J. Microbiol. Biotechnol. (2007), **17**(9), 1550–1553

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Functional Characteristics of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. BL-31 Highly Specific for Intermolecular Transglycosylation of Bioflavonoids

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Received: January 10, 2007 Accepted: April 23, 2007

Abstract The functional characteristics of a β-cyclodextrin glucanotransferase (CGTase) excreted from alkalophilic *Bacillus* sp. BL-31 that is highly specific for the intermolecular transglycosylation of bioflavonoids were investigated. The new β-CGTase showed high specificities for glycosyl acceptor bioflavonoids, including naringin, rutin, and hesperidin, and especially naringin. The transglycosylation of naringin into glycosyl naringin was then carried out under the conditions of 80 units of CGTase per gram of maltodextrin, 5 g/l of naringin, 25 g/l of maltodextrin, and 1 mM Mn^{2+} ion at 40°C for 6 h, resulting in a high conversion yield of 92.1%.

Keywords: Cyclodextrin glucanotransferase, alkalophilic *Bacillus* sp. BL-31, intermolecular transglycosylation, bioflavonoids, glycosyl naringin

Bioflavonoids are a group of natural substances containing various phenolic structures and are found in fruit, vegetables, and green leaves. Rutin, hesperidin, and naringin are the most representative bioflavonoids, and widely used as food additives, antioxidants, and skin protectants from ultraviolet radiation. Bioflavonoids also have other useful properties, such as anticarcinogenic, cholesterol lowering, and free radical scavenging activities [2, 3, 18, 20]. However, they do not dissolve well in water and have a strong bitter taste, limiting their use in the food and pharmaceutical industries.

To improve the physicochemical properties of bioflavonoids, the intermolecular transglycosylation of bioflavonoids using a few starch-related enzymes has drawn much attention [6, 8–10, 13, 15]. Cyclodextrin glucanotransferase (CGTase, E.C. 2.4.1.19), which catalyzes the coupling and disproportionation reactions that transfer glycosyl residues from starch or cyclodextrin (CD) to acceptor molecule bioflavonoids, have been the most commonly used. Furthermore, since the solubility of bioflavonoids [5, 9] in water increases significantly with an alkaline pH rather than a neutral or acidic pH, a new type of CGTase needs to be developed that exhibits a high transglycosylation activity and stability at alkaline pHs.

The transglycosylation of various bioflavonoids, including naringin and neohesperidin, was carried out by Kometani *et al.* [8–10] using a CGTase from alkalophilic *Bacillus* sp. A2-5a. The transglycosylation of bioflavonoids, such as hesperidin, naringin, and rutin, using a commercial CGTase from *Bacillus macerans* was conducted in previous work by the current authors [14, 19]. However, the transglycosylation yield remained at a lower level. A new thermophilic bacterium, *Geobacillus thermosacchalytycus*, excreting an α-CGTase showing a high coupling activity for the transglycosylation of glucosides has also been screened [12].

In this work, alkalophilic *Bacillus* sp. BL-31 excreting a β-CGTase showing high specificity for the intermolecular transglycosylation of bioflavonoids was newly screened from soil. The β-CGTase was purified by ultrafiltration and β-CD polymer affinity chromatography to near homogeneity. The bioflavonoid specificity of the purified β-CGTase was then investigated with naringin, rutin, and hesperidin. The reaction conditions for the transglycosylation of naringin were determined, including the enzyme amount, substrate concentration, mixing ratio of the glycosyl donor and acceptor, and metal ion additive. The transglycosylation reaction of naringin was then carried out using the new β-CGTase from alkalophilic *Bacillus* sp. BL-31.

Bacterial strains excreting CGTase were selected on an alkaline Horikoshi II medium [17] after incubation at 37°C for 48 h. The transglycosylation yield of naringin was analyzed by TLC using ethyl acetate/acetic acid/water (3:1:1, v/v/v) as the solvent, and then further analyzed by HPLC. A new strain, BL-31, excreting a CGTase with high transglycosylation specificity for naringin was finally selected

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from soil, and classified according to the partial 16S rDNA sequence.

The new strain *Bacillus* sp. BL-31 was cultivated for the excretive production of CGTase in a liquid medium composed of 10 g/l of soluble starch, 5 g/l of polypeptone, 5 g/l of yeast extract, 1 g/l of K_2HPO_4, and 0.2 g/l of $MgSO_4 \cdot 7H_2O$ containing 1% (w/v) Na_2CO_3 (autoclaved separately) at 37°C, pH 10.0, for 48 h. The excreted β-CGTase was then purified by a two-step procedure: ultrafiltration and β-CD polymer affinity chromatographies, as described in our previous work [11]. The starch hydrolyzing activity was measured using Kitahata's method [7], and one unit of activity defined as the amount of CGTase corresponding to a 1% increase in transmittance at 660 nm per min.

Hesperidin, naringin, and rutin (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used as glycosyl acceptors, whereas glucose, maltose, maltotriose (Sigma Chemical Co.), α-, β-, and γ-CDs (CycloLab, Ltd., Budapest, Hungary), dextrin, maltodextrin, corn starch, potato starch, and soluble starch (Sigma Chemical Co.) were used as glycosyl donors. The intermolecular transglycosylation reaction of bioflavonoids into glycosyl-flavonoids was carried out using 80 units of CGTase per gram of maltodextrin, 5 g/l of bioflavonoids as the glycosyl acceptor, and 25 g/l of carbohydrates as the glycosyl donor in a 50 mM Tris-HCl buffer (pH 8.5) at 40°C and 200 rpm for 12 h.

The bioflavonoid and transglycosyl-bioflavonoid amounts were measured using an HPLC (Gilson Co., France) equipped with an Inertsil ODS-2 column (GL Science Inc., Tokyo, Japan) under a mobile phase of acetonitrile/water (20/80), flow rate of 0.8 ml/min, 40°C, and UV detector at 280 nm. The conversion yield of naringin into glycosyl naringin was then calculated as the conversion yield (%)= [pure glycosyl naringin produced]/[initially added naringin] ×100.

The glycosyl naringin in the reaction mixture was digested by the amyloglucosidase (E.C. 3.2.1.3., Sigma Chemical Co.) from *Rhizopus* at 55°C for 6 h to obtain a pure form of glycosyl naringin. The molecular weight of the glycosyl naringin was measured by a LC/ESI-MS analysis using an API 2000 System (Applied Biosystems, Foster City, CA, U.S.A.) in a negative mode and scanned from m/z 100 to 1,800 within an acquisition time of 2.7 s at 4.5 kV.

Table 1. Bioflavonoid specificity of β-CGTase from alkalophilic *Bacillus* sp. BL-31.

Bioflavonoid	Conversion yield (%)
Rutin	70.8
Hesperidin	71.2
Naringin	76.3

Reaction conditions: 1 g/l of each glycosyl acceptor, 5 g/l of soluble starch, 50 units of CGTase per gram of soluble starch at 40°C, pH 8.5, and 200 rpm for 12 h.

The isolate was a Gram-positive rod-shaped bacterium, and revealed a high 16S rDNA homology with strains that belonged to the genus *Bacillus*, and particularly high homology with *Bacillus agaradhaerens* GSP78 (AY553094). The strain was then named *Bacillus* sp. BL-31, and deposited as a new strain under the accession number KACC91294P.

The β-CGTase excreted from the new alkalophilic *Bacillus* sp. BL-31 was purified by ultrafiltration and β-CD polymer affinity chromatographies [11], achieving a 17.8-fold purification with an overall recovery yield of 21.7%. The molecular mass was 92 kDa, a relatively high value compared with other known CGTases, except for the β-CGTase from *B. agaradhaerens* LS-3C at 110 kDa [16]. The optimum pH for the purified β-CGTase was an alkaline pH of 9.0, similar to those of other known alkalophilic β-CGTases [4, 16]. The thermal stability increased substantially in the presence of 1 mM Ca^{2+} ion and 1% (w/v) soluble starch, similar to other work [16].

The specificities for glycosyl acceptor bioflavonoids, such as rutin, hesperidin, and naringin, were compared using

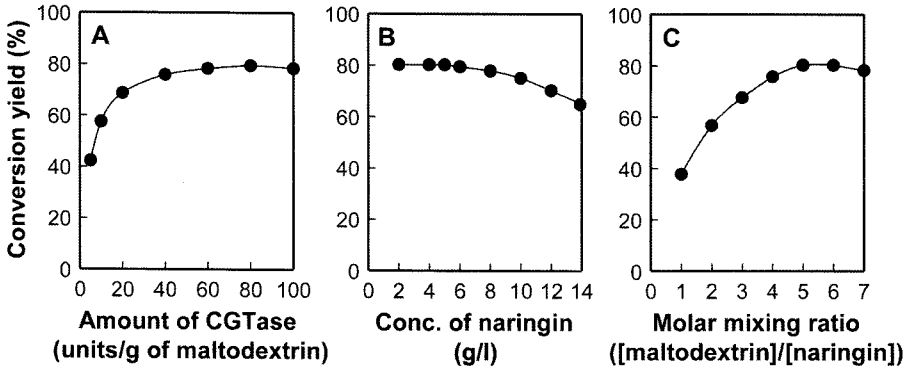

Fig. 1. Effect of reaction conditions on intermolecular transglycosylation of naringin into glycosyl naringin.
The enzyme reaction was carried out in a 50 mM Tris-HCl buffer (pH 8.5) at 40°C and 200 rpm for 12 h. **A**. Amount of β-CGTase. **B**. Concentration of naringin. **C**. Molar mixing ratio between maltodextrin and 5 g/l of naringin.

soluble starch as the glycosyl donor. The transglycosylation yields of rutin, hesperidin, and naringin after 12 h were measured as 70.8%, 71.2%, and 76.3%, respectively, as shown in Table 1. Oligomeric and polymeric saccharides, such as maltodextrin, soluble starch, and potato starch, showed better results compared with low molecular weight carbohydrates, such as glucose, maltose, maltotriose, α-, β-, and γ-CDs, and dextrin. The highest conversion yield of 78.2% was obtained when maltodextrin was used as the intermolecular glycosyl donor for naringin.

The conversion yield increased as the amount of CGTase increased up to 80 units per gram of naringin (Fig. 1A), whereas an excess amount of CGTase seemed to produce several undesirable side reactions, such as the formation of an excess amount of intermediate compound CDs and the disproportionation of the formed glycosyl naringin. The effect of the amount of naringin and the mixing ratio between naringin and maltodextrin were also investigated, as shown in Figs. 1B and 1C. A conversion yield of 80.2% was achieved with the following reaction conditions: 5 g/l of naringin, 25 g/l of maltodextrin, and a mixing ratio of 5 between the glycosyl donor and acceptor. Furthermore, most of the divalent metal ions known as cofactors of CGTase showed positive effects on the transglycosylation of naringin. In particular, the Mn^{2+} ion significantly enhanced the conversion yield from 80.2% up to 92.1%.

Fig. 2 illustrates the progression of the intermolecular transglycosylation reaction of naringin into glycosyl naringin when using the β-CGTase from alkalophilic *Bacillus* sp. BL-31. The transglycosylation reaction was mostly completed

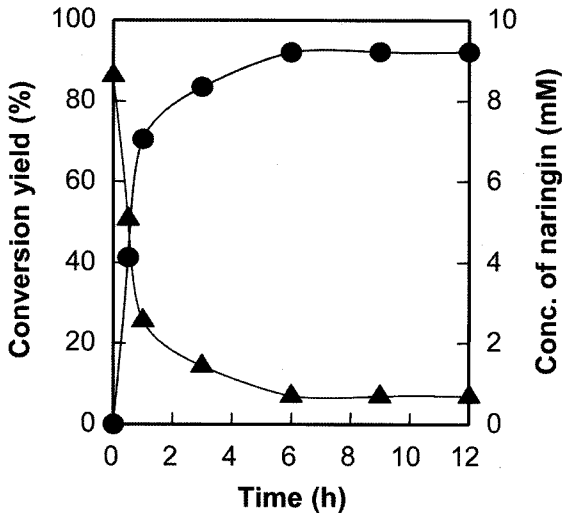

Fig. 2. Production of glycosyl naringin using β-CGTase from alkalophilic *Bacillus* sp. BL-31.
The transglycosylation reaction was carried out in a 50 mM Tris-HCl buffer (pH 8.5) containing 80 units of CGTase per gram of starch, 5 g/l of naringin, 25 g/l of maltodextrin, and 1 mM $MnCl_2$ at 40°C for 12 h. ●: conversion yield of naringin into glycosyl naringin, ▲: concentration of unreacted naringin.

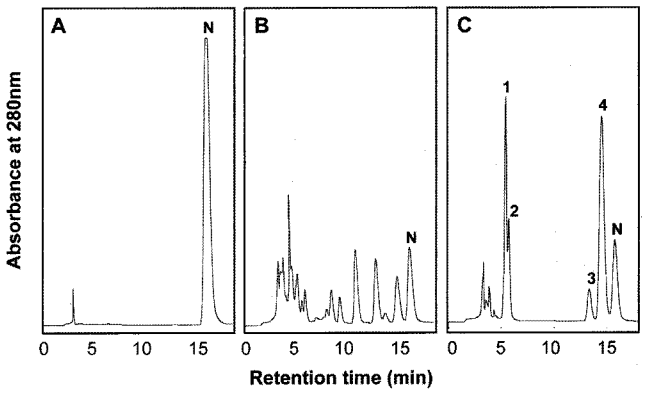

Fig. 3. HPLC chromatogram of the reaction mixture after transglycosylation of naringin using β-CGTase from alkalophilic *Bacillus* sp. BL-31.
A. Before reaction; **B**. After 6 h; and **C**. 6 h mixture after amyloglucosidase treatment. Peak 1: diglycosyl naringin; peaks 2, 3, and 4: monoglycosyl naringin; peak N: naringin or unreacted naringin.

after 6 h, and the final conversion yield was 92.1%, which is exceptionally high when compared with the 40% for a known CGTase for naringin excreted from alkalophilic *Bacillus* sp. A2-5a [9].

The final reaction mixture after 6 h was analyzed using HPLC, and the naringin and putative mono-, di-, and oligoglycosyl naringin profiles are illustrated in Figs. 3A and 3B. The reaction mixture was further treated with amyloglucosidase to hydrolyze the oligoglycosyl naringin into a monoglycosyl naringin, resulting in four peaks (1, 2, 3, and 4) in addition to the unreacted residual naringin peak N (Fig. 3C). The molecular weights of each glycosyl naringin were measured by LC/ESI-MS, plus one diglycosyl naringin (peak 1) and three monoglycosyl naringins (peaks 2, 3, and 4) were identified (data not shown).

In a previous structural analysis of enzymatically modified naringin, Akiyama *et al.* [1] reported that peak 1 represents the glycosylation of both the hydroxyl group of the glucose moiety and the phenolic group in naringin, whereas peak 2 represents the glycosylation of the phenolic group, and peaks 3 and 4 represent the glycosylation of the hydroxyl group of the glucose moiety. Thus, the strong appearance of peaks 1 and 2 for the newly isolated β-CGTase from alkalophilic *Bacillus* sp. BL-31 (Fig. 3C) indicated that the high transglycosylation yield of naringin seemed to stem from the additional positional specificity of the phenolic group. However, the structural feature related to the specific position of the glucose moiety linkage in naringin also needs to be further elucidated.

Acknowledgments

This work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Korean Ministry

of Science & Technology (Grant MG 05-0301-5-0), and a grant from the Korea Health 21 R&D Project, Korean Ministry of Health & Welfare (0405-VN05-0702-0004).

REFERENCES

1. Akiyama, T., M. Yamada, T. Yamada, and T. Maitani. 2000. Naringin glycosides α-glucosylated on ring B found in the natural food additive, enzymatically modified naringin. *Biosci. Biotechnol. Biochem.* **64:** 2246–2249.
2. Bok, S. H., S. H. Lee, Y. B. Park, K. H. Bae, K. H. Son, T. S. Jeong, and M. S. Choi. 1999. Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: Cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J. Nutr.* **129:** 1182–1185.
3. Chen, Y. T., R. L. Zheng, Z. J. Jia, and Y. Ju. 1990. Flavonoids as superoxide scavengers and antioxidants. *Free Radic. Biol. Med.* **9:** 19–21.
4. Gawande, B. N. and A. Y. Patkar. 2001. Purification and properties of a novel raw starch degrading-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* AS-22. *Enzyme Microb. Technol.* **28:** 735–743.
5. Kang, S., S. Lee, C. Kwon, and S. Jung. 2006. Solubility enhancement of flavonoids by cyclosophoraose isolated from *Rhizobium meliloti* 2011. *J. Microbiol. Biotechnol.* **16:** 791–794.
6. Kim, J. H., B. G. Kim, J. A. Kim, Y. H. Park, Y. J. Lee, Y. H. Lim, and J. H. Ahn. 2007. Glycosylation of flavonoids with *E. coli* expressing glycosyltransferase from *Xanthomonas campestris*. *J. Microbiol. Biotechnol.* **17:** 539–542.
7. Kitahata, S. and S. Okada. 1974. Action of cyclodextrin glycosyltransferase from *Bacillus megaterium* strain No. 5 on starch. *Agric. Biol. Chem.* **38:** 2413–2417.
8. Kometani, T., T. Nishimura, T. Nakae, H. Takii, and S. Okada. 1996. Synthesis of neohesperidin glycosides and naringin glycosides by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species. *Biosci. Biotechnol. Biochem.* **60:** 645–649.
9. Kometani, T., Y. Terada, T. Nishimura, H. Takii, and S. Okada. 1994. Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species and transglycosylation at alkaline pHs. *Biosci. Biotechnol. Biochem.* **58:** 517–520.
10. Kometani, T., Y. Terada, T. Nishimura, H. Takii, and S. Okada. 1994. Transglycosylation to hesperidin by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species in alkaline pH and properties of hesperidin glycosides. *Biosci. Biotechnol. Biochem.* **58:** 1990–1994.
11. Lee, K. W., H. D. Shin, and Y. H. Lee. 2003. Catalytic function and affinity purification of site-directed mutant β-cyclodextrin glucanotransferase from alkalophilic *Bacillus firmus* var. *alkalophilus*. *J. Mol. Catal. B Enzym.* **26:** 157–165.
12. Lee, M. S., H. D. Shin, T. K. Kim, and Y. H. Lee. 2004. Purification of α-cyclodextrin glucanotransferase excreted from thermophilic *Geobacillus thermosacchalytycus* and characterization of transglycosylation reaction of glucosides. *Kor. J. Microbiol. Biotechnol.* **32:** 29–36.
13. Lee, S. J., J. C. Kim, M. J. Kim, M. Kitaoka, C. S. Park, S. Y. Lee, M. J. Ra, T. W. Moon, J. F. Robyt, and K. H. Park. 1999. Transglycosylation of naringin by *Bacillus stearothermophilus* maltogenic amylase to give glycosylated naringin. *J. Agric. Food Chem.* **47:** 3669–3674.
14. Lee, Y. H. and D. C. Park. 1999. Novel heterogeneous carbohydrase reaction systems for the direct conversion of insoluble carbohydrates: Reaction characteristics and their applications. *J. Microbiol. Biotechnol.* **9:** 1–8.
15. Lee, Y. J., B. G. Kim, Y. H. Park, Y. H. Lim, H. G. Hur, and J. H. Ahn. 2006. Biotransformation of flavonoids with *O*-methyltransferase from *Bacillus cereus*. *J. Microbiol. Biotechnol.* **16:** 1090–1096.
16. Martins, R. F. and R. Hatti-Kaul. 2002. A new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* isolate: Purification and characterization. *Enzyme Microb. Technol.* **30:** 116–124.
17. Nakamura, N. and K. Horikoshi. 1976. Characterization and some cultural conditions of a cyclodextrin glycosyltransferase-producing alkalophilic *Bacillus* sp. *Agric. Biol. Chem.* **40:** 753–757.
18. Nijveldt, R. J., E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren, and P. A. van Leeuwen. 2001. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74:** 418–425.
19. Park, D. C., T. K. Kim, and Y. H. Lee. 1998. Characteristics of transglycosylation reaction of cyclodextrin glucanotransferase in the heterogeneous enzyme reaction system using extrusion starch as a glucosyl donor. *Enzyme Microb. Technol.* **22:** 217–222.
20. Ryu, J. Y. and H. G. Hur. 2005. Comparative analyses of flavonoids for *nod* gene induction in *Bradyrhizobium japonicum* USDA110. *J. Microbiol. Biotechnol.* **15:** 1280–1285.