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ORIGINAL ARTICLE

Effect of ethanol on the synthesis of large-ring cyclodextrins by cyclodextrin glucanotransferases

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Abstract Cyclodextrin glucanotransferase (EC 2.4.1.19, CGTase) synthesizes cyclodextrins (CD) composed of 6 to more than hundred glucose units from amylose by an intramolecular transglycosylation reaction. The addition of ethanol to the reaction medium resulted in an increase of the yield of large-ring CD obtained with a CGTase from *Bacillus sp*. BT3-2 and *Bacillus macerans*. The presence of 15% ethanol in the reaction mixture with the CGTase from *Bacillus sp*. BT3-2 resulted in a 30% increase of the amounts of CD_{10}–CD_{13} synthesized after 5 h of reaction. The addition of 20% ethanol increased the yield of CD_{14}–CD_{21} up to 1000%. The hydrolysis of the large-ring CD by the CGTases was suppressed in the presence of ethanol. The ring-opening coupling cyclization reactions of the CGTase were effected differently by the organic solvent which may contribute to the observed increase of the yield and size of the CD obtained in the synthesis reactions.

Keywords Ethanol effect · Coupling · Cyclization · Cyclodextrin glucanotransferase (CGTase) · Large-ring cyclodextrins (LR-CD) · *Bacillus sp.* BT3-2 · *Bacillus macerans*

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Introduction

Cyclodextrin glucanotransferase (CGTase) catalyzes the conversion of starch and linear α-1,4-glucans to cyclodextrins (CD). The molecular structures of CD resemble a hollow, truncated cone with a hydrophobic cavity allowing the formation of inclusion complexes with suitable guest molecules [1]. CD_6, CD_7 and CD_8, which are commercially available, are therefore widely applied mainly in the food and pharmaceutical industries. The structure and properties of large-ring CD composed of nine to 21 glucose units have been characterized recently. The molecular structure of CD_9 resembles a distorted boat shape [2]. CD_{10} and CD_{14} show a butterfly-like structure to reduce steric strain with twisting of some glucose units to form flips and kinks [3]. The structure of CD_{26} contains two single helices with 13 glucose units each in antiparallel direction [4]. Due to their different sizes and structural features distinct from the small CD, large-ring CD could find applications as novel host compounds in molecular recognition processes in nanobiotechnology [5].

CGTase is a member of the family of amylase enzymes. CD are produced from α-1,4-glucan substrates by an intramolecular transglycosylation (cyclization) reaction [1]. However, CGTase also catalyzes further reactions, including the coupling reaction by which the CD is cleaved and the linear glucan is transferred to an acceptor molecule. In a disproportionation reaction, a linear oligosaccharide is cleaved and transferred to a linear acceptor molecule. In addition, CGTase also catalyzes a hydrolytic reaction by which oligosaccharides or CD are hydrolysed using water as the reaction acceptors.

The ability of CGTases to synthesize CD with a degree of polymerization (DP) of 9 to more than 60 at

the initial stage of the reaction has been reported recently [6, 7]. During prolonged incubation times, the amount of large-ring CD decreased due to their conversion to CD_6–CD_8. The size of CD obtained in synthesis ractions with CGTases is largely depending on the type of CGTase, the reaction conditions and the production process chosen [8]. By modifying the production process, for example by the addition of complexing agents or organic solvents like ethanol, toluene, or 1-butanol to the reaction medium, the total yield and the size of the small CD can be manipulated [9]. Lee and Kim [10], and also Tomita and co-workers [11] have reported a significant increase in the yield of CD_7 and CD_8, respectively, in the presence of ethanol. Furthermore, ethanol has been shown to affect the ratio of CD_6–CD_8 produced by CGTases [12].

The catalytic mechanism leading to the formation of large-ring CD by CGTases is not well understood. Likewise, the synthesis of these compounds in any larger amounts has not been accomplished yet, which prohibits their further evaluation for applications in nanobiotechnology. In this paper, we report the effect of ethanol on the synthesis of large-ring CD by CGTases from *Bacillus sp.* BT3-2 and *Bacillus macerans*.

Materials and methods

Chemicals and materials

Phenolphthalein and soluble starch were from Merck AG (Darmstadt, Germany). CD_6–CD_8 were a kind gift of Wacker-Chemie GmbH, Germany. Methyl-α-D-glucose (MαDG) was from Sigma-Aldrich Chemie GmbH (Munich, Germany). Synthetic amylose with an average molecular weight of 280.9 kDa was prepared by the method of Kitamura et al. [13]. Samples of CD_9–CD_{21} were kindly provided by T. Endo and H. Ueda, Hoshi University, Tokyo, Japan. *Bacillus sp.* BT3-2 isolate was from the culture collection of the Department of Microbiology and Bioprocess Technology, University of Leipzig, Germany. CGTase from *Bacillus macerans* was obtained from Amano Enzyme Inc. (Aichi, Japan) and *Rhizopus sp.* glucoamylase was from Toyobo Co., Ltd. (Osaka, Japan).

Preparation of CGTase from *Bacillus* sp. BT3-2

Crude enzyme solution (11 U/mg), produced by *Bacillus sp.* BT3-2 grown in a medium containing 2% (w/v) soluble starch, 0.3% yeast extract, 0.5% peptone, 0.3% NaCl, 0.02% $MgSO_4$, 0.1% KH_2PO_4, pH 7.0, at 37°C for 36 h, was collected by centrifugation (4000 rpm for 30 min at 4°C). The enzyme solution was precipitated with ammonium sulfate (70%). The precipitate was collected by centrifugation and dissolved in 0.05 M phosphate buffer at pH of 7.0. It was dialyzed for 48 h against distilled water. The CGTase was purified by DEAE Sepharose 6 Fast Flow anion exchange chromatography (Amersham Biosciences Europe GmbH). The enzyme was eluted with a linear gradient between 0 and 0.4 M NaCl in 50 mM sodium phosphate buffer (pH 7.8). The fractions containing the CGTase were applied to a CD_7-activated Sepharose 6B affinity chromatography column (Amersham Biosciences Europe GmbH) and eluted with 25 mM phosphate buffer (pH 7.0) containing 15 mM CD_6. The CGTase was applied to a Resource Q anion exchange column (Amersham Biosciences Europe GmbH) and was eluted with a linear gradient of NaCl (0–0.5 M) in phosphate buffer (25 mM, pH 7.2). The protein concentration was determined by the Bradford method [14]. The specific activity of the purified CGTase was 116 U/mg (31.9% yield). SDS-polyacrylamide gel electrophoretic analysis indicated a single band and a molecular weight of the enzyme of 72 kDa.

Synthesis of large-ring cyclodextrins

Synthetic amylose (0.2 g) was dissolved in 10 ml of 90% aqueous DMSO. To remove the DMSO, 0.5 ml of amylose solution was mixed with 0.5 ml of distilled water and loaded onto a PD10 column (Amersham Biosciences Europe GmbH). After washing with 2 ml of distilled water, the synthetic amylose was eluted with a further 1.5 ml of distilled water and used immediately [7]. The CD synthesis reaction was performed using 0.5% (w/v) synthetic amylose and 2.4 U/ml CGTase from *Bacillus sp.* BT3-2 and *Bacillus macerans* in acetate buffer (50 mM, pH 5.5) for 5 h at 50°C (total volume, 2 ml). The reaction was stopped by boiling for 10 min in a water bath. Glucoamylase (3.85 U/ml) was added and the mixture was incubated for 24 h to convert the linear oligosaccharides to glucose. The reaction was stopped by boiling for 10 min in a water bath. To test the effect of organic solvents, ethanol (15–25%) was added at the beginning of the reaction.

Analysis of synthesized cyclodextrins

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to analyse the cyclic reaction products [15]. A model DX600 (Dionex, Sunnyvale USA) was used with a Carbopac PA-100 column (4 by 250 mm,

Dionex Sunnyvale). A sample (25 µl) was loaded onto the column and 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. The amounts of CD_6–CD_{21} synthesized were quantified by comparison with standard curves prepared with authentic CD_6–CD_{21} samples.

Determination of CGTase activities

The cyclization activity of the CGTase from *Bacillus sp.* BT3-2 was determined using synthetic amylose as the substrate [6]. A synthetic amylose solution (0.5% w/v) was incubated at 50°C for 10 min in 50 mM acetate buffer (pH 5.5) with the enzyme at a concentration of 2.4 U/ml. The reaction was stopped by boiling (10 min) the mixture in a water bath. The amount of synthesized CD_7 was determined spectrophotometrically by measuring the inclusion of phenolphthalein [8]. An aliquot (100 µl) of the reaction solution was added to a mixture containing 100 µl phenolphthalein in 800 µl sodium hydroxide (0.03 M). One unit of the enzyme activity was defined as the amount of the enzyme that produced 1 µmol of CD_7 per min.

The coupling activity between CD_8 and methyl-α-D-glucopyranoside (MαDG) of the CGTase was measured as described previously [8]. A reaction mixture (100 µl) containing 0.5% (w/v) CD_8 as a donor substrate and 60 mM M&DG as an acceptor substrate was incubated with appropriately diluted CGTase solutions for 10 min at 50°C in 50 mM acetate buffer (pH 5.5). The reaction was stopped by boiling (10 min) the mixture in a water bath. The reaction product was incubated with 3.85 U/ml glucoamylase for 24 h to convert the linear oligosaccharides formed to glucose, then, it was stopped by boiling for 10 min in a water bath. The amount of glucose was determined with glucose oxidase [16]. One unit of the enzyme activity was defined as the amount of enzyme coupling 1 µmol of CD_8 to MαDG per min.

To test the effect of organic solvents, ethanol in different concentrations (5–30%) was added at the beginning of the reaction. The cyclization and coupling activities were measured after 10 min of incubation at 50°C.

The hydrolytic activity of the CGTase from *Bacillus sp.* BT3-2 with large-ring CD was determined by incubating the enzyme with CD_9–CD_{21} in the absence and presence of 15% ethanol. Each reaction medium (100 µl) containing 0.5% (w/v) of a single large-ring CD and appropriately diluted CGTase solution in 50 mM acetate buffer was incubated for 10 min at 50°C. The reaction was stopped by boiling the mixture in a water bath for 10 min. The reaction mixtures were treated with 3.85 U/ml glucoamylase for 24 h, then it was stopped by boiling for 10 min in a water bath. The liberated glucose was determined with glucose oxidase [16]. One unit of the enzyme activity was defined as the amount of the enzyme that produced 1 µmol of glucose per min.

Results

The addition of ethanol to the reaction mixture containing the CGTase from *Bacillus sp*. BT3-2 and synthetic amylose had a marked effect on the yield of large-ring CD synthesized (Fig. 1). The presence of 15% ethanol in the reaction mixture resulted in a 30% increase of the amounts of CD_{10}–CD_{13} synthesized after 5 h of reaction. The addition of 20% ethanol increased the yield of CD_{14}–CD_{21} up to 1000%. With an ethanol concentration of 25%, lower amounts of CD_{14}–CD_{20} were formed while CD_{21} was obtained with a further 5% increase in yield. As shown in Table 1, the addition of ethanol had a similar effect on large-ring CD yield obtained with the CGTase from *Bacillus macerans*. A total yield of 47% CD in the size range from CD_9 to CD_{21} was obtained in the presence of 25% ethanol compared to 19.7% large-ring CD produced without ethanol.

Analysis of the effect of ethanol on the different transglycosylation reactions of the CGTase from *Bacillus sp.* BT3-2 revealed that the coupling activity showed a higher susceptibility to ethanol compared to the cyclization activity. At an ethanol concentration of 5% in the reaction mixture, the coupling activity was

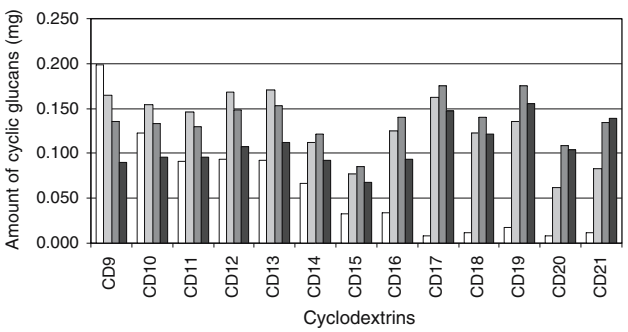

Fig. 1 Effect of the addition of ethanol (0%, 15%, 20% and 25%) on the amount of CD_9–CD_{21} synthesized by the CGTase from *Bacillus sp.* BT3-2 with synthetic amylose as substrate. Ethanol 0%, white bars; ethanol 15%, light shaded bars; ethanol 20%, dark shaded bars; ethanol 25%, black bars

Table 1 Effect of the addition of ethanol to the reaction solutions on the yield of large-ring CD ($CD_{9–21}$) synthesized by the CGTase from *B. macerans*

Ethanol (%)	Large-ring CD yield (%)
0	19.7
10	34.5
15	42.8
20	45.7
25	47.0

reduced to 65% while the cyclization activity was still retained at almost 100%. With 10% and 15% ethanol present in the reaction mixture, the cyclization activity was still 15% higher than the coupling activity.

The hydrolysis rates of the large-ring CD from CD_9 to CD_{21} were also determined in the absence and presence of 15% ethanol in the reaction mixture (Table 2). The results show that the hydrolysis rates of the large-ring CD increased with their DP. By the addition of 15% ethanol, the hydrolysis rates of individual large-ring CD were 32–65% lower compared to reactions performed without ethanol.

Discussion

Our results show that by the addition of ethanol, the yield of large-ring CD synthesized by CGTases can be increased significantly. Ethanol has been previously shown to affect the synthesis of CD_7 by the formation of an inclusion complex, thereby shifting the equilibrium of the synthesis reaction resulting in higher product yields [10]. Ethanol may also affect the function of the CGTase itself. The formation of an inclusion complex with a polar organic solvent may prevent its binding to the active site of the CGTase [12]. It is also possible that the addition of an organic solvent reduces the amount of water or acceptor molecules at the surface of the enzyme thereby preventing the degradation of the large-ring CD by the CGTase.

It has been suggested that the coupling reaction of CGTase can be inhibited by an organic co-solvent [12]. Our results show that the suppression of the coupling and hydrolysis activity by ethanol will result in an increased yield of large-ring CD. The yield of large-ring CD can also be controlled by the temperature of the synthesis reaction which is likewise differently affecting the hydrolysis and coupling activity compared to the cyclization activity [8]. The susceptibility of the large-ring CD to hydrolysis by the CGTase which is increasing with their size contributes to their conversion to smaller CD observed during longer reaction times [7, 9]. By the addition of ethanol, the hydrolysis of large-ring CD could be partially inhibited resulting in significantly higher yields.

The control of the ring-opening hydrolysis and coupling reactions is therefore an important factor for increasing the yield of large-ring CD in the synthesis reaction of the CGTase. By the addition of ethanol, the yield of large-ring CD in a synthesis reaction can be increased by suppressing the secondary degradation reactions of the CGTase resulting in their conversion to smaller CD.

Table 2 Hydrolysis of $CD_{9–21}$ by the CGTase from *Bacillus sp.* BT3-2 in the absence and presence of 15% ethanol in the reaction solutions

CD	Hydrolysis rate (μmol glucose min^{-1})	
	Without ethanol	With 15% ethanol
CD_9	298	204
CD_{10}	406	234
CD_{11}	347	261
CD_{12}	395	261
CD_{13}	590	347
CD_{14}	600	408
CD_{15}	885	426
CD_{16}	694	466
CD_{17}	1180	479
CD_{18}	1042	466
CD_{19}	1151	582
CD_{20}	1527	592
CD_{21}	1632	612

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