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Synthesis of epicatechin glucosides by a β-cyclodextrin glycosyltransferase

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ABSTRACT

Enzymatic synthesis of (−)-epicatechin (EC) glucosides was performed through the transglucosylation reaction catalyzed by the cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. RB01. The enzyme showed the same product specificity for the three donor substrates, starch, β-cyclodextrin and maltoheptaose (G7). Using β-cyclodextrin as the glucosyl donor, several EC glucoside products were obtained at an overall minimal yield of 18.1%. The structures of the four main products were elucidated by MS and NMR techniques as (−)-EC-3′-*O*-α-D-glucopyranoside (EC3A), (−)-EC-3′-*O*-α-D-diglucopyranoside (EC3B), (−)-EC-3′-*O*-α-D-triglucopyranoside (EC3C) and (−)-EC-4′-*O*-α-D-glucopyranoside (EC4A). Of these, EC3A was the major product while EC4A, unique for this CGTase, was formed in the lowest amount. The water solubility and stability against UV irradiation of EC3A were significantly higher than that of EC. Although the antioxidant activity was 1.5-fold lower, the advantage of the enhanced solubility and stability makes the EC3A glucoside more beneficial as food ingredient than its parent EC.

1. Introduction

Catechins are polyhydroxylated flavonoids [1] that are found in various plant species, especially in the tea-plant *Camellia sinensis* [2], and in other plant derived human dietary components, such as fruits, vegetables, chocolate and red wine [3]. The green tea catechins are composed of six kinds of catechin and its derivatives: (+)-catechin, (+)-gallocatechin (GC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCg). The composition and level of catechins in each plant may vary considerably between cultivars and cultivation conditions. For example, the catechins in red wine, which are mainly catechin and EC, are found at a variable ratio and total amount depending on the grape cultivars [4].

EC (cis-3,3′,4′,5,7-pentahydroxyflavane, an epimer of catechin, Fig. 1), a strong antioxidant, has an insulin mimic action and improves heart health. It also reduces lipid peroxidation, inhibits platelet aggregation [5] and prevents gastric cancer [6]. Although EC, and indeed catechins in general, have potential health benefits and are being used widely in food and drinks, they are poorly soluble in water, have a bitter taste, and are easily oxidized and degraded by light irradiation [7]. Therefore, they have limitations upon their use as a natural food additive or medicine [8]. However, these problems are mostly overcome by modifying them with sugar molecules. Glucosylation of catechins, through enzyme catalysis using members of the glycosyltransferase, sucrose phosphorylase or glucansucrase families, has been reported [7,9–12]. (+)-Catechin 3′-*O*-α-D-glucopyranoside was synthesized via transglucosylation using the CGTase from *Bacillus macerans* (α-CGTase) [9]. While EGCg-4″-monoglucopyranoside, EGCg-7,4″-diglucopyranoside and EGCg-4′, 4″-di-*O*-α-D-glucopyranoside were all synthesized by transglucosylation using the glucansucrase from *Leuconostoc mesenteroid* [12]. The most common glucoside derivatives of catechins prepared by biotransformation reactions are the α-D-glucosides [9], while, in contrast, very few reports on the enzymatic synthesis of the β-D-glucosides exist, the most notable exception being by catalysis of enzymes from plant extracts, e.g. eucalyptus, barley and lentil [13,14]. Under these conditions the obtained structure and yield of the catechin glucosides were dependent on the type of enzyme and the conditions used in the transglucosylation reaction.

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) catalyzes the production of cyclodextrin from starch and related α-(1,4)-linked glucose polymers or oligomers via a transglycosylation

Abbreviations: β-CD, β-cyclodextrin; CGTase, cyclodextrin glycosyltransferase; DPPH, 2,2-diphenyl-1-picryhydrazyl; EC, (−)-epicatechin; EC3A, (−)-epicatechin-3′-*O*-α-D-glucopyranoside; EC3B, (−)-epicatechin-3′-*O*-α-D-diglucopyranoside; EC3C, (−)-epicatechin-3′-*O*-α-D-trigluco-pyranoside; EC4A, (−)-epicatechin-4′-*O*-α-D-glucopyranoside; EGCg, (−)-epigallocatechin gallate; IC, inhibitory concentration; Rt, retention time.

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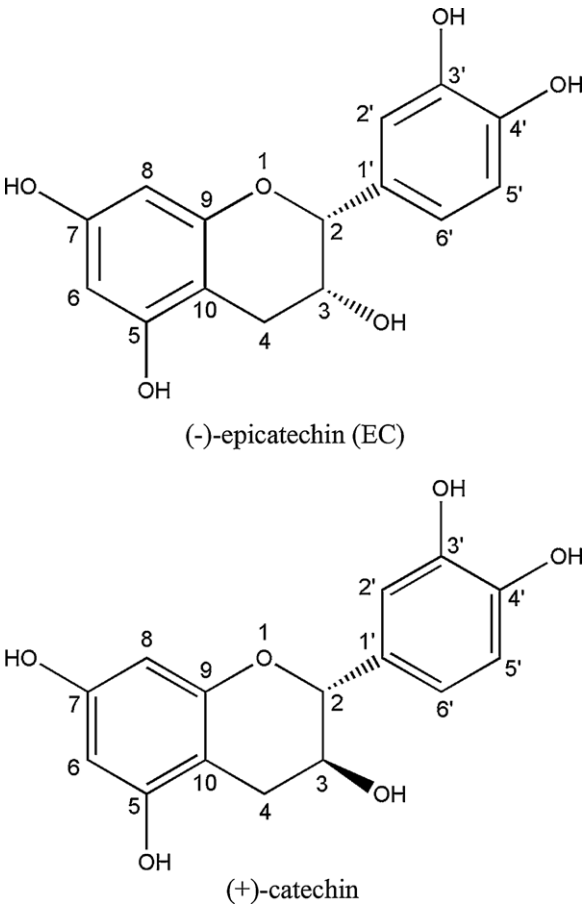

Fig. 1. Structures of (−)-epicatechin (EC) and (+)-catechin.

reaction. CGTase catalyzes four related reactions: cyclization, coupling, disproportionation and hydrolysis [15–18]. Several reports on the transglycosylation of interesting compounds to make useful glucosides by the action of CGTase are available. For example, the industrial production of glucosyl stevioside, a sweetener from a plant stevioside with a very bitter taste, is catalyzed by the CGTase from *Bacillus stearothermophilus* FERM-P No. 2222 [19]. In addition, the 3^G-α-D-glucopyranosyl neohesperidin, synthesized by transglucosylation with the CGTase from an alkalophilic *Bacillus* species, was found to be 1500-fold more soluble and 10-fold less bitter than its parent neohesperidin, a unique flavanoid of oranges [20].

The present work aims to use a β-CGTase, derived from *Paenibacillus* sp. RB01, which was isolated from a hot spring area in Thailand, to enzymatically synthesize EC glucosides, to obtain new product and to improve properties of EC that are of beneficial use for industrial applications. This is the first report on the synthesis of EC glucosides using the enzyme CGTase, as well as their characterization.

2. Materials and methods

2.1. Materials

(+)-Catechin, (−)-epicatechin (EC), β-cyclodextrin (β-CD) and maltoheptaose (G7) were purchased from Sigma. Soluble starch (potato), glucoamylase (*A. niger*), α-glucosidase (*S. cerevisiae*), Sephadex-LH-20 and 2,2-diphenyl-1-picryhydrazyl (DPPH) were obtained from Merck Co. All other chemicals used were commercially available and of analytical grade.

2.2. Enzyme preparation

Paenibacillus sp. RB01 screened from the hot-spring area in Ratchburi Province of Thailand [21] was grown in an enriched Medium I [18] at 37 °C for 18 h, before transferring into Horikoshi's medium [22] and further cultivation at 40 °C for 72 h. Cells were then removed by centrifugation (3000 × *g* for 30 min) and the culture broth, as a crude CGTase source, was partially purified by starch adsorption. The CGTase activity at each stage was determined by starch degrading assay [23].

2.3. Synthesis of EC glucosides

2.3.1. Donor specificity

Selection of the appropriate donor was performed by incubating 0.5% (w/v) EC and CGTase (30 U/ml) in 50 mM phosphate buffer (pH 6.0) with 1.8% (w/v) of the different glucosyl donors: starch, β-CD or G7, in a reaction volume of 1 ml at 40 °C for 24 h. The products were analyzed using TLC and HPLC resolution (see below) with reference to each type of catechins as standard.

2.4. Detection of products

2.4.1. Thin layer chromatography (TLC)

The products were analyzed by silica gel 60 (Merck, Co.) based TLC using a 3:1:1 (v/v) ratio of ethyl acetate: acetic acid: water as the mobile phase solvent. Spots were detected by spraying with 1:2 (v/v) ratio of sulfuric acid: methanol as reported [9], followed by heating at 120 °C for 20 min.

2.4.2. High performance liquid chromatography (HPLC)

Glucosylated products were detected using a C18-reverse phase HPLC column (Shimadzu, Japan; 5.0 μm; 4.6 mm × 250 mm) with 22% (v/v) methanol as the mobile (solvent) phase at a flow rate of 0.5 ml/min and at 35 °C. The eluted products were monitored by UV detection at 279 nm.

2.4.3. Determination of the transglucosylation efficiency

The efficiency of the transglucosylation reaction was judged by evaluating the transglucosylation product yield, determined from the HPLC profile by two methods. Firstly, the yield was calculated from the difference between the amount of EC at each time point and the initial amount added [24]. Secondly, the product yield was measured directly from the ratio of the peak area of each product relative to that of the initial EC concentration added [7].

2.5. Optimization of transglucosylation reaction

The optimum conditions for the synthesis of EC glucosides were considered in terms of obtaining the highest percentage yield of the transglucosylated products, as analyzed by HPLC. The effects of varying the pH (5.0–8.0), enzyme concentration (20–120 U/ml), EC concentration (0.06–2.0% (w/v)), β-CD concentration (0.125–1.8% (w/v)), temperature (30–70 °C) and incubation time (12–60 h) were investigated.

2.6. Larger scale preparation and isolation of glucosylated products

The synthesis of EC glucosides was then performed under the optimized conditions obtained above in a reaction volume of 50 ml. After completion, the reaction mixture was concentrated by rotary evaporation to ~5 ml and applied onto a Sephadex LH-20 column (2.5 cm × 55 cm) equilibrated with distilled water. The glucoside

products were separated from the sugars and enzyme by elution with distilled water (2 l, 0.5 ml/min). Thereafter, EC was then eluted by changing the mobile phase to 50% (v/v) methanol. The fractions containing the EC-glucosides were concentrated and further separated by HPLC. Each glucoside-containing peak was collected for further characterization.

2.7. Characterization of glucoside products

2.7.1. Enzyme treatment

Glucoamylase from *A. niger* was added to the reaction mixture (18.3 U/ml) after the CGTase catalyzed synthesis was finished, and incubated at 40 °C for 1 h. After that, to one half of this reaction mixture, α-glucosidase (18.3 U/ml) from *S. cerevisiae* was then added and further incubated at 40 °C for 3 h. The products of all three enzyme treatments (CGTase, CGTase – glucoamylase and CGTase – glucoamylase – α-glucosidase) were then analyzed by TLC.

2.7.2. Mass spectrometry

Mass analysis of the major glucoside products was performed on an Electrospray Ionization-MicroTOF Mass spectrometry (ESI-TOF MS).

2.7.3. Nuclear magnetic resonance

For structural elucidation, ^{1}H-, ^{13}C- and two-dimensional (homonuclear proton correlation COSY and heteronuclear HSQC and HMBC) NMR spectra were performed in CD_3OD on a Varian Gemini 400 MHz spectrometer. The chemical shifts in ppm were expressed with respect to tetramethylsilane as an internal reference.

2.8. Determination of the properties of EC glucosides

2.8.1. Solubility in water

Excess amounts of EC and EC glucosides were mixed with 200 μl of water, vortexed for 5 min at room temperature to disperse into solution, and then each sample was incubated at 30 °C for 15 min to allow the solvation equilibrium (saturation) to be attained. The samples were then filtered through a 0.45 μm membrane to remove insoluble particles and subjected to quantitative reverse phase C18 HPLC analysis in order to determine the soluble EC and EC-glucoside concentrations.

2.8.2. Browning resistance

Browning resistance was examined in an aqueous solution (1.0 ml) containing 0.1% (w/v) of either EC or the respective EC glucoside. The samples were then exposed to UV irradiation from a 30 W UV lamp (254 nm) at a distance of 35 cm for 10 h. Following this treatment, the absorbance at 460 nm of the solutions were then determined to evaluate the concentration of EC or EC glucoside remaining, and so from this its photostability, as a % of that remaining compared to that initially present, was evaluated.

2.8.3. Antioxidant activity

The antioxidant activity of EC and each EC glucoside was evaluated using the DPPH radical scavenging reaction [25]. Each of the samples was dissolved, or diluted, in the required volume of ethanol to yield the final EC or EC glucoside concentration range of 0–800 μM, then DPPH was added to a final of 100 μM. After 10 min in the dark, the absorbance of the mixture was measured at 517 nm. The DPPH radical scavenging activity was evaluated by analyzing the percentage decrease in the absorbance of the sample compared to the blank (ethanol). The IC_{50} value designates the inhibitory concentration at which the absorbance was reduced by 50%.

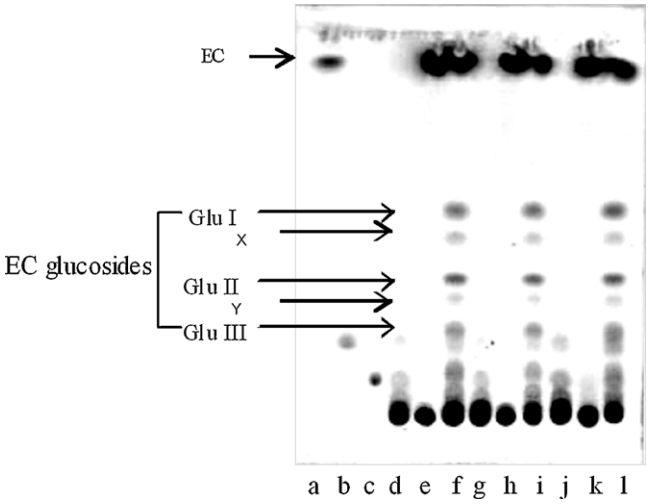

Fig. 2. TLC chromatogram of the reaction products catalyzed by CGTase between EC and starch, β-CD and G7 as donors. Standards of (a) EC, (b) glucose and (c) maltose compared to the (d) control (starch + CGTase) (e and f) starch as donor, 0 and 24 h incubation, (g) control (beta-CD + CGTase), (h and i) beta-CD as donor, 0 and 24 h incubation, (j) control (G7 + CGTase) and (k and l) G7 as donor, 0 and 24 h incubation.

3. Results and discussion

3.1. Donor specificity

The donor specificity of the CGTase from *Paenibacillus* sp. RB01 was evaluated using starch, β-CD and G7 as the glucosyl donors in the transglucosylation reaction with EC as the acceptor. The products were then resolved and analyzed by TLC (Fig. 2). All three different glucosyl donors gave the same three major products (Glu I, Glu II and Glu III in Fig. 2), as well as two minor products (X and Y in Fig. 2), which were not observed at the initial time of incubation. The products were likely to be glucoside derivatives with the relative intensities of Glu I > Glu II > Glu III > X > Y. The transglucosylation yield of the main products Glu I to Glu III was determined from the ratio of disappearance of the amount of EC acceptor to the initial amount, as analyzed by HPLC. The obtained yields were 22.6, 22.4 and 21.4% for starch, β-CD and G7 as donors, respectively. This suggests that these substrates were equally effective as a glucosyl donor for this CGTase from *Paenibacillus* sp. RB01. For all subsequent experiments, β-CD was accordingly chosen as the appropriate donor since it is the specific substrate of the CGTase.

The reaction products of EC and β-CD when catalyzed by the CGTase from *Paenibacillus* sp. RB01 were analyzed by HPLC (Fig. 3), revealing at least four EC glucoside products with a Rt of 27, 30, 45 and 48 min, respectively. Surprisingly, two of the main glucosides, with a Rt of 45 and 48 min, were eluted after the less polar EC at 38 min. In addition, the elution of the standard catechin, which appeared to be less polar than EC when resolved by either TLC (data not shown) or Sephadex-LH20 column chromatography (Fig. 4), had a Rt of 19 min in the HPLC peak. After 24 h of incubation, the catechin peak at Rt 19 min was increased due to epimerization from EC. The epimerization of EC to catechin was observed at an alkaline pH, high temperature and longer incubation time [26], and brings about a decrease in the EC substrate level and consequently a lower yield of EC glucosides. Small product peaks around Rt 15–23 min might be glucosides of catechin as by-products. Therefore, optimization of the reaction conditions for effective transglucosylation of EC is required.

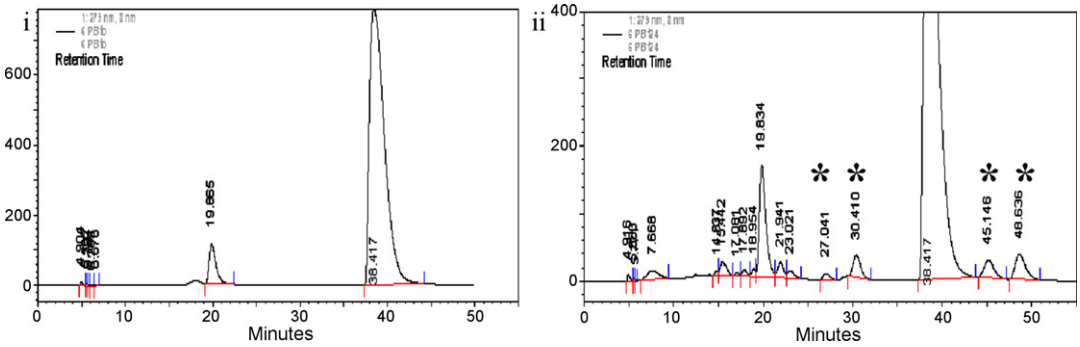

Fig. 3. HPLC chromatogram of the reaction products of EC and β-CD catalyzed by the CGTase from *Paenibacillus* sp. RB01 after (i) 0 and (ii) 24 h incubation. The asterix (*) marks the EC-glucosides.

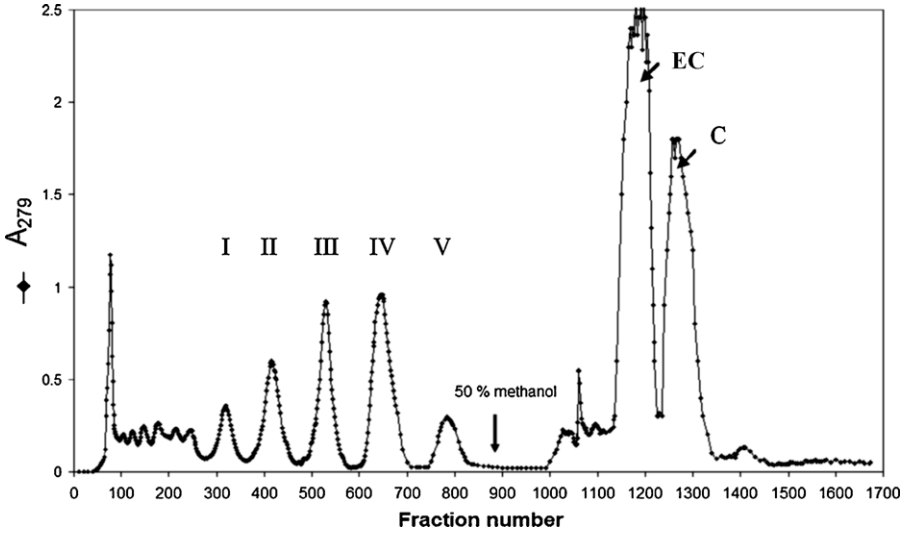

Fig. 4. Sephadex LH-20 column chromatography elution profile of the EC and β-CD reaction products, catalyzed by CGTase from *Paenibacillus* sp. RB01. Two ml fractions were collected from the column (55 cm × 2.5 cm), eluted with water initially and then (arrow) with 50% (v/v) methanol at a flow rate of 30 ml/h. Fractions labeled I–V are EC glucosides, and are discussed in the text, while EC and C are epicatechin and catechin, respectively.

3.2. Optimization of transglucosylation reaction

In order to increase the product yield, the transglucosylation reaction was optimized by sequential independent variable analysis (data not shown). The optimal conditions obtained were incubation of 0.1% (w/v) β-CD and 0.5% (w/v) EC with 80 U/ml of CGTase in 10 mM acetate buffer (pH 6.0) at 50 °C for 24 h. Under these conditions, the product yield, which was calculated from peak area of the product to that of EC at time t_0, increased over 2.3-fold to 18.1% (Table 1). This increased yield was found for all four EC glucoside products and, especially for that of the main product at a Rt of 48, which showed a 2.7-fold increase in yield up to a 8.33% yield. It was found that the total yield of EC glucosides obtained in this study (18.1%) was approximately the same as that reported previously for catechin glucosides (18.3%) when using the *Bacillus macerans* α-CGTase to catalyze the transglucosylation [27].

3.3. Isolation of EC glucoside products

The reaction products were separated by Sephadex LH-20 column chromatography on the basis of size and polarity differences [28]. The collected fractions were detected spectrophotometrically for EC at A_{279} (Fig. 4). When the column was eluted with distilled water, the enzyme CGTase (the first sharp peak) and five main separate product peaks (peaks I–V) were obtained. Subsequent elution by 50% (v/v) methanol yielded two more peaks with a high A_{279} absorbance that were subsequentially identified by comparative HPLC analysis to be EC and catechin, respectively.

From the HPLC analysis, peaks I–V were composed of more than one product, as summarized in Table 2. The main EC glucoside products, with a Rt of 48 and 45 min, were eluted in peaks IV and III, respectively. Peak IV also contained a minor product with a Rt of 27. The minor product at a Rt of 30 min existed in both peaks II and V, and were named 30(1) and 30(2), respectively, and later clarified

Table 1
Yields of EC glucoside products before and after optimization. Data are shown as the mean ± 1 SD and are derived from three independent repeats. Means within a column with a different letter are significantly different ($p < 0.05$).

	Product yield (%)				Total yield (%)
	Rt 27	Rt 30	Rt 45	Rt 48	
Before optimization	0.47 ± 0.07[a]	2.01 ± 0.40[a]	2.04 ± 0.21[a]	3.13 ± 0.09[a]	7.65 ± 0.47[a]
After optimization	2.17 ± 0.20[b]	3.39 ± 0.17[b]	4.18 ± 0.31[b]	8.33 ± 0.19[b]	18.1 ± 0.48[b]

Table 2
Identification of peaks I–V, resolved by Sephadex LH-20 column chromatography, by their relative retention time in the C18 reverse phase HPLC column.

Peak	Retention time (Rt)
I	15, 18, 22
II	15, 20, 30(1)[a]
III	15, 23, 45[a]
IV	18, 27[a], 48[a]
V	20, 30(2)[a]

[a] –EC glucoside products (see Fig. 3).

by TLC (Fig. 5). Other peaks at Rt 15–23 min (see Fig. 3) should be products of catechin. Each peak eluted from the Sephadex LH-20 column was then pooled and repeatedly purified by reverse phase C18 HPLC.

3.4. Characterization of EC glucosides

To correlate the four main glucoside products from the HPLC peaks with a Rt of 27, 30, 45 and 48 min, respectively, to the products resolved as TLC spots, the HPLC-resolved EC products at each of these respective Rt values were collected and then analyzed by TLC (Fig. 5). It was found that the peaks with a Rt of 48, 45 and 30(1) corresponded to Glu I, Glu II and Glu III, respectively. Due to the close migration with the major products with a Rt of 48 and 45 min, Glu I might also contain the compound with a Rt of 30(2), while Glu II might contain that with a Rt of 27 min. The minor products X and Y observed in lane b were not matched with any of the HPLC peaks tested. They might be those products with Rt of 15–23 min which were proposed to be glucosides of catechin.

3.4.1. Enzyme treatment

Product characterization was preliminary investigated by enzymic treatment of the reaction products with glucoamylase and α-glucosidase followed by TLC resolution of the final deglucosylated products. Prior to glucoamylase treatment, three main glucoside products were observed (Glu I, II and III), but after treatment with glucoamylase the Glu II and III spots disappeared while the intensities of the Glu I and glucose spots increased (Fig. 6). This result suggests that Glu I is an EC-monoglucoside while Glu II and Glu III are both EC-oligoglucosides. To further hydrolyze the glucoside products, α-glucosidase was added to the reaction after the glucoamylase, and this resulted in no glucoside spots being detected (Fig. 6), indicating that the first glucosyl residue attached to the EC is α-linked. The result agrees with the fact that the CGTase enzyme is specific for α-1,4-linkage transfer [29]. It was also observed that the unidentified product *Y* was disappeared in lane d and the X was not seen in lane e, this suggests that Y should be an oligoglucoside and X monoglucoside of catechin.

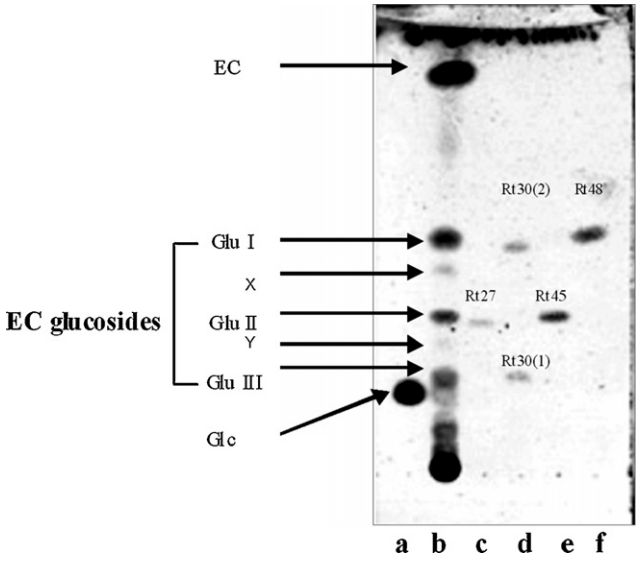

Fig. 5. TLC chromatogram of EC glucoside products separated by HPLC. (a) Standard glucose, (b) reaction products after 24 h, and fractions (peaks) from the HPLC separation of reaction products with Rt of (c) 27, (d) 30, (e) 45 and (f) 48 min (referred to as Rt27, Rt30 (1) and Rt30 (2), Rt45 and Rt48, respectively, in the text).

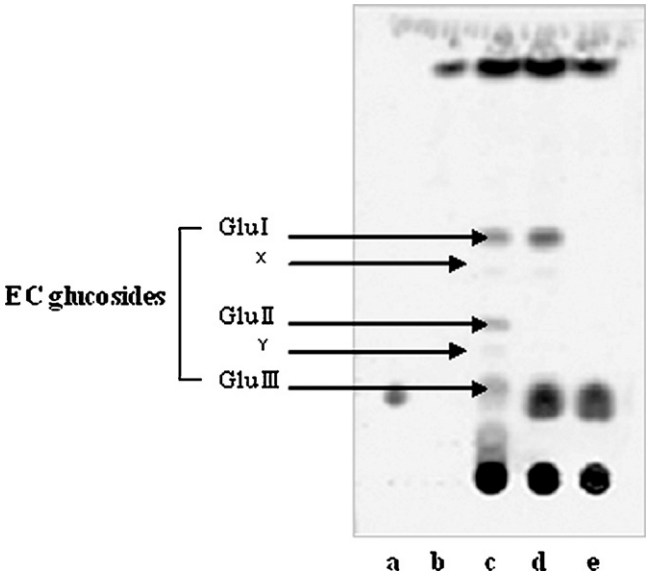

Fig. 6. TLC chromatogram of the reaction products of EC and β-CD incubated with *Paenibacillus* sp. RB01 CGTase for 24 h. Standard (a) glucose and (b) EC, plus the reaction products after 24 h (c) untreated, (d) treated with glucoamylase and (e) treated with glucoamylase and then α-glucosidase.

3.4.2. Mass spectrometry and nuclear magnetic resonance

From the mass spectra, the molecular weight of the EC glucoside products with a Rt of 48 and 30(2) min corresponded to the expected size for an EC monoglucoside, while those at a Rt of 45 and 30(1) min were an EC diglucoside and an EC-triglucoside, respectively (Table 3).

NMR analysis was used to postulate the structures of the EC-glucoside products (Table 4). From the proton [H^1] signal of the compound with a Rt of 48 min, a doublet signal at 7.31 ppm and a doublet-doublet signal at 6.96 ppm were assigned to the H-2′ and H-6′ of the EC moiety, respectively. Due to the glucosylation of EC, these proton signals showed downfield shifts by 0.44 and 0.26 ppm, respectively, when compared with the standard EC. This supports the presence of a glycosidic bond on the 3′-hydroxyl group of the catechol ring of the EC moiety [7]. The signal for the anomeric proton of the sugar moiety appeared at 5.28 ppm with a coupling constant (*J*) of 3.6 Hz. This glucosyl residue was, therefore, connected to the EC by an α-linkage. Integrated with the product size analyzed by MS, the compound with a Rt of 48 min was identified as (−)-EC-3′-*O*-α-D-glucopyranoside (EC3A) (Fig. 7i). Similarly,

Table 3
Molecular weight of the four main resolved EC glucoside products by mass spectrometry.

Glucoside product	$[M+Na]^+$ at *m*/*z*	MW (Da)
Rt 48	475	452
Rt 45	637	614
Rt 30(1)	798	775
Rt 30(2)	475	452

Table 4
^{1}H, ^{13}C NMR data of the EC-glucosides.

Position	$\delta^{1}H$ (ppm, in CD_3OD); *J* (Hz)					$\delta^{13}C$ (ppm, in CD_3OD); *J* (Hz)	
	EC	Rt48 min (EC3A)	Rt45 min (EC3B)	Rt30(1) min (EC3C)	Rt30(2) min (EC4A)	EC	Rt30(2) min (EC4A)
Aglycone							
2	4.72 (s)	4.78 (s)	4.87 (s)	4.88 (s)	4.85 (s)	78.2	78.2
3	4.08 (s)	4.06 (m)	4.19 (s)	4.09 (m)	4.19 (s)	65.8	66.0
4	2.63 (dd 16.8, 2.6)	2.64 (dd 16.8, 2.3)	2.74 (dd 16.8, 2.0)	2.73 (dd 16.8, 2.2)	2.72 (dd 16.9, 1.5)	27.7	27.8
4a	2.76 (dd 16.7, 4.5)	2.77 (dd 16.6, 4.5)	2.86 (dd 16.7, 4.4)	2.86 (dd 17.2, 4.6)	2.87 (dd 16.5, 4.1)	98.4	98.6
6	5.82 (d 2.2)	5.82 (d 2.0)	5.92 (d 1.9)	5.91 (d 2.17)	5.92 (d 5.7)	94.3	95.0
8	5.84 (d 2.2)	5.85 (d 2.2)	5.94 (d 2.0)	5.93 (d 1.65)	5.92 (d 5.7)	93.8	94.4
2′	6.87 (d 1.5)	7.31 (d 1.31)	7.42 (d 1.4)	7.41 (s)	7.04 (s)	114.1	114.5
5′	6.66 (d 8.1)	6.75 (d 8.3)	6.84 (d 8.2)	6.84 (d 8.1)	7.23 (d 8.4)	114.3	117.2
6′	6.70 (dd 8.1, 1.6)	6.96 (dd 8.1, 1.2)	7.06 (dd 8.2, 1.3)	7.05 (dd 8.1, 1.7)	6.89 (d 8.3)	117.8	117.8
Glycoside							
1″		5.28 (d 3.6)	5.35 (d 3.6)	5.21 (d 3.4)	5.32 (d 3.4)		100.0
2″		3.35 - 3.76 (m)	3.39 - 3.90 (m)	3.34 - 3.91 (m)	3.40 - 3.89 (m)		72.0
3″							73.4
4″							69.9
5″							73.0
6″							60.9

s, singlet; d, doublet; dd, doublet of doublet; m, multiplet.

due to the downfield shift of the proton signals at the H-2′ and H-6′ and also the MS result, the compounds with Rt of 45 and 30(1) min were identified as (−)-EC-3′-*O*-α-D-diglucopyranoside (EC3B)) (Fig. 7ii) and (−)-EC-3′-*O*-α-D-triglucopyranoside (EC3C)) (Fig. 7iii), respectively. All the structures with the 3′-*O* linkage were confirmed by comparison with literature data [7,11,27]. For the product at Rt 30(2) min, a downfield shift of 0.57 ppm at the H-5′ of the EC moiety with a *J* value of the anomeric proton (5.32 ppm) of 3.4 Hz was observed. This product was identified as a monoglucoside from the MS analysis, the proposed structure was (−)-EC-4′-*O*-α-D-glucopyranoside (EC4A) (Fig. 7iv). Since this product was new for CGTase reaction, analysis by ^{13}C- and two-dimension NMR (COSY, HSQC and HMBC) was performed. The result confirmed the signal shift of 2.9 ppm at the C-5′ position of EC (Table 4).

The structures of these four EC-glucoside products showed either the 3′-*O*- (EC3A, EC3B and EC3C) or a 4′-*O*-linkage (EC4A). Since the characteristics of the glucosylated products of EC have never been reported before, here we compare them with the reported glucoside products of catechin, which is an epimer of EC. Catechin-3′-*O*-α-D-glucopyranoside has been synthesized by transglucosylation using (i) the α-CGTase from *B. macerans* [27], (ii) the sucrose phosphorylase from *L. mesenteroids* [9], and (iii) by transferase activity from *X. campestris* WU-9701 [11], while catechin 4′-*O*-α-D-glucopyranoside has been synthesized using the glucosyltransferase from *Streptococcus* sp. [10] and *S. sobrinus* [30]. However, the formation of a 4′-*O*-glucoside by the action of a CGTase has never previously been reported. Thus, our unique finding of a 4′-*O*-glucoside derivative of EC shows that *Paenibacillus* sp. RB01 CGTase, a β-CGTase, acts differently from the established α-CGTase of *B. macerans* in its transglucosylation of EC and catechin.

As mentioned, the catechin glucoside products obtained from enzymatic transglucosylation reactions were mostly in the α-configuration, whereas the β-glucosides are mainly found in the natural glucosides from plants. For example, the catechin 7-*O*-β-D-glucopyranoside isolated from barley (*Hordeum vulgare* L.) [31] and buckwheat (*Fagopyrum*) [32], while 4′-*O*-β, 5-*O*-β and 3-*O*-β isomers also coexisted in those plants.

3.5. Properties of EC3A glucoside

3.5.1. Solubility, browning resistance and antioxidant activity

The water solubility of EC was 4.9 mg/ml (0.017 mol/l) whereas that of EC3A was found to be significantly higher at 216.6 mg/ml (0.479 mol/l). Likewise, the solubility of catechin-3′-*O*-α-D-glucopyranoside has been reported to be between 50- and 100-fold higher than that of catechin [8,11], and the solubility of 4″ EGCg-monoglucoside was 69 times higher than that of EGCg [12]. Although the discrepancy between these solubility properties may come from the different structures of the catechin derivatives, it may also be a result of variation in the experimental methods used to determine their solubility.

The browning of EC was found to increase with increasing exposure time to the UV irradiation, while under the same conditions the EC3A derivative showed resistance to browning, even after 10 h of irradiation (Fig. 8A). The EC3A glucoside was thus more stable than its parent EC, and thus suggests that the glucosyl moiety helps pro-

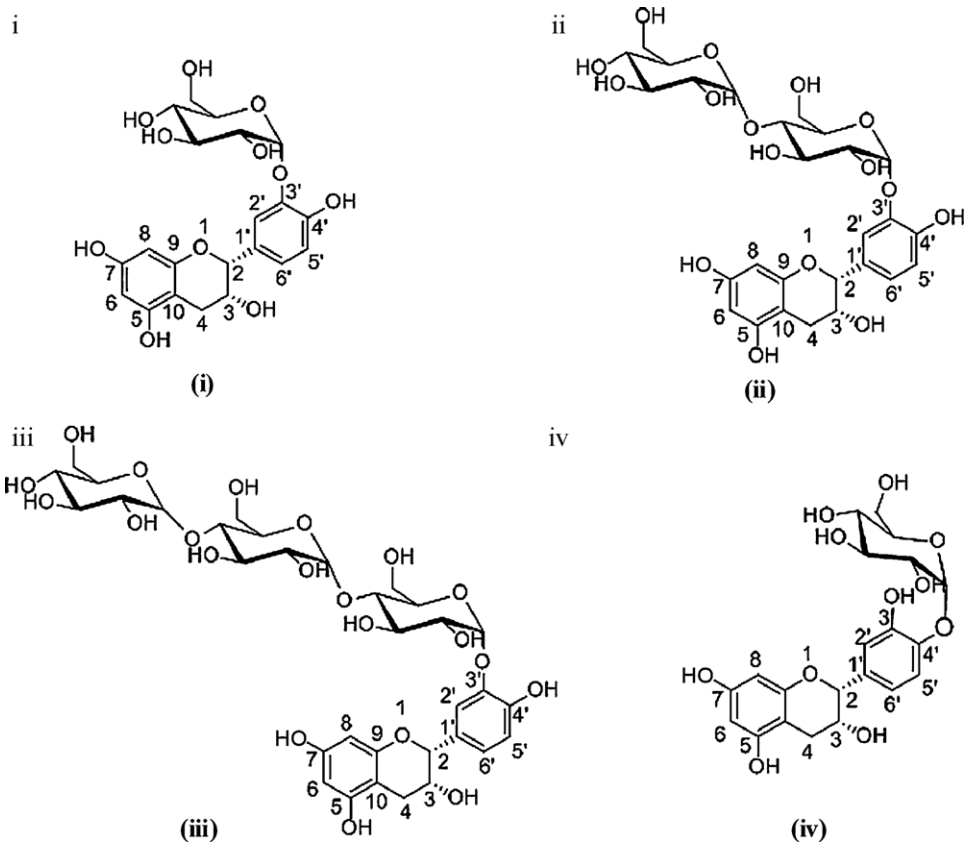

Fig. 7. Proposed structure of the products (i) (−)-EC-3′-*O*-α-D-glucopyranoside (EC3A), (ii) (−)-EC-3′-*O*-α-D-diglucopyranoside (EC3B), (iii) (−)-EC-3′-*O*-α-D-triglucopyranoside (EC3C) and (iv) (−)-EC-4′-*O*-α-D-glucopyranoside (EC4A).

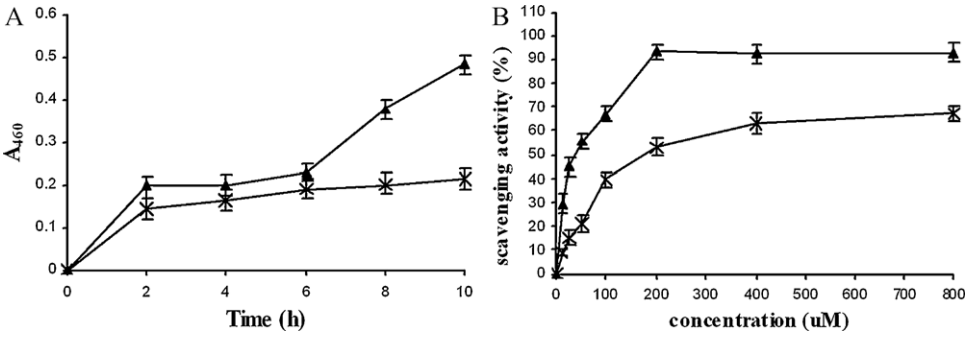

Fig. 8. (A) Browning resistance to UV irradiation. A solution containing 0.1% (w/v) EC (▲) or EC3A (×) was exposed to UV irradiation (254 nm) for the indicated periods before the absorbance at 460 nm was monitored. (B) DPPH radical-scavenging activities. EC (▲) and EC3A (×) at various concentrations mixed with 100 μM DPPH and left for 10 min in the dark were then monitored for their absorbance at 517 nm. Data are shown as the mean ± 1 SD and are derived from three independent repeats.

tect the EC catechol rings from polymerization, which is the cause of the browning phenomena [8]. Catechin was also reported to be easily degraded with rapid browning activated by UV irradiation [7]. In addition, catechin solution was shown to turn brown after standing for 24 h in contact with air at 20 °C while the monoglucoside solution showed no color change [11].

The free-radical scavenging activity was determined using the stable free radical, DPPH where the IC_{50} (inhibitory concentration at which the absorbance at 517 nm was reduced by 50%) of EC and EC3A were 76.5 and 115 μM, respectively, making the IC_{50} of EC3A 1.5-fold higher than that of the parental EC (Fig. 8B). This reduced scavenging activity for the EC3A glucoside might be due to the presence of an ortho-dihydroxyl group in the flavonoid B ring that is important for achieving a high radical scavenging activity [33]. The relationship between scavenging ability and the ring B structure of tea catechins was later confirmed [34]. The antioxidative molecular mechanism of catechins through NMR approach was presented whereby the same mechanism for catechin and epicatechin was proved [35]. Our result corresponds well with previous report on the decrease in the antioxidant activity when catechins were in the glycosylated form [36].

4. Summary

The overall results demonstrated that the CGTase from *Paenibacillus* sp. RB01 can be used to synthesize four to five different EC glucosides, as analyzed by HPLC, which after optimization had a total yield of 18.1%. The main product (EC3A) was a 3′-O-monoglucoside with a higher water solubility and browning resistance to UV irradiation than EC, but its antioxidant property

was decreased. The other products whose structures were analyzed by NMR were EC3B and EC3C (3′-O-α-di- and tri-glucosides, respectively), plus EC4A. EC4A is a unique product with the monoglucosyl α-linked to EC at the 4′-position of the catechol ring. Although it is produced in a relatively low amount, its synthesis has never been previously reported for other CGTases.

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