Original Article

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Galactosylation of caffeic acid by an engineered β-galactosidase

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Summary **Glycosylation is useful for improving the chemical properties and physiological functions of biologically and pharmacologically important compounds. The glycosylation of phenolic compounds can increase their solubility and stability in water. The addition of galactose residue has special meaning as it facilitates targeted delivery of drugs to the liver cancer cells with abundant galactose acceptors on the cell surface. In this work, the engineered β-galactosidase W980F from *Lactobacillus bulgaricus* L3 was utilized for the glycosylation of caffeic acid, a well-known phenolic phytochemical with broad bioactivities. The reaction was performed by incubation of the enzyme with 200 mM of lactose and 100 mM of caffeic acid at 45°C for 1 h. The product was purified and analyzed by MS and NMR spectra. The MS revealed a signal of $[M-H]^-$ at *m/z* 341.09, suggesting monogalactosylated products of caffeic acid (M_r 342). The NMR spectra further identified the products to be caffeic acid 3'-*O*-β-galactopyranoside and caffeic acid 4'-*O*-β-galactopyranoside in a ratio of 1:3. This was the first discovery that caffeic acid could be galactosylated by the engineered glycosidase.**

Keywords: *Lactobacillus bulgaricus* L3, β-galactosidase, galactosylation, caffeic acid

1. Introduction

Caffeic acid, 3,4-dihydroxycinnamic acid, is a well-known phenolic phytochemical present in plants such as coffee and honey. It reportedly has broad bioactivities including anti-oxidant, anti-cancer, anti-inflammatory, immunomodulatory, neuroprotective and tissue reparative effects (*1-10*). The clinical and experimental findings demonstrated anticancer properties of caffeic acid against both ER (estrogen receptor-α) positive and ER negative breast cancer (*1*). Additional data showed it attenuated solar UV-induced skin carcinogenesis (*2*) and reduced the risk of patocellular carcinoma caused by hepatitis C virus (*3*). Also, caffeic acid inhibited the multiplication of viruses such as influenza A virus, herpes simplex virus, and polio virus (*4*), and suppressed the growth of bacteria such as *Pseudomonas aeruginosa* commonly isolated from wound infections (*5,6*). Besides, caffeic acid exhibited an immunomodulatory action in human monocytes with no cytotoxic effects (*7*). Moreover, caffeic acid showed inhibitory activity against α-synuclein fibrillation, which would be helpful for design of novel therapeutic drugs for Parkinson's disease (*8*). More importantly, caffeic acid exerted neuroprotective and antidementia effects, at least in part, by preventing the loss of neural cells and synapses in ischemic brain injury (*9,10*). Despite the broad biological activities, the low stability and poor solubility of caffeic acid reduced its applicability as a pharmaceutical product (*6*).

Glycosylation is considered to be a very useful method for improving the chemical properties and physiological functions of biologically and pharmacologically important compounds (*11-15*). The introduction of the glycosyl residue into the phenolic compounds can increase their solubility and stability in water and protect them from oxidation (*11,12*). Also, glycosylation can improve biological and pharmacological functions, including the decrease of toxicity and side effects, as well as the increase of bioavailability of drugs that need to pass through the blood-brain barrier (*11*). For instance, hydroquinone is toxic while its glucoside arbutin has antibacterial and skin whitening effects (*12*). Similar finding is about the eugenol with function as a hair restorer but liable to sublimate, the glucoside of which can be gradually degraded into eugenol by the indigeneous microorganisms of human skin and acts as a pro-drug

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additive in commercial hair-restorers (*13*). Another example is that the glucosides of quercetin exhibit unexpectedly improved bioavailability (*14*). In a word, new glycosides of phenolic compounds could have novel pharmacological properties (*12*).

Variation of the introduced glycosyl residues shows different impact on the properties of phenolic compounds. The galacosylated hydroquinone is 1.19 times higher antioxidant than the glucoside counterpart, arbutin (*15*). More interestingly, there exist abundant galactose acceptors on the hepatocyte surface that could bind with galactose derivatives (*16*). Thus, the glycosylation with galactose could aid targeted delivery of drugs to liver cancer cells but not to nearby normal cells. For example, the galatosylated lithocholic acid shows high specificity to mouse liver cells *in vivo* (*17*).

Generally, there are two basic classes of methods for the glycosylation (*18*). One is the chemical method that requires laborious protection and deprotection steps to control the stereo- and regio-specificity of products, along with the use of toxic catalysts and solvents. The other is the enzymatic glycosylation that possesses the advantages of high stereo- and regio-selectivity and can be accomplished in one step under mild conditions. Glycosyltransferases (EC 2.4) and glycosidases (EC 3.2.1) are two classes of enzymes responsible for this application (*19*). Typically, glycosyltransferases are effective and catalyze stereo/regio-selective reactions, but they have strict substrate selectivity and require costly glycosyl donors in the one-step reactions. As for large-scale synthesis, the glycosidases exhibit obvious advantages including the readily available enzyme source, the simple and inexpensive donor substrates, as well as the wide acceptor tolerance (*20*).

β-Galactosidases (EC 3.2.1.23) are among the most important glycosidases that can catalyze the galactosylation process (*21*). However, only one natural β-galactosidase from *Kluyveromyces lactis* was reported to be able to galactosylate hydroquinone (*15*). The low nucleophilicity of phenolic hydroxyl groups might be related to the difficulty of their glycosylation by the enzymes. In the previous work, the β-galactosidase from *Lactobacillus bulgaricus* L3 had been engineered to possess high transglycosylation activity toward a series of single phenolic-ring compounds including phenol, hydroquinone, catechol and pyrogallol (*22*). In this work, the enzyme was extendedly used to glycosylate the caffeic acid with a more complex phenolic structure, resulting in caffeic acid galactosides potentially with novel pharmacological properties.

2. Materials and Methods

2.1. *Strains*

The β-galactosidase gene from *L. bulgaricus* L3 had been submitted to GenBank with accession No. EU734748.1. This gene was inserted into the pET-21b vector (pET-21b-bga) and subjected to site mutation (W980F) in the previous work (*22*). The *Escherichia coli* BL21(DE3) carrying the recombinant wild-type and mutant genes were cultured in LB medium containing 10 g of peptone, 5 g of yeast extract and 5 g of NaCl in 1,000 mL of water (pH 7.5), supplemented with ampicillin at 100 μg/mL. The solid medium additionally included 15 g/L agar.

2.2. *Preparation of the W980F enzyme*

The *E. coli* BL21(DE3) carrying the recombinant mutant genes was grown in 30 mL of LB medium containing ampicillin at 37°C for 12 h, and then transferred into 1 L of fresh medium in a 1:100 (v/v) ratio. The enzyme was induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG) when the cell density reached 0.6-1.0 at 600 nm. After continuous cultivation for 3 h, the cells were harvested and disrupted by ultrasonic treatment. The lysate was centrifuged at 12,000 rpm for 30 min at 4°C and the enzyme was purified from the suspension by Ni^{2+} chelation chromatography. The column with 5 mL of Ni Sepharose (GE Healthcare) was equilibrated with phosphate buffer (50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0). The column was loaded with samples and washed with phosphate buffer and subsequent with washing buffer (50 mM sodium phosphate, 500 mM sodium chloride, 50 mM imidazole, pH 7.0). Bound protein was eluted with elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 300 mM imidazole, pH 7.0). Aliquots of the fractions were run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue to analyze their purity. Fractions containing the pure protein were pooled, dialyzed and stored at -20°C. The wild-type enzyme was prepared with the same procedures.

2.3. *Enzyme and protein assays*

The β-galactosidase activity was measured by adding 50 μL of enzyme solution to 450 μL of 2 mM *o*NPGal. The reaction was performed at 37°C for 10 min and then stopped by adding 1 mL of 500 mM Na_2CO_3. The amount of *o*-nitrophenol released was measured at 420 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmol of *o*-nitrophenol per minute under the assay conditions. The amount of protein was quantified by the method of Lowry with bovine serum albumin as the standard.

2.4. *Transglycosylation reactions*

The transglycosylation reactions were performed at 45°C for 1h in 50 μL mixtures (pH 7.0) containing 0.2 μg of pure enzyme, 200 mM of lactose and 100 mM of caffeic acid. The control reactions followed the same

conditions except for the use of inactivated enzyme. All the reactions were terminated by heating at 100°C for 5 min. Glycoside products were detected by TLC and HPLC as described below.

2.5. *TLC and HPLC analysis*

TLC was performed with Silica gel 60 F254 plates (Merck, Germany). The developing solvent was a mixture of *n*-butanol:ethanol:water (5:3:2, v/v/v). Sugars on the TLC plate were detected by spraying with a solution of 0.5% (w/v) 3,5-dihydroxytoluene dissolved in 20% (v/v) sulfuric acid and subsequent heating at 120°C for 5 min. HPLC was performed by Agilent 1200 series equipped with Agilent Zorbax carbohydrate analysis column (4.6 × 250 mm). The column temperature was maintained at 30°C. Samples were eluted with 85% (v/v) acetonitrile at a flow rate of 1.0 mL/min, through a refractive index detector (G1362A) for the sugar analysis or a UV detector (G1314B) at 254 nm for the analysis of phenolic derivatives. Conversion yield of aromatic product (%): [product concentration (mM)/concentration of lactose used (mM)] × 100 (*15*).

2.6. *Purification of glycoside products*

Ten milliliters of transglycosylation reactions were performed at 45°C for 1 h. The resulting glycosylated products were concentrated by vacuum freeze dehydration. Then, the samples were subjected to a Bio-Gel P2 column (1.5 × 100 cm) and subsequently loaded on preparative Silica gel 60 F_{254} 1-mm plates (Merck, Germany) for purification.

2.7. *MS and NMR analysis*

Mass spectra were recorded on a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan) equipped with an ESI source in negative ion mode at a resolution of 10,000 full width at half-maximum. ^{1}H and ^{13}C NMR spectra were recorded at 26°C with a Bruker DRX Advance-600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts were given in ppm downfield from internal TMS of D_2O. Chemical shifts and coupling constants were calculated from a first-order analysis of the spectra. Assignments were fully supported by homo- and hetero- nuclear correlated 2D techniques, including COSY (correlation spectroscopy), HSQC (hetero-nuclear single quantum coherence) and HMBC (hetero-nuclear multiple band correlation) experiments following standard Bruker pulse programs.

3. Results and Discussion

The wild-type and mutant (W980F) recombinant β-galactosidases from *L. bulgaricus* L3 were expressed in *E. coli* BL21 (DE3), and subsequently purified to electrophoresis purity through Ni^{2+} chelation chromatography. The ability of their transglycosylation toward caffeic acid were tested by incubation of the enzymes with lactose and caffeic acid at 45°C for 1 h. Then the reactions were detected by TLC. As shown in Figure 1a, an obvious novel product spot appeared in the reaction catalyzed by the mutant W980F when compared with that catalyzed by the wild-type enzyme. In the β-galactosidase-catalyzed reactions, the side products, glucose and galactose, were formed by the enzymatic hydrolysis of lactose, while the prebiotic galactooligosaccharides (GOS) were produced by the enzymatic self-condensation of lactose (*21,22*). The migration distance of the novel product was larger than the monosaccharides resulting from the hydrolysis reaction, consistent with the characteristic of the phenolic glycoside. HPLC analysis of the reaction mixture revealed a 7% conversion yield of the new product.

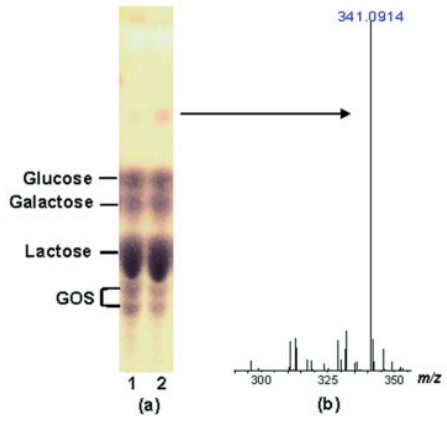

Figure 1. TLC analysis of the transglycosylation reactions catalyzed by wild-type and mutant enzymes (a) as well as MS analysis of the purified novel product (b). Lane 1, reaction catalyzed by the wide-type enzyme; Lane 2, reaction catalyzed by the W980F enzyme.

The novel product was purified and analyzed by negative ESI-MS. Figure 1b showed a characteristic signal of $[M-H]^-$ at *m/z* 341.09, confirming that the newly produced glycoside was monogalactoside (M_r 342) of caffeic acid (M_r 180). Although the TLC result revealed one spot that was monoglycosylated as determined by MS, NMR analysis of the derivative displayed signals of two products (Figures S1-S5, *http://www.ddtjournal.com/docindex.php?year=2015&kanno=2*). The signals of galactose were overlapped while those of caffeic-acid residues were separate. In the ^{1}H NMR spectra, chemical shifts of the galactose protons located at δ 4.92 to 3.64 ppm while those of caffeic acid were in the range of δ 7.27 to 6.22 ppm (Figure S1). The anomeric H-1 peaks at 4.92 ppm as well as the coupling constant of H-1 and H-2 (J = 7.8 Hz) confirmed the β-linkage between the sugar and caffeic acid. In ^{13}C NMR spectrum, chemical shifts of the sugar carbons located at δ 101.1 to 60.5 ppm while those

from δ 181.4 to 114.6 ppm belonged to the caffeic-acid residues (Figure S2).

The complete structural characterization was achieved using 2D-NMR analysis, including ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and HMBC experiments, to assign the chemical shifts and configurations. In HMBC, the C-3′ (δ 144.9) and C-4′ (δ 145.9) in two separate phenolic rings displayed correlation signals with the anomeric H-1 of the sugar residue, respectively (Figure S5). Based on the above analysis, the chemical structure of the glycoside products were identified to be caffeic acid 3'-*O*-β-galactopyranoside and caffeic acid 4'-*O*-β-galactopyranoside, respectively. According to the peak area of phenolic H-5' in the ^{1}H NMR spectra, the ratio of the two products was 1:3. Thus, their structure data were summarized in Table 1.

Figure 2 showed the outline of the biosynthesis of these compounds using the engineered β-galactosidase from *L. bulgaricus* L3. The caffeic acid was galactosylated for the first time *via* glycosidase-mediated catalysis. Although glycosidases have great advantage of relaxed substrate specificity for acceptors in the enzymatic synthesis, the glycosylation of phenolic compounds were still challenging for these enzymes. The low nucleophilicity of phenolic hydroxyl groups might be responsible for the difficulty in the enzymatic glycosylation when compared to the alcoholic hydroxyl nucleophiles that are easily glycosylated by the enzymes. Currently, only a few natural glycosidases were discovered to be able to glycosylate phenolic compounds. They were glucose-transferred glycosidases in most cases, such as α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) from *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Xanthomonas campestris* and *Xanthomonas maltophilia* (*12,13, 23-25*). Only one natural β-galactosidase was available from *K. lactis* for the galactosylation of simple hydroquinone (*15*).

The β-galactosidases have attracted particular interest due to their production of promising galactose-containing chemicals, including diverse oligosaccharides, alkyl-glycoside, glycoconjugates and others that play important roles in the industries of food additives, cosmetics, and medicines (*21*). However, these enzymes still have limitations in the glycosylation of phenolic compounds that have many physiological functions. The β-galactosidase from *L. bulgaricus* L3 was proved to be a wonderful tool for glycoside synthesis in the previous work. It could efficiently synthesize galacto-oligosaccharides as well as 6'-galactosyl sucralose with combined functions of prebiotics and sweeters (*26-28*). In the recent work, the site-saturation mutagenesis of the β-galactosidase from *L. bulgaricus* L3 generated a W980F mutant with broadened substrate specificity toward pyrogallol. In this work, the W980F was found to be able to glycosylate caffeic acid, suggesting the change of tryptophan into phenylalanine made it possible for glycosylation of more complex polyphenol structure. The acceptor specificity of the enzyme was further expanded. This was a breakthrough in the galactosylation of unusual acceptors by glycosidases. Also there reported other successful examples of glycosidases engineering that optimized enzyme properties including broadening the acceptor specificity. One example was that the nucleophile variant of cellulase (E197S) from *Humicola insolens* displayed transglycosylation activity towards flavonoid acceptors, which were not part of its normal substrate (*29*).

It is also worth noting that glycosylation of the caffeic acid was of great meaning considering that glycosylation of phenolic compounds could bring improved properties such as increased solubility and stability in water (*11-15*). The solubility of caffeic acid in water as well as its stability toward light irradiation

Table 1. ^{1}H and ^{13}C NMR data assignment for the caffeic-acid glycosides produced by the W980F enzyme

C-atom	CA-4'-*O*-β-Gal*		CA-3'-*O*-β-Gal	
	δC	δH	δC	δH
C-1	101.1	4.92	101.1	4.92
C-2	70.3	3.72	70.3	3.72
C-3	72.3	3.64	72.3	3.64
C-4	68.3	3.86	68.3	3.86
C-5	75.4	3.73	75.4	3.73
C-6	60.5	3.64	60.5	3.64
C-1'	130.9	-	127.9	-
C-2'	114.6	7.04	114.9	7.27
C-3'	145.6	-	144.9	-
C-4'	145.9	-	147.3	-
C-5'	116.0	7.02	116.5	6.82
C-6'	120.8	7.00	123.9	7.08
C-7'	175.9	-	181.4	-
C-8'	122.9	6.25	121.8	6.22
C-9'	140.0	7.14	140.4	7.16

* CA, caffeic acid.

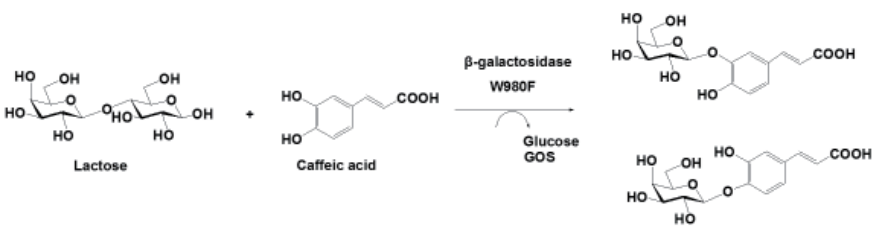

Figure 2. W980F-catalyzed glycosylation of caffeic acid and chemical structures of the glycoside products.

had been improved by the glucosylation without the loss of any biological activity, such as its antiozidative and antimutagenic properties (*23*). This confirmed the positive effects of glycosylation on the optimization of phenolic compounds. As properties of glycosides could vary with the type of sugars attached, it is of particular meaning to modify phenolic compounds with novel sugars. The W980F-mediated galactosylation provided an alternative to the existing enzymatic glucosylation method for the modification of caffeic acid. The enzymatic introduction of galactose into the caffeic acid would bring promising properties and widen the scope of their applications such as the design of liver-targeted drugs. Thus, the caffeic acid galactosides obtained in this work not only possess inherent values by themselves but also could act as intermediates for further modification to be highly valuable products.

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