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Enzymatic Synthesis of Puerarin Glucosides Using *Leuconostoc* Dextransucrase

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Puerarin (P), an isoflavone derived from kudzu roots, has strong biological activities, but its bioavailability is often limited by its low water solubility. To increase its solubility, P was glucosylated by three dextransucrases from *Leuconostoc* or *Streptococcus* species. *Leuconostoc lactis* EG001 dextransucrase exhibited the highest productivity of puerarin glucosides (P-Gs) among the three tested enzymes, and it primarily produced two P-Gs with a 53% yield. Their structures were identified as α-D-glucosyl-(1→6)-P (P-G) by using LC-MS or ^{1}H- or ^{13}C-NMR spectroscopies and α-D-isomaltosyl-(1→6)-P (P-IG2) by using specific enzymatic hydrolysis, and their solubilities were 15- and 202-fold higher than that of P, respectively. P-G and P-IG2 are easily applicable in the food and pharmaceutical industries as alternative functional materials.

Keywords: Puerarin, dextransucrase, *Leuconostoc lactis*, water solubility, transglucosylation

Puerarin (P, daidzein 8-*C*-glucoside, Fig. 1), the most abundant *C*-glucosyl isoflavone isolated from the traditional Asian medicinal herb *Pueraria lobata*, is a bioactive material that has been used to prevent or treat osteoporosis [14], alcoholism [1], and cancer because of its antioxidant properties [2]. Although P contains one glucosyl residue attached to the parent isoflavone, its water solubility is still low. Therefore, its applications in the pharmaceutical and food industries are very limited because of its poor absorption after oral administration and difficulty in food processing. In an effort to develop highly water-soluble P derivatives, P has been modified using chemical and biological methods [11]. The chemical approach has the disadvantages of low stereospecificity and the generation of secondary toxic waste products. Conversely, enzymatic or microbial transformation of P has been reported to result in higher regioselectivity under eco-friendly conditions. Li *et al*. [11] developed two P derivatives, glucosyl and maltosyl P, by using maltogenic amylase, and Jiang *et al*. [8] synthesized 7-position-specific fructosyl and isomaltosyl P by using *Microbacterium oxydans*. The newly synthesized puerarin glucosides (P-Gs) with improved water solubility exhibited the same antioxidant activity [6] and vasorelaxant effect [8] as the parent P. These improved properties of P-Gs have revealed their potential as pharmacophores and their potentially increased absorption in humans. These modifications induced by transglucosyltransferase produce various highly soluble structures.

Dextransucrase (DexT), an alternative well-known transglucosyltransferase, has been previously used to modify a variety of bioactive substances; for example, for salicin, to improve its anticoagulant activity [18], and for catechin, to increase its water solubility [13]. In a previous study performed in our laboratory, we have shown that *Leuconostoc lactis* EG001 DexT (LLDexT) can transform ascorbic acid to glucosyl ascorbic acid, resulting in enhanced stability and improved water solubility [9]. In our studies on the biotransformation of P by LLDexT, we found that the enzyme produces novel P-Gs containing α-(1→6)-glucosidic linkages, such as α-D-glucosyl-(1→6)-P (P-G) and α-D-isomaltosyl-(1→6)-P (P-IG2), which exhibit superior water solubility.

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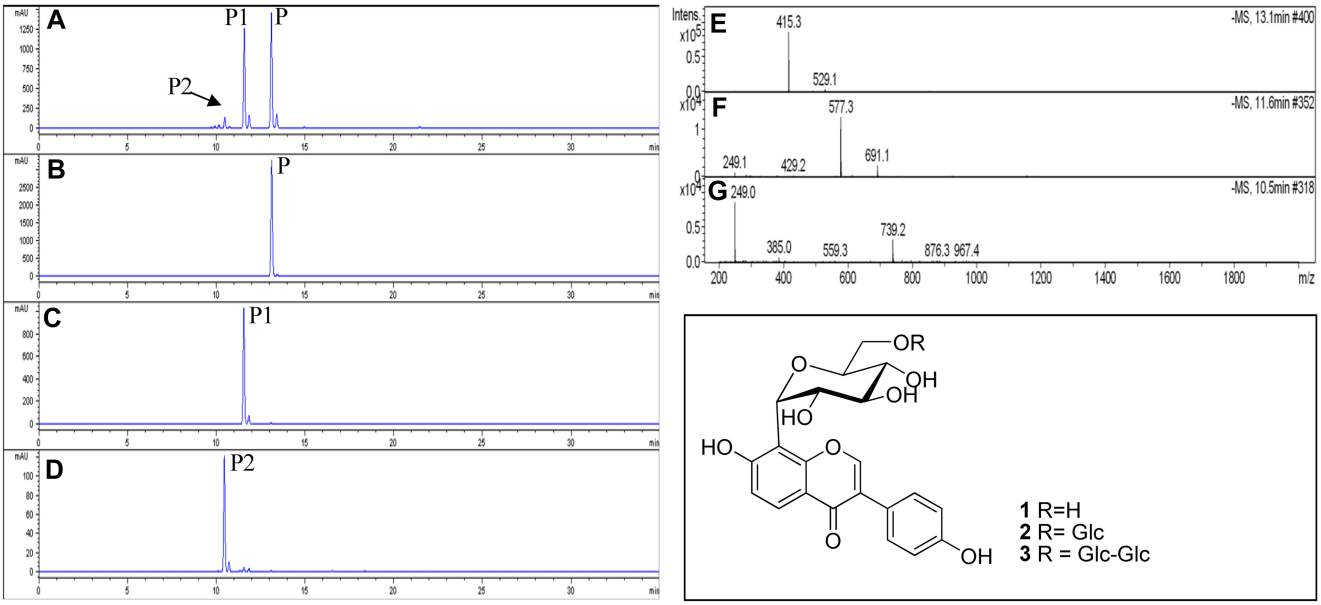

Fig. 1. HPLC and LC-MS spectra of two products of P.
(**A**) Reaction mixture of P and LLDexT; (**B**) P standard; (**C**) purified P1; (**D**) purified P2; (**E**) LC-MS spectra for P; (**F**) LC-MS spectra for P1; and (**G**) LC-MS spectra for P2.

Materials and Methods

Preparation of Recombinant DexTs

Leuconostoc mesenteroides B-512F DexT (512FDexT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant *Streptococcus mutans* DexT (SMDexT) and LLDexT were overexpressed in *Escherichia coli* (*E. coli*) BL21-(DE3)-CodonPlus RIL (Novagen, Madison, WI, USA) *via* the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 12 h at 20°C, as described previously [9, 19]. The cultivated cells were harvested by centrifugation and suspended in 50 ml of lysis buffer containing 100 mM sodium phosphate buffer (pH 6.0). Thereafter, SMDexT and LLDexT were purified using Ni-Sepharose affinity chromatography (GE Healthcare, Piscataway, NJ, USA). The suspended cells were sonicated and centrifuged at 12,000 ×*g* for 30 min, and the supernatant was loaded on an Ni-Sepharose resin. The column was washed with buffer containing 20 mM sodium phosphate (pH 6.8), 0.5 M NaCl, and 20 mM imidazole. The target protein was eluted from the column by using the same buffer but with 250 mM imidazole. The elution buffer was then changed to 50 mM sodium acetate buffer (NAB, pH 5.0 for LLDexT and pH 5.2 for SMDexT), and the protein concentration was determined by the Bradford method [3] with bovine serum albumin as the standard.

Enzyme Assay

DexT activity was measured at 28–30°C in a reaction mixture consisting of 100 mM sucrose and 50 mM NAB (pH 5.0 for LLDexT, pH 5.2 for 512FDexT and SMDexT) containing 200 ml of enzyme. The fructose liberated was measured using the dinitrosalicylic acid assay [12]. One unit of enzyme activity was defined as the quantity of enzyme required to generate 1 μmol of fructose per minute under the given reaction conditions.

Enzymatic Synthesis and Measurement of P-Gs

For analytic-scale reactions (1 ml), a reaction mixture consisting of 2% (w/v) P, 2% (w/v) sucrose, and 1.2 U of each DexT was incubated in 50 mM NAB (pH 5.0 or 5.2) at 30°C for 24 h. For preparative-scale reactions (100 ml), a reaction mixture consisting of 2% (w/v) P, 4% (w/v) sucrose, and 52 U LLDexT was incubated in 50 mM NAB (pH 5.0) at 30°C for 72 h. The reaction mixture was placed in a water bath for 5 min to stop the enzyme activity. At designated time intervals, 30 μl aliquots were withdrawn to analyze the reaction products *via* thin layer chromatography (TLC). The P-Gs were analyzed by TLC performed in a solvent system consisting of acetonitrile:water in a ratio of 85:15 (v/v) with carbohydrate standards. Carbohydrates were visualized on the TLC plate as described previously [20]. Chromatographic separation for quantitative analysis was achieved using a high-performance liquid chromatography (HPLC; Agilent Technologies, Palo Alto, CA, USA) system equipped with a quaternary HPLC pump, a degasser, an autosampler, a UV detector, and a ZORBAX SB-C_{18} column (5 μm, 150 × 4.6 mm). The mobile phase for HPLC consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The solvent gradient was as follows (relative to solvent A): 0 min, 30% B; 10 min, 45% B; 20 min, 55% B; 30 min, 70% B; 40 min, 85% B; 50 min, 100% B; and 70 min, 100% B. The flow rate was 0.5 ml/min, and the injection volume was 10 μl. The eluent was detected at 210 nm and all HPLC analyses were performed at 30°C.

Isolation and Structural Elucidation of P-Gs

During the purification step, the carbohydrate composition of the fractions was analyzed by TLC. Reaction mixtures with volumes of approximately 100 ml were added to 2 volumes of chilled ethanol and then kept at −20°C for 2 h. After separation by centrifugation,

the supernatant was passed through a C_{18} Sepak cartridge to remove oligosaccharides, unreacted sucrose, and salts. Thereafter, the reaction product was concentrated under vacuum to 2 ml by using a rotary evaporator at 55°C. The resultant products (50 mg/ml) were loaded onto a Bio-Gel P2 column (3 × 120 cm) and eluted with deionized water at a flow rate of 0.05 ml/min, with 1.0 ml fractions collected as described previously [18]. Finally, the compounds obtained were concentrated to approximately 1 ml by vacuum rotary evaporation.

We used several methods to determine the structure of the P derivatives. For the structural elucidation of P-G, approximately 30 mg of the purified P-G was dissolved in 250 µl of deuterated water and was placed into 3 mm nuclear magnetic resonance (NMR) tubes. NMR spectra were acquired on a Unity INOVA 500 spectrometer (Varian, Palo Alto, CA, USA) operated at 500 MHz for ^{1}H and at 125 MHz for ^{13}C at 25°C. Linkages between P and glucose were evaluated using the spectra obtained *via* homonuclear correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation. In addition, P-IG2 was digested with endo- or exo-glycosidases for structural analysis. Endodextranase from *Thermoanaerobacter pseudethanolicus* hydrolyzes the α-(1→6)-D-glucosidic linkages in dextran [16], and highly purified α-glucosidase from *Aspergillus niger* releases the glucose moiety from the nonreducing terminal of α-glucosidic linkages such as maltose or aryl glucosides [10]. The hydrolyzate was also identified by TLC and compared with the original compounds.

Water-Solubility Determination

Purified P-G and P-IG2 were tested for their water solubility as described by Li *et al.* [11]. Briefly, each compound was mixed with 0.2 ml of distilled water in an Eppendorf tube at 25°C. An ultrasonic cleaner was used to maximize their solubility. After centrifugation at 10,000 ×*g* for 10 min to remove the insoluble material, the sample solutions were diluted, filtered through a 0.45 µm membrane, and used for HPLC analysis to determine the concentrations of the compounds in the solution.

RESULTS AND DISCUSSION

Enzymatic Synthesis and Purification of P-Gs

Three enzymes, namely SMDexT, 512FDexT, and LLDexT, were examined for their acceptor specificity toward P. Using SMDexT, 512FDexT, and LLDexT, 5.3, 8.2, and 10.5 mg of P-Gs, respectively, were synthesized from 20 mg of P and 20 mg of sucrose (Table 1). LLDexT was found to be the most efficient enzyme for modifying P, and this enzyme was used in the subsequent reactions. The HPLC analysis showed at least two significant peaks (P1 and P2 in Fig. 1), suggesting that LLDexT produced a series of transfer products from P.

To optimize the reaction conditions, the transglucosylation reaction was performed using a fixed P concentration of 2% (w/v) and a sucrose concentration ranging from 0.5% to 5% (w/v). The highest production of transfer products, 15.2 mg (53.4% yield of the P supplied), was obtained with a sucrose concentration of 4% (w/v), and the conversion ratio of P1 to P2 was 90%:10%. On a 100 ml reaction-scale, 1.45 g of P-Gs was obtained using 52 U of LLDexT with 4 g of sucrose and 2 g of P at 30°C for 72 h. After discarding the glucan formed by ethanol precipitation, the P derivatives were purified by single Bio-gel P2 column chromatography after C_{18} cartridge purification. Finally, the major transfer products (P1 and P2 in Fig. 1) were obtained with a purification yield of 78% and were used in additional experiments to determine their structures. Consequently, the purification yield of P-Gs was approximately 39% of the initially supplied P. In this study, two P-Gs were synthesized with a high yield of 53% by using LLDexT. This yield is similar to that in the production of transfer products by *Bacillus subtilis* maltogenic amylase [4] and the yield was high relative to its concentration of 2.3 mg/g in kudzu root [15].

Structural Determination of the Two Products

After purification by Bio-gel P2 column chromatography, the molecular weights and structures of the P-Gs were analyzed by LC-MS, 1D- and 2D-NMR, and specific enzymatic hydrolysis. The molecular formula of P1, $C_{27}H_{30}O_{14}$, was determined by electron ionization mass spectrometry [m/z = 578, P (416) + glucose (180) – H_2O = 578]. The ^{1}H NMR spectrum of P1 exhibited signals related to a pentasubstituted aromatic ring [δ_H 7.89 (H-5, 1H, d, J = 8.79 Hz); δ_H 6.94 (H-6, 1H, d, J = 8.08 Hz)], a 1,4-disubstituted aromatic ring [δ_H 7.39 (H-2,6, 2H, d, J = 8.5 Hz); δ_H 6.81 (H-3,5, 2H, d, J = 8.52 Hz)], and an α,β-unsaturated enone moiety [δ_H 8.28 (H-2, 1H, s)]. The position of the *C*-glucopyranoside group in the parent isoflavone was determined by heteronuclear multiple-bound correlation (HMBC) between the anomeric proton G-1 (δ_H 4.80) and C-7 (δ_C 163.0) and between C-8 (δ_C 112.7) and C-8a (δ_C 156.8) (Fig. 2A). Importantly, the presence of a glycosyl group (P-G) was deduced from the HMBC correlation of G-1 (δ_H 4.82) with G-6 (δ_C 66.4)

Table 1. Reaction conditions for glucosylation with three types of DexT.

DexT	Optimum pH/temperature (°C)	Product concentration (mg)[a]	Composition of P-G and P-IG2[b]
SMDexT	5.2/28	5.3 ± 0.13	80/20
512FDexT	5.2/28	8.2 ± 0.11	83/15
LLDexT	5.0/30	10.5 ± 0.21	90/10

[a]2.0% (w/v) sucrose, 2.0% (w/v) P, 1.2 U/ml.

[b]Conversion ratio was calculated *via* HPLC analysis.

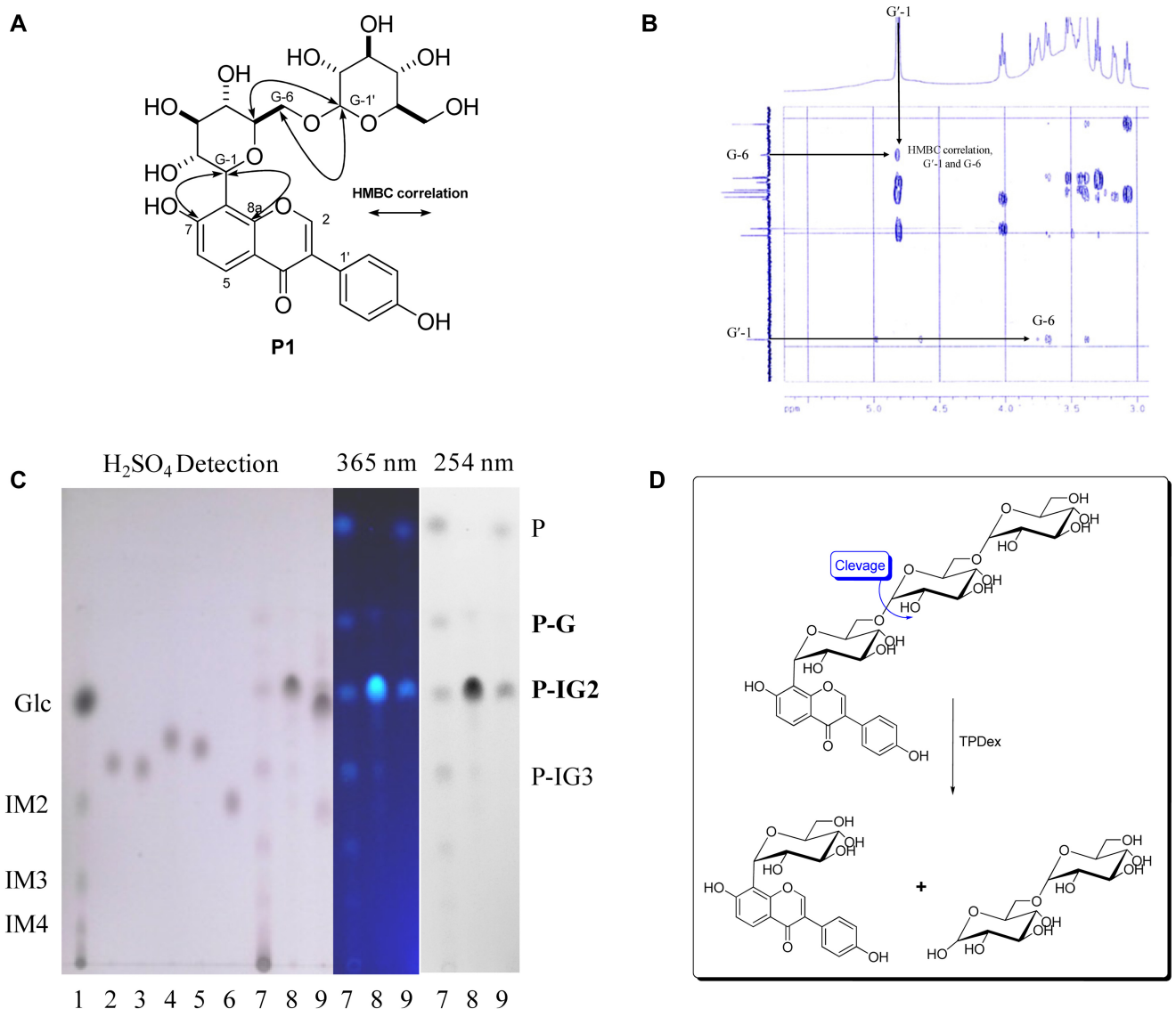

Fig. 2. (**A**) HMBC correlation of P1, (**B**) HMBC spectrum of P1 in the presence of a glycosyl group in P, (**C**) analysis of the hydrolysis products of P-IG2, obtained on treatment with endodextranase (TPDex), and (**D**) schematic representation of the hydrolysis of P-IG2 by TPDex.

The reaction mixture containing 330 μg/ml TPDex and 20 mM P-IG2 was incubated in 30 mM NAB (pH 5.2) at 60°C for 24 h. The hydrolysis products were analyzed by TLC. The carbohydrates were determined using H_2SO_4 in 5% phenol, and P-Gs were assayed using a UV detector at 365 and 254 nm. (**C**) Lane 1, isomaltooligosaccharides (IM2-IM7); lane 2, trehalose ; lane 3, kojibiose; lane 4, nigerose; lane 5, maltose; lane 6, isomaltose; lane 7, P-Gs; lane 8, purified P-IG2; lane 9, TPDex-treated P-IG2.

(Fig. 2A and 2B). Thus, these observations indicated that the structure of P1 was α-D-glucosyl (1→6)-puerarin.

To elucidate the position of glucosylation of P2, P2 was degraded *via* enzymatic hydrolysis [m/z = 740, P (416) + 2 molecules of glucose (360) – H_2O = 740] by dextranase into isomaltose (lane 9 for H_2SO_4 detection, Fig. 2C and 2D) and P-G (lane 9 at UV 365 and 254 nm, Fig. 2C and 2D). These results suggest that P2, which was identified as α-D-isomaltosyl (1→6)-P (P-IG2), is connected by α-(1→6) glucosidic linkages between isomaltose and P-G. The possible reaction scheme of P and sucrose catalyzed by LLDexT is illustrated in Fig. 3. LLDexT attacks and hydrolyzes sucrose to form an intermediate complex between the hydrolyzed glucose and the enzyme [17]. Subsequently, the hydroxyl group of P attacks the intermediate complex immediately in the reaction mixture. The three-dimensional structure of DexT led us to propose that the formation of a large acceptor-binding pocket composed of subsites +1 and +2, as described for P, between the acceptor molecule and the enzyme is easily

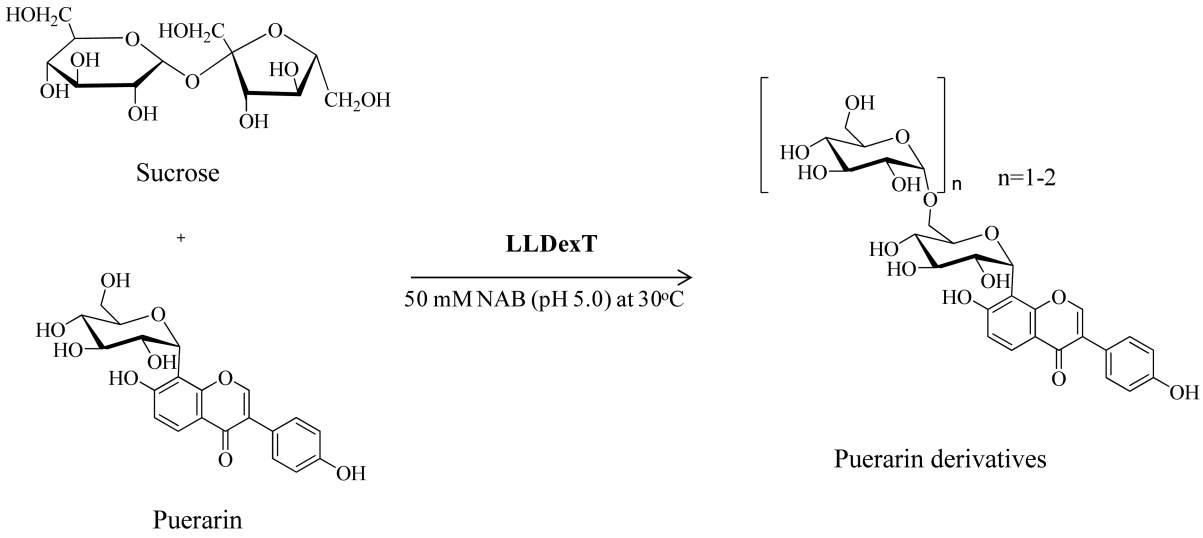

Fig. 3. Schematic representation of P derivatives produced by LLDexT.

Table 2. Solubility of P and its transfer products.

Isoflavones	Solubility in water (mM)[a]	Relative solubility
P	130.1	1
P-G, α-D-glucosyl (1→6)-P	1,950.12	15
P-IG2, α-D-isomaltosyl (1→6)-P	262,630.4	202

[a]Mean standard deviation (n = 3).

induced. In particular, because subsite +2 plays an important role in determining the orientation of the acceptor product [7], it may be involved in the transglucosylation reaction catalyzed by LLDexT. In fact, DexT is also capable of transferring various non-sugar moieties such as salicin [18], catechin [13], and ascorbic acid [9]. LLDexT preferentially transfers moieties to an acceptor molecule by forming an α-(1→6)-glucosidic linkage between the glucosyl moiety and P [9]. This acceptor specificity led us to infer that glucose generated from sucrose molecules was transferred to P *via* the formation of a α-(1→6)-glucosidic linkage. Furthermore, the safety of maltosyl P synthesized by maltogenic amylase was confirmed in rats [5]. The 2 P derivatives produced in this study, P-G and the novel structure P-IG2, could be used in *in vivo* experiments as newly identified bioactive compounds.

Solubility of the P-Gs

The solubility of the transfer products in water was compared with that of P. The solubility of P in water was 13 mM, whereas that of P-G and P-IG2 was 0.19 and 2.62 M, respectively. This finding suggests that the glucosylation of a glucosyl or isomaltosyl residue to P by LLDexT results in greatly enhanced water solubility (Table 2). The newly synthesized P-G and P-IG2 exhibited greatly improved water solubility (202-fold increase) compared with that of the maltosyl P (168-fold increase) produced by maltogenic amylase [11] and puerarin-7-*O*-isomaltoside (100-fold increase) modified by *M. oxydans* [8, 21]. This improved water solubility is the result of the structural flexibility of α-(1→6) glucosidic linkages as opposed to the rigidity of α-(1→4) linkage. The glucosyl position of P also appears to perform another important role in enhancing the solubility of the compound.

In summary, this study showed that the attachment of a glucosyl or isomaltosyl residue to P results in increased water solubility. The number of glucosyl residues attached to P clearly determined the degree of water solubility of P derivatives. P-Gs synthesized by LLDexT are also expected to maintain the antioxidant activity [6] and the vasorelaxant effect [8] of the parent P. Therefore, this improved water solubility of P-Gs suggests that these compounds have great potential for development into new pharmaceutical agents for treating osteoporosis and menopause-associated symptoms as well as for use as functional food additives.

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