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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 °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.

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1. 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

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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.

2. Results and discussion

2.1. 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 *Escherichia 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 data). 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]

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

Under the initial transformation conditions (temperature 37 °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.

Figure 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]

Figure 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 °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 recom-

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.

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