Biosynthesis of (+)-catechin glycosides using recombinant amylucrase from Deinococcus geothermalis DSM 11300

Hyun-Kug Cho a, Hee-Hang Kim a, Dong-Ho Seo a, Jong-Hyun Jung a, Ji-Hae Park a, Nam-In Baek a, Myo-Jeong Kim b, Sang-Ho You c, Jaeho Cha d, Young-Rok Kim a, Cheon-Seok Park a,∗

a Graduate School of Biotechnology and Institute of Life Sciences & Resources, Kyung Hee University, Yongin 446-701, Republic of Korea
b Food Research Institute and School of Food and Life Science, and Biohealth Products Research Center, Inje University, Gimhae 621-749, Republic of Korea
c Department of Food Science and Technology, BK21 Project Team, and Carbohydrate Bioprodut Research Center, Sejong University, Seoul 143-747, Republic of Korea
d Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 22 December 2010
Received in revised form 29 March 2011
Accepted 6 May 2011

Keywords:
Amylosucrase
Biotransformation
(+)-Catechin
(+)-Catechin glycoside
Sucrose
Transglycosylation

ABSTRACT

Amylosucrase (ASase, EC 2.4.1.4) is a glucosyltransferase that hydrolyzes sucrose into glucose and fructose and produces amylose-like glucan polymers from the released glucose. (+)-Catechin is a plant polyphenolic metabolite having skin-whitening and antioxidant activities. In this study, the ASase gene from Deinococcus geothermalis (dgas) was expressed in Escherichia coli, while the recombinant DGAS enzyme was purified using a glutathione S-transferase fusion system. The (+)-catechin glycoside derivatives were synthesized from (+)-catechin using DGAS transglycosylation activity. We confirmed the presence of two major transglycosylation products using TLC. The (+)-catechin transglycosylation products were isolated using silica gel open column chromatography and recycling-HPLC. Two (+)-catechin major transfer products were determined through 1H and 13C NMR to be (+)-catechin-3-O-α-D-glucopyranoside with a glucose molecule linked to (+)-catechin and (+)-catechin-3-O-α-D-maltoside with a maltose linked to (+)-catechin. The presence of (+)-catechin maltooligosaccharides in the DGAS reaction was also confirmed via recycling-HPLC and enzymatic analysis. The effects of various reaction conditions (temperature, enzyme concentration, and molar ratio of acceptor and donor) on the yield and type of (+)-catechin glycosides were investigated.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Phenolic compounds are universally distributed in nature, especially in the plant kingdom [1]. These natural products are known to be essential for the growth and reproduction of plants. In fact, these compounds provide reproductive benefit as attractants of pollinators and seed dispersers [2]. Furthermore, one of the important roles of phenolic compounds is in the defense against plant pathogens and predators such as harmful insects [3,4]. Recent interest has increased in the potential protective effects of phenolic compounds against oxidative damage diseases (cardiovascular diseases and cancers) caused by reactive oxygen species (ROS) and other free radicals [5,6]. The importance of the antioxidant activities of various phenolic compounds as natural antioxidants and their possible usage in processed foods have also attracted worldwide attention in recent years.

(+)-Catechin (C15H14O6) belongs to flavan-3-ols, a class of flavonoids that use the 2-phenyl-3,4-dihydro-2H-chromen-3-ol as a structural skeleton [7]. This plant secondary metabolite is a polyphenolic flavonoid that has been isolated from a variety of natural sources including grape seeds, and tea leaves as well as the wood and bark of trees such as acacia and mahogany [8,9]. Mostly (+)-catechin is found with its other stereoisomers, (−)-catechin or ent-catechin [10]. There are many studies that describe the bio-functional activities and health benefits of (+)-catechin. In addition to its well-known bio-functional antioxidant activity [11,12], (+)-catechin was reported to induce longevity in the nematode Caenorhabditis elegans [13]. Also, (+)-catechin exhibited antitumor activities by inhibiting intestinal tumor formation in mice [14]. Furthermore, this polyphenolic antioxidant metabolite has been shown to reduce atherosclerotic lesion development in apoE-deficient mice in transcriptomic studies [15]. However, (+)-catechin is unstable, losing its antioxidant activity through a browning reaction with the exposure to light [16,17]. The low water solubility of (+)-catechin may limit its application in food industry.

∗ Corresponding author at: Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Biotechnology, Graduate School of Biotechnology, Kyung Hee University, Tel.: +82 31 201 2631; fax: +82 31 204 8116.
E-mail address: cspark@khu.ac.kr (C.-S. Park).

0141-0229/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmitec.2011.05.007
Puerarin, to their NMR. Biochemistry glycosylated to amylase 2.1. Puerarin, a functional isoflavone, was modified by maltogenic amylase to produce α-α-glucosyl-(1→6)-puerarin and α-α-maltosyl-(1→6)-puerarin, which have 14 and 168 times higher solubility than that of puerarin, respectively [21]. Catharanthus roseus cell suspension cultures were effectively employed to convert exogenously supplied curcumin to a series of glucosides. In fact, the water solubility of synthesized curcumin-4'-4"-O-β-p-digentiobioside was 20 million-fold greater than that of curcumin [22]. Importantly, the numerous glycosylated products retained their original bio-activities in vitro [19,23]. For example, the transglycosylated puerarins generated by maltogenic amylase from archeaeon Thermoﬂium pendens fully maintained their antioxidant activities [20].

Amylosucrase (ASase, EC 2.4.1.4) is a glycosyltransferase that belongs to the glycosyl hydrolase family 13 (GH 13). This enzyme not only catalyzes the hydrolysis of the glucosidic linkage in sucrose to release glucose and fructose, but also synthesizes α-glucan polymers by reacting with the released glucose molecules [26–28]. Recently, it was found that ASase can employ various biomaterials other than glucose as acceptor molecules to synthesize various transglycosylation products [24,25]. Furthermore, a semirational engineering approach was successfully applied to engineer ASase of Neisseria polysaccharae to engender this enzyme with the ability to regiospecifically glucosylate protected unnatural acceptors [29].

In this study, we employed ASase of Deinococcus geothermalis to modify (+)-catechin to (+)-catechin glycosides. Various transformed (+)-catechin glycosides were analyzed using TLC, HPLC, and NMR. In addition, the optimal condition for the superior production of (+)-catechin glycosides was determined. The manipulation of the (+)-catechin transglycosylation products via control of the donor and acceptor molecules was also investigated.

2. Material and method

2.1. Chemicals and enzymes

Sucrose and (+)-catechin hydrate were purchased from Duchefa Biochemistry (Haarlem, Netherlands) and Sigma–Aldrich (St. Louis, MO, USA), respectively. To determine the glucosidic bonds of the transglycosylation products, various glycoside hydrolases including α-glucosidase from yeast (OYC Americas, Andover, MA, USA), Sulfolobus shibatae β-glucosidase [30], and Lactobacillus gasseri maltogenic amylase (LGMA) [31] were employed. All other chemicals used in this study were of analytical reagent grade.

2.2. Strain and culture conditions

Escherichia coli BL21 [F-, ompT, hsdSR(B- m-), dcm, gal, (DE3)] harboring the cloned dgaS (D. geothermalis amylosucrase) in pGEX vector (pGEX-DGAS) was utilized for the production of DGAS in this experiment [28]. The broth medium of Luria–Bertani (LB) containing 1% tryptone (w/v), 0.5% yeast extract (w/v) and 1% sodium chloride (w/v) with ampicillin (100 μg/ml) was used for the growth of E. coli BL21 cells.

2.3. Preparation of the recombinant DGAS

Recombinant E. coli BL21 harboring pGEX-DGAS cells were grown in 500 ml LB culture with 0.1 mg/ml ampicillin in 37 °C for 3 h to obtain an optical density of 0.5–0.6 at 600 nm. The final concentration of 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction. After induction, cells were further incubated for 14 h at 18 °C. The induced cells were harvested via centrifugation at 10,000 × g for 10 min. The pellet was resuspended in phosphate buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4), and the cells were disrupted using ultra-sonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, six times for 30 s, constant duty). Finally, the extracted proteins in the solution were collected via centrifugation at 15,000 × g for 20 min.

The purification of the recombinant DGAS was performed using Glutathione-Sepharose® High Performance affinity chromatography (GE Healthcare Bio-Science AB, Uppsala, Sweden) with an affinity chromatography column of 0.8 × 4 cm (Bio-RAD, Hercules, CA, USA); PBS (pH 7.4) through which a Tris–HCl buffer (pH 8.0) containing 30 mM of reduced glutathione were used for purification. The specific purification steps were described previously [28]. The collected recombinant DGAS were dialyzed with dialysis seamless cellulose tube (Sigma–Aldrich) with optimum buffer (50 mM Tris-HCl, pH 7.0). After dialysis, the purified DGAS were concentrated with a Vivaspin column (30,000 MWCO; Sartorius Stedim Biotech GmbH, Goettingen, Germany) using centrifugation until the final protein concentration reached 12 mg/ml in optimum buffer.

2.4. Determination of the enzyme and its activity

The cell extract and purified enzyme were confirmed using SDS-PAGE. A 10% polyacrylamide gel was prepared for SDS-PAGE analysis. A 5 μl aliquot of each sample was transferred to a fresh microcentrifuge tube, to which 3 μl of 3× loading dye had been added. The samples were mixed, boiled for two minutes, and then loaded onto the polyacrylamide gel. The gel was run for 45 min at 200 V/6.5 cm and stained using Coomassie Brilliant Blue R-250.

The DGAS activity assay was performed with 146 mM sucrose and enzyme in 50 mM Tris-HCl pH 7.0 at 45 °C for 10 min. After the enzyme reaction, the 3,5-dinitrosalicylic acid (DNS) method was used to measure the released fructose. The fructose concentration in the reaction mixture was calculated using fructose as a standard. The specific activity was defined as the amount of enzyme needed to hydrolyze sucrose to 1 μmol of fructose per min in the reaction condition.

2.5. Biosynthesis of (+)-catechin glycosides

Transglycosylation reactions on (+)-catechin were carried out with three different concentrations of sucrose. The basic reaction mixture for the transglycosylation consisted of 25 mM of (+)-catechin as an acceptor, 25 mM sucrose as a donor, and 50 units of DGAS/mg of sucrose in a 50 mM Tris-HCl buffer (pH 7.0). The (+)-catechin concentration was fixed at 25 mM as a final concentration in the reaction mixture, whereas the sucrose concentrations were varied. The three molar ratios between (+)-catechin and sucrose chosen in this study were 1:1, 1:5 and 1:10 ((+)-catechin:sucrose). For the production of (+)-catechin glycoside products, the transglycosylation reaction was performed in a 50 mM Tris–HCl buffer (pH 7.0) at 30 °C instead of 45 °C since the transglycosylation activity of DGAS was known to be higher at 30 °C than it was at 45 °C [24].

2.6. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses

TLC and HPLC analyses were carried out to detect the transglycosylation products. For TLC analysis, a TLC silica gel Plate 60F254 (Merck, Whitehouse Station, NJ, USA) was activated at 110 °C for 30 min, and the separation solvent of ethyl acetate:acetic acid:water (3:1:1, v:v:v) was employed. Development of the
sample on the TLC plate was allowed after the TLC plate was completely dried in an oven. The visualization under a Reprostar 3 UV detector (CAMAG, Muttenz, Switzerland) at 280 nm was carried out to define the origin of the (+)-catechin glycosides. Finally, the TLC plate was soaked rapidly in 0.3% (W/V) N-(-1-naphthyl)-ethylenediamine and 5% (v/v) H2SO4 in methanol and then dried in 110°C oven until visible spots were clearly observed.

For the HPLC analysis, reaction mixtures were centrifuged, filtered through a 0.2 μm Nylon 66 syringe filter (Whatman, Kent, UK), and analyzed with Luna 5 μ NH2 (Phenomenex, Torrance, CA, USA) connected to a Shimadzu model SCL-10 system with a Shimadzu LC10 pump and a Shimadzu SPD-10A UV detector (Shimadzu, Kyoto, Japan) at 280 nm. Separation of products was achieved with a mobile solvent of acetonitrile and water with 1% phosphoric acid (7:3, v/v) at flow rate of 1.0 ml/min.

2.7. Open column chromatography and recycling-HPLC for separation of transglycosylated products

Large scale preparations of (+)-catechin transglycosylation products were performed with open column chromatography, whereas other small quantities of minor transglycosylation products were obtained using recycling-HPLC. The stationary phase used was silica gel 60 (Merck) powder, and the mobile phase solvent solution was composed of ethyl acetate:methanol:water (40:4:1, v/v/v). The reaction mixture sample was dried with silica gel powder in a rotary evaporator (EYELA, Tokyo, Japan). The fully dried sample was loaded flat onto the silica gel (3.5 cm diameter, 12 cm high) in the open column, and the flow rate of the mobile phase was 1.5 ml per minute. Each fraction (10 ml) was collected in a test tube, and the separation of the transglycosylation products was verified through TLC analysis. The separated compounds in the eluent were dried using a freeze-dryer (Il-shin Lab, Suwon, Korea) and were collected for further analysis.

A recycling preparative HPLC system (LC-9104, JAI, Tokyo, Japan) was used for isolation of minor (+)-catechin glycosides. Three milliliters of sample were applied to a JAIHEL-W252 (2 cm × 50 cm, JAI) column connected in tandem with a JAIHEL-W251 (2 cm × 50 cm, JAI) and guard columns. The sample was eluted with deionized water at a flow rate of 1.8 ml/min. The fractions corresponding to the detected peaks were collected and freeze-dried. The purity of each sample was confirmed via TLC analysis.

2.8. Structure determination based on the enzymatic hydrolysis of the transglycosylation products

The structures of various (+)-catechin glycosides were presumed based on enzymatic digestion with α-glucosidase, β-glucosidase, and maltogenic amylase. The (+)-catechin transglycosylation reaction mixtures were further reacted with three enzymes at the optimum reaction conditions of each enzyme [30,31]. The α-glucosidase reaction was performed at 37°C, whereas the β-glucosidase reaction was carried out at 90°C. The reaction temperature of LGMA was 50°C.

2.9. NMR analysis

The 1H and 13C NMR spectra of (+)-catechin and the purified (+)-catechin glycosides were obtained with a Varian Inova AS 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA). The sample was dissolved in DMSO-d6 at 24°C and used tetramethylsilane (TMS) as the chemical shift reference.

3. Results and discussion

3.1. Transglycosylation of (+)-catechin by DGAS using sucrose as a donor molecule

ASase is particularly useful for glycosylation of bio-materials due to its ability to utilize sucrose, an inexpensive and abundant renewable substrate, as a glucose donor molecule [24–28]. Until now, there were relatively few microbial sources of ASases, including Alteromonas macedionii, D. geothermalis, and N. polysacchara [26–28]. Among those, ASase from D. geothermalis (DGAS) is known to be the most active and thermostable [28]. In this study, DGAS was employed to modify (+)-catechin to its glycoside using sucrose as the sole donor molecule. As shown in Fig. 1, (+)-catechin could be successfully used as an acceptor for the transglycosylation activity of DGAS. Two spots (CT1 and CT2) appeared in TLC analysis (Fig. 1). These spots were also detected under UV light (data not shown), implying that they originated from (+)-catechin, since (+)-catechin was the only fluorescent chemical in the reaction. The HPLC analysis also confirmed the presence of two transglycosylation reaction products of (+)-catechin (Fig. 2). When ASase of N. polysacchara was employed to transglycosylate salicin, two salicin glycoside transfer products were detected in TLC and HPLC analyses. Those salicin glycoside transfer products were determined to be glucosyl-salicin and maltosyl-salicin [25]. Likewise, two glycosylated (+)-catechin products observed in the DGAS transglycosylation reaction were believed to be glucosyl-(+)-catechin and maltosyl-(+)-catechin.

The possible structures of the two glycosylated (+)-catechin products were determined using enzymatic analysis (Fig. 3). Three glycoside hydrolyses, including α-glucosidase, β-glucosidase, and maltogenic amylase, were used to hydrolyze the DGAS reaction products of (+)-catechin. α-Glucosidase (EC 3.2.1.20) acts on α-1,4 glycosidic linkages in many α-glucoside substrates to release glu-

![Fig. 1. TLC analysis of the transglycosylation activity of DGAS in a mixture.](image-url)
cose. The β-glucosidase catalyzes the hydrolysis of β-1,4 glycosidic bonds in the β-bond linked glucoside molecules and liberates glucose [30]. Unlike these enzymes, maltogenic amylase hydrolyzes α-1,4 glycosidic linkages similar to α-glucosidase but produces maltose instead of glucose [31].

After the reaction of α-glucosidase, a spot equivalent to glucose appeared while the spot corresponding to the compound CT1 was no longer present (Fig. 3, lane 2). There were no changes in the spots in the reaction of β-glucosidase (Fig. 3, lane 3). In contrast, the catalytic action of LGMA did not produce the compound CT2 but showed a spot for maltose (Fig. 3, lane 4).

These results indicated that the glucose molecules released by the hydrolysis of DGAS were attached to (+)-catechin through α-1,4 glycosidic linkages due to the transglycosylation activity of DGAS. Furthermore, the compound CT1 was presumed to be a glucosyl-(+)-catechin, in which one glucose molecule was linked to (+)-catechin. The compound CT2 seemed to be a maltosyl-(+)-catechin, in which two glucose molecules were connected to (+)-catechin (Fig. 4).

Interestingly, the CT2 spot was not completely absent in the presence of α-glucosidase, while no CT1 spot was produced. This situation is most likely due to the substrate specificity of α-glucosidase, which was greater for maltose than it was for maltotriose [32]. Similarly, CT1 might be a more favorable substrate than is CT2, resulting in a small quantity of CT2 remaining after the reaction with α-glucosidase.

3.2. Separation of (+)-catechin glycosides and structural analysis

To determine the exact chemical structures, the two compounds (CT1 and CT2) that appeared in TLC analysis were purified using open column chromatography as described in Section 2. Two (+)-catechin transglycosylation products were successfully separated as shown in Fig. 5, and their structures were determined using NMR analysis: 1H NMR and 13C NMR results are shown in Table 1.

The molecular mass of CT1 was calculated to be 452 daltons according to FAB mass spectrometry analysis. This result implied that CT1 was a (+)-catechin monogluco side, in which one molecule of glucose is attached to (+)-catechin. 1H NMR and 13C NMR confirmed the structure of CT1 as a (α)-catechin monogluco side (Table 1). The binding position of glucose was C-3' from the results of 1H NMR, and 13C NMR results showed that the chemical shift of C-3' (146.1) was basically unchanged and that of H-2' (δ 7.29) was lower than that of free (+)-catechin. The α-configuration of the anomeric carbon in glucose was revealed by the coupling constant (J = 3.6 Hz) in the 1H NMR spectrum. We therefore concluded that the structure of the compound CT1 was (+)-catechin-3'-O-α-D-glucopyranoside, in which a glucose molecule was linked to the C3' position of (+)-catechin (Fig. 6).

The molecular mass of compound CT2 was determined to be 614 daltons, since two peaks appeared at m/z 615 ([M + H]+) and m/z 637 ([M + Na]+) in FAB mass analysis. Similar to the structure
Fig. 4. The scheme for the (+)-catechin transglycosylation reaction of DGAS and the enzymatic assay with α-glucosidase, β-glucosidase, and LGMA for structure analyses of (+)-catechin glucoside and (+)-catechin maltoside.

Table 1

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>$^{13}$C NMR</th>
<th>$^{1}$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-catechin</td>
<td>(+)-catechin</td>
<td>(+)-catechin</td>
</tr>
<tr>
<td>CT1</td>
<td>CT2</td>
<td>CT1</td>
</tr>
<tr>
<td>2</td>
<td>82.69</td>
<td>82.7</td>
</tr>
<tr>
<td>3</td>
<td>68.70</td>
<td>68.7</td>
</tr>
<tr>
<td>4</td>
<td>28.43</td>
<td>29.0</td>
</tr>
<tr>
<td>4a</td>
<td>100.71</td>
<td>101.5</td>
</tr>
<tr>
<td>5</td>
<td>157.32</td>
<td>157.4</td>
</tr>
<tr>
<td>6</td>
<td>96.19</td>
<td>96.3</td>
</tr>
<tr>
<td>7</td>
<td>157.55</td>
<td>157.6</td>
</tr>
<tr>
<td>8</td>
<td>95.41</td>
<td>95.5</td>
</tr>
<tr>
<td>8a</td>
<td>156.67</td>
<td>156.7</td>
</tr>
<tr>
<td>1′</td>
<td>132.01</td>
<td>132.1</td>
</tr>
<tr>
<td>2′</td>
<td>115.08</td>
<td>116.8</td>
</tr>
<tr>
<td>3′</td>
<td>145.98</td>
<td>146.1</td>
</tr>
<tr>
<td>4′</td>
<td>146.00</td>
<td>148.6</td>
</tr>
<tr>
<td>5′</td>
<td>115.96</td>
<td>118.5</td>
</tr>
<tr>
<td>6′</td>
<td>119.91</td>
<td>123.9</td>
</tr>
<tr>
<td>α-D-Glucopyranose</td>
<td>1′′</td>
<td>100.09</td>
</tr>
<tr>
<td>2′′</td>
<td>73.4</td>
<td>73.07</td>
</tr>
<tr>
<td>3′′</td>
<td>74.4</td>
<td>74.74</td>
</tr>
<tr>
<td>4′′</td>
<td>71.2</td>
<td>80.88</td>
</tr>
<tr>
<td>5′′</td>
<td>74.8</td>
<td>74.68</td>
</tr>
<tr>
<td>6′′</td>
<td>62.2</td>
<td>61.68</td>
</tr>
<tr>
<td>α-D-Glucopyranose</td>
<td>1′′′</td>
<td>102.81</td>
</tr>
<tr>
<td>2′′′</td>
<td>74.24</td>
<td></td>
</tr>
<tr>
<td>3′′′</td>
<td>75.05</td>
<td></td>
</tr>
<tr>
<td>4′′′</td>
<td>71.52</td>
<td></td>
</tr>
<tr>
<td>5′′′</td>
<td>73.07</td>
<td></td>
</tr>
<tr>
<td>6′′′</td>
<td>62.73</td>
<td></td>
</tr>
</tbody>
</table>

of CT1, the chemical structure of CT2 was undoubtedly confirmed through $^{1}$H NMR and $^{13}$C NMR. Both NMR spectra showed that the molar ratio of (+)-catechin to glucose in compound CT2 was 1:2 (Table 1). In addition, the bond between the two glucose molecules was determined to be α-1,4-glycosidic linkage according to the coupling constant ($J = 3.6$ Hz) in the $^{1}$H NMR spectrum. From these results, we concluded that the structure of compound CT2 was (+)-catechin-3′-O-α-D-maltoside, in which two glucose molecules (maltose) were connected to the C3′ position of (+)-catechin (Fig. 6). The structures of the two (+)-catechin transglycosylation products corresponded exactly to the expected chemical structures obtained from enzymatic analysis (Figs. 3 and 4).

Previously, the transglycosylation mechanism of ASase had been shown to transfer a glucose molecule released from the hydrolysis of sucrose to the sugar moieties of various acceptor molecules. Such a transfer was reported in the formation of amylose-like polymer, salcin-, and arbutin-transglycosylation products [24–26]. As a result, an α-1,4-glycosidic linkage between the transferred glucose and a sugar moiety in the acceptor molecule was formed. In this study, we have clearly demonstrated that a phenolic aglycone compound can also act as an acceptor molecule of ASase transglycosylation activity.

3.3. Reaction conditions for the production of (+)-catechin transglycosylation products

Various reaction conditions were examined in order to obtain a maximum yield of (+)-catechin transglycosylation products. First,
the reaction temperatures were investigated in the temperature range of 25–45 °C. At higher temperatures (40 and 45 °C), the yields of (+)-catechin transglycosylation products from DGAS were significantly lower than that was at 35 °C (data not shown), as was expected based on the characteristics of DGAS [24,28]. DGAS showed varied dual responses (hydrolysis and transglycosylation) based on the reaction temperature. Transglycosylation occurred preferentially at lower temperatures, while hydrolysis was predominant at higher temperatures. In general, glycoside hydrolyses are supposedly capable of creating as well as breaking glycosidic bonds in the substrate molecule depending on the thermodynamic equilibrium. A hydrolysis reaction which breaks glycosidic bonds by glycoside hydrolyses is more favorable at a high temperature until it reaches to an unfolding temperature [33]. However, under suitable conditions, such as with an optimal water content, the addition of BSA, the presence of an organic solvent, and a high acceptor concentration, hydrolytic enzymes can be induced to form glycosides. However, the reaction temperature could be decreased lower than 35 °C in this experiment. At temperatures lower than 30 °C, the solubility of (+)-catechin decreased, yielding a white (+)-catechin precipitant. Therefore, the reaction temperature of DGAS transglycosylation of (+)-catechin was set at 35 °C.

The effect of the ratio between acceptor and donor concentrations was investigated. Two ratios, 1:1 and 1:10 (acceptor:donor, molar basis), were employed and the reaction products were examined using TLC and HPLC analyses. The concentration of (+)-catechin was fixed at 25 mM due to its low solubility. With the ratio of 1:1, the TLC analysis showed a major transglycosylation products of CT1 and a relatively small quantity of CT2 (Fig. 3, lane 1). However, at the ratio of 1:10, the CT1 spot disappeared and a spot corresponding to CT2 was the most prevalent (Fig. 3, lane 5). Interestingly, some smears were observed above the fructose spot, indicating that there may have been some other transglycosylation products synthesized by DGAS.

The presence of (+)-catechin transglycosylation products other than CT1 and CT2 were confirmed through recycling preparative HPLC analysis (Fig. 7). The peaks that appeared in the HPLC chromatogram showed a regular interval between peaks. In addition, those compounds were apparently derived from (+)-catechin, based on the use of UV as a detection method. Therefore, we presumed that those peaks corresponded to various (+)-catechin maltooligosaccharides, in which maltooligosaccharides (maltotriose, maltotetraose, and maltopentaose) were attached to the (+)-catechin molecule. The enzymatic digests of these compounds by maltogenic amylase produced (+)-catechin and (+)-catechin-3′-O-α-D-glucopyranoside spots in the TLC analysis, supporting our assumption of structure. We concluded that DGAS mainly produced (+)-catechin-3′-O-α-D-glucopyranoside at the low donor to acceptor ratio (1:1). With the increase of donor concentration, comparatively more (+)-catechin-3′-O-α-D-maltoside and other (+)-catechin maltooligosaccharides were synthesized (Fig. 8). This observation implies that DGAS transfers a glucose moiety released from the hydrolysis of sucrose to another glucose molecule. The resulting product, (+)-catechin-3′-O-α-D-glucopyranoside, can be used as an acceptor when it is produced in proper quantities. This result indicates that the profile of the transglycosylation products can be controlled by modulating the ratio of acceptor to donor molecules in the DGAS reaction.
Therefore, a selective synthesis of transglycosylation product is possible to simplify separation.

The effect of the enzyme concentration on the DGAS transglycosylation reaction was investigated. The higher was the enzyme (DGAS) concentration used in the reaction, the faster was the transglycosylation. The increase in DGAS concentration from 50 U/ml to 500 U/ml reduced the final reaction time such that the same quantity of transglycosylation reaction products was produced in only 1 h rather than 24 h. However, the profile of (+)-catechin–transglycosylation products was unchanged at the different concentrations of enzyme.

Previously, several carbohydrate-active enzymes have been reported to produce (+)-catechin glycosides. An α-amylase from Trichoderma viride JCM22452 was able to glucosylate a wide range of natural flavonoids, particularly (+)-catechin [34]. The glucose donor molecule of this reaction was a dextrin. The (+)-catechin glycosides synthesized were diverse, and their structures were determined to be (+)-catechin 5-O-α-D-glucopyranoside, (+)-catechin 5-O-α-D-maltoside, and (+)-catechin 4’-O-α-D-maltoside [34]. A cellulase from Aspergillus niger was employed to synthesize a (+)-catechin β-D-fucopyranoside using p-nitrophenyl-β-D-fucopyranoside as a donor molecule. An α-glucosidase from Bacillus stearothermophilus was able to produce a mixture of (+)-catechin 7-O-α-D-glucopyranoside and (+)-catechin 5-O-α-D-glucopyranoside, present in approximately equal quantities, using maltose as a donor molecule [35]. The chemical structures of these resulting products were different from those of the transglycosylation products (CT1 and CT2) found in the DGAS reaction. These results show that each enzyme has a specific transglycosylation mechanism on the same acceptor molecule, and that each enzyme synthesizes a diverse range of transglycosylation products. In addition, there is an obvious advantage to the use of DGAS in that the donor molecule is relatively inexpensive.

The transglycosylation yield of (+)-catechin β-D-fucopyranoside by a cellulase from A. niger was measured as 26%, whereas that of a mixture of (+)-catechin 7-O-α-D-glucopyranoside and (+)-catechin 5-O-α-D-glucopyranoside by α-glucosidase from B. stearothermophilus was determined to be 20% [35]. The yield of (+)-catechin transglycosylation caused by DGAS was more than 97% on the basis of (+)-catechin added and it was independent of the concentration of donor molecule at acceptor to donor ratios greater than 1:1. The efficiency of DGAS transglycosylation was higher than those of all other published sources.

**Fig. 8.** Catechin transglycosylation reaction by DGAS with different acceptor to donor ratio.
Markings on the TLC plate represent: sucrose (S) and fructose (F) standards (lane 1), control reaction (lane 2), reaction with acceptor to donor ratio of 1:1 (molar ratio, lane 3), reaction with acceptor to donor ratio of 1:5 (molar ratio, lane 4), and reaction with acceptor to donor ratio of 1:10 (molar ratio, lane 5).

**4. Conclusions**

The biosynthesis of (+)-catechin glycosides with DGAS was successfully accomplished using sucrose, an inexpensive substrate, as a donor molecule. The structures of main transglycosylated products were determined by ^1^H and ^13^C NMR to be (+)-catechin-3’-O-α-D-glucopyranoside and (+)-catechin-3’-O-α-D-maltoside. The enzymatic digestion experiment confirmed these product identifications. The presence of (+)-catechin-3’-O-α-D-maltooligosaccharides was also confirmed through recycling preparative HPLC and maltogenic amylase digestion. The effects of various reaction conditions on the profile of the transglycosylated products revealed that the ratio between acceptor and donor was the most critical factor on the control of the (+)-catechin products. Also, the reaction temperature and enzyme concentration affected the yield of the transglycosylation products in the DGAS reaction.
Acknowledgements

This work was supported by a Korea Research Foundation grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-313-F00145).

References