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PRODUCTION OF LARGE-RING CYCLODEXTRINS COMPOSED OF 9 ~ 21 α -D-GLUCOPYRANOSE UNITS BY CYCLODEXTRIN GLUCANOTRANSFERASE —EFFECTS OF INCUBATION TEMPERATURE AND MOLECULAR WEIGHT OF AMYLOSE

Tomohiro Endo,^{a,*} Noriko Ogawa,^a Hiromasa Nagase,^a Haruyo Sambe,^b Takeshi Takaha,^b Yoshinobu Terada,^b Wolfgang Zimmermann,^c and Haruhisa Ueda^a

a) Department of Physical Chemistry, Hoshi University, Tokyo 142-8501, Japan;

b) Biochemical Research Laboratory, Ezaki Glico Co. Ltd., Osaka 555-8502, Japan;

c) Department of Microbiology and Bioprocess Technology, University of Leipzig, Leipzig 04103, Germany. E-mail: endoh@hoshi.ac.jp

Abstract – Cyclodextrin glucanotransferase (CGTase) is the only enzyme that can produce large-ring cyclodextrins (LR-CDs) composed of 9 ~ 21 α -D-glucopyranose units (CD₉ ~ CD₂₁). In this study, two amyloses with different average molecular weights (14.6 and 280 kDa) were incubated with CGTase at three different temperatures, and the yield and composition of the LR-CDs were examined. The composition was not significantly affected by the size of amylose used, but was greatly affected by the reaction temperature.

INTRODUCTION

Cyclodextrin (CD) is the common name for cyclomaltooligosaccharides and α -, β -, and γ -CDs are composed of 6, 7, and 8 α -D-glucopyranose units, respectively. CDs have been extensively studied, and have found application in various fields. These CDs are now produced industrially in fairly large quantities by an enzyme reaction of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), which can convert α -glucan into a mixture of CDs and maltodextrins. However, it is not yet generally recognized that CGTase also has the ability to produce CDs composed of more than 9 α -D-glucopyranose units.¹ These compounds are named large-ring CDs (LR-CDs) and significant progress has been achieved in this area of research over the last two decades. To date, CD₉ ~ CD₃₉ (CD_n, where *n* indicates the number of

α -D-glucopyranose units in the CD ring), have been purified and the physicochemical properties evaluated.² It has been shown that CD₉ ~ CD₁₇ and CD₂₆ have the ability to form inclusion complexes,³⁻⁹ although the molecular structures are different from the truncated cone structures.¹⁰⁻¹³ Since the discovery of effective LR-CD production enzymes at the end of the 1990's, the production method has developed enormously. Disproportionating enzyme from potato (potato D-enzyme, EC 2.4.1.25), heat resistance amylomaltase from *Thermus aquaticus* (EC 2.4.1.25), and glycogen debranching enzyme from *Saccharomyces cerevisiae* (EC 2.4.1.25/EC 3.2.1.33) can produce LR-CDs starting from CD₁₇, CD₂₂, and CD₁₁, respectively.¹⁴⁻¹⁶ Furthermore, a higher yield of LR-CD mixture is obtained when synthetic amylose of a higher average molecular weight is used as a substrate.¹⁷ A production system for obtaining an LR-CD mixture that contains CD₂₂ and larger has been established and such a mixture is now commercially available. On the other hand, CD₉ to CD₂₁ are still difficult to produce. So far, CGTase is the only enzyme known to produce CD₉ to CD₂₁. However, CGTase preferentially produces conventional CDs rather than LR-CDs and thus, the projected yield of LR-CDs is very low. In order to establish an effective system for the production of CD₉ ~ CD₂₁, we investigated the effects of incubation temperature and the molecular weight of amylose on the generation of LR-CDs by CGTase.

RESULTS AND DISCUSSION

It is reported that the yields of LR-CD by potato D-enzyme from synthetic amylose with average molecular weights of 10, 30, 100, and 320 kDa, were 62.2, 81.4, 95.5, and 97.5%, respectively.¹⁷ This indicates that the production of an LR-CD mixture using potato D-enzyme is considerably affected by the average molecular weight of amylose. In the case of CGTase from *Bacillus macerans*, the total CD yields of amylose EX-III (average molecular weight 14.6 kDa) and synthetic amylose (average molecular weight 280 kDa) did not differ by more than 4%, as shown in Table 1. On the other hand, the total CD yield decreased at elevated temperatures. The total yields at 40 °C and 60 °C differed by about 20% for all experimental conditions. One reason for this could be due to inactivation of CGTase during incubation, since the CGTase used in this study was not thermostable. Alternatively, this effect may be due to differences between the rates of production and hydrolysis of CDs by CGTase depending on the incubation temperatures, because CGTase also catalyzes the opposing activities of cyclization and coupling.¹⁸

In order to confirm the cyclic structure of glucan products by CGTase reaction, they were subjected to matrix assisted laser desorption/ionization-time of flight-MS (MALDI-TOF-MS). A non-cyclic glucan with α -D-glucopyranose units of n has a molecular mass of $(162.1425 \times n + 18.0153)$ Da, whereas a CD should have a molecular mass of $162.1425 \times n$ Da. A mass spectrum of the CD mixture from amylose EX-III incubated at 60 °C for 24 h is shown in Figure 1 and the molecular mass of each component is

listed in Table 2. Several peaks were observed in the mass spectrum and the molecular masses were close to the theoretical values for a CD composed of 6 ~ 36 α -D-glucopyranose units, though not to those of non-cyclic glucans. From these results, and considering the resistance to glucoamylase, which hydrolyzes maltodextrins from the non-reducing end, we concluded that these components were CDs and the smallest was α -CD.

Table 1. Effect of incubation time and temperature on the CD yields from synthetic amylose and amylose EX-III

Incubation temperature (°C)	CD Yield (%)			
	(Synthetic amylose)		(Amylose EX-III)	
	Incubation time (h)		Incubation time (h)	
	2	24	2	24
40	85.71 $\pm$ 0.26	85.10 $\pm$ 0.56	82.86 $\pm$ 0.67	81.59 $\pm$ 0.35
50	83.07 $\pm$ 0.30	84.78 $\pm$ 0.26	80.45 $\pm$ 0.25	82.36 $\pm$ 0.10
60	61.14 $\pm$ 3.11	62.41 $\pm$ 1.44	62.66 $\pm$ 1.87	63.14 $\pm$ 0.63

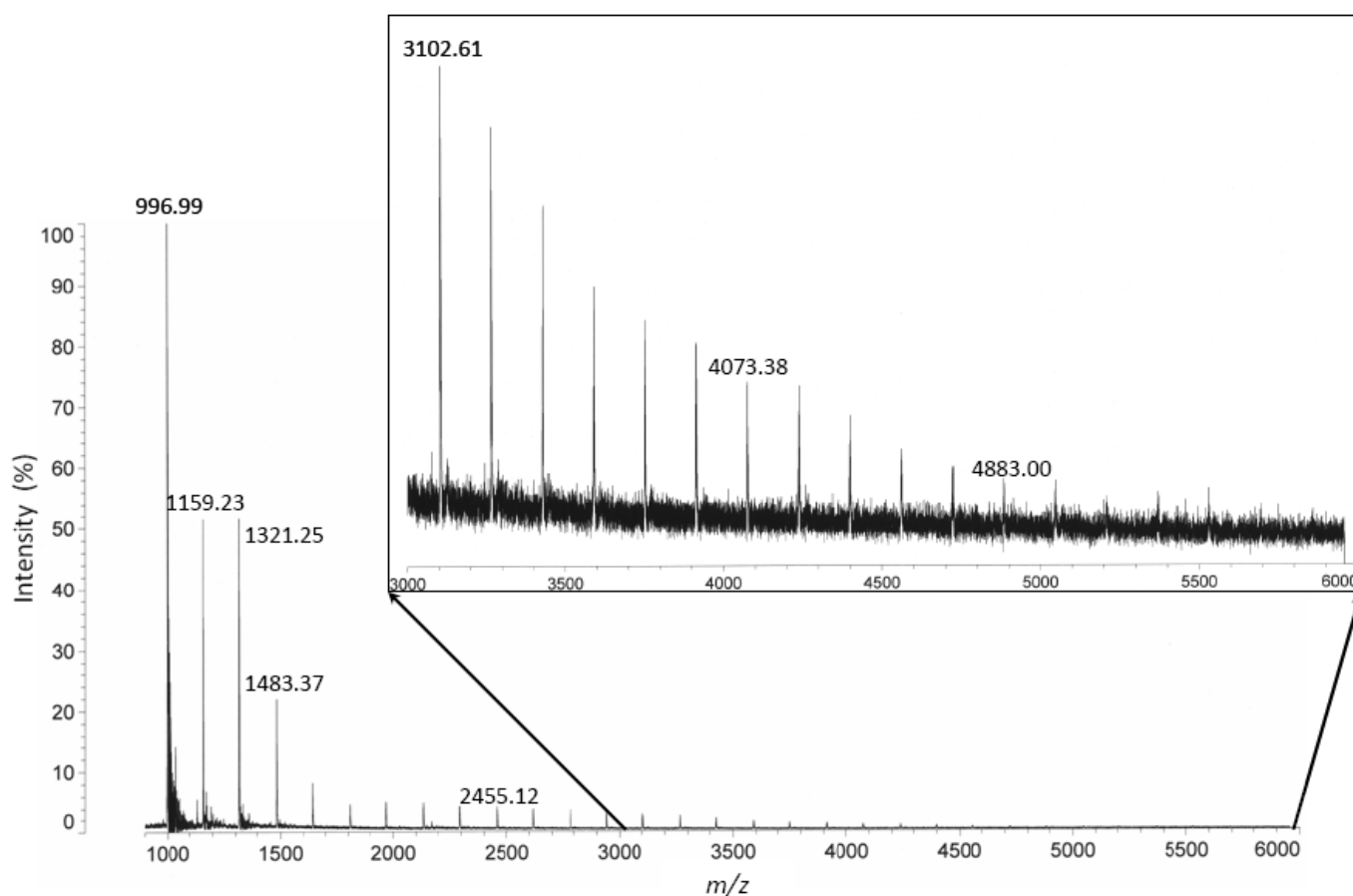


Figure 1. Mass spectrum of a CD mixture by MALDI-TOF-MS

Sample: CD mixture from amylose EX-III at 60 °C for 24 h.

Numbers above selected peaks indicate the molecular mass (in Dalton) of the molecular mass plus the mass of Na^+ (22.9898 Da).

Table 2. Theoretical and experimental masses of glucans

DP (n)	Theoretical		Experimental
	Non-cyclic glucan	Cyclic glucan (CD)	
6	1013.86	995.84 (α -CD)	996.99
7	1176.00	1157.99 (β -CD)	1159.23
8	1338.15	1320.13 (γ -CD)	1321.25
9	1500.29	1482.27 (CD ₉)	1483.37
10	1662.43	1644.41 (CD ₁₀)	1645.37
11	1824.57	1806.56 (CD ₁₁)	1807.24
12	1986.72	1968.70 (CD ₁₂)	1969.44
13	2148.86	2130.84 (CD ₁₃)	2131.30
14	2311.00	2292.98 (CD ₁₄)	2293.10
15	2473.14	2455.13 (CD ₁₅)	2455.12
16	2635.29	2617.27 (CD ₁₆)	2616.98
17	2797.43	2779.41 (CD ₁₇)	2778.96
18	2959.57	2941.55 (CD ₁₈)	2940.73
19	3121.71	3103.70 (CD ₁₉)	3102.61
20	3283.86	3265.84 (CD ₂₀)	3264.43
21	3446.00	3427.98 (CD ₂₁)	3426.26
22	3608.14	3590.12 (CD ₂₂)	3588.08
23	3770.28	3752.27 (CD ₂₃)	3749.81
24	3932.43	3914.41 (CD ₂₄)	3910.96
25	4094.57	4076.55 (CD ₂₅)	4073.38
26	4256.71	4238.69 (CD ₂₆)	4235.17
27	4418.85	4400.84 (CD ₂₇)	4396.88
28	4581.00	4562.98 (CD ₂₈)	4558.59
29	4743.14	4725.12 (CD ₂₉)	4719.20
30	4905.28	4887.26 (CD ₃₀)	4883.00
31	5067.42	5049.41 (CD ₃₁)	5045.85
32	5229.57	5211.55 (CD ₃₂)	5206.93
33	5391.71	5373.69 (CD ₃₃)	5366.98
34	5553.85	5535.83 (CD ₃₄)	5529.81
35	5715.99	5697.98 (CD ₃₅)	5691.10
36	5878.14	5860.12 (CD ₃₆)	5855.60

Theoretical masses of cyclic and non-cyclic glucans were calculated as $(162.1425 \times n + 22.9898)$ and $(162.1425 \times n + 22.9898 + 18.0153)$, respectively. DP (n), number of α -D-glucopyranose unit; 162.1425, mass of α -D-glucopyranose unit; 22.9898, mass of sodium ion; 18.0153, mass of H₂O.

The composition of each CD mixture was subsequently analyzed by high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) and the chromatograms are shown in Figure 2. In general, the retention time of a homologous series of carbohydrates on a column using

HPAEC increases as the degree of polymerization increases. However, the elution order and pattern of CDs composed of less than 11 α -D-glucopyranose units is due to the hydrophobic interaction between CDs and the packing in the column. The differences in the molecular weights of amylose did not affect the composition of CDs produced under the same incubation time and temperature. The production ratios of CD₉ to CD₂₁ (ratio of peak area between CD from amylose EX-III and CD from synthetic amylose) at 60 °C for 2 h ranged from 1.4 to 1.2. Moreover, there were small differences in the amounts of each CD produced from synthetic amylose and amylose EX-III under the same conditions. On the other hand, the incubation temperature clearly affected the kinds and amounts of LR-CDs produced.

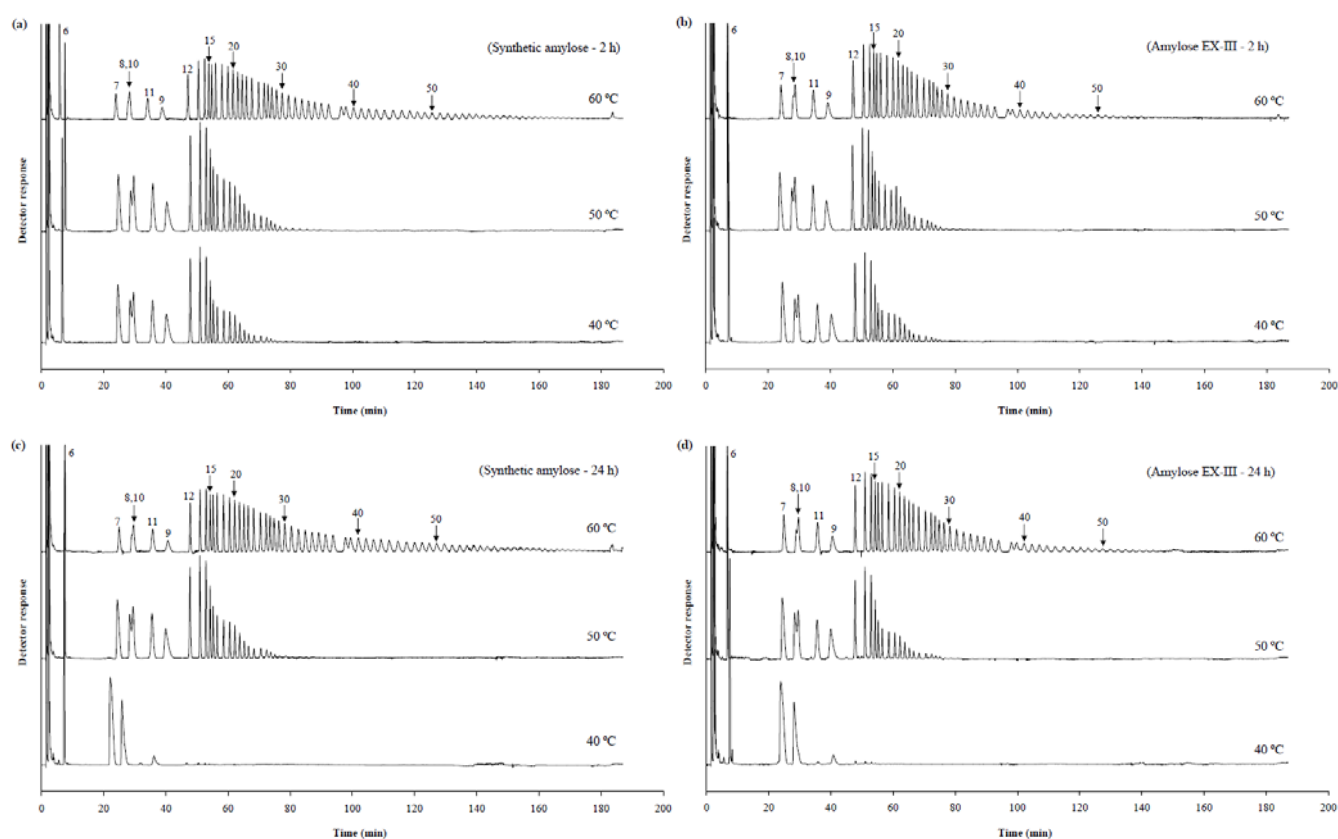


Figure 2. Chromatograms of each CD mixture by HPAEC-PAD

(a) CD mixture from synthetic amylose incubated for 2 h, (b) CD mixture from amylose EX-III incubated for 2 h, (c) CD mixture from synthetic amylose incubated for 24 h, (d) CD mixture from amylose EX-III incubated for 24 h. Numbers above selected peaks indicate the number of α -D-glucopyranose units in the CD ring.

The present results showed that the compositions of LR-CDs produced by CGTase were not significantly affected by the size of the amylase substrate, but were greatly affected by the reaction temperature. This finding is very interesting in both practical and scientific terms. The prime objective of this project is to establish a system for producing an LR-CD mixture with a large proportion of CD₉ to CD₂₁, and these

results should therefore be directly applicable to achieving this aim. On the other hand, the results suggest that the reaction specificity or product selectivity of CGTase is affected by reaction temperature. Further investigation should provide a better understanding of this useful and important enzyme.

EXPERIMENTAL

Materials

CGTase from *B. macerans* was donated from Amano Enzyme Inc. and used without further purification. The activity of CGTase was assayed using soluble starch as the substrate by measuring the decrease in iodine-staining power as described previously.¹⁹ Glucoamylase from *Rhizopus* sp. was purchased from Toyobo Co., Ltd. Synthetic amylose with an average molecular mass of 280 kDa was prepared at Ezaki Glico. Amylose with an average molecular mass of 14.6 kDa (Amylose EX-III) was purchased from Hayashibara Biochemical Laboratories Inc. All other chemicals were from reliable commercial sources and used without further purification. Milli-Q water was used in all experiments.

Preparation of CDs

A mixture (1 mL) containing about 5 mg of amylose, 50 mM acetate buffer (pH 5.5) and 2.3 units of CGTase from *B. macerans* was incubated at 40, 50 and 60 °C for 2 or 24 h and then boiled for 10 min to terminate the reaction. The product mixture was incubated with 20 units of glucoamylase for 24 h at 40 °C to hydrolyze a linear maltodextrin remaining, and then boiled for 10 min to terminate the reaction.

Determination of CD yield

The CD yield was calculated by subtracting the amount of glucose released by glucoamylase in the product mixture from that of total amylose without the CGTase reaction. The amount of glucose was measured by the mutarotase-glucose oxidase method.²⁰

Identification of each CD in CD mixture by MS

After removing glucose and ions from the reaction product with an ODS cartridge, the molecular mass of each CD was analyzed by MALDI-TOF-MS (Axima CFRplus, Shimadzu Co.) using 2,5-dihydroxybenzoic acid (Aldrich) as a matrix. Calibration was performed by using 2,4-dihydroxybenzoic acid and insulin (bovine, Sigma) and the accuracy was tested by α -CD.

Analysis of CD mixture by HPLC

The CDs in the reaction product were determined by HPAEC-PAD on the basis of α -D-glucopyranose unit oxidation. HPAEC-PAD was carried out on the DX-500 system (Dionex) with a pulsed amperometric detector (ED-50) using a Carbowac PA-100 with a guard column (250 $\times$ 4 mm I.D., Dionex). A sample was eluted with a gradient of sodium nitrate in 150 mM sodium hydroxide with a flow rate of 1 mL/min. The gradient program of sodium nitrate was used as in a previous report with a minor modification.²¹

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