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Synthesis of Aesculetin and Aesculin Glycosides Using Engineered *Escherichia coli* Expressing *Neisseria polysaccharea* Amylosucrase

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Because glycosylation of aesculetin and its 6-glucoside, aesculin, enhances their biological activities and physicochemical properties, whole-cell biotransformation and enzymatic synthesis methodologies using *Neisseria polysaccharea* amylosucrase were compared to determine the optimal production method for glycoside derivatives. High-performance liquid chromatography analysis of reaction products revealed two glycosylated products (AGG1 and AGG2) when aesculin was used as an acceptor, and three products (AG1, AG2, and AG3) when using aesculetin. The whole-cell biotransformation production yields of the major transfer products for each acceptor (AGG1 and AG1) were 85% and 25%, respectively, compared with 68% and 14% for enzymatic synthesis. These results indicate that whole-cell biotransformation is more efficient than enzymatic synthesis for the production of glycoside derivatives.

Keywords: Aesculetin, aesculin, amylosucrase, biotransformation, *Neisseria polysaccharea*, transglycosylation

Chinese herbal medicine has been widely used for centuries in treating various diseases. Cortex Fraxini, a commonly used traditional oriental medicine comprising the stem bark of *Fraxinus rhynchophylla* Dence (Oleaceae), is used for treating leucorrhea, gout, and arthritis [1]. Aesculetin (6,7-dihydroxycoumarin) and its derivative aesculin (6,7-dihydroxycoumarin-6-*O*-β-glucopyranoside) are major active ingredients in Cortex Fraxini [2] and have been reported to be effective antioxidant, anti-inflammatory, and anticancer molecules [3–5]. Aesculetin reduces inflammation by preventing the adhesion of leukocytes and endothelial cells by inhibiting the secretion of soluble intercellular adhesion molecule [6]. Aesculetin also inhibits the production of proinflammatory cytokines during the interaction between adipocytes and macrophages. Finally, aesculin inhibits oxidative damage in liver and lung inflammation in an LPS-induced acute lung injury model in mice by suppressing NF-κB, a key proinflammatory transcription factor [7, 8]. The pharmacokinetic properties of aesculetin and aesculin have been examined in rats to determine the bioavailability of these chemicals by monitoring their appearance in plasma following oral administration [9]. The oral bioavailability of aesculin was only 0.62%, indicating that it is barely absorbed via the oral route.

The glycosylation of various phytochemicals by bacterial glycosyltransferases (GTFs) is widely known to enhance their bioavailability. Amylosucrase, one of the various non-Leloir GTFs, exhibits a remarkable ability to synthesize glycoside derivatives via transglycosylation reaction, and various phytochemicals such as rutin, baicalein, arbutin, catechin, and salicin have been glycosylated by this enzyme, and the glycosylated products display enhanced solubility and stability compared with that displayed by the native compounds [10–14]. However, the utility of these enzymatic syntheses is limited by low conversion rates. Furthermore, the conversion yield is highly dependent on the phytochemicals being glycosylated. The aim of this study was to identify a more efficient process to increase the yield of the glycoside derivatives. To this effect, the ability of *Neisseria polysaccharea* amylosucrase (NPAS) to glycosylate aesculetin and aesculin was examined, and this showed that whole-cell biotransformation is a more

convenient and efficient method for the production of aesculetin and aesculin glycosides.

Aesculetin, aesculin, and sucrose were purchased from Sigma-Aldrich (USA). Recombinant *Escherichia coli* BL21(DE3) expressing NPAS on a pET21a plasmid was grown overnight at 37°C in Luria–Bertani medium containing 100 μg/ml ampicillin. Expression and purification of recombinant NPAS (rNPAS) was carried out as previously described [15]. Transglycosylation (0.1 ml) was carried out in 20 mM Tris-HCl buffer (pH 7.5) at 30°C for 12 h, using 5 μg of purified enzyme with 10 mM sucrose as substrate and 1 mM aesculetin or aesculin as the acceptor. The reaction was terminated by heating the mixture in boiling water for 10 min and then placing the reaction tube on ice. For whole-cell biotransformation, NPAS-expressing *E. coli* cells were grown as described above in 100 ml of medium. The cells were induced at an optical density of 0.6 by adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside, and growth was continued at 18°C for 12 h. The cells were then harvested by centrifugation at 8,000 ×*g* for 10 min, and the cell pellets were thoroughly washed with M9 minimal medium followed by resuspension in the same medium containing 0.4% glucose and 100 μg/ml ampicillin. The resuspended cells were incubated with 1 mM aesculetin or aesculin and 10 mM sucrose with rotation at 150 rpm at 30°C. Aliquots were withdrawn at various time points and the cells pelleted by centrifugation at 12,000 ×*g* for 10 min. Supernatant fractions were filtered using 0.2 μm syringe filters (Pall, USA) and the glycosylated derivatives were analyzed by high-performance liquid chromatography (HPLC) using a Nanospace SI-2 HPLC system (Shiseido, Japan) equipped with a UV detector (Shodex, Japan). Separations were carried out on a reversed phase Capcell Pak C_{18} MG (4.6 × 250 mm) column (Shodex, Japan) at a flow rate of 1.0 ml/min. The mobile phases consisted of buffer A (0.5% acetic acid in water) and buffer B (methanol), and chromatography was conducted isocratically in 22% buffer B. Each compound was detected at 340 nm, which corresponds to the absorbance maximum of aesculetin. The conversion yield was defined as the ratio of the synthesized aesculetin or aesculin glycosides to the initial aesculetin or aesculin added. Purification of the major glycosylated aesculin was carried out using a preparative recycling HPLC system (LC-918; JAI, Japan) equipped with a refractive index detector as previously described [16]. Approximately 3 ml of the reaction mixture was loaded onto a combination JAIGEL-W252/W251 gel filtration column (2 cm × 50 cm) and eluted with deionized water at a flow rate of 3 ml/min. The chemical structure of the purified major aesculin glycosylation product was analyzed by nuclear magnetic resonance (NMR).

Previous studies optimized reaction conditions for the hydrolysis and transglycosylation activities of rNPAS with respect to substrate concentration, temperature, enzyme concentration, and pH [15]. Furthermore, the mechanism of glucosyl transfer to the acceptor molecule by this enzyme was also elucidated when salicin was used as an acceptor. The molecular structures of the salicin transfer products analyzed by NMR indicated that rNPAS exclusively transfers glucose released from sucrose hydrolysis to an acceptor salicin to form the α-(1,4)-glycosidic linkage [15]. In this study, the newly synthesized aesculetin or aesculin

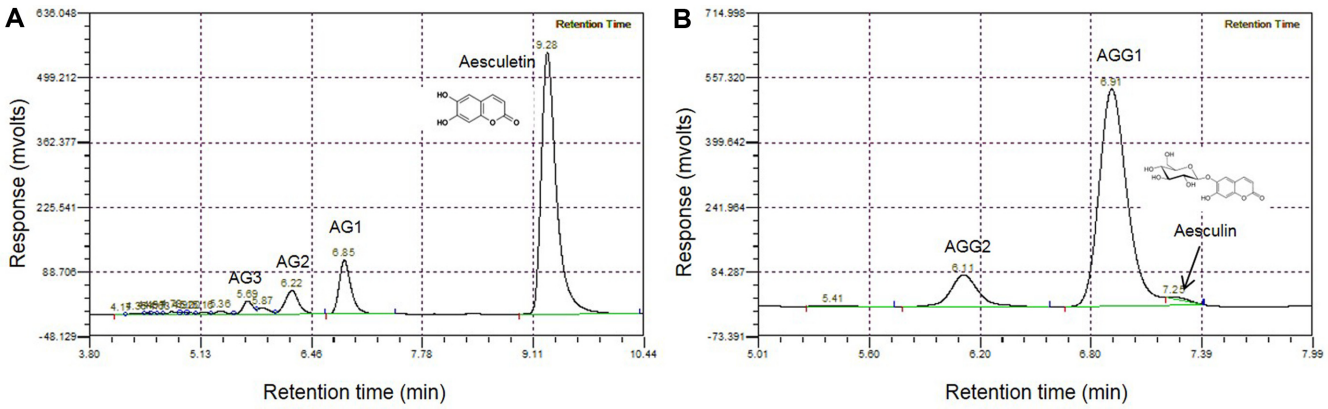

Fig. 1. High-performance liquid chromatography analysis of aesculetin (**A**) and aesculin (**B**) transfer products synthesized by whole-cell biotransformation using engineered *Escherichia coli* expressing *Neisseria polysaccharea* amylosucrase.

The conversion rates of aesculetin and aesculin were the highest at 36 and 30 h, respectively. AG1, AG2, and AG3 were aesculetin transfer products, and AGG1 and AGG2 were aesculin transfer products.

transfer products were also produced via rNPAS-catalyzed transglycosylation reaction. In the case of the enzymatic method, 1 mM of aesculetin was added at the start of the reaction, whereas in the whole-cell biotransformation method, 100 μM aesculetin was added to the reaction mixture at 0, 4, 8, 12, and 18 h, and finally 500 μM at 24 h, to give a total aesculetin concentration of 1 mM in the reaction because a high concentration of aesculetin was toxic in *E. coli*. At different time points, an aliquot (200 μl) was collected and the reaction products were analyzed by HPLC. Three transfer products (AG1, AG2, and AG3) were detected when using aesculetin as an acceptor, whereas two transfer products (AGG1 and AGG2) were detected when using aesculin (Fig. 1), with AG1 or AGG1 being the major transfer products for the two respective acceptors. Much higher yields of glycoside derivatives were obtained when aesculin was used as the acceptor. In the case of aesculin composed of aesculetin linked with a glucose in 6 position, a glucose moiety would be a good acceptor unit for the transglycosylation reaction of NPAS. This phenomenon was also observed in studies of amylosucrase-mediated synthesis of resveratrol and its 3-glucoside, piceid [12]. There was no difference in the structures of the glycoside derivatives synthesized by the two different methods (data not shown).

The molecular structure of the major transglycosylated

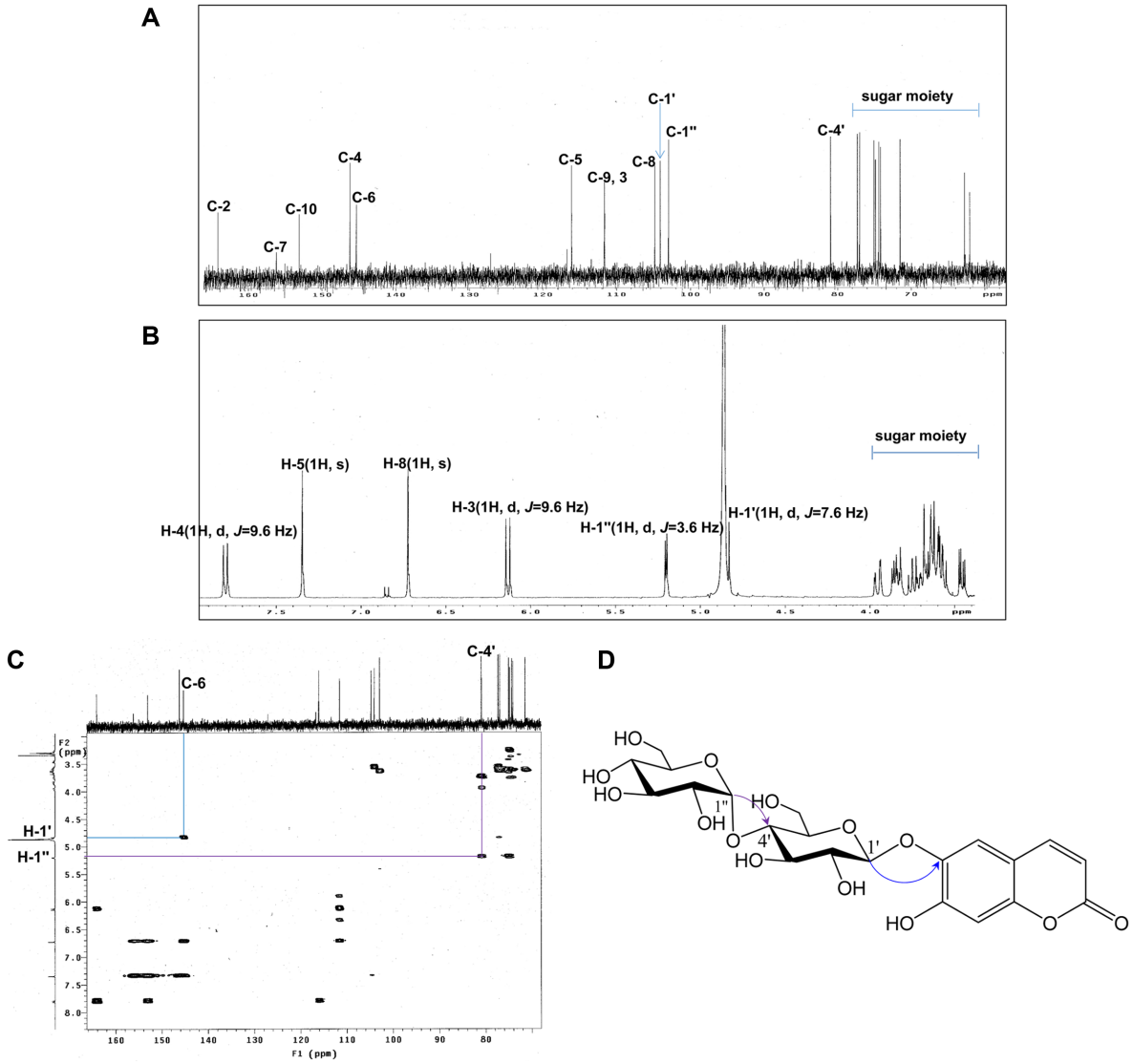

Fig. 2. Nuclear magnetic resonance analysis of the major glycosylated product of aesculin, AGG1.
(**A**) ^{13}C NMR, (**B**) ^{1}H NMR, and (**C**) heteronuclear multiple bond connectivity spectra, and (**D**) the chemical structure of AGG1.

product of aesculin, AGG1, was elucidated by NMR analysis. ^{1}H and ^{13}C NMR spectra showed that the position of the terminal glucose linked to aesculin was determined through the shift of the C-4′ signal (72 to 81.0 ppm), confirming that the transferred glucosyl group was connected to the C-4′ carbon of the glucose unit of aesculin (Fig. 2A). In addition, the ^{1}H NMR spectrum indicated that the bond was in an α-anomeric configuration based on the coupling constant (J = 3.6 Hz) of the glucose anomeric proton signal observed at 5.2 ppm (Fig. 2B). The two-dimensional heteronuclear multiple bond correlation spectrum confirmed the molecular structure of the compound, which was identified as 4-*O*-α-D-glycosyl aesculin (Fig. 2C). Based on this structural analysis of AGG1, NPAS catalyzed the transfer of glucose from sucrose to an aesculin acceptor to form an α-1,4 linkage. The transfer specificity of NPAS to aesculin glycosides was in good agreement with previously reported specificities of NPAS-mediated synthesis of other compounds [11, 15, 17]. Aesculetin has two hydroxyl groups at the 6th and 7th carbon positions of coumarin. If glucose is attached to the 6th hydroxyl group, it is aesculin, and if glucose is linked to the 7th position, it will be α-cichoriin. The retention time of AG1 by HPLC is different from that of aesculin. Furthermore, the transglycosylation of aesculetin was carried out by using amylosucrase from the bacterium *Deinococcus geothermalis* DSM 11300 previously in our hands. A newly synthesized aesculetin glycoside was identified as aesculetin 7-α-D-glucoside (α-cichoriin) by NMR (data not shown). Therefore, AG1 is considered to be α-cichoriin.

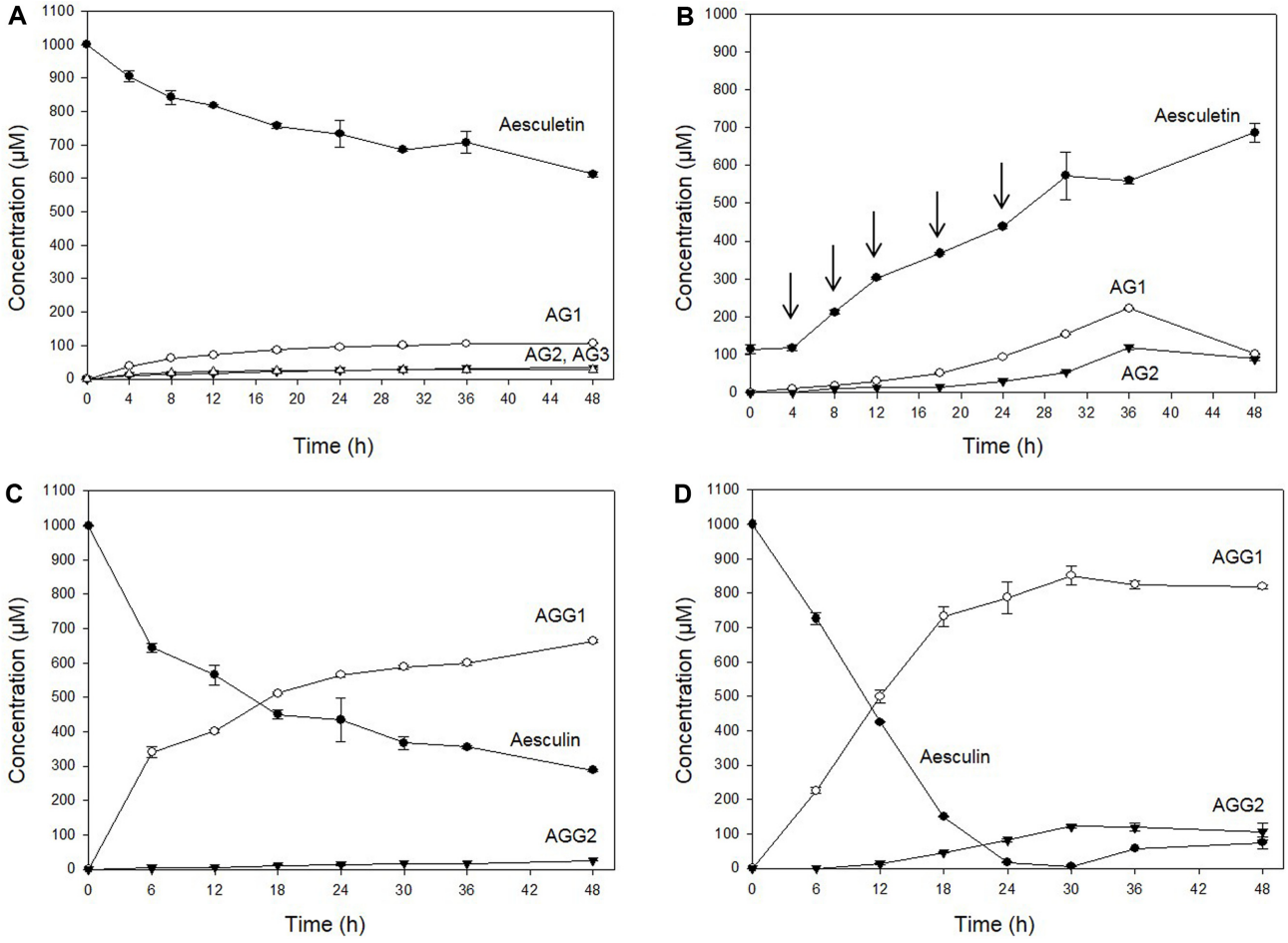

Fig. 3. Production of aesculetin (**A** and **B**) and aesculin (**C** and **D**) transfer products by enzymatic (**A** and **C**) and whole-cell biotransformation methods (**B** and **D**).

For the enzymatic synthesis method, aesculetin or aesculin (1 mM) was added to the reaction mixture at 0 h. In the case of biotransformation, aesculetin (100 μM) was added to the reaction mixture at 0, 4, 8, 12, and 18 h, and finally 500 μM was added at 24 h. An aliquot (200 μl) of the reaction mixture was collected at each time point, and the reaction products were analyzed by HPLC. Arrows indicate the time of aesculetin

To determine the conversion yields of both methods using both aesculetin and aesculin as acceptors, the relative amounts of the different synthesized glycoside derivatives were determined by HPLC (Fig. 3). The amounts of all reaction products increased with reaction time for both methods, but whole-cell transformation exhibited better yields than the enzymatic synthesis method. The production yields of AGG1 and AG1 by whole-cell biotransformation were 85% and 25%, respectively, whereas the corresponding yields from the enzymatic method were 68% and 14%. The conversion rates of aesculetin and aesculin in whole-cell biotransformation were the highest at 36 and 30 h, respectively. After 36 h, the decrease in AG1 and AG2 concurrent with the increase in aesculetin seems to be due to the increased solubility of aesculetin over a longer reaction time and the conversion of AG1 and AG2 to more glycosylated products. The results suggest that whole-cell biotransformation was more efficient at producing glycoside derivatives. Furthermore, this method did not require purification of the enzyme, and extraction and purification of the glycoside derivatives from the cells was unnecessary because they were generally secreted into the medium. Therefore, compared with enzymatic processes, whole-cell biotransformation methods using engineered cells expressing NPAS are more suitable for the production of transglycosylation products. In general, bioconversion using bacterial strains expressing various GTFs may offer more benefits for the production of glycoside derivatives than alternative enzymatic synthesis methods.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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