Bioconversion of Piceid to Piceid Glucoside Using Amylosucrase from *Alteromonas macleodii* Deep Ecotype

Park, Hyunsu¹, Jieun Kim¹, Ji-Hae Park², Nam-In Baek², Cheon-Seok Park², Hee-Seob Lee³, and Jaeho Cha¹*

¹Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea
²Graduate School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Yongin 446-01, Korea
³Department of Food and Nutrition, Pusan National University, Busan 609-735, Korea

Received: August 9, 2012 / Accepted: August 13, 2012

Resveratrol, or its glycoside form piceid, is a dietary antioxidant polyphenolic compound, found in grapes and red wine that has been shown to have protective effects against cardiovascular disease. However, very low water solubility of the compound may limit its application in the food and pharmaceutical industries. The amylosucrase (AMAS) of *Alteromonas macleodii* Deep ecotype was expressed in *Escherichia coli* and showed high glycosyltransferase activity to produce the glucosyl piceid when piceid was used as an acceptor. The conversion yield of piceid glucoside was 35.2%. Biotransformation using culture of the *E. coli* harboring the *amas* gene increased the yield up to 70.8%. The transfer product was purified by reverse phase chromatography and recycling preparative HPLC, and the molecular structure of the piceid glucoside was determined using NMR spectroscopy. The piceid glucoside was identified as glucosyl-α-(1→4)-piceid. The solubility of glucosyl piceid was 5.26 and 1.14 times higher than those of resveratrol and piceid, respectively. It is anticipated that dietary intake of this compound is more effective by enhancing the bioavailability of resveratrol in the human body because of its hydrophilic properties in the intestinal fluid.

**Keywords:** Amylosucrase, *Alteromonas macleodii* Deep ecotype, biotransformation, piceid, resveratrol, transglycosylation

---

*Corresponding author
Phone: +82-51-510-2196; Fax: +82-51-514-1778; E-mail: jhcha@pusan.ac.kr

However, resveratrol mostly exists as its glycoside form, *trans*-piceid (5,4-dihydroxystilbene-3-O-α-D-glucopyranoside), in wine [15]. After oral administration in humans, the *trans*-piceid is hydrolyzed by a β-glucosidase in the small intestine, and the converted resveratrol is efficiently absorbed across intestinal Caco-2 cells and metabolized to glucuronides or sulfated derivatives [6, 18].

Even though *trans*-resveratrol and *trans*-piceid have therapeutic benefits, their water-insolubility limits their pharmacological exploitation in industry. Much effort has been made to increase the solubility of natural polyphenols. One method is to use cyclodextrin, which is widely employed as an excipient to increase the solubility and stability of drugs and chelate isoflavone. Gu [4] reported that a cyclodextrin-isoflavone complex increased solubility by 2–6 times. However, the use of this complex is problematic because of the cloudiness of its solution. The other way to enhance the water solubility is to add a sugar moiety to the compound by enzymatic transglycosylation or biotransformation using cultured cells. Although chemical synthesis of glycides is reasonable, it generates a lot of waste. The method also includes several protection and deprotection steps of the substrates and products, unlike enzymatic synthesis. Higher selectivity and specificity of enzymatic synthesis can overcome the limitation of chemical synthesis and provide an important green tool to obtain glycosylated products without toxic solutions and heavy metals [16]. Phenolic compounds such as arbutin, naringin, neohesperidin, and salicin have been glycosylated using bacterial glycosidases and/or glycosyltransferases [3, 8, 9, 12]. α-Oligoglucosylation of a sugar moiety by enzymatic synthesis enhances the bioavailability of quercetin glucosides in humans [13]. Enzymatic and microbial transformations of puerarin have also been examined, which rendered higher stereoselectivity and produced some specific compounds that suffered difficulty acquiring through chemical approaches [11, 20]. Puerarin glucosides

---

*trans*-Resveratrol (*trans*-3,5,4-trihydroxystilbene) is a polyphenolic phytoalexin with strong antioxidative activity, which is isolated from a variety of plant species and synthesized. Resveratrol has been reported to have potential chemopreventive activity against cardiovascular disease and a variety of cancers in model systems [2, 18, 19].
synthesized by bioconversion using Microbacterium oxydans CGMCC 1788 exhibited superior water solubility and improved pharmacokinetic properties, while maintaining original bioactivity [7].

Alteromonas macleodii Deep ecotype is a Gram-negative, mesophilic, heterotrophic bacterium. It belongs to the genus of Proteobacteria and is an obligate marine bacterium isolated at a depth of 3,500 m in the Urania Basin [5]. The genome of A. macleodii has been sequenced and made available at the National Center for Biotechnology Information (NCBI). A putative amylolyscrose (ASase) tagged MADE_00676 is located in the genome of the A. macleodii Deep ecotype. To purify the putative ASase and obtain the recombinant E. coli cells bearing the gene, the putative MADE_00676 was isolated from A. macleodii Deep ecotype. Based on the fact that ASase forms an α-1,4-linked glucan polysaccharide, we hypothesized that the putative ASase could synthesize soluble piceid glucoside via tranlgcosylation [8]. In this study, we expressed the gene for ASase from the A. macleodii Deep ecotype by transforming E. coli BL21(DE3) and used the glycolyl transfer activity of ASase to synthesize a piceid glucoside. The transglycosylation reaction was successfully conducted with piceid as the acceptor and sucrose as the donor using the purified AMAS and the recombinant E. coli cell culture. The molecular structure of the tranlgcosylation product, piceid glucoside, was determined and its water solubility was examined.

MATERIALS AND METHODS

Chemicals and Reagents

trans-Piceid and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Water and methanol [high-performance liquid chromatography (HPLC)-grade] were purchased from Burdick & Jackson (Morristown, NJ, USA) for purification of transfer products. All other chemicals were of reagent grade and purchased from Sigma Chemical Co.

Construction of amas Gene Expression Vector

In order to detect a potential amylolyscrose gene (amas), Alteromonas macleodii Deep ecotype ORF (MADE_00676)-encoded ASase was compared with other genes through a BLAST search. PCR amplification of amas was performed with A. macleodii Deep ecotype genomic DNA as a template, using Takara prime STAR polymerase and the amas-specific primers AmaASU-Nde1-f (5'-GTGAGCTCAAGCAGCTGCTG-3') and AmaASU-Xho1-R (5'-AGACCTCGAGGAGCTGGCTGCAAAC-3'), which respectively contained Nde1 and Xho1 restriction enzyme sites (underlined). The amas-specific internal primers were designed based on the A. macleodii Deep ecotype putative ASase nucleotide sequence. The PCR conditions were as follows: 30 cycles at 98°C for 10 s (denaturation), 55°C for 15 s (annealing), and 72°C for 2 min (extension). The 1,947 bp product was purified with a PCR purification kit and then digested with Nde1 and Xho1. This was then ligated into the expression vector pET29b(+).

AMAS Assay

The AMAS transglycosylation activity was determined by the dinitrosalicylic acid (DNS) method. The standard assay mixture (0.3 ml) was assayed at 45°C for 10 min in a reaction mixture containing 50 mM buffer, 500 mM sucrose as a substrate, and 100 μg of AMAS. To identify the effect of pH on transglycosylation activity of AMAS, 50 mM buffer was used as follows; citrate-NaOH (pH 3.0–6.0), sodium phosphate (pH 6.0–7.0), Tris-HCl (pH 7.0–9.0), and glycine-NaOH (9.0–11.0). The influence of temperature on the activity of AMAS was examined in 50 mM Tris-HCl buffer (pH 8.0) in a range from 25°C to 55°C for 10 min. After enzyme reaction, the enzyme samples were mixed with 450 μl of DNS solution, and then the reaction mixtures were incubated at 80°C for 15 min. Color development was measured at 575 nm and the specific activity was calculated using fructose as a standard. One unit was defined as the amount of enzyme that catalyzes the production of 1 μmol fructose per minute in the assay conditions. All enzyme assays were performed by preincubation for 10 min before adding enzyme.

Enzymatic Synthesis of Piceid Glucoside

The reaction mixture containing 5 mM piceid, 5 mM sucrose, and 50 mM Tris-HCl (pH 8.0) was preincubated at 30°C for 10 min, and
then AMAS (0.1 mg/ml) was added and incubation of the reaction mixture (10 ml) was continued for 12 h. The reaction was stopped by boiling for 10 min and placing the mixture tube in ice. The reaction mixture was centrifuged at 2,000 \( \times g \) for 10 min to get rid of the insoluble substance. The supernatant fraction was filtered using a 0.45 \( \mu \)m syringe filter.

**Biotransformation of Piceid by \( E. \ coli \)**

\( E. \ coli \) BL21(DE3) containing pET29b-amas was cultured as described above in 10 ml of LB medium. After IPTG induction for 3 h, the \( E. \ coli \) cells were separated by centrifugation at 8,000 \( \times g \) for 10 min to remove the supernatant. The cells were washed twice with 20 mM sodium phosphate buffer (pH 7.4), and resuspended in the M9 medium (10 ml) containing kanamycin (30 \( \mu \)g/ml), 0.1 mM IPTG, 1 mM piceid, and 1 mM sucrose. Experiment with resting cells was carried out at 150 rpm at 30°C in 250 ml Erlenmeyer flasks for 12 h. After incubation, the supernatant was obtained by centrifugation at 12,000 \( \times g \) for 10 min. The supernatant fraction was filtered using a 0.2 \( \mu \)m syringe filter (Pall, Port Washington, NY, USA) and analyzed by HPLC.

**TLC and HPLC Analyses**

The purified transfer products were analyzed using TLC as described previously [14]. HPLC analysis was performed using a Shiseido Nanospace SI-2 (Shiseido, Tokyo, Japan) HPLC system, which includes a UV detector (Shodex, Tokyo, Japan). Separation of piceid glucoside was carried out by reversed phase HPLC using a C18 (4.6 \( \times \) 250 mm) column (Shodex). Separation was performed at a flow rate of 1 ml/min with a mobile phase composed of buffer A (1% acetic acid in water) and buffer B (1% acetic acid in methanol). The run was set as 22% buffer B. The column temperature was 40°C and the injection volume was 30 \( \mu \)l. The analysis was observed at 306 nm, where trans-resveratrol shows absorbance maxima.

**Purification of Piceid Glucoside**

The transfer products were separated by C18-T cartridge (100 mg/ml, Strata) and recycling preparative HPLC equipped with RI detector (JAI, Tokyo, Japan). A C18-T cartridge, previously activated using methanol and water, was used to absorb the piceid glucoside in the transglycosylation reaction mixture and to remove any sucrose and salts. The transglycosylation reaction mixture was filtered by a 0.45 \( \mu \)m syringe filter and subjected to C18-T cartridge. After washing twice, elution of transfer products was carried out with methanol. The main transfer products in methanol were purified using a combination W-252/W-251 polymeric gel filtration column (2 cm \( \times \) 50 cm; JAI) in the recycling preparative HPLC. The mobile phase was 100% methanol at a flow rate of 3 ml/min. The fractions corresponding to the detected peaks were collected and freeze-dried. The purity of each sample was confirmed using TLC analysis.

**\( \alpha \)-Glucosidase Hydrolysis**

The purified transfer product was dissolved in McIlvaine buffer (pH 5.0). *Sulfolobus acidocaldarius* \( \alpha \)-glucosidase (MalA; 0.5 \( \mu \)g) was added to the solution containing 10 mM glucosyl piceid and incubated at 85°C for 60 min. The reaction mixture was analyzed by TLC at 254 nm.

**Nuclear Magnetic Resonance Analysis**

The \( ^{1}H \) and \( ^{13}C \) nuclear magnetic resonance (NMR) spectra of piceid and purified piceid glucoside were obtained with a Varian Inova AS 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA). The sample was dissolved in DMSO-d\(_6\), at 24°C with tetramethylsilane (TMS) as the chemical shift reference.

**Solubility Determination**

Solubility of piceid or piceid glucoside was determined as described previously [14].

**RESULTS AND DISCUSSION**

**Purification of Recombinant AMAS**

The glycosyltransferase, designated AMAS, of the *A. macleodii* Deep ecotype was expressed in \( E. \ coli \) BL21(DE3) cells harboring pET29b-amas. AMAS was efficiently purified by Ni-NTA chromatography. Crude cell-free extracts gained from sonication were filtered and loaded onto a Ni-NTA affinity column. The size of the purified AMAS estimated by SDS-PAGE was approximately 74 kDa, which is close to the expected molecular mass deduced from the amino acid sequence of *A. macleodii* AMAS (Fig. 1). The yield of purified AMAS was about 14.5%.

**Characterization of AMAS**

\( \alpha \)-Sase is highly useful for glycosylation of biomaterials owing to its ability to utilize sucrose, an inexpensive and abundant renewable substrate, as a glucose donor. The deepsea eco-environment is obviously different from other environments. Therefore, it is important to find out the optimal condition of AMAS for substrate hydrolysis and transglycosylation. To optimize transglycosylation conditions...
for synthesis of piceid glucoside, the pH range at which the recombinant AMAS was active was determined using sucrose as the substrate. The maximum activity was observed at pH 8.0 (Fig. 2A). The optimal temperature of AMAS was 37°C, and the activity was sharply decreased above 42°C (Fig. 2B). Based on these results, the transglycosylation reaction was carried out at 37°C and pH 8.0. ASase is known for having unique substrate specificity, strictly to transfer glucosyl units and form amylose or other glucosyl transfer products. It has been reported that ASase (DGAS) from *Deinococcus geothermalis* transferred a glucose moiety of sucrose to the C-4 position in the glucose residue of arbutin and salicin [8, 17]. AMAS also produced amylose when sucrose was used as a substrate. These results indicate that AMAS from *A. macleodii* Deep ecotype is indeed an ASase that transfers the glucosyl unit exclusively, even though DGAS has more outstanding transglycosylation activity compared with AMAS.

Glucosylation of Piceid by AMAS

AMAS was reacted with piceid in the presence of sucrose as a donor molecule. On the TLC plate, one major transglycosylated spot, possibly newly produced piceid glucoside, was observed after the AMAS reaction (Fig. 3). This spot was detected under UV light (data not shown), implying that it originated from piceid, since piceid was the only chromophore in the reaction. TLC analysis of the AMAS reaction with sucrose and piceid suggested that the glucosyl-transfer activity of AMAS was successfully used to produce the piceid glucoside. Other polyphenols such as aesculin and arbutin were also good acceptors for transglycosylation by AMAS (data not shown). When the DGAS of *Deinococcus geothermalis* was used to transglycosylate arbutin, the major arbutin glycoside transfer product was determined to be glucosyl salicin [8]. Likewise, the newly produced piceid glucoside product observed in the AMAS transglycosylation reaction was believed to be a glucosyl unit attached to piceid. The conversion yield of piceid glucoside was 35.2% through enzymatic synthesis by the purified AMAS. Bioconversion using recombinant *E. coli* cells harboring the *amas* gene showed approximately 71% yield of the glucosyl piceid.

![Fig. 2. Effects of pH (A) and temperature (B) on the transglycosylation activity of AMAS. The hydrolysis activity of the AMAS was identified by the DNS method.](image)

![Fig. 3. TLC analysis of the reaction product of AMAS with sucrose and piceid. Lane M, maltooligosaccharide standard solution including glucose, maltose, and maltotriose; lane 1, sucrose; lane 2, glucose; lane 3, piceid; lane 4, reaction product with sucrose and piceid. The arrow indicates the glucosyl piceid.](image)
Enzymatic Synthesis of Piceid Glucoside by Alteromonas macleodii Amylosucrase

The yield by biotransformation was much higher than that of enzymatic synthesis. If the reaction condition of biotransformation is more investigated and optimized, we can expect that the functional glycosides will be easily produced and obtained for large-scale production.

Structural Analysis of Glucosyl Piceid

The sugars included in the transglycosylation reaction mixture were firstly discarded by a reverse phase C$_{18}$-T column, and then the mixture of resveratrol glucosides in methanol was separated by using recycling preparative HPLC combined with a W-251 and W-252 polymeric gel filtration column. The presence of the piceid and piceid glucoside was detected by a refractive index detector (Fig. 5). The anomer proton signals were detected and the bonds between the 2 glucose molecules were determined to be $\alpha$-glucosidic linkages according to the coupling constant ($J = 4.0$ Hz) in the $^1$H NMR spectrum. The coupling constant value ($J = 8.0$ Hz) of one anomeric proton signal indicates the $\beta$-linkage between the glucose moiety and aglycone resveratrol in piceid. The assignment of the carbon signal showed that the transferred glucosyl group was connected to C-4" of piceid (Fig. 6). Based on the result, the transfer product of piceid by AMAS was defined to be $\alpha$-D-glucosyl-(1→4)-piceid. These results mean that the $\alpha$-1,4-glycosidic linkage formed in the transglycosylation reaction is easily hydrolyzed by various glycosyl hydrolases, such as $\alpha$-glucosidase, implying that the human body metabolizes piceid glucoside in the same way as piceid or resveratrol itself. This suggests that the bioavailability of piceid glucoside might not change greatly.

Water Solubility of Piceid and Piceid Glucoside

The solubility of glucosyl piceid in water was evaluated by comparing the retention time of glucosyl piceid with that of piceid by HPLC analysis combined with C$_{18}$ column. We could imply that glucosyl piceid (8.59 min) is more soluble than piceid (9.30 min), which is known as a natural resveratrol glucoside owing to its short retention time compared with piceid (Fig. 4). This implies that the attachment of a

---

Fig. 4. HPLC analysis of the piceid transfer product after biotransformation.

The 1 mM piceid and 1 mM sucrose were cultured with the recombinant E. coli cell in M9 medium containing kanamycin and 0.1 mM IPTG at 30°C for 12 h. The sample was injected after removal of sugars by C$_{18}$-T cartridge column. The piceid and the glucosyl piceid appeared at 9.30 and 8.59 min, respectively.

Fig. 5. TLC and recycling preparative HPLC analysis of the piceid transfer products by the transglycosylation of AMAS. The inset shows the purified transfer product. R, piceid and sucrose reaction products; 1, the piceid transfer product; 2, unreacted piceid.
glucosyl residue to piceid by AMAS enhanced the water solubility of the original compound. Excess piceid or glucosyl piceid was suspended and sonicated in water to investigate its water solubility. We found that the solubility of piceid and glucosyl piceid was identified as 0.069 g/l and 0.079 g/l, respectively. The solubility of glucosyl piceid was 1.14 times higher than that of natural piceid. Compared with the resveratrol, which has no glucose moiety, the solubility was increased by 5.26-fold. After oral administration, unmetabolized resveratrol remains at low a level in human plasma because of poor absorption and ready conversion to its metabolites (glucuronidated or sulfated forms), even though resveratrol and piceid have beneficial biological activities in humans [19]. To apply the resveratrol as one of the effective medicinal supplements, it is necessary to increase the amount of unmetabolized compound at the target site at which resveratrol exerts biological activity. In our study, the enzymatic transglycosylation or biotransformation by AMAS or AMAS-expressed cells succeeded to produce glucosyl piceid, but was not effective to enhance solubility dramatically. We are trying to use maltosyltransferase to increase the solubility of piceid [14]. In preliminary result, it seems to increase the solubility of resveratrol more efficiently.

To summarize, the enzymatic transglycosylation using AMAS and/or biotransformation using AMAS-expressed E. coli cells are powerful techniques to produce water-soluble natural compound derivatives that have industrial potential in the commercial market. Various polyphenolic compounds such as aesculin or genistin are being examined to develop effective drugs having hydrophilic property. In the future, we anticipate that the glycosylation of biologically active compounds will be an alternative to pre-existing lipophilic drugs to increase bioavailability and delay the rapid conversion of those compounds.

Acknowledgment

This work was supported by a Bio-Scientific Research Grant funded by the Pusan National University (PNU, Bio-Scientific Research Grant) (PNU-2008-101-204).

REFERENCES


