Cyclodextrins Are Not the Major Cyclic α-1,4-Glucans Produced by the Initial Action of Cyclodextrin Glucanotransferase on Amylose

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The initial action of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from an alcalophilic Bacillus sp. A2-5a on amylose was investigated. Synthetic amylose was incubated with purified CGTase then terminated in the very early stage of the enzyme reaction. When the reaction mixture was treated with glucoamylase and the resulting glucamylase-resistant glucans were analyzed with high performance anion exchange chromatography, cyclic α-1,4-glucans, with degree of polymerization ranging from 9 to more than 60, in addition to well known α-, β-, and γ-cyclodextrin (CD), were detected. The time-course analysis revealed that larger cyclic α-1,4-glucans 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 large cyclic α-1,4-glucans except that the final major product was α-CD. Based on these results, a new model for the action of CGTase on amylose was proposed, which may contradict the widely held view of the cyclization reaction of CGTase.

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19), found in several bacterial species, catalyzes the inter- and intramolecular transglycosylation of α-1,4-glucan. Such activity of CGTase on inter- and intramolecular transglycosylation of α-1,4-glucan is called the disproportionation reaction and the cyclization reaction, respectively. It is also known that CGTase catalyzes the transglycosidic linearization (coupling reaction) of cyclic α-1,4-glucan in the presence of a suitable acceptor molecule to produce linear α-1,4-glucan. The cyclization reaction of CGTase has been of great interest since this is the only enzyme that can produce α-, β-, and γ-cyclodextrin (CD), which are generally known as the cyclic α-1,4-glucan with DP of 6, 7, or 8. These CDs all have a hydrophobic central cavity, incorporate various inorganic or organic compounds, and form inclusion complexes (1). Therefore, these CDs are widely used in the pharmaceutical, food, agricultural, and cosmetic industries (2).

Extensive analyses on various CGTases indicated that all CGTases convert amylose or amylopectin into a mixture of α-, β-, and γ-CD and remaining dextrans; differences, however, were found in their product specificities (α-, β-, and γ-CD ratios). Thus, CGTase is sometimes classified into three types (α-, β-, and γ-CGTase), depending on the major CD produced. Since α-, β-, and γ-CD all have a dimensionally distinct central cavity and different specificity for guest molecules, recent studies on CGTase have focused on trying to understand the mechanism of the cyclization reaction and to find or engineer a CGTase that produces a specific type of CD.

Several approaches have been carried out to obtain the structural explanation of the cyclization reaction of CGTase. Analyses of the three-dimensional structure of CGTase have been carried out using several types of CGTases (3–6). Additionally, the structures of CGTases with substrates (5, 7–9) and with inhibitor molecules (9, 10) were also analyzed. From these studies, models of CGTase activity cleaving the target α-1,4-linkage were proposed. In the case of CGTase from Bacillus circulans strain 251, three active site residues, Asp-229, Glu-257, and Asp-328, which are conserved in all CGTase primary sequences, play important roles for this step of reaction (8, 10).

A similar catalytic mechanism has been reported from structural studies on amylases (11–14) that hydrolyze α-1,4-glucan. Several amino acid residues involved in substrate binding or in the determination of product specificity have also been proposed by three-dimensional structure analyses (5, 6, 8, 9) or by protein engineering approaches (15–17). However, it is less clearly understood how CGTase catalyzes the following intramolecular transfer reaction to produce cyclodextrins.

Although α-, β-, and γ-CD are the major products of CGTase, it has been known that trace amounts of larger cyclic glucans (δ-, ε-, ζ-, η-, and θ-CD) were also present in the reaction mixture of CGTase on starch (18, 19). The structures of these larger cyclic glucans are still not well understood, because they seem to be a mixture of cyclic α-1,4-glucans, outer-branched cyclic α-1,4-glucans, and inner-branched cyclic glucans (18). Kobayashi and Yamazaki (20) carried out further structural analyses on putative δ-, ε-, ζ-, η-, and θ-CD fractions and reported that the δ-CD fraction contained a large amount of unbranched cyclic α-1,4-glucan with DP 9. However, the proportion of unbranched cyclic α-1,4-glucan in the following fraction decreased dramatically (50% in ε-CD, 25% in ζ-CD, and almost 0% in η- and θ-CD fractions). From this study, it is thought that the presence of cyclic α-1,4-glucan with DP larger than 12 in the CGTase reaction products is unlikely. Recently, however, we found that potato D-enzyme (disproportionating enzyme or 4-α-glucanotransferase, EC 2.4.1.25) catalyzes an intramolecular transglycosylation reaction on amylose to produce cyclic α-1,4-glucans with DP range from 17 to several hundred (21). The time-course analysis of D-enzyme action on amylose revealed that large cyclic α-1,4-glucans were preferentially produced in the initial stage of the cyclization reaction, and subsequently converted into small cyclic α-1,4-glucans, although α-, β-, and γ-CD were never produced (21). D-enzyme also catalyzes disproportionating reactions on malto-oligosaccharides (22) and transglycosidic linearization of cyclic α-1,4-glucans in the presence of a suitable acceptor (21). In all these
respects, D-enzyme and CGTase both seem to catalyze the same reaction, with the major difference being the DP of the cyclic α-1,4-glucan produced. Furthermore, we also reported that the glycogen-branching enzyme (EC 2.4.1.18) from Bacillus steareothermophilus also catalyzes the intramolecular trans-glycosylation of amylose and amylpectin to produce branched cyclic glucans with DP larger than 18 (23, 24). From these studies on glycosyltransferases other than CGTase, we are interested in the action of CGTase in producing cyclic glucans larger than CDs. In this paper, we investigated the initial action of CGTase from an alkalophilic Bacillus sp. A2-5a (25) on synthetic amylose and found that the CGTase also produced cyclic α-1,4-glucans with DP ranging from 9 to more than 60, in addition to α-, β-, and γ-CD, from synthetic amylose in the very early stage of the reaction.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—Synthetic amylose with an average molecular mass of 30 kDa (amylose AS-30) and soluble starch were purchased from Nakano Vinegar Co., Ltd. (Aichi, Japan) and E. Merck AG (Darmstadt, Germany), respectively. Glucoamylase from Rhizopus sp. was purchased from Toyobo Co., Ltd. (Osaka, Japan). CGTase from an alkalophilic Bacillus sp. A2-5a was purified to a homogeneous state (25). CGTases from B. macerans was purchased from Amano Pharmaceutical Co., Ltd. (Aichi, Japan) and was 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 (25).

Preparation of Amylose Solution—Amylose AS-30 (8 mg) was dissolved in 200 μl of 1 N NaOH solution then neutralized by adding 200 μl of 1 M sodium acetate buffer (pH 5.5), 200 μl of 1 N HCl, and 400 μl of distilled water. The solution was used immediately after neutralization.

Analysis of Reaction Products of CGTase on Amylose—CGTase (0.75 unit/ml) was incubated at 40 °C with amylose AS-30 (0.4% (w/v)) in 0.2 M sodium acetate buffer, pH 5.5, and reactions were terminated by boiling the solutions for 10 min. The reaction mixture containing 20 μg of glucan was incubated with glucoamylase (0.2 units) in 20 mM sodium acetate buffer (pH 5.5) for 16 h at 40 °C and then boiled for 5 min. The products in the reaction mixture were determined with high performance anion exchange chromatography (HPAEC, see below). The amounts of α-, β-, and γ-CD were measured with high performance liquid chromatography (HPLC, see below). The amount of glucoamylase-resistant molecules was calculated by subtracting the amount of glucose released by glucoamylase from that of total glucan in the reaction mixture. The amount of glucose was measured by the glucose oxidase method (26).

HPAEC—HPAEC was carried out based on the DX-300 system (Dionex) with a pulsed amperometric detector (model PAD-II, Dionex) using a CarboPac PA-100 column (4 mm × 250 mm). A sample (25 μl) containing 40 μg of glucan was injected and eluted with a gradient of sodium acetate (0–2 min, 50 mM; 2–37 min, increasing from 50 mM to 350 mM with the installed gradient program 3; 37–45 min, increasing from 350 mM to 850 mM with the installed gradient program 7; 45–47 min, 850 mM) in 150 mM NaOH with a flow rate of 1 ml min⁻¹.

HPLC—HPLC was carried out based on the DX-300 system (Dionex) using an Aminex HPX-42A column. To remove glucose from the reaction mixtures, a sample (50 μl) containing 80 μg of glucan was charged on a Waters Sep-Pak C₁₈ cartridge (Millipore), washed with 10 ml of H₂O and eluted with 1.5 ml of 50% methanol. The eluate was dried up in vacuo, and dissolved in 50 μl of water. The sample was then injected and eluted with water with a flow rate of 0.6 ml min⁻¹ at 80 °C. The eluate from the column was mixed with 0.3 M LiOH using an anion micromembrane suppressor (model AMMS-II, Dionex), after which the carbohydrate in the eluate was detected with a pulsed amperometric detector (model PAD-II, Dionex).

“Time of Flight” Mass Spectrometry (TOF-MS)—A reaction mixture (5 ml) containing 10 mg of amylose AS-30 and CGTase (0.7 unit) was incubated in 0.2 M sodium acetate buffer, pH 5.5, at 40 °C for 1 h, and then boiled for 10 min. The reaction mixture was incubated with 10 units of glucoamylase at 40 °C for 16 h, and then boiled for 5 min. After removing glucose with the Waters Sep-Pak C₁₈ cartridge, the molecular masses of glucoamylase-resistant glucans were determined with a Kompact Maldi I TOF-MS system (Shimadzu, Kyoto, Japan).

RESULTS

Analysis of Initial Glucoamylase-resistant Molecules of CGTase on Amylose—Synthetic amylose AS-30 was incubated...
with CGTase from alkalophilic *Bacillus* sp. A2-5a. The enzyme reaction was terminated at the early stage of the reaction (10 min), and then the reaction mixture was incubated with glucoamylase to digest the linear amyllose into glucose. When the glucoamylase-resistant molecules thus obtained were analyzed by HPAEC, many peaks were detected (Fig. 1A). Note that these peaks were not found in a control experiment with heat-inactivated CGTase (result not shown). Most of these peaks were eluted in the region where cyclic $\alpha$-1,4-glucans with DPs of over 17, produced by potato D-enzyme on synthetic amylose (Fig. 1F), were eluted. This result suggests that CGTase produced such cyclic $\alpha$-1,4-glucans. The large peak around 42 min may indicate the presence of glucoamylase-resistant molecules with DP more than 60, since these molecules were not resolved in this HPAEC condition and eluted together.

Then to prove the cyclic structure of glucoamylase-resistant molecules produced by CGTase, their molecular masses were determined with TOF-MS (Fig. 2). A non-cyclic glucan with DP of n has a molecular mass of $162.1436n + 18.01534$ Da, whereas a cyclic glucan should have a molecular mass of $162.1436n$ Da. The molecular mass of each glucoamylase-resistant molecule was compared with the theoretical value for non-cyclic and cyclic glucans (Table I). As shown in Table I, experimental values of glucoamylase-resistant molecules were consistent with theoretical values for cyclic glucan but not with those for non-cyclic glucan.

To confirm that the glucoamylase-resistant molecules produced by CGTase are $\alpha$-1,4-glucans, the structure of these glucans was further examined by treatment with several enzymes. $\alpha$-Amylase from *Bacillus subtilis*, an endo-type amylase, completely degraded these molecules to glucose and maltose, and isoamylase and pullulanase, which degrade $\alpha$-1,6-linkage of glucans, did not (data not shown). These results indicate that the glucoamylase-resistant molecules produced by CGTase were $\alpha$-1,4-glucans.

Based on all the results mentioned above, we concluded that the molecules produced by the CGTase reaction on amyllose, as shown in Fig. 1A, were cyclic $\alpha$-1,4-glucans with DPs ranging from 6 to more than 60.

**Time Course of Reaction of CGTase on Amylose**—It is known that the glucoamylase-resistant products of the CGTase reaction on amyllose, after a prolonged reaction time, are $\alpha$-, $\beta$-, and $\gamma$-CD and negligible amounts of other glucoamylase-resistant glucans. Our result apparently contradicts this widely held view since the major cyclic $\alpha$-1,4-glucans produced in the initial stage of the CGTase reaction were not $\alpha$-, $\beta$-, and $\gamma$-CD but were those with high DPs. To investigate how large cyclic $\alpha$-1,4-glucans, which were found in the initial stage of CGTase reaction, were replaced by $\alpha$-, $\beta$-, and $\gamma$-CD, the time course of the reaction of CGTase were monitored. Amylose AS-30 was incubated with CGTase from alkalophilic *Bacillus* sp. A2-5a for up to 960 min. Each sample was treated with glucoamylase and was analyzed by HPAEC (Fig. 1). As shown in Fig. 1 (A–E), cyclic $\alpha$-1,4-glucans with DPs of over 60 were most prevalent after a reaction time of 10 min (Fig. 1A). However, with prolonged reaction, cyclic $\alpha$-1,4-glucans with high DPs gradually decreased, and those with low DPs increased. At the end of the reaction, the main product of CGTase was $\beta$-CD (Fig. 1E).

Fig. 3 shows the time course of the amount of cyclic $\alpha$-1,4-glucans produced by CGTase on amyllose AS-30. The amount of total cyclic $\alpha$-1,4-glucan (○), and total CD (the sum of $\alpha$-, $\beta$-, and $\gamma$-CD, □) were determined as described under “Experimental Procedures.” Cyclic $\alpha$-1,4-glucans apart from $\alpha$-, $\beta$-, and $\gamma$-CD (●) were calculated by subtracting the amount of total CD from that of total cyclic $\alpha$-1,4-glucan.
decreased thereafter.

Analysis of Initial Action of Other CGTases—CGTases found in many bacterial species are classified into three types, α-, β-, or γ-CGTase, depending on the major product of the cyclization reaction. The CGTase employed above is β-CGTase because it mainly produces β-CD (25). To examine whether the production of a large cyclic α-1,4-glucan is the specific feature only found in this CGTase or is the common feature for others, similar experiments were carried out by using CGTases from B. macerans, which is classified as α-CGTase. This enzyme also produced large cyclic α-1,4-glucans in the initial stage of reaction (Fig. 4, A and B), which were subsequently converted into small cyclic α-1,4-glucans. However, the final major cyclic product was α-CD (Fig. 4E).

DISCUSSION

It is generally believed that the cyclization reaction of CGTase on amylose is an exo-type attack (2), 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 (Fig. 5A). This view was only confirmed from the analysis of CGTase action on 14C-labeled linear α-1,4-glucans with DP 7–12 (27), but not investigated in high molecular weight glucans. If this view can be applied to the CGTase action on high molecular weight glucans, cyclic products throughout the reaction on amylose are expected to be only α-, β-, and γ-CD. However, the results presented in this paper clearly demonstrate that the cyclic glucans produced in the initial stage of cyclization reaction of CGTase are not only α-, β-, and γ-CD, but are cyclic α-1,4-glucans with various DP ranging from 6 to more than 60. Large cyclic α-1,4-glucans were preferentially produced in the initial stage of cyclization reaction, which were subsequently converted into small cyclic α-1,4-glucans and into the final major products, α-CD or β-CD. Thus these findings apparently contradict the widely held view of the action model of CGTase, and so we propose a new model for the action of CGTase as shown in Fig. 5B. CGTase probably 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, Fig. 5B). This random cyclization reaction produces wide ranges of cyclic α-1,4-glucans with DP 6 to more than 60. The reversibility of 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.

Both CGTase and D-enzyme catalyze the cyclization and disproportionation of α-1,4-glucan and transglucosidic linear-
ization of cyclic α-1,4-glucan in the presence of a suitable acceptor molecule. During the cyclization reaction, large cyclic α-1,4-glucans were preferentially produced in the initial stage, but were subsequently converted into smaller cyclic α-1,4-glucans in both cases. Thus both enzymes seem to catalyze the same reaction, with the major difference being in the smallest size of the cyclic α-1,4-glucans produced. The DP of the smallest cyclic α-1,4-glucan produced by CGTase is 6. On the other hand, D-enzyme never produced α-, β-, and γ-CD and the smallest cyclic α-1,4-glucan has DP of 17. It is very interesting to know how the specificities of these products are determined differently between D-enzyme and CGTase. In our previous paper (23), we discussed that cyclic α-1,4-glucans differently between D-enzyme and CGTase. In our previous analyses.

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