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The Application of Cyclodextrin Glycosyltransferase in Biological Science

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Abstract

Cyclodextrin glycosyltransferase is an important enzyme of cyclodextrin synthesis. This article mainly discusses the recent progress of the application of cyclodextrin glycosyltransferase in biological science. These applications included that synthesis of large-ring CD and enzyme engineering to produce a specific type of CD. Through these applications, the cyclodextrin glycosyltransferase will develop a great contribution to the new type of CD synthesis research area.

Keywords: Cyclodextrin glycosyltransferase; Large-ring CD; Enzyme-engineering; CD synthesis

Introduction

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) plays a role in the starch utilization pathway of some bacteria and catalyzes various glucan transfer reactions with starch. The action of CGTase starts with the cleavage of one α-1, 4-linkage within the glucan molecule. The newly produced reducing end is then transferred either to the nonreducing end of another molecule (disproportionation reaction) or to its own nonreducing end (cyclization reaction). CGTase also catalyzes the reverse reaction of cyclization, in which cycloamylose is opened by the enzyme and a linearized fragment is transfered to an acceptor (coupling reaction). At a certain frequency, the newly produced reducing end is transferred not to a carbohydrate acceptor but rather to a water molecule, which results in either the hydrolysis of amylose or the linearization of cycloamyloses (hydrolytic reaction).

Application in synthesis of large-ring cyclodextrins

Large-ring cyclodextrins (LR-CDs) are composed of 9 to more than several hundred glucopyranose units. LR-CDs have cavity geometries different from the smaller CD, which may expand the range of macrocyclic host molecules available for molecular inclusion processes. Up to date, LR-CDs composed of between 9 and 32 glucopyranose units (CD_9 to CD_{32}) have been described [1,2]. However, due to the low yields and difficulties in their isolation from commercial CD mixtures, only a few of them have been characterized [3]. The crystal structure of CD_9 showed that its macrocycle is distorted, forming a boat-shaped molecule and CD_9 is more flexible than CD_6, CD_7 or CD_8 [4,5]. 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 [6-10]. The structure of CD_{26} contains two single helices with 13 glucose units each in antiparallel direction [11-13]. Due to their structural features which are distinct from the small CD, LR-CDs could find applications as novel host compounds in molecular recognition processes [14].

The formation of CD_6 to CD_9 in different proportions at the beginning of the reaction, depending on the type of enzymes obtained from various bacterial isolates, has also been shown [15]. In addition to CD_6 to CD_8, Okada and coworkers could demonstrate that the CGTase from the alkalophilic *Bacillus sp.A2-5a*, *B.stearothermophilus* and *B. macerans* produced LR-CD from CD_9 to more than CD_{60} in an early stage of the reaction when synthetic amylose (average molecular mass of 30 kDa) was used as the substrate [16,17]. Zheng et al. [18] report the production of LR-CDs by crude CGTase preparations from four bacterial isolates with soluble potato starch and synthetic amylose as substrates. The four bacterial isolates were named BT3, BT4, BT25 and BT57 respectively. It was found that the time courses of CD synthesis by the CGTases from BT3 and BT4 were similar. Both enzyme preparations synthesized small amounts of LR-CDs after short 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-CDs during incubation with the enzymes. The yield of LR-CDs 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 23h. The amount and size of LR-CDs synthesized depended on the reaction time and on the particular CGTase preparation used [18]. These results suggest that the initial formation of LR-CDs by a random cyclization reaction is a common feature of CGTases. The formation of LR-CDs is, therefore, dependent on the substrate specificity of the coupling and hydrolytic activities, and on the product specificity of the cyclization reaction of the CGTase.

It is generally believed that the cyclization reaction of CGTase on amylose is an exo-type attack, where the enzyme recognizes the 6-8 glucose units from the non-reducing end, attacks the adjacent α-1,4-linkage, and transfers it to the C-4 position of the non-reducing end to produce α-,β-,or γ-CD [19] (Figure 1A).

Terada et al. [16] proposed a new model for LR-CDs production [16]. They investigated the initial action of cyclodextrin glycanotransferase from an alkalophilic *Bacillus sp.* A2-5a on amylose. They incubated the synthetic amylose with purified CGTase then terminated the enzyme reaction in the very early stage. Then the reaction mixture was treated with glucoamylase and the resulting glucoamylase-resistant glucans were cyclic α-1, 4-glucans, with degree of polymerization ranging from

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9 to more than 60, in addition to well known α-, β-, and γ-CD. The time-course analysis revealed that LR-CDs were preferentially produced in the initial stage of the cyclization reaction and were subsequently converted into smaller cyclic α-1, 4-glucans and into the final major product, β-CD. CGTase from *Bacillus macerans* also produced LR-CDs except that the final major product was α-CD. Based on these results, they put forward a new model for the action of CGTase on amylose. It goes like the following:

CGTase attacks any α-1,4-linkage within the amylose molecule, and then transfers the newly formed reducing end of the substrate either to the non-reducing end of a separate linear acceptor molecule or glucose (the intermolecular transglycosylation or disproportionation reaction), or to its own non-reducing end (the intramolecular transglycosylation or cyclization reaction, Figure 1B). This random cyclization reaction produces wide range of cyclic α-1, 4-glucans with DP 6 to more than 60. The reversibility or these reactions allows large cyclic molecules to be linearized again by transglycosylation, and smaller cyclic molecules to be subsequently produced. The equilibrium of the whole reaction tends toward the formation of α- or β-CD as the final major products [16].

Further evidence for a random cleavage of soluble starch by CGTase similar to an endo-type of attack has also been provided using fluorescent-labelled starch as substrate [20].

The yield of LR-CDs could be controlled by the temperature of the synthesis reaction which was probably inhibiting the hydrolysis and

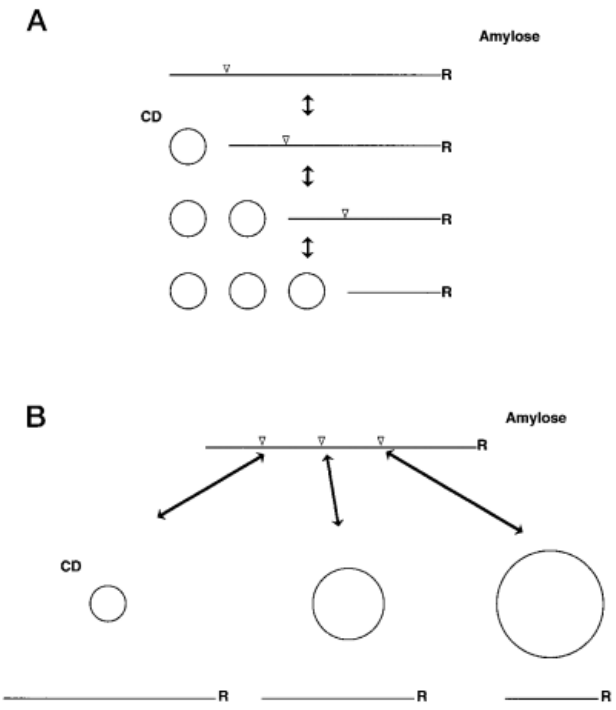

Figure 1: Models of cyclization reaction of CGTase on amylose. A is a conventional model for the action of CGTase. B is a proposed model. Solid lines and circles indicate α-1, 4-glucan chains, where the relative length represents their relative DP. R, glucosyl residue with reducing end; ∆, α-1, 4 linkage attacked by CGTase; CD, α-, β-, γ-CD. Reprinted from [16]. Cyclodextrins are not the major cyclic α-1, 4-glucans produced by the initial action of cyclodextrin glucanotransferase on amylose, 25 pp 15729-15733, Reprinted with permission. Copyright (1997) The American Society for Biochemistry and Molecular Biology, Inc.

coupling activity compared to the cyclization activity. For example, the synthesis of cyclodextrins with from 6 to more than 50 glucose units by cyclodextrin glucanotransferase from *Bacillus macerans* was investigated. It was found that a higher yield of large-ring cyclodextrins were obtained with a reaction temperature of 60°C compared to 40°C. It was shown from the yield of large-ring cyclodextrins obtained at 60°C that about 50% of the total glucans participated in the reaction. Further analysis indicated that cyclization reaction mainly occurred at 60°C while coupling reaction mostly occurred at 40°C, which suggested that cyclization reaction was related to cyclodextrin forming but coupling reaction could degrade larger cyclic α-1, 4-glucans [21].

The hydrolysis of the LR-CDs by CGTases would be also suppressed in the presence of ethanol. For instance, adding ethanol to the reaction medium resulted in an increase of the yield of LR-CDs 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 5h of reaction. Adding 20% ethanol increased the yield of CD_{14}-CD_{21} up to 1000%. It has been suggested that the coupling reaction of CGTase can be inhibited by an organic co-solvent. By the addition of ethanol, the hydrolysis of LR-CDs could be partially inhibited resulting in significantly higher yields.

Application in enzyme engineering to produce a specific type of CD

All known CGTases produce a mixture of α-, β-, and γ-cyclodextrins. For the industrial production of pure cyclodextrins, β-CD is selectively crystallized and α- and γ-CD are complexed with organic solvents. The industrial production of cyclodextrins might be improved by the construction of mutant CGTase with improved product specificity.

Site-directed mutagenesis of CGTase

Cyclodextrin glycanotransferase (CGTase) constitute a group of transglycosidases that belong to family 13 of the glycoside hydrolases (α-amylase family). A distinctive feature of CGTases is the existence of the so-called cyclization axis (generally an aromatic residue, either Phe or Tyr) which is crutial for cyclodextrin formation. Two carboxylic residues (the catalytic nucleophile Asp229 and the acid/base catalyst Glu257) are involved in a combined attack on a glycosidic bond that results in the release of the reducing end of amylose.

It had been demonstrated for CGTase that the transglycosylation reaction operates by a ping-pong mechanism [22]. Veen et al. [23] proposed a scheme for the cyclization reaction as depicted in Figure 2C. In this scheme, the involvement of the maltose binding sites (MBS) located in the E-domain is included. The first step is binding of a starch chain at MBS1, after which this binding is extended to the active site via MBS2. Cleavage of the starch chain results in formation of the nonreducing end of the covalently linked intermediate to the acceptor site, resulting in the formation of a CD (Figure 2C). Cyclic and linear compounds can bind closely to each other in the active site (Figure 2B). Thus, the starch chain bound to the MBSs does not have to move far to allow ring closure. Upon dissociation of the synthesized CD, the same starch chain can immediately migrate further down in to the active site. Alternatively at MBS1 the bound starch chain may be replaced by another chain at any time during the reaction, without affecting the reaction taking place in the active site. And it is clear that the binding sites of the linear donor and acceptor substrates used overlap at least partially. The cleaved-off part of the donor substrate has to dissociate

from the active site before the acceptor substrate can bind at these acceptor subsites (Figure 2A) [24].

Y195W: A mutant in which residues Tyr195 is replaced by a tryptophan. Tyr195 plays a central role in curling glycosyl chain back because it participates in a guideway near the active site. If this residue was mutated to a tryptophan, a substrate guide would favor longer chains. The left picture of Figure 3 showed 3D structure of CGTase from *Bacillus circulans* strain 251. After it was mutated with Tyr195 to Trp, its 3D structure (Right of Figure 3) was changed greatly and

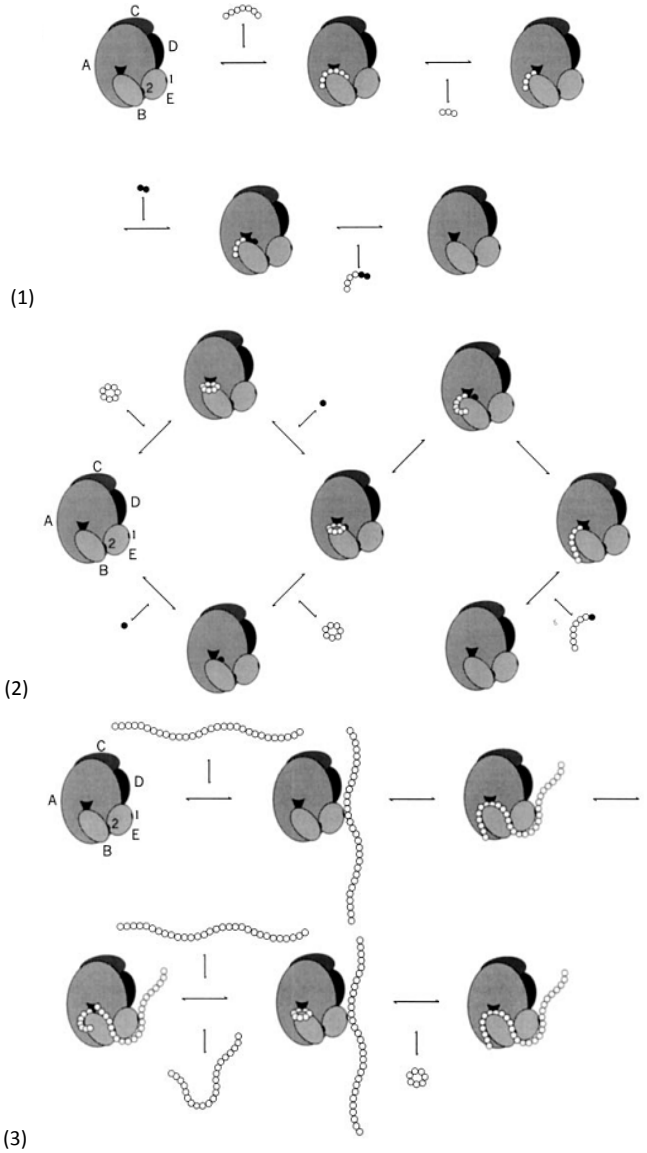

Figure 2: Proposed model of the events taking place in the CGTase-catalyzed reactions. (1) Disproportionation, (2) Coupling, (3) Cyclization. The different CGTase domains are indicated (A, B, C, D, and E). 1 and 2 indicate the maltose binding sites on the E-domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residues; acceptor residues are represented in black. Reprinted from [23].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) FEBS.

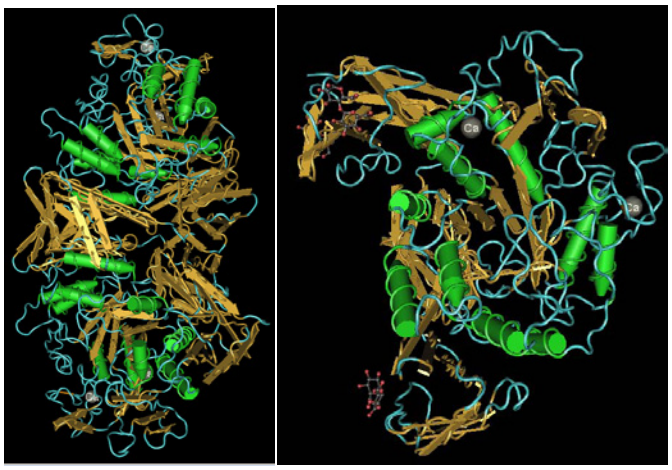

Figure 3: Left: CGTase(E.C.2.4.1.19) from *Bacillus circulans* strain 251(Klein, 1991).Right: CGTase (E.C.2.4.1.19) from *Bacillus circulans* strain 251 mutant with Tyr 195 replaced by Trp (Y195w)(Penninga, 1995). Reprinted from Chen et al., MMDB: Entrez's 3D-structure database, 31: pp474-477. Reprinted with permission. Copyright (2000) NCBI.

it seems that its topological structure would become much looser than that of the natural CGTase. Indeed, mutant Y195W made CD_8 the main product. But there was such a limitation of Y195W that the mutation decreased the absolute CD_8 formation rate [25].

Mutant Δ (145–151) D: A mutant in which residues 145 through 151 are deleted and replaced by an aspartate. Subsites from residue 145 to residue 151 are responsible for binding the preferred α-glucan and provide a stereoselectivity of the substrates. The deletion was thought to produce more space for the bound glycosyl chain, the aspartate could perform the shortcut and could also help binding saccharides. The structure analysis of this mutant Δ (145-151) D showed only local changes. The newly introduced aspartate had clear electron density, indicating a defined conformation. Mutation Δ (145-151) D could change the product spectrum in favor of CD_8, but not as drastically as mutation Y195W. But there was such an advantage of Δ (145-151) D mutant that the mutation could increase the absolute CD_8 formation rate [25].

Mutant Arg47→Leu or Arg47→Gln: Recent structural studies have identified Arg47 in the *Bacillus circulans* strain 251 CGTase as an active-site residue interacting with cyclodextrins, but not with linear oligosaccharides. Arg47 thus may specifically affect CGTase reactions with cyclic substrates or products. It was shown that mutations in Arg47 (to Leu or Gln) indeed have a negative effect on the cyclization and coupling activities; Arg47 specifically stabilizes the oligosaccharide chain in the transition state for these reactions. As a result, the mutant proteins display a shift in product specificity towards formation of larger cyclodextrins. As expected, both mutants also showed lower affinities for cyclodextrins in the coupling reaction, and a reduced competitive (product) inhibition of the disproportionation reaction by cyclodextrins. Both mutants also provide valuable information about the processes taking place during cyclodextrin production assays. Mutant Arg47→Leu displayed an increased hydrolyzing activity, causing accumulation of increasing amounts of short oligosaccharides in the reaction mixture, which resulted in lower final amounts of cyclodextrins produced from starch. Interestingly, mutant Arg47→Gln displayed an increased ratio of cyclization/coupling and a decreased hydrolyzing activity. Due to the decreased coupling activity, which

especially affects the production of larger cyclodextrins, this CGTase variant produced the various cyclodextrins in a stable ratio in time. This feature is very promising for the industrial application of CGTase enzymes with improved product specificity [24].

Crosslinking imprinted protein engineering

Recently, a technique of crosslinking imprinted proteins (CLIP) has been described. In the first step, the enzyme is derivatized with itaconic acid anhydride and then imprinted with ligands such as substrate analogs or inhibitors in aqueous medium [26,27]. Subsequently, the manipulated enzyme conformation is fixed by polymerizing it in a water-free organic solvent. The ligand is removed in the final step, and the CLIP enzyme can be used either in aqueous medium or organic solvent. The CLIP enzymes show altered substrate or product specificity and enhanced stability in high concentrations of organic solvents. CLIP enzymes are also more enantioselective than the native enzyme. Furthermore, they are insoluble and can be separated and recycled many times, increasing their productivity [28].

Some researchers used various amounts of itaconic anhydride to acylate *Panehibacillys sp.*A11 (A11 CGTase) and *Bacillus macerans* CGTase (BM CGTase) in potassium phosphate buffer (pH 6.0). Then they used gel filtration to remove nonreacted itaconic anhydride and other low molecular mass compounds. The fractions containing CGTase activity were combined and lyophilized. Dry derivatized enzyme and CD_8 were dissolved in potassium phosphate buffer. The mixture was incubated at 25°C and then the CGTase-CD_8 complex was precipitated by adding n-propanol (-20°C) and kept on ice for 10min. The precipitate was collected and the pellet was washed with n-propanol (-20°C), freeze-dried and kept at -20°C. Imprinted derivatized CGTase were suspended in dry cyclohexane by using an ultrasonication bath. 2, 2'-azobis and ethylene glycol dimethacrylate were added to the suspension. The radical polymerization was initiated by UV irradiation at 25°C. The resulting polymer was washed with cyclohexane and potassium phosphate buffer and lyophilized. At last The CD_8-imprinted CLIP CGTase was obtained. The CD_8-imprinted CLIP CGTase showed optimal pH and temperature similar to those of the native CGTase. However, the pH and temperature stability of CLIP CGTase were higher than those of the native CGTase. The catalytic activity of GLIP CGTase for CD_8 synthesis was increased 10-fold that of native CGTase. Comparison of the product ratios by HPAEC (High Performance Anion Exchange Chromatography) showed that the native A11 CGTase and BM CGTase produced CD_6:CD_7:CD_8:≥CD_9 ratios of 15:65:20:0 and 43:36:21:0 after 24h of reaction at 40°C with starch substrates respectively. In contrast, the CLIP CGTase from A11 and BM produced cyclodextrin in ratios of 15:20:50:15 and 17:14:49:20, respectively. The size of the synthesis products formed by CLIP CGTase was shifted towards CD_8 and ≥CD_9, and the overall cyclodextrin yield was increased by 12% compared to the native enzymes [29].

Conclusions

Large ring cyclodextrins could be synthesized by CGTase. The key point of such a technique might be the enzyme activity; furthermore, all research presently focused on the strain with high CGTase activity breeding. Another problem is the recovery of large ring cyclodextrin from the fermentation broth.

Specific type of CD could be synthesized by mutational CGTase. Mutagenesis technology might produce the most required CD product, but the cost is very high and the properties of these mutants are not very stable, whereas crosslinking imprinted protein engineering might reduce the cost and get the high stability, but the synthesized product need a lot of continuous separation work to obtain the high purity CD product. Even different mutants have different transferring capacities. Among them, Mutant Arg47→Leu or Arg47→Gln is the most effective one which could get the high pure CD product, next one is Mutant Δ (145−151) D, and the last one is Y195W. Further studies should be focused on what reason caused their different transferring capacities.

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