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Effect of the reaction temperature on the transglycosylation reactions catalyzed by the cyclodextrin glucanotransferase from *Bacillus macerans* for the synthesis of large-ring cyclodextrins

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Abstract—The synthesis of cyclodextrins with from 6 to more than 50 glucose units by cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from *Bacillus macerans* was investigated. Analysis of the synthesized cyclic α-1,4-glucan products showed that a higher yield of large-ring cyclodextrins were obtained with a reaction temperature of 60 °C compared to 40 °C. The yield of large-ring cyclodextrins obtained at 60 °C represented about 50% of the total glucans employed in the reaction. Analysis of the cyclodextrin-forming cyclization reaction and of the coupling reaction of the CGTase resulting in the degradation of mainly the larger cyclic α-1,4-glucans indicated higher rates of the cyclization reaction at 60 °C compared to 40 °C while the opposite was found for the coupling reaction.

1. Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme involved in the conversion of starch. The enzyme is a member of the α-amylase family of glycosyl hydrolases (family 13).[1] CGTase can also hydrolyze glucan chains to some extent similar to α-amylases, but differs in its ability to form cyclodextrins (CD), cyclic α-1,4-glucans, as reaction products. While CGTase has been previously employed for the industrial production of CD_6, CD_7, and CD_8 which are composed of six, seven and eight glucose units (α-, β-, and γ-CD), the enzyme is also capable of synthesizing much larger rings. The CGTases from various *Bacillus* species including *Bacillus macerans* convert starch into a mixture of CD with a degree of polymerization from 6 to more than 60.[2,3]

The synthesis and properties of industrially produced CD have been studied intensively in the last years (for a review, see Szejtli 1998).[4] The molecular structures of CD resemble a hollow, truncated cone with a hydrophobic cavity.[5,6] Due to this structure, CD can form inclusion complexes with suitable guest molecules. Various properties of the guest molecules, such as their water solubility, stability, and bioavailability can thereby be manipulated. The commercially available CD are therefore widely applied in the food and pharmaceutical industries. The structure and properties of large-ring CD composed of nine to 21 glucose units have only been characterized recently (for a review, see Endo et al. 2002).[7] The molecular structure of CD_9 resembles a distorted boat shape because CD_9 is more flexible than CD_6, CD_7 or CD_8.[8,9] CD_{10} and CD_{14} have a butterfly-like structure to reduce steric strain with twisting of some glucose units to form flips and kinks.[10–14] The structure of CD_{26} contains two single helices with 13 glucose units each in antiparallel direction.[15–17] Due to their structural features which are distinct from the small CD, large-ring CD could find applications as novel host compounds in molecular recognition processes.[7]

The formation of CD by CGTase proceeds by an α-retaining double displacement mechanism.[18,19] Two catalytic amino acid residues, the catalytic acid/base residue Glu257 and the nucleophile Asp229 are involved in the reaction (Fig. 1). The glycosidic scissile bond oxygen of the bound α-1,4-glucan donor is protonated by Glu 257 resulting in the formation of an oxo-carbonium ion transition state. The transition state collapses into a stable covalent glycosyl-enzyme intermediate linked to Asp 229. The acceptor activated by Glu257 acting as a base then attacks the intermediate and a new α-glycosidic bond is formed via another oxo-carbonium ion transition state (Fig. 1).

CGTase performs three transglycosylation reactions and a hydrolysis reaction. Cyclic α-1,4-glucans are formed by an intramolecular transglycosylation reaction where the terminal 4-OH group of the intermediate acts as an acceptor (cyclization reaction, Fig. 2A). The reverse reaction also occurs where the CD ring is opened and the linear oligosaccharide is transferred to a linear glucan acceptor (coupling reaction, Fig. 2B). Linear α-1,4-glucan products

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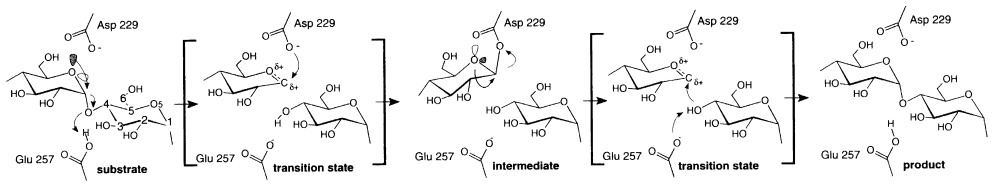

Figure 1. Scheme of the CGTase reaction mechanism. Reprinted from Uitdehaag, J.; van der Veen, B. A.; Dijkhuizen, L.; Dijkstra, B. W. *Enzyme Microb. Technol.*. Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the alpha-amylase family, *30* pp 295–304, Reprinted with permission. Copyright (2002) Elsevier.

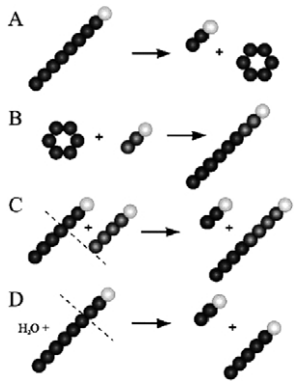

Figure 2. Scheme of the transglycosylation reactions catalyzed by CGTase*. Cyclization (A); coupling (B); disproportionation (C); and hydrolysis (D). The dark circles represent glucose residues, the white circles denote glucose residues with free reducing ends. *Reprinted from van der Veen, B. A.; van Alebeek, G. J.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.*. The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms, *267* pp 658–665, Reprinted with permission. Copyright (2000) Blackwell Publishing Ltd.

are also formed in the third reaction, an inter-molecular transglycosylation where a linear oligosaccharide is transferred to another linear glucan acceptor (disproportionation reaction, Fig. 2C). In a hydrolysis reaction, a glucan is cleaved and the reducing end is transferred to water (Fig. 2D).

The amounts and size distribution of CD formed by CGTase will be strongly influenced by the combined effects of the three transglycosylation reactions, as well as by the hydrolytic activity of the enzyme. CGTases isolated from wild type bacterial strains have been shown to exhibit different product profiles with respect to the size and amount of the CD synthesized.[20–22] A comparison of the large-ring CD synthesis activity of CGTases from *Bacillus* spp. also indicated that the enzymes exhibited different product specificity. The CGTases differed in their hydrolytic activities affecting the ratio of small CD to large-ring CD products synthesized.[3]

The three transglycosylation reactions catalyzed by CGTases show distinct differences in their kinetic mechanisms.[3,23] The differences observed have been explained by the way the substrates bind to the enzyme. Site-directed mutagenesis and kinetic studies have suggested the possibility to independently modify the cyclization and coupling activities of CGTase.[24] Thereby, the product range of CGTase could be manipulated for the synthesis of CD of specific sizes.

In this study, we compare the effect of different reaction temperatures on the cyclization and coupling reactions of the CGTase from *B. macerans* influencing the yield of large-ring CD synthesis products.

2. Results and discussion

Using synthetic amylose, a linear α-1,4-glucan with an average molecular weight of 280.9 kDa as substrate, large-ring CD are formed at an early stage of the reaction with CGTases from *Bacillus* species.[2,3,22] It has been observed that the amount of large-ring CD decreased during a prolonged incubation with the enzyme. The yield and size distribution of large-ring CD in the course of a synthesis reaction will be strongly influenced by the extent of the coupling and hydrolysis reactions by which the large-ring CD are converted to smaller CD. Since CD_7 and CD_8 were found to be poor substrates for the coupling and hydrolytic reactions, they will accumulate during longer reaction times.[3]

To further investigate the factors influencing the synthesis of large-ring CD by CGTase, we compared the yield and size distribution of CD obtained at different reaction temperatures and incubation times. An analysis of the cyclic α-1,4-glucan products obtained from synthetic amylose as substrate catalyzed by the CGTase from *B. macerans* is shown in Figure 3. Prior to analysis by high performance anion exchange chromatography with pulsed amperometric detection, the reaction mixtures were treated with glucoamylase to convert non-cyclic glucans to glucose. At a reaction temperature of 40 °C, large-ring CD were detected after a reaction time of 1 h (Fig. 3A). After a reaction time of 20 h, almost all of them were converted to CD_6, CD_7 and CD_8 as reported previously.[2] In contrast, at a reaction temperature of 60 °C, large-ring CD were formed after an incubation time of 30 min and could still be detected after a reaction time of 20 h (Fig. 3B). A time course over 480 min of the total production of CD, of CD_6–CD_8, large-ring CD, and of the reducing power at different reaction temperatures is shown in Figure 4. The yield of large-ring CD reached a maximum of 35% of the total glucan after 2 h of incubation at 40 °C and decreased during longer incubation times. Due to the conversion of the large-ring CD to smaller CD by coupling or hydrolysis reactions, their amount increased during longer incubation times keeping the amount of total CD produced almost constant (Fig. 4A). The maximum yield of large-ring CD after 2 h of incubation at 60 °C reached about 50% of the total glucan and remained almost constant during longer incubation times (Fig. 4B).

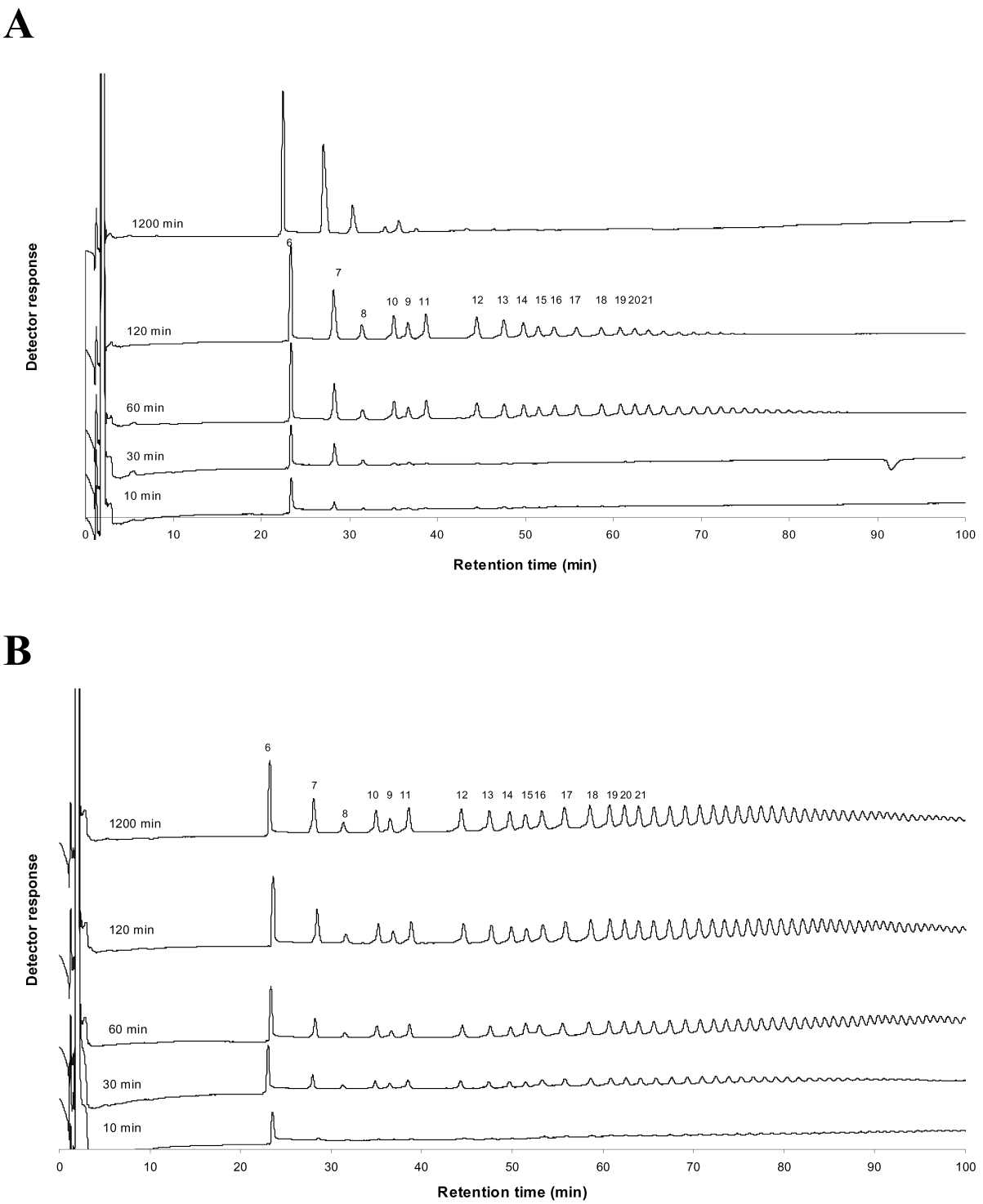

Figure 3. HPAEC analysis of CD synthesized after 10, 30, 60, 120 and 1200 min by the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase. Peak numbers indicate the degree of polymerization of identified CD.

Beside the coupling reaction, the hydrolytic activity of CGTase has also been considered to influence the conversion of large-ring CD to smaller CD.[3] However, it is difficult to distinguish between the hydrolysis and the coupling activity of the enzyme since it catalyzes both reactions at the same catalytic site. After opening of the CD ring, the resulting linear oligosaccharide will be transferred to water in the case of hydrolysis. In the presence of other oligosaccharides or glucose in the enzyme reaction mixture these will also serve as acceptors resulting in a coupling reaction. The amount of reducing power detected in the reaction mixtures representing the amounts of reducing sugars formed also depended on the reaction temperature. In reaction mixtures incubated at 40 °C, it steadily increased and reached higher levels compared to reactions performed at 60 °C. The observed higher amounts of reducing sugars formed at 40 °C may reflect the increased formation of linear oligosaccharides due to hydrolysis reactions.

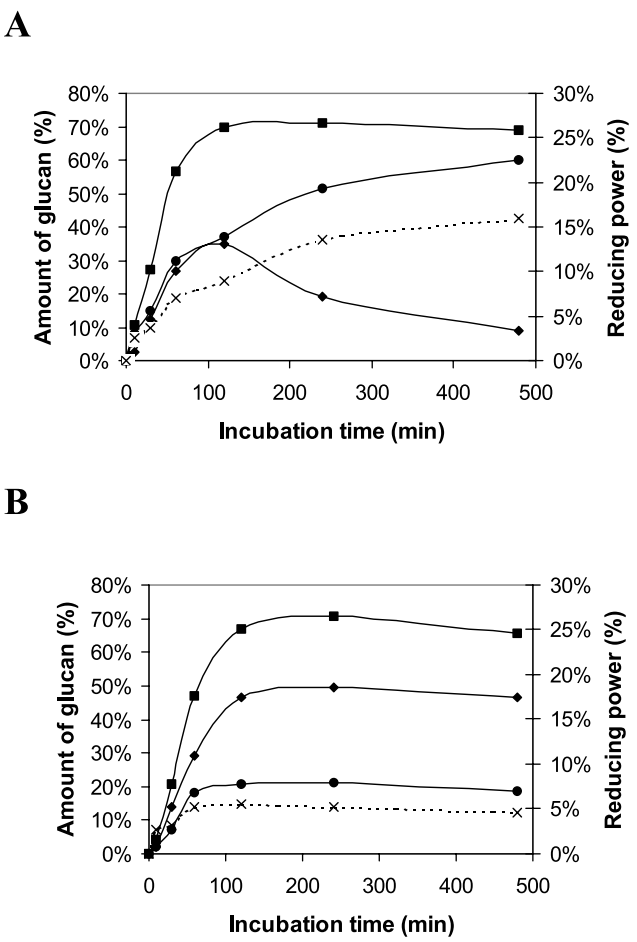

Figure 4. Time course of the amounts of the total CD, CD_6 to CD_8, large-ring CD and the reducing power produced by the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). Total CD, ■; CD_6–CD_8, ●; large-ring CD, ◆; reducing power, ×. Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase.

The amounts of large-ring CD with a degree of polymerisation from 9 to 21 synthesized during 4 h of incubation with the CGTase at the two reaction temperatures were quantified (Table 1). The amounts of each single large-ring CD formed at 60 °C reached about 20–40 nmol ml^{-1}, corresponding to conversion yields of 3–5% of the total glucan employed in the reaction. Reactions performed at 40 °C yielded much lower amounts of large-ring CD, and CD with a degree of polymerisation above 13 could not be detected. A comparison of the size distribution of all CD formed at different temperatures also shows that the ratio of large-ring CD to small CD was higher at 60 °C than at 40 °C (Fig. 5).

Table 1. Amounts of large-ring CD obtained by incubating 0.5% synthetic amylose with CGTase from *B. macerans* at 40 and 60 °C

Cyclodextrin	40 °C (nmol ml^{-1})	60 °C (nmol ml^{-1})
CD_9	34.66	45.71
CD_{10}	11.43	49.03
CD_{11}	4.63	44.33
CD_{12}	4.70	45.35
CD_{13}	2.11	41.59
CD_{14}	nd[a]	29.74
CD_{15}	nd	18.88
CD_{16}	nd	33.58
CD_{17}	nd	45.33
CD_{18}	nd	33.48
CD_{19}	nd	39.68
CD_{20}	nd	24.26
CD_{21}	nd	30.25

[a] nd, not detectable.

To further investigate the influence of the reaction temperature on the amount and size of CD synthesized, the optimum temperature for the cyclization and coupling activities of the CGTase was compared. The results show an optimum of the cyclization reaction at 60 °C, while the optimum for the coupling activity with CD_8 as substrate was found around 45 °C (Fig. 6). A comparison of the kinetic parameters of the cyclization reaction of the CGTase showed a higher rate of CD_7 formation at 60 °C compared to 40 °C (Table 2). The K_m value for synthetic amylose could not be determined since the CD detection assay was not sensitive enough to reliably measure the low amounts of CD_7 obtained at the low amylose concentrations required.

Analysis of the kinetic properties of the coupling activity of the CGTases from *B. circulans* and *Thermoanaerobacterium thermosufurigenes* has revealed a random-order reaction mechanism involving a ternary complex, while the CGTase from *Bacillus sp.* 1101 showed a non-sequential ping-pong reaction mechanism.[23,25–26] Our experimental data for the CGTase from *B. macerans* also indicated a random-order reaction mechanism. The Lineweaver–Burk plots of the kinetic data obtained where the reciprocal of the velocities was plotted against the reciprocal of the CD_8 concentration at different acceptor concentrations did not result in parallel lines (Fig. 7). In a random-order reaction, any of the two substrates, CD_8 and methyl-α-D-glucopyranoside (MαDG), can bind first to the enzyme in a sequential mechanism resulting in two apparent affinity constants: K_m in the absence and K'_m in the presence of the second substrate. The kinetic parameters determined at different temperatures indicated higher rates of the coupling reaction at 40 °C compared to 60 °C (Table 3). The affinity constants K'_m for both substrates in the presence of the other substrate were lower at 40 °C than at 60 °C, while in the absence of the second substrate the reverse effect was found. The affinity for CD_8 was decreased in the presence of the acceptor at both temperatures. The decrease of affinity was more pronounced at 60 °C compared to 40 °C. In contrast, the affinity of the CGTase from *B. circulans* (strain 251) for CD_8 was not affected by the presence of the acceptor.[23]

Structural studies of the CGTases from *B. circulans* strain 251 complexed with CD_8 and of *B. circulans* strain 8 complexed with a CD_7 derivative have indicated different binding modes at the acceptor site for the CD and the linear substrate.[27] These differences may affect the binding of linear oligosaccharides and CD at the active site of the CGTase at different reaction temperatures favouring the coupling reaction at the lower reaction temperature.

The cyclization reaction of CGTase with amylose as substrate has been previously described as an exo-type of attack occurring at the sixth to the eighth glycosidic linkage from the non-reducing end of a α-1,4-glucan chain. However, recent results have shown that CGTases can attack any α-1,4-linkage within the amylose molecule

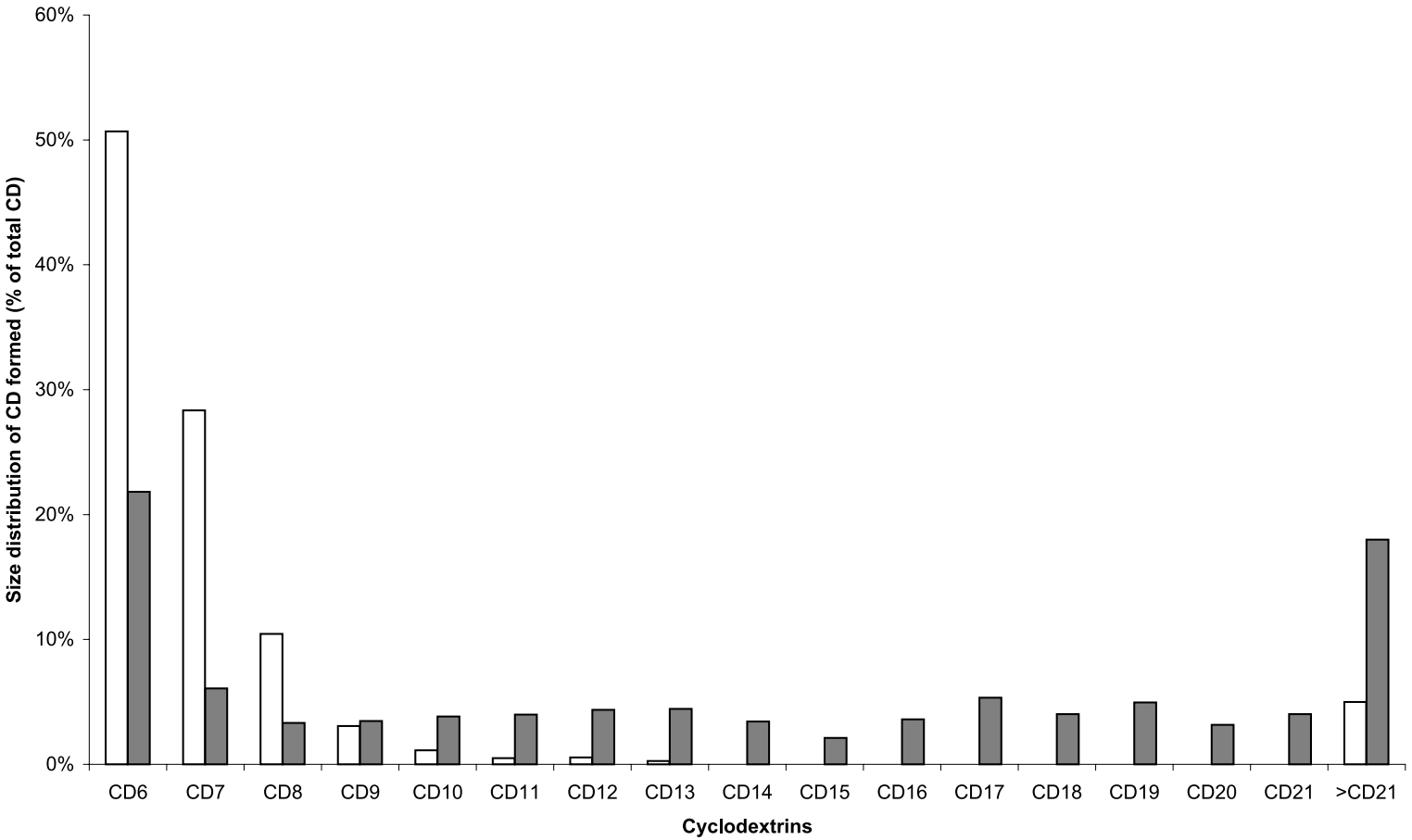

Figure 5. Size distribution of CD synthesized by the CGTase from *B. macerans* at 40 °C (open bars) and 60 °C (solid bars). Synthetic amylose (0.5%) was incubated with 2 U ml^{-1} CGTase for 480 min.

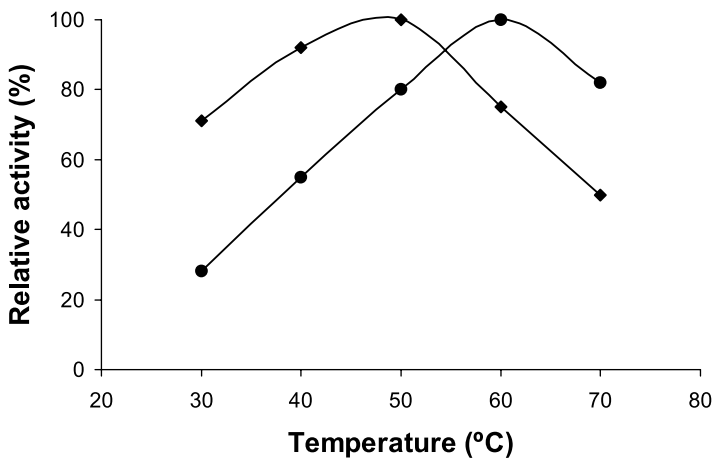

Figure 6. Comparison of the coupling activity (◆) and the cyclization activity (●) of the CGTase from *B. macerans* at different temperatures.

Table 2. Kinetic parameters of the CD_8 cyclization reaction catalyzed by the CGTase from *B. macerans* at 40 and 60 °C

Parameter	40 °C	60 °C
V_{max} (U mg^{-1})	44.6±4.1	56.3±5.2
k_{cat} (s^{-1})	51.2±4.9	64.7±6.0

resulting in the synthesis of a wide range of cyclic α-1,4-glucans by a random cyclization reaction. By adjusting the reaction conditions such as temperature and incubation time, the yield of large-ring CD in the synthesis reaction can be effectively increased.

3. Experimental

3.1. Chemicals and enzymes

Synthetic amylose with an average molecular weight of 280.9 kDa was prepared by the method of Kitamura et al.[28] Soluble starch and α-amylase was from E. Merck AG (Darmstadt, Germany) and methyl-α-D-glucose (MαDG) was from Sigma-Aldrich Chemie GmbH (Munich, Germany). Glucoamylase was from Toyobo Co., Ltd. (Osaka, Japan) and *B. macerans* CGTase was from Amano Enzyme Inc. (Aichi, Japan). The enzyme was further purified using DEAE Sepharose Fast Flow media (Amersham Biosciences Europe GmbH). The enzyme was eluted with a linear gradient between 0 and 0.5 M NaCl in Tris–HCl buffer, pH 7.8. Standards of large-ring CD (CD_9 to CD_{21}) were kindly provided by H. Ueda, Hoshi University, Tokyo, Japan.

3.2. Synthesis of large-ring CD

Synthetic amylose was dissolved in 10 ml of 90% DMSO (v/v). To remove the DMSO, 0.5 ml of amylose solution was mixed with 0.5 ml of distilled water and loaded on a PD10 column (Amersham Biosciences Europe GmbH). After eluting the column with 2 ml of distilled water, the synthetic amylose was eluted with a further 1.5 ml of distilled water and used immediately.[3] The CD synthesis reaction was performed using 0.5% synthetic amylose and 2 U ml^{-1} CGTase in acetate buffer (50 mM, pH 5.5). The reaction was terminated by boiling the mixture for 10 min. Glucoamylase (3.85 U ml^{-1}) was added and the mixture was incubated for 24 h to convert the linear oligosaccharides to glucose. The amount of total CD in the sample was calculated by subtracting the amount of glucose released by glucoamylase from the total amount of glucose released by a combined treatment with glucoamylase and α-amylase.[3]

3.3. Analysis of large-ring CD

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out using a DX-600 system (Dionex Corp., Sunnydale, USA) to analyze and quantify the CGTase synthesis products and to monitor the production process of large-ring CD. A Carbopac PA-100 analytic column (4×250 mm, Dionex Corp., Sunnydale, USA) was used. Samples (25 μl) were centrifuged before analysis. Products were eluted with a linear gradient of sodium nitrate (0–5 min, 1%; 5–49 min, increasing from 1% to 18%; 49–89 min, increasing from 18% to 35%; 89–100 min, increasing from 35% to 39%) in 200 mM NaOH containing 8% MeCN with a flow rate of 1 ml min^{-1}. Retention times (min) of each CD obtained were: CD_6, 21.1; CD_7, 27.3; CD_8, 30.8; CD_9, 36.3; CD_{10}, 34.7; CD_{11}, 38,4; CD_{12}, 44.3; CD_{13}, 47.5; CD_{14}, 49.8; CD_{15}, 51.6; CD_{16}, 53.5; CD_{17}, 53.0; CD_{18}, 59.0; CD_{19}, 61.2; CD_{20}, 62.9; CD_{21}, 64.5). The amounts of CD_6 to CD_{21} obtained were quantified by preparing standard curves with concentrations of 0.1, 0.25, 0.5, 0.75 and 1 mM of each CD.

3.4. Assay for starch-hydrolyzing activity

The starch-hydrolyzing activity of the CGTase was determined by the iodine method with some modifications.[29] The reaction mixture (250 μl) contained 1.2% soluble starch and 50 μl of the enzyme solution. After incubation at 40 °C for 10 min, the reaction was stopped by the addition of 500 μl of 0.5 M acetic acid–0.5 M HCl (5:1, v/v). Then 5 ml of 0.005% I_2 in 0.05% KI solution was added to 100 μl of the mixture and the absorbance was measured at 660 nm. One unit of the enzyme was defined as the amount of enzyme producing a 10% reduction of the intensity of the colour of the iodine complex per minute under the conditions described.

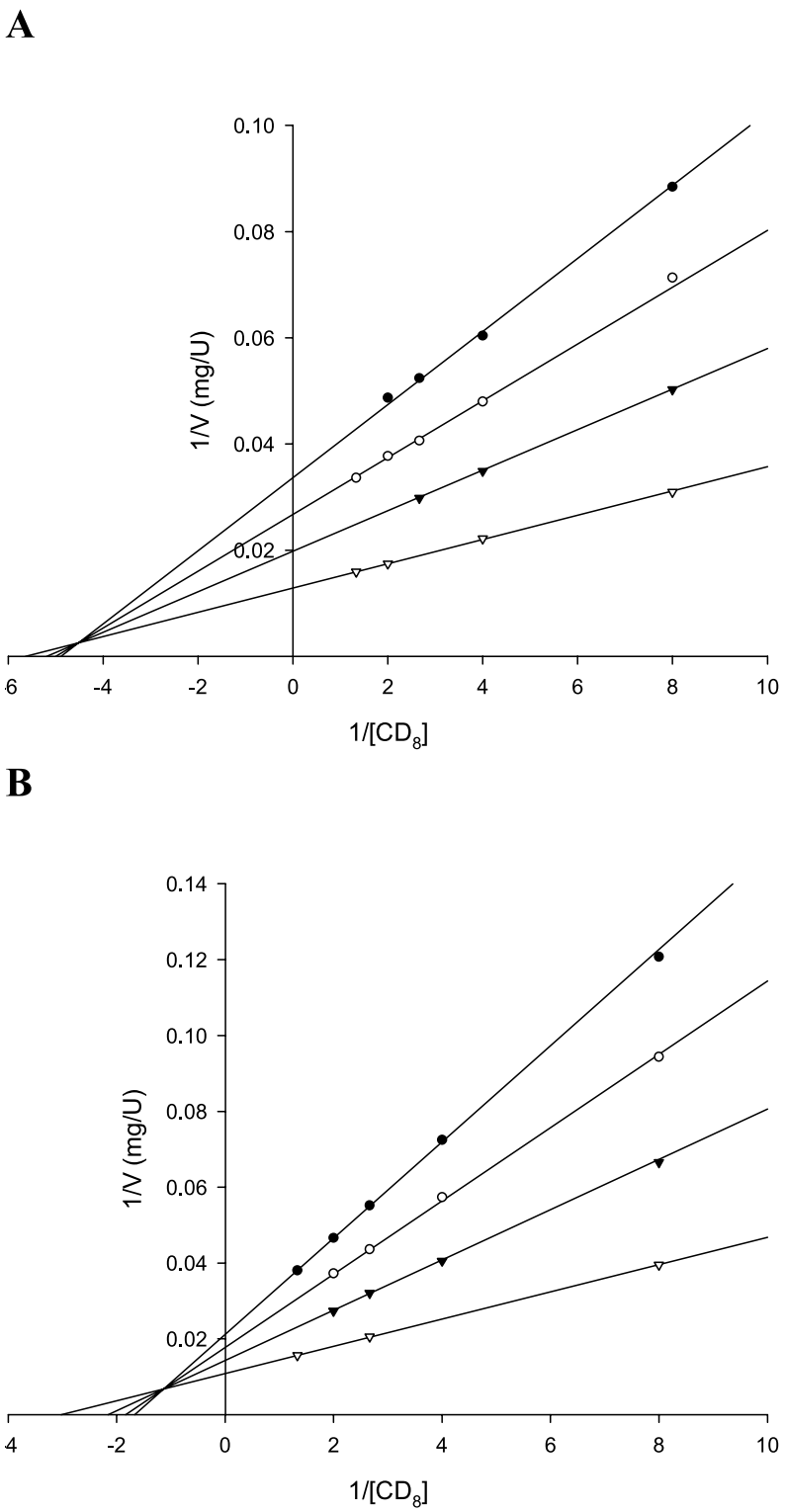

Figure 7. Lineweaver–Burk plots of the coupling reaction of the CGTase from *B. macerans* at 40 °C (A) and 60 °C (B). The reciprocal of the velocities is plotted against $1/[CD_8]$ at MαDG concentrations of 15 mM (●), 20 mM (○), 30 mM (▼), and 60 mM (▽).

Protein concentrations were determined using the Bradford method[30] and the reducing power by a modified Park–Johnson method.[31]

3.5. Cyclization activity

Cyclization activity was determined using synthetic amylose (MW 280.9 kDa, 0.5%) as the substrate. The synthetic amylose was incubated in 50 mM acetate buffer (pH 5.5) with appropriately diluted CGTase. At time intervals (0.5–1 min), 100 μl samples were taken and added to a mixture containing 100 μl phenolphthalein in 800 μl sodium hydroxide (0.03 M). One unit of activity was defined as the amount of enzyme able to form 1 μmol of CD_7 per min.

Table 3. Kinetics parameters of the coupling reaction of CD_8 with MαDG catalyzed by the CGTase from *B. macerans* at 40 and 60 °C

Parameter	40 °C	60 °C
$K_{m\ CD8}$ (mM)	0.125±0.01	0.074±0.02
$K'_{m\ CD8}$ (mM)	0.22±0.01	0.88±0.04
$K_{m\ M\alpha DG}$ (mM)	69.87±1.67	27.6±0.7
$K'_{m\ M\alpha DG}$ (mM)	122.8±16.7	326.6±46.0
V_{max} (U mg^{-1})	168.1±5.0	134.3±2.7
k_{cat} (s^{-1})	196.1±5.8	156.6±3.1

Kinetic analysis was performed with Sigmaplot software (SPSS Inc., Chicago, USA) using the Michaelis–Menten equation.

3.6. Coupling activity

The coupling activity between CD_8 and methyl-α-D-glucopyranoside (MαDG) was measured as described previously.[23] CD_8 at concentrations of 1.5, 2, 2.5, 4 and 8 mM was used as donor substrate and up to 60 mM methyl-α-D-glucopyranoside (MαDG) as acceptor substrate. The substrates were incubated in 10 mM phosphate buffer (pH 5.5) for 5 min with appropriately diluted CGTase. The reaction products were incubated with 3.85 U ml^{-1} glucoamylase to convert the linear oligosaccharides formed to glucose. The amount of glucose was determined with the glucose oxidase method.[32] One unit of enzyme activity was defined as the amount of enzyme coupling 1 μmol of CD_8 to MαDG per min.

Kinetic analysis was performed with Sigmaplot software (SPSS Inc., Chicago, USA). The following equation for a random-order reaction mechanism was used to fit the experimental data.[33]

$$v = V_{max}\cdot a\cdot b/(K'_{mA}\cdot K_{mB} + K_{mB}\cdot a + K_{mA}\cdot b + a\cdot b) \quad (1)$$

v=reaction rate, V_{max}=maximum rate; A and B=donor and acceptor substrate; a and b=substrate concentrations; K_m and K'_m=affinity constants for the substrates in the absence and in the presence and of the second substrate.

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References and notes

1. Henrissat, B.; Davies, G. *Curr. Opin. Struct. Biol.* **1997**, *7*, 637–644.
2. Terada, Y.; Yanase, M.; Takata, H.; Takaha, T.; Okada, S. *J. Biol. Chem.* **1997**, *272*, 15729–15733.
3. Terada, Y.; Sanbe, H.; Takaha, T.; Kitahata, S.; Koizumi, K.; Okada, S. *Appl. Environ. Microbiol.* **2001**, *67*, 1453–1460.
4. Szejtli, *J. Chem. Rev.* **1998**, *98*, 1741–1753.
5. Lindner, K.; Saenger, W. *Acta Crystallogr. Sect. B* **1982**, *38*, 203–210.
6. Lindner, K.; Saenger, W. *Carbohydr. Res.* **1982**, *99*, 103–105.
7. Endo, T.; Zheng, M.; Zimmermann, W. *Aust. J. Chem.* **2002**, *55*, 39–48.
8. Fujiwara, T.; Tanaka, N.; Kobayashi, S. *Chem. Lett.* **1990**, 739–742.
9. Harata, K.; Akasaka, H.; Endo, T.; Nagase, H.; Ueda, H. *Chem. Commun.* **2002**, *17*, 1968–1969.
10. Ueda, H.; Endo, T.; Nagase, H.; Kobayashi, S.; Nagai, T. *J. Inclusion. Phenom. Mol. Recognit. Chem.* **1996**, *25*, 17–20.
11. Harata, K.; Endo, T.; Ueda, H.; Nagai, T. *Supramol. Chem.* **1998**, *9*, 143–150.
12. Jacob, J.; Geßler, K.; Hoffman, D.; Sanbe, H.; Koizumi, K.; Smith, S. M.; Takaha, T.; Saegner, W. *Angew. Chem. Rev.* **1998**, *37*, 605–609.
13. Jacob, J.; Geßler, K.; Hoffman, D.; Sanbe, H.; Koizumi, K.; Smith, S. M.; Takaha, T.; Saegner, W. *Carbohydr. Res.* **1999**, *322*, 228–246.
14. Endo, T.; Nagase, H.; Ueda, H.; Kobayashi, S.; Shiro, M. *Anal. Sci.* **1999**, *15*, 613–614.
15. Gessler, K.; Uson, I.; Takaha, T.; Krauss, N.; Smith, S. M.; Okada, S.; Sheldrick, G. M.; Saenger, W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4246–4251.
16. Nimz, O.; Gessler, K.; Uson, I.; Saenger, W. *Carbohydr. Res.* **2001**, *336*, 141–153.
17. Nimz, O.; Gessler, K.; Uson, I.; Laettig, S.; Welfle, H.; Sheldrick, G. M.; Saenger, W. *Carbohydr. Res.* **2003**, *338*, 977–986.
18. Withers, S. G. *Carbohydr. Polym.* **2001**, *44*, 325–337.
19. Uitdehaag, J.; van der Veen, B. A.; Dijkhuizen, L.; Dijkstra, B. W. *Enzyme Microb. Technol.* **2002**, *30*, 295–304.
20. Kobayashi, S. In *Enzymes for Carbohydrate Engineering*; Park, K. H., Robyt, J. F., Choi, Y. D., Eds.; Elsevier: Amsterdam, 1996; pp 23–41.
21. Larsen, K. L.; Duedahl-Olesen, L.; Jørgens, H.; Christensen, S.; Mathiesen, F.; Pedersen, L. H.; Zimmermann, W. *Carbohydr. Res.* **1998**, *310*, 211–219.
22. Zheng, M.; Endo, T.; Zimmerman, W. *J. Inclusion Phenom.* **2002**, *44*, 387–390.
23. van der Veen, B. A.; van Alebeek, G. J.; Uitdehaag, J. C.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2000**, *267*, 658–665.
24. Leemhuis, H.; Uitdehaag, J. C.; Rozeboom, H. J.; Dijkstra, B. W.; Dijkhuizen, L. *J. Biol. Chem.* **2002**, *277*, 1113–1119.
25. Leemhuis, H.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2003**, *270*, 155–162.
26. Nakamura, A.; Haga, K.; Yamane, K. *FEBS Lett.* **1994**, *337*, 66–70.
27. Schmidt, A. K.; Cottaz, S.; Driguez, H.; Schulz, G. E. *Biochemistry* **1998**, *37*, 5909–5915.
28. Kitamura, S.; Kobayshi, K.; Tanahashi, H.; Ozaki, T.; Kuge, T. *Denpun Kagaku* **1989**, *36*, 257.
29. Kometani, T.; Terada, Y.; Nishimura, T.; Takii, H.; Okada, S. *Biosci. Biotech. Biochem.* **1994**, *58*, 517–520.
30. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
31. Takeda, Y.; Guan, H.-P. Preiss. *J. Carbohydr. Res.* **1993**, *240*, 253–263.
32. Barham, D.; Trinder, P. *Analyst* **1972**, 97–101.
33. Cornish-Bowden, A. Fundamentals of Enzyme Kinetics; Portland: London, 1995.