Potato D-enzyme was purified from recombinant Escherichia coli, and its action on synthetic amylose (average M, of 320,000) was analyzed. D-enzyme treatment resulted in a decrease in the ability of the amylose to form a blue complex with iodine. Analysis of the products indicated that the enzyme catalyzes an intramolecular transglycosylation reaction on amylose to produce cyclic α-1,4-glucan (cycloamylose). Confirmation of the cyclic structure was achieved by demonstrating the absence of reducing and nonreducing ends, resistance to hydrolysis by glucoamylase (an exoamylase), and by “time of flight” mass spectrometry. The degree of polymerization of cycloamylose products was determined by time of flight mass spectrometry analysis and by high-performance anion-exchange chromatography following partial acid hydrolysis of purified cycloamylose molecules and was found to range from 17 to several hundred. The yield of cycloamylose increased with time and reached >95% D-enzyme did not act upon purified cycloamylose, but if glucose was added as an acceptor molecule, smaller cyclic and linear molecules were produced. The mechanism of the cyclization reaction, the possible role of the enzyme in starch metabolism, and the potential applications for cycloamylose are discussed.

EXPERIMENTAL PROCEDURES

Materials—Synthetic amylose (average molecular masses of 5, 10, 30, 70, 110, 320, and 1000 kDa) was obtained from Nakano Vinegar Co., Ltd. (Aichi, Japan). Isoamylase and pullulase were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Bacterial saccharifying α-amylase was obtained from Nagase Biochemicals (Kyoato, Japan). Glucoamylose from Rizopus sp. was purchased from Toyobo Co., Ltd. (Osaka, Japan).

Preparation of Recombinant Potato D-enzyme from E. coli—E. coli cells carrying the plasmid pKK233-DPE (8) were cultured in LB medium at 37°C until late log phase, and then the inducer isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added and incubated for 6 h at 15°C. Cells were harvested by centrifugation and disrupted by sonication in 20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer A) at 4°C. This crude extract was centrifuged, and the supernatant was filtered through a 0.45-μm membrane and then loaded onto a Q-Sepharose fast flow column (16 × 100 mm; Pharmacia Biotech Inc.) and washed with 150 mM NaCl in buffer A. D-enzyme was eluted with buffer A containing 450 mM NaCl and dialyzed against buffer A. Concentrated Tris buffer, ammonium sulfate, and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate, and 5 mM 2-mercaptoethanol. The solution was loaded onto a phenyl-TOYOPEARL 650M column (10 × 100 mm; TOSOH, Tokyo, Japan) and washed with 150 mM NaCl in buffer A. D-enzyme was eluted with buffer A containing 450 mM NaCl and dialyzed against buffer A. Concentrated Tris buffer, ammonium sulfate, and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate, and 5 mM 2-mercaptoethanol. The solution was loaded onto a phenyl-TOYOPEARL 650M column (10 × 100 mm; TOSOH, Tokyo, Japan) and eluted with a linear gradient of 500 to 0 mM ammonium sulfate in buffer A. Active fractions were pooled and dialyzed against buffer A. The dialysate was loaded onto a Resource Q column (6 ml; Pharmacia Biotech Inc.). The enzyme was eluted with buffer A containing 450 mM NaCl and dialyzed against buffer A. Concentrated Tris buffer, ammonium sulfate, and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate, and 5 mM 2-mercaptoethanol. The solution was loaded onto a phenyl-TOYOPEARL 650M column (10 × 100 mm; TOSOH, Tokyo, Japan) and eluted with a linear gradient of 500 to 0 mM ammonium sulfate in buffer A. Active fractions were concentrated with an Amicon Centricon 30 and dialyzed against 20 mM sodium citrate buffer (pH 7.0).

Assay of D-enzyme—D-enzyme activity was assayed in a 100-μl reaction mixture containing 180 mM Tris-Cl (pH 7.0), 1% (w/v) maltotriose, and enzyme. The reaction mixture was incubated at 37°C for 10

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min, and the reaction was terminated by immersing the reaction tubes in boiling water for 3 min. Released glucose was measured by the glucose oxidase method (12). One unit of activity is defined as the amount of enzyme that produces 1 μmol of glucose-6-phosphate under these assay conditions.

Preparation of Amylase Solution—Synthetic amylase (20 mg) was dissolved in 2 ml of 1 N NaOH solution and then neutralized by adding 4 ml of distilled water, 2 ml of 1 N sodium citrate buffer, and 2 ml of 1 N HCl. The solution was used immediately after neutralization.

Absorption Spectrum of Glucan–Iodine Complex—One-hundred μl of 0.1% (w/v) glucon solution was mixed with 2 ml of iodine reagent, and the absorption spectrum was monitored using a Shimadzu UV-240 spectrophotometer. Iodine reagent was made daily from 0.5 ml of iodine stock solution (0.26 g of I₂ and 2.6 g of KI in 10 ml of water) mixed with 0.5 ml of 1 N HCl and diluted to 130 ml with distilled water.

Gel Filtration Chromatography—Gel filtration chromatography was carried out using a Superose 6 prep grade column (10 × 300 mm; Pharmacia Biotech Inc.) plus a Superdex 30 column (10 × 300 mm; Pharmacia Biotech Inc.) with a flow rate of 1 ml/min. The eluant used was 150 mM sodium acetate. The molecular mass of the glucon was estimated using a standard curve produced with synthetic amylase with an average molecular mass of 5, 10, 30, 70, 320, or 1000 kDa as a standard.

Determination of Yield of Glucoamylase-resistant Molecules—The amount of glucoamylase-resistant molecules in the presence of linear glucon was determined as follows. The glucon (100 mg) was incubated either with glucoamylase (10 units) or with glucoamylase (10 units) and α-amylase (10 units) in 100 mM acetate buffer (pH 5.5) for 1 h at 40 °C. After terminating the reaction by boiling for 5 min, released glucose was measured by the glucose oxidase method (12). The amount of glucoamylase-resistant molecules was calculated by subtracting the amount of glucose released by glucoamylase and α-amylase from that released by glucoamylase only.

High-performance Anion-exchange Chromatography (HPAEC)—HPAEC was carried out with a Dionex DX-300 system with a pulsed amperometric detector (Model PAD-II, Dionex Corp., Sunnyvale, CA). For analytical purposes, a Carbopac PA-100 column (4 × 250 mm) was used. A sample (25–250 μl) containing 25–100 μg of glucon was injected and eluted with a gradient of sodium acetate (0–2 min, 50 mM; 2–37 min, increasing from 50 to 350 mM with installed gradient program 3; 37–45 min, increasing from 350 to 850 mM with installed gradient program 7; and 45–47 min, 850 mM) in 150 mM NaOH with a flow rate of 1 ml/min.

Quantitation of Reducing and Non-reducing Ends of Glucan—Reducing and non-reducing ends were quantitated using a modified Park-j ohnson method (see Ref. 13) and the rapid Smith degradation method (see Ref. 14), respectively.

Preparation of Glucoamylase-resistant Molecules and Their Size Fractionation by Gel Filtration Chromatography—A reaction mixture (10 ml) containing 20 mg of synthetic amylose AS-320 (Nakano Vinegar Co., Ltd.), 50 mM sodium citrate buffer (pH 7.0), 100 mM NaCl, and 68 units of purified D-enzyme from recombinant E. coli was incubated at 30 °C. At the indicated time points, 1 ml of the reaction mixture was removed, boiled for 5 min to stop the reaction, and then centrifuged. Fifty μl of the supernatant was mixed with 50 μl of distilled water and 2 ml of iodine reagent to measure the absorbance at 660 nm, and 200 μl was used for reducing power measurement by a modified Park-j ohnson method (see Ref. 13). Reducing power when all the amylose was broken down to glucose was defined as 100%.

RESULTS

Action of D-enzyme on Synthetic Amylose—Synthetic amylose was chosen to investigate the action of D-enzyme on high molecular weight starch since it is a linear α-1,4-glucan free from α-1,6-branches or other modifications. Purified potato D-enzyme from recombinant E. coli was incubated with synthetic amylose AS-320 (average Mr of 320,000). During the reaction, the ability of amylose to form a blue complex with iodine (the blue value) decreased, and a brown product resulted instead. To understand how this decrease in the blue value was caused by D-enzyme, samples were taken at time points during the reaction, and the blue value (A₆₆₀) and reducing power were measured. As shown in Fig. 1, A₆₆₀ decreased rapidly during the initial few hours, but more slowly subsequently. If this decrease in the blue value was caused by the hydrolysis of amylose, an increase in reducing power should be expected. However, no increase in reducing power was detected. These results indicate that the decrease in the blue value was not caused by hydrolytic activity on the amylose molecule. It is well known that a similar change in the absorption spectrum of amylose–iodine complexes can be produced by starch branching enzyme introducing branches into the amylose molecule. We measured the number of α-1,6-linkages during the incubation period by quantitative reducing sugars after isoamylase treatment (13), but none were detected.

To determine how the decrease in the blue value is caused by D-enzyme, possible structural changes in the amylose molecule were analyzed by gel filtration chromatography. As shown in Fig. 2A, the narrow size distribution of amylose AS-320 was changed to a broad distribution during the initial few minutes, but subsequently, the products showed a narrow size distribution in the low molecular weight region (average Mr of 15,000). Similar results were obtained using other high molecular weight amyloses (AS-1000 and AS-110) as substrates, in each case yielding products with a Mr of 15,000 (data not shown).

Since no increase in reducing power was detected, the most

The abbreviations used are: HPAEC, high-performance anion-exchange chromatography; DP, degree of polymerization; TOF-MS, time of flight mass spectrometry.
The likely explanation for this observation is that D-enzyme catalyzes an intramolecular transglycosylation reaction (cyclization reaction) to produce cyclic $\alpha$-1,4-glucans.

The possibility that D-enzyme could have catalyzed the formation of cyclic $\alpha$-1,4-glucans was examined by glucoamylase treatment. Glucoamylase is an exo-type amylase and hydrolyzes both $\alpha$-1,4- and $\alpha$-1,6-linkages in starch to produce glucose from the non-reducing end of the substrate. Thus, linear or branched glucans are completely broken down to glucose by glucoamylase. However, glucan with cyclic structure should be resistant to glucoamylase. The samples shown in Fig. 2A were treated with glucoamylase, and the resultant glucoamylase-resistant glucan was precipitated with ethanol and then analyzed with the same gel filtration column (Fig. 2B). The peak of intact amylose AS-320 (0 min) was completely hydrolyzed by glucoamylase, but molecules produced by the action of D-enzyme were resistant to glucoamylase. The amount of glucoamylase-resistant molecules and their average molecular weights are shown in Fig. 3. The yield of glucoamylase-resistant molecules increased to its maximum level (>95%) within 30 min and then remained constant. The average molecular weight of glucoamylase-resistant molecules estimated by gel filtration was initially ~70,000, but decreased with time to ~15,000. The presence of these glucoamylase-resistant molecules strongly suggested that D-enzyme had catalyzed the cyclization of amylose AS-320 and produced cyclic $\alpha$-1,4-glucans.

Analysis of Structure of Glucoamylase-resistant Molecules—Further structural analysis of the final products of D-enzyme action on amylose AS-320 was carried out by HPAEC. The elution pattern of the products after 18 h of D-enzyme treatment is shown in Fig. 4A, and $\alpha$-1,4-glucan standards are shown in Fig. 4D. The minor peaks that eluted between 3 and 17 min had the same retention times as linear $\alpha$-1,4-glucans with a DP of between 1 and 14, but the major peaks that eluted after 17 min had different retention times than $\alpha$-1,4-glucans. The nature of the products was first analyzed by treatment with glucoamylase. The minor products of D-enzyme reaction that eluted between 3 and 17 min were completely hydrolyzed to glucose by glucoamylase (Fig. 4B). However, the major products with retention times >17 min were resistant to glucoamylase. It should also be noted that no peaks corresponding to cyclodextrins (15) were detected in the glucoamylase-resistant molecules (Fig. 4B). The products were next analyzed by treatment with $\alpha$-amylase, an endoamylase that hydrolyzes $\alpha$-1,4-glucans to produce only glucose and maltose. The products of D-enzyme action were completely hydrolyzed to glucose and maltose (Fig. 4B), indicating that such products are all $\alpha$-1,4-glucans without any modification or branching.

The glucoamylase-resistant products were next purified from glucose and then size-fractionated by gel filtration chromatography. The putative cyclic $\alpha$-1,4-glucans in each fraction were
precipitated with ethanol and then lyophilized. Quantitation of reducing and nonreducing ends was carried out, but none were detected (data not shown), consistent with the proposed cyclic structure. Further evidence for the cyclic nature of the products of D-enzyme action was obtained by partial acid hydrolysis. The gel filtration fractions that contained the lower molecular weight glucoamylase-resistant glucans were next separated by HPAEC (Fig. 5A). Peaks G–J were purified by further HPAEC and then partially hydrolyzed with 0.1 N HCl. Products from peak G hydrolysis eluted as linear molecules with a DP of 1–23 (Fig. 5B). These products were hydrolyzed to glucose by glucoamylase (data not shown). The largest product of partial acid hydrolysis, with a DP of 23 (G23), is assumed to have resulted from hydrolysis of one glucosidic linkage of the peak G molecule. Similar results were obtained for all other peaks, except that the DP of the largest linear product was 1 unit larger for each successive peak (C–E). The retention time of each peak was less than that of the largest linear product.

Final confirmation of the cyclic nature of the glucoamylase-resistant products of D-enzyme action and determination of their molecular masses were obtained by TOF-MS, which can determine the molecular mass not only of single molecules, but also of several molecules in a mixture. A glucan with a DP of n in any non-cyclic structure should have a molecular mass of $162^n + 18$, whereas a glucan with a DP of n in a cyclic structure should have a mass of $162^n$. The fraction containing the smallest glucoamylase-resistant molecules (Fig. 5A) was subjected to TOF-MS (Fig. 6). Several peaks were obtained in the mass spectrometry spectrum, and the molecular masses agreed with the theoretical values for cyclic glucans with DPs of 17 and greater, but not with those for linear glucans. From this and previous results, we conclude that the smallest glucoamylase-resistant molecule (peak A) is cyclic $\alpha$-1,4-glucan with a DP of 17 (cG17) and that the consecutive peaks (B, C, D, etc.) are cyclic glucans with DPs of 1 unit longer for each successive peak (cG18, cG19, cG20, etc.). Therefore, our results show that D-enzyme catalyzes an intramolecular transglycosylation reaction on amylose to produce cyclic $\alpha$-1,4-glucans (cycloamylose) with DPs ranging from a minimum of 17 (peak A) to a few hundred (Fig. 3).

**Fig. 4.** HPAEC analysis of products of D-enzyme action on amylose AS-320. The products of D-enzyme treatment (18 h) of amylose AS-320 were analyzed by HPAEC using the conditions described under “Experimental Procedures.” Fifty μg of glucan before any treatment (A), after glucoamylase treatment (B), and after $\alpha$-amylase treatment (C) was analyzed. Short-chain amylose was mixed with glucose and malto-digiose saccharides and used as an $\alpha$-1,4-glucan marker (D). Positions where $\alpha$, $\beta$, and $\gamma$-cyclodextrins were eluted are indicated by arrows. Numbers above and beside peaks (G1, G5, etc.) indicate the DPs of the products. The amylase treatments were carried out at pH 5.5 and 40°C. The amounts of glucoamylase and $\alpha$-amylase used were 50 and 10 units/mg of glucan, respectively.

**Fig. 5.** Partial acid hydrolysis of glucoamylase-resistant molecules. Glucoamylase-resistant molecules were size-fractionated by gel filtration chromatography as described under “Experimental Procedures,” and the fractions containing the smallest glucans were analyzed by HPAEC (A). Glucoamylase-resistant peaks G–J were purified by HPAEC and partially hydrolyzed as described under “Experimental Procedures.” The elution profiles of each purified peak before (upper trace) and after (lower trace) partial acid hydrolysis are shown (B–E). Numbers above peaks (G1, G5, etc.) indicate the DPs of the products, with the largest product indicated by an arrow.
molecular weight cyclic \(\alpha\)-1,4-glucan (average \(M_r\) of 30,000) prepared by gel filtration was incubated with D-enzyme in the presence or absence of glucose as an acceptor molecule. No lower molecular weight products were produced in the absence of glucose (Fig. 7A), but lower molecular weight products were produced in the presence of glucose (Fig. 7, B–E). As the proportion of glucose was increased, the amount of lower molecular weight molecules produced also increased (Fig. 7, B–E). The presence of lower molecular weight cyclic \(\alpha\)-1,4-glucan was confirmed by analyzing the glucoamylase-resistant molecules in each reaction (Fig. 7, F–J). As the proportion of glucose was increased, the amount of cyclic \(\alpha\)-1,4-glucan decreased (Fig. 7, G–J). This result is dependent on the ratio of glucose to amylose, not on concentrations. These results are consistent with a transglycosidic mode of conversion of high molecular weight cyclic \(\alpha\)-1,4-glucans into lower molecular weight cyclic \(\alpha\)-1,4-glucans.

**DISCUSSION**

D-enzyme has previously been shown to catalyze a transglycosylation reaction on malto-oligosaccharides, and the action of this enzyme on small oligosaccharides has been extensively...
Cyclization Activity of D-enzyme on Amylose

Fig. 8. Diagrammatic representations of action of D-enzyme on amylose and cycloamylose. Lines and circles indicate α-1,4-glucan chains, where the relative length represents their relative DP. Ø, glucosyl residue at reducing end. Reaction 1 is the disproportionation reaction (intermolecular transglycosylation). Reaction 2 is the cyclization reaction (intramolecular transglycosylation). Reaction 3 is the transglycosidic linearization reaction (intermolecular transglycosylation).

Fig. 9. Diagrammatic representations of cyclization of amylose in antiparallel double (A) and single (B) helix configurations. Ø, glucosyl residue at reducing end. Open and closed arrowheads indicate the sites attacked by D-enzyme (donor site) and those to which the α-1,4-glucan chain will be transferred (acceptor site), respectively.

analyzed (3, 4). However, little is known about the action of this enzyme on amylose or amylpectin, although it has been demonstrated that maltodi-oligosaccharide units (a maltose unit is preferred) can be transferred from starch to glucose (2, 4). The present results clearly demonstrate novel activities of D-enzyme. First, it can catalyze an intramolecular transglycosylation reaction on high molecular weight amylose to produce cyclic α-1,4-glucans (cycloamylose) with a DP ranging from 17 to several hundred. Second, amylose can serve as donor and acceptor. Third, very long α-1,4-glucan units can be transferred by the enzyme. Fourth, D-enzyme catalyzes the transglycosidic linearization of cycloamylose when an acceptor is present. These activities of D-enzyme are explained in Fig. 8. The reaction starts with D-enzyme attacking an α-1,4-linkage. The enzyme then transfers the newly formed reducing end of the substrate either to the nonreducing end of a separate linear acceptor molecule or glucose (the intramolecular transglycosylation or disproportionation reaction) or to its own nonreducing end (the intramolecular transglycosylation or cyclization reaction). The reversibility of these reactions allows high molecular weight cyclic molecules to be linearized again by transglycosylation and lower molecular weight cyclic molecules to be produced subsequently (Fig. 8). Apparently, the equilibrium tends toward the formation of cycloamylose with a M₉₀ of ~15,000 (DP of 90), as shown in Fig. 3.

Cyclodextrin glucanotransferase also catalyzes cyclization and disproportionation reactions on α-1,4-glucans (15). The transglycosidic linearization of cyclodextrin in the presence of a suitable acceptor has also been demonstrated (the "coupling reaction") (15). In all these respects, D-enzyme and cyclodextrin glucanotransferase seem to catalyze the same reaction, but the major difference is the DP of the cyclic α-1,4-glucans produced. Cyclodextrin glucanotransferase produces cyclodextrins with DPs of 6, 7, or 8. Larger cyclodextrins with DPs of 9–13 have been reported, but only in trace amounts (16). D-enzyme produces cycloamylose with DPs ranging from 17 to several hundred. This observation suggests that there may be fundamental differences between cyclodextrins and cycloamylose and the enzymes that act upon them.

Cyclic α-1,4-glucans can potentially adopt antiparallel double helix, single helix, or nonhelical conformations. Cyclodextrins are known to adopt a nonhelical structure (17). Energy calculations of cyclic α-1,4-glucans in the nonhelical conformation indicated that cyclodextrin with a DP of 6 has the minimum energy and that as cyclodextrin size increases, so does the energy (18). The observation that cyclodextrins with DPs of 6 and 7 are the major products of cyclodextrin glucanotransferase activity agrees well with such calculations (19). It has been reported that linear amylose can occur in a double-stranded or single-stranded helix conformation (20). Fig. 9 shows that either conformation in linear amylose could potentially allow cyclization to form cycloamylose in either configuration. The Monte Carlo simulation suggested that the antiparallel double helix structure is the most likely conformation of amylose in solution (21), and crystallographic analysis has shown that linear amylose with a DP of 6 adopts a left-handed antiparallel double helix conformation (22, 23). It can be seen how such a conformation would readily allow cyclization by D-enzyme since donor and acceptor sites of amylose are juxtaposed (Fig. 9A) and so may be readily accommodated by the active site of the enzyme. Energy minimization calculations suggest that cycloamylose in the antiparallel double helix conformation is more stable than in the single helix configuration. We therefore favor the view that cycloamylose adopts the antiparallel double helix structure, but confirmation awaits structural analysis.

The function of D-enzyme in plants remains unknown. Preliminary results showed that D-enzyme can also catalyze intramolecular transglycosylation reactions on amylpectin in vitro. A role for D-enzyme in starch breakdown can be considered in which linear or cyclic glucans are produced as substrates for hydrolytic or phosphorolytic enzymes. We have not yet been able to detect cycloamylose in vivo, which could be explained if it has a short half-life. However, cycloamylose may not be produced in vivo if the ratio of acceptor (eg. glucose) to amylose is high (Fig. 7), but we have no information on such a ratio in vivo. Alternatively, D-enzyme could modify starch structure through its glucanotransferase activity. The function may be revealed when mutants lacking D-enzyme can be obtained.

Due to the high efficiency of cyclization of amylose by D-enzyme in vitro and the production of recombinant D-enzyme in E. coli, the large-scale production of cycloamylose is feasible. Preliminary experiments have shown that cycloamylose has several interesting properties. It is nonreducing, is highly soluble in cold water, and can form inclusion complexes with several inorganic and organic compounds (data not shown). Cycloamylose may have different dimensions and tertiary structure than cyclodextrins, so that different specificities for

2 J. Shimada, personal communication.
3 T. Takaha, M. Yanase, H. Takata, S. Okada, and S. M. Smith, unpublished data.
guest molecules can be anticipated. Therefore, there is great potential for the exploitation of cycloamylose in chemical, pharmaceutical, and food industries to safely achieve the solubilization, increased stability, sequestration, or altered reactivity of molecules with which it can form inclusion complexes.

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