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RESEARCH ARTICLE

Biosynthesis of 2-O-α-D-glucopyranosyl-L-ascorbic acid from Maltodextrin Catalyzed by Cyclodextrin Glucanotransferase from *Bacillus* sp. SK13.002

Ahmed Eibaid[1,2], Ming Miao[1], Mohanad Bashari[1,2], Abubakr Musa[1,3] and Bo Jiang[1*]

[1]State Key Laboratory of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, 1800 Lihu Ave., Wuxi, Jiangsu 214122, China; [2]Dept. of Food Science and Technology, Faculty of Engineering and Technology, University of Gezira, P. O. Box 20, Wad Madani, Sudan; [3]Sugar Institute, University of Gezira, P. O. Box 20, Wad Madani, Sudan
bjiang@jiangnan.edu.cn*; Tel.: 86-510-85329055; Fax: 86-510-85919625

Abstract

Cyclodextrin glucanotransferase from *Bacillus* sp. SK13.002 has specificity to maltodextrin as a glycosyl donor for production of 2-O-α-D-glucopyranosyl-L-ascorbic acid. In the present investigation, the product was analyzed by HPLC and the reaction parameters were optimized. The results showed that the optimum reaction condition was pH 8 at 37°C for 24 h with 1:1 maltodextrin to L-ascorbic acid ratio and 80 U/mL of Cyclodextrin glucanotransferase. Under the above optimal conditions, the production of AA-2G was 6.3 g/L, indicates that Cyclodextrin glucanotransferase from *Bacillus* sp. SK13.002 is an effective producer of AA-2G compared to previous studies.

Keywords: Cyclodextrin glucanotransferase, maltodextrin, 2-O-α-D-glucopyranosyl-L-ascorbic acid.

Introduction

Stable form of L-ascorbic acid (AA) was enzymatically synthesized via transglycosylation by mammalian α-glucosidase and identified as 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) (Yamamoto *et al*., 1990). Unlike AA, AA-2G is stable against enhanced oxidative degradation by heat, light Cu^{2+} ion or ascorbate oxidase, and it was found to have no reducing activity toward radicals. AA-2G was available *in vitro* and *in vivo* as AA (Kumano *et al*., 1998; Nakamura and Oku, 2009) and it was applied in cosmetics, medicine and foods industries (Yamamoto and Tai, 1997). AA-2G was found in Kimchi, a traditional Korean fermented cabbage (Jun *et al*., 1998), it was effectively produced by five different enzymes, cyclodextrin glucanotransferase (CGTase) is considered to be the best enzyme for large-scale production of AA-2G due to its high substrate specificity (Han *et al*., 2012). Cyclodextrins (α and β) were found as best glycosyl donor to AA for production of AA-2G by CGTases but they found limitation for large-scale production of AA-2G due to the cost of α-cyclodextrin and low solubility of β-cyclodextrin (Han *et al*., 2013a). Maltodextrin, with its low cost and high solubility in aqueous solution, has great potential in AA-2G production, but the production is weak due to low specificity (Markosyan *et al*., 2007). Maltodextrin specificity of CGTase from *Paenibacillus macerans* has been improved for AA-2G synthesis, AA-2G productions was 1.2 g/L and 2.23 g/L for wild and mutant CGTases respectively (Liu *et al*., 2013a). These results were too low compared to 13 g/L of AA-2G that produced by recombinant CGTase using β-cyclodextrin as a glycosyl donor (Zhang *et al*., 2011).

So, finding of CGTase utilizing maltodextrin for large AA-2G production will assist to narrow the wide gab with β-cyclodextrin. In this study, maltodextrin was found as best glycosyl donor to AA for AA-2G biosynthesis by CGTase from *Bacillus* sp. SK13.002. AA-2G production was improved by optimization of reaction parameters.

Materials and methods

*Materials***:** AA-2G standard was from Hayashibara Biochemical Laboratories (Okayama, Japan), AA and glucoamylase were from Sigma. Maltodextrins (DE 10-15) were from Grain Processing Corporation (Muscatine, USA). All other analytical grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Bacterial strain and enzyme production: Bacillus sp. SK13.002 originally was isolated from soil sample in our laboratory. The 16S rRNA gene sequences for this strain have been deposited to the National Center for Biotechnology Information (NCBI) GenBank database under accession number GU570959 and it has been characterized as alkaliphilic cyclodextrin glucanotransferase (CGTase) producing strain (Sun *et al*.**,** 2011a). CGTase from *Bacillus* sp. SK13.002 was partially purified and freeze-dried (Sun *et al*.**,** 2011b).

Enzymatic production of AA-2G: The reaction mixture contained AA, maltodextrin or other substrates as glycosyl donor and CGTase in 0.1 M acetate buffer (pH 6). The reaction mixture was incubated at 40°C for 24 h in the dark in shaking water bath.

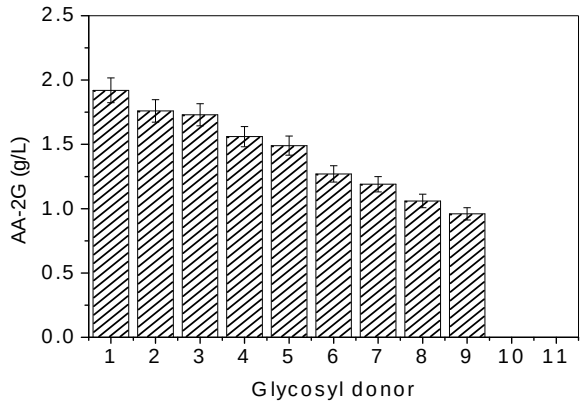
AA-2-oligosaccharides (AA-2G$_n$) were hydrolyzed to AA-2G and glucose by addition of glucoamylase (10 U/mL) to the reaction mixture and incubated at 65°C and pH 5.5 for 6 h.

HPLC analysis of AA-2G: HPLC system consisted of Agilent Technologies 1200 Series (USA) connected with ODS-HYPERSIL column (4X250 mm). The assay conditions were a detection wavelength of 240 nm, mobile phase of 0.1 M KH_2PO_4/H_3PO_4 (pH 2) 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. AA-2G concentration was calculated on the basis of peak area from standard curve with coefficient of correlation (r^2) equal 0.9993. All the experimental data were conducted at least twice.

Results and discussion

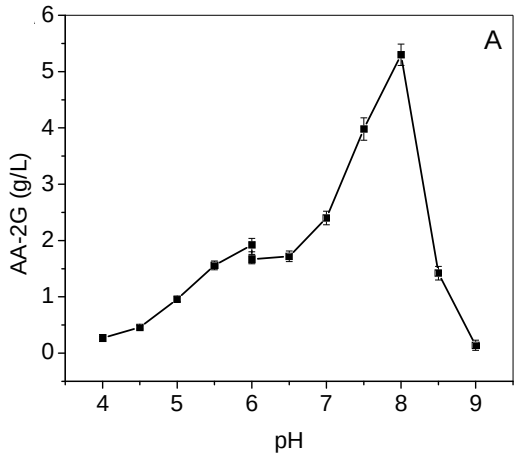
Enzymatic production of AA-2G and glycosyl donor specificity: Standard AA and AA-2G have a retention time of 6.5 and 7.5 min respectively in HPLC results, the reaction mixtures were analyzed by HPLC for AA-2G production. Maltodextrins in general were the best glycosyl donor (Fig. 1) especially maltodextrin (MALTRIN M150) with highest AA-2G production (1.92 g/L) and was used in the following steps. All substrates produced AA-2G except glucose and corn starch by CGTase from *Bacillus* sp. SK13.002. Maltodextrin previously was used as glycosyl donor for transglycosylation of rutin by CGTase from *Bacillus* sp. SK13.002 (Sun *et al*., 2011b). Production of AA-2G by maltodextrin (MALTRIN M150) was confirmed by LC-MS/MS (results not shown).

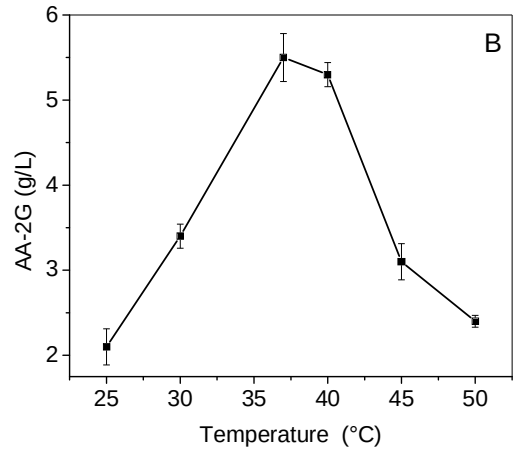
Fig. 1. Glycosyl donor specificity for AA-2G biosynthesis by CGTase from *Bacillus* sp. SK13.002. Reaction mixture contained AA (0.016 g/mL), glycosyl donor (0.016 g/mL) and CGTase (75 U/mL) in 0.1 M acetate buffer pH 6 incubated at 40°C for 24 h. 1, maltodextrin (MALTRIN M150); 2, maltodextrin (MALTRIN M180); 3, maltodextrin (MALTRIN M100); 4, β-cyclodextrin; 5, dextrin; 6, rice; 7, sweet potato starch; 8, maltose; 9, potato starch; 10, glucose; 11, corn starch. Data represent the means of two experiments and error bars represent the standard deviation.



Effect of pH and temperature on AA-2G production: The effect of pH (4-9) was studied using different buffers. AA-2G production was reached maximum (5.3 g/L) on pH 8 (Fig. 2A), one of two isozymes of CGTase from *Bacillus* sp. SK13.002 has optimum hydrolysis activity on pH 8 (Sun *et al*., 2011a). This result is consistent with other results in which transglycosylation maximum pH for AA-2G production was same as optimum cyclization activity pH (5.5) (Zhang *et al*., 2011). AA-2G production has an optimum (5.5 g/L) at 37°C (Fig. 2B) in pH 8, other CGTase showed same maximum temperature (Zhang *et al*., 2011). On the other hand, CGTase from *Bacillus stearothermophilus* showed a maximum transglycosylation temperature for AA-2G production at 70°C (Tanaka *et al*., 1991).

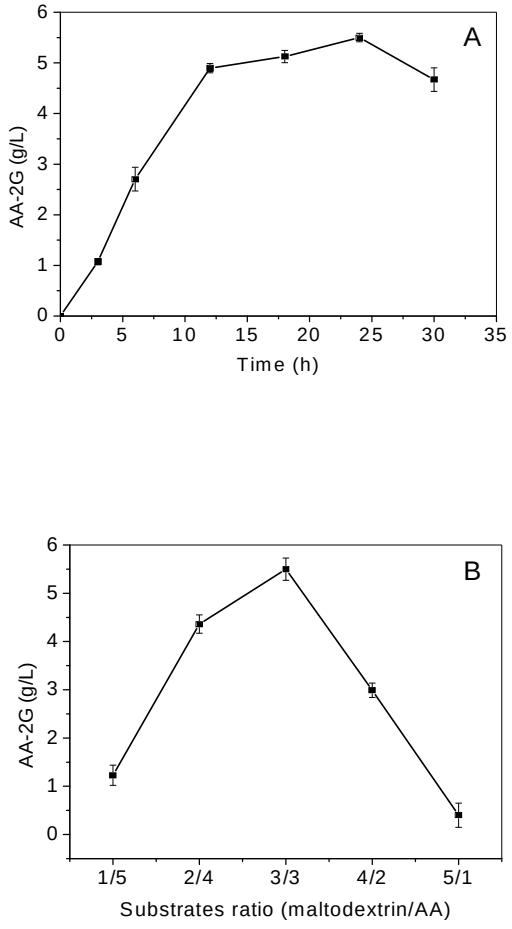
Fig. 2. The effect of reaction pH (A) and temperature (B) on biosynthesis of AA-2G. (A) Reaction mixture contained AA (0.016 g/mL), maltodextrin (0.016 g/mL) and 75 U/mL of CGTase from *Bacillus* sp. SK13.002 incubated at 40°C in 0.1 M different pH, sodium acetate buffer (pH 4-6), sodium phosphate buffer (pH 6-8), and HCl–Tris buffer (pH 8.5 and 9) for 24 h. (B) Same reaction mixture as in (A) in 0.1 M sodium phosphate buffer (pH 8) incubated at different temperature for 24 h. Data represent the means of two experiments and error bars represent the standard deviation.





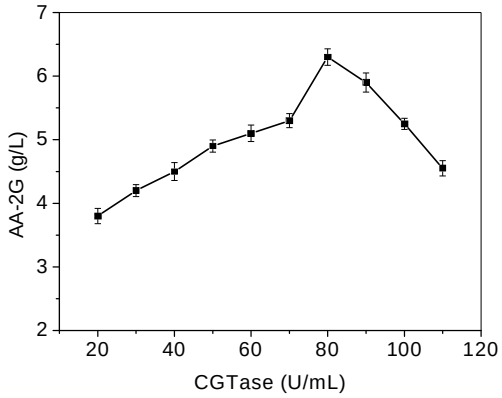
Effect of time duration and substrate ratio on AA-2G production: The effect of reaction time (0-30 h) was shown in Fig. 3A, the production of AA-2G exponentially increased within the first 12 h, then the rate of increasing slowed down till it reached the maximum at 24 h. Wild and mutant CGTases from *Paenibacillus macerans* showed maximum AA-2G yield at 24 h (Liu *et al*., 2013a). The best substrate mass ratio (maltodextrin/AA) on AA-2G biosynthesis was achieved at 1:1 (Fig. 3B), other CGTase showed same result (Liu *et al*., 2013b). Reaction time (24 h) and substrate ratio (1:1) was selected from the beginning of the experiment depending on previous studies.

Fig. 3. Influence of reaction time (A) and substrates ratio (B) on biosynthesis of AA-2G. (A) AA (0.016 g/mL) and maltodextrin (0.016 g/mL) incubated at 37°C with 75 U/mL of CGTase from *Bacillus* sp. SK13.002 in 0.1 M sodium phosphate buffer (pH 8) for different times. (B) Different substrate ratios (maltodextrin/AA), 1/5 (0.013 g/0.067 g), 2/4 (0.027 g/0.053 g), 3/3 (0.04 g/0.04 g), 4/2 (0.053 g/0.027 g), and 5/1 (0.067 g/0.013 g) in 2.5 mL 0.1 M sodium phosphate buffer (pH 8) incubated at 37°C with 75 U/mL of CGTase from *Bacillus* sp. SK13.002 for 24 h. Data represent the means of two experiments and error bars represent the standard deviation.



Effect of enzyme concentration on AA-2G production: The effect of enzyme concentration on AA-2G production was investigated by varying the enzyme concentration from 20 U/mL to 110 U/mL and 1:1 substrate ratio for 24 h (Fig. 4). AA-2G production increased with increasing the enzyme concentrations up to 80 U/mL. However, above 80 U/mL, AA-2G production was decreased, same trend was observed for other CGTase (Zhang *et al*., 2011). At optimum conditions, AA-2G production by CGTase from *Bacillus* sp. SK13.002 reached 6.3 g/L, CGTase from *Paenibacillus* sp. produced 3 g AA-2G/L using dextrin as glycosyl donor (Jun *et al*., 2001), mutant CGTase produced 1.12 g AA-2G/L using maltose as glycosyl donor (Liu *et al*., 2013b). On the other hand, in three separate studies, one study of site engineering mutagenesis and two studies of site engineering saturation studies, maltodextrin specificity of CGTase from *Paenibacillus macerans* was improved, AA-2G production were 1.2, 1.92, 1.97 and 2.23 g/L for wild and mutant CGTases respectively (Han *et al*., 2013a,b; Liu *et al*., 2013a). Our result (6.3 g AA-2G/L) is 2.83-fold of last result (2.23 g AA-2G/L) that produced by mutant CGTase from *Paenibacillus macerans.* The results suggested that CGTase from *Bacillus* sp. SK13.002 is an effective producer of AA-2G.

Fig. 4. Effect of different concentrations of CGTase from *Bacillus* sp. SK13.002 on biosynthesis of AA-2G from maltodextrin (0.016 g/mL) and AA (0.016 g/mL) in 0.1 M sodium phosphate buffer (pH 8) incubated at 37°C for 24 h. Data represent the means of two experiments and error bars represent the standard deviation.



Conclusion

CGTase from *Bacillus* sp. SK13.002 used maltodextrin as best glycosyl donor for AA-2G biosynthesis. The reaction optimization results was pH 8 at 37°C for 24 h with 1:1 maltodextrin to L-ascorbic acid ratio and 80 U/mL of Cyclodextrin glucanotransferase at which AA-2G production was 6.3 g/L indicated that CGTase from *Bacillus* sp. SK13.002 is an effective producer of AA-2G compared to previous studies.

Acknowledgements

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