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A Novel Synthesis Method for Cyclodextrins from Maltose in Water-Organic Solvent Systems

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ABSTRACT

A novel enzymatic synthesis method of cyclodextrin (CD) from low-mol-wt maltose using cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus macerans* has been developed in various water-organic solvent systems. A β-CD was synthesized in a two-phase system consisting of water and cyclohexane. However, no CDs could be synthesized in an aqueous buffer solution. A maximal yield of β-CD has been obtained at a cyclohexane content volume of 44%. This synthesis has been obtained only at low temperatures, i.e., 7°C, and did not take place at 50°C. In addition, various organic solvents have been used for the enzymatic synthesis of CD from maltose. Consequently, β-CD could be synthesized in various water-organic solvent systems, e.g., cyclohexane, benzene, xylene, and chloroform, but no enzymatic reaction occurred using aliphatic *n*-hydrocarbon solvents such as hexane, dodecane, and hexadecane. Furthermore, α- and β-CD could be synthesized in water mixture solutions using organic solvents having an alcoholic group (e.g., ethanol, propanol, butanol, and pentanol) in a wide range of the reaction temperatures, typically 7–50°C. In this temperature range, α- and β-CD were also formed and the maximal yield from maltose to β-CD of approx 13% was reached in 60 h.

Index Entries: Cyclodextrin; enzymatic synthesis in organic media; maltose; cyclohexane; CGTase from *Bacillus macerans*.

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INTRODUCTION

In general, enzymes function in aqueous solutions, and enzymatic activity in total absence of water has not been observed. However, full solution conditions are not necessary since enzyme crystals have been found, both in water (*1*) and in organic solvents (*2*). Recently, many enzymatic catalysis experiments have been performed in predominantly organic media (*3–9*). In particular, when using almost anhydrous organic solvents, enzymes acquire remarkable novel properties (*6*) such as large stability enhancement (*10,11*), radical substrate specificity alteration (*12*), and the ability to catalyze new reactions (*13*). Moreover, the thermodynamic equilibrium of hydrolase-catalyzed reactions has been shifted to favor synthesis over hydrolysis (*14*). The aforementioned references primarily concern reactions with water as one of the products, such as synthesis of esters, peptides, oligosaccharides, or glycerides (*15–17*). Enzymes are used in monophasic organic systems, emulsified in water-immiscible solvents, or directly suspended in anhydrous organic solvents (*7,18,19*).

Cyclodextrins (CDs) were first isolated in 1891 by Villiers as degradation products of starch (*20*), and they were characterized as cyclic oligosaccharides in 1904 by Schardinger (*21*). CDs are cyclic and nonreducing oligosaccharides consisting of glucopyranose units joined together by an α-1,4-glucosidic linkage, and they are designated α-, β-, and γ- according to the number of glucose units (α- for 6 U, β- for 7 U, and γ- for 8 U) (*22,23*). Each of these molecules is a torus (doughnut-shape), having a hydrophilic shell and a hydrophobic cavity. The most important feature of this structure is the ability of the CDs to form inclusion complexes with organic substances (*24*) like fatty acids, vitamins, and flavor compounds (*25*). Using this ability, CDs might transform volatile substances into nonvolatile or mask unpleasant odors, CDs are therefore applicable in a variety of fields (e.g., for use in medicinal products, foodstuffs, cosmetics, and the like).

Cyclodextrins have conventionally been produced from starch using cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19) excreted from microorganisms such as *Bacillus macerans* (*26*), *Bacillus circulans* (*27,28*) *Bacillus megatherium* (*29*), and alkalophilic *Bacillus* sp. (*30,31*). CGTase catalyzes reactions mainly by (1) cyclization, (2) coupling, (3) disproportionation, and (4) hydrolysis (*32*).

The substrate concentration should preferably be high from an industrial point of view, which involves an increase in viscosity of the reaction solution resulting in stirring difficulty in combination with a lower reaction rate. To that end a method has been proposed, comprised of preliminary subjecting starch to a pretreatment process with α-amylase to decrease he viscosity of solution. Because the produced reaction product is a mixture of 3 CDs, namely α-, β-, and γ- type, purification is needed to obtain each CD, logically resulting in a decrease of the yield. To our knowledge,

no report has been proposed of the production of CDs via CGTase using maltose as the substrate.

Furthermore, CDs produced by traditional methods have a ring-shaped structure. Making the selective introduction of different functional groups difficult, such differential functionalization of CD might enhance its functions, such as increasing water its solubility or increasing the affinity of CD for specific tissues present in certain human internal organs.

Therefore, in this report, we investigated the enzymatic synthesis of CDs in various organic solvent systems using maltose as a substitute for starch. A low-mol-wt compound is used in this synthesis, capable of forming an inclusion complex with CD or bonding of two glucose molecules. This prevents the formation of products with high viscosity and facilitates the purification of CDs. Furthermore, the use of maltose might make it possible to introduce different functional groups into a CD ring, affording derivatives that are more useful in drug-delivery applications.

MATERIALS AND METHODS

Enzymes

Cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19), from *B. macerans*, was obtained from Amano Pharmaceuticals, Co., Ltd. (Japan) as a crude enzyme solution having a specific activity of 600 U/mL. Glucoamylase (EC 3.2.1.3), from *Rhizopus niveus*, was purchased from Seikagaku Kogyo, Co., Ltd. (Japan) as a lyophilized powder with a specific activity of 12.1 U/mg dry weight.

Chemicals

α-, β-, and γ-Cyclodextrin were purchased from Sigma. Cyclohexane, cyclooctane, hexane, decane, dodecane, benzene, ethylbenzene, o-, m-, and p-xylene, ethanol, propanol, butanol, and other organic solvents were obtained from Waco (Japan). All other chemicals and solvents used in this work were of the highest purity commercially available. Water was of Milli Q purity.

Analysis of Reactants and Products

The concentrations of all products of the enzymatic reactions were determined by high-performance liquid chromatography (HPLC) analysis. A chromatography system equipped with a Shimadzu Inc. model LC-6AD pump, a CTO-6A column oven with a 20 μL loop, and a RID-6A refractive index detector were used. The detector signal was mathematically integrated by a Shimadzu C-R6A Chromatopac. Samples were analyzed using either an Asahipak NH2P-50 (Asahi Chemical) or TSKgel Amide-80 (Tosoh

Inc.) column (4.6 × 250 mm) protected by a guard column (30 mm) with the same packing. All samples were eluted with acetonitrile:water (55:45% [v/v]) at a flow rate of 0.8 mL/mi. Column temperature was kept at 30°C.

Nuclear magnetic resonance (NMR) spectra were recorded on a Joel JNM-A500 spectrometer. ^{13}C NMR analyses were carried out on standard β-CD and the experimentally synthesized compound in deuterium oxide (D_2O) at 19.4°C.

Enzymatic Reaction

CGTase-catalyzed reactions were performed by placing 20% (w/w) of maltose into 44% (v/v) cyclohexane or other organic solvent systems. The reaction was initiated by adding CGTase at a concentration of 300 U/g maltose, while constantly stirring (400 rpm) with a magnetic stirrer at 7°C. After the reaction, an aliquot of the sample to be analyzed was boiled for 5 min to completely inactivate the CGTase. Subsequently, glucoamylase was added to the reaction medium for hydrolysis of by-products such as linear malto-origosaccharides. These samples were then subjected to HPLC, as described.

Experimental Precipitation Percentage in Various Organic Solvents

To each of the solutions of α-, β-, and γ-CD (1% [w/w] in water phase), an organic solvent was added at an excess amount followed by stirring for 30 min at 20°C. The organic solvents were each adjusted to a concentration of 44% [v/v]. The aqueous phase was used for HPLC analysis to determine by depletion the amount of the precipitate. No free CD was detected in the organic phase using HPLC. Complete precipitation of a CD was defined at 100%. Cyclododecane, naphthalene, and anthracene, which were solid at room temperature, were dissolved in hexane prior to use. Hexane and the above three organic solvents were all adjusted to 0.95:0.05% (w/w). The amount of solvents dissolved in hexane were adjusted enough to make an inclusion complex with CDs possible.

RESULTS AND DISCUSSION

HPLC and ^{13}C NMR Analyses of Enzymatic Products in the Water-Cyclohexane Systems

Figure 1 shows the HPLC chromatograms of the reaction products without (A) and with (B) 44% (v/v) of cyclohexane in the enzymatic reaction medium. CGTase catalyzes various kinds of reaction such as coupling and disproportionation besides the cyclization; therefore, many peaks appeared on the chromatograms. At the retention time of 17.8 min on the chromatogram, a peak was observed as shown in Fig. 1B, which was

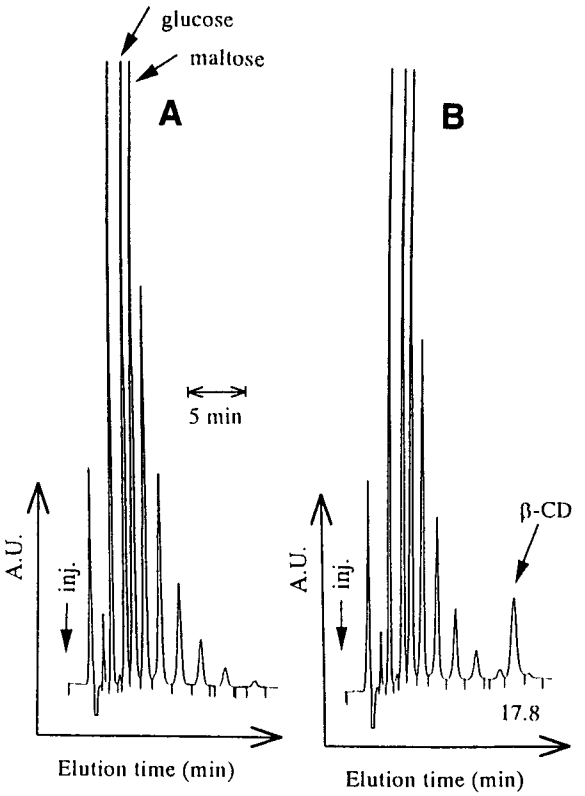

Fig. 1. Chromatograms of the reaction products using CGTase from *B. macerans* in different media; (**A**) no cyclohexane added; (**B**) 44% (v/v) cyclohexane added. (RI-signal in A.U.)

absent in Fig. 1A. Both reactions (A and B) have been carried out at 50 and 7°C; however, the peak at the retention time of 17.8 min has only been observed from the sample which has been carried out at 7°C in the water-cyclohexane system (Fig. 1B). This retention time of 17.8 min corresponded to that of standard β-CD.

Subsequently, glucoamylase was added to the above reaction medium to hydrolyze linear malto-oligosaccharides to glucose, since it is well known that CDs are not easily decomposed with glucoamylase. The chromatograms of the reaction products after adding glucoamylase are shown in Fig. 2. The peak corresponding to the standard β-CD remained in Fig. 2B. The second chromatography was also performed using the precipitated fraction from Fig. 2B. The retention time of the precipitant was the same as that of standard β-CD, and the single peak was observed (data not shown). In addition, ^{13}C NMR analysis of the peak observed at 17.8 has been performed on the precipitated fraction (from Fig. 2B) and a standard β-CD in D_2O. Both materials indicated the same data (*see* Appendix) and they also corresponded to the reported ^{13}C NMR data for β-CD (33).

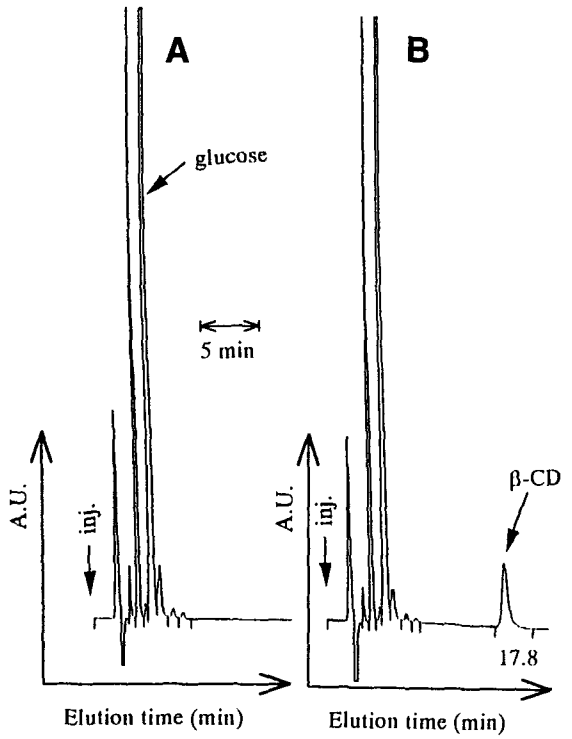

Fig. 2. Chromatograms of the reaction products after addition of glucoamylase to the reaction media of (**A**) and (**B**). (**A**) no cyclohexane added; (**B**) 44% (v/v) cyclohexane added. (RI-signal in A.U.)

These results indicate clearly that only β-CD has been synthesized, by using maltose as the substrate through the reaction of CGTase in the water-cyclohexane biphasic system. As many workers pointed out, CGTase converts (liquefied) starch to α-, β-, and γ-CD and linear malto-oligosaccharides in conventional aqueous systems (*34–36*). However, specific synthesis of β-CD from maltose was never achieved in such systems.

Throughout the enzymatic reaction in the water-cyclohexane biphasic solvents system, gradual accumulation of white precipitant was observed in the reaction medium, which appeared to be β-CD when subjected to HPLC analysis. Moreover, the glucose concentration in the reaction medium also increased as the reaction proceeded. From these results, it might be speculated that each maltose molecule might be cleaved to glucose and one of them used for the synthesis of malto-oligosaccharides by CGTase. The other glucose molecule might be released to the medium and subsequently cycled to β-CD before or after forming an inclusion complex with cyclohexane. Consequently, precipitation of β-CD might be observed in the reaction medium. Next, we will describe several factors effecting on the β-CD synthesis.

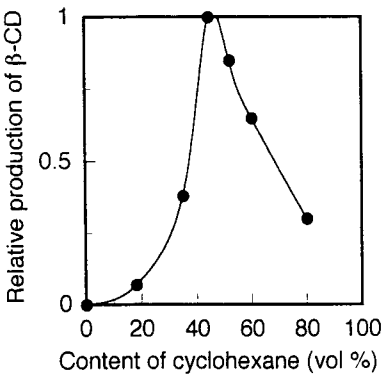

Fig. 3. Effect of cyclohexane content on the synthesis of β-CD from maltose as substrate in water-cyclohexane biphasic solvent systems.

Effect of Cyclohexane Content

The relative yield of β-CD (maximal yield of β-CD was defined as 1.0) after reaction of 66 h at 7°C, as a function of the cyclohexane concentration (cyclohexane % (v/v) to the total volume of the reaction solution), is shown in Fig. 3. A bell-shaped curve was obtained with respect to the cyclohexane content. This result indicates that maximal β-CD production is generated in the water-cyclohexane system containing 44% (v/v) cyclohexane. With respect to the water content, similar bell-shaped curves were reported in our previous work on the hydrolysis of various polysaccharides in water-immiscible organic two-phase solvent systems (*37,38*). The use of water-immiscible organic solvent systems to enhance the enzymatic reaction of saccharification polysaccharides suggests that the structure (i.e., the surface area of the substrate) might be altered favoring enzyme attack. For the synthesis of β-CD from maltose, however, it is unlikely that an alteration of substrate structure is responsible since maltose is a small molecule with a simple structure. The most acceptable explanation of the bell-shaped curve observed is (1) a favorable concentration of cyclohexane to form an inclusion complex with cyclodextrin, and (2) an adequate ratio of water:cyclohexane to maintain the CGTase activity.

Effect of pH

The effect of pH on the β-CD synthesis in the water-cyclohexane system containing 44% cyclohexane, using 20% (w/w) maltose as substrate, is shown in Fig. 4. Each pH was adjusted before the enzymatic reaction was carried out. The results indicate that the optimal pH is around 6.0, which is almost the same optimal pH value as the conventional synthesis of CDs from starch in an aqueous solution using *B. macerans* (*36,39,40*).

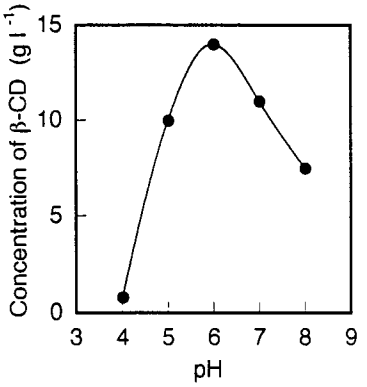

Fig. 4. Effect of pH in the aqueous phase of water-cyclohexane system on the CGTase catalyzed synthesis of β-CD from maltose.

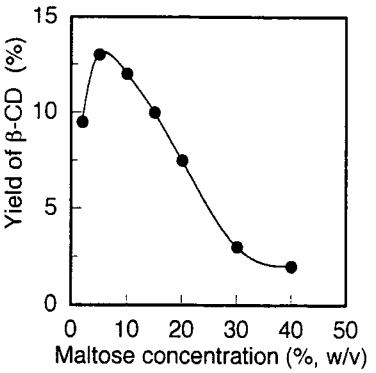

Fig. 5. Effect of maltose concentration on the enzymatic synthesis of β-CD in the water-cyclohexane system.

Effect of Maltose Concentration

As shown in Fig. 5, β-CD has been synthesized at various concentrations of the substrate. The enzyme concentration was maintained at a constant level, 300 U/g maltose. Cyclohexane (44% (v/v) was used as an organic solvent, and the enzymatic reaction had been carried out for 48 h under the same conditions as given in Fig. 3. The results indicated that the elevation of the maltose concentration increased the yield of β-CD, and the maximal yield of β-CD, 13% (w/w) of initial maltose was obtained at a concentration of 5% (w/w) maltose. However, the yield quickly decreased when the maltose concentration exceeded above 5% (w/w).

It has been reported (*26*) that CGTase cannot only produce but also decompose CDs in the presence of cosubstrates such as glucose, maltose, and sucrose. In addition, the effect of maltose concentration on the hydrolysis of β-CD by CGTase in the presence or absence of organic solvent has been reported as well (*41*). From these reports, it might be possible that the inhibition of CGTase by maltose caused the reduction of β-CD yield at a high concentration range of maltose (Fig. 5).

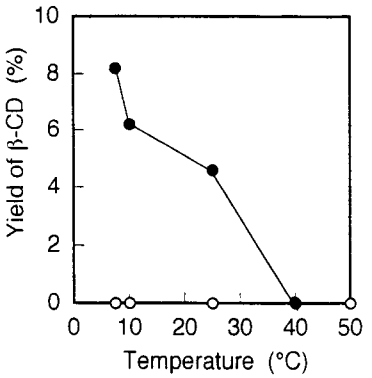

Fig. 6. Effect of temperature on the enzymatic synthesis of β-CD in the water-cyclohexane system. Water-cyclohexane (●) and water (○) systems.

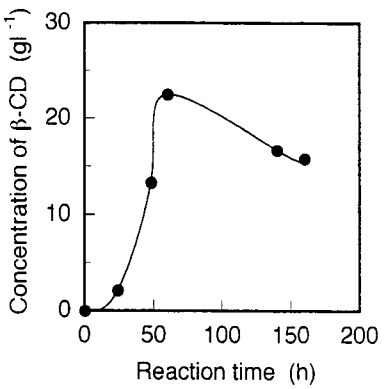

Fig. 7. Time course of β-CD synthesis by CGTase in the water-cyclohexane system.

Effect of Temperature

The effect of temperature on the β-CD synthesis was also examined (Fig. 6). No synthesis of β-CD occurred at 40°C in the water-cyclohexane system, but that reaction occurred at temperatures below 40°C. The maximal yield of β-CD in this experiment was obtained at the reaction temperature, 7°C. On the other hand, no synthesis of β-CD has been observed in an aqueous solution at any of these temperatures. A conventional method for the enzymatic synthesis of CDs from starch in aqueous solution uses CGTase around 50°C. On the other hand, it is known that the binding of CGTase to starch is stronger at low temperatures when the reaction is directed to cyclic synthesis (42). At a low temperature, the precipitation of synthesized β-CD might be accelerated in the water-cyclohexane system by the reduction of β-CD solubility to water.

Time Course of β-CD Production

Experiments were also performed to relate the yield of β-CD in the water-cyclohexane system to the reaction time (Fig. 7). As can be seen,

the β-CD yield reached a maximum after 60 h. However, beyond 60 h, the yield of β-CD gradually decreased. The reaction of β-CD hydrolysis by CGTase might be an explanation for this phenomenon, as described in the introduction.

Enzymatic Synthesis in Various Water-Organic Solvent Systems

From all of these experiments, the following questions arose:

1. Why is β-CD synthesized only in two-phase solvent systems?
2. Why is only the β-CD synthesized?
3. What is the mechanism of the synthesis of β-CD? and
4. Can other organic solvents be used in the enzymatic synthesis of CDs from maltose?

The most remarkable property of the cyclodextrins is their ability to form inclusion complexes with a variety of molecules that apparently only have to satisfy one condition: they must fit into the cyclodextrin cavity (*25*). CDs easily form inclusion complexes with organic substances or solvents of low-mol wt (*43,44*), which might lead to a conformational change and precipitation of the CDs. Thus, it seems possible that CDs are protected from hydrolytic reactions by CGTase once they form an inclusion complex with organic solvent (*45*). Throughout our experiments, a gradual increase in precipitant with time was observed in the water-cyclohexane reaction system.

To confirm the effect of organic solvent on the enzymatic synthesis of CDs using maltose as substrate, various organic solvents have been used in accordance with the above experiments. As shown in Table 1, the solvents that show a high precipitation ratio of β-CD (more than 87.5% except for cyclooctane) in the precipitation tests in the absence of CGTase, also synthesized only β-CD in the enzymatic reaction medium. With respect to α- and γ-CD, there were some organic solvents that could precipitate a higher proportion of α- and γ-CD; however, no production of α- and γ-CD was observed in the enzymatic reaction medium.

Water-miscible alcohols, such as ethanol and propanol, did not show any precipitation of CD, but both α- and β-CD were obtained in the enzymatic reaction medium. Some reports describe the enhancement of CD synthesis from starch in ethanol or propanol mixture solutions (*45–47*). The main reason for the promotion of CD production by addition of an alcoholic solvent is thought to be a decreased activity of water in the reaction mixture, specifically decreasing the hydrolytic reaction of the CGTase. Hydrolysis, as an occasional side reaction on the active site, might be less probable in the presence of any substantial amount of other solvents than water (*45*).

Table 1
Effect of Various Organic Solvents
on the Experimental Precipitation Percentage by Forming an Inclusion Complex with Cyclodextrins, and the Relationship with the Production of Cyclodextrins from the Enzymatic Reaction in Each of the Organic Solvents

Organic solvents	Precipitation percentage			Type of CD produced
	α-CD	β-CD	γ-CD	
Hexane	39.2	50.9	17.9	×
Decane	49.0	14.1	11.6	×
Dodecane	60.7	25.8	17.1	×
Tridecane	62.8	16.2	17.2	×
Hexadecane	69.5	19.2	17.8	×
Cyclohexane	84.1	94.0	0.0	β-
Cyclooctane	8.7	56.4	20.0	β-
Benzene	11.1	87.5	11.8	β-
Ethyl benzene	13.3	93.3	94.9	β-
o-Xylene	0.0	97.7	96.6	β-
m-Xylene	0.0	93.8	88.6	β-
p-Xylene	10.1	96.7	27.0	β-
o-Dichlorobenzene	5.3	98.9	99.2	β-
Tetrachloroethylene	8.0	98.4	93.9	β-
Chloroform	2.1	89.5	92.2	β-
Cyclododecane + Hexane	35.7	91.0	0.0	β-
Naphthalene + Hexane	55.4	99.5	85.5	β-
Anthracene + Hexane	34.0	90.8	0.0	β-
Ethanol	0.0	0.0	0.0	α-, β-
Propanol	0.0	0.0	0.0	α-, β-
Butanol	0.0	0.0	0.0	α-, β-
Pentanol	0.0	0.0	0.0	α-, β-

×, none observed.

Three phenomena were observed from the aforementioned experiments:

1. production of many malto-oligosaccharides both in water and water-cyclohexane systems;
2. precipitation proceeded gradually only in the water-cyclohexane system, and was proportional to the enzymatic reaction time; and
3. accumulation of glucose was observed both in water and water-cyclohexane systems.

From these phenomena we propose the following hypothesis. Initially, a maltose molecule ws trapped at the substrate binding site of the CGTase and hydrolyzed into two molecules of glucose by the hydrolytic reaction

of the CGTase. At that time, one glucose molecule was released to the reaction medium, and another glucose molecule remained at the binding site of CGTase. Subsequently, another maltose molecule remained at the binding site and was combined to that remaining glucose molecule to form a molecule of malto-triose. This malto-triose was released to the reaction medium or used for a next synthesis reaction with maltose. Thus, the concentrations of glucose and many kinds of malto-oligosaccharides were increased, as indicated in both water and water-cyclohexane systems (Fig. 1A,B). In water-cyclohexane systems, CD was synthesized by the enzymatic reaction of cyclization of CGTase, using these malto-oligosaccharides as substrates. It might be possible to synthesize CD in water (*see* Fig. 1A), where many malto-oligosaccharides were formed. However, these CD might be degraded quickly into malto-oligosaccharides because of the disproportion reaction or hydrolyzation of CGTase. In water-organic solvent systems, CDs form inclusion complexes with organic solvent, and subsequent precipitation prohibits degradation by CGTase.

CONCLUSIONS

Our study clearly demonstrates, for the first time, a novel enzymatic synthesis method for CDs from maltose in various two-phase organic solvent systems, but does not provide direct evidence for the reaction mechanism. The enzymatic reaction in an aqueous solution, using CGTase and maltose, might also briefly produce CDs in the reaction mixture; however, their degradation might be too fast and therefore difficult to detect.

Furthermore, our research suggests a different synthesis mechanism of CDs using either hydrophilic or hydrophobic organic cosolvents. No precipitation and specificity of β-CD has been observed in hydrophilic cosolvents, but β-CD could be synthesized in both hydrophilic and hydrophobic organic cosolvents. Further work is aimed at elucidating the mode of action of both organic solvent systems on the CDs synthesis, and synthesizing novel hetero-CDs consisting of glucose and other kinds of saccharides in their cyclic structure.

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APPENDIX

^{13}C NMR (D_2O) data of precipitated fraction and standard β-CD. Both data were the same as shown below.

C-1 (102.56 ppm), C-2 (72.47), C-3 (73.75), C-4 (81.80), C-5 (72.75), C-6 (60.94).

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