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Biosynthesis of 2-O-α-D-glucopyranosyl-L-Ascorbic Acid from Maltose by Cyclodextrin Glucanotransferase from Bacillus sp. SK 13.002

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Abstract In this work 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) was synthesized by Cyclodextrin glucanotransferase (CGTase) from *Bacillus* sp. SK 13.002 with L-ascorbic acid (AA) as an acceptor and maltose as a glycosyl donor. AA-2G production was analyzed by HPLC and was confirmed by LC/MS results. The reaction parameters, such as pH (4.0-9.0), temperature (25-50°C), time (0-30 h), substrate ratios and enzyme concentration were optimized. The results showed that the optimum condition was pH 8.0 at 37°C for 24 h, 1:1 maltose to AA substrates mass ratio, and 200 U/mL of CGTase. Under these conditions, the production of AA-2G was 5.5 g/L, this result indicate that CGTase from Bacillus sp. SK 13.002 can effectively uses maltose as a glycosyl donor to produce AA-2G in high yield.

Keywords: *Cyclodextrin glucanotransferase, 2-0-α-D-glucopyransyl-L-ascorbic acid, maltose*

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1. Introduction

Vitamin C is the common name for L-ascorbic acid (AA), a six-carbon sugar derivative of L-threo-hex-2-enono-1,4-lactone [1]. The nutritional importance of vitamin C as an essential water-soluble vitamin is well established. It has long been known that a nutritional deficiency in vitamin C causes scurvy, a disease characterized by bleeding gums, impaired wound healing, anemia, fatigue, and depression, which, without proper care, can eventually be fatal [2]. AA can also act as a cofactor for the hydroxylation of proline and lysine residues in collagen, a major protein component of the body [3]. However, vitamin C is extremely unstable in aqueous solution, especially in the presence of heat, light, Cu2+, and ascorbate oxidase, which reduces its biological activity and limits its applications [4,5].

Different AA derivatives have been chemically synthesized as attempts to improve the stability of AA, such as AA-2-O-phosphate (AA-2P) [6], AA-2-O-sulfate (AA-2S) [7], and AA-2-methyl ether (AA-2M) [8]. Some vitamin glycosides are present in nature [9], but AA glycosides are only available as chemicals or via enzymatic synthesis [10]. The molecule 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) was enzymatically synthesized by Yamamoto et al. [11] via transglycosylation using α-glucosidase. AA-2G is stable against enhanced oxidative degradation by heat, Cu^{2+} ion, or ascorbate oxidase, and it was found to have no reducing activity [4]. AA-2G is extremely stable and is considered to be superior to other chemically synthesized AA derivatives in terms of reaction specificity and efficiency in large-scale production [4]. AA-2G is available as AA well [12]; therefore it has found wide application in cosmetics, medicines and foods [5].

Although AA-2G can be produced by different enzymes [11,13,14], but studies have shown that cyclodextrin glucanotransferase (CGTase) is the most preferable either mostly in free [15], immobilized [16], recombinant [17], or in mutant state [18]. CGTase transfer glucose unit to C_2 in AA via transglycosylation from glycosyl donor, different substrates were used as glycosyl donor except glucose [15,17]. Cyclodextrins (α and β) are best substrates for CGTase for the production of AA-2G, but they found limitation for industrial applications due to the cost of α-cyclodextrin and low solubility of β-cyclodextrin. Maltose was used as substrate, it represents best choice due to it is low cost and high solubility compared to cyclodextrins, but the production of AA-2G was weak due to low specificity [15,17]. CGTase specificity for synthesis AA-2G using maltose was improved by site-saturation engineering of lysine 47 and the yield was 1.12 g/L [19]. This yield is too low compared to 13 g/L of AA-2G that produced by recombinant CGTase using β-cyclodextrin as a glycosyl donor [17]. Therefore finding of CGTase with high

specificity for synthesis AA-2G from maltose will assist to narrow the wide gab with β-cyclodextrin. In this research work, maltose was used as a glycosyl donor for production of AA-2G by CGTase from *Bacillus* sp. SK 13.002.

2. Materials and Methods

2.1. Materials

AA-2G standard was from Hayashibara Biochemical Laboratories (Okayama, Japan), AA and glucoamylase were from Sigma. All other analytical grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Methods

2.2.1. Bacterial Strain and Enzyme Production

Bacillus sp. SK 13.002 originally was isolated from soil sample in our laboratory. The 16S rRNA gene sequences for this strain have been deposited in the National Center for Biotechnology Information NCBI GenBank database under accession number GU570959 [20]. After cultivation for 96 h, cells were removed by centrifugation at 10,000 rpm and 4°C for 15 min. The supernatant was used as crude enzyme solution, which was concentrated using Millipore ultra-filtration system (Millipore Company, USA) with a molecular cut-off point of 10 kDa. The concentrated enzyme solution was precipitated with 70% saturated ammonium sulfate at 4°C. The mixture was centrifuged at 10,000 rpm, 4°C for 15 min and the pellets were dissolved in 20 mM Tris-HCl buffer (pH 8.0) and dialysed against the same buffer overnight at 4°C. The solution obtained after dialysis was freeze-dried and the resulting CGTase powder was used in carrying out all experiments.

2.2.2. Enzymatic Production of AA-2G

The reaction mixture consisted of AA, maltose as a glycosyl donor, and CGTase in 0.1 M acetate buffer (pH 6.0). The reaction mixture was incubated at 40°C for 24 h in the dark in a shaking water bath. Glucoamylase (10 U/mL) was added to the mixture and incubated at 65°C and pH 5.5 for 6 h to hydrolyze the AA-2-oligosaccharides (AA-2G_n) to AA-2G and glucose.

2.2.3. HPLC analysis of AA-2G

Agilent Technologies 1200 Series (USA) connected with ODS-HYPERSIL column (4x250 mm) was used in this study. The assay conditions were a detection wavelength of 240 nm, mobile phase of 0.1 M KH_2PO_4/H_3PO_4 (pH 2.0), and a flow rate of 0.5 mL/min. The reaction mixture was centrifuged at 13000 rpm for 15 min and the supernatant was filtered through a 0.45 µm membrane before injection. The AA-2G concentration was calculated on the basis of peak area from standard curve with coefficient of correlation (r2) equal 0.9993. All experiments were conducted at least twice.

2.2.4. LC/MS/MS Analysis of AA-2G

LC/MS/MS (Waters Acquity UPLC and PDA; Waters Maldi Synapt Q-T of MS) was operated in negative ion detection mode; ultra pure synthetic air was used as a nebulisation desolvation gas (flow rate = 500 l/h) and MS fragment ions were obtained with 15 eV collision energy. The eluent consisted of gradient, A 100% methanol and B 0.1 formic acid using BEH C18 column (2.1×50 mm 1.7 µm) at 0.3 m/l flow rate, 245 nm, and at 45°C.

3. Results and Discussion

3.1. Enzymatic Production of AA-2G

The reaction mixture consists of, AA, maltose, and CGTase from *Bacillus* sp. SK13.002 in 0.1 M acetate buffer. HPLC profile of the reaction mixture (Figure 1 A) showed production of AA-2G (peak 2) at 7.5 min followed by AA-2-oligosaccharides; AA-2G_2 and AA-2G_3 at peaks 3 and 4 respectively. AA-2G production was 0.68 g/L, after addition of glucoamylase (10 U/mL), AA-2-oligosaccharides were hydrolyzed and AA-2G production was increased to 1.06 g/L (Figure 1 B). However, glucoamylase in addition to hydrolyze AA-2-oligosaccharides, it will also hydrolyze AA-2G; previously glucoamylase concentration was optimized [17]. Formation of AA-2-oligosaccharides by CGTase from *Bacillus* sp. SK 13.002 when maltose was used as a glycosyl donor is consistent with the result of CGTase from *Paenibacillus sp* [15]. On the other hand result of CGTase from *Paenibacillus macerans* showed that AA-2-oligosaccharides were not produced when maltose was used as a glycosyl donor [19].

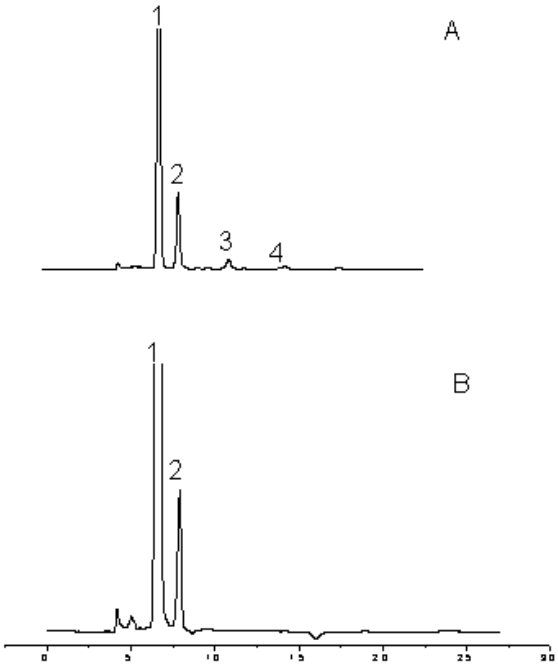

Figure 1. HPLC profiles: (A) Reaction mixture product of CGTase with AA and maltose at 40°C for 24 h. (B) Reaction mixture after addition of glucoamylase to reaction mixture. 1: AA, 2: AA-2G, 3: AA-2G_2, and 4: AA-2G_3

3.2. Confirmation of AA-2G Production by LC-MS/MS

LC-MS/MS results (Figure 2) showed that mass spectrum of sample AA-2G (Standard) revealed a protonated molecule [M-H]- at 337 m/z, and this

molecular ion produced ions at 277, 174, and 114 m/z. The reaction mixture showed a protonated molecule [M-H]- at 337 m/z, and this molecular ion produced ions at 277, 174, and 114 m/z, this result confirmed the production of AA-2G in the reaction of AA with maltose catalyzed by CGTase from *Bacillus* sp. SK13.002. The reaction mixture also showed other two peaks (data not shown) with protonated molecule [M-H]- at 499 and 661 m/z, these peaks related to AA-2G_2, and AA-2G_3 respectively. LC-MS/MS results are summarized in Table 1.

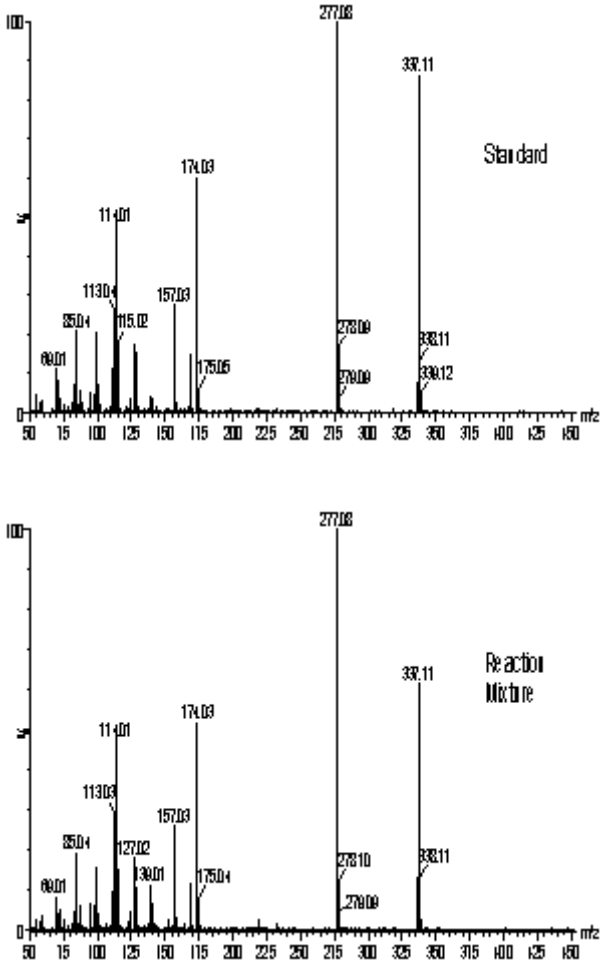

Figure 2. LC-MS/MS profile for standard (AA-2G) and reaction Mixture (AA-2G) product of AA, maltose and CGTase

Table 1. LC-MS/MS Feature of the ascorbic acid transglycosylated products

Product	Retention time (min)	$[M-H]^-$
AA-2G	1.892	337.1
AA-2G_2	2.556	499.2
AA-2G_3	3.308	661.2

3.3. Optimization of AA-2G Production

3.3.1. Influence of Reaction pH and Temperature

The influence of pH was studied using different buffers, sodium acetate (4.0-6.0), sodium phosphate (6.0-8.0), and Tris- HCl (8.5 - 9.0). AA-2G production with acetate buffer showed gradual increase but, with phosphate buffer showed semi exponential increase and reached the maximum at pH 8.0 (Figure 3 A). With Tris-HCl buffer AA-2G production rapidly was decreased. AA-2G optimally was produced by CGTases with pH from 5.5-6.5. On the other hand, AA-2G was produced at pH 7.5 by Sucrose phosphorylase [21], but this is first time AA-2G is produced at pH 8.0. One of two isozymes of CGTase from *Bacillus* sp. SK 13.002 has an optimum hydrolysis activity in pH 8.0 [20]. This result is consistent with result of α-CGTase from recombinant *Escherichia coli*, which was showed transglycosylation maximum pH for AA-2G production same as optimum cyclization activity at pH 5.5 [17].

The influence of temperature was studied with pH 8.0 at 25-50°C (Figure 3 B), the optimum temperature for AA-2G production was at 37°C, other CGTase showed same maximum temperature [17] while other CGTases showed optimum transglycosylation temperature for AA-2G production around 37°C [18,19,22]. On the other hand, CGTase from *Bacillus stearothermophilus* showed a maximum transglycosylation temperature for AA-2G production at 70°C [23].

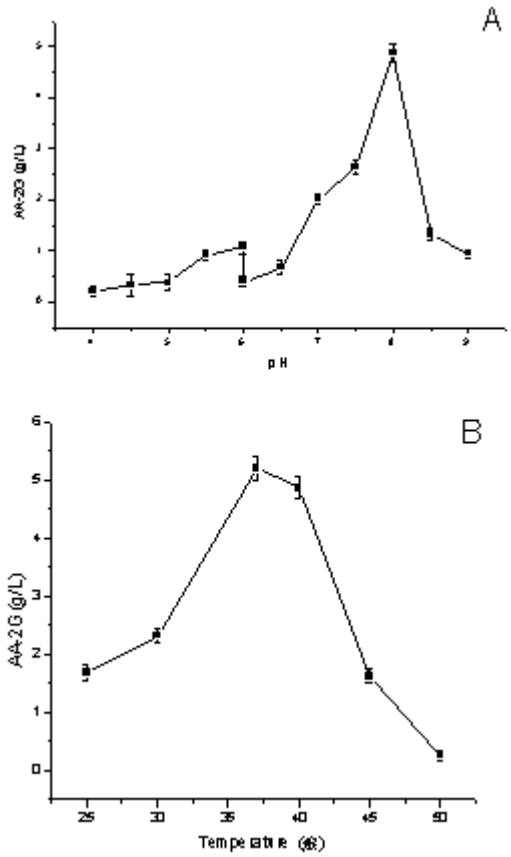

Figure 3. Effect of reaction pH (A) and temperature (B) on biosynthesis of AA-2G by CGTase from Bacillus sp. SK13.002 with AA (0.016 g/mL) and maltose (0.016 g/mL) for 24 h.

3.3.2. Influence of Time and Substrate Ratio on Production of AA-2G

Figure 4 A shows the time course profile of AA-2G synthesis by CGTase from *Bacillus* sp. SK13.002 in pH 8.0 at 37°C. From the initial state (3 h), AA-2G production was exponentially increased with time till reached the maximum at 24 h and stayed constant till 30 h. Other wild and mutant CGTase showed same results [18]. The effects of substrate mass ratio (maltose/AA) on AA-2G biosynthesis were shown in Figure 4 B, maximum AA-2G production by CGTase from *Bacillus* sp. SK 13.002 was obtained at a 1:1 maltose (0.016 g/L) to AA

(0.016 g/L) mass ratio. This result is in agreement with results obtained by other CGTases [17,19].

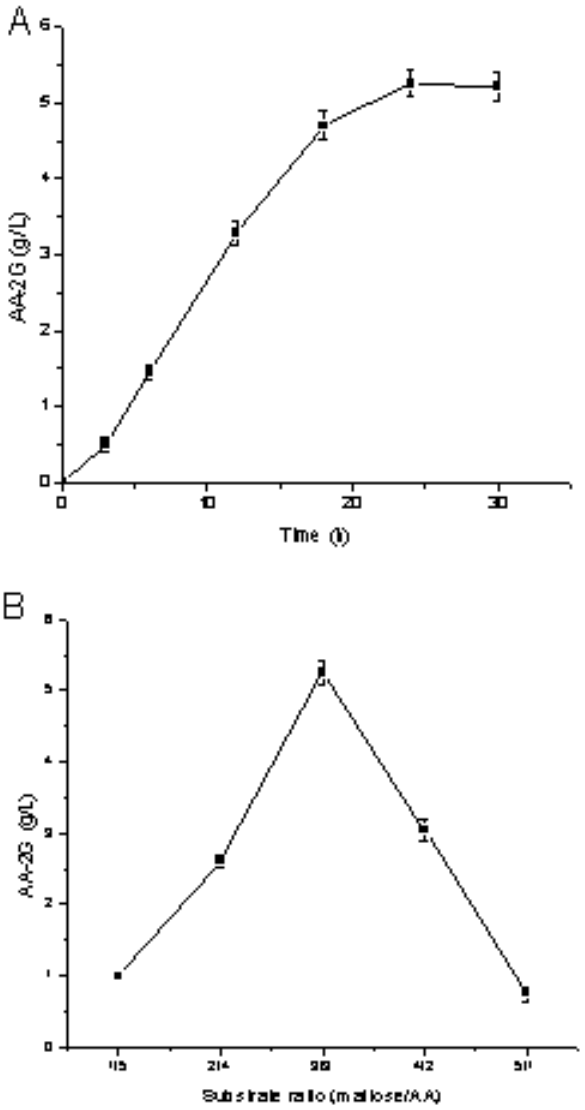

Figure 4. Influence of reaction time (A) and substrate ratios (B) on biosynthesis of AA-2G by CGTase from Bacillus sp. SK13.002 with AA and maltose in pH 8.0 incubated at 37°C.

3.3.3. Influence of Enzyme concentration

The effect of concentration of CGTase from *Bacillus* sp. SK 13.002 on synthesis of AA-2G is shown in Figure 5. The results show that, maximum AA-2G production is reached when the enzyme concentration is 200 U/mL. Further increase in enzyme concentration after 200 U/mL showed decrease in AA-2G yield. Decrease of yield after reaching maximum was observed in production of AA-2G by α-CGTase from recombinant *Escherichia coli*, which has maximum AA-2G production at enzyme concentration of 160 U/mL [17]. These results may indicate that some CGTases may exert hydrolyzing effect on formed AA-2G, but this need to be confirmed. On the other hand, CGTase from *Bacillus stearothermophilus* hardly was hydrolyzed AA-2G compared to rat and rice α-glucosidases [23].

At maximum enzyme concentration the AA-2G yield by CGTase from *Bacillus* sp. SK13.002 using maltose as a glycosyl donor was 5.5 g/L. Recombinant CGTase showed maximum AA-2G production (13 g/L) using β-cyclodextrin as a glycosyl donor [17] and CGTase from *Paenibacillus* sp. approximately showed a yield of 3 g/L AA-2G using dextrin as a glycosyl donor [15]. On the other hand, wild and mutant CGTases from *Paenibacillus macerans* showed a maximum AA-2G production of 0.85 g/L and 1.12 g/L respectively using maltose as a glycosyl donor [19]. Comparing our result with last results, the production of AA-2G by CGTase from *Bacillus* sp. SK13.002 was 6.5 and 4.9-fold of that produced by wild and mutant CGTases from *Paenibacillus macerans* respectively.

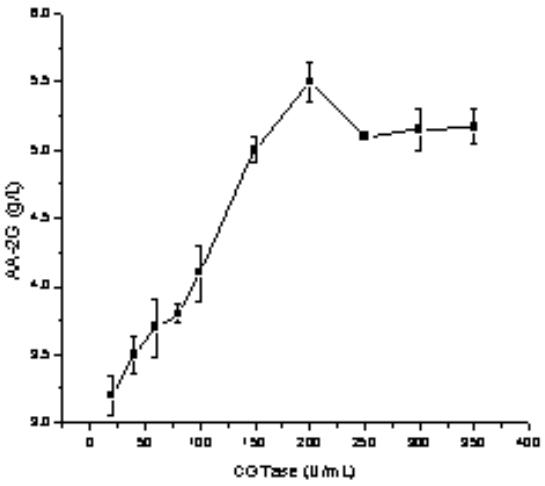

Figure 5. Influence CGTase from *Bacillus* sp. SK13.002 concentration on biosynthesis of AA-2G from maltose (0.016 g/mL) and AA (0.016 g/mL) in pH 8.0 incubated at 37°C for 24 h.

4. Conclusions

The biosynthesis of 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) from maltose; the cheap and highly soluble substrate as a glycosyl donor by CGTases was previously reported. However the yield was poor due to low specificity, specificity for maltose was previously improved by site-saturation engineering and the yield increased from 0.85 to 1.12 g/L. In this study we biosynthesized 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) by CGTase from *Bacillus* sp. SK 13.002 using ascorbic acid as acceptor and maltose as a glycosyl donor, the results were analyzed by HPLC and confirmed by LC-MS/MS. The AA-2G yield was 1.06 g/L in first step, the reaction conditions such as, pH, temperature, time, substrates ratio and enzyme concentration were optimized. At optimum conditions the maximum AA-2G production by CGTase from *Bacillus* sp. SK 13.002 was 5.5 g/L. This result indicated that CGTase from Bacillus sp. SK 13.002 is effective in production of AA-2G compared to other CGTases.

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Statement of Competing Interests

The authors have no competing interests.

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