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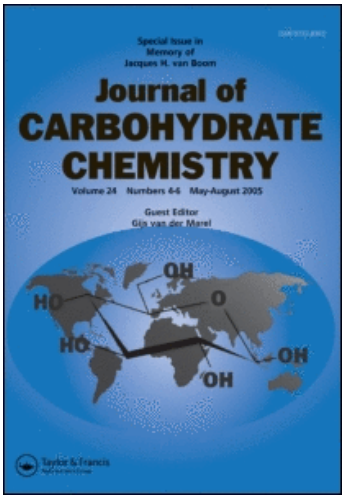


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J. CARBOHYDRATE CHEMISTRY, 10(4), 701-709 (1991)

# ACTION OF CYCLODEXTRIN GLUCANOTRANSFERASE ON DIMALTOSYL- AND DIGLUCOSYL- α -CYCLODEXTRINS[1]

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## ABSTRACT

The mixture of $(G_2)_2$- α -CD[2] and $G_2$[2] was reacted with cyclodextrin glucanotransferase (CGTase). Doubly branched CDs which have longer glucan branches than maltose were formed by the transfer action of CGTase. The mixture of $(G_1)_2$- α -CD[2] and glucose was also reacted with the enzyme. Branched glucan having 9 glucose units (branched $G_9$)[2] was produced by the coupling action of CGTase. The branched $G_9$ was separated and reacted again with CGTase to form $(G_1)_2$- α -CD and glucose. The mixture of $(G_1)_2$- α -CD[3] and glucose was reacted with the combined enzyme system of CGTase and glucoamylase to obtain glucose and residual $(G_1)_2$- α -CD, which was completely resistant to the action of the enzyme system. Thirty seven % of $(G_1)_2$- α -CD was nonreactable $(G_1)_2$- α -CD. From the enzyme model which we propose, the structure of the CD might be an AD type of $(G_1)_2$- α -CD, and the structure of reactable $(G_1)_2$- α -CD might be AB and AC types.

## INTRODUCTION

The action of CGTase (cyclodextrin glucanotransferase, EC 2.4.1.19) has been investigated,[4,5] and it is known that various branched maltooligosaccharides are formed from $G_1$- α -CD and glucose by the coupling action of the enzyme.[6] From the results obtained during investigation of the action of the enzyme, an enzyme model explaining formation of such oligosaccharides was presented.[7] According to the enzyme model, the CD ring of branched CDs having a maltotriosyl or longer glucosyl unit chain will be opened by the coupling action of the enzyme, whereas the CD ring of branched CDs not



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having these moieties will not be opened. Moreover, the maltose or longer maltooligosaccharide branches possibly fit on the enzyme active site as substrates to form branches of various length, by the transfer action of the enzyme. Recently, branched CDs with 2 branches on the CD ring have been prepared,[8] and these authors have prepared $(G_2)_2$-CDs and $(G_1)_2$-CDs.[9]

In this report, the authors have tried to elucidate the action of the enzyme on such branched CDs.

## RESULTS AND DISCUSSION

Action of CGTase on the mixture of $(G_2)_2$- $\alpha$ -CD[2] and maltose.

Various branched $\alpha$ -CDs were formed by the transfer action of CGTase on the mixture of $(G_2)_2$- $\alpha$ -CD and maltose and the mixtures analysed by HPLC (Fig. 1). Branched glucans having more than 10 glucose units (peak 2 group) were also detected between 10-15 min retention time. These glucans might be formed by the coupling and transfer action of CGTase. The amount of these glucans was low as compared with the coupling products from maltose and $\alpha$ - CD. Accordingly, the branches may effectively depress the coupling action of CGTase.

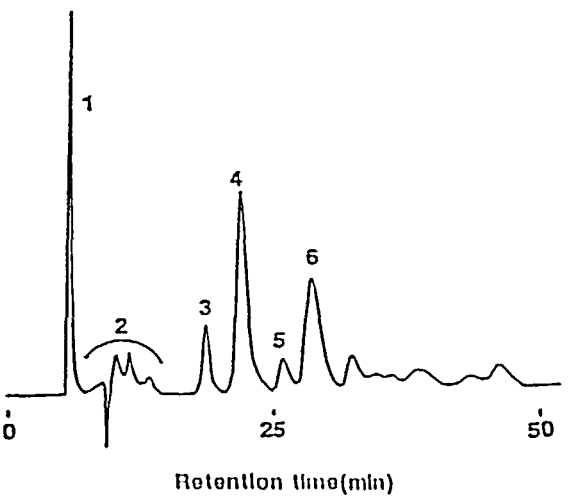


Fig. 1 HPLC profile showing production of longer chain branched $\alpha$ -CD from $(G_2)_2$- $\alpha$ -CD and maltose by the transfer action of CGTase. 1: maltose, 2: branched dextrins, 3: $(G_1)_2$- $\alpha$ -CD and $G_1$- $\alpha$ -CD, 4: $(G_2)_2$- $\alpha$ -CD, 5: $G_2$-,$G_2$- $\alpha$ -CD, 6: $(G_3)_2$- $\alpha$ -CD.

Longer chain branched $\alpha$ -CD was prepared (see text, EXPERIMENTAL, Action of CGTase on the mixture of $(G_2)_2$- $\alpha$ -CD and maltose), and 10 $\mu$L of the reaction mixture was injected. HPLC conditions column:Inertsil ODS-2, solvent: aqueous 1% ethanol, flow rate: 0.7 mL/min, temperature: 50 °C.

All sugar peaks between 15 and 50 min retention time were collected and concentrated to obtain the branched $\alpha$ -CD. To the branched CD (10 mg), 100 $\mu$L of 50 mM acetate buffer (pH 5.0) was added 0.1 IU of pullulanase and the

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mixture incubated at 50 °C for 24 h. The reaction mixture was heated at 100 °C to inactivate the enzyme, then deionized and filtered. Water (100 $\mu$L) was added and the mixture was injected to obtain the HPLC profile as shown in Fig. 2A. $\alpha$ -CD (peak 2) might be formed from branched $\alpha$ -CD having maltose or longer maltooligosaccharides branches, because pullulanase splits maltose and longer chained branches from branched CDs. Peak 3 is the mixture of $G_1$- $\alpha$ -CD and $(G_1)_2$- $\alpha$ -CD, and peak 4 might be unreacted $(G_2)_2$- $\alpha$ -CD which has a comparatively resistant structure to the pullulanase action. $(G_2)_2$- $\alpha$ -CD hardly reacted with CGTase without an acceptor such as maltose. This may be due to the CD ring not fitting the active site of CGTase and preventing ring opening associated with the coupling action. On the other hand, the maltosyl branch might easily fit the active site of CGTase and exchange the moiety to maltose to form a longer branched $\alpha$ -CD.

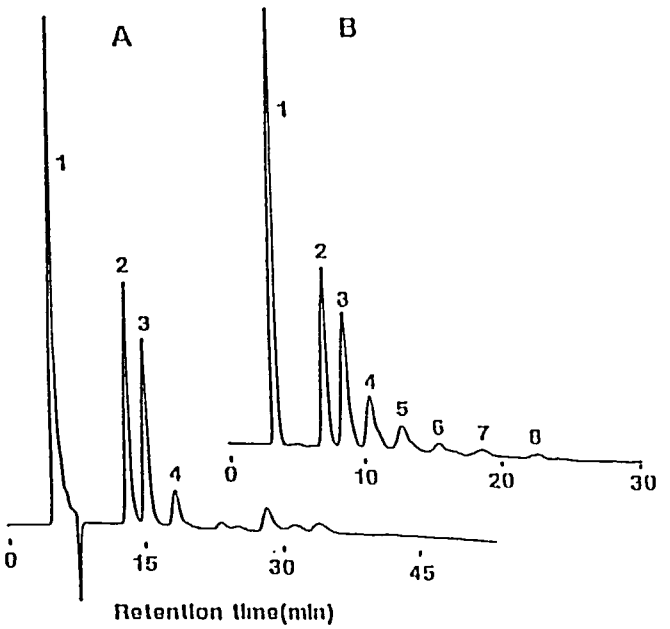


Fig. 2 Composition of branches of longer chain branched $\alpha$ -CD. 2A 1: open chain maltooligosaccharides, 2: $\alpha$ -CD, 3: $(G_1)_2$- $\alpha$ -CD and $G_1$- $\alpha$ -CD, 4: $(G_2)_2$- $\alpha$ -CD. 2B 1: $H_2O$, 2: maltose, 3: $G_3$, 4: $G_4$, 5: $G_5$, 6: $G_6$, 7: $G_7$, 8: $G_8$. All sugar peaks (branched $\alpha$ -CD) between 15 and 50 min retention time in Fig. 1 were collected. Peak 1 in Fig. 2A was collected and injected to obtain a profile 2B (separation of maltooligosaccharides). 2A HPLC conditions column: Inertsil ODS-2, eluant: 1% aqueous ethanol flow rate: 0.7 mL/min, temperature: 50 °C. 2B HPLC conditions column: Lichrospher $NH_2$, eluant: 55% acetonitrile, flow rate: 0.8 mL/min, temperature: 20 °C.

Peak 1 in Fig. 2A contained several kinds of maltooligosaccharides. The maltooligosaccharides were collected, and injected on a different HPLC column for the analysis. Peaks of $G_2$-$G_8$ were obtained as in Fig. 2B. These maltooligosaccharides were from longer branched $\alpha$ -CDs by the action of

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pullulanase, because maltose and α-CD were formed from peak 4 (in Fig. 1) in a molar ratio of 2:1; $G_2$, $G_3$ and α-CD from peak 5 in a molar ratio of 1:1:1; $G_3$ and α-CD from peak 6 in a molar ratio of 2:1.

Action of CGTase on the mixture of $(G_1)_2$-α-CD[3] and glucose. The mixture of glucose and $(G_1)_2$-α-CD was reacted with CGTase, and the reaction mixture injected to obtain HPLC profiles as in Fig. 3. The main product formed by the coupling action of CGTase was branched $G_9$. To study the structure of the branched oligosaccharides, peak 5 in Fig. 3B was separated and reacted with glucoamylase, and the reaction mixture was periodically sampled and injected to obtain HPLC profiles as in Fig. 4. From the profiles, the possible structure of the branched $G_9$ was $6^6,6^4$-O-α-diglucosyl maltoheptaose and $6^5,6^4$-O-α-diglucosyl maltoheptaose, because glucoamylase can more rapidly degrade α-1,4 linkages from the non-reducing end of glucan molecules than α-1,6 linkages.

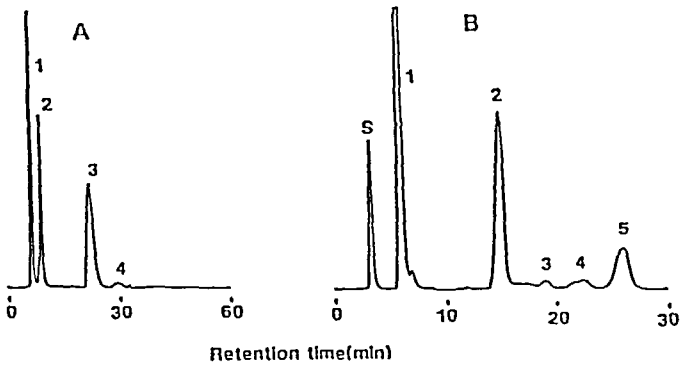


Fig. 3 Formation of branched $G_9$ from the mixture of glucose and $(G_1)_2$-α-CD by the coupling action of CGTase. 3A 1: glucose, 2: branched $G_9$, 3: $(G_1)_2$-α-CD, 4: unknown (presumably $(G_1)_3$-α-CD). 3B S: solvent, 1: glucose, 2: $(G_1)_2$-α-CD, 3: unknown (presumably $(G_1)_3$-α-CD), 4: unknown (presumably branched $G_8$), 5: branched $G_9$. HPLC conditions are same as in Fig. 2.

Peak 4 in Fig. 4 (branched $G_8$) was separated, reacted again with glucoamylase, and injected to obtain HPLC profile having peaks of glucose, branched $G_5$, and unreacted branched $G_8$. Peak 3 (branched $G_7$) gave an HPLC profile having glucose, branched $G_5$, and unreacted branched $G_7$. From the results, branched $G_8$ and $G_7$ were assumed to be $6^6,6^4$-O-α-diglucosyl maltohexaose and $6^5,6^4$-O-α-diglucosyl maltopentaose, respectively. Unknown peak 4 in Fig. 3A is possibly $(G_1)_3$-α-CD[2], although the structure has not been verified, it was completely resistant to the action of glucoamylase.

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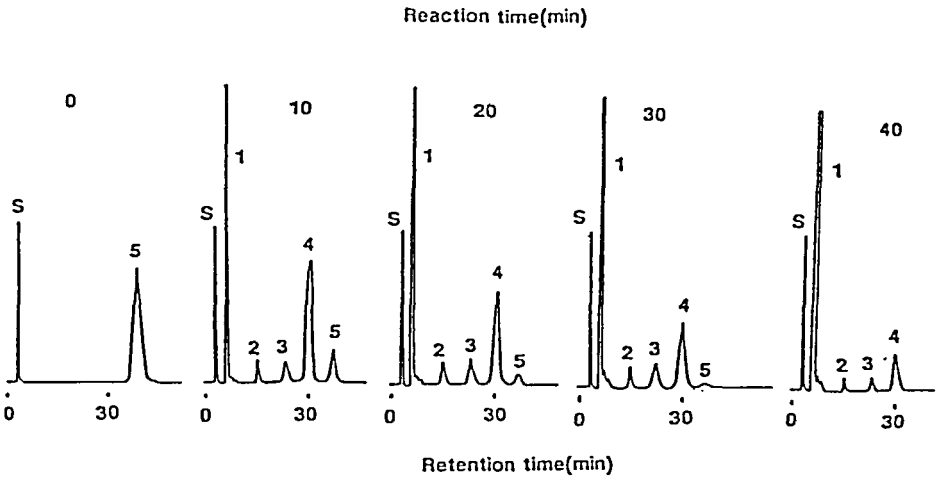


Fig. 4 Time course of branched $G_9$ degradation by the action of glucoamylase. S: solvent, 1: glucose, 2,3,4: presumably branched $G_5$, branched $G_7$, branched $G_8$, 5: branched $G_9$. HPLC conditions column: Lichrospher-$NH_2$, solvent: aqueous 55-60% acetonitrile, flow rate:0.8 mL/min, temperature: 20 °C.

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Unreacted $(G_1)_2$- $\alpha$ -CD which was separated from peak 2 of Fig. 3B was again reacted with CGTase on addition of glucose to obtain branched $G_9$ again, but the amount of branched $G_9$ formed in this second reaction was less than half of that obtained in the first reaction. After repeating this treatment several times, about 37% (from $(G_1)_2$- $\alpha$ -CD preparation) of completely nonreactive $(G_1)_2$- $\alpha$ -CD was obtained. We deduce that the structure of nonreactive $(G_1)_2$- $\alpha$ -CD is an AD type of doubly branched $\alpha$ -CD. According to our enzyme model, which was proposed in 1980,[7] any part of the molecule of an AD type of $(G_1)_2$- $\alpha$ -CD can't fit on the catalytic site.

A fragment of peak 5 (Fig. 3B) was collected and reacted with CGTase, and two new peaks appeared as shown in Fig. 5. Fragment of peak 1 (Fig. 5) was determined to be glucose from its HPLC retention time, peak 2 was unreacted branched $G_9$, and the molecular weight and HPLC retention time of peak 3 were the same as that of $(G_1)_2$- $\alpha$ -CD, the latter fragment being completely resistant to the action of glucoamylase. The molar ratio of fragments 1 and 3 (Fig. 5) was 1:1. Thus, fragment 3 was determined to be $(G_1)_2$- $\alpha$ -CD. Moreover, the type of the CD should be AC and AB, as described above. This reaction was reversible as shown in Fig. 6, and more than 50% of $(G_1)_2$- $\alpha$ -CD was formed from the branched $G_9$ by no addition of glucose.

Addition of yeast itself to the reaction mixture was considerably effective in increasing the yield of $(G_1)_2$- $\alpha$ -CD, whereas addition of glucose decreased the yield.

Action of the mixed enzyme system of CGTase and glucoamylase on $(G_1)_2$- $\alpha$ -CD.[3] To a solution of $(G_1)_2$- $\alpha$ -CD preparation was added the mixed enzyme solution of CGTase and glucoamylase, and the reaction mixture was analyzed. Only glucose was formed (Fig. 7), and 37% of the $(G_1)_2$- $\alpha$ -CD preparation remained as completely nonreactable $(G_1)_2$- $\alpha$ -CD. This completely nonreactable $(G_1)_2$- $\alpha$ -CD should be type AD. Fragments formed by the hydrolyzing and coupling action of CGTase were simultaneousl degraded to glucose by the action of glucoamylase. Therefore, the pure AD type of $(G_1)_2$- $\alpha$ -CD was prepared by use of the combined enzyme as in Fig. 8.

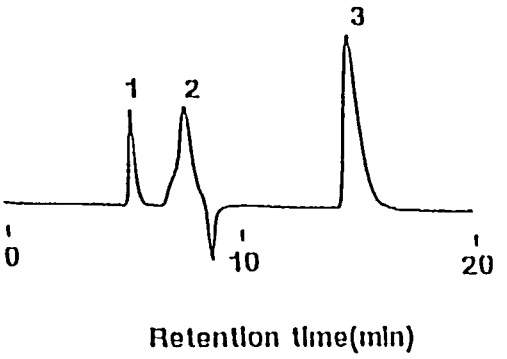



Fig. 5 Reversible formation of $(G_1)_2$- $\alpha$ -CD from branched $G_9$ by cyclization of CGTase. 1: glucose, 2: branched $G_9$, 3: $(G_1)_2$- $\alpha$ -CD. HPLC conditions column: Inertsil ODS-2, solvent: aqueous 1% ethanol, flow rate: 0.7 mL/min, temperature: 50 °C.

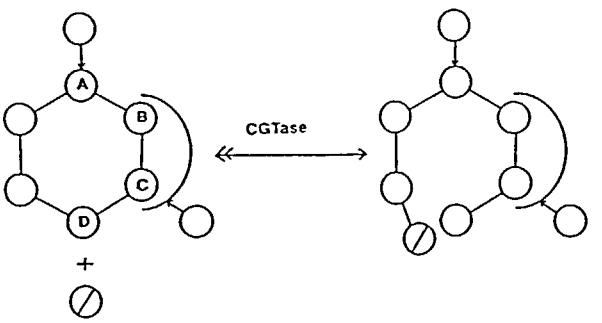
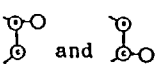



Fig. 6 Schematic explanation of branched $G_9$ formation from $(G_1)_2$- $\alpha$ -CD and glucose by the coupling action of CGTase. ○:glucose residue, -: $\alpha$ -1,4 linkage, ↓: $\alpha$ -1,6 linkage, ∅:reducing end,  stands for a mixture of  and 

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$\beta$ -CD series were also investigated, and preliminary results were obtained. Almost all of $(G_2)_2$- $\beta$ -CD molecules were degraded to branched maltooligosaccharides by the action of CGTase with maltose, but the composition was very complicated and has not been analyzed yet. The details will be reported elsewhere.

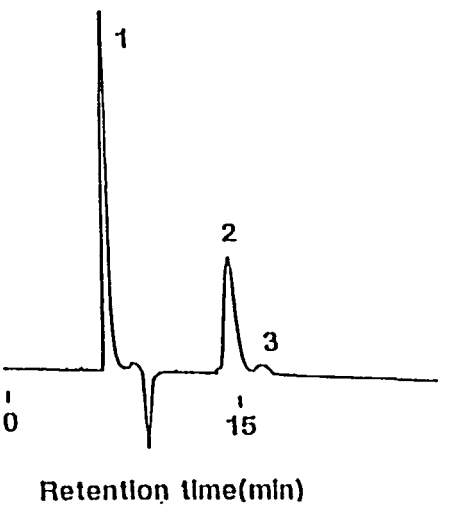


Fig. 7 Formation of a resistant $(G_1)_2$- $\alpha$ -CD by the combined action of CGTase and glucoamylase. 1: glucose, 2: $(G_1)_2$- $\alpha$ -CD, 3: unknown (presumably $(G_1)_3$- $\alpha$ -CD). HPLC conditions column: Inertsil ODS-2, solvent: aqueous 1% ethanol, flow rate: 0.7 mL/min, temperature: 50 °C.

## EXPERIMENTAL

Maltose (purity 99%) and glucose (purity 99%) were commercial products. CGTase (from *Bacillus macerans*), kindly donated by Amanoseiyaku Co.,Ltd., was purified by ammonium sulfate fractionation and adsorption on starch, and dialyzed against 100 mM acetate buffer (pH 6.0) before use. The enzyme activity (THU) was determined by the method of Tilden and Hudson.[10] Glucoamylase (pure grade 35 IU/mg, commercial product of Seikagaku Kogyo Co.,Ltd.) was dissolved in 100 mM acetate buffer (pH 5.2) to give 100 IU/mL.

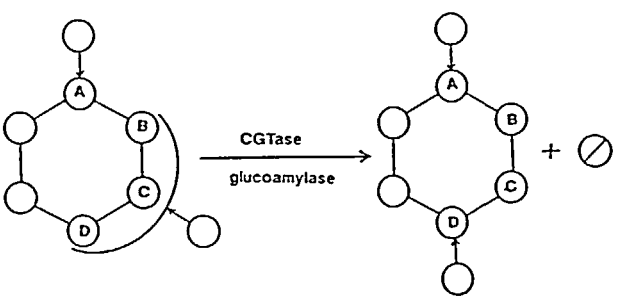


Fig. 8 Schematic explanation of the AD type of $(G_1)_2$- $\alpha$-CD formation by the combined action of CGTase and glucoamylase. Symbols are same as in Fig. 6.

$(G_2)_2$- $\alpha$ -CD and $(G_1)_2$- $\alpha$ -CD were prepared by the previously reported method.[9]

Determination of the structure of $(G_2)_2$- $\alpha$ -CD and $(G_1)_2$- $\alpha$ -CD. The molecular weights of the branched CDs were analyzed by secondary ion mass spectrometry (SIMS, using Hitachi model M-80B), and those were 1,296 and 1,620 respectively. By the action of glucoamylase, $(G_2)_2$- $\alpha$ -CD was converted to $(G_1)_2$- $\alpha$ -CD which was not further degraded by the enzyme. $(G_1)_2$- $\alpha$ -CD and $G_1$- $\alpha$ -CD were separated by use of a LiChrospher $NH_2$ column.

Column chromatography on HPLC. Purification of the branched CDs were carried out by HPLC using a JASCO 880-PU pump equipped with an 880-51 degasser, an 860-CO column oven, a 7125 Rheodyne injector, an 830-RI differential refractometer, and an SIC chromatocorder. The column (20 × 250 mm) contained Inertsil PREP-ODS (10 $\mu$m, Gaskurokogyou); solvent system, distilled water at 2.0 mL/min at 50 °C; column pressure, < 50 kg/cm$^2$. The refractometer was maintained at 35 °C.

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The purity of branched CDs in the fractions was checked by HPLC on a LiChrospher $NH_2$ (4 × 250 mm, Merck) with a solvent of aqueous 55-60% acetonitrile at a flow rate 0.8 mL/min at 20 °C, and on a Inertsil ODS-2 (6 × 250 mm, Gaskurokogyou) with a solvent of aqueous 1% ethanol at a flow rate 0.7 mL/min at 50 °C. LiChrospher $NH_2$ column is suitable for the analysis of open chained maltooligosaccharides, whereas the Inertsil ODS-2 column is suitable for the analysis of cyclic maltooligosaccharides. Each appropriate fraction having a purity of more than 95% was combined and freeze dried to give $(G_2)_2$- $\alpha$ -CD and $(G_1)_2$- $\alpha$ -CD.

Action of CGTase on the mixture of $(G_2)_2$- $\alpha$ -CD and $G_2$. To a solution of $G_2$ (purity 99%, 5 mg), and $(G_2)_2$- $\alpha$ -CD (purity 95%, 20 mg) in 20 mM acetate-phosphate buffer (200 $\mu$L, pH 6.0) 3 THU of CGTase (300 THU/mL) was added, and the mixture was kept at 40 °C for 24 h. The mixture was treated at $\sim$ 100 °C for 10 min, deionized, and filtered (Milipore 0.45 $\mu$m), to give branched $\alpha$ -CD with two long chains.

Action of CGTase on the mixture of $(G_1)_2$- $\alpha$ -CD and glucose. To a solution of glucose (purity 99%, 15 mg), and $(G_1)_2$- $\alpha$ -CD (purity 95%, 15 mg) in 20 mM acetate-phosphate buffer (250 $\mu$L, pH 6.0) 3 THU of CGTase (300 THU/mL) was added, and the mixture was kept at 40 °C for 24 h. The mixture was treated at $\sim$ 100 °C for 10 min, deionized, filtered (0.45 $\mu$m), and 10 $\mu$L was subjected to HPLC.

Action of the combined enzyme of CGTase and glucoamylase on $(G_1)_2$-$\alpha$-CD. To a solution of $(G_1)_2$-CD (purity 95%, 20 mg) in 20 mM acetate-phosphate buffer (200 $\mu$L, pH 5.5) both 2 THU of CGTase (300 THU/mL) and 2 IU of glucoamylase (100 IU/mL) were added, and the mixture was kept at 40 °C for 12 h. The mixture was treated at ~ 100 °C for 10 min, deionized, filtered through a milipore filter (0.45 $\mu$m), and 10 $\mu$L was subjected to HPLC.

ACKNOWLEDGMENTS

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1. Presented at the XVth International Carbohydrate Symposium, Yokohama, Japan, August 12-17, 1990.
2. $G_2$ ~ $G_9$, $G_1$-CD, $(G_1)_2$-CD, $(G_1)_3$-CD, and $(G_2)_2$-CD are abbreviations for maltose ~ maltononaose, glucosyl-cyclodextrin, doubly branched glucosyl-cyclodextrin, triply branched glucosyl-cyclodextrin, and doubly branched maltosyl-cyclodextrin.
3. $(G_1)_2$-$\alpha$-CD used in this experiments contains AB, AC, AD types of $(G_1)_2$-$\alpha$-CD whose structures are $6^A$, $6^B$-di-O-$\alpha$-glucosyl-cyclodextrin, $6^A$, $6^C$-di-O-$\alpha$-glucosyl-cyclodextrin, and $6^A$, $6^D$-di-O-$\alpha$-glucosyl-cyclodextrin.
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