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Biosynthesis of 2-O-D-glucopyranosyl-L-ascorbic acid from maltose by an engineered cyclodextrin glycosyltransferase from *Paenibacillus macerans*

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Abstract: In this work, the specificity of cyclodextrin glycosyltransferase (CGTase) of *Paenibacillus macerans* towards maltose was improved by the site-saturation engineering of lysine 47, and the enzymatic synthesis of 2-O-D-glucopyranosyl-L-ascorbic acid (AA-2G) with L-ascorbic acid and maltose as substrates was optimized. Compared to the AA-2G yield of the wild-type CGTase, that of the mutants K47F (lysine→ phenylalanine), K47P (lysine→ proline), and K47Y (lysine→ tyrosine) was increased by 17.1, 32.9, and 21.1%, respectively. Under the optimal transformation conditions (pH 6.5, temperature 36 ^{o}C, the mass ratio of L-ascorbic acid to maltose 1:1), the highest AA-2G titer by the K47P reached 1.12 g/L, which was 1.32-fold of that (0.85 g/L) obtained by the wild-type CGTase. The reaction kinetics analysis confirmed the enhanced maltose specificity of the mutants K47F, K47P, and K47Y. It was also found that compared to the wild-type CGTase, the three mutants had relatively lower cyclization activities and higher disproportionation activities, which was favorable for AA-2G synthesis. As revealed by the interaction structure model of CGTase with substrate, the enhancement of maltose specificity may be due to the removal of hydrogen bonding interactions between the side chain of residue 47 and the sugar at -3 subsite. The obtained mutant CGTases, especially the K47P, has a great potential in the large-scale production of AA-2G with maltose as a cheap and soluble substrate.

Keywords: Cyclodextrin glycosyltransferase (CGTase), L-ascorbic acid, 2-O-glucopyranosyl-L-ascorbic acid, maltose, site-saturation engineering

Introduction

Vitamin C (VC) is an essential nutrient, which means that our body cannot synthesize it and must absorb it from foods and supplements. [1] VC usually exists *in vivo* in its reduced form L-ascorbic acid (L-AA) and plays vital roles in numerous biological processes. For example, many sicknesses such as scurvy, heart disease, cancer, and eye diseases can occur if we lack VC. [2] VC can also improve collagen formation, carnitine synthesis, and iron absorption. [2-4] However, VC is extremely instable in aqueous solution, especially in the presence of heat, light, Cu^{2+}, and ascorbate oxidase, and this reduces its biological activity and limits its applications. [5]

To improve its stability, various VC derivatives such as ascorbyl phosphate, [6] ascorbyl palmitate [7], and ascorbyl glucoside [6] have been synthesized by chemical or biological approaches. Particularly, as one of the ascorbyl glucosides, 2-O-glucopyranosyl-L-ascorbic acid (AA-2G), is considered to be the best one among all the VC derivatives due to its non-reducibility, anti-oxidation, and effortless release of L-AA and glucose. [8, 9] AA-2G has found wide applications in cosmetics, [10] medicine, [11] husbandry, and aquaculture fields . [12, 13]

Currently, AA-2G is produced by enzymatic transformation, and the enzymes used for synthesis of AA-2G include α-glucosidase, [14] cyclodextrin glycosyltransferase (CGTase), [15] amylase, [16] sucrose phosphorylase [17], and α-isomaltosyl glucosaccharide-forming enzyme. [18] Among these enzymes, CGTase is considered to be the most effective catalyst for AA-2G production. [15, 19, 20] CGTase mainly catalyzes transglycosylation reaction (cyclization, coupling and disproportionation) and hydrolysis reaction. [21] AA-2G biosynthesis is catalyzed by CGTase via transferring a glycosyl residue from a glycosyl donor to the C-2 position of VC with α-1, 2-linkage. Many saccharides (except glucose) can be used as glycosyl donors. [22] It has been demonstrated that α- and β-cyclodextrin were the best glycosyl donors for AA-2G production, while maltose is a weaker one because of the low specificity[19,

[20, 23]. However, due to the high cost of α-cyclodextrin and low solubility of β-cyclodextrin in water, both of them are unsuitable for industrial production of AA-2G. [24] On the other hand, maltose is cheap and can be easily dissolved in aqueous solution. In addition, with maltose as a glycosyl donor, the by-products AA-2Gn ("n" means the number of glycosyls attached to the L-AA) will be avoidable due to the use of disaccharide donor. Therefore, maltose is a potentially ideal glycosyl donor for the enzymatic synthesis of AA-2G if the specificity of CGTase towards maltose can be improved by some strategies such as fusion protein and site-directed mutagenesis. The site-directed mutagenesis is a common and convenient method and many site-directed mutations of CGTase have been conducted with a purpose of changing the cyclization of CGTase and the cyclodextrin product specificity. [25-29] It was found that the mutation of Lys 47 of CGTase from *Bacillus circulans* strain 251 into tryptophan resulted in the decrease of cyclization activity and the increase of disproportionation and hydrolysis activities.[30, 31] It was indicated that the residue 47 of CGTase may play a key role in affecting the interrelations among the four reactions of cyclization, coupling, disproportionation and hydrolysis, and thus in this work the Lys47 was selected as an engineering site to improve the binding capacity of CGTase with maltose.

First, the Lys47 in the CGTase of *Paenibacillus macerans* was replaced with other nineteen amino acids, and it was found that the AA-2G titer of three mutants K47F (lysine→ phenylalanine), K47P (lysine→ proline), and K47Y (lysine→ tyrosine) with maltose as the glycosyl donor was increased compared to that of the wild-type CGTase. The transformation conditions (pH, temperature, and the mass ratio of L-AA to maltose) were optimized to improve the yield of AA-2G by the positive mutant CGTases. Furthermore, the reaction kinetics of the wild-type and three mutant CGTases were explored to verify the enhanced binding capacity of mutant CGTases with maltose and to clarify

which reaction (cyclization, disproportionation and hydrolysis) was mainly involved in the synthesis of AA-2G. Finally, the possible mechanism responsible for the increased maltose specificity was explored by modeling the interaction of CGTase with the substrate. This is the first report about the site-saturation engineering of CGTase for the enhanced maltose specificity to improve AA-2G yield, and also deepens our understanding about the role of Lys47 in the catalysis of CGTase with disaccharide as the substrate.

Results and discussion

Construction, expression, and purification of the wild-type and mutant CGTases

The *cgt* gene amplified from the genomic DNA of *P. macerans* strain JFB05-01 by PCR (without its own signal peptide) was ligated into the vector pET-20b(+) with the restriction sites of *Bam*H I and *Xho* I, and the obtained recombinant plasmid cgt/pET-20b(+) contained the *pelB* signal peptide upstream and six histidine codons downstream. The constructed recombinant plasmid was further confirmed by DNA sequencing, and the result showed that the 2061 bp open reading frame of *cgt* gene (without the stop codon) corresponded to the published *cgt* gene (GenBank accession no. AF047363. 1). In addition, the 687 amino acid residues encoded by the *cgt* gene was the same with the published CGTase amino acid sequence (NCBI accession number: P04830), indicating that the plasmid cgt/ pET-20b (+) was successfully constructed.

All the other 19 mutants were also successfully constructed by site-directed mutagenesis and verified by DNA sequencing. The correct recombinant plasmids contained the wild-type and mutant *cgt* genes were transformed into *E. coil* BL21 (DE3) for expression. It was found that the mutation had no significant influence on the expression level of CGTases compared to the wild-type CGTase,

(Table S1 in supplementary materials). The crude CGTases solution was purified by one-step nickel affinity chromatography on Ni-NTA resin and a relatively high purity (more than 95%) of CGTase proteins were obtained. By the analysis of sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), it was found that there was no difference in the molecular mass (about 75 kDa) between the wild-type and mutant CGTases, and this result was similar to what previously reported. [32]

Enzymatic synthesis of AA-2G by the wild-type and mutant CGTases with maltose as glycosyl donor

Under the initial transformation conditions (temperature 37 ^{o}C, pH 5.5, and the mass ratio of L-AA to maltose, 1:1), the wild-type and mutant CGTases were used for AA-2G biosynthesis with maltose as the glycosyl donor. As shown in Table 1, the yield of AA-2G was 0.76 g/L by the wild-type CGTase. Compared to the wild-type CGTase, three mutants K47P, K47F, and K47Y exhibited higher AA-2G yield among all of the mutants, and their yields were 1.01, 0.89 and 0.92 g/L, respectively (Table 1). All the other sixteen mutants produced lower AA-2G yield than the wild-type CGTase (data not shown). Compared to the wild-type CGTase, the mutants K47P, K47F, and K47Y showed 32.9, 17.1, and 21.1% increase in AA-2G yield, respectively. To further increase the titer of AA-2G, three key factors (pH, temperature, and substrate ratio) were further optimized.

Fig. 1 shows the influence of reaction pH, temperature, and substrate ratio on AA-2G biosynthesis by the wild-type and mutant CGTases (K47F, K47P and K47Y) with maltose as the glycosyl donor. Both the wild-type CGTase and mutants showed the highest AA-2G yield at pH 6.5 (Fig. 1(A)), which was different from the optimal pH (pH 5.5) of AA-2G biosynthesis by the recombinant α-CGTase with β-cyclodextrin as the substrate. [22, 29]

Fig. 1(B) shows the influence of reaction temperature on AA-2G biosynthesis by the wild-type and mutant CGTases (K47F, K47P, and K47Y). The optimal temperature of the wild-type and mutant CGTases for AA-2G synthesis was 36 ^{o}C, which was the same with that for α-CGTase-catalyzed AA-2G biosynthesis with β-cyclodextrin as the glycosyl donor, [22] whereas lower than that of the recombinant α-CGTase for the cyclization activity (45^{o}C). [32] There was only a little AA-2G when the reaction temperature was lower than 20 ^{o}C, while the yield of AA-2G kept above 60% of the highest within the rage from 28 to 52^{o}C. This suggested that the wild-type and mutant CGTases were stable within this temperature range.

The effects of mass ratio of substrates (L-AA: maltose) on AA-2G biosynthesis were also examined. As shown in Fig. 1(C), for the wild-type and the mutant CGTases (K47F, K47P, and K47Y), the maximal AA-2G yield was attained at a 1:1 mass ratio of L-AA to maltose.

Fig. 1(D) shows the time profiles of AA-2G synthesis by the wild-type and mutant CGTases. The amount of AA-2G formed by the wild-type and mutant CGTases (K47F, K47Y, and K47P) reached the maximal values at 20, 16, 20, and 20 h, respectively. It was also observed that the mutant K47F reached the highest AA-2G yield earlier than the wild-type and the mutants K47P and K47Y. The possible reason was that the thermostability of the K47F was lower than that of the wild-type and other two mutants.

Under the optimal conditions (pH 6.5, temperature 36 ^{o}C, and the mass ratio of L-AA to maltose, 1:1), the AA-2G titer of the wild-type and mutants (K47F, K47Y, and K47P) reached 0.85, 0.92, 1.06, and 1.12 g/L, respectively. The AA-2G titer by the mutant K47P was 1.32-fold of that produced by the wild-type CGTase.

Influence of mutation on the reaction kinetics and thermal stability of CGTase

The experimental data of kinetics reaction for the wild-type and mutant CGTases were best fitted by the Equation (1) [33] (data not shown), indicating a normal ping-pong type of kinetics. The kinetic parameters were obtained by plotting the experiment data based on the Equation (1) and were listed in Table 2. It could be seen that the maximal reaction rate (V_{max}) of the mutant CGTases (K47P, K47F, and K47Y) was higher than that of the wild-type CGTase. Meanwhile, compared to the wild-type CGTase, the K_m (with maltose as the substrate) for the mutants K47P, K47F, and K47Y was decreased by 19.3, 9.7, and 11.7%, while the k_{cat}/K_m was increased by 57, 20, and 23%, respectively. The reaction kinetics indicated that the binding capacity and catalytic efficiency of these mutants towards maltose were increased compared to those of the wild-type CGTase.

$$v = V_{max} \cdot a \cdot b / (K_{mA} \cdot b + K_{mB} \cdot a + a \cdot b) \qquad \text{(Equ. 1)}$$

where v is the reaction rate (the amount of AA-2G formed by 1 mg enzyme per hour, $mM \cdot mg^{-1} \cdot h^{-1}$), and V_{max} is the maximal reaction rate ($mM \cdot mg^{-1} \cdot h^{-1}$), and a and b are the acceptor (L-AA) and donor (maltose) concentrations (mM), respectively, and K_{mA} and K_{mB} are the affinity constants for the substrates L-AA and maltose, respectively.

The influence of mutations on the cyclization, hydrolysis, and disproportionation activities of the CGTase was investigated. As shown in Table 1, compared to that of the wild-type CGTase, the cyclization (α-cyclodextrin forming) activity of the mutants K47P, K47F, and K47Y was decreased by 2.6, 44.9, and 52.5%, respectively, while the hydrolysis (starch-degrading) activity of the mutants K47P and K47Y was decreased by 68.7 and 62.2%, respectively. Nevertheless, the mutant K47F showed about 6 % increase in hydrolysis activity (Table 1). This might be due to the fact that the instable covalent reaction intermediates of the mutant K47F collapsed faster to form a hydrolysis

product than the wild-type CGTase and the mutants K47P and K47Y. [31]

All the three mutants K47P, K47F, and K47V showed an increased disproportionation activity by 30.2, 12.5, and 18.1%, respectively. The simultaneous increase of the disproportionation activity and AA-2G yield of three mutants indicated that the disproportionation of CGTase may be the main reaction for the biosynthesis of AA-2G.

Table 2 shows the thermal stabilities of the wild-type and mutant CGTases. It could be seen that the half-life ($t_{1/2}$) of the wild-type CGTase at 40 ^{o}C was a little longer than that of K47P, K47F, and K47Y, indicating that the thermal stability of the mutants K47P, K47F, and K47Y decreased.

The crystal structure of CGTase revealed that this enzyme had five domains (A-E) and nine sugar binding subsites (labeled -7 to +2) [21, 34], and the residue 47 occupied an important position in substrate binding at -3 subsite. [20, 35] By the sequence comparisons of CGTases from different sources, it was observed that the amino acid residue at the 47 position had a clear discrimination among different groups of CGTases (α-, β-, and γ-CGTase). [30, 36] This suggested that the amino acid residue at the 47 position of CGTase played a key role in the product specificity. In addition, when the Arg47 of CGTase in *Bacillus circulans* strain 251 CGTase was replaced with tryptophan, the cyclization activity decreased, while the disproportionation and hydrolysis activities increased, [30, 31] indicating that the residue 47 also affected the different reaction activities of CGTase. Therefore, the residue 47 of CGTase might be a crucial site for all the reactions of CGTase and was selected as the mutation site in this work.

Because both maltose and maltohexaose are short linear oligosaccharides, here we constructed theoretical structures of the wild-type and mutant CGTases and modeled complex structures of the CGTase with maltohexaose to explore the possible mechanism responsible for the enhanced

substrate specificity at molecular level (Fig. 2). The X-ray structural analysis of CGTase from *T. thermosulfurigenes* with a bound semicyclic maltohexaose inhibitor revealed that the bent conformation was stabilized by a hydrogen-bonding between the Lys 47 and the sugar at subsite -3.[37] Therefore, in *P. macerans* CGTase, a hydrogen bond might also exist between Lys47 and the sugar substrate (Fig. 2(A)). However, phenylalanine, proline, and tyrosine removed all possible hydrogen-bonding interactions with the sugar at -3 subsite due to their lower hydrophilicity and shorter side chain. In the present work, the decreased cyclization activity of the mutants K47F, K47Y, and K47P was observed (Table 1), possibly due to the removal of all possible hydrogen-bonding interactions with intermediate at subsite -3 (Fig. 2(B), (C) and (D)). Compared to K47P and K47Y, the mutant K47F had a higher hydrolysis reaction activity (Table 1).

It was previously reported that the Arg47 of wild-type CGTase from *B. circulans* strain 251 could form hydrogen bonding with cyclic compounds and interact with cyclodextrins but not linear oligosaccharides, [30] and thus the residue 47 may affect CGTase reactions with cyclic or linear substrates. Therefore, we could suppose that the preferred substrate for the wild-type CGTase was the bent substrates such as cyclodextrins but not linear oligosaccharides such as maltose due to the possible hydrogen-bonding interactions. This might explain why the wild-type CGTase had a higher AA-2G production with cyclodextrins rather than maltose as glycosyl donors. Due to the fact that there was larger room and less obstruction for binding the linear saccharides when the long side chain lysine was replaced by short chain amino acids (Fig. 2), the mutants K47F, K47Y, and K47P might enhance the affinity between the residue 47 and maltose. Therefore, the AA-2G production with maltose as the glycosyl donor was increased by the three mutants. Furthermore, the decrease of the K_m (maltose) and the increase of k_{cat} /K_m (maltose) for the mutants (K47P, K47F, and K47Y)

(Table 2) confirmed that the binding capacity with maltose and catalytic efficiency of the mutant CGTases were enhanced.

In addition, the increased disproportionation activities of the three mutants were also favorable for the synthesis of AA-2G. Disproportionation was the main reaction in the AA-2G synthesis. Many reports indicated that the mutations of the residue 47 could increase the disproportionation activity of CGTase. [31, 32] It was reported that the increased hydrolysis activity was attributed to the decreased cyclization and disproportionation activities. [32] Therefore, we could suppose that the decreased cyclization and hydrolysis activities enable the increase of the disproportionation activity, because the intermediate had more chances to be attacked by the glycosyl acceptor.

Conclusion

In summary, in this work three CGTase mutants with enhanced maltose specificity were obtained by site-saturation engineering strategies and the AA-2G yield was significantly improved when using these mutant CGTase as biocatalysts. The enhanced substrate specificity towards maltose was confirmed by reactions kinetics and the inherent mechanism was revealed by structure modeling. Although the yield of AA-2G produced by the three positive mutants was lower compared to that with α-cyclodextrin or β-cyclodextrin as the glycosyl donor, the results obtained here indicated that the maltose specificity of CGTase could be enhanced by site-directed mutagenesis and it was possible to produce AA-2G with maltose as the cheap substrate. The AA-2G yield could be further improved by systems engineering of the other substrate binding sites of CGTase, enzyme immobilization, and transformation conditions optimization in the future.

Experimental

Bacterial strains, plasmids and materials

P. macerans strain JFB05-01(CCTCC M203062) was used as the source of the wild-type CGTase gene. *E. coil* JM109 was used for recombinant DNA manipulations. *E. coil* BL 21(DE3) was used as the host for the expression of the wild-type and mutant CGTases. The pMD19-T vector used for *cgt* gene cloning and sequencing was purchased from Takara (Dalian, China). The pET-20 (+) vector was used for expression of α-CGTase in *E. coli* BL 21 (DE3).

PrimeSTAR HS DNA polymerase, restriction endonucleases, PCR reagents, Genomic Extraction Kit, and MutanBEST kit were purchased from TakaRa (Dalian, China). DNA sequencing was performed by Sangon (Shanghai, China). AA-2G was purchased from Wako Pure Cheemical (Wako, Japan), and L-AA was purchased from Jiangshan Pharmaceutical (Jiangsu, China), and maltose was purchased from Sangon (Shanghai, China). All other chemicals and reagents were of analytical grade.

Construction of the recombinant plasmid cgt/ pET-20b (+)

The genomic DNA of *P. mancerans* strain JFB05-01 extracted by the Genomic Extraction Kit was used for PCR as the DNA model. The primers were designed by Primer Premier 5 from the published *cgt* gene (GenBank accession no. AF047363.1). The restriction sites of *Bam*H I and *Xho* I (underlined letters) were introduced into the forward primer (5'-CGCGGATCCGTCACCCGATACGAGCGTGGACA-3') and reverse primer (5'-CCGCTCGAGATTTTGCCAGTCCACCGTCACC-3'), respectively. The gel-purified PCR product was digested with *Bam*H I and *Xho* I and then ligated into the similarly restriction-digested expression vector pET-20b (+) to construct recombinant plasmid cgt/pET-20b (+). The plasmid containing the correct insert was confirmed by DNA sequencing and used for site-directed mutagenesis.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the MutanBEST kit (TakaRa, Dalian, China). One-step PCR method was carried out by PrimeSTAR HS DNA polymerase using plasmid cgt/pET-20b (+) as the template DNA and oligonucleotide primers. The sequences of the mutagenic primers are shown in Table 3. The PCR products were treated with blunting kination enzyme and Ligation Solution I, and ligated into circular plasmids, and then were transformed into *E. coli* JM109. These plasmids were confirmed by DNA sequencing and the correct ones were transformed into *E. coli* BL21 (DE3) for expression.

Preparation and purification of the mutant CGTases

The mutant CGTases were prepared according to what previously reported. [22, 34] The recombinant *E. coil* BL 21(DE3) were inoculated into 20 mL Luria-Bertani (LB) medium containing 100 μg/mL ampicillin and grown at 37 ^{o}C overnight. Then the seed culture was inoculated into the flask with an inoculum ratio of 4 % (v/v). The fermentation medium contained (g/L): glucose 8, lactose 0.5, peptone 12, yeast extract 24, K_2HPO_4 16.43, KH_2PO_4 2.31, $CaCl_2$ 0.28, ampicillin 0.1, pH 7.0. The flask culture was then incubated on a rotary shaker (200 rpm) at 25 ^{o}C for 90 h. The expression of CGTases was induced with 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm (OD_{600}) reached 0.6. The culture broth was centrifuged at 10, 000 × *g* and 4 ^{o}C for 5 min, and then the supernatant was purified and used for the subsequent transformation. The purification of the crude enzyme solution was carried out by an Ni-NTA agarose column (Qiagen, Chatsworth, CA) as described previously. [36]

Biosynthesis and analysis of AA-2G

The purified wild-type or mutant CGTase was diluted with acetic acid-sodium acetate buffer (pH 5.5) to a protein concentration of 1 mg/mL, and then was mixed with the substrates (L-AA and maltose) with a final concentration of 2 % (w/v). The mixture was incubated at 37 ^{o}C for 24 h under the dark and oxygen-free conditions. AA-2G was analyzed by the HPLC method[22] using an Amethyst C18-H column (4.6×250 mm, Sepax, America). Samples were filtered through a 0.22 μm membrane before injection. The detection wavelength was 238 nm. The mobile phase consisted of 0.05 M KH_2PO4/H_3PO_4 (pH 2.0) and the flow rate was 0.6 mL/min. Under these conditions, the standard AA-2G was separated reproducibly with a retention time of 10 min. The AA-2G concentration was calculated on the basis of peak area of standard sample.

On the basis of the initial transformation conditions (temperature 37 ^{o}C , pH 5.5, and the mass ratio of L-AA to maltose, 1:1), the influence of temperature (20, 28, 36, 44 and 52^{o}C), pH (pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0), and the mass ratio of substrates (L-AA: maltose, 1:5, 2:4, 3:3, 4:2, and 5:1) on the synthesis of AA-2G by the wild-type and mutant CGTases was investigated.

The kinetic analysis of the wild-type and mutant CGTases for AA-2G biosynthesis was performed by measuring the amount of AA-2G with different concentrations of one substrate while fixing the concentration of the other substrate, and the obtained results were subjected to kinetic analysis using SigmaPlot (Jandel Scientific). The thermostability of the wild-type and mutant CGTases was determined by incubating the purified enzyme at 40 ^{o}C. At a regular time interval (2 h), the samples were taken to catalyze the synthesis of AA-2G.

Analysis of CGTase

The α-cyclodextrin forming activity of CGTase was determined using the methyl orange method

described by Li et al.[29] Namely, 0.1 mL of the purified wild-type or mutant CGTase was diluted with 50 mM phosphate buffer to 1 mg/mL and then was added into 0.9 mL of 3 % (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0). After incubating at 40 °C for 10 min, the reaction was terminated by the addition of 1.0 M HCl (1.0 mL). Finally, 1.0 mL of 0.1 mM methyl orange in 50 mM phosphate buffer (pH 6.0) was added, and the absorbance at 505 nm was measured after incubating at 16 °C for 20 min. One unit of α-cyclodextrin-forming activity was defined as the amount of enzyme that was able to produce 1 μmol α-cyclodextrin per minute. The hydrolyzing activity was analyzed by the starch-degrading method.[31] The purified CGTase (1 mg/mL) was incubated with 1 % soluble starch solution at 50 °C for 10 min. One unit of hydrolyzing activity was defined as the amount of enzyme producing 1 μmol reducing sugar per minute. The disproportionation activity was determined as previously described,[31] and 1 mM 4-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene (EPS: Megazyme, County Wicklow, Ireland) and 10 mM maltose were used as donor and acceptor substrates, respectively. One unit of activity was defined as the amount of enzyme converting 1 μmol of EPS per minute.

Protein concentrations were determined with the Bradford method using Bradford Protein Assay Kit with bovine serum albumin as a standard (Beyotime, Jiangsu, China)

Structure modeling of the (mutant) CGTases

The homology modeling of wild-type and mutant CGTases form *P. macerans* was performed using the crystal structure of *B. circulans* strain 251 CGTase (PDB 1D3C, 1.81 Å resolution) as the template by the SWISS-MODEL protein-modeling sever (http://swissmodel.expasy.org/).[38] The similarity of *P. macerans* CGTase with the template was 68.37 %. Structural alignment was done according to the

combinatorial extension method by using the server http://cl.sdsc.edu/. The root mean square deviation between the template and model alpha carbon backbones (RMSD 0.1 Å) was calculated by the combinatorial extension method. All graphical molecular representations were generated using Accelrys Discovery Studio Client 2.5. The proposed complex structures of the (mutant) CGTases with a maltohexaose were modeled by means of superpositioning of the above theoretical structure and the complex structure of *Thermoanaerobacterium thermosulfurigenes* CGTase with a maltohexaose inhibitor in the active site (PDB accession codes 1A47 [37]) , followed by least-squares fitting of the Cα atoms. [27, 30]

Statistical analysis

All the experiments were performed at least three times, and the results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Student's *t* test. *P* values less than 0.05 were considered statistically significant.

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Figure captions:

Fig. 1. Influence of reaction temperature, pH and substrate ratio on AA-2G synthesis by the wild-type and mutant CGTases with maltose as the glycosyl donor. (pH 4.5~6.0 with acetic acid-sodium acetate buffer; pH 6.0~7.0 with phosphate buffer. There are two dots at pH 6 because two different buffers were used at this pH. ■, wild-type CGTase; ▯, K47F; ▲, K47Y; □, K47P)

Fig. 2. Different conformations of amino acid side chain at position 47 in the structural models of the wild-type and mutant CGTases complexed with a maltohexaose inhibitor in the active site. (Atom display style: Ball and stick, amino acid; line, maltohexaose inhibitor. A: wild-type CGTase; B: mutant K47F; C: mutant K47Y; D: mutant K47P.)

Table 1. Comparison of the cyclization, hydrolysis, disproportionation and AA-2G titer of the wild-type and the three positive CGTase mutants [a]

CGTase	Relative activity (%)[b]			AA-2G titer [c] (g/L)
	Cyclization (α-cyclodextrin-forming activity)	Hydrolysis (starch-degrading activity)	Disproportionation	
Wild-type	100	100	100	0.76±0.03
K47F	55.1±1.2	106.5±0.6	112.5±1.4	0.89±0.05
K47P	97.4±1.5	31.3±1.1	130.2±1.5	1.01±0.03
K47Y	47.5±1.0	37.8±0.8	118.1±0.9	0.92±0.04

a: Each reaction of the (mutant) CGTases was measured with the same concentration of zymoproteins;

b: The reaction activity of the wild-type CGTase was defined as 100 %;

c: AA-2G biosynthesis with maltose as the glycosyl donor.

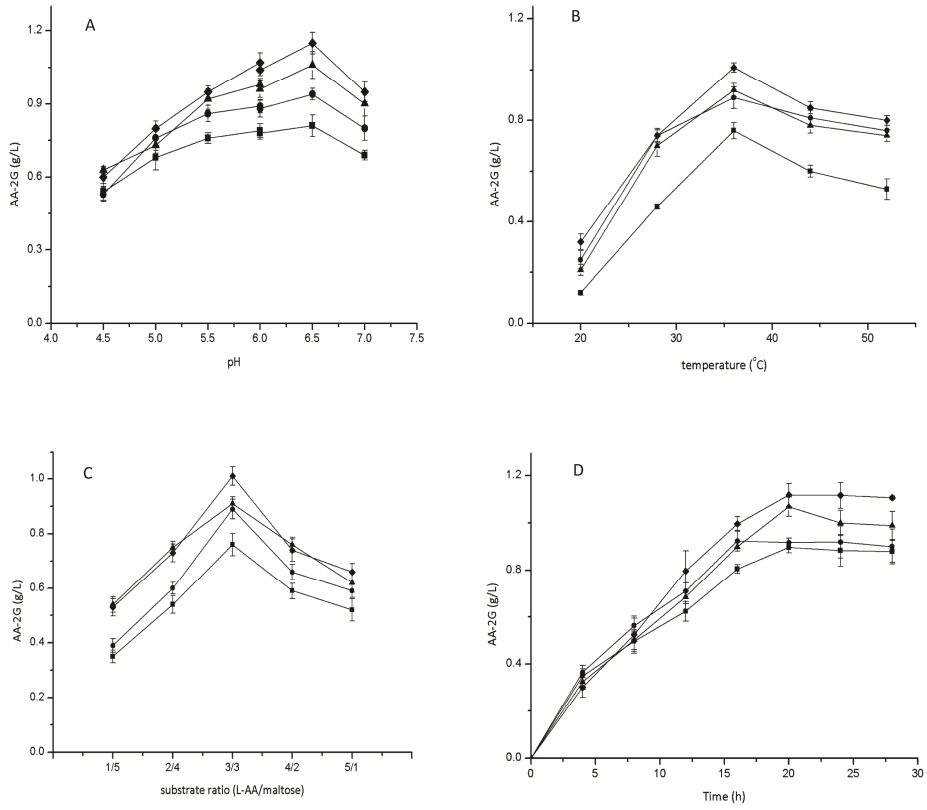
Table 2 Kinetic parameters and thermal stability (40 °C) of the wild-type and three CGTase mutants at pH 6.5

enzyme	V_{max} (mM/mg/h)	k_{cat} (h^{-1})	K_m (L-AA) (mM)	k_{cat}/K_m(L-AA) ($mM^{-1}h^{-1}$)	K_m(maltose) (mM)	k_{cat}/K_m(maltose) ($mM^{-1}h^{-1}$)	$t_{1/2}$ (h)
Wild type	0.11±0.01	8.25	40.15±0.52	0.20	5.55±0.11	1.49	7.6
K47F	0.12±0.02	9.00	40.20±0.62	0.22	5.01±0.21	1.80	6.8
K47P	0.14±0.02	10.50	40.02±0.48	0.26	4.48±0.08	2.34	7.1
K47Y	0.12±0.01	9.00	40.11±0.74	0.22	4.90±0.19	1.84	7.2

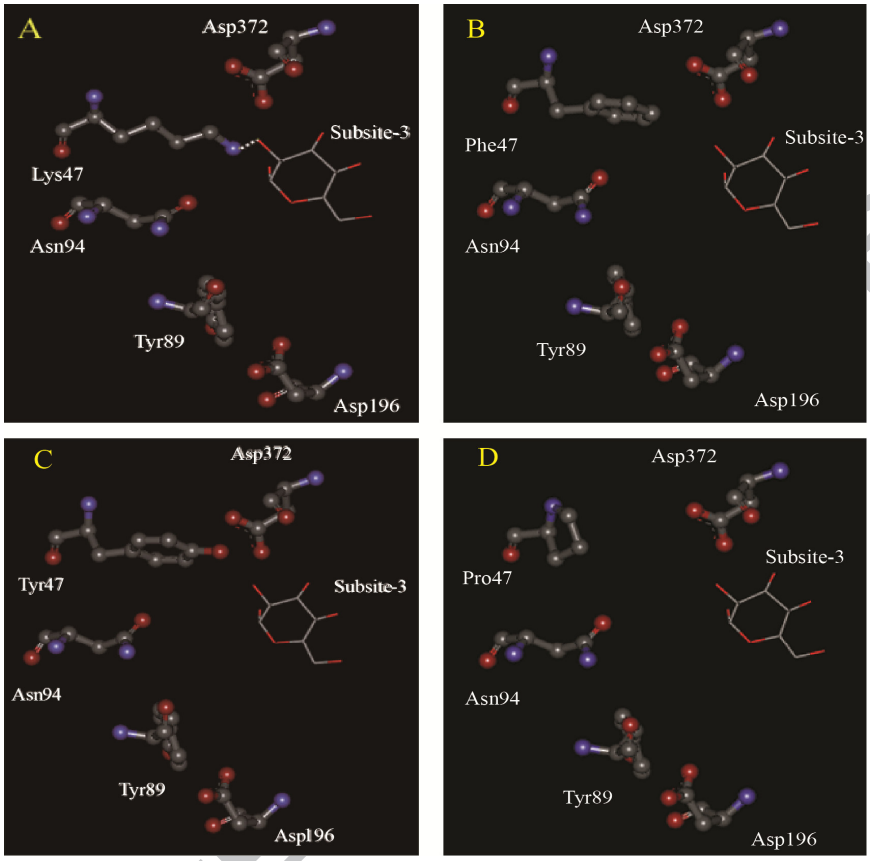
Table 3. Oligonucleotide primers used for saturation site-directed mutagenesis

primer		Sequence* (5′ to 3′ direction)
Forward Primer	K47F	CCAATTTGTTCCTCTATTTCGGGGG
	K47I	CCAATTTGATCCTCTATTTCGGGGG
	K47M	CCAATTTGATGCTCTATTTCGGGGG
	K47V	CCAATTTGGTTCTCTATTTCGGGGG
	K47S	CCAATTTGTCTCTCTATTTCGGGGG
	K47P	CCAATTTGCCGCTCTATTTCGGGGG
	K47T	CCAATTTGACCCTCTATTTCGGGGG
	K47A	CCAATTTGGCGCTCTATTTCGGGGG
	K47Y	CCAATTTGTACCTCTATTTCGGGGG
	K47H	CCAATTTGCACCTCTATTTCGGGGG
	K47Q	CCAATTTGCAGCTCTATTTCGGGGG
	K47N	CCAATTTGAACCTCTATTTCGGGGG
	K47D	CCAATTTGGACCTCTATTTCGGGGG
	K47E	CCAATTTGGAACTCTATTTCGGGGG
	K47C	CCAATTTGTGCCTCTATTTCGGGGG
	K47W	CCAATTTGTGGCTCTATTTCGGGGG
	K47R	CCAATTTGCGTCTCTATTTCGGGGG
	K47G	CCAATTTGGGTCTCTATTTCGGGGG
	K47L	CCAATTTGCTGCTCTATTTCGGGGG
Reverse primer	47-Rev	ATCGGTCGCCGCTGAACGCA

(*The underlined letters denote the coding sequences of the mutated amino acid)

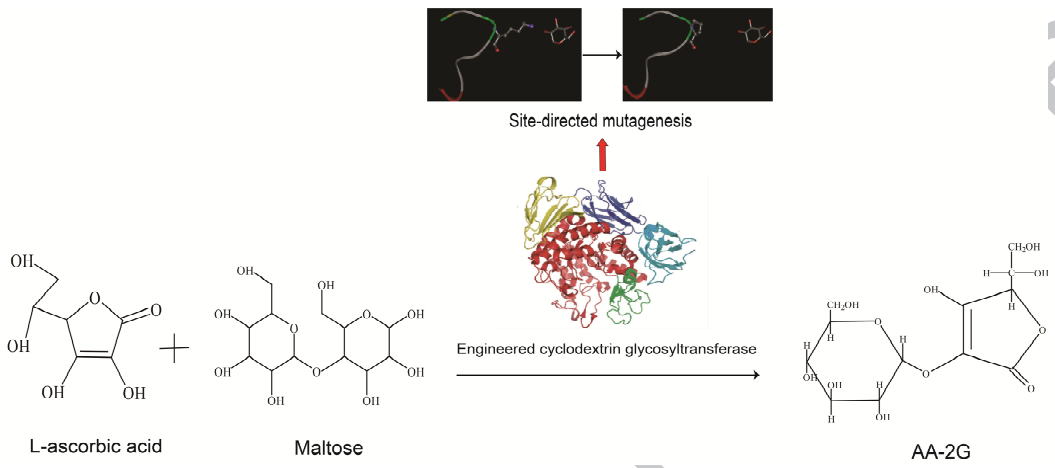


(Fig. 1)



(Fig. 2)

Graphical abstract



Highlights

1. The specificity of CGTase towards maltose was improved by site-saturation engineering of lysine 47;
2. The mechanism for enhanced specificity was clarified by structure modeling the of CGTase;
3. Enzymatic synthesis of AA-2G with maltose as glycosyl donor was optimized;
4. The obtained CGTase mutants have great potential in the large-scale production of AA-2G.