 40 by the action of CITase.3 Maltose was purchased from Wako Pure Chem-

*Corresponding author.
icals. The series of isomaltooligosaccharides from isomaltose to isomaltohexaose used as standard were purchased from Seikagaku Co.; those from isomaltooctaose to isomaltodecaose were obtained from Funakoshi Co.

Time course of synthesis of branched CIs by the action of CGTase. Six hundred μl of a 2% solution of CI-7, 300 μl of a 18% solution of maltose, 120 μl of 100 mM acetate buffer (pH 5.5), 150 μl of deionized water, and 30 μl of CGTase solution (300 U/ml) were mixed and incubated at 40°C for 24 h. One hundred μl aliquots of the reaction mixture were withdrawn at appropriate times and boiled for 10 min. After centrifugation at 8000 × g for 10 min, the supernatant was analyzed by HPLC using TSKgel Amide 80 column (4.6 mm i.d. × 250 mm; Tosoh Co., Japan).

Synthesis and purification of the branched CIs by the action of CGTase. The reaction condition for synthesis of branched CI was as follows: 4.5 g of maltose, 1.0 g of CI-7, 10 ml of 100 mM acetate buffer (pH 5.5), 85 ml of deionized water, and 3 ml of CGTase solution (300 U/ml) were mixed and incubated at 40°C for 24 h. After boiling for 10 min and chilling, 0.45 ml of β-amylase solution (252 U/ml) was added to the reaction mixture and incubated at 40°C for 4 h to remove linear maltooligosaccharides which were also synthesized from maltose by the disproportionation action of CGTase. The reaction was stopped by boiling for 10 min, and the supernatant was dried in vacuo and dissolved in 10 ml of deionized water. The resulting solution was then used as the branched CI solution.

The purification of the main products from the branched CI solution was carried out by HPLC using a preparative CAPCELL PAK C18 column (20 mm i.d. × 250 mm; Shiseido, Japan) and YMC-Pack PA-43 column (20 mm i.d. × 250 mm; YMC Co., Ltd., Japan). The operation conditions for HPLC were as follows: solvent, water/methanol (93: 7, v/v) for CAPCELL PAK, water/acetonitrile (45: 55, v/v) for PA-43 column; flow rate, 5 ml/min; fraction size, 5 ml/fraction; column temperature, 35°C for CAPCELL PAK, 25°C for PA-43 column; injection volume, 500 μl; detection, refractive index (model RI-8010; Tosoh Co., Japan). The branched CI fractions were pooled and dried using a rotary evaporator. The purities of the branched CIs were analyzed by three kinds of HPLC columns using Dionex Carbopac™ PA1, YMC Pack ODS AQ-303, and TSKgel amide 80 column. High performance anion exchange column chromatography (HPAEC) was conducted with a DIONEX DX500 chromatography system (Dionex, CA, USA) under the following conditions: detector, ED40 electrochemical detector; column, Carbopac™ PA1 (4 mm i.d. × 250 mm); solvent, 100 mM NaOH; elution, a linear gradient of sodium acetate (0-175 mM over 120 min); flow rate, 0.8 ml/min; temperature, 25°C; injection volume, 20 μl. Apart from the solvent system (water/methanol; 93: 7, v/v), the conditions for HPLC analysis using the AQ-303 column (4.6 mm i.d. × 250 mm; YMC Co., Ltd., Japan) were the same as those when using the TSKgel Amide 80 column.

Determination of the structures of the branched CIs. The structures of the main products were determined by mass, 1H-NMR, and 13C-NMR spectroscopies, acid degradation, and enzymatic analysis using glucoamylase and glucoamylase.

Fast atom bombardment mass spectrometry (FAB-MS) was performed with JMS-SX102A (JEOL, Japan), and secondary ion mass spectrometry (SIMS) was carried out with M-80B (Hitachi, Japan). The mass spectrometer was equipped with a FAB gun that produced a 6 keV xenon beam. The mass scale was calibrated with Ulramark 1621 (fluorinated phosphazene) at a resolution of 3000. Glycerol was used as matrix. 13C-(150.8 MHz) and 1H-(600.1 MHz) NMR including DQF-COSY, NOESY, ROESY, 2D-HOHAHA and C-H COSY were run on a JNM-A600 (JEOL, Japan) in D2O at 50°C. Chemical shifts were expressed in ppm downfield from the signal of external tetramethylsilane. 13C-NMR spectra were also recorded at 25°C in dimethylsulfoxide on JNM-FX200.
Synthesis of Branched Cyclodextran (JEOL, Japan) at 50 MHz.

Two μl of glucoamylase solution (150 U/ml) or glucodextranase solution (12.4 U/ml), 36 μl of each branched CI solution (10 mg/ml), and 2 μl of 100 mM acetate buffer (pH 5.5) were mixed and incubated at 40°C for 24 h. The aliquots (12 μl) which were withdrawn from the reaction mixture at appropriate times were boiled for 10 min. The supernatant obtained via centrifugation was analyzed by HPLC.

Acid degradation was performed as follows. After 160 μg of P2 was dissolved into 15 μl of 0.2 M HCl, the solution was incubated at 95°C for 1 h. After cooling, the solution was then analyzed by HPLC.

Action of endo-dextranase on the branched CIs. Six μl of endo-dextranase solution (333 U/ml), 30 μl of 1% branched CI or CI-7, and 6 μl of 100 mM acetate buffer (pH 6.0) were mixed and incubated at 40°C for 16 h. The reaction mixture was analyzed by HPLC after boiling and centrifugation.

RESULTS AND DISCUSSION

The time course of the production of the branched CI is shown in Fig. 1. While CI-7 decreased linearly, two unknown products, P1 and P2, were gradually produced. The HPLC profile of 6 h reaction products is shown in Fig. 2. The amount of P1 was about two times that of P2. The yield of P1 and P2 to initial CI-7 was about 40% after 24 h reaction. If reaction time is extended, the final yield of P1 and P2 may increase, although several kinds of minor unknown products may also increase.

All profiles of HPLC analyses using various columns showed that each of the isolated products, P1 and P2, was obtained at more than 99% purity. P1 and P2 may be branched CI-7s, since glucoamylase and glucodextranase digestion of the products gave glucose and CI-7 (Fig. 3). As shown in Fig. 3-A-1, P1 produced equimolar amounts of glucose and CI-7.
Fig. 3. Time courses of hydrolysis of branched Cls by glucoamylase (1) and glucodextranase (2).

Details of the enzyme reaction and analytical conditions are described in the text. Panels A and B are reaction mixture of P1 and P2, respectively. Symbols: CI-7 (▲), glucose (○), P1 (●), P2 (■).

Fig. 4. Carbon NMR spectra of branched Cls and CI.

Panels 1, 2, and 3 are 13C-NMR spectra of P1, P2, and CI-7, respectively. R-, R’, S- and S’- show the C atoms of each d-glucopyranosyl unit depicted in the schematical diagrams.
In Fig. 4, $^{13}$C-NMR spectra of P1, P2, and CI-7 are compared. Assignments of signals were carried out using the 2D methods. Each C-4 signal (R'-4) of the CI-ring glucoses in the spectra of P1 and P2 shifted downfield by -7.5 ppm from the other C-4s (R-4), appearing at $\delta \sim 77.5$. Moreover, one C-4 signal (S-4) of the two side-chain glucoses in the spectrum of P2 appeared at $\delta -78$, and shifted downfield by -8.3 ppm from another C-4 signal of the side-chain glucose (S'-4). Therefore, P1 and P2 were thought to be 4-0-$\alpha$-d-glucosyl-Cl-7 and 4-0-$\alpha$-maltosyl-Cl-7, respectively. C-1 signals of the side-chain glucose residues (S-1 and S'-1) of P1 and P2 were observed at a lower field than $\alpha$-1,6 linked C-1 signals of CI ring glucoses (R-1 and R'-1), and thus supported the structures of P1 and P2 mentioned above. Moreover, P2 was hydrolyzed into maltose and CI-7 by the acid degradation, although glucose and P1 were produced simultaneously. This result also supports the proposed structures.

As shown in Fig. 5, molecular weights of P1 and P2 were determined as 1296 and 1458, respectively, by FAB-MS. These values agreed with the data obtained by SIMS, and completely coincided with the calculated molecular weights of glucosyl-Cl-7 and maltosyl-Cl-7, respectively. It was reported that galactobiosyl CDs (two galactose residues linked linearly with one end of the array bound to the mother ring) showed fragment ion peaks [M-2Gal-H]$^-$ along with [M-Gal-H]$^-$, while digalactosyl CDs (two galactose residues individually linked with the mother ring) showed fragment ion peaks [M-Gal-H]$^-$ only. The spectrum of P2 showed both fragment ion peaks [M-Glc-H]$^-$ at $m/z$ 1295 and [M-2Glc-H]$^-$ at $m/z$ 1133. Furthermore, the intensity of the signal at $m/z$ 1133 was higher than that of the signal at $m/z$ 1295. This result also seemed to suggest that P2 consisted of CI-7 and maltose. Consequently, it also seemed to be elucidated that P2 was not 4', 4''-di-0-($\alpha$-d-glucosyl)-Cl-7 but 4-0-$\alpha$-maltosyl-Cl-7. From these results, we identified P1 as 4-0-$\alpha$-d-glucosyl-Cl-7 and P2 as 4-0-$\alpha$-maltosyl-Cl-7.

We also investigated resistances of 4-0-$\alpha$-d-glucosyl-Cl-7, 4-0-$\alpha$-maltosyl-Cl-7 and Cl-7 to hydrolysis by endo-dextranase. The branched CIs showed stronger resistance to the action of endo-dextranase than that of the parent CI-7 (Table 1). We consider these resistance to be derived from the steric hindrance of branched glucosyl and maltosyl residue.

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### Table 1. Hydrolysis of cyclic sugars by endo-dextranase.

<table>
<thead>
<tr>
<th>Cyclic sugars</th>
<th>Remaining cyclic sugars (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-7</td>
<td>45.5</td>
</tr>
<tr>
<td>P1</td>
<td>75.6</td>
</tr>
<tr>
<td>P2</td>
<td>68.0</td>
</tr>
</tbody>
</table>

$^a$ Details of the enzyme reaction and analytical condition are described in the text.

### REFERENCES


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サイクロデキストリン合成酵素の作用による
分岐サイクロイソマルトオリゴ糖の合成
小熊哲哉，黒川滋子，永田忠博1，
亀山直雄1，北尾 悟2，
戸辺光一郎2，小林浩彦1

財団法人野田産業科学研究所（278 千葉県
野田市野田 399）
1農林水産省食品総合研究所（305 茨城県
つくば市観音台 2-1-2）
2キッコーマン株式会社研究本部（278 千葉県
野田市野田 399）

グルコースのα-1.6 結合よりなるサイクロデキスト
ラン（サイクロイソマルトオリゴ糖；CI）とマル
トースからサイクロデキストリン合成酵素の作用によ
り，2種類の分岐 CI が合成されることを見出した．
これらの分岐 CI（P1，P2）は，グルクロアミラーゼの
作用により，P1 は等モルのグルコースと CI-7 で分
解され，P2 は，2:1 のモル比で，グルコースと CI-7
に分解された．また P2 は，酸分解によりマルトース
と CI-7 に水解された．一方，質量分析により，P1 と
P2 の分子量は各々 1296，1458 と算出された．また
1H-NMR および 13C-NMR 分析によりいずれの環状
オリゴ糖 α-1,4 結合の存在が示された．これらの
結果より，P1 を 4-O-α-グルコシル CI-7，P2 を 4-O-
α-マルトシル CI-7 と同定した．これらの分岐 CI は，
エンドタイプキスタナーゼの作用に対して，CI-7 よ
りも強い抵抗性を示した．