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Synthesis of Large-Ring Cyclodextrins by Cyclodextrin Glucanotransferases from Bacterial Isolates

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Abstract

The synthesis of large-ring cyclodextrins (LR-CD) with cyclodextrin glucanotransferase (CGTase) preparations from the bacterial isolates BT3, BT4, BT25 and BT57 was investigated. All CGTase preparations catalyzed the synthesis of LR-CD with 5% soluble starch or 2% synthetic amylose as substrate. The amount and size of LR-CD synthesized depended on the reaction time and on the particular CGTase preparation used. The yield of LR-CD obtained with the enzyme preparation from BT3 and synthetic amylose as substrate was about 18% of the total glucan amount after a reaction time of 23 h.

Introduction

Cyclodextrins (CD) are cyclic α-1,4-glucans with hydrophobic cavities whose sizes and shapes are determined by the number of glucopyranose units forming the macrocycles. CD can form inclusion complexes with various guest compounds [1]. Large-ring cyclodextrins (LR-CD) are composed of 9 to more than several hundred glucopyranose units [2, 3]. Since LR-CD have cavity geometries different from the smaller CD, they may expand the range of macrocyclic host molecules available for molecular inclusion processes. Only in the past 10 years, LR-CD composed of between 9 and 32 glucopyranose units (CD_9 to CD_{32}) have been described. However, due to the low yields and difficulties in their isolation from commercial CD mixtures, only a few of them have been characterized [4, 5].

Cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) catalyze the synthesis of CD from starch by a transglycosylation reaction [6]. Recently, Terada *et al.* [3, 7] have reported the formation of LR-CD from CD_9 to larger than CD_{60} in addition to CD_6 to CD_8 when synthetic amylose was used as the substrate by CGTases from three *Bacillus* species. The LR-CD were formed in the early stage of the reaction and were subsequently converted to smaller CD.

In this paper, we report the production of LR-CD by crude CGTase preparations from four bacterial isolates with soluble potato starch and synthetic amylose as substrates.

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Experimental

Micro-organisms and chemicals

The bacterial isolates were from the culture collection of the Department of Bioprocess Technology, Chemnitz University of Technology, Germany.

Methyl orange, bromcresol green and carboxymethylcellulose were obtained from Sigma Chemical Co., USA. Phenolphthalein and soluble potato starch were from E. Merck, Germany. CD_6, CD_7 and CD_8 were a kind gift from Wacker-Chemie GmbH, Germany. Synthetic amylose with an average molecular weight of 300 kDa was prepared by the method of Kitamura *et al.* [8]. CD_9 to CD_{17} were kindly provided by H. Ueda, Hoshi University, Tokyo, Japan.

Enzyme production

The bacterial isolates were cultivated at 37 °C in 2 L flasks containing 400 mL medium on a rotary shaker with 200 rpm. For the detection and production of CGTase, the liquid medium consisted of (w/v) 2% soluble starch, 0.5% yeast extract, 0.5% tryptone, 0.1% K_2HPO_4, 0.1% NaH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.1% $(NH_4)_2SO_4$, pH 7.0. For the culture of alkalophilic isolates, the medium was adjusted to pH 10 after autoclaving by the addition of sterile 20% Nav_2CO_3 and 20% $NaHCO_3$ to a final concentration of 0.42% and 0.53%, respectively.

Culture broth was harvested after 24 h of incubation and centrifuged at 4 °C. Ammonium sulphate was added to the supernatant to 90% saturation. The precipitate was collected by centrifugation and dissolved in 0.05 M phosphate buffer, pH 7.0. The solution was dialyzed overnight against buffer. Crude enzyme preparations (CD_7 synthesis activity 0.2 U/mL) were obtained by concentrating the solution in dialysis bags with carboxymethylcellulose overnight.

Table 1. CGTase and starch-degrading activity of the bacterial isolates

Isolate No.	CD_6 synthesis activity (U/mL)	CD_7 synthesis activity (U/mL)	CD_8 synthesis activity (U/mL)	Starch-degrading activity (U/mL)
BT3	0.0097	0.0206	0.0132	0.756
BT4	0.0089	0.0158	0.0118	0.672
BT25	0.0021	0.0035	0.0045	0.012
BT57	0.0036	0.0081	0.0058	0.401

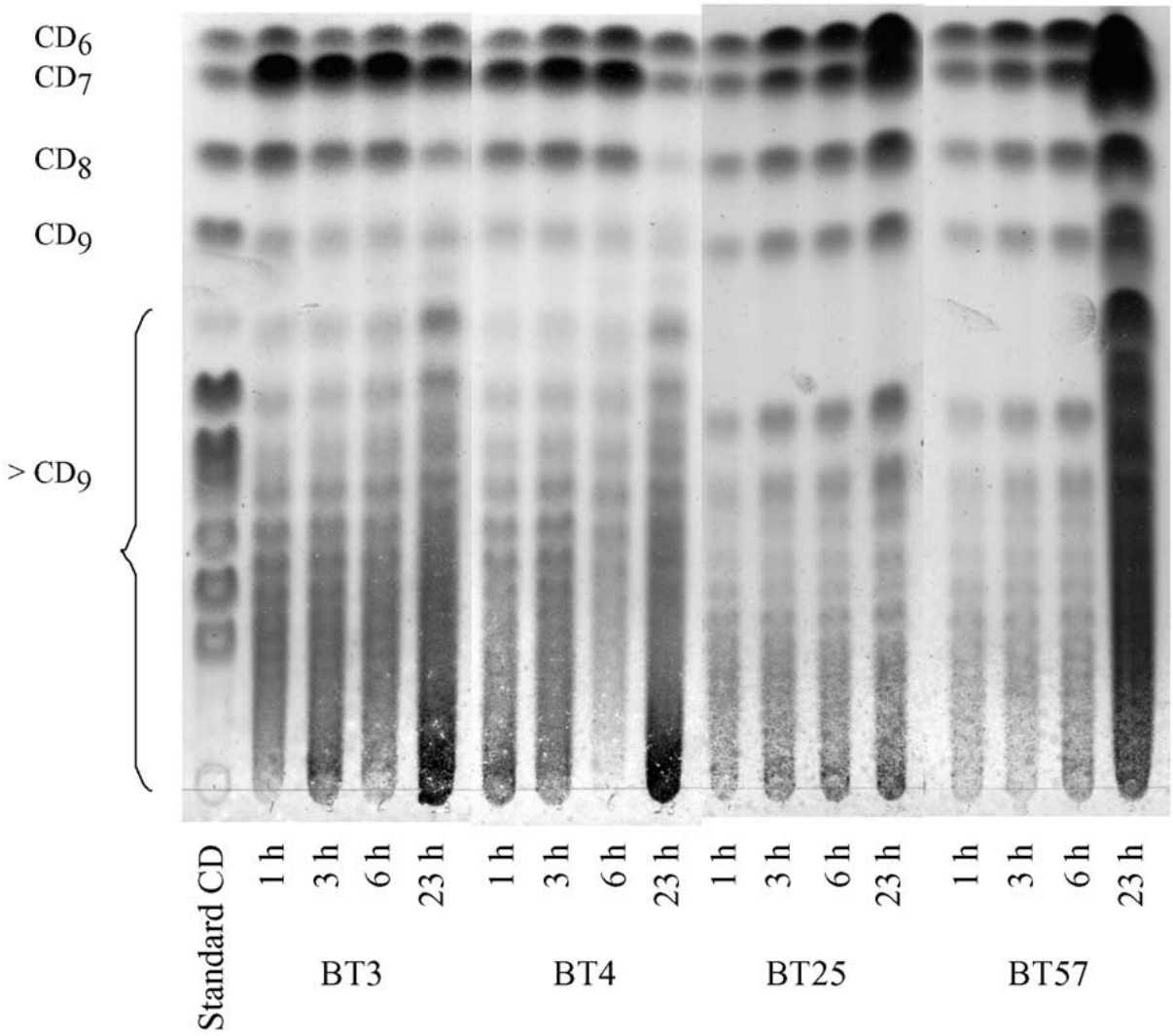

Figure 1. Time course of LR-CD synthesis by CGTase preparations from BT3, BT4, BT25 and BT57. Soluble potato starch (5%) was used as substrate. CD were analyzed by TLC.

Detection of CGTase activity

CD_6 synthesis activity was measured spectrophotometrically by complex formation of CD_6 with methyl orange [9]. One unit of CD_6 synthesis activity was defined as the amount of enzyme which synthesized 1mmol of CD_6 per min. CD_7 synthesis activity was measured spectrophotometrically by complex formation of CD_7 with phenolphthalein [10]. One unit of CD_7 synthesis activity was defined as the amount of enzyme which synthesized 1 μmol of CD_7 per min. CD_8 synthesis activity was measured spectrophotometrically by complex formation of CD_8 with bromcresol green [11]. One unit of CD_8 activity was defined as the amount of enzyme which synthesized 1 μmol of CD_8 per min.

Starch-degrading activity was measured by determining the decrease in absorbance of an iodine starch complex [12]. One unit of starch-degrading activity was defined as the amount of enzyme which decreased the absorbance at 660 nm by 1% per min.

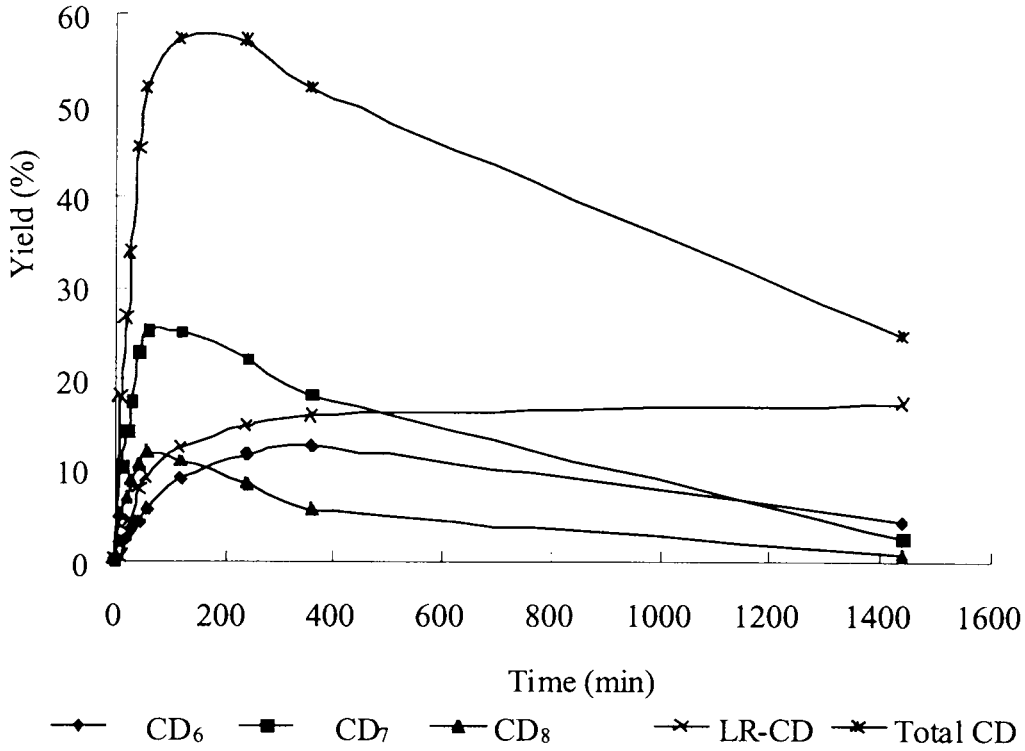

Figure 2. Time course of CD synthesis by the CGTase preparation from BT3 with synthetic amylose (2%) as substrate.

Production of LR-CD

The enzyme preparations (0.2 U/mL) were incubated at 50 °C with 5% soluble potato starch in 50 mM phosphate buffer, pH 7.0. Synthetic amylose (0.5 g) was dissolved in 5 mL of 1 N NaOH solution and neutralized by adding 5 mL of 1 M sodium phosphate buffer (pH 6.0), 5 mL of 1 N HCl, and 10 mL of distilled water. The solution was used immediately after neutralization. The CGTase preparation (0.2 U/mL) from BT3 was incubated at 50 °C with 2% synthetic amylose (w/v) in 50 mM phosphate buffer, pH 7.0. The reactions were stopped by boiling the solutions for 10 min.

Analytical methods

LR-CD production was analyzed by thin layer chromatography (TLC). Protein and starch were removed by the addition of twice the volume of ethanol, followed by centrifugation. The supernatant was evaporated under reduced pressure, and the residue was dissolved in distilled water. To remove linear oligosaccharides and salts, the solution was loaded onto an ODS column (ODS-AQ-120-S50, YMC Co., Ltd., Japan) and washed with distilled water. CD were eluted with 50% methanol. After removing the methanol under reduced pressure, the residue was dissolved in a small volume of distilled water and spotted on a TLC plate (Silica Gel 60, Merck Co., Germany). The plate was developed twice with dioxane-25% ammonia solution (1 : 1, v/v). The products were detected by spraying the plate with 50% ethanolic sulphuric solution and heating at 120 °C for 10 min.

The amounts of LR-CD synthesized by the CGTase preparation from BT3 with synthetic amylose as substrate were determined according to Terada *et al.* [3]. The reaction mixtures were incubated with glucoamylase (120 U, Toyobo Co., Ltd., Japan) in 50 mM phosphate buffer (pH 6.0) for 5 h at 40 °C and then boiled for 5 min. The amounts of CD_6, CD_7 and CD_8 were determined spectrophotometrically as described above. The amount of glucoamylase-resistant products was calculated by subtracting the amount of glucose released by glucoamylase from that of the total amount of glucan in the reaction mixture. The amount of glucose was measured according to Trinder [13].

Results and discussion

For the detection of CGTase-producing bacterial strains, 57 bacterial isolates cultivated in a starch-containing medium were analyzed for CGTase activity. The isolates BT4 cultivated at pH 10, and the isolates BT3, BT25 and BT57 cultivated at pH 7 were identified as CGTase producers. Their CGTase and starch-degrading activities are shown in Table I.

The analysis of the CD reaction products obtained with the crude CGTase preparations from BT3, BT4, BT25 and BT57 and 5% soluble potato starch as substrate by TLC is shown in Figure 1. The time courses of CD synthesis by the CGTases from BT3 and BT4 were similar. Both enzyme preparations synthesized small amounts of LR-CD after short reaction times. With extended reaction times, the amounts of CD_6 to CD_9 decreased with a concomitant increase of the amounts of CD_{10} to CD_{17}. In contrast, the CGTase preparations from BT25 and BT57 both formed increasing amounts of CD_6 to CD_9 and LR-CD during incubation with the enzymes.

Previous research on CGTases has mostly considered the synthesis of CD_6 to CD_8 [2]. The enzyme catalyzes the formation of CD by an intramolecular transglycosylation (cyclization) reaction. However, CGTase catalyzes also additional reactions. By the reverse reaction, a transglycosidic linearization, the CD is cleaved and transferred to an acceptor substrate. Intermolecular transglycosylation also occurs, in which a linear oligosaccharide is cleaved and transferred to a linear acceptor substrate. Furthermore, distinct CGTases show to various extents hydrolytic activities. The CD-forming cyclization reaction has been suggested to occur as an exo-type of reaction leading predominantly to the synthesis of CD_6 to CD_8 [14]. However, Terada and co-workers could show that CGTases also readily produce LR-CD after short reaction times suggesting the cyclization reaction to occur randomly following an endo-type of attack within the amylose molecule [3, 7]. Our data confirm these results which were obtained using synthetic amylose, an unbranched α-1,4-glucan of high molecular weight as substrate and indicate that the CGTases also synthesized LR-CD with soluble potato starch as substrate.

LR-CD were also obtained when the enzyme preparation from BT3 was incubated with synthetic amylose with an average molecular weight of 300 kDa (Figure 2). The amount of LR-CD increased during reaction with the enzyme preparation reaching a maximum yield of 18% of the total glucan amount after 23 h. The amounts of CD_6 to CD_8 reached a maximum after 2 h of incubation decreasing to about 10% of the total glucan amount after 23 h of reaction. Terada *et al.* found when using the same substrate with three other CGTases that LR-CD were produced after short incubation times and were subsequently converted to CD_6 to CD_8 [7]. The rates of this conversion differed however depending on the CGTase used.

Although only crude enzyme preparations were used with the possibility to contain other starch converting activities influencing the product composition, our results indicate that the diverse amounts and sizes of CD synthesized are a result of variances in the extent of the different transglycosylation and hydrolysis reactions of the individual CGTases investigated.

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