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Enhanced synthesis of 2-*O*-α-D-glucopyranosyl-L-ascorbic acid from α-cyclodextrin by a high disproportionating CGTase

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ABSTRACT: 2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) is an industrially important derivative of vitamin C (L-ascorbic acid; L-AA). A useful synthetic route towards AA-2G is the selective glucosylation of L-AA by cyclodextrin glucanotransferase (CGTase). However, the cyclodextrin donor substrate is utilized rather inefficiently, because only one of its constituent glucosyl residues is coupled to the L-AA acceptor. A CGTase catalyzing disproportionation of the linear maltooligosaccharide chain formed in the initial coupling reaction might utilize a

greater portion of the substrate for L-AA glucosylation and thus boost the AA-2G yield on cyclodextrin conversion. We present here a detailed characterization of the transfer reactions involved in AA-2G formation from α-cyclodextrin by a commercial CGTase preparation from *Thermoanaerobacter* sp. (Toruzyme 3.0L). We demonstrate that besides coupling, disproportionation constitutes a major route of glucosylation of L-AA by this enzyme. L-AA glucosides with oligoglucosyl chains between 1 and 12 units long were produced in the reaction. After chain-trimming hydrolysis with glucoamylase, however, AA-2G was recovered as sole product of the enzymatic transglucosylation. The molar yield of AA-2G from cyclodextrin was 1.4, thus clearly exceeding the maximum yield of 1 for the coupling reaction. Using conditions optimized for transfer efficiency and productivity, we obtained AA-2G in the highest concentration (143 g/L; 424 mM) so far reported from an enzymatic glucosylation of L-AA. The synthetic yield was 30% based on L-AA (250 g/L; 1420 mM) offered in ≤ 4.6-fold molar excess over α-cyclodextrin.

Key words: *Ascorbic acid glucoside*, *AA-2G production, CGTase, Thermoanaerobacter sp., disproportionation, α-cyclodextrin, starch*

INTRODUCTION

Biocatalytic transglycosylations (Scheme 1) constitute important synthetic routes towards glycosides as industrial chemicals.[1-4] Their optimization typically requires a carefully integrated development, where the enzyme used and the reaction scheme applied are strongly intertwined key factors of overall process performance.[5-9] Here we focus on AA-2G synthesis through CGTase-catalyzed transglucosylation from α-cyclodextrin to L-AA (Figure 1).

L-AA is an important commodity chemical having wide applications with main uses in foods and dietary supplements.[10-13] However, L-AA is highly prone to degradation, thus limiting the scope of its applications.[14] Modification at the 2-OH (e.g. glycosylation,[14] phosphorylation[15]) effectively stabilizes the L-AA. AA-2G has drawn high interest for application in the cosmetic, food and health-care areas.[16] Currently, AA-2G is mainly used as an active ingredient in skin-care products.

In order to form the α-glucoside of L-AA by transglucosylation (Scheme 1), an α-configured glucoside must be used as the donor substrate of the enzymatic reaction. Starch-derived malto-oligosaccharides are therefore promising in particular. Not only do they constitute a group of relatively inexpensive α-glucosidic substrates, but there are also industrial enzymes available as possibly useful, L-AA glucosylating catalysts.[17] High site-selectivity of the enzyme used is however essential to prevent glucosylation of L-AA at positions other than the 2-OH (Scheme 2).[18–20] Furthermore, enzymes catalyzing transglucosylation normally also catalyze, to a widely-varying degree, the reaction with water (hydrolysis; Scheme 1).[21] The class of glycoside hydrolases reflects a continuum of enzyme selectivities between the (scarcely hydrolyzing) transglycosidases and the true hydrolases.[22,23] An enzyme low in α-glucoside hydrolase activity is therefore needed to avoid "unproductive" utilization of donor substrate during synthesis of AA-2G. Among different options considered, including α-glucosidase and disproportionating 4-α-glucanotransferase, CGTase was a promising candidate catalyst.[18,21,24,25]

CGTase (EC 2.4.1.19) is a special 4-α-glucanotransferase widely known for its application to the industrial synthesis of cyclodextrins from starch.[26] Besides cyclization, CGTase also catalyzes coupling, disproportionation, and hydrolysis reactions, as shown in Scheme 1.[27] Considering transglucosylation from cyclodextrin, both coupling and disproportionation are

synthetically relevant reactions. The known CGTases differ in the extent to which disproportionation participates in the overall enzymatic reaction, the synthesis of alkyl glucosides for example.[28] Transglucosylation exclusively via coupling is limited in that just a single glucosyl residue is attached to the L-AA from the cyclic oligo-glucosyl donor substrate. The product formed initially is thus a 2-*O*-α-oligo-glucosyl derivative of L-AA.[19] Hydrolysis of the oligo-glucosyl chain by either the endogenous exo-glucosidase activity of the CGTase[29] or a subsequently added glucosidase[19] is used to recover the AA-2G product. However, all non-coupled glucosyl residues are consequently lost and cyclodextrin substrate is therefore utilized inefficiently.[29] Transglucosylation via disproportionation of oligo-glucosyl chains derived through coupling or hydrolysis could thus enhance significantly the yield of AA-2G on cyclodextrin converted.

Previous studies of CGTase-catalyzed synthesis of AA-2G focused on the coupling reaction from cyclodextrin.[29] Thermostable CGTases from *Bacillus* sp. were mainly used. The problem of incomplete utilization of the glucosyl residues from the cyclodextrin substrate was recognized. Protein engineering was applied to broaden the substrate scope of *Paenibacillus macerans* CGTase to less expensive and, from the perspective of glucosyl content, more completely utilizable substrates (e.g. maltose).[30-32] It was noted that increased disproportionation activity of enzyme variants could be favorable for the AA-2G synthesis.[32] However, the possible useful involvement of disproportionation in AA-2G synthesis was not examined, whereas the study we report here characterizes its role in detail, specifically transglucosylation from α-cyclodextrin to L-AA by a high disproportionating CGTase (from *Thermoanaerobacter* sp.; Toruzyme 3.0L).[33,34] The evidence presented is relevant in showing significant (1.4-fold) enhancement in AA-2G yield due to disproportionation, which is demonstrated to occur from intermediary oligo-

glucosylated forms of L-AA to free L-AA. By exploiting this type of disproportionation, we report enzymatic AA-2G synthesis in the highest concentrations and yields so far disclosed.

EXPERIMENTAL SECTION

Materials and Assays: Toruzyme 3.0L, a commercial liquid preparation of CGTase from *Thermoanaerobacter* sp., was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). *Aspergillus niger* glucoamylase, *A. niger* α-glucosidase and rice α-glucosidase were from Sigma-Aldrich (Schnelldorf, Germany). α-Cyclodextrin, L-AA, maltohexaose and AA-2G (>98%) were from TCI Deutschland GmbH (Eschborn, Germany). Dowex 1X2 (chloride form, 50-100 mesh) and calcined Celite 501 were from Sigma-Aldrich (Vienna, Austria). Activated charcoal was from Merck (Darmstadt, Germany). All other chemicals were from Carl Roth GmbH+Co. KG (Karlsruhe, Germany) unless specified otherwise.

Protein concentration was determined using Roti-Quant assay (Roth) referenced against bovine serum albumin. The Toruzyme preparation contained 5 mg/mL of protein content and its activities were 114 U/mL and 161 U/mL of coupling and cyclization respectively as previously determined.[35,36] Toruzyme is a technical-grade enzyme preparation. Its enzymatically active component is the CGTase from *Thermoanaerobacter* sp., as declared by the supplier and demonstrated in several previous studies.[28,35,37-39] The α-glucosidase and glucoamylase from *A. niger* were commercially supplied as powder with enzyme activity of 892 U/g and 70,000 U/g respectively. Stock solutions of *A. niger* α-glucosidase (134 U/mL) and glucoamylase (1000 U/mL) were prepared by dissolving 150 mg and 14.3 mg of α-glucosidase and glucoamylase respectively in one mL of 100 mM sodium citrate buffer (pH 4.5). Commercial preparation of

rice α-glucosidase was supplied as ammonium sulfate suspension with activity and protein content of 134 U/mL and 1.68 mg/mL respectively.

Reaction Set-up For Enzymatic Transglucosylation: Transglucosylation of L-AA was performed in 1.5 mL Eppendorf tubes using 100 mM sodium citrate buffer (pH 4.5), containing 2.6 mM thiourea as reducing agent. Using Toruzyme, α-cyclodextrin (up to 310 mM) or maltohexaose (310 mM) was used as donor substrate. Using *A. niger* or rice α-glucosidase, maltose (310 mM) was used as donor substrate. L-AA (up to 1420 mM) was used as acceptor substrate. The ratio of acceptor to donor substrate was varied as part of the reaction optimization. Temperature was also varied in the range 30 - 70 °C. Reaction was started by adding 50 µL of Toruzyme 3.0L or 300 µL of α-glucosidase stock solution (134 U/mL). To determine the initial reaction rates, Toruzyme 3.0L was diluted 162-fold and 50 µL of diluted enzyme were added to the reaction mixture. The total reaction volume was always 1 mL. The tubes were incubated in a thermomixer at the specified temperature at an agitation rate of 300 rpm in the dark. The reaction mixture was equilibrated for 10 min to the desired temperature before adding the enzyme. Samples were withdrawn at certain times and the reaction was quenched by diluting with 1.2 M HCl to a final acid concentration of 100 mM. After 10 min, the samples were neutralized with dilute aqueous NaOH. The samples were appropriately diluted and subsequently analyzed by HPLC for qualitative and quantitative determination of the L-AA transglucosylation products formed.

Trimming of Oligoglucosylated Forms of L-AA by Glucoamylase: Reaction mixtures (2 mL) from Toruzyme-catalyzed transglucosylation for 24 h were divided in two parts. One part (1 mL) was heated to 95°C for 30 min to inactivate the CGTase. The other part was kept on ice during that time and served as a control. Both samples were brought to 60 °C. The sample that

had undergone heat treatment was supplied with 50 µL of glucoamylase stock solution (1000 U/mL). The untreated sample received the same volume of buffer. Both samples were incubated for 2 h at 300 rpm and in the dark. They were then analyzed by HPLC as described above. Thus the effect of oligoglucosyl chain trimming was determined by glucoamylase. The control was designed to evaluate the intrinsic trimming activity of Toruzyme.

Isolation of AA-2G: A procedure adapted from Aga et al.[19] was used. The glucoamylase-treated reaction mixture (~140 g/L AA-2G; 10 mL) was loaded onto an anion-exchange chromatography column (XK 50/30, GE Healthcare Life Sciences, Vienna, Austria; bed volume of 150 mL) comprising a Dowex 1X2 (chloride form) resin packed at a flow rate of 15 mL/min and 0.3 MPa. Elution was performed at the same flow rate, starting with water (1.2 l) followed by three steps of NaCl concentration (0.1 M, 0.25 l; 0.25 M, 0.15 l; 0.5 M, 0.20 l). Glucose and other uncharged carbohydrates were eluted with water. AA-2G and L-AA eluted together at 0.1 and 0.25 M NaCl. Fractions containing AA-2G were pooled and concentrated 10-fold in vacuo at 40 °C. The sample was then loaded onto a column (XK 50/30, bed volume of 150 mL) packed with a 1:1 mixture of activated charcoal and calcined Celite 501. Elution was performed with a flow rate of 15 mL/min employing a three-step gradient starting with water (0.5 l) followed by 10% (0.5 l) and 25% (1.0 l) of ethanol. Salts were eluted with 10% ethanol. Fractions eluted at 25% ethanol contained most of the AA-2G. They were pooled and concentrated to one-tenth of volume in vacuo. After lyophilization, pure AA2G (98%, by HPLC) was obtained as a white powder. Its structure was confirmed by ^{1}H and ^{13}C NMR analyses.

Transglucosylation Product Analysis: L-AA and glucosylated forms of L-AA were analyzed using HPLC coupled to UV detection at 243 nm. A Shimadzu (Tokyo, Japan) HPLC 20A system equipped with a UV-Vis detector was used. Separation was performed with a Lichrosper 100

C18 reverse-phase column (250 × 4.6 mm, 5 μm; Macherey-Nagel; Düren, Germany) operated at 30 °C. Gradient elution was performed at a constant flow rate of 1 mL/min using potassium phosphate buffer (10 mM; pH 2.0) that contained varied amounts of acetonitrile (solvent B) in different steps: 0-2 min B = 0%; 2-10 min B = 2%; 10-20 min B = 0%. Separation of oligoglucosylated L-AA having side chains of up to 12 glucosyl residues could thus be achieved. Authentic standards of L-AA and AA-2G were used for quantification. Oligoglucosylated forms of L-AA were quantified based directly on the UV response factor of AA-2G.

The transglucosylation products were also analyzed and their masses identified by LC-MS. Sample analysis was conducted with an Agilent Technologies (Santa Clara, CA, USA) 1200 HPLC system coupled to a 6120 Quadrupole mass spectrometer (also from Agilent Technologies) equipped with an electro spray ionization source. The separation was performed using the Lichrosper column under the conditions described above, except that 10 mM ammonium acetate supplemented with 0.5 mM LiCl (pH 6.75) replaced the phosphate buffer. The mass spectrometer was used under the following instrument settings: athmospheric pressure ionization-electro spray positive scan mode, 10.5 L/min drying gas flow (N_2), 35 psig nebulizer pressure, 350 °C drying gas temperature, 3 kV capillary voltage and 30 V fragmentor voltage.

Determination of Site-selectivity of Transglucosylation: Although L-AA offers a total of four hydroxy groups as potential glucosylation sites, it was known from prior work in the field[19,20,29] that enzymatic transglucosylations give AA-6G, AA-2G or both. To distinguish between AA-2G and AA-6G (Scheme 2), an HPLC analysis was used that employed an Aminex® HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) operated at 25 °C. The UV detection described above was used. An isocratic elution with 20 mM H_2SO_4 in water was used. A constant flow rate of 0.4 mL/min was applied. Samples of

Toruzyme-catalyzed reactions underwent treatment with glucoamylase to eliminate effects of longer oligoglucosyl side chains on product separation and identification. Samples from reactions of the α-glucosidase from rice (known to produce both AA-2G and AA-6G)[20,19] and the α-glucosidase from *A. niger* (known to produce mainly AA-6G)[40] were used without treatment.

NMR spectroscopy: A Varian (Agilent Technologies) INOVA 500-MHz NMR spectrometer and the VnmrJ 2.2D software were used for all measurements. ^{1}H NMR spectra (499.98 MHz) were measured on a 5 min indirect detection pulsed-field-gradient probe, while a 5 mm dual-direct-detection probe with *z*-gradients was used for collecting ^{13}C NMR spectra (125.71 MHz).

RESULTS AND DISCUSSION

High-temperature Reaction Conditions For AA-2-G Synthesis by Toruzyme: Despite previous studies of Toruzyme[41,42] it was necessary to find suitable reaction conditions for AA-2G synthesis. L-AA stability was considered important in particular. Reported syntheses of AA-2G using CGTase[29] or another transglucosidase[14] differed in whether thiourea was added to prevent oxidative degradation of L-AA. An acidic pH (4.5) was chosen because the transglycosylase activity of Toruzyme and the stability of L-AA were both high under these conditions.

Figure 2 (panel A) shows the effect of temperature in the range 30 to 70 °C on the amounts of AA-2G formed and L-AA consumed in reactions with and without thiourea (2.6 mM). At temperatures of up to 50 °C, the AA-2G formation was enhanced by about 20 to 30% on addition of thiourea. The benefit of thiourea vanished gradually at the higher temperatures. When thiourea was absent, the L-AA consumption exceeded the AA-2G release at all temperatures, suggesting loss of L-AA due to instability. On addition of thiourea, a close mass balance for the two

compounds was obtained in the temperature range 30 - 50 °C. At 60 °C and higher, however, effect of thiourea on was strongly attenuated. Because the highest AA-2G amounts were obtained at 50 °C (Figure 2, panel A), all further experiments were performed at this temperature in the presence of thiourea.

Reaction Time Course Analysis: A typical time course of AA-2G synthesis is displayed in Figure 2 (panel B). Initially in the reaction, there was concurrent release of AA-2G and different oligoglucosylated forms of L-AA, in short AA-2G_{n+1} where $n \geq 1$ is the number of additional glucosyl residues attached. We discuss later the implication that n was observed to be as high as 11, despite the fact that a donor substrate comprising only 6 glucosyl residues was applied. Figure 2 (panel B) shows that the summed-up amount of AA-2G_{n+1} passed through a maximum shortly after the start of the reaction, only to decrease slowly afterwards. By contrast, AA-2G concentration increased in a more gradual manner, levelling out at a time when the AA-2G_{n+1} products had already been depleted from the reaction mixture to a substantial degree. HPLC analysis that resolved L-AA oligoglucosides according to their n-value (Figure 3) revealed Toruzyme-catalyzed degradation of the AA-2G_{n+1} products through "exo-mode" hydrolysis of their oligoglucosyl chains. (Note: MS analysis was used to confirm the masses of AA-2G_{n+1} products ($n \leq 5$), as shown in Figure S1 of Supporting Information.) Longer chain products disappeared faster from the reaction mixture than others comprising smaller chains, as shown in Figure 3. Even though conversion of AA-2G_{n+1} into AA-2G by Toruzyme-intrinsic hydrolase activity was relatively slow, it was suggested from literature[19] and confirmed in our experiments (see later) that post-synthetic processing of AA-2G_{n+1} by addition of glucoamylase was highly effective. Therefore, optimization for conditions of synthesis focused on maximizing not only the

amount of AA-2G$_6$ but also the total amount of transglucosylation product, that is, AA-2G plus AA-2G$_{n+1}$.

Effect of Acceptor and Donor Concentration, and of the Acceptor/Donor Ratio: In enzymatic transglucosylations, the acceptor is commonly used in a suitable molar excess over the donor to suppress the competing hydrolysis.[43] To define a useful "operational window" for AA-2G synthesis, experiments were performed at a variable molar ratio L-AA/α-cyclodextrin in the range 1.4 to 22. The total initial concentration of α-cyclodextrin and L-AA was constant at 37 g/L. Product formation after 24 h exhibited a broad plateau at L-AA/α-cylcodextrin ratios between around 4 and 13 (Figure S2, Supporting Information). Analysis of the initial rates of transglucosylation gave a similar result, as shown in Figure S3 and Table S1 in Supporting Information. Because utilization of the acceptor becomes progressively less efficient as the acceptor/donor ratio rises, we chose a ratio at the lower end (L-AA/α-cylcodextrin = 4.6) of the range explored for the following experiments.

Figure 4 shows the effect of varying α-cyclodextrin concentration in the range 31 – 310 mM on the formation of AA-2G (panel A) and total AA-2G$_{n+1}$ products (panel B) over time. Production was enhanced through increase in donor substrate concentration with the highest AA-2G output being obtained when initial α-cyclodextrin concentration was 248 mM. A greater initial α-cyclodextrin concentration brought about no further increase. However, the highest amount of total AA-2G$_{n+1}$ products was obtained at an initial α-cyclodextrin concentration of 310 mM. In contrast to AA-2G formation, which except for reaction at low α-cyclodextrin concentration increased steadily over time (Figure 4, panel A), total AA-2G$_{n+1}$ concentration increased only shortly into the reaction (≤ 6 h) and stopped sharply afterwards (Figure 4, panel B). On the other hand, AA-2G formation continued, albeit at a rate that was significantly slower

than in the early reaction phase. A plausible explanation for this behavior is that: (a) AA-2G and other AA-2G_{n+1} products were synthesized only early in the reaction; (b) any further increase in AA-2G results from degradation of the oligoglucosylated chains in AA-2G_{n+1} by the intrinsic hydrolase activity of CGTase. The molar product ratio ΣAA-2G_{n+1}/AA-2G after exhaustive reaction (24 h) increased from a value of 0.15 at a low α-cyclodextrin concentration to a value of 1.03 at a high α-cyclodextrin concentration, suggesting that elevated donor substrate concentrations drive the overall synthesis towards oligoglucosylation of L-AA.

Trimming of the Oligoglucosyl Chain in AA-2G_{n+1} Products by Glucoamylase Boosts the AA-2G Yield: It was known from literature,[19] and was also suggested by results of this study (see Figure 2, panel B) that AA-2G_{n+1} products can be converted to AA-2G through hydrolysis of their oligoglucosyl chains. Rather than relying on the low hydrolase activity of the CGTase itself, use of a second hydrolase has the advantage of speeding up the process considerably. Here the glucoamylase from *A. niger* was used. The enzyme is a known exo-hydrolase that degrades α-1,4-oligoglucosyl chains in a processive manner, cleaving glucose residues from the non-reducing chain end.[19,39] AA-2G was shown here not to be a substrate of the glucoamylase. Figure 5 (panel A) shows HPLC traces that illustrate the composition of a product mixture before and after glucoamylase treatment. It is shown that all peaks of AA-2G_{n+1} products disappeared and the peak of AA-2G increased strongly on incubation with the glucoamylase. Panel B of the same Figure 5 shows the impact of the glucoamylase "oligoglucosyl chain trimming" on AA-2G recovery from different transglucosylation reactions. As expected from the relative abundance of oligoglucosylated L-AA present in the mixtures (see Figure 4), the glucoamylase trimming was relatively more effective in reactions where a high α-cyclodextrin concentration had been used. It was possible to double the AA-2G concentration by means of the glucoamylase treatment

(Figure 5, panel B). Notably, the amount of AA-2G thus obtained exceeded on a molar basis that of the α-cyclodextrin employed. For example, a reaction starting from 310 mM α-cyclodextrin furnished 424 mM AA-2G, demonstrating the effect to be substantial and also highly significant regarding the overall AA-2G yield from α-cyclodextrin. This reflects boost of the donor substrate utilization by a factor of 1.37 (= 424/310) compared to transglucosylation by coupling only. Note: the theoretical molar yield of coupling is 1. Therefore, a CGTase having high disproportionation activity should be a very useful catalyst for a yield-enhanced synthesis of AA-2G. Using α-cyclodextrin concentrations lower than 310 mM this enhancement decreased gradually but was significant in all reactions, as shown in Figure 5.

Site-selectivity of Glucosylation of L-AA by Toruzyme: Figure 6 shows results of an analysis to characterize the site selectivity of Toruzyme-catalyzed glucosylation of L-AA under the conditions used. Contrary to reactions catalyzed by α-glucosidases that gave the wrong product regioisomer AA-6G (*A. niger* enzyme) or a mixture of AA-2G and AA-6G (rice enzyme) as expected, absence of AA-6G from reactions of Toruzyme was demonstrated within detection limits. Therefore, Toruzyme presents a highly site-selective catalyst for the formation of 2-*O*-α-D-glucosyl linkages on glucosylation of L-AA. After glucoamylase treatment, as shown in Figure 5 (panel A), AA-2G was obtained as a single glucosyl transfer product from L-AA.

Characterization of Glucosylation of L-AA via Disproportionation: The distribution of oligoglucosylated L-AA products in samples from the transglucosylation at extended reaction time reflects the complex interplay between the different reactions catalyzed by the CGTase (Scheme 1). To distinguish between product formation due to direct coupling and disproportionation, it was therefore necessary to analyze the reaction at an early stage (≤ 30 min) where the distribution of transglucosylation products showed strong variation with time and was

not primarily shaped by secondary conversion processes. Figure 7 (panel A) reveals that besides AA-2G, which was the predominant glucosylation product at all times, oligoglucosylated forms (AA-2G_2 - AA-2G_{12}) were also present in appreciable amounts after just 10 min. Reactions performed under initial rate conditions (Figure S3 and Table S1, Supporting Information) confirmed the presence of different oligoglucosylated forms of L-AA right from the beginning of the conversion. The largest transglucosylation product was AA-2G_6 under these conditions and it accounted for about one-sixth to one-fifth of the total amount of L-AA glucosides formed (Table S1, Supporting Information). However, already very early in the reaction (Figure S3, Supporting Information) the release of AA-2G_6 leveled off and started to decrease even. Therefore, these results clearly indicated that direct coupling, to produce AA-2G_6 (Scheme 1), was not the preferred reaction path of glucosylation of L-AA. Instead it seemed that disproportionation, probably involving maltohexaose formed through rapid hydrolysis of α-cyclodextrin, constituted the main reaction path, as shown in Scheme 3.

Evidence in Figure 7 (panel B) confirmed maltohexaose to be active as donor substrate of Toruzyme-catalyzed glucosylation of L-AA. However, the chain-length distribution of transglucosylation products from maltohexaose was much steeper, that is, shifted strongly toward products having smaller chains, as compared to reaction using α-cyclodextrin. Product distribution obtained from maltohexaose was independent of reaction time whereas in the case of α-cyclodextrin, the distribution appeared somewhat bimodal in the beginning (10 - 20 min) with maxima at AA-2G and AA-2G_6, only to shift later to a unimodal distribution with a maximum at AA-2G. However, even at a longer time (1 h), a decline in the relative abundance from AA-2G to longer-chain transglucosylation products remained less pronounced in the reaction with α-cyclodextrin as compared to reaction with maltohexaose. We also noted that reaction from

maltohexaose was somewhat (about 6.5-fold) slower than that from α-cyclodextrin and produced a smaller (about 3.6-fold) amount of transglucosylation products. Therefore, this suggested that a "secondary disproportionation" where oligoglucosylated L-AA products served as donor substrates for another glucosylation of L-AA also contributed to product formation and to the shaping of the product distribution obtained from α-cyclodextrin (Figure 7, panel A). Secondary disproportionation provides a reasonable explanation for the high preponderance of AA-2G as transglucosylation product, as follows. CGTase is thought to cleave linear donor substrates with release of G_2 units from the reducing end.[44,45] Assuming the same cleavage pattern for L-AA glucosides whereby L-AA is recognized as the reducing-end residue, AA-2G_6 for example would be converted into AA-2G and the remaining maltopentosyl moiety might be transferred to another L-AA acceptor to give AA-2G_5. Knowing the product distribution due to disproportionating transglucosylation from maltohexaose (Figure 7, panel B), it was possible to roughly estimate that the relative contributions of direct coupling to formation of L-AA glucosides from α-cyclodextrin was only about one-sixth that of disproportionation (Scheme 3). Note that direct coupling to L-AA gives AA-2G_6 as a single product. The remaining products are formed through disproportionation reactions.

As already shown in Figure 4 (panel B), the formation of L-AA transglucosylation products except that of AA-2G proceeded only for a few hours until oligoglucosyl chain hydrolysis became the predominant reaction. Hydrolysis was estimated to have been 20 times slower than the transglucosylation. More detailed analysis of the product distribution in the "hydrolysis phase" (6 - 24 h; data not shown) revealed that, shorter-chain oligoglucosides ($n \leq 3$) were observed to accumulate apparently at the expense of products having longer side chains.

Toruzyme Prefers AA-2G Over L-AA As Acceptor Substrate For Transglucosylation: It was known from earlier studies[38,45–47] that Toruzyme utilizes different glucosides (e.g. steviosides, steroidal saponins and others) as acceptor substrates, thus converting them into oligoglucosylated products. To determine if glucosylation of AA-2G contributed to the amount and the distribution of L-AA glucosides formed, we performed a "competing acceptor" experiment in which both L-AA (142 mM) and AA-2G (24 mM) were present at start of the reaction to become glucosylated from α-cyclodextrin. A control experiment lacking AA-2G was performed. Figure 8 shows the L-AA glucoside product distribution in samples from the two reactions. When AA-2G was initially present, the abundance of oligoglucoside products having n ≥ 6 was enhanced in a pronounced manner compared to the control. The relative amount of products having 6 to 8 glucosyl residues increased up to eightfold (Figure 8, panel B). Time-resolved analysis (data not shown) revealed that the AA-2G concentration dropped within the first 6 h from the initial value of 24 mM to 15 mM, only to remain constant afterwards until 24 h. About 11 mM AA-2G was produced in the control. These results implicate AA-2G as acceptor substrate for Toruzyme-catalyzed transglucosylation and they also reveal clear preference of the enzyme for glucosylation of AA-2G compared to glucosylation of L-AA. They also show the potential for tuning the composition of the product mixture towards longer-chain oligoglucosides (Figure 8, panel B). It appears that AA-2G competes much more efficiently with water for reaction with the glucosylated CGTase than L-AA is able to, thus promoting the formation of long-chain oligoglucosylated products through enhanced direct coupling from α-cyclodextrin and also "primary" disproportionation reactions. This is the first report describing transglucosylation of AA-2G with detailed product analysis.

The Benefit of Disproportionation - Highly Efficient Synthesis of AA-2G by Toruzyme-Catalyzed Transglucosylation From α-Cyclodextrin: A summary of the reactions involved in the Toruzyme-catalyzed transglucosylation of L-AA is shown in Scheme 3. Through effectively combining coupling with disproportionation, Toruzyme enabled a highly efficient utilization of α-cyclodextrin as donor substrate for AA-2G production. Table 1 enables a comparison of the results from this and earlier studies of AA-2G synthesis by transglucosylation. CGTases from *Bacillus* sp. were recognized for their relatively high coupling activity from α-cyclodextrin, and the enzyme from *B. stearothermophilus* was regarded as the most suitable biocatalyst for commercial AA-2G production. To replace cyclodextrin with a less expensive and in terms of glucosyl content more completely utilizable donor substrate (e.g. starch, maltodextrin, maltose), other CGTases in native or engineered form were considered. Variants of *Paenibacillus macerans* CGTase showed enhanced specificity towards these non-cyclic substrates.[30-32] However, the AA-2G yields thus obtained were still much lower than the "benchmark" yield using cyclodextrin.[30,32,48,49] Interestingly, the increased disproportionation activity of the CGTase variants proved beneficial for the AA-2G yield from cyclodextrin.[32,50,51] However, the engineered CGTases often showed a substantially lowered efficiency in the reaction with L-AA as compared to the wild-type enzyme. Table 1 indicates strong variation in the degree of utilization of L-AA in the different studies on AA-2G synthesis performed. With a 30% utilization of L-AA, the enzymatic reactions performed herein appear to be relatively efficient. CGTase variants utilized just a few percent of the L-AA offered. Recent developments notwithstanding, there appears to be still a long way to an optimally engineered CGTase for efficient AA-2G synthesis.

Advances made with these alternative systems notwithstanding, transglucosylation from α-cyclodextrin remains the strongest candidate for CGTase-catalyzed synthesis of AA-2G (Table 1). An important limitation of the previous studies was their exclusive focus on optimization of the AA-2G concentration, thus not considering the formation of L-AA oligoglucosides and the importance of these "AA-2G precursor products" for an optimized utilization of the donor substrate. The current study is the first to take into account the total amount of L-AA glucosides formed in the reaction as a target for process optimization and to recognize the significant potential of CGTase-catalyzed primary and secondary disproportionations for enhanced AA-2G production. Thus, to our knowledge, this study discloses AA-2G concentrations that exceed those so far reported, obtaining them by means of practical conditions and consideration of the mass ratio of donor and L-AA used. Toruzyme and glucoamylase are commercially available enzymes and the above process conditions can be used for large-scale production of AA-2G.

After glucoamylase treatment the reaction mass contains AA-2G along with unreacted L-AA and sugars, mostly glucose. Using anion exchange and activated carbon chromatographies, we isolated AA-2G at gram scale in ≥98% purity with a yield of 52% or higher. The expected product structure was confirmed by ^{1}H and ^{13}C NMR (Figure S4, Supporting Information).

CONCLUSIONS

Exploiting the high disproportionation activity of Toruzyme, the efficiency of CGTase-catalyzed synthesis of AA-2G by transglucosylation from α-cyclodextrin could be enhanced significantly in terms of both product concentration and degree of utilization of donor substrate. The improved use of α-cyclodextrin is considered especially in reducing the cost contribution of the donor substrate to total production cost. Using detailed analysis of the distribution of L-AA glucosides

formed in the reaction over time, reaction paths involved in the transglucosylation were revealed and their relative importance to the formation of product was identified.

FIGURES

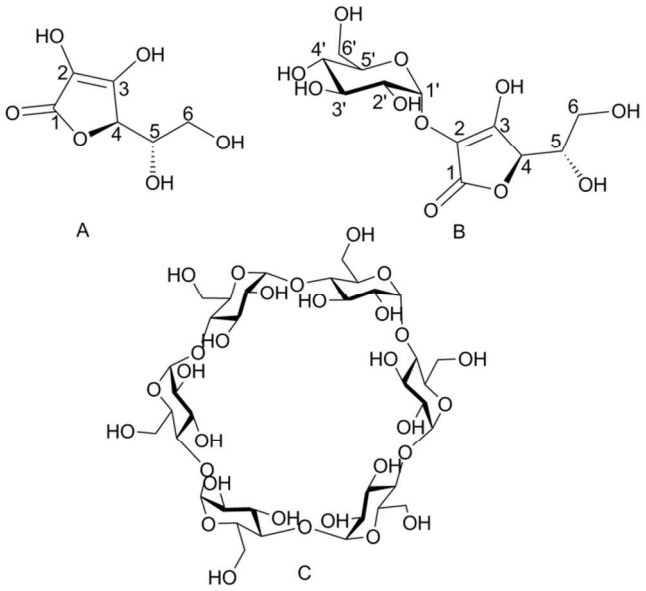


Figure 1. The chemical structures of L-ascorbic acid (L-AA) (A), 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) (B), and α-cyclodextrin (C) are shown.

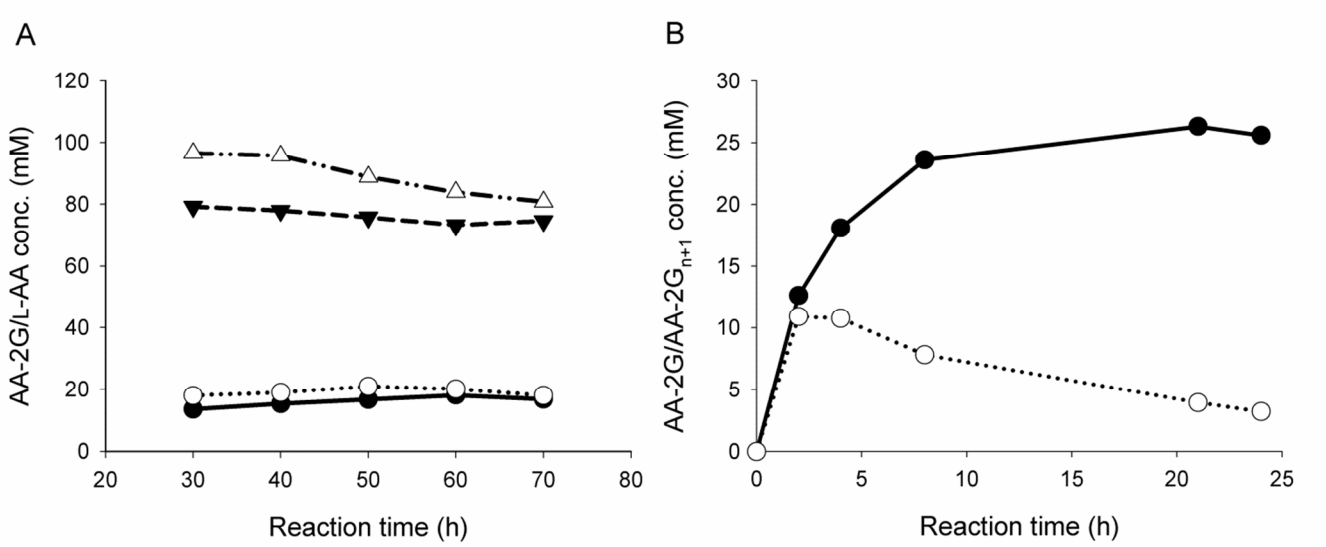


Figure 2. Toruzyme-catalyzed glucosylation of L-AA is shown. A. Temperature dependence of AA-2G formation (circles) and the corresponding L-AA depletion (triangles) in the absence (closed symbols) and presence (open symbols) of 2.6 mM thiourea. The reaction time was 4 h. B. Time course of AA-2G (closed circles) and AA-2G$_{n+1}$ (open circles) production during reaction at 50 °C in the presence of 2.6 mM thiourea. All reactions were performed at pH 4.5 and used 30 mM α-cyclodextrin, 135 mM L-AA, and a Toruzyme loading of 50 µL/mL. The symbols show the measured data. The lines indicate the data trend. Results are mean values of at least triplicate determination and have an S.D. of 6% or smaller.

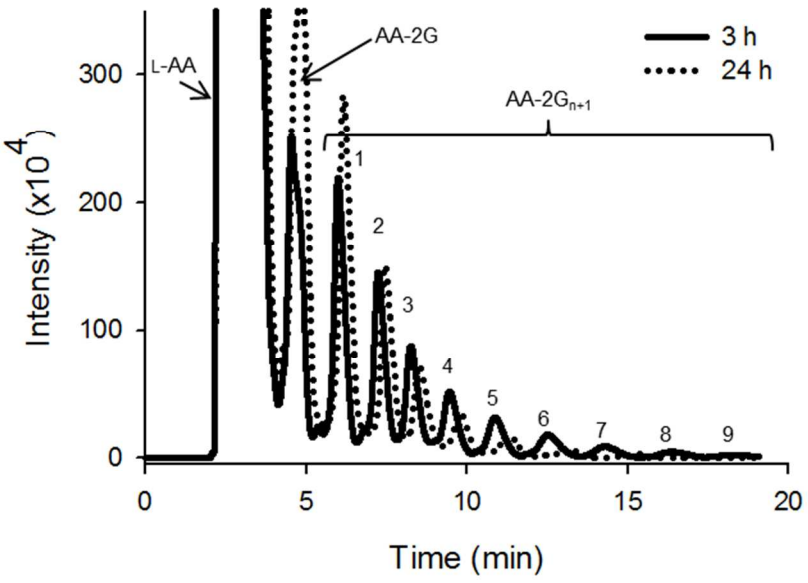


Figure 3. Degradation of transglucosylation products (AA-2G$_{n+1}$ where n ≥ 1) through a low "exo-trimming" activity of Toruzyme is shown. The HPLC traces show the product composition after 3 h and 24 h of reaction using conditions of Figure 1 (panel B). Peaks of AA-2G$_{n+1}$ products were designated with numbers according to their n-values.

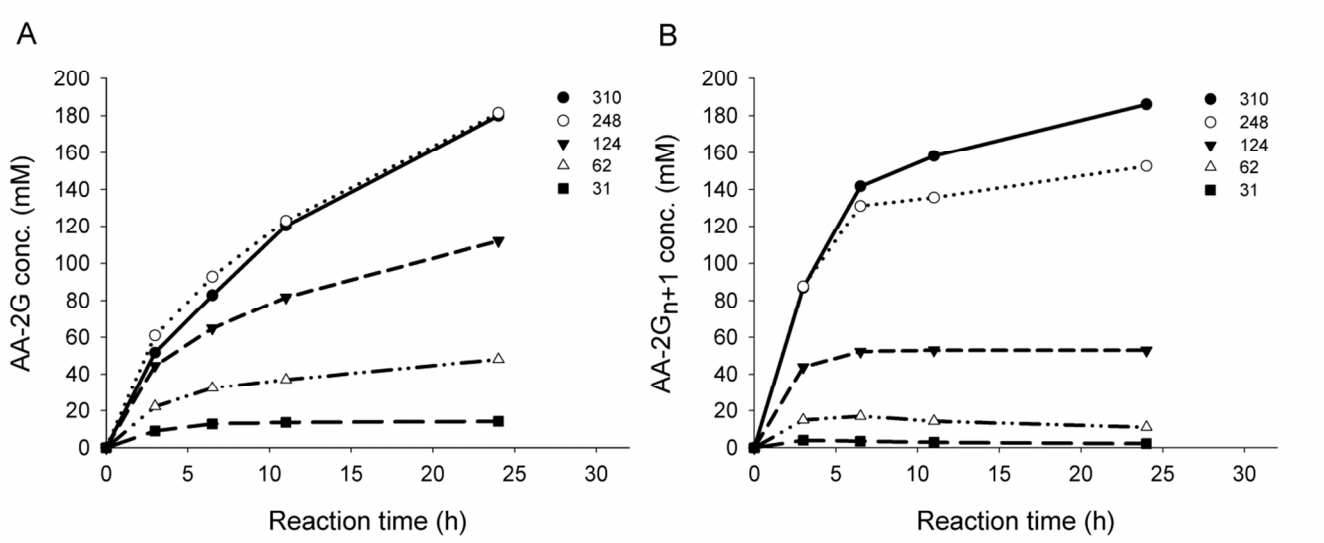


Figure 4. Time courses of AA-2G (panel A) and total AA-2G_{n+1} (panel B) formation at the different α-cyclodextrin concentrations (mM) indicated using a constant L-AA/ α-cyclodextrin ratio of 4.6. The AA-2G_{n+1} products analyzed and included in panel B have n-value between 1 and 11. Reactions conditions: 50 µL Toruzyme/mL, 2.6 mM thiourea, pH 4.5 and 50 °C. The symbols show the measured data and the dotted lines indicate the data trend. Results are mean values of at least triplicate determination and have an S.D. of 6% or smaller.

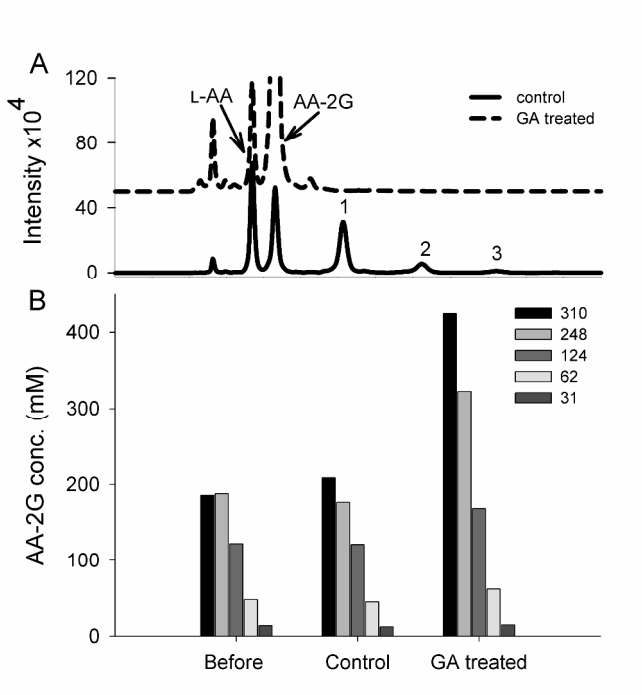


Figure 5. Effect of glucoamylase (GA) treatment on the recovery of AA-2G from AA-2G$_{n+1}$ transglucosylation products is shown. A. Superimposition of HPLC traces of reaction sample before (control) and after the GA treatment. Peak numbering 1 – 3 indicates the value of n in AA-2G$_{n+1}$. B. Change in AA-2G concentrations in GA-treated and untreated (control) samples obtained from transglucosylations performed at different α-cyclodextrin concentrations (mM) as indicated. AA-2G$_{n+1}$ formation was done for 24 h under the reaction conditions of Figure 3. One sample was treated with GA (50 U/mL) for 2 h. The control was incubated identically without GA.

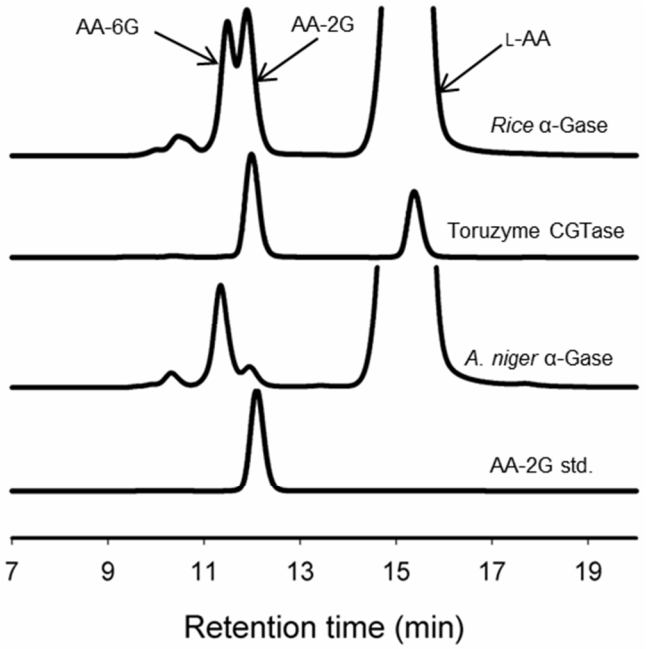


Figure 6. Superimposition of HPLC traces from the analysis of reaction samples of transglucosylations catalyzed by Toruzyme and the α-glucosidases (α-Gase) from rice and *A. niger*. The trace of an authentic standard of AA-2G is also shown. The enzyme from *A. niger* is known to synthesize mainly AA-6G whereas the rice enzyme produces both AA-2G and AA-6G. Thus absence of AA-6G from Toruzyme-catalyzed reactions was shown clearly.

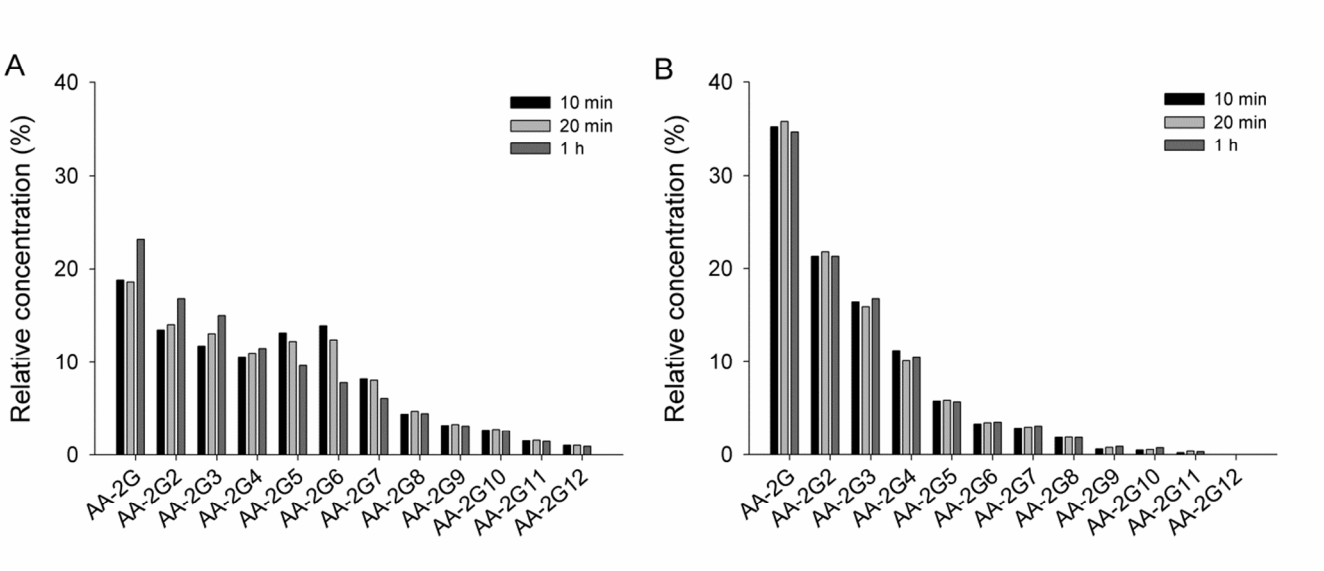


Figure 7. Distribution of L-AA glucosides formed in transglucosylations from α-cyclodextrin (panel A) and maltohexaose (panel B) at early reaction times. The reaction conditions of Figure 3 were used. Reactions used 310 mM donor substrate and 1420 mM L-AA.

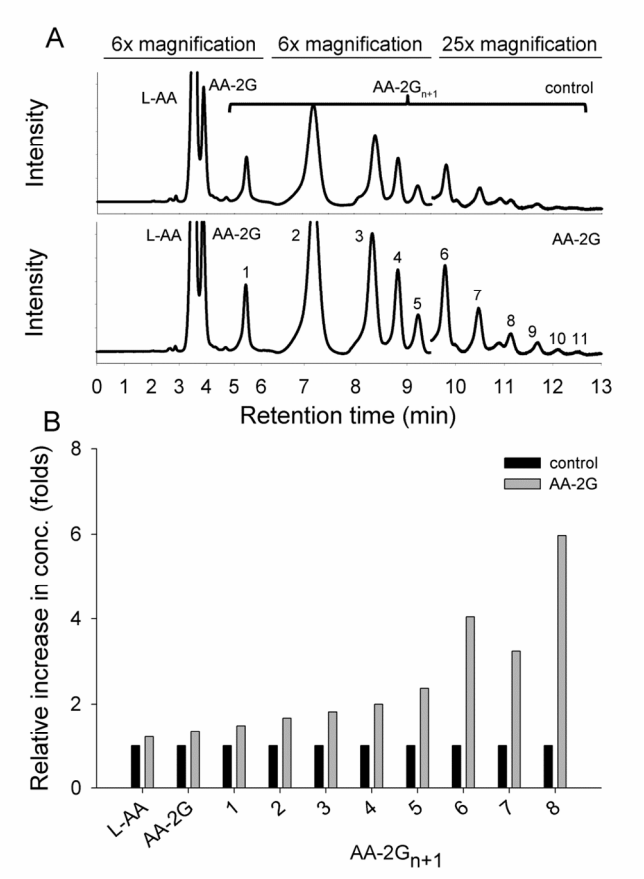
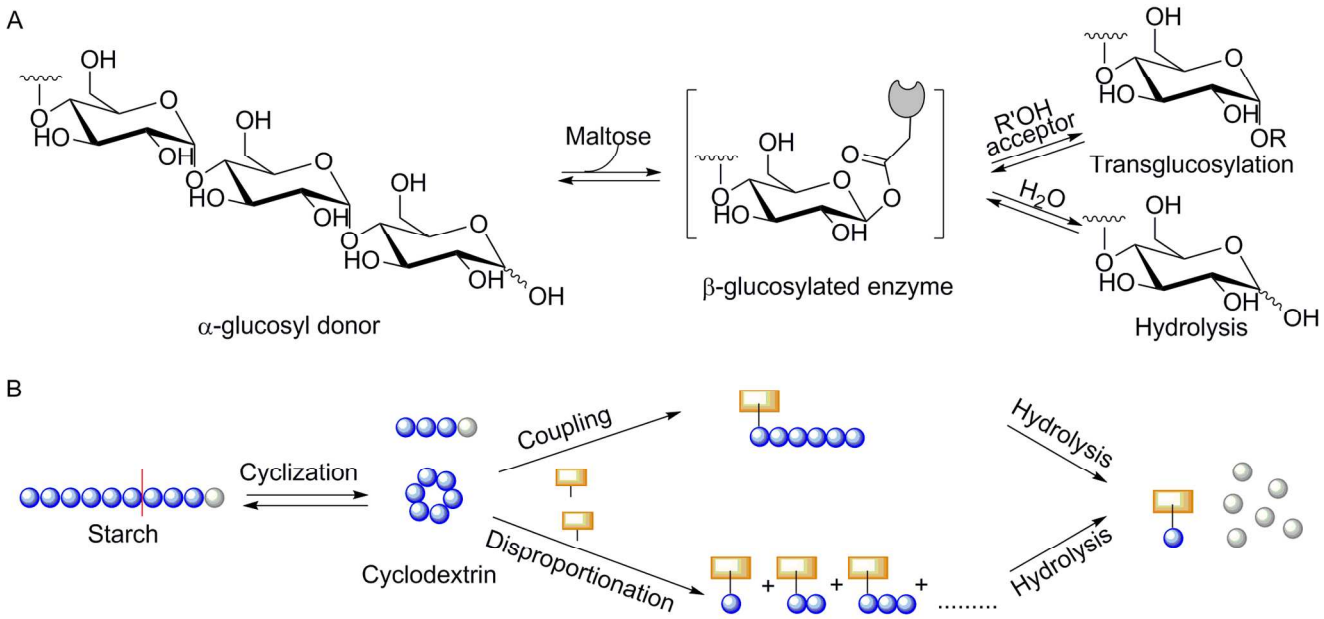
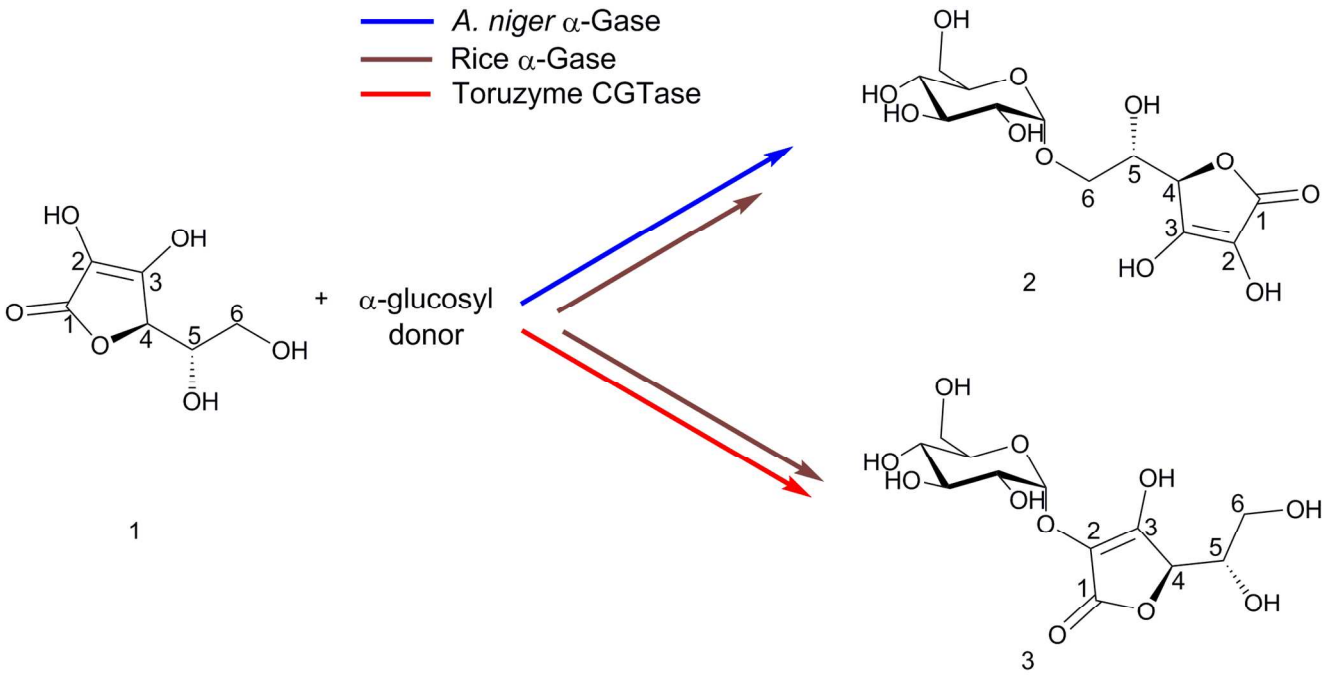


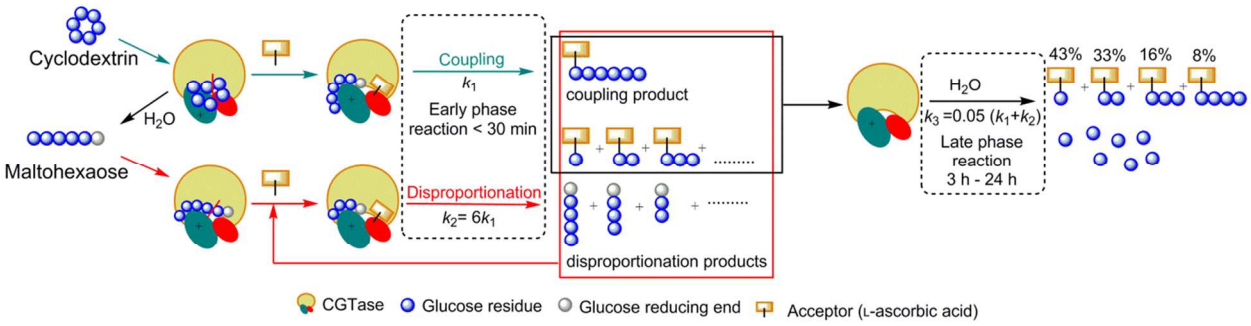
Figure 8. The acceptor specificity of Toruzyme in the transglucosylation reaction is shown. A. HPLC traces showing the effect of externally added AA-2G at reaction start on the distribution of transglucosylation products formed within 6 h. B. Relative increase in the concentration of each transglucosylation product due to the initial presence of AA-2G. Reactions were performed under conditions of Figure 1 (panel B) using 31 mM α-cyclodextrin and 142 mM L-AA in the absence (control) and presence of AA-2G (24 mM).



Scheme 1. Tranglucosylation catalyzed by CGTases. A. Double displacement-like mechanism of the reaction (transglucosylation to a suitable acceptor, hydrolysis). B. Reaction pathways via cyclization, coupling, disproportionation and hydrolysis. Blue circles show glucosyl residues, the white circle is the reducing-end glucose or free glucose. The acceptor substrate glucosylated in the reaction is shown as a yellow frame. The red line indicates an enzymatic cleavage site. Maltose is a preferred cleavage product in the first (glucosylation) step of the reaction of CGTase, as shown in panel A.



Scheme 2. Synthesis of AA-2G by biocatalytic transglucosylation to L-AA (**1**) requires a suitably site-selective enzyme. Transglucosylation from the disaccharide maltose gives a product mixture of AA-6G (**2**) and AA-2G (**3**) when the rice α-glucosidase (α-Gase) is used or mainly AA-6G when the *A. niger* α-glucosidase (α-Gase) is used. Use of CGTase (Toruzyme) in combination with α-cyclodextrin as donor substrate could result in a completely site-selective glucosylation of L-AA. **2** – 6-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-6G); **3** – 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G)



Scheme 3. The proposed pathway of transglucosylation of L-AA with Toruzyme is shown. The symbols used are those from Scheme 1. The donor and acceptor binding domains of the CGTase molecule are highlighted in dark cyan and red, respectively. Transglucosylation via coupling and disproportionation is distinguished from the product distribution in the initial reaction phase (≤ 30 min). Hydrolysis occurs in the late reaction phase. Its rate was estimated from the slow increase of AA-2G and AA-2G_2 in the time after 3 h of reaction.

TABLES

Table 1. Summary of results of AA-2G synthesis in different CGTase-catalyzed transglucosylations

Source	Donor substrate	Donor/L-AA (wt/wt)	AA-2G/donor (wt/wt)	AA-2G (g/L)	AA-2G/L-AA (molar yield %)	Reference
Paenibacillus macerans	Maltose	1	0.06	1.12	3	30
Paenibacillus macerans	Maltodextrin	1	0.04	1.97	2	48
Paenibacillus macerans	Soluble Starch	1	0.61	3.03	32	32
Paenibacillus macerans	β-Cyclodextrin	1	0.42	21[a]	22	49
Bacillus stearothermophilus	α-Cyclodextrin	2	0.25	67	27	19
Thermoanaerobacter sp.	α-Cyclodextrin	2.4	0.11	6.9	14	42
Thermoanaerobacter sp.	α-Cyclodextrin	1.2	0.47	143	30	Present study

[a] Reaction with immobilized enzyme

ASSOCIATED CONTENT

Supporting Information

Supporting information is available: Mass spectra of transglucosylated products of L-AA formed during the reaction catalyzed by Toruzyme (Figure S1); effect of the L-AA/α-cyclodextrin molar ratio on the total amount of transglucosylated products of L-AA synthesized by Toruzyme (Figure S2); analysis of the early phase of the transglucosylation of L-AA from α-cyclodextrin catalyzed by Toruzyme (Figure S3); ^{1}H and ^{13}C NMR spectra of purified AA-2G (Figure S4); and initial rates of formation of L-AA transglucosylation products by Toruzyme (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

R.K.G., A.T., S.V. and B.N. designed the research. R.K.G. conducted the experiments and analyzed data. The manuscript was prepared through contributions of all authors. R.K.G. and B.N. wrote the paper. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CGTase, cyclodextrin glucanotransferase; α-Gase, α-glucosidase; GA, glucoamylase; L-AA, L-ascorbic acid; AA-2G, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid; AA-6G, 6-*O*-α-D-glucopyranosyl-L-ascorbic acid.

REFERENCES:

(1) De Roode, B. M.; Franssen, M. C. R.; Van Der Padt, A.; Boom, R. M. *Biotechnol. Prog.* **2003**, 19, 1391–1402.

(2) Hehre, E. J. *Carbohydr. Res.* **2001**, 331, 347–368.

(3) Weijers, C. A. G. M.; Franssen, M. C. R.; Visser, G. M. *Biotechnol. Adv.* **2008**, 26, 436–456.

(4) Desmet, T.; Soetaert, W. *Biocatal. Biotransform.* **2011**, 29, 1–18.

(5) De Bruyn, F.; Maertens, J.; Beauprez, J.; Soetaert, W.; De Mey, M. *Biotechnol. Adv.* **2015**, 33, 288–302.

(6) Leemhuis, H.; Kelly, R. M.; Dijkhuizen, L. *Appl. Microbiol. Biotechnol.* **2010**, 85, 823–835.

(7) Leemhuis, H.; Dijkhuizen, L. *Biocatal. Biotransform.* **2003**, 21, 261–270.

(8) Desmet, T.; Soetaert, W.; Bojarová, P.; Kařen, V.; Dijkhuizen, L.; Eastwick-Field, V.; Schiller, A. *Chem. Eur. J.* **2012**, 18, 10786–10801.

(9) Bojarová, P.; Křen, V. *Trends Biotechnol.* **2009**, 27, 199–209.

(10) Kumari, J.; Sahoo, P. K. *Mol. Cell. Biochem.* **2005**, 280, 25–33.

(11) Zümreoglu-Karan, B. *Coord. Chem. Rev.* **2006**, 250, 2295–2307.

(12) Englard, S.; Seifter, S. *Annu. Rev. Nutr.* **1986**, 6, 365–406.

(13) Tripathi, R.; Singh, B. *Curr. Org. Chem.* **2009**, 13, 99–122.

(14) Yamamoto, I.; Muto, N.; Nagata, E.; Nakamura, T.; Suzuki, Y. *Biochim. Biophys. Acta.* **1990**, 1035, 44–50.

(15) Smaoui, S.; Hlima, H. Ben; Kadri, A. *J. Chem. Soc. Pak.* **2013**, 35, 1096–1102.

(16) Kumano, Y; Sakamoto, T; Egawa, M; Tanaka, M and Yamamoto, I. *Biol. Pharm. Bull*. **1998**, 21, 662-666.

(17) van der Maarel, M. J. E. C.; van der Veen, B.; Uitdehaag, J. C. M.; Leemhuis, H.; Dijkhuizen, L. *J. Biotechnol.* **2002**, 94, 137–155.

(18) Han, R.; Liu, L.; Li, J.; Du, G.; Chen, J. *Appl. Microbiol. Biotechnol.* **2012**, 95, 313–320.

(19) Aga, H.; Yoneyama, M.; Sakai, S.; Yamamoto, I. *Agric. Biol. Chem.* **1991**, 55, 1751–1756.

(20) Muto, N.; Suga, S.; Fujii, K.; Goto, K.; Yamamoto, I. *Agric. Biol. chem.*, **1990**, 54, 1697–1703.

(21) Kuriki, T.; Imanaka, T. *J. Biosci. Bioeng.* **1999**, 87, 557–565.

(22) Sinnott, M. L. *Chem. Rev.* **1990**, 90, 1171–1202.

(23) Cantarel, B. I.; Coutinho, P. M.; Rancurel, C.; Bernard, T.; Lombard, V.; Henrissat, B. *Nucleic Acids Res.* **2009**, 37, 233–238.

(24) Knegtel, R. M. K.; Strokopytov, B.; Penninga, D.; Faber, O. G.; Rozeboom, H. J.; Kalk, K. H.; Dijkhuizen, L.; Dijkstra, B. W. *J. Biol. Chem.* **1995**, 270, 29256–29264.

(25) van der Veen, B. A.; Leemhuis, H.; Kralj, S.; Uitdehaag, J. C. M.; Dijkstra, B. W.; Dijkhuizen, L. *J. Biol. Chem.* **2001**, 276, 44557–44562.

(26) Qi, Q.; Zimmermann, W. *Appl. Microbiol. Biotechnol.* **2005**, 66, 475–485.

(27) van Der Veen, B. A.; Van Alebeek, G. J. W. M.; Uitdehaag, J. C. M.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2000**, 267, 658–665.

(28) Svensson, D.; Ulvenlund, S.; Adlercreutz, P. *Biotechnol. Bioeng.* **2009**, 104, 854–861.

(29) Tanaka, M.; Muto, N.; Yamamoto, I. *Biochim. Biophys. Acta* **1991**, 1078, 127-132.

(30) Liu, L.; Han, R.; Shin, H. D.; Li, J.; Du, G.; Chen, J. *Carbohydr. Res.* **2013**, 382, 101–107.

(31) Han, R.; Liu, L.; Shin, H. D.; Chen, R. R.; Li, J.; Du, G.; Chen, J. *Appl. Environ. Microbiol.* **2013**, 79, 672–677.

(32) Han, R.; Li, J.; Shin, H. D.; Chen, R. R.; Du, G.; Liu, L.; Chen, J. *Appl. Environ. Microbiol.* **2013**, 79, 3234–3240.

(33) Alcalde, M.; Plou, F. J.; Andersen, C.; Martín, M. T.; Pedersen, S.; Ballesteros, A. *FEBS Lett.* **1999**, 445, 333–337.

(34) Martin, M. T.; Alcalde, M.; Plou, F. J.; Dijkhuizen, L.; Ballesteros, A. *Biocatal. Biotransform.* **2001**, 19, 21–35.

(35) Lu, T.; Xia, Y. M. *Food Chem.* **2014**, 159, 151–156.

(36) Tardioli, P. W.; Zanin, G. M.; de Moraes, F. F. *Enzyme Microb. Technol.* **2006**, 39, 1270–1278.

(37) Rather, M. Y.; Ara, K. Z. G.; Nordberg Karlsson, E.; Adlercreutz, P. *Process Biochem.* **2015**, 50, 722–728.

(38) Zhou, W. Bin; Feng, B.; Huang, H. Z.; Qin, Y. J.; Wang, Y. Z.; Kang, L. P.; Zhao, Y.; Wang, X. N.; Cai, Y.; Tan, D. W.; Ma, B. P. *Carbohydr. Res.* **2010**, 345, 1752–1759.

(39) Miranda-Molina, A.; Marquina-Bahena, S.; López-Munguía, A.; Álvarez, L.; Castillo, E. *Carbohydr. Res.* **2012**, 360, 93–101.

(40) Yamamoto, I.; Muto, N.; Murakami, K.; Suga, S.; Yamaguchi, H. *Chem. Pharm. Bull* **1990**, 38, 3020–3023.

(41) Norman, B. E.; Jørgensen, S. T. *J. Japanese Soc. Starch Sci.* **1992**, 39, 101–108.

(42) Tai, A.; Iwaoka, Y.; Ito, H. *J. Mol. Catal. B Enzym.* **2013**, 92, 19–23.

(43) Goedl, C.; Sawangwan, T.; Mueller, M.; Schwarz, A.; Nidetzky, B. *Angew. Chemie - Int. Ed.* **2008**, 47, 10086–10089.

(44) Börner, T.; Roger, K.; Adlercreutz, P. *ACS Catal.* **2014**, 4, 2623–2634.

(45) Leemhuis, H.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2003**, 270, 155–162.

(46) Mathew, S.; Adlercreutz, P. *Biochem. Eng. J.* **2013**, 79, 187–193.

(47) Rendleman, J. A. *Biotechnol. Appl. Microbiol.* **1996**, 24, 121–127.

(48) Han, R.; Liu, L.; Shin, H.; Chen, R. R.; Du, G.; Chen, J. *Appl. Microbiol. Biotechnol.* **2013**, 97, 5851–5860.

(49) Zhang, Z.; Li, J.; Liu, L.; Sun, J.; Hua, Z.; Du, G.; Chen, J. *J. Mol. Catal. B Enzym.* **2011**, 68, 223–229.

(50) Han, R.; Liu, L.; Shin, H. D.; Chen, R. R.; Li, J.; Du, G.; Chen, J. *Appl. Environ. Microbiol.* **2013**, 79, 7562–7568.

(51) Han, R.; Li, J.; Shin, H.; Chen, R. R.; Liu, L.; Du, G. **2014**, 80, 4717–4724.

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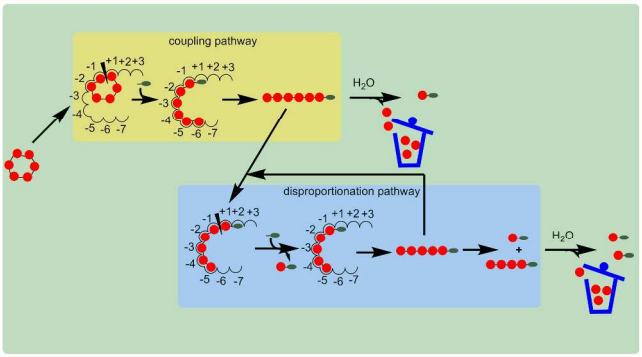

Site-selective transfer of glucosyl residues (red symbols) from α-cyclodextrin to L-ascorbic acid (grey symbols) catalyzed by a commercial cyclodextrin α-glucanotransferase preparation (Toruzyme 3.0L) provided 2-*O*-α-D-glucopyranosyl L-ascorbic acid in good yield and in highest so far reported concentrations (143 g/L). Besides reaction via coupling (see the Scheme), reaction via disproportionation due to the specificity of the enzyme was essential to enable efficient utilization of the α-cyclodextrin donor substrate. Enzymatic trimming via hydrolysis of oligoglucosyl side chains gave the singly glucosylated L-ascorbic acid. The numbers show the subsites for substrate binding in the enzyme, and chain cleavage occurs between subsites -1 and +1.