Synthesis of 2-*O*- α -D-Glucopyranosyl L-Ascorbic Acid by Cyclomaltodextrin Glucanotransferase from *Bacillus stearothermophilus*

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Cyclomaltodextrin glucanotransferase (CGTase) [EC 2.4.1.19] was found to catalyze the transglycosylation from α -cyclodextrin (α -CD) to L-ascorbic acid (AA). A main product formed by this reaction was identified as 2-*O*- α -D-glucopyranosyl L-ascorbic acid (AA-2G) with the enzymatic hydrolysis and ultraviolet absorption spectra using standard AA-2G. A series of maltooligosaccharide substituted 2-*O*-derivatives of AA were also detected by HPLC. These were thoroughly hydrolyzed to AA-2G and glucose by treatment with glucoamylase [EC 3.2.1.3].

A large amount of AA-2G was prepared with 500 g of AA and 1000 g of α -CD. Two hundred and fifty grams of purified AA-2G, the content of which was 97%, was obtained through enzyme reactions and purification with HPLC using an ion-exchange resin.

L-Ascorbic acid (AA) is well known as vitamin C, an antiscorbutic reagent. Current studies have demonstrated that AA has multiple functions in various kinds of cells. However, its instability under oxidative conditions is disadvantageous. To improve the chemical stability or lipophilicity, various AA derivatives such as acyl-,¹⁾ -phosphate,²⁾ and -sulfate³⁾ have been chemically synthesized.

Some reports describe enzymatic synthesis of AA derivatives.^{4–13)} Recently, Yamamoto *et al.* found a nonreducible L-ascorbic acid glucoside formed by the transglucosylation of α -glucosidase [EC 3.2.1.20] from rat intestine⁴⁾ or rice seed,⁵⁾ and identified this glucoside as 2-*O*- α -D-glucopyranosyl L-ascorbic acid (AA-2G).⁶⁾ AA-2G is chemically stable like L-ascorbic acid 2-*O*-phosphate or L-ascorbic acid 2-*O*-sulfate. AA-2G is hydrolyzed to AA and glucose by the action of mammalian α -glucosidase,⁵⁾ and has biological activities like AA *in vivo*⁸⁾ and in tissue culture.⁹⁾

Cyclomaltodextrin glucanotransferase

(CGTase) [EC 2.4.1.19] catalyzes intermolecular transglycosylation in which glycosyl residues are transferred from an α -1,4-glucan or a cyclodextrin to a suitable acceptor such as glucose or sucrose.¹⁴⁾ However, there has been no report on the formation of AA derivatives by CGTase.

In this paper, we describe how CGTase was found to catalyze transglycosylation from cyclodextrins and other glycosyl donors to AA, and we provide a method for the preparation of AA-2G in a large amount.

Materials and Methods

1. *Materials.* L-Ascorbic acid (AA) and glucose were purchased from Wako Pure Chemicals (Osaka). Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, α -cyclodextrin (α -CD), β -cyclodextrin, γ -cyclodextrin, amylose EX-I (DP 18.5), and CGTase from *Bacillus stearothermophilus* were obtained from Hayashibara Biochemical Laboratories (Okayama). Dextrins [Pinedex #100 (DP 44.6), #1 (DP 15.6), and #4 (DP 6.6)] were purchased from Matsutani Chemicals (Hyogo).

Rhizopus niveus glucoamylase [EC 3.2.1.3] and sweet potato β -amylase [EC 3.2.1.2] were purchased from Seikagaku Kogyo (Tokyo). CGTases from *B. circulans* and *B. macerans* were prepared in our laboratory. AA-2G formed by rat intestinal α -glucosidase was prepared by the method described previously.⁶⁾ 6-O- α -D-Glucopyranosyl L-ascorbic acid (AA-6G) was prepared by the method of Suzuki.¹¹⁾ Other reagents used were of analytical grade.

2. *Assay for CGTase activity.* The CGTase was assayed as the dextrinogenic activity by the method of Shiosaka *et al.*¹⁵⁾

3. *Assay for AA derivatives.* Assay for AA derivatives was done by HPLC with a multichannel UV/VIS detector MULTI-340 (JASCO) and a refractive index detector ERC-7520 (Erma) attached. Samples were previously filtered through a Molcut-II UFPI-LGC (Millipore). HPLC was done in a ODS-M column (4.6 $\times$ 250 mm, Shimadzu Techno-Research) with the mobile phase of 0.1 M potassium phosphate-phosphoric acid (pH 2.0) at the flow rate of 0.5 ml/min and/or in a column of Wakopak WB-T-330 (10 $\times$ 300 mm, Wako Pure Chemicals) with the mobile phase of 100 ppm nitric acid at the flow rate of 0.5 ml/min.

Results

1. Enzymatic formation of glycosylated AA

Transglycosylation products were examined in detail with CGTase from *B. stearothermophilus*. As shown in Fig. 1A, CGTase produced a series of transglycosylation products of AA. Peak 1 corresponded to AA as glycosyl acceptor. The peaks X₁, X₂, X₃, X₄, X₅, and X₆ were the transglycosylation products. Although some α -CD seemed to remain in this reaction mixture, no peak of it was detected under these HPLC conditions. AA and AA-6G showed a UV $\lambda_{\max}$ of 243 nm at pH 2.0 with detection by a multichannel UV/VIS detector for HPLC, but every transglycosylation product showed at 238 nm (data not shown). The UV $\lambda_{\max}$ and the retention time of X₁ coincided with AA-2G formed by rat intestinal α -glucosidase,⁴⁾ and a compound that had the same retention time as the standard AA-2G was observed in the analysis on a Wakopak column. X₁, which was purified by the method mentioned afterward, had no reducing activity on cytochrome *c*.^{4,7)} X₁ was hydrolyzed by rat intestinal α -

Fig. 1. HPLC Profiles of Transglycosylation Products to AA by CGTase.

Samples were analyzed by HPLC using the ODS-M column. (A) A reaction mixture of CGTase. CGTase (25 units) was added to 1 ml of reaction mixture consisted of 100 mg of AA and 100 mg of α -CD, and incubated at 60°C, pH 5.5, for 1 hr. (B) A reaction mixture after treatment with β -amylase. β -Amylase (2 units) was added to the reaction mixture of CGTase and was incubated at 50°C, pH 5.5, for 20 hr. (C) A reaction mixture after treatment with glucoamylase. Glucoamylase (4 units) was added to the reaction mixture of CGTase and was incubated at 50°C, pH 4.5, for 20 hr. 1, AA; 2, maltose; 3, glucose; X₁, X₂, X₃, X₄, X₅, and X₆, transglycosylation products of AA; $\uparrow$, position of standard AA-2G.

glucosidase and cytochrome *c* was reduced by liberated AA. So X₁ was identified as AA-2G.

2. Enzymatic hydrolysis of the transglycosylation products

To investigate the structure of transglycosylation products, the reaction mixture of CGTase was treated with β -amylase or glucoamylase. Figure 1B shows the chromatogram of the reaction mixture treated with β -amylase. Peaks X₃, X₄, X₅, and X₆ disappeared. Instead of them, peak 2, which corresponded to maltose, appeared and the content of X₁ (AA-2G) and X₂ slightly increased. Figure 1C shows the chromatogram of the reaction mixture treated with glucoamylase. In this case, the peaks X₂, X₃, X₄, X₅, and X₆ disappeared with increasing contents of X₁ (AA-2G) and the formation of glucose

(peak 3). X_2 , X_3 , X_4 , X_5 , and X_6 were assumed that maltooligosaccharides are combined with the C2-hydroxyl group of AA for the results of these hydrolysis patterns and the UV $\lambda_{\max}$. The glucoamylase treatment following the transglycosylation reaction by CGTase is efficient for the mass production of AA-2G.

3. Formation of the derivatives of AA with various CGTase

The formation of transglycosylation products of AA (AA-2Gs) was examined with three CGTases from *B. stearothermophilus*, *B. circulans*, and *B. macerans*. AA-2Gs-forming reactions were done under these conditions: 1 ml of reaction mixture composed of 217 mg of α -CD, 100 mg of AA, and 109 units of CGTase was incubated at 40°C for 24 hr. The resultant mixtures were analyzed by HPLC. As shown in Table I, CGTase from *B. stearothermophilus* had the highest AA-2Gs-forming activity.

4. Condition of transglycosylation reaction

1) *Effects of the enzyme concentration.* The effects of enzyme concentration for AA-2Gs formation were examined. One hundred μ l of enzyme solution was added to the mixture (4.9 ml) of AA (1.0 g) and α -CD (1.0 g) and incubated at 60°C, pH 5.5, for 20 hr. As shown in Table II, large molecular weight AA-2Gs, which were observed to form at the low enzyme concentration, disappeared with increasing amounts of enzyme. At 500 units/g- α -CD, AA-2Gs production was the largest and 45.6% of the AA was used for transglycosylation.

2) *Ratio of substrates.* The effects of the donor (α -CD)/acceptor (AA) ratio on AA-2G formation were examined. Figure 2 shows the composition of AA-2G and the rate of AA used for AA-2G formation. Equal amounts of the substrates gave the maximum AA-2G composition of 38.7%. The rate of AA used rose with a decreasing AA ratio.

3) *Specificity of glycosyl donor.* The substrate

Table I. MOLAR RATIO OF TRANSGLYCOSYLATION PRODUCTS BY VARIOUS CGTASES

Source	Ratio* of product (%)							
	AA	AA-2G	X_2	X_3	X_4	X_5	X_6	X_7
<i>B. stearothermophilus</i>	67.0	17.0	7.8	4.3	2.0	1.0	0.5	0.4
<i>B. circulans</i>	84.3	7.4	3.0	3.3	1.1	0.9	0.0	0.0
<i>B. macerans</i>	89.8	8.4	1.2	0.6	0.0	0.0	0.0	0.0

* Initial AA as 100%.

X_2 , X_3 , X_4 , X_5 , X_6 , and X_7 are calculated tentatively as AA-maltoside, AA-maltotriose, AA-maltotetraose, AA-maltopentaose, AA-maltohexaose and AA-maltoheptaose, respectively.

Table II. MOLAR RATIO OF TRANSGLYCOSYLATION PRODUCTS BY CGTASE FROM *B. stearothermophilus*

Enzyme concentration (units/g- α -CD)	Ratio* of products (%)									
	AA	AA-2G	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9
10	92.2	1.9	1.5	1.1	0.9	0.7	0.6	0.4	0.4	0.3
50	73.9	10.4	5.7	3.6	2.5	1.9	1.1	0.6	0.3	0.0
100	68.3	15.0	8.7	4.7	2.3	0.7	0.3	0.0	0.0	0.0
500	54.4	32.4	9.4	2.8	0.8	0.2	0.0	0.0	0.0	0.0
1000	56.0	33.6	7.9	2.0	0.5	0.0	0.0	0.0	0.0	0.0
2500	60.3	32.2	6.3	1.2	0.0	0.0	0.0	0.0	0.0	0.0
5000	66.8	27.8	4.5	0.9	0.0	0.0	0.0	0.0	0.0	0.0

* Initial AA as 100%.

X_2 , X_3 , X_4 , X_5 , X_6 , and X_7 are indicated in Table I.

X_8 and X_9 are calculated tentatively as AA-maltooctaose and AA-maltononaose, respectively.

Fig. 2. Effects of Substrate Ratio on Transglycosylation to AA.

The reaction mixture consisted of 40% (w/w) substrate. AA and α -CD were incubated with 500 units/g- α -CD of CGTase at 60°C, pH 5.5, for 20 hr. AA-2Gs were hydrolyzed by glucoamylase. HPLC analysis was done with Wakopak column. Composition is expressed as weight percentage of AA-2G. The AA used up is calculated from AA-2G formed and AA added.

specificity of the enzyme toward various glycosyl donors is shown in Fig. 3. The enzyme acted on maltooligosaccharides, cyclodextrins, and dextrans, but not on glucose. Among them, α -CD was the best donor for AA-2G formation. Of the maltooligosaccharides and dextrans, AA-2G increased with increasing the length of the sugar chain.

5. Large scale preparation of AA-2G

The following is a procedure for a large scale preparation of AA-2G. The reaction mixture consisting of 500 g of AA, 1000 g of α -CD, and 400,000 units of CGTase from *B. stearothermophilus* was incubated at 60°C, pH 5.5, for 24 hr. CGTase was removed from the mixture by ultrafiltration (AIL-1010, Asahi Chemical Industry). Subsequently, 15,000 units of glucoamylase was added to the reaction mixture and incubated at 50°C, pH 4.5, for 24 hr. Glucoamylase was removed as well as CGTase. The AA-2G content was approximately 33%. AA-2G was purified by a preparative liquid chromatography with an ion-exchange resin (column size 3 $\times$ 400 cm, Amberlite HFS-471X, Japan Organo). AA-2G was finally obtained as 250 g-DS with the purity

Fig. 3. Glycosyl Donor Specificity for AA-2G Formation.

Five ml of reaction mixture consisting of 1.0 g of AA, 1.0 g of glycosyl donor, and 500 units of CGTase was incubated at 60°C, pH 5.5, for 20 hr. AA-2Gs were hydrolyzed by glucoamylase, and HPLC analysis was done with the Wakopak column. G₁, glucose; G₂, maltose; G₃, maltotriose; G₄, maltotetraose; G₅, maltopentaose; G₆, maltohexaose; G₇, maltoheptaose; A, amylose; α , α -CD; β , β -cyclodextrin; γ , γ -cyclodextrin; #100, Pinedex #100; #1, Pinedex #1; #4, Pinedex #4. Composition of AA-2G is indicated in Fig. 2.

of 97%.

Discussion

CGTase is a unique enzyme that catalyzes the conversion of starch to cyclodextrin by intramolecular transglycosylation. In the presence of a suitable acceptor (such as glucose or sucrose), glycosyl residues are transferred from starch or cyclodextrin to the acceptor and form an α -1,4-glycosidic linkage.¹⁴⁾ To investigate the enzyme structure and reaction mechanism, studies for amino acid sequence¹⁶⁾ and X-ray crystal structure analysis¹⁷⁾ have been done.

Kitahata *et al.*^{18,19)} reported that the structure of an effective acceptor of transglycosylation by CGTase is the pyranose structure having the same configuration of the free C2-, C3-, and C4-hydroxyl groups as D-glucopyranose. In this sense, the structure of AA is quite different from the structures of effective acceptors they mentioned. Nevertheless, AA was confirmed as an efficient acceptor of transglycosylation by CGTase. This is the first report on transglycosylation to AA with

CGTase. These results suggest that the acceptor specificity of CGTase may be widely extended.

Two types of glucosylated AA, namely AA-2G⁴⁻⁹⁾ and AA-6G,¹⁰⁻¹²⁾ have been reported to be synthesized enzymatically. AA-6G was synthesized in the presence of AA and maltose with α -glucosidase from *Aspergillus niger* by Suzuki *et al.*^{10,11)} However, AA-6G is not satisfactorily improved in chemical stability over AA in aqueous solutions.⁶⁾ Our assay system can distinguish AA-2G and AA-6G from each other. With the ODS-M column, AA-2G and AA-6G elute at almost the same retention time, but they have the different UV $\lambda_{\max}$ of 238 nm and 243 nm, respectively, and they are efficiently separated with the Wakopak column.

Various enzymes were screened to form AA-2G and AA-6G referring to AA-2G formed by rat intestine α -glucosidase and AA-6G formed by *Asp. niger* α -glucosidase, respectively. AA-2G was detected in the reaction mixtures of α -glucosidase from rat intestine, dog intestine, porcine intestine, rice seed, buckwheat seed, *Mucor rouxianus*, *M. circinelloides*, *Penicillium chrysogenum*, *Asp. niger*, or *Asp. awamori* with AA and maltose as the substrate. AA-6G was also detected in the mixture of rice seed and buckwheat seed slightly, and *Asp. niger* and *Asp. awamori* largely (data not shown). Other amylases are also reported to catalyze transglycosylation.²⁰⁾ Among of them, saccharifying α -amylase from *B. subtilis* [EC. 3.2.1.1] formed AA-2G with AA and maltotriose as the substrate, and *Rh. niveus* glucoamylase catalyzed the synthesis of AA-2G and AA-6G from AA and glucose by the condensation reaction (data not shown). It has been shown that AA-2G forming enzymes are common in nature.

One of us⁷⁾ described how an α -glucosidase that formed α -1,4-glucosidic linkages produced AA-2G, and one that formed α -1,6-glucosidic linkages produced AA-6G. These results are sure to explain the regioselectivity in glycosylation of AA. Various enzymes, not only α -glucosidase but also α -amylase, glucoamylase

and CGTase, which form α -1,4-glycosidic linkages, produce AA-2G.

We showed a process for a large-scale preparation of AA-2G by CGTase from *B. stearothermophilus* and glucoamylase. CGTase is commercially available for the production of cyclodextrins or glycosyl sucrose. Glucoamylase is also commercially available for the production of glucose. It is very advantageous to use these enzymes for the production of AA-2G on the manufacturing scale.

We have succeeded in crystallization of AA-2G from the purified aqueous solution. Studies on the crystal structure and physico-chemical properties of AA-2G are in progress. AA-2G can be advantageously purified by crystallization.

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