J Incl Phenom Macrocycl Chem (2007) 57:39–46
DOI 10.1007/s10847-006-9163-5

ORIGINAL ARTICLE

Production of 2-*O*-α-glucopyranosyl L-ascorbic acid from ascorbic acid and β-cyclodextrin using immobilized cyclodextrin glycosyltransferase

Manchumas Hengsakul Prousoontorn · Supranee Pantatan

Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 18 January 2007

Abstract Cyclodextrin glycosyltransferase (CGTase) isolated and purified from *Paenibacillus* sp. A11 was immobilized on various carriers by covalent linkage using bifunctional agent glutaraldehyde. Among tested carriers, alumina proved to be the best carrier for immobilization. The effects of several parameters on the activation of the support and on the immobilization of enzyme were optimized. The best preparation of immobilized CGTase retained 31.2% of its original activity. After immobilization, the enzymatic properties were investigated and compared with those of the free enzyme. The optimum pH of the immobilized CGTase was shifted from 6.0 to 7.0 whereas optimum temperature remained unaltered (60°C). Free and immobilized CGTase showed similar pH stability profile but the thermal stability of the immobilized CGTase was 20% higher. Kinetic data (K_M and V_{max}) for the free and immobilized enzymes were determined from the rate of β-CD formation and it was found that the immobilized form had higher K_M and lower V_{max}. The immobilized CGTase also exhibited higher stability when stored at both 4°C and 25°C for 2 months. The enzyme immobilized on alumina was further used in a batch production of 2-*O*-α-glucopyranosyl-L-ascorbic acid (AA-2G) from ascorbic acid and β-cyclodextrin. The yield of AA-2G was 2.92% and the immobilized CGTase retained its activity up to 74.4% of the initial catalytic activity after being used for 3 cycles. The immobilized CGTase would have a promising application in the production of various transglycosylated compounds and in the production of cyclodextrin by the hydrolysis of starch.

Keywords Alumina · Covalent immobilization · Cyclodextrin glycosyltransferase · 2-*O*-α-D-glucopyranosyl L-ascorbic acid · *Paenibacillus* sp. · Transglycosylation

Abbreviations

AA	Ascorbic acid
AA-2G	2-*O*-α-glucopyranosyl-L-ascorbic acid
AANa	L-Ascorbic acid sodium salt
APTS	Aminopropyltriethoxysilane
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
GA	Glutaraldehyde

Introduction

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) is one of the considerable important amylolytic enzymes with potential applications in the production of cyclodextrins (CDs), α-1,4-linked cyclic oligosaccharides. CGTase produces CDs from starch and related α-1,4-glucans via an intramolecular transglycosylation reaction known as cyclization [1]. These cyclic products have an ability to form inclusion complexes with various compounds resulted in changes of their physical and chemical properties which make CDs having numerous applications in food, pharmaceutical, chemical and cosmetic industries [2]. In the

M. H. Prousoontorn (✉) · S. Pantatan
Department of Biochemistry, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
e-mail: manchumas.h@chula.ac.th

presence of suitable acceptors, CGTase also catalyzes other transglycosylation reactions: coupling (opening of the CD ring and transfer a linear oligosaccharide to an acceptor) and disproportionation (transfering a linear oligosaccharide to another oligosaccharide chain) [1]. Therefore, besides its application to produce CDs, CGTase has recently been widely used for the modification and development of new functional properties of various substances such as sugars, flavanoids, vitamins and natural sweet glycosides through its transglycosylation action [3, 4].

There is an increasing interest in the utilization of the transglycosylase activity of CGTase for the purpose of improving the stability of vitamin C or ascorbic acid (AA). AA is a well-known antioxidant which is commonly used in various industries to maintain organoleptic quality and protect other components from oxidation. It also has been shown to have a beneficial role against skin aging [5]. However, its instability against oxidative environment limits their usage in such applications. Thus, a highly soluble vitamin C derivative, 2-*O*-α-glucopyranosyl-L-ascorbic acid (AA-2G), was synthesized from AA and glycosyl donor by the transglucosylation enzyme such as α-glucosidase [6–8] and CGTase [9, 10]. This AA-2G is markedly stable in vitro and exhibits vitamin C activity in vivo after enzymatic degradation to produce free AA by the action of mammalian α-glucosidase [8]. Many studies are currently carried out on the biochemical properties, nutritional values and clinical effects of AA-2G in order to replace AA in several usages [11–14]. However, to the best of our knowledge, there has been no report focusing on the mass production of AA-2G using immobilized CGTase. The immobilization of enzyme constitutes an interesting strategy to achieve stabilization, reusage and recovery of the enzyme. Different approaches have been applied for the immobilization of CGTases based on adsorption [15], entrapment [16] and covalent binding [17–20]. Among these, the covalent binding of enzymes seems to be the most attractive method due to its high operational stability.

The activities of the immobilized CGTase reported so far are still relatively low. In this work, we then investigated the immobilization of CGTase from *Paenibacillus* sp. A11 on various supports using a covalent coupling method and the performance of the immobilized enzyme was evaluated with regard to the production of AA-2G. Characterization of the immobilized enzyme for catalytic properties in comparison with the free enzyme was also investigated.

Experimental

Materials

The supports used for enzyme immobilization were: alumina (Sigma, USA), silica (BDH, England), activated carbon (Aldrich, USA) and chitosan, a gift from Dr. Rath Pichayankura. 3-Aminopropyltriethoxysilane (APTS), glutaraldehyde (25% aqueous solution, GA) β-cyclodextrin, L-ascorbic acid sodium salt (AANa) and soluble starch were obtained from Sigma, USA. AA-2G was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). All other chemicals were of analytical grade.

Bacteria and enzyme production

Paenibacillus sp. A11 with CGTase activity was isolated from South-East Asian soil [21] and used for the production of extracellular CGTase. The bacteria was cultured at 37°C for 72 h in Horikoshi's medium [22] containing 1.0% (w/v) rice starch, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.75% (w/v) Na_2CO_3 with initial pH of 10.2. The culture broth was centrifuged (1380*g*) for 30 min at 4°C and the cell-free supernatant with crude CGTase was partially purified by starch adsorption [21]. The enzyme was concentrated by ultrafiltration and dialyzed against 50 mM acetate buffer (pH 6.0) containing 10 mM $CaCl_2$. This partially purified enzyme (specific activity 119 U/mg protein) obtained was used as a source of enzyme for immobilization.

Enzyme immobilization

Several inorganic and organic carrier materials were tested to find a suitable support for the immobilization of CGTase. Immobilization was carried out according to the method described by Weetall [23]. One gram of inorganic support was activated through contact with 10 ml of 1–10% (v/v) aqueous solution of APTS for 3 h with gentle stirring at room temperature. The silanized carrier was washed thoroughly with distilled water, and further reacted with 10 ml of 0.1–2.5% (v/v) GA in 0.1 M phosphate buffer (pH 7.0) under mild agitation for 1 h at room temperature. After the GA-activated support was washed with distilled water, it was incubated with 5 ml of enzyme solution (2–20 U of CGTase per gram support) in 50 mM acetate buffer (pH 6.0) containing 10 mM $CaCl_2$ under mild agitation for 0–12 h at 4°C. The unbound CGTase was removed by centrifugation at 1380*g* for 10 min at 4°C. The immobilized enzyme was thoroughly washed with 1 M NaCl in 50 mM acetate buffer (pH 6.0)

containing 10 mM $CaCl_2$ and followed by several washes with the same buffer without NaCl. The covalently bound enzyme was stored at 4°C until further use.

Enzyme activity assay

CGTase activity was measured as β-CD forming activity by a modification of phenolphthalein method described by Abdel-Naby [17]. The CGTase solution or weight sample of immobilized enzyme was incubated with 1 ml of 4% (w/v) soluble starch solution in 50 mM phosphate buffer (pH 6.0) for 10 min at 60°C. The reaction was terminated by the addition of 3.5 ml of 40 mM NaOH solution, and 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 was added. After incubating at room temperature for 15 min, the decrease in absorbance at 550 nm caused by the inclusion complex of phenolphthalein with CD was measured. One unit of enzyme activity (U) is defined as the amount of the enzyme forming 1 mg of CD per minute under the assay conditions.

Protein determination

Protein content was determined by the method of Bradford [24] using bovine serum albumin as standard. The amount of immobilized enzyme was estimated by subtracting the amount of protein recovered in supernatant and washings after immobilization from the amount of protein added.

Properties of enzyme preparation

Optimum pH

The effect of pH on the free and immobilized CGTase activities was determined by assaying the preparations at different pH values (4.0–11.0) using: acetate buffer (50 mM, pH 4.0–5.5), phosphate buffer (50 mM, pH 6.0–7.5), Tris–HCl buffer (50 mM, pH 8.0–9.5) and glycine–NaOH buffer (50 mM, pH 10.0–11.0).

pH stability

The pH stability of the free and immobilized enzyme was examined by preincubating the enzyme samples at room temperature for 1 h at different pH values (4.0–11.0). The residual activity was assayed under standard assay conditions.

Effect of temperature

The effect of temperature on the activities of free and immobilized enzyme was investigated in 50 mM phosphate buffer (pH 6) at temperatures ranging from 30–90°C. The energy of activation (E_a) was determined from the data obtained by using Arrhenius equation plot: Slope = $-E_a/2.303R$, where R is the gas constant (1.98 kcal/mol/K).

Thermal stability

The thermal stability of the free and immobilized CGTase was performed by incubating the enzyme samples in 50 mM acetate buffer (pH 6.0) in the presence of 10 mM $CaCl_2$ at a designated temperature (30–90°C) for 20 min. The residual activity was measured under the standard conditions.

Kinetic parameters

The rate of β-CD formation was measured at starch concentration ranging from 0.05 to 0.5 mg/ml for the free CGTase and from 25 to 40 mg/ml for immobilized enzyme preparation. K_M and V_{max} values of the soluble and immobilized enzyme were determined from the Lineweaver–Burk plots.

Storage stability

The free and immobilized CGTase samples were stored in 50 mM acetate buffer (pH 6.0) with 10 mM $CaCl_2$ at 4°C and 25°C for 60 days. The remaining activity was measured every 5 days and compared with the value at time zero.

Discontinuous AA-2G production

The enzyme reaction was performed by the method of Tanaka et al. [9] with slight modifications. The soluble or immobilized CGTase samples (280 units) were incubated at 40°C with 4% (w/v) β-CD, 4% (w/v) AANa and 0.2% (w/v) thiourea in a total volume of 20 ml of 10 mM acetate buffer (pH 6.0) containing 10 mM $CaCl_2$. After 24-h incubation with continuous shaking in the dark, a 200 μl-aliquot was with drawn and mixed with 800 μl of 1.06% metaphosphoric acid to terminate the reaction. Immobilized enzyme was collected by centrifugation (1380g) for 10 min and the amount of AA-2G in the reaction mixture was further analyzed by HPLC.

The reusability of the immobilized CGTase for AA-2G production was studied in repeated batch experiments up to three times in Erlenmeyer flask. The reaction was carried out under the appropriate optimum conditions. At the end of each cycle the immobilized enzyme, which was separated and assayed

for AA-2G content by HPLC, was thoroughly washed with 50 mM acetate buffer (pH 6.0) containing 10 mM $CaCl_2$ before subjected to activity assay. After that the immobilized enzyme was resuspended in freshly prepared substrate to start a new cycle.

HPLC analysis

The determination of AA-2G content was carried out by HPLC (Hewlett PACKARD series 1050) equipped with UV detector set at 240 nm using a C_{18} column (4.6 × 250 mm, Phenomenex, USA). Samples were previously filtered through a 0.45 μm membrane (Whatman) before injection. Samples were eluted with 0.1 M KH_2PO_4–H_3PO_4 (pH 2.0) at a flow rate of 0.3 ml/min. The amount of AA-2G was calculated on the basis of its standard curve of peak area.

Results and discussion

Optimization of immobilization procedure

CGTase from *Paenibacillus* sp. A11 was covalently immobilized on solid support using APTS to activate the surface of the support which, after reaction with GA, is able to link the enzyme through the carbonyl derivative (Fig. 1). Four different types of supports, alumina, silica, activated carbon and chitosan, were tested to find a suitable support efficiently immobilize the enzyme (Table 1). The adsorption of CGTase on these supports was also investigated. Among these supports, alumina was chosen as the most appropriate support for the immobilization of CGTase because high immobilized activity was achieved through covalent binding and not physical adsorption. Hence, further work was continued using alumina as immobilization support. However, to obtain higher immobilization efficiencies, several parameters such as the concentration of APTS and GA, the contact time and the concentration of enzyme per support ratio have yet to be optimized. These parameters were studied by varying some of the conditions of standard immobilization process described in ''Experimental''.

The degree of functionalization of support surface is related to the amount of alkylamino groups incorporated during incubation of alumina with APTS. Figure 2A shows the influence of APTS concentration on immobilized CGTase activity on alumina. The results showed that it was enough to use low concentration of APTS (2% (v/v)) to achieve the highest immobilization yield on alumina.

The optimum GA concentration was found to be 1% (v/v), and the activity yield decreased with the enhancement of GA concentration (Fig. 2B). This finding can be explained by assuming that the increase in GA concentration creates more bonds per enzyme molecule and, consequently, may cause molecular structure deformation. Also, at higher GA concentration the probability for covalent modifications close to the active site increases leading to enzyme inactivation.

The effect of enzyme concentration on the efficiency of immobilization is shown in Fig. 2C. It was found that as the amount of CGTase added per gram of carrier increased, the activity of immobilized enzyme increased and reached equilibrium when 14 U/g carrier was added. The activity yield at this point was 32%. At the enzyme concentration per alumina ratios above 14 U/g support, the immobilized enzyme activity did not increase due to the reactive groups on the support appear to be saturated. Moreover, high

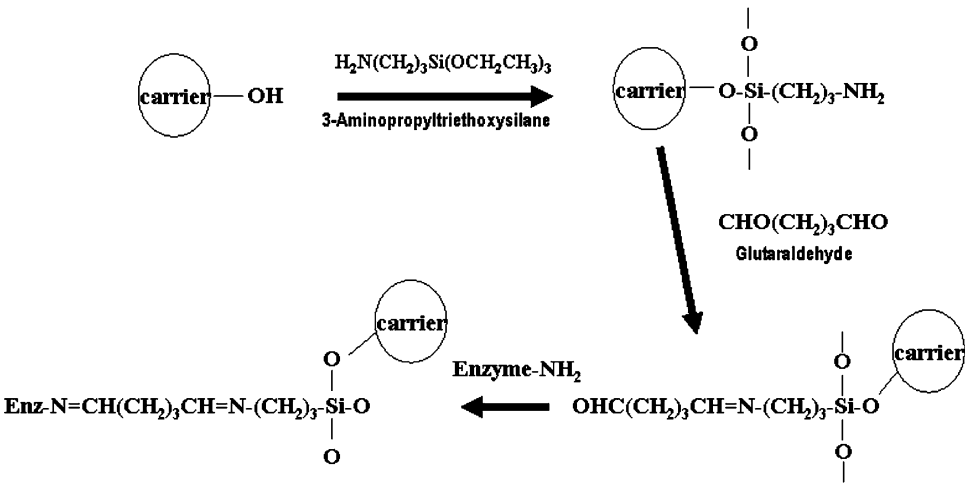

Fig. 1 Schematic diagram showing covalent attachment of CGTase onto inorganic carrier

Table 1 Immobilization of CGTase from *Paenibacillus* sp. A11 on alumina, silica, chitosan and activated carbon[a]

Support	Enzyme added (U/g carrier)	Immobilized activity (U/g carrier)		Activity yield[b] (%)	
		Adsorption	Covalent	Adsorption	Covalent
Alumina	10	0.04	1.65	0.4	16.5
Silica	10	2.06	1.56	20.6	15.6
Chitosan	10	0.38	0.61	3.8	6.1
Activated carbon	10	0.04	0.27	0.4	2.7

[a] Conditions: (APTS) = 2% (v/v); (GA) = 0.25% (v/v); 4°C; coupling time = 12 h

[b] The activity yield was expressed as the percentage of immobilized activity to added activity

enzyme loading on the support generally leads to low activity yield due to steric hindrance preventing access of the substrate to the active sites of the enzyme and/or diffusional effect of the substrate to the immobilized enzyme molecule. In subsequent studies, an initial enzyme concentration/support ratio of 14 U/g was used.

Influence of coupling time between CGTase and alumina is shown in Fig. 2D. It was found that the retaining activities of the immobilized enzymes were notably dependent on the immobilization time. The coupling time in the range of 2–12 h did not show much difference in the activity yield, except at 6 h-incubation which gave the highest immobilization yield. Therefore, in this study the incubation time of 6 h was selected in order to achieve high immobilized activity yield.

Properties of immobilized CGTase on alumina

After immobilization some properties of the CGTase immobilized on alumina were studied and compared with those of the free enzyme (Table 2). The optimum pH of the CGTase immobilized on alumina exhibited a shift of 1.0 pH unit towards the alkaline values as compared to the free enzyme (from 6.0 to 7.0). These effects might be dependent on proton partition around the enzyme active site. Other investigations described the displacement of the optimum pH to lower pH values when CGTase was immobilized on supports [17, 25].

The pH stability of free and immobilized CGTase was also examined. Both free and immobilized CGTase were stable over the range of pH 5.0–9.0 upon incubation at room temperature for 1 h and

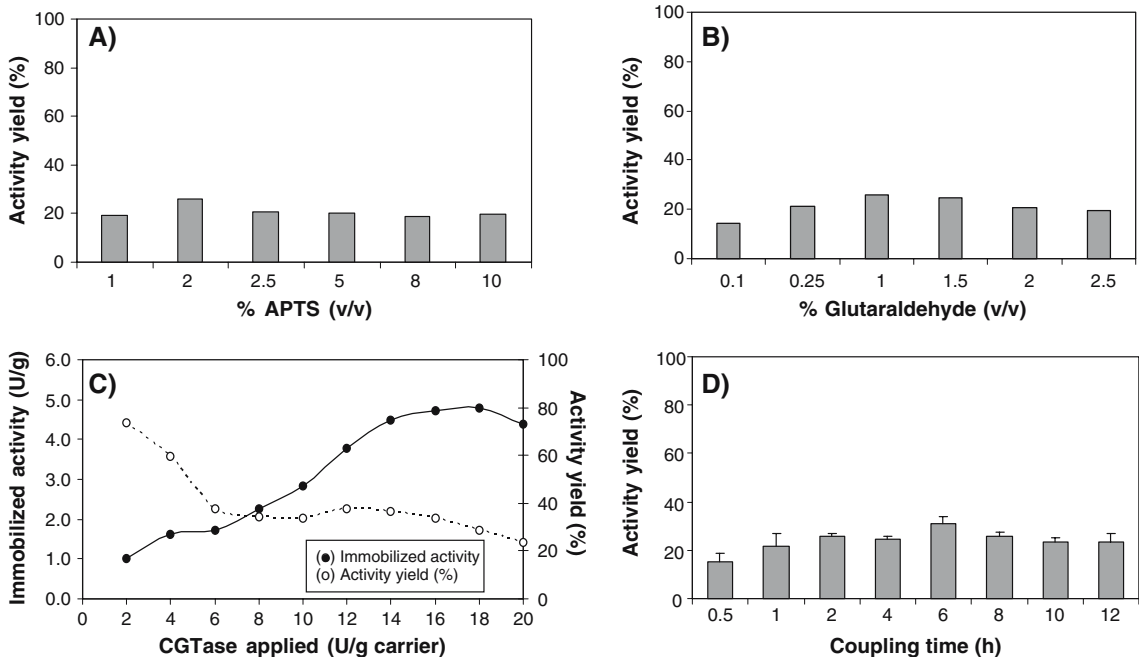

Fig. 2 Effects of (**A**) APTS, (**B**) GA, (**C**) enzyme and (**D**) coupling time on the immobilized CGTase activity on alumina. Various APTS (1–10%, v/v), GA (0.1–2.5%, v/v) and CGTase (2–20 units per gram support) concentrations were tested. The coupling time between the enzyme and the support was also examined during 2–12 h. The reactions were carried out at 4°C with gentle agitation. The activity yield was expressed as the percentage of immobilized activity to added activity

Table 2 Summary of some properties of the free and immobilized CGTase on alumina

Parameters	Free CGTase	Immobilized CGTase
pH optimum	6.0	7.0
pH stability (25°C 60 min)	5.0–9.0	5.0–9.0
Temperature optimum (°C)	60	60
Thermal stability (20 min) (°C)	≤40	≤50
Residual CGTase activity after 2 month storage at 4°C and 25°C (%)	80, 68	96, 79
K_M (mg/ml starch)	0.50 ± 0.25	5.62 ± 0.20
V_{max} (U/mg protein)	9.69 ± 1.70	5.82 ± 0.13
E_a (kcal/mol)	2.50	3.62

immobilization did not affect the pH stability of the enzyme. The wide range in pH stability of the immobilized enzyme offers a valuable characteristic for use in industrial application.

The optimum reaction temperature for CD-forming activity was found to be 60°C for both free and immobilized CGTase. The immobilization process with respect to the free enzyme introduced no major differences, only a slight protective effect of the immobilized enzyme was detected at temperature over 60°C.

The activation energy (E_a), determined form the Arrhenius plots at low temperature (30–60°C), increased from 2.50 kcal/mol for the free CGTase to 3.62 kcal/mol for the immobilized CGTase. The higher value of the activation energy obtained for the immobilized CGTase indicated that the applied immobilization process caused in the changes of the enzyme structure which impeded the enzyme catalyzed reaction. The increases in activation energy of other immobilized CGTase were also previously reported [16, 17].

The thermal stability of the immobilized enzyme was the important criteria with respect to commercial applications. The immobilized CGTase on alumina showed an increase in thermal stability when compared to the free enzyme. This characteristic depends on the source of enzyme and immobilization process applied [26]. An increase in thermal stability was also observed when CGTase immobilized on PVC [17] and Eupergit C [18].

For kinetic determination, the apparent K_M of the immobilized enzyme determined from Lineweaver–Burk plot was found to be 10 times higher than that of soluble enzyme. The large increase in apparent K_M value may be due to the conformational changes caused by enzyme fixation to the support and/or the limitation on substrate accessibility to the active site [25]. On the other hand, the V_{max} value of the immobilized CGTase was lower than that of the free form. A similar change was also observed in the immobilization of other CGTase [17].

The storage stability of the immobilized enzyme preparations is an important additional factor that determines their usefulness. Hence, the stability of the immobilized CGTase at 4°C and 25°C was studied. When alumina bound enzyme was stored at 4°C, it was found to be stable without any activity loss for more than 30 days and lost only 21% of its initial activity after 2 months. In comparison with the soluble enzyme, the stability of the immobilized enzyme increases about 20%. The result indicated that immobilization of CGTase on alumina increased the stability of enzyme preparation which could be due to the stiffening of the protein tertiary structure.

Batch production of AA-2G by immobilized CGTase

The production of AA-2G using immobilized CGTase was investigated to evaluate the potential application of immobilized enzyme. Enzymatic production of AA-2G by soluble and immobilized CGTase was determined at pH 5.0 and 40°C in the dark during 0–48 h using β-CD as a substrate (Fig. 3). The amount of AA-2G formed in the reaction mixture by both enzyme samples increased as the incubation time increased. The % conversion of AA-2G by soluble and immobilized CGTase was comparable to previous reports when β-CD was used as a glycosyl donor [10, 27]. The best glycosyl donors for AA-2G production by *Bacillus stearothermophilus* CGTase [27] and *Paenibacillus* sp. CGTase [10] were α-CD and dextrin, respectively. However, the production yield obtained by

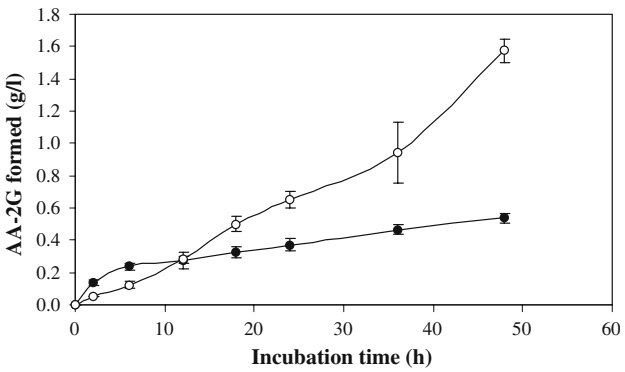

Fig. 3 Time course of AA-2G production with soluble (○) and immobilized CGTase (•). Reaction was carried out at 40°C in the reaction mixture containing 4% (w/v) of AANa, 4% (w/v) of β-CD and 280 units of CGTase. AA-2G was analyzed by HPLC with a C_{18} column

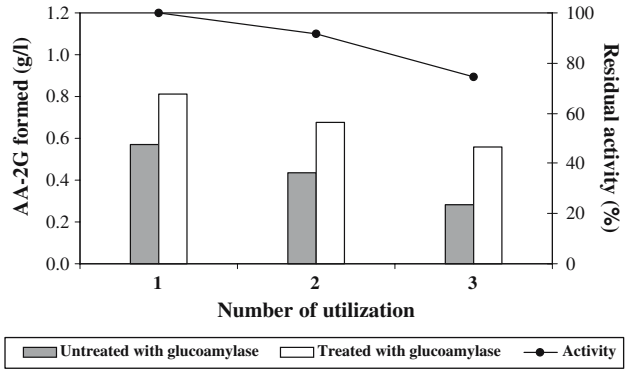

Fig. 4 Batch reusability of immobilized CGTase on alumina for AA-2G production under optimal condition. The immobilized CGTase on alumina (280 units) was incubated with the mixture containing 4% (w/v) of β-CD and 2% (w/v) AANa at pH 5.0 with shaking at 40°C for 24 h

immobilized enzyme was lower than that obtained by soluble CGTase (Fig. 3). This could be due to the adsorption of the compound in the reaction mixture onto the immobilized supports which may have blocked its active site. Thus, low product was observed. To test whether the compounds were adsorbed onto the immobilized carrier, the immobilized enzyme was then washed after the production of AA-2G. The content in the washing solution was then determined by HPLC and it was found that AA and AA-2G adsorbed on immobilized CGTase (data not shown).

In order to obtain optimum conversion parameters, the influence of AANa concentration (0.5–10%, W/V), the effect of pH (5.0, 5.5, 6.0) and temperature (30, 40, 50°C) were investigated over 48 h in shaking water-bath. The best condition yielding maximum amount of AA-2G formed by each parameter was then selected as the optimal condition for the production of AA-2G. The best working condition for the immobilized enzyme to produce AA-2G were to incubate 280 units of immobilized enzyme with 4% (w/v) of β-CD and 2% (w/v) of AANa at pH 5.0 and 30°C for 24 h. Under the optimum condition, the immobilized CGTase produced AA-2G with the yield of 2.92% (0.584 g/l) (Fig. 4). For mass production of AA-2G, glucoamylase was used to hydrolyze other transglycosylation products [27]. After treatment with glucoamylase (20 units/ml), the yield of AA-2G could be improved by 30–40% (Fig. 4).

One of the most important criteria for evaluating the possibility of a practical application of the immobilized enzyme is its reusability. The operational stability of the immobilized CGTase was then evaluated in a batch process (Fig. 4). The alumina bound CGTase produced AA-2G with 2.92% yield, with 74.4% of its original activity after three repeated uses of 24 h. It was found that the immobilized CGTase can be reused without significant loss of its activity.

The results revealed that the *Paenibacillus* sp. A11 CGTase immobilized on alumina exhibited a resistance against thermal denaturation, and has a promising operational stability for the production of AA-2G. CGTase is of great value in various industries not only for the production of CD, but also for the production of other glycosylated compounds, using other acceptors than AANa. This would widen the commercial developments of various compounds with some new functions.

Acknowledgments This work was supported by a grant from Starch and Cyclodextrin Unit Cell, Chulalongkorn University (Bangkok, Thailand). We are grateful to Dr. Rath Pichayankura for providing chitosan used in this study.

References

1. Tonkova, A.: Bacterial cyclodextrin glucanotransferase. Enzyme Microb. Technol. **22**, 678–686 (1998).
2. Szejtli, J.: Cyclodextrin Technology. Kluwer, Dordrecht (1988), Chapters 1 and 5.
3. Kometani, T., Nishimura, T., Nakae, T., Takii, H., Okada, S.: Synthesis of neohesperidin glycosides and naringin glycosides by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species. Biosci. Biotech. Biochem. **60**, 645–649 (1996).
4. Shibuya, T., Miwa, Y., Nakano, M., Yamauchi, T., Chaen, H., Sakai, S., Kurimoto, M.: Enzymatic synthesis of novel trisaccharide, glucosyl lactoside. Biosci. Biotech. Biochem. **57**, 56–60(1993).
5. Bae, H.K., Lee, S.B., Park, C.S., Shim, J.H., Lee, H.Y., Kim, M.J., Baek, J.S., Roh, H.J., Choi, J.H., Choe, E.O., Ahn, D.U., Park, K.H.: Modification of ascorbic acid using transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase to enhance its oxidative stability. J. Agric. Food Chem. **50**, 3309–3316 (2002).
6. Muto, N., Suga, S., Fuji, K., Goto, K., Yamamoto, I.: Formation of a stable ascorbic acid 2-glucoside by specific transglucosylation with rice seed α-glucosidase. Agric. Biol. Chem. **54**, 1697–1703 (1990).
7. Muto, N., Nakamura, T., Yamamoto, I.: Enzymatic formation of a nonreducing L-ascorbic acid α-glucoside: Purification and properties of a α-glucosidase catalyzing site-specific transglucosylation from rat small intestine. J. Biochem. **107**, 222–227 (1990).
8. Yamamoto, I., Muto, N., Nagata, E., Nakamura, T., Suzuki, Y.: Formation of a stable L-ascorbic acid α-glucosidase by mammalian α-glucosidase-catalyzed transglucosylation. Biochim. Biophys. Acta**1035**, 44–50 (1990).
9. Tanaka, M., Muto, N., Yamamoto, I.: Characterization of *Bacillus stearothermophilus* cyclodextrin glucanotransferase in ascorbic acid 2-*O*-α-glucoside formation. Biochim. Biophys. Acta. **1078**, 127–132 (1991).
10. Jun, H.K., Bae, K.M., Kim, S.K.: Production of 2-*O*-α-D-glucopyranosyl L-ascorbic acid using cyclodextrin glucanotransferase for *Paenibacillus* sp. Biotechnol. Lett. **23**, 1793–1797 (2001).
11. Kumano, Y., Sakamoto, T., Egawa, M., Tanaka, M., Yamamoto, I.: Enhancing effect of 2-*O*-α-glucopyranosyl L-

ascorbic acid, a stable ascorbic acid derivative, on collagen synthesis. Biol. Pharm. Bull. **21**, 662–666 (1998).
12. Wakamiya, H., Suzuki, E., Yamamoto, I., Akiba, M., Otsuka, M., Arakawa, N.: Vitamin C activity of 2-*O*-α-glucopyranosyl L-ascorbic acid in guinea pigs. J. Nutr. Sci. Vitaminol. **38**, 235–245 (1992).
13. Yamamoto, I., Muto N.: Bioavailability and biological activity of L-ascorbic acid 2-*O*-α-glucoside. In: Proceeding of the 1st International Congress on Vitamins and Biofactors in Life Science in Kobe, 1991. J. Nutr. Sci. Vitaminol. Special Issue, 161–164 (1992).
14. Yamamoto, I., Suga, S., Mitoh, Y., Tanaka, M., Muto, N.: Antiscorbutic activity of L-ascorbic acid 2-glucoside and its availability as a vitamin C supplement in normal rats and guinea pigs. J. Pharmacobio. Dyn. **13**, 688–695 (1990).
15. Kato, T., Horikoshi, K.: Immobilized cyclodextrin glucanotransferase of an alkalophilic *Bacillus* sp. No. 38-2. Biotechnol. Bioeng. **24**, 595–598 (1984).
16. Lee, S.H., Shin, H.D., Lee, Y.H.: Evaluation of the immobilization methods for cyclodextrin glucanotransferase and characterization of its enzymatic properties. J. Microbiol. Biotechnol. **1**, 54–62 (1991).
17. Abdel-Naby, M.: Immobilization of *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme. Process Biochem. **34**, 399–405 (1999).
18. Martin, M.T., Plou, F.J., Alcalde, M., Ballesteros, A.: Immobilization on Eupergit C of cyclodextrin glucosyltransferase (CGTase) and properties of the immobilized biocatalyst. J. Mol. Catal. B: Enzym. **21**, 299–308 (2003).
19. Choi, S.H., Kim, M.S., Ryoo, J.J., Lee, K.P., Shin, H.D., Kim, S.H., Lee, Y.H.: Immobilization of a cyclodextrin glucanotransferase (CGTase) onto polyethylene film with a carboxylic acid group and production of cyclodextrins from corn starch using CGTase immobilized PE film. J. Appl. Polym. Sci. **85**, 2451–2457 (2002).
20. Sobral, K.A., Rodrigues, R.O., Oliveira, R.D., deMoraes, F., Zanin, G.M.: Evaluation of supports and methods for immobilization of enzyme cyclodextrin glycosyl transferase. Appl. Biochem. Biotechnol. **105–108**, 809–819 (2003).
21. Pongsawasdi, P., Yagisawa, M.: Screening and identification of a cyclomaltodextrin glucanotransferase producing bacteria. J. Ferment. Technol. **65**, 463–467 (1987).
22. Horikoshi, K.: Production of alkaline enzymes by alkalophilic microorganisms. Agric. Biol. Chem. **35**, 1783–1791 (1971).
23. Weetal, H.H.: Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. Appl. Biochem. Biotechnol. **41**, 157–188 (1993).
24. Bradford, M.M.: A rapid and sensitive method for the quantitatively of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254 (1976).
25. Chibata, I.: Properties of immobilized enzymes and microbial cells III. In: Chibata, I. (ed.) Immobilized Enzymes, pp. 108–147. Halsted Press, New York (1978).
26. Yang, C.P., Su, C.S.: Study of cyclodextrin production using cyclodextrin glycosyltransferase immobilized on chitosan. J. Chem. Technol. Biotechnol. **46**, 283–294 (1989).
27. Aga, H., Yoneyama, M., Sakai, S., Yamamoto, I.: Synthesis of 2-*O*-α-Dglucopyranosyl L-ascorbic acid by cyclodextrin glucanotransferase from *Bacillus stearothermophilus*. Agric. Biol. Chem. **55**, 1751–1756 (1991).