Efficient α-glucosylation of epigallocatechin gallate catalyzed by cyclodextrin glucanotransferase from *Thermoanaerobacter* sp.

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Abstract

The glycosylation of plant polyphenols may modulate their solubility and bioavailability, and protect these molecules from oxygen, light degradation and during gastrointestinal transit. In this work, the synthesis of various α-glucosyl derivatives of (–)-epigallocatechin gallate (EGCG), the predominant catechin in green tea, was performed in water at 50 °C by a transglycosylation reaction catalyzed by cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp. The molecular weight of reaction products was determined by HPLC-MS. Using hydrolyzed potato starch as glucosyl donor, two main monoglucosides were obtained with conversion yields of 58% and 13%, respectively. The products were isolated and chemically characterized by combining 2D-NMR methods. The major derivative was epigallocatechin gallate 3'-*O*-α-D-glucopyranoside (***1***) and the minor epigallocatechin gallate 7-*O*-α-D-glucopyranoside (***2***).

Keywords: Glycosylation; Tea polyphenols; Antioxidants; Catechins; Cyclodextrin glucosyltransferase; Enzymatic glucosylation.

INTRODUCTION

Plant polyphenols are gaining importance owing to their capacity to delay the appearance of several degenerative illnesses and pathologic processes such as Alzheimer's and Parkinson's diseases, schizophrenia, cancer, chronic inflammatory disease, atherosclerosis or myocardial infarction.[1-3] Their action is based on the enhancement of the antioxidant system due to their ability to lower the level of reactive oxygen species (ROS).[4] Most polyphenols are hydrophobic molecules that exhibit poor absorption *in vivo*, giving rise to a negligible concentration in the circulatory streams.[5]

Several polyphenols appear glycosylated in nature[6] and the sugar moiety seems to play a significant function in their human absorption.[7,8] Glycosylation of polyphenols may thus modulate their bioavailability,[9] bioactivity,[10] and various physicochemical features such as the solubility[11] and the partition coefficient.[12] Glycosylation may also facilitate the entrance of several flavonoids into intestinal enterocytes.[9] For instance, 3-*O*-glycosides of quercetin displayed improved bioavailability compared with the aglycon.[13] However, it has been reported that a prior hydrolysis of the glycosides is critical to obtain an efficient cellular uptake.[14,15] In this context, the main advantage of glycosylation could be thus related with the increase of polyphenol stability, in particular during storage and gastrointestinal transit after ingestion.[16]

Glycosylation may also exert other benefits to the polyphenols including a protection from oxygen and/or light degradation by masking certain phenolic groups, or an increased efficiency for the prevention of skin photo-ageing damages.[17] Due to the excellent specificity of enzymes and to the mild conditions required, the enzymatic glycosylation is preferred over other methodologies based on traditional

chemistry, which require various steps of protection/deprotection of functional groups.[18-22]

(–)-Epigallocatechin gallate (EGCG) is the prevailing flavanol (≥ 50%) in green tea (*Camellia sinensis*). Its antioxidant,[23] antihypertensive,[24] antitumoral,[25,26] bactericidal[27] and anti-inflammatory[28] properties, among others, have been widely studied. However, the bioavailability of EGCG is low[29] as it also undergoes rapid degradation in aqueous solutions.[30,31] EGCG has arisen a lot of attention as a potential therapeutic substance for the prevention, among others, of neurodegenerative diseases.[32-34] Various investigations proved the potential of EGCG to promote healthy aging, suppress cognitive dysfunction, boost learning ability and minimize oxidative damage in the brain.[35,36]

The enzymatic glycosylation of EGCG has been explored as a means to increase its stability, solubility, bioavailability and browning resistance,[37] as well as to reduce its astringency for food applications.[38] Thus, the α-glucosylation of EGCG has been described using a sucrose phosphorylase[39] or a glucansucrase[37,40,41] from *Leuconostoc mesenteroides,* and an α-amylase from *Trichoderma viride*.[38]

In this article, we report the enzymatic preparation of various α-glucosylated derivatives of EGCG by a transglucosylation reaction catalyzed by a cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) from *Thermoanaerobacter* sp.[42] This biocatalyst was anteriorly employed in our laboratory for the α–glucosylation of resveratrol[43] and pterostilbene.[44] A variant of the same enzyme from *Bacillus macerans* was reported to glucosylate catechin but with low yield (1.2%).[45] Our objective was to develop a friendly and efficient process for α-glucosylation of EGCG.

MATERIALS AND METHODS

Enzyme and reagents

(-)-Epigallocatequin gallate (EGCG) was acquired from Zhejiang Yixin Pharmaceutical Co. (Zhejiang, China). Toruzyme 3.0L, a commercial preparation of cyclodextrin glucanotransferase (CGTase) from *Thermoanaerobacter* sp., was gently provided by Novozymes. It was partially purified using a PD-10 desalting column (GE Healthcare). Partially hydrolyzed starch from potato (Passelli SA2) was from Avebe (Foxhol, The Netherlands). All other reagents and solvents were of the maximum available purity and used as acquired.

General procedure for enzymatic glucosylation

Epigallocatequin gallate (9.2 mg, 20 mM) and starch (20 mg) were dissolved in 1 mL of water. Desalted CGTase from *Thermoanaerobacter* sp was incorporated to a final concentration of 10% (v/v). The mixture was held at 50 °C with 150 rpm orbital stirring (model SI50, Stuart Scientific). Aliquots (100 μL) were withdrawn at intervals, filtered with 0.45 μm nylon filters (Cosela) and the formation of products was followed by TLC and HPLC.

Thin-Layer Chromatography (TLC)

TLC analysis was carried out on silica gel plates with fluorescent indicator (Polygram SIL G/UV254, Macherey-Nagel) using ethyl acetate/methanol (9:1, v/v) as eluent. Phenolic compounds were observed under UV light (UV transiluminator, UVP, USA) and the carbohydrates were stained with a solution containing $(NH_4)_6Mo_7O_{21}\cdot 4H_2O$ and $Ce(SO_4)_2$ in 10% (v/v) H_2SO_4.

High-Performance Liquid Chromatography (HPLC)

HPLC analyses were performed employing a quaternary pump (model 600, Waters) coupled to an autosampler (Varian ProStar, model 420). The injection volume was 10 µL. The column was maintained at 40 °C. The detection of peaks was carried out using a photodiode array detector (ProStar, Varian) and integration was performed using the Varian Star LC workstation 6.41. The column was a Zorbax Eclipse Plus C-18 (4.6 x 100 mm, 5 µm, Agilent Technologies) and the mobile phase was H_2O/methanol 85:15 (v/v), degassed with helium. The solvents were acidified with formic acid (0.1% v/v). The flow rate was 0.5 mL/min.

HPLC coupled to Mass Spectrometry (HPLC-MS)

The molecular weight of synthesized derivatives was determined by HPLC-MS using a HPLC 1100 (Agilent Tecnologies) coupled to a photodiode array detector and a mass spectrometer (Maxis II, Bruker) with hybrid QTOF analyzer. Samples were ionized by electrospray (with nitrogen to desolvate the mobile phase) and analyzed in positive reflector mode. The column and elution conditions were the same as described above, except for the flow rate that was 0.3 mL/min.

Purification of glucosylated derivatives of EGCG by semipreparative HPLC

The glucosylation reaction was scaled up. The reaction mixture was formed by EGCG (92 mg), soluble starch (200 mg), partially purified Toruzyme 3.0 L (1 mL), and 9 ml of water. The mixture was maintained at 50 °C for 2 h with orbital shaking (150 rpm). Then it was cooled and concentrated by rotary evaporation, and the glucosylated derivatives of EGCG were isolated by semipreparative HPLC. A Zorbax Eclipse XDB C-18 column (9.4 x 250 mm, Agilent) and a three-way flow splitter (Accurate, LC Packings) were used. The mobile phase was H_2O/methanol 85:15 v/v (both solvents containing 0.1% of acetic acid) at 7.0 mL/min. The column was kept at 40 °C. A

photodiode array detector (PDA, Varian Prostar) was used. After collecting the glucosylated derivatives of epigallocatechin gallate, the solvents were evaporated in an R-210 rotavapor (Buchi). The purified products were further characterized by mass spectroscopy and NMR.

Mass spectrometry (MS)

The molecular mass of synthesized EGCG glucosides was determined employing a mass spectrometer coupled to a hybrid QTOF analyzer (model QSTAR, Pulsar i, AB Sciex). The compounds were analyzed by direct infusion and ionized by electrospray (ESI) in negative reflector mode. The ionizing phase was methanol basified with 1% of NH_4OH.

Nuclear Magnetic Resonance (NMR) analysis

The structure of the glucosylated derivatives was assessed using a combination of 1D (^1H, 1D-selective NOESY experiments) and 2D (COSY, DEPT-HSQC, NOESY) NMR techniques. The compounds were solubilized in deuterated water (ca. 10 mM). The spectra were recorded on a Bruker IVDr 600 spectrometer equipped with a BBI probe with gradients in the Z axis, at 300 or 313 K. Chemical shifts were expressed in parts per million (ppm) with respect to the 0 ppm point of DSS (4-dimethyl-4-silapentane-1-sulfonic acid), employed as internal standard. All the pulse sequences were provided by Bruker. For the DEPT-HSQC experiment, values of 7 ppm and 2K points, for the ^1H dimension, and 160 ppm and 256 points for the ^{13}C dimension, were utilized. For the homonuclear COSY and NOESY experiments, 7 ppm windows were used with a 2K x 256 point matrix. For the NOESY and 1D-selective NOESY experiments, the mixing times were 500-600 ms.

RESULTS AND DISCUSSION

EGCG glycosylation and optimization of the reaction

It is well reported that the stability of EGCG in aqueous solutions is rather limited.[39] The two main processes involved in the degradation of EGCG are epimerization and oxidative coupling.[30] The stability of EGCG is concentration-dependent and can be also influenced by temperature, pH and the amount of oxygen in the solution, among other parameters.[31] In order to avoid interferences in the enzymatic glycosylation of EGCG, the stability of this compound was studied in different buffers at room temperature and 50 °C (Fig. 1).

In presence of phosphate buffer (pH 7.6), the degradation of EGCG was fairly fast, especially at 50 °C (38% degradation in 24 h). This process was concomitant with the appearance of (-)-gallocatechin gallate (GCG) as a result of EGCG epimerization (Fig. 2, chromatogram I). In addition, the color of the solutions became brown upon incubation, as a consequence of the formation by oxidative coupling of dimers and compounds of higher molecular-weight.[46] At lower pH values (e.g. 5.6) the stability of EGCG was notably improved (Fig. 1), in accordance with previous reports.[47,48] However, the maximum stability of EGCG was found in water, with negligible degradation during 24 h even at 50 °C. Considering that most glycosidases display certain degree of activity at neutral pH,[49] we selected water as reaction medium to screen the glycosylation of EGCG.

Amongst the glycosidases and glycosyltransferases tested by TLC analysis, only cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp.[50,51] resulted in a significant formation of glycosylated EGCG derivatives. Previously, CGTases had been successfully employed in the glycosylation of other polyphenols such as

157 resveratrol,[43,52] genistein,[53] pterostilbene[44] or hydroquinone.[54] Fig. 2 (chromatogram
158 II) illustrates a typical reaction mixture with this enzyme after 72 h. It is worth noting
159 that the major product (Glc-EGCG-1) presented a higher retention time than EGCG in
160 a C-18 column. A secondary product (Glc-EGCG-2) was also observed.

161 With a view to optimize the production of glucosylated derivatives, we analyzed
162 the effect of starch concentration (10-300 mg/mL). Fig. 3 illustrates the maximum
163 conversion yield of Glc-EGCG-1 and Glc-EGCG-2. As shown, the differences were not
164 very substantial. Under the optimal conditions (9.2 mg of EGCG and 20 mg/mL of
165 partially hydrolyzed starch), the conversions to Glc-EGCG-1 and Glc-EGCG-2 were
166 39% and 10%, respectively. We also found that decreasing the enzyme concentration
167 from 10% to 5% (v/v) no significant effect on the reaction rate was observed.

168 The reaction mixture was characterized in detail by HPLC coupled to mass
169 spectrometry (Fig. 4). As shown, we detected the formation of at least four
170 monoglucosides and four diglucosides (see mass spectra of the different peaks in
171 Supplementary Material, part A).

172 ***Kinetics of EGCG glucosylation***

173 The progress of formation of the two glucosides under the optimal conditions was
174 studied by HPLC (Fig. 5). The reaction was quite fast; after 3 hours, the concentration
175 of the two main products remained stable. The conversion yield of Glc-EGCG-1 and
176 Glc-EGCG-2 were 58% (7.2 mg/mL) and 13% (1.6 mg/mL), respectively.

177 Using a similar EGCG concentration and a sucrose phosphorylase from *L.*
178 *mesenteroides*, Kitao *et al.* reported 30% yield of the EGCG 4'-*O*-α-D-glucopyranoside
179 and 40% of the 4',4''-*O*-α-D-diglucopyranoside derivative.[39] With a glucansucrase
180 from the same microorganism, Moon *et al.* achieved 20% yield of EGCG 4'-*O*-α-D-
181 glucopyranoside, 9% of the 7-*O*-α-D-glucopyranoside and approximately 9% of a

182 derivative glucosylated at 4'- and 7- positions.[40] More recently, Kim *et al.* reported a
183 91% conversion yield with the dextransucrase from *L. mesenteroides;* however,
184 selectivity was low, as nine different glucosides were isolated and characterized.[41]

185 ***Characterization of the main glucosylated derivatives***

186 The monoglucosides Glc-EGCG-1 and Glc-EGCG-2 were purified by semipreparative
187 HPLC as described. Their molecular weights were confirmed by ESI-MS employing a
188 QTOF analyzer in negative mode (see Supplementary Material, part B). In both cases
189 we observed the presence of a major peak at *m/z* 619.1 that corresponded to the M-
190 $[H]^+$ ion.

191 The glycosylation position was deduced by NMR. By comparing the HSQC spectra
192 of EGCG and Glc-EGCG-1, no significant differences were observed in the NMR cross
193 peaks belonging to the A- and D-rings, whilst the cross peak assigned to the B-ring of
194 EGCG was split into two new signals in the monoglucosylated derivative (Fig. 6, top).
195 This fact implies that the substitution has taken place at B-ring, namely at positions
196 3'- or 5'- (chemically equivalent). This substitution causes a loss of symmetry in this
197 ring, and positions 2- and 6- become non-equivalent providing two differentiated
198 signals. NOE experiments confirmed the vicinity between the anomeric proton H1Glc
199 and one of the aromatic protons at B-ring (see Supplementary Material, part C).
200 Taking into account the described results, the proposed non-ambiguous structure for
201 Glc-EGCG-1 is epigallocatechin gallate 3'-*O*-α-D-glucopyranoside (Fig. 7, compound ***1***),
202 first described by Nanjo *et al*.[46]

203 A similar analysis was performed for the monoglucoside Glc-EGCG-2. In this case,
204 the perturbed cross peak corresponds to that of A-ring (Fig. 6, bottom). A selective
205 1D–NOE experiment was further performed by inverting the H1-Glc signal, showing
206 NOEs with H8 and H6 of A-ring besides the obvious intra-residue NOE with H2-Glc

(see Supplementary Material, part C). The observed NOEs can only be explained if the Glc substitution has taken place at position 7 of the A-ring. On this basis, the proposed non-ambiguous structure for Glc-EGCG-2 is epigallocatechin gallate 7-*O*-α-D-glucopyranoside (Fig. 7, compound ***2***), which was first reported by Moon et al.[40]

In summary, we synthesized two α-glucosides of EGCG under gentle conditions (aqueous medium, 50 °C) employing pretreated starch as glucose source and CGTase as biocatalyst. The major product ***1*** (58%) contained a glucosyl moiety at C-3' in the B-ring. A derivative glucosylated at C-7 of A-ring (compound ***2***) was also isolated at lower yield.

The synthesized derivatives are unlikely to reach the blood in their intact form, because they are expected to be first deglycosylated at the intestinal surface before diffusing into the enterocytes. However, glucosylation could increase the stability during processing, storage and gut transit after ingestion. These α-glucosides of EGCG could act as prodrugs releasing the EGCG in the intestine, as has been seen for other catechin glucosides.[16] However, to determine their full potential, further studies regarding bioavailability are necessary. The synthesized compounds could be thus useful for nutraceutical, cosmetic and biomedical applications, as is the case of other enzymatically-synthesized glycosides of flavonoids, such as hesperidin α-glucoside.[55]

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SUPPORTING INFORMATION

(A) HPLC-MS analysis of the reaction of EGCG and partially hydrolyzed starch catalyzed by the CGTase from *Thermoanaerobacter* sp. **(B)** ESI-MS of the isolated

231 EGCG 3'-*O*-α-D-glucopyranoside and EGCG 7-O-α-D-glucopyranoside. **(C)** 1D-NOE
232 spectrum of EGCG 3'-*O*-α-D-glucopyranoside and EGCG 7-*O*-α-D-glucopyranoside.

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FIGURE CAPTIONS

Figure 1. Stability of EGCG under different experimental conditions: (●) Distilled water; ( ) Sodium acetate buffer (10 mM, pH 5.6); ( )Sodium phosphate buffer (10 mM, pH 7.6). Graphic **(A)** corresponds to room temperature, and graphic **(B)** to 50 °C.

Figure 2. HPLC chromatograms showing: **(I)** the degradation of EGCG in 10 mM sodium phosphate buffer (pH 7.6); **(II)** the reaction mixture after 72 h with the CGTase from *Thermoanaerobacter* sp. in water. Glc-EGCG-1 and Glc-EGCG-2 are the two main synthesized products. Reaction conditions: EGCG (20 mM), soluble starch (100 mg/mL), partially purified Toruzyme 3.0L (10% v/v), 50 °C, 150 rpm.

Figure 3. Effect of starch concentration on the yield of EGCG glucosylated products. Reaction conditions: EGCG (20 mM), soluble starch (10-100 mg/mL), partially purified Toruzyme 3.0L (10% v/v), 50 °C, 150 rpm.

Figure 4. HPLC-MS chromatogram of the reaction mixture after 72 h obtained with the CGTase from *Thermoanaerobacter* sp. Peaks: (1) Unknown; (2) Monoglucoside; (3) Diglucoside; (4) Monoglucoside (Glc-EGCG-2); (5) Diglucoside; (6) Diglucoside; (7) EGCG; (8) Monoglucoside; (9) Monoglucoside (Glc-EGCG-1); (10) Diglucoside; (11) Mixture of products. Reaction conditions: EGCG (20 mM), soluble starch (20 mg/mL), partially purified Toruzyme 3.0L (5% v/v), 50 °C, 150 rpm.

Figure 5. Kinetics of formation of EGCG glucosides under optimal conditions. Glc-EGCG-1 and Glc-EGCG-2 are the two main monoglucosides. Reaction conditions as described in Fig. 4.

Figure 6. DEPT-HSQC NMR spectra superposition of EGCG with the monoglucosides Glc-EGCG-1 **(Top)** and Glc-EGCG-2 **(Bottom)**.

Figure 7. Structure of the two main EGCG monoglucosides synthesized: epigallocatechin gallate 3'-*O*-α-D-glucopyranoside **(*1*)** and epigallocatechin gallate 7-*O*-α-D-glucopyranoside **(*2*)**.

Fig. 1

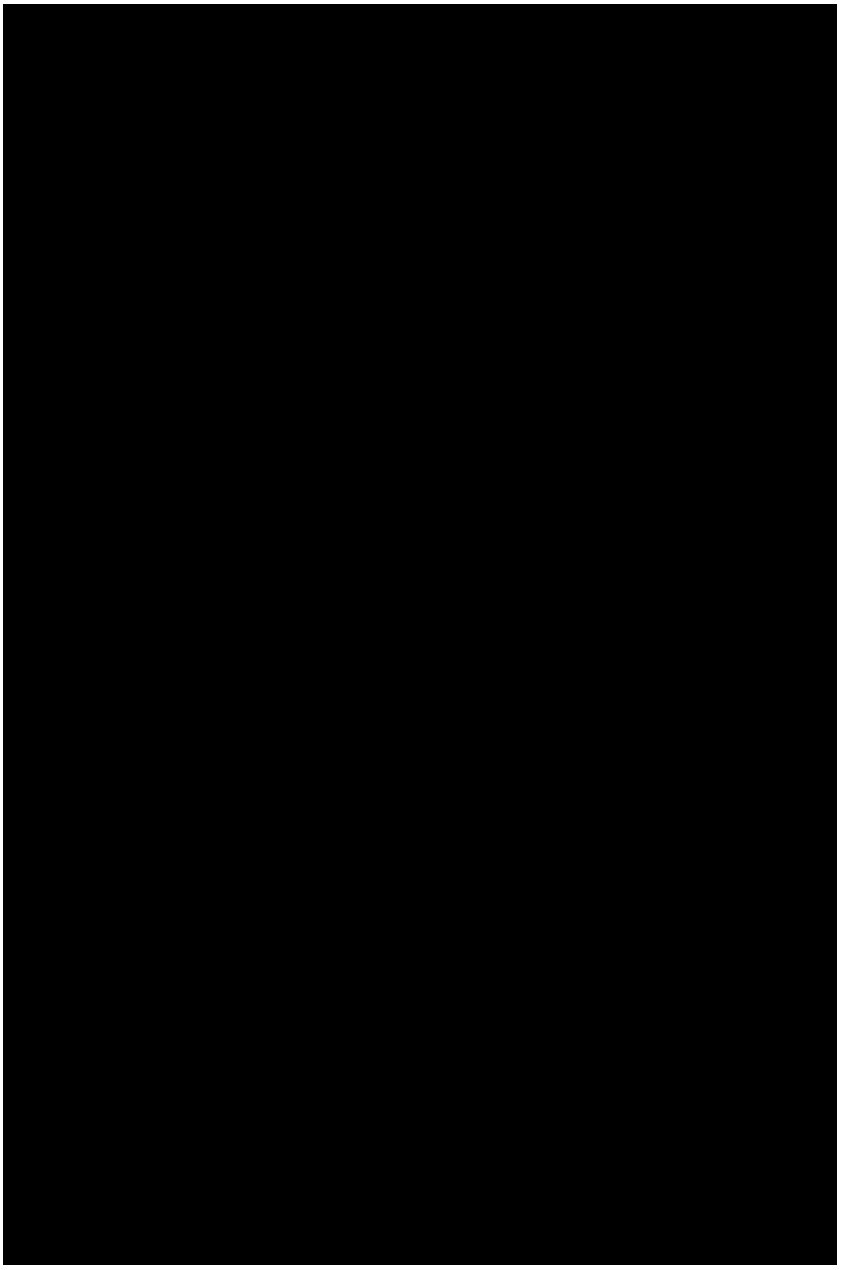


Fig. 2

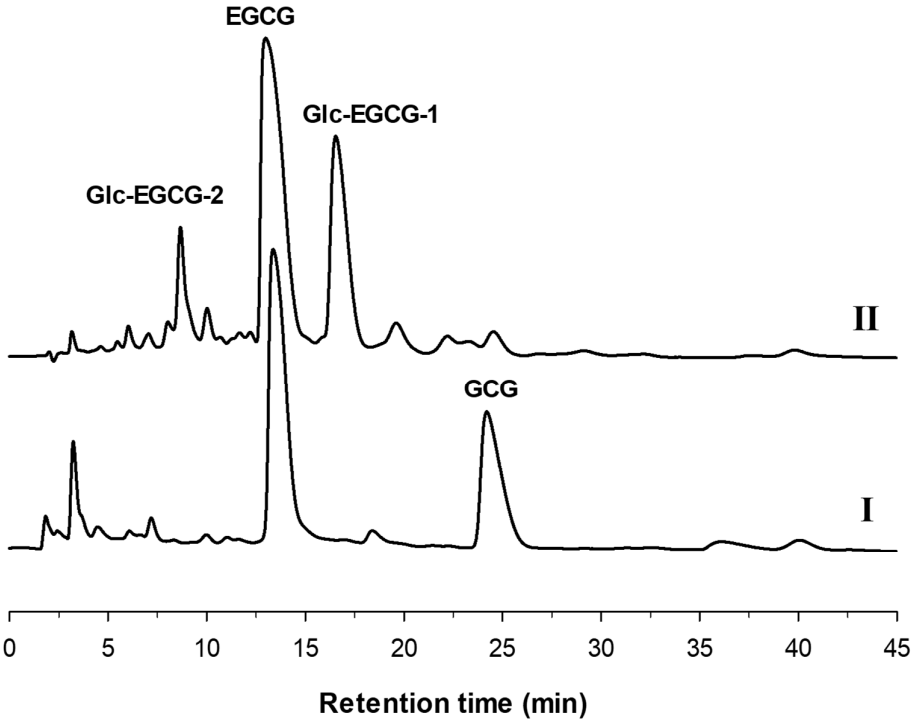

EGCG
Glc-EGCG-1
Glc-EGCG-2
II
GCG
I
0
5
10
15
20
25
30
35
40
45
Retention time (min)

Fig. 3

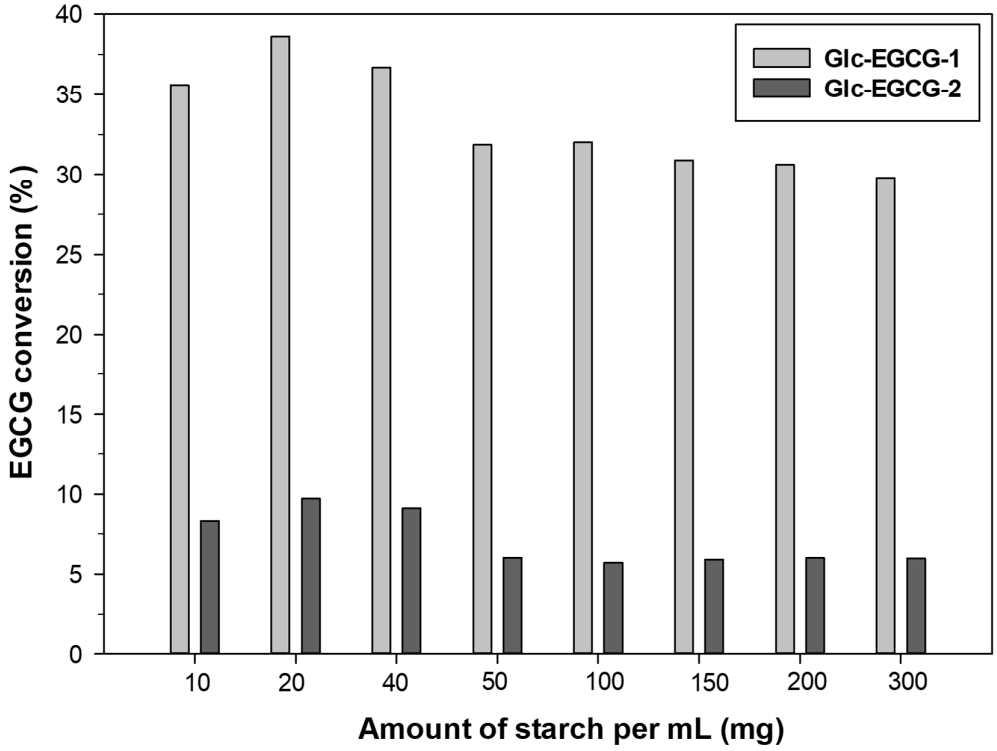

Glc-EGCG-1
Glc-EGCG-2
EGCG conversion (%)
0
5
10
15
20
25
30
35
40
10
20
40
50
100
150
200
300
Amount of starch per mL (mg)

Fig. 4

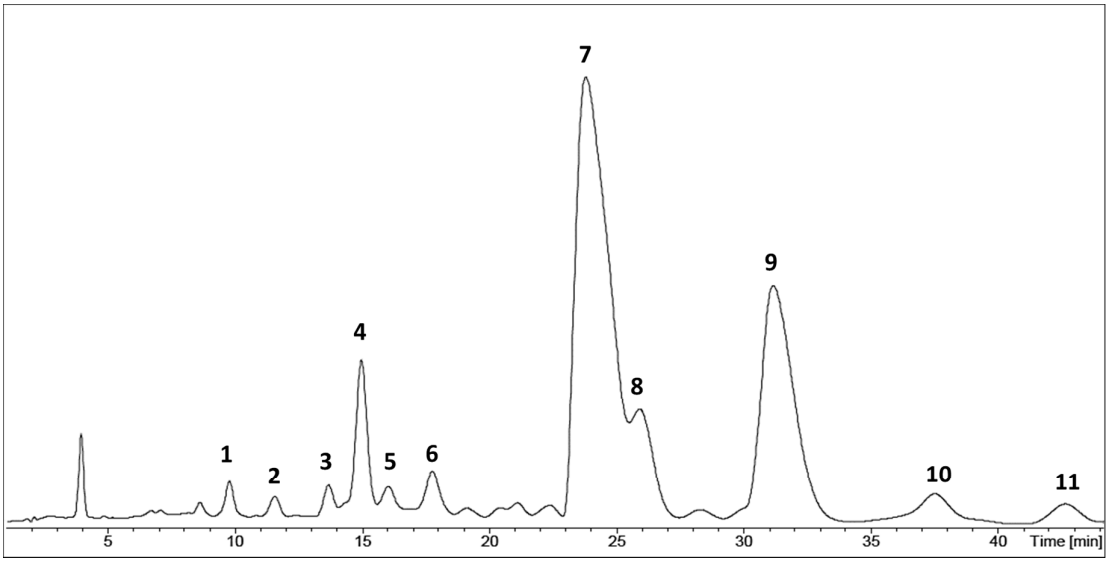


Fig. 5

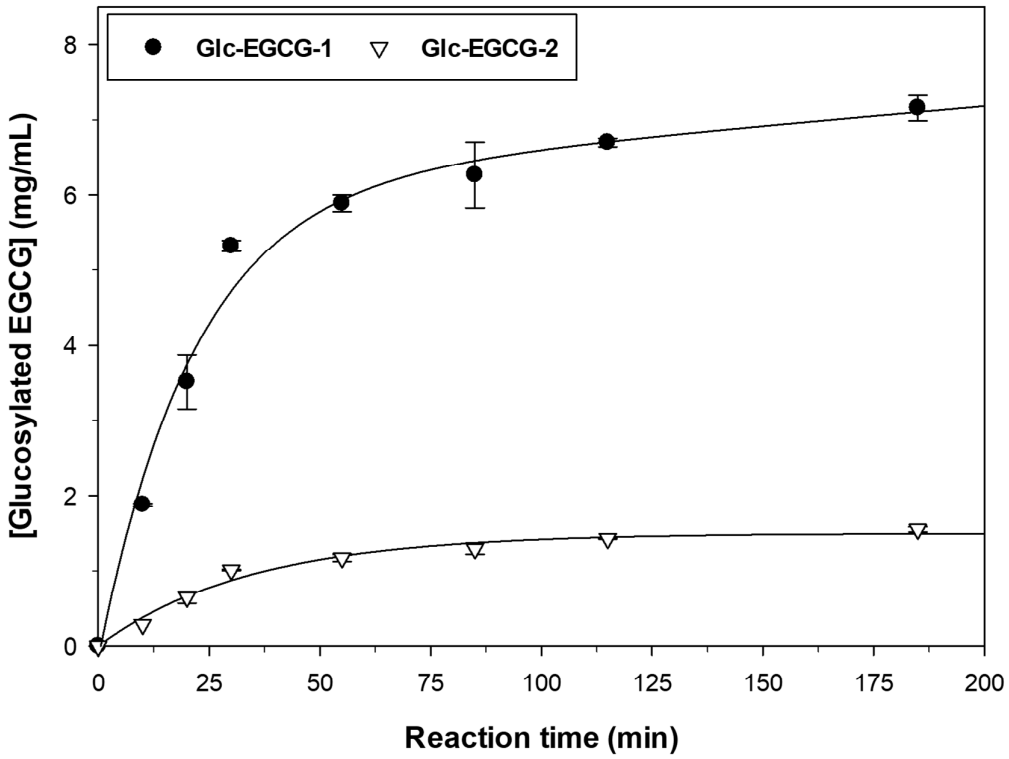

Glc-EGCG-1
Glc-EGCG-2
[Glucosylated EGCG] (mg/mL)
Reaction time (min)
0
2
4
6
8
0
25
50
75
100
125
150
175
200

Fig. 6

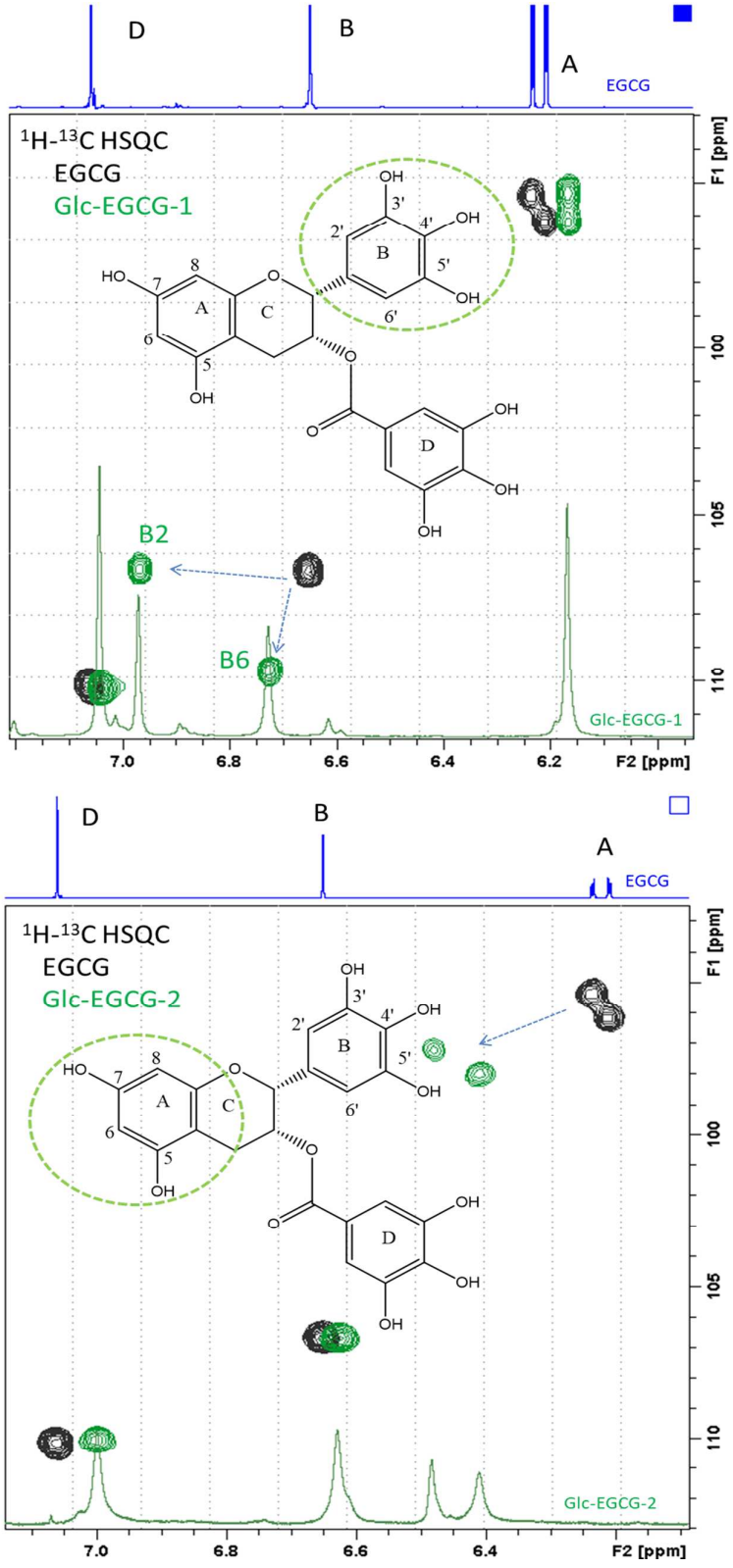
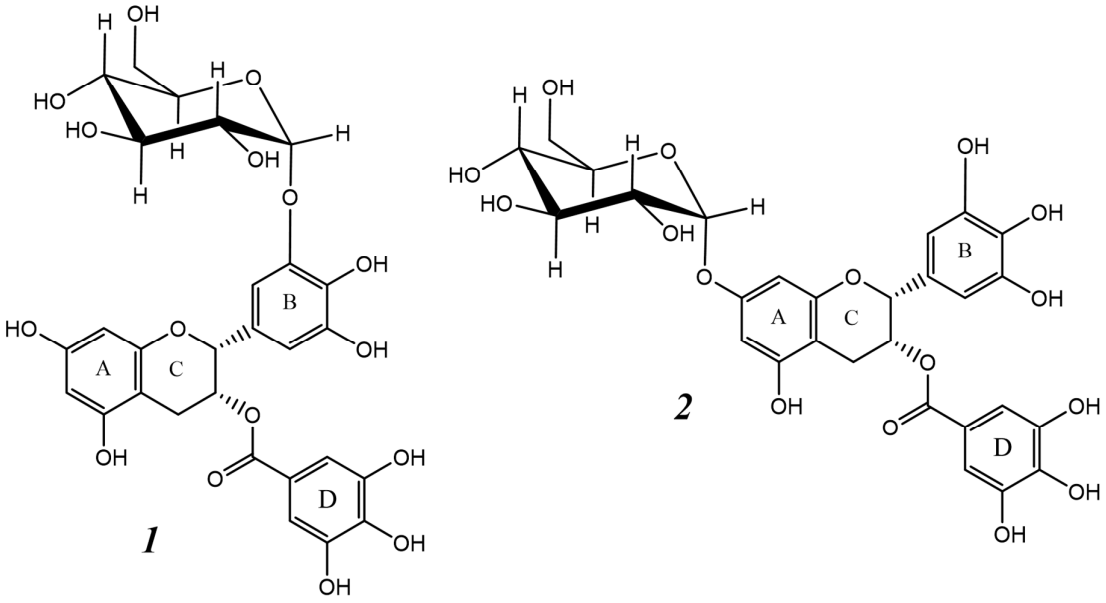
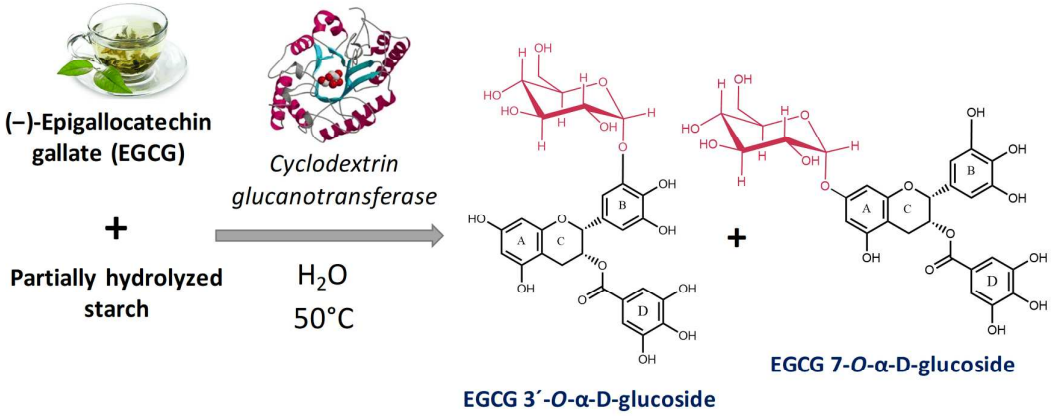

D
B
A
EGCG
^{1}H-^{13}C HSQC
EGCG
Glc-EGCG-1
B2
B6
Glc-EGCG-1
F1 [ppm]
F2 [ppm]
7.0
6.8
6.6
6.4
6.2
100
105
110
D
B
A
EGCG
^{1}H-^{13}C HSQC
EGCG
Glc-EGCG-2
Glc-EGCG-2
F1 [ppm]
F2 [ppm]
7.0
6.8
6.6
6.4
100
105
110

Fig. 7



TOC Graphic


(–)-Epigallocatechin gallate (EGCG)
+
Partially hydrolyzed starch
Cyclodextrin glucanotransferase
H2O
50°C
+
EGCG 3´-O-α-D-glucoside
EGCG 7-O-α-D-glucoside