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Bacillus macerans cyclomaltodextrin glucanotransferase transglycosylation reactions with different molar ratios of D-glucose and cyclomaltohexaose☆

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Dedicated to Professor Derek Horton, on the occasion of his 70th birthday, and in recognition of his long and dedicated service as an Editor of *Carbohydrate Research*, from its inception in 1965 to the present

Abstract

It was found that *Bacillus macerans* cyclomaltodextrin glucanotransferase (CGTase) reacts with cyclomaltohexaose (α-cyclodextrin, α-CD) to give a series of cyclomaltooligosaccharides (cyclomaltodextrins, CDs), having seven to more than 20 D-glucose residues and maltooligosaccharides (maltodextrins, MDs) from G5 to G12+. When D-glucose (Glc) was added to the α-CD at very low molar ratios (1:100) of Glc to α-CD, the predominant products (95%) were CDs, some of which were macrocyclic MDs with 20–60 D-glucose residues, along with MDs that also had high molecular weights, containing 10–75 D-glucose residues and gave a blue iodine–iodide color. As the molar ratio of Glc to α-CD was increased, the amount of CDs progressively decreased and MDs proportionately increased in the range of G2–G12. At 25 mM α-CD and Glc to α-CD molar ratio of 1:1, a 75% yield of MDs, G1–G12, each in approximately equal amounts, was obtained; and at 20 mM and a 5:1 ratio, a 97% yield of MDs, G2–G9, was obtained but in unequal amounts. At higher ratios (10:1), the CDs completely disappeared, and at very high ratios (50:1 to 100:1) only low-molecular-weight MDs, G2–G4, were formed.

Keywords: Cyclomaltodextrin glucanotransferase; Cyclomaltooligosaccharides; Cyclomaltodextrins; Maltooligosaccharides; Maltodextrins; Macrocyclic maltooligosaccharides; Macrocyclic maltodextrins; Amylose; Transglycosylation reactions; Acceptor reactions; Coupling reactions

1. Introduction

Bacillus macerans cyclomaltodextrin glucanotransferase [(1→4)-α-D-glucan 4-α-D-(1→4)-α-D-glucano)-transferase cyclizing, CGTase, EC 2.4.1.19)] catalyzes three types of reactions: (1) reaction with the nonreducing ends of starch chains to give a mixture of cyclomaltooligosaccharides (cyclomaltodextrins, CDs) primarily composed of 6, 7, and 8 D-glucose residues, linked α-(1→4) in a nonreducing cyclic structure, designated as α-, β-, and γ-cyclomaltodextrins (α-, β-, γ-CDs),[1,2] respectively; (2) 'coupling reactions' that occur when the CD rings are opened and the resulting MD is transferred to carbohydrate acceptors, such as D-glucose, maltose, sucrose, and so forth,[3] or to water, which gives a very low degree of hydrolysis; and (3) disproportionation reactions, which occur between two MD molecules in which part of one is transferred to the nonreducing end of the other.[2,4]

Demonstration of higher CDs, composed of 9, 10, 11, and 12 D-glucose residues was first made by Pulley and French[5] in 1961, and the structures were determined by French, et al.[6] in 1965. CDs, having 9 – 13

☆ An oligosaccharide made up of α-(1→4)-linked glucose units is named according to IUPAC as a 'maltooligosaccharide'. Older names include the terms 'dextrin' or 'maltodextrin' (abbreviated as 'MD'). A cyclic maltooligosaccharide is known by its common name as a 'cyclodextrin' (abbreviated 'CD'). A group of 'macrocyclic maltodextrins' are termed 'macrocyclic MDs'. The abbreviated terminology (CD, MD, macrocyclic MD) is used throughout this paper.

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D-glucose residues were isolated in larger amounts, and their properties were determined by Endo, et al.[7,8] in 1995. Very large CDs, having up to 60 D-glucose residues, were reported in 1997 to have been formed by the reaction of CGTase with amylose.[9]

The present study reports on the qualitative and quantitative distribution of products of the CGTase coupling reactions between different molar ratios of 1:100 to 100:1 of D-glucose (Glc) to cyclomaltohexaose (α-CD). A comparison is also made for different concentrations of α-CD. It was found that the type of products formed and their amounts varied for the different ratios and concentrations.

2. Experimental

Materials.—*Bacillus macerans* CGTase was elaborated by growing *B. macerans* ATCC 8517 on wheat bran media[10] and purified by a modification of the method of Kobayashi et al.,[10] as previously described.[11] Barley β-amylase was obtained from Megazyme International, Wicklow, Ireland. It was a highly purified enzyme with a specific activity of 1400 IU/mg with starch as the substrate and reported to have <0.33 ppm of α-amylase. α-CD was available in the laboratory, prepared by methods previously reported;[2] β- and γ-CD were obtained from Ensiuko Sugar Co., Yokohama, Japan. The three CDs were pure by TLC analysis.

Assay of the activity of CGTase.—CGTase activity was measured by a modification of the method of Thoma et al.[12] as previously described.[11] One CGTase unit (an International Unit, IU) was the number of μmoles of D-glucose, divided by 6, that is formed per min in the reaction of α-CD with methyl α-D-glucopyranoside in the presence of excess glucoamylase.

Reaction conditions and analysis.—CGTase (4.2 μL, 200 mIU) was added to substrate solutions (200 μL), containing 25 mM imidazole·HCl buffer (pH 6.0), D-glucose and α-CD in various molar ratios from 1:100 to 100:1. Two major concentrations of α-CD were used, 100 mM and 50 mM in molar ratios of 1:100–5:1; other concentrations of 25, 20, 10, 5, 2, and 1 mM were used to obtain specific molar ratios from 1:1 to 100:1. The reactions were carried out at 37 °C, and 30-μL aliquots were taken after 24 h; the reaction was stopped by heating in a boiling water bath for 5 min, diluted 5-fold with water, and analyzed by TLC. An amount (1–3 μL) was spotted onto a 10×20 cm Whatman K5 silica-gel plate. The plate was irrigated 3-times (18 cm path length for each irrigation) with 85:25:55:50 volume proportions of CH_3CN–EtOAc–PrOH–H_2O. The carbohydrates on the plate were visualized by dipping into a methanol solution, containing 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) sulfuric acid, dried, and heated at 120 °C for 10 min. They were quantitatively determined by scanning densitometry.[13]

Large-scale preparation of insoluble product.—The insoluble product obtained from the reaction of CGTase with 1 mM D-glucose and 100 mM α-CD was prepared on a larger scale by adding 10 IU of CGTase to 10 mL of the substrate in 25 mM imidazole·HCl buffer (pH 6.0), containing 1 mM $CaCl_2$. The reaction was carried out at 37 °C for 24 h, and was stopped by heating for 5 min in a boiling water bath. The insoluble material (~ 0.5 g) was obtained by centrifuging at $4000 \times g$ for 20 min and was washed with water (3×8 mL).

β-Amylase digestion of CGTase reaction products.—β-Amylase was used to remove MDs in the CGTase reaction products. The carbohydrate (10–100 mg in 4.5 mL of 25 mM imidazole·HCl, pH 6.0) was diluted to 17 mL with water; 45 IU (3 mL, 15 IU/mL) of barley β-amylase was added, and the reaction allowed to go 24 h at 37 °C.

Fluorescent-assisted capillary electrophoresis (FACE)[14].—Insoluble carbohydrates (10 mg) were dissolved in 1 mL of 1 M NaOH, and then diluted with water to 8 mL. The solution was neutralized (~ 1 mL of 1 M HCl), and the final volume was adjusted to 10 mL; 40 μL of this solution, containing 40 μg of carbohydrate, was taken to dryness, using a Speedvac evaporator; 2 μL of APTS (5 mg of 8-amino-1,3,5-pyrenetrisulfonic acid in 48 μL of 15% acetic acid) and 2 μL 1 M sodium cyanoborohydride in tetrahydrofuran was added, and the mixture was allowed to react for ~ 15 h at 42 °C; 46 μL of Milli Q-pure water was added, and the solution was centrifuged for 2 min. The supernatant (5 μL) was diluted with 195 μL of Milli Q-pure water and transferred to a 0.5-mL tube for capillary electrophoresis for 60 min using a P/ACE MDQ Glycoprotein Electrophoresis System (Beckman Coulter, Fullerton, CA).

Analysis of CDs by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS)[15,16].—The carbohydrate samples were treated with β-amylase to hydrolyze the MDs to maltose and maltotriose; 1 mg of each sample was dissolved in 1.0 mL of pure water and filtered through a 0.2-μm membrane; 10 μL of the filtered solution was mixed with 10 μL of 0.1 M 2,5-dihydroxybenzoic acid in CH_3CN; 1 μL of this solution was transferred to the probe, and the solvent was evaporated under vacuum. The masses of the compounds in the sample were analyzed by MALDI–TOF MS[15,16] using a Voyager-DE Pro instrument (Perseptive Biosystem, Framingham, MA) with a nitrogen laser (337 nm). Ions were detected in a positive-ion mode at an acceleration voltage of 20 kV. The theoretical mass numbers of the CDs were calculated as $162 \times \mathrm{dp} + 23$ for sodium-bound molecules or $+40$ for potassium-bound molecules.

3. Results

CGTase reacted with α-CD in the absence of any acceptor. Two concentrations of α-CD were used, 100 mM and 50 mM. The results (column I, Tables 1 and 2) show that the primary products were CDs, α- to κ-CDs, having 6 to 16 D-glucose residues, comprising 82.2% and 73.3%, respectively. The predominant products were β-CD (cyclomaltoheptaose) and γ-CD (cyclomaltooctaose).

The addition of D-glucose to the reaction with 100 mM α-CD to give a Glc to α-CD molar ratio of 1:100 gave a series of products of CDs and MDs (lane 3, Fig. 1) that were quantitatively determined by TLC densitometry.[13] This mixture was treated with β-amylase to convert the MDs to maltose and maltotriose and CDs (lane 4, Fig. 1). The quantitative values for the CDs from lane 4 of Fig. 1 were subtracted from the values obtained for lane 3 of Fig. 1 to give the amounts of individual MDs. The quantitative amounts for CDs and MDs are given in column II of Tables 1 and 2 for 100 mM and 50 mM α-CD, respectively. A 1:100 molar ratio of Glc to α-CD gave 95% CDs and 5% MDs with dp values of 7 to >12 for 100 mM α-CD and 84% CDs and 16% MDs for 50 mM α-CD, with dp values of 6 to >12. After β-amylase treatment (lane 4, Fig. 1), there remained compounds that were believed to be CDs because of their resistance to hydrolysis by β-amylase, which requires a nonreducing end for action. Their molecular sizes were analyzed by MALDI–TOF MS[14,15] and were found to be a series of CDs from α-CD to ν-CD, having 6–25 D-glucose residues (see, Fig. 2). The predominant CDs were α-, β-, γ-, δ-, ε-, ζ-, η-, having 6 to 12 D-glucose residues.

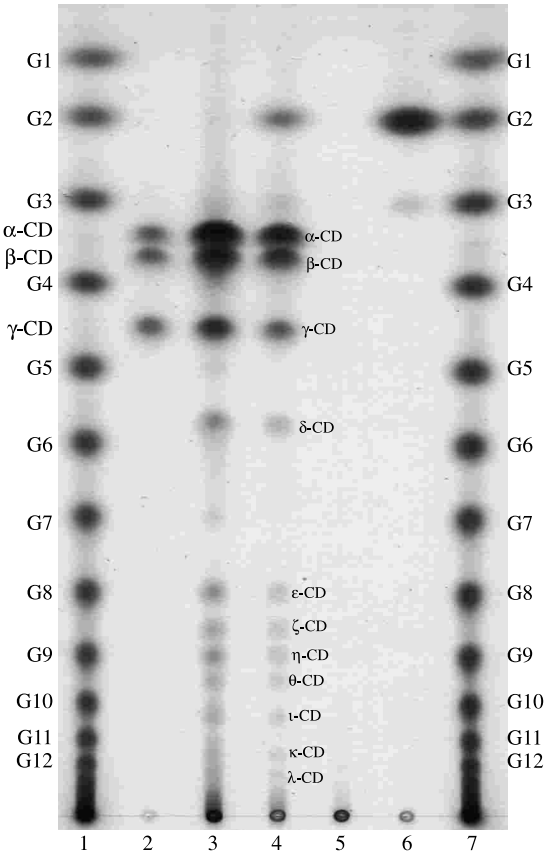

Fig. 1. TLC analysis of the reaction of CGTase with a Glc to α-CD molar ratio of 1:100. Lanes 1 and 7 are MD standards. Lane 2 is α-, β-, and γ-CD standards. Lane 3 is the reaction digest. Lane 4 is the reaction digest after treatment with β-amylase. Lane 5 is the insoluble product formed that was dissolved in 0.1 M NaOH, diluted to 1 mg/mL and neutralized. Lane 6 is the treatment of the material in Lane 5 with β-amylase.

An insoluble material was produced in the reactions with 100 mM and 50 mM α-CD with molar ratios of 1:100 to 1:5. The largest amount was produced in the 100 mM α-CD with a molar ratio of 1:100. This insoluble material was dissolved in 0.1 M NaOH, followed by dilution to 1 mg/mL and neutralization. The material did not migrate on the TLC plate (lane 5, Fig. 1) and gave a dark blue color with iodine–iodide, indicating relatively large MDs, approaching the size of amylose. Analysis of this solubilized material by fluorescence-assisted capillary electrophoresis (FACE),[14] showed a broad distribution of MDs (CDs were not detected because of the absence of a reducing end, which reacts with the fluorescent dye) from dp 4 to dp 75 (see, Fig. 3). When this solubilized material was treated with β-amylase to remove the MDs, G2 + G3 and a material that did not migrate from the origin of the TLC plate were obtained (lane 6, Fig. 1). This soluble, β-amylase-resistant material was composed of CDs of high molecular weight, which were precipitated with 2 volumes of ethanol, dried, and dissolved in water and analyzed by MALDI–TOF MS[15,16] to determine their number and size. The results show CDs with molecular sizes from 10 to 60 D-glucose residues (Fig. 4). CDs, dp 6–25 in Fig. 2, gave a weak wine color with iodine–iodide, and the macrocyclic MDs, dp 10–60 in Fig. 4, gave a weak purple color with iodine–iodide.

The products of the reactions, using different ratios of Glc to α-CD, were analyzed by TLC densitometry, both before treatment with β-amylase and after treatment, and the quantitative amounts of CDs and MDs were determined for each ratio by densitometric TLC. As the molar ratios of Glc to α-CD were increased from 1:50 to 1:1, the amount of CDs decreased from 93% to 2.3% for 100 mM α-CD (Table 1, columns III–VII). The major difference between the two concentrations of α-CD was the much higher amount of MDs obtained (98%) for the 1:1 molar ratio for 100 mM α-CD compared to 76% obtained for 50 mM α-CD (compare, columns VII in Table 1 and Table 2). The individual amounts of the MDs obtained from the 50

Table 1
Percent molar composition of the products from the reaction of CGTase with various molar ratios of D-glucose to 100 mM cyclomaltohexaose (α-CD) after 24 h at 37 °C[a]

Experiment	I	II	III	IV	V	VI	VII
	100 mM α-CD only	1 mM G1+100 mM α-CD	2 mM G1+100 mM α-CD	5 mM G1+100mM α-CD	10 mM G1+100 mM α-CD	20 mM G1+100 mM α-CD	100 mM G1+100 mM α-CD
[G1]/[α-CD]	0	1/100	1/50	1/20	1/10	1/5	1/1
α-CD	28.1	34.8	34.0	30.0	19.2	16.1	1.1
β-CD	22.6	24.2	24.3	24.1	26.4	21.5	0.8
γ-CD	10.7	13.6	14.0	14.6	12.9	10.6	0.4
δ-CD	4.1	3.4	3.6	3.3	1.9	1.1	–
ε-CD	1.0	1.8	2.1	1.7	1.0	0.8	–
ζ-CD	1.5	1.4	1.4	1.2	0.3	0.4	–
η-CD	0.9	1.4	1.6	1.3	0.6	0.7	–
θ-CD	0.7	1.6	1.6	1.5	0.4	0.3	–
ι-CD	0.5	0.9	1.1	0.9	0.5	0.4	–
κ-CD+	12.1	11.7	8.8	8.9	9.2	6.4	–
Total CDs	82.2	94.9	92.5	87.6	72.3	58.3	2.3
G1	–	–	–	–	–	1.0	21.1
G2	–	–	–	–	1.0	1.6	20.0
G3	1.0	–	–	–	1.0	2.8	15.8
G4	1.1	–	–	–	4.0	4.0	11.3
G5	0.6	–	–	1.4	2.1	2.9	9.7
G6	0.5	–	0.9	1.1	1.7	2.9	7.3
G7	1.4	0.5	0.7	0.9	1.9	3.8	5.1
G8	1.3	0.5	0.2	0.5	1.7	2.5	2.6
G9	1.4	0.2	0.3	0.4	2.1	2.6	1.9
G10	0.8	0.5	0.3	0.5	1.4	2.0	1.1
G11	1.1	0.9	1.0	1.2	1.4	1.9	0.7
G12+	8.6	2.5	4.1	6.4	9.2	13.7	1.2
Total MDs	17.8	5.1	7.5	12.4	27.7	41.7	97.7

[a] D, cyclodextrin; MD, maltodextrin; G*n*, n = number of glucose units.

Table 2
Percent molar composition of the products from the reaction of CGTase with various molar ratios of D-glucose to 50 mM cyclomaltohexaose (α-CD) after 24 h at 37 °C[a]

Experiment	I	II	III	IV	V	VI	VII	VIII
	50 mM α-CD only	0.5 mM G1 + 50 mM α-CD	1 mM G1 + 50 mM α-CD	2.5 mM G1 + 50 mM α-CD	5 mM G1 + 50 mM α-CD	10 mM G1 + 50 mM α-CD	50 mM G1 + 50 mM α-CD	250 mM G1 + 50 mM α-CD
[G1]/[α-CD]	0	1/100	1/50	1/20	1/10	1/5	1/1	5/1
α-CD	23.0	19.4	19.8	17.0	19.9	16.6	8.7	–
β-CD	27.6	29.3	15.3	16.2	22.8	23.4	11.3	–
γ-CD	14.1	15.1	13.5	13.8	14.8	11.8	3.6	–
δ-CD	1.6	3.5	2.8	3.2	2.3	1.6	0.4	–
ε-CD	1.3	1.9	1.8	1.3	1.0	0.9	–	–
ζ-CD	0.8	1.8	1.6	1.5	0.9	0.9	–	–
η-CD	0.8	1.5	1.5	1.1	0.7	0.6	–	–
θ-CD	0.5	1.4	1.5	1.0	0.9	0.9	–	–
ι–CD	0.5	0.9	1.0	0.7	0.4	0.3	–	–
κ-CD+	3.2	9.1	14.6	11.3	3.8	2.8	–	–
Total CDs	73.3	83.9	73.5	67.3	67.5	59.8	24.1	0.0
G1	1.4	–	1.1	2.6	2.0	2.6	4.4	61.2
G2	1.8	–	1.7	2.7	3.0	3.6	6.7	23.1
G3	2.7	–	1.9	2.7	1.8	1.6	7.8	12.8
G4	2.8	–	9.2	6.6	5.6	7.4	6.8	2.6
G5	1.8	–	1.8	2.7	2.5	3.1	8.3	0.3
G6	1.1	2.6	2.6	2.7	2.8	2.9	7.3	–
G7	1.2	2.1	2.1	2.8	1.9	2.7	6.9	–
G8	0.6	1.5	1.4	1.9	1.5	1.7	4.2	–
G9	0.6	1.3	1.0	1.3	1.4	1.8	4.0	–
G10	0.9	0.9	1.1	1.5	1.2	1.6	3.2	–
G11	1.0	2.0	1.9	2.1	1.3	1.5	3.3	–
G12+	11.1	5.7	0.5	3.1	7.5	9.7	13.1	–
Total MDs	26.7	16.1	26.5	32.7	32.5	40.2	75.9	100.0

[a] CD, cyclodextrin; MD, maltodextrin; G*n*, *n* = number of glucose units.

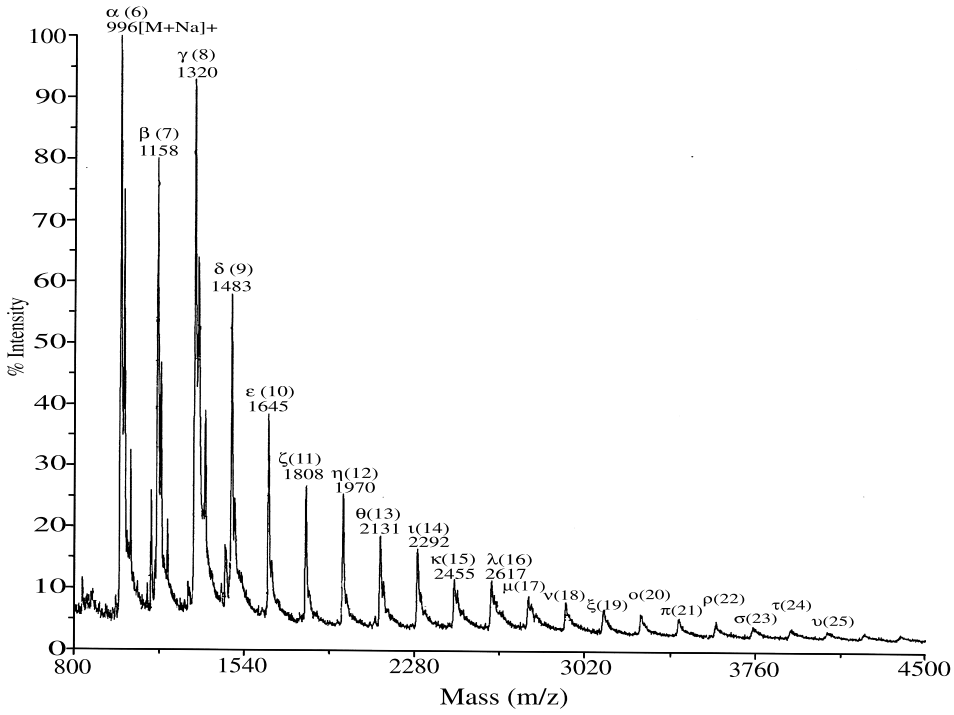

Fig. 2. MALDI–TOF MS analysis of the CDs that resulted from the β-amylase treatment of the CGTase reaction with a 1:100 molar ratio of Glc to α-CD. The Greek symbol for each CD is given next to the number of D-glucose residues in the CD. The mass (m/z) is given below the Greek symbol and the number of D-glucose residues.

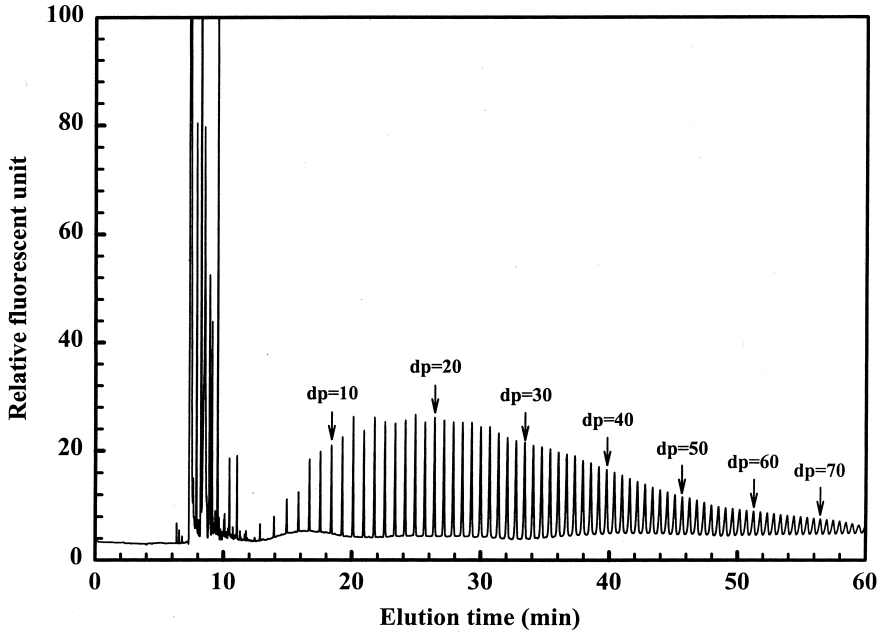

Fig. 3. FACE analysis of the MDs formed from the CGTase reaction with a 1:100 molar ratio of Glc to α-CD. Each peak is an individual MD with the indicated degree of polymerization (dp).

mM α-CD were closer to each other and in greater amounts than the MDs obtained from 100 mM α-CD. The major MDs for the latter were G2–G5, making up 64% of the total, while the major MDs of the former were G2–G12+, all in about the same amounts. Unreacted G1 also comprised 21% for the 100 mM α-CD reaction and only 4.4% for the 50 mM α-CD reaction.

When the molar ratio of Glc to α-CD was increased to 10:1, the CDs were completely absent, and the MDs increased to 100%, with G2–G5 predominating, and together G6–G8, making up 3.3–2.9% of the total

Table 3
Comparision of the percent molar composition of the products from the reaction of CGTase with various concentrations of D-glucose and cylomaltohexasoe (α-CD) in molar ratios of 1:1 to 100:1[a]

Experiment	I 100 mM G1 + 100 mM α-CD	II 50 mM G1 + 50 mM α-CD	III 25 mM G1 + 25 mM α-CD	IV 100 mM G1 + 20 mM α-CD	V 250 mM G1 + 50 mM α-CD	VI 100 mM G1 + 10 mM α-CD	VII 50 mM G1 + 5 mM α-CD	VIII 100 mM G1 + 5 mM α-CD	IX 100 mM G1 + 2 mM α-CD	X 100 mM G1 + 1 mM α-CD
[G1]/[α-CD]	1/1			5/1		10/1		20/1	50/1	100/1
α-CD	1.1	8.7	7.1	1.5	–	–	–	–	–	–
β-CD	0.8	11.3	12.3	0.8	–	–	–	–	–	–
γ-CD	0.4	3.6	4.8	0.4	–	–	–	–	–	–
δ-CD	–	0.4	1.3	–	–	–	–	–	–	–
ε-CD	–	–	–	–	–	–	–	–	–	–
ζ-CD	–	–	–	–	–	–	–	–	–	–
η-CD	–	–	–	–	–	–	–	–	–	–
θ-CD	–	–	–	–	–	–	–	–	–	–
ι-CD	–	–	–	–	–	–	–	–	–	–
κ-CD +	–	–	–	–	–	–	–	–	–	–
Total CDs	2.3	24.1	25.5	2.7	0.0	0.0	0.0	0.0	0.0	0.0
G1	21.1	4.4	6.6	22.7	61.2	36.6	40.0	52.4	69.4	83.7
G2	20.0	6.7	9.5	22.7	23.1	30.5	29.1	29.7	23.4	13.1
G3	15.8	7.8	7.7	16.6	12.8	17.3	15.4	11.9	6.4	1.7
G4	11.3	6.8	8.0	11.7	2.6	9.0	8.5	0.0	0.8	1.5
G5	9.7	8.3	6.7	9.2	0.3	3.2	4.1	4.1	–	–
G6	7.3	7.3	6.6	5.7	–	2.1	1.7	1.6	–	–
G7	5.1	6.9	5.5	3.5	–	0.8	0.8	0.4	–	–
G8	2.6	4.2	5.0	2.1	–	0.3	0.4	–	–	–
G9	1.9	4.0	3.9	1.1	–	0.1	–	–	–	–
G10	1.1	3.2	3.0	0.6	–	–	–	–	–	–
G11	0.7	3.3	2.5	0.4	–	–	–	–	–	–
G12 +	1.2	13.1	9.6	0.9	–	–	–	–	–	–
Total MDs	97.7	75.9	74.5	97.3	100.0	100.0	100.0	100.0	100.0	100.0

[a] CD, cyclodextrin; MD, maltodextrin; G*n*, *n* = number of glucose units.

(Table 3, columns VI and VII). As the molar ratios were increased further from 20:1 to 100:1 (Table 3, columns VIII–X), the size of the MDs decreased so that at a ratio of 100:1, G2 was the predominant (13%) product with G3 and G4 together making up 3.2%, and the D-glucose was only utilized to the extent of 16.3%.

A comparison of the effect of the concentration of α-CD shows that when the ratio of Glc to α-CD is 1:1, there is not much difference in the products that are formed from 50 mM and 25 mM α-CD (Table 3, columns II and III). But, for 100 mM α-CD, there was a 90% decrease in the amount of CDs that were formed for the 1:1 ratio and a very large amount of G1 and G2 made up the MDs (Table 3, column I). When the molar ratio of Glc to α-CD was increased to 5:1 and the concentration of α-CD was 20 mM, the majority (97.3%) of the products were MDs, and for the 50 mM concentration, 100% of the products were MDs (see Table 3, columns IV and V). The significant difference was in the distribution of the MDs: 20 mM α-CD gave a much broader distribution of products that ranged from G1 to $>$G12 in relatively equal amounts, while 50 mM α-CD gave only G1–G5, with 61.2% being G1. As the molar ratios of Glc to α-CD were increased further from 10:1 to 100:1 and the concentrations of α-D were 10 mM to 1 mM, the CDs completely disappeared, and the number of MDs progressively decreased with the amount of unreacted G1 progressively increasing (see, Table 3, columns VI–X).

4. Discussion

The reaction of pure CD with *B. macerans* CGTase was previously observed in the trapping of a covalent CGTase intermediate.[11] The enzyme opens the CD ring and in so doing, forms a covalent linkage to the reducing end of the resulting MD. The formation of products from the reaction of CGTase with only α-CD has not previously been reported. α-CD gave a mixture of CDs, having 6–11 D-glucose residues and MDs 5–12+ D-glucose residues. The presence of CDs was identified by treating the sample with β-amylase, which hydrolyzes MDs to maltose plus a small amount of maltotriose. The material that was resistant to β-amylase hydrolysis was considered to be CDs in that CDs do not have a nonreducing end glucose, which is required by β-amylase to carry out hydrolysis. CDs were further identified by their only giving a weak triiodide color. The size and number of the CDs were determined by their relative migration on TLC and by MALDI–TOF MS. The size and number of the MDs were determined by fluorescent-assisted capillary electrophoresis.

When different carbohydrates, called acceptors, are added to α-CD, CGTase opens the CD ring and transfers the resulting MD to the acceptor.[2,3] Qualitatively, we had observed that different molar ratios of the acceptor and α-CD gave different distributions of products.

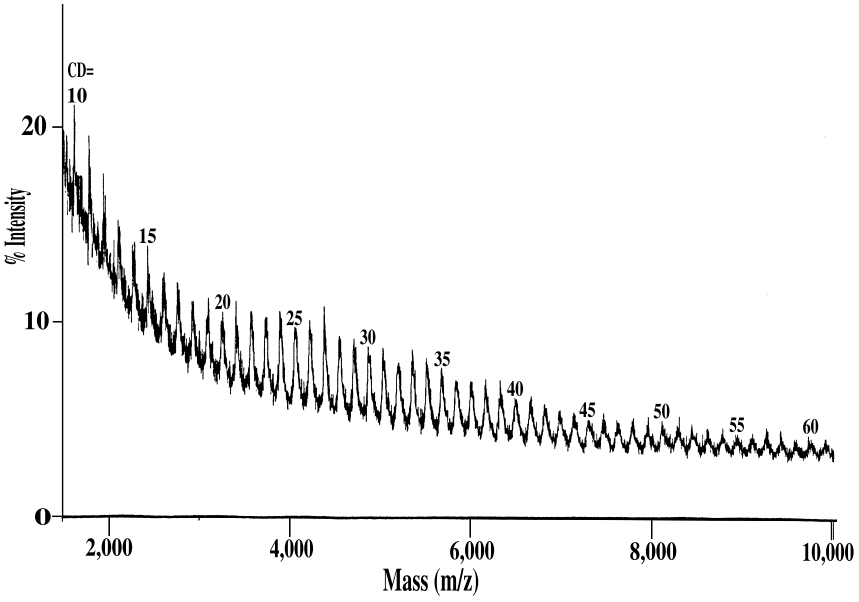

Fig. 4. MALDI–TOF MS analysis of the CDs that were present in the insoluble precipitate that formed from the reaction of CGTase reaction with a 1:100 molar ratio of Glc to α-CD. The precipitate was dissolve in 0.1 M NaOH, diluted to 1 mg/mL and neutralized, and then treated with β-amylase, which removes the MDs. The soluble, β-amylase resistant material (macrocyclic CDs), was precipitated with 2 volumes of ethanol and then dissolved in pure water to give 1.0 mg/mL for analysis. The dp's of the macrocyclic MDs are indicated above the peaks.

We have shown in the present study, that when the molar ratio of Glc to α-CD was low (e.g. 1:100), a large percentage and number of CDs were formed, including high-molecular-weight CDs (macrocyclic MDs), along with MDs, some of which also were of high molecular weight that gave a dark blue iodine–iodide color. Macrocyclic MDs were previously reported when CGTase reacted with amylose.[9] When the Glc to α-CD ratio was low in the CGTase reaction, 95% of the products were CDs, having 6–60 D-glucose residues. When the ratios of Glc to α-CD were increased, the amount of CDs progressively decreased and the amount of MDs proportionately increased. When the ratio was 1:5 for an α-CD concentration of 100 mM, the percent of CDs equaled 58.3% and MDs equaled 41.7%. When the ratio was 1:1, only 2.3% were CDs (α-, β-, γ-, and δ-CDs) and 97.7% were MDs, with 2–12+ D-glucose residues. When the ratio was increased to 10:1 and the α-CD was 10^{-5} mM, the CDs were completely absent and 100% of the products were MDs, G2–G9. When the ratio was increased to 50:1 and 100:1, no CDs were formed and the MDs were exclusively G2–G4.

The highest yield (75%) with the widest distribution of MDs, G1–G12+, which had relatively equal amounts for the individual MDs, occurred when the concentration of α-CD was 25 mM and the molar ratio of Glc to α-CD was 1:1. This concentration and ratio, however, still gave 25.5% α- to δ-CD. The next best condition for producing MDs was 20 mM α-CD and a molar ratio of Glc to α-DC of 5:1. This reaction only gave 2.7% CDs and 97.3% MDs, but the distribution of the individual MDs was much less equal, with 22.7% G2, 16.6% G3, and 11.7% G4. The amount of CDs and MDs and their distribution were nearly equal to that obtained for 100 mM α-CD and a molar ratio of 1:1.

Previously, it had been shown that the acceptor, D-glucose in this study, appears exclusively at the reducing end of the MD acceptor products,[17] and when the D-glucose is labeled with ^{14}C, reducing end labeled maltodextrins result[18] and have been used in the study of the mechanism of action of α-amylases.[19,20]

The main product of the reaction of *B. macerans* CGTase with starch is α-CD.[2,21] The reaction of *B. macerans* CGTase with β-CD only gave a small amount of α-CD and γ-CD; likewise the reaction with γ-CD also only gave a small amount of α-CD and β-CD. β-CD and γ-CD were not good substrates for the acceptor reactions with D-glucose and other acceptors catalyzed by *B. macerans* CGTase (data not presented).

CGTases from *B. megaterium*,[22] *B. circulans*,[23–25] and *B. stearothermophilus*[26] formed β-CD as the main product from starch, and *Brevibacterium* sp. CGTase[27] formed γ-CD as the main product from starch. It would be of interest to see if these CGTases would react with their specific products, β-CD and γ-CD, to produce a large number of CDs, including macrocyclic CDs. Further, it would be of interest to see if these CGTases would also catalyze the acceptor reactions with their specific CD-products, β-CD and γ-CD, as *B. macerans* CGTase-catalyzed acceptor reactions with α-CD and acceptors in different molar ratios.

In this study, we have put the acceptor (coupling) reaction of *B. macerans* CGTase on firmer ground by qualitatively and quantitatively determining the different kinds of products that result when D-glucose reacts with α-CD in different molar ratios and concentrations. We show that the types and amounts of the products are dependent on the concentration of the substrates and on the molar ratio of the acceptor to α-CD. Thus, the types and amounts of the products can be selected and controlled by the selection of the concentration and the molar ratio of the acceptor to α-CD.

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