Deglycosylation of the Isoflavone C-Glucoside Puerarin by a Combination of Two Recombinant Bacterial Enzymes and 3-Oxo-Glucose

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ABSTRACT A human intestinal bacterium strain related to *Dorea* species, PUE, can metabolize the isoflavone C-glucoside puerarin (daidzein 8-C-glucoside) to daidzein and glucose. We reported previously that 3"-oxo-puerarin is an essential reaction intermediate in enzymatic puerarin degradation, and we characterized a bacterial enzyme, the DgpB-DgpC complex, that cleaved the C-glycosidic bond in 3"-oxo-puerarin. However, the exact enzyme catalyzing the oxidation of the C-3" hydroxyl in puerarin has not been identified. In this study, we demonstrated that recombinant DgpA, a Gfo/Idh/MocA family oxidoreductase, catalyzed puerarin oxidation in the presence of 3-oxo-glucose as the hydride acceptor. In the redox reaction, NAD(H) functioned as the cofactor, which bound tightly but noncovalently to DgpA. Kinetics analysis of DgpA revealed that the reaction proceeded via a ping-pong mechanism. Enzymatic C-deglycosylation of puerarin was achieved by a combination of recombinant DgpA, the DgpB-DgpC complex, and 3-oxo-glucose. In addition, the metabolite derived from the sugar moiety in the 3"-oxo-puerarin-cleaving reaction catalyzed by the DgpB-DgpC complex was characterized as 1,5-anhydro-D-erythro-hex-1-en-3-ulose, suggesting that the C-glycosidic linkage is cleaved through a β -elimination-like mechanism.

IMPORTANCE One important role of the gut microbiota is to metabolize dietary nutrients and supplements such as flavonoid glycosides. Ingested glycosides are metabolized by intestinal bacteria to more-absorbable aglycones and further degradation products that show beneficial effects in humans. Although numerous glycoside hydrolases that catalyze O-deglycosylation have been reported, enzymes responsible for C-deglycosylation are still limited. In this study, we characterized enzymes involved in the C-deglycosylation of puerarin from a human intestinal bacterium, PUE. Here, we report the purification and characterization of a recombinant oxidoreductase involved in C-glucoside degradation. This study provides new insights for the elucidation of mechanisms of enzymatic C-deglycosylation.

KEYWORDS C-glucoside, Gfo/Idh/MocA, deglycosylation, intestinal bacterium, oxidoreductase, puerarin

More than 1,000 species of bacteria colonize the human gut and affect host health and disease (1, 2). One important role of the gut microbiota is to metabolize dietary nutrients and supplements such as flavonoids (3, 4). Many natural flavonoids in plants are stored in the form of glycosides. In general, ingested glycosides are poorly absorbed in the human small intestine because of their hydrophilicity but are reported to be metabolized by intestinal bacteria to more-absorbable aglycones and further degradation products (3, 4). For example, the isoflavone O-glucoside daidzin (daidzein 7-O-glucoside) is hydrolyzed to aglycone, and the resulting daidzein is reduced to

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FIG 1 Proposed puerarin degradation pathway catalyzed by DgpA and the DgpB-DgpC complex.

(*S*)-equol, which shows beneficial effects in humans by preventing hormone-related diseases (4–6). From this perspective, naturally occurring glycosides are considered to be a type of prodrug activated by the intestinal bacterial metabolism (7).

C-Glucoside is a kind of naturally occurring glycoside in which the anomeric carbon of glucose is directly connected to the aglycone via carbon-carbon bonding. Because of the stability of C-glucosyl bonds, C-glucosides are resistant to glycoside hydrolase and acid treatments, in contrast to O-glucosides. Although the catalytic mechanisms of enzymatic C-deglycosylation have not been well characterized, some intestinal bacteria have been reported to metabolize C-glucosides to the corresponding aglycones (8–14). Braune et al. reported that heterologous expression of five *Eubacterium cellulosolvens* genes (*dfgABCDE*) in *Escherichia coli* led to the metabolization of flavone C-glucosides to aglycone (15). This was the first study in which the genes involved in C-deglycosylation were cloned; however, the roles of these five gene products in the reaction remain unclear.

We previously isolated a human intestinal bacterium, PUE (with 98% similarity in the 16S rRNA gene sequence to *Dorea longicatena* strains LCR19 [GenBank accession no. [HQ259728](#)] and Marseille-P2116 [GenBank accession no. [LT223662](#)]), that metabolizes the isoflavone C-glucoside puerarin (daidzein 8-C-glucoside) to daidzein and glucose (10, 16). Enzymatic studies have revealed that more than three bacterial enzymes are involved in the multistep reaction of C-deglycosylation (17). Moreover, a putative puerarin-metabolizing operon composed of eight genes (*dgpABCDEFGH*) from strain PUE has been identified (GenBank accession no. [LC422372](#)), and a recombinant DgpB-DgpC complex has been shown to cleave the C-glycosidic bond in 3''-oxo-puerarin but not in puerarin (Fig. 1) (18). These results indicated that 3''-oxo-puerarin is an essential reaction intermediate in the puerarin degradation reaction, and an unidentified oxidoreductase that catalyzes oxidation at the C-3'' hydroxyl of puerarin was predicted to be encoded in the operon.

In this study, we demonstrated that recombinant DgpA catalyzed puerarin oxidation in the presence of 3-oxo-glucose as the hydride acceptor (Fig. 1). In addition, enzymatic C-deglycosylation of puerarin was achieved by a combination of DgpA, the DgpB-DgpC complex, and 3-oxo-glucose. Furthermore, the real metabolite derived from the sugar moiety in 3''-oxo-puerarin by the reaction catalyzed by the DgpB-DgpC complex was characterized as 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1).

RESULTS

Purification of recombinant DgpA, a Gfo/Idh/MocA family oxidoreductase.

3''-Oxo-puerarin is a key intermediate in the enzymatic C-deglycosylation of puerarin (Fig. 1); however, the exact enzyme catalyzing the oxidation of the C-3'' hydroxyl in puerarin has not been identified. We reported previously the putative puerarin-metabolizing operon, composed of eight genes (*dgpA* to *dgpH*), from the intestinal bacterium strain PUE (18). A BLAST search with the deduced amino acid sequences of

FIG 2 HPLC analysis of DgpA-bound NAD(H). Purified DgpA was denatured with cold methanol, and the dissociated cofactors were analyzed by HPLC. (a) Authentic mixture of cofactors NAD⁺, NADH, NADP⁺, and NADPH; (b) DgpA-bound cofactors.

the eight genes in the operon revealed that both of the gene products DgpA (NCBI Protein accession no. [BBG22493.1](#)) and DgpF (NCBI Protein accession no. [BBG22498.1](#)) might be oxidoreductases of the Gfo (glucose-fructose oxidoreductase)/Idh (inositol 2-dehydrogenase)/MocA (rhizopine catabolism protein MocA) protein family (19). Particularly, DgpA was implicated in puerarin oxidation because the N terminus of the amino acid sequence deduced from the *dgpA* gene was identical to that of a previously reported protein involved in puerarin metabolism (17).

To characterize the enzymatic activity of DgpA, the encoding gene *dgpA* was heterologously expressed in *E. coli*, and the recombinant protein was purified by two-step column chromatography. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the purified DgpA appeared as a single band with an apparent molecular mass of 42 kDa, showing good agreement with the calculated molecular mass of 40,161 Da (see Fig. S1 in the supplemental material). The previously characterized DgpB-DgpC complex (18), which catalyzes the deglycosylation of 3''-oxo-puerarin, was also analyzed (Fig. S1), and its purity was found to be 94% by using ImageJ software.

Determination of the cofactor(s) bound to DgpA. In the UV-visible (UV-vis) spectrum of purified DgpA, a broad shoulder peak at approximately 340 nm was observed, suggesting that nicotinamide cofactors such as NAD(H) or NADP(H) were bound to the enzyme (Fig. S2). To characterize the cofactors, high-performance liquid chromatography (HPLC) analysis was performed after the protein was treated with cold methanol to dissociate the cofactors. As shown in Fig. 2b, two major peaks were observed, at 10.9 and 13.5 min, in HPLC analysis of DgpA-bound cofactors. These two peaks were characterized as NAD⁺ and NADH by comparing the retention times to those of authentic nicotinamide cofactors (Fig. 2a). These results indicate that NAD(H) functioned as the cofactor that binds tightly but noncovalently to DgpA. The amounts of NAD⁺ and NADH dissociated from 25 nmol (1 mg) of DgpA were determined by the HPLC method as 3.4 and 11 nmol, respectively, indicating that the estimated stoichiometry of NAD(H) per enzyme was approximately 0.58:1 [mol of NAD(H) per mol of enzyme]. Some Gfo/Idh/MocA family proteins contain strongly bound NAD(P)(H), and the stoichiometry of NAD(P)(H) per enzyme was 1:1 [mol of NAD(P)(H) per mol of enzyme] (20, 21). Therefore, the purified DgpA used in this study might be a mixture of the apoenzyme and the holoenzyme.

Oxidation of puerarin by DgpA and 3-oxo-glucose. To confirm the oxidation of puerarin catalyzed by DgpA, recombinant DgpA was incubated with 0.5 mM puerarin in potassium phosphate buffer (pH 7.4) at 37°C for 30 min. According to HPLC analysis,

FIG 3 HPLC analysis of metabolites in the enzymatic reaction catalyzed by DgpA and the DgpB-DgpC complex. Enzymatic reaction mixtures were incubated at 37°C for 30 min, and the reaction solutions were analyzed by HPLC. Sugars cannot be detected by UV absorbance at 256 nm. The compositions of the reaction mixtures were as follows: puerarin and DgpA (a), puerarin, DgpA, and 3-oxo-glucose (b), 3''-oxo-puerarin and DgpA (c), 3''-oxo-puerarin, DgpA, and glucose (d), and puerarin, DgpA, the DgpB-DgpC complex, and 3-oxo-glucose (e).

no metabolites were detected under this condition (Fig. 3a). The same result was obtained when 1 mM NAD^+ and 1 mM MnCl_2 were added to the reaction mixture, even though these two cofactors have been reported to increase enzymatic activity during puerarin C-deglycosylation (17). As shown in Fig. 1, DgpA may require 3-oxo-glucose for the oxidation of puerarin, since the ultimate sugar metabolite in puerarin degradation should be glucose rather than an oxo-sugar derivative (16). Based on this assumption, 3-oxo-glucose instead of NAD^+ was added to the reaction mixture including DgpA and puerarin, resulting in the detection of two metabolite peaks, at 8.1 and 8.6 min, in HPLC analysis (Fig. 3b). Eighty percent of the puerarin was converted to the metabolites. The retention times and elution profiles of the metabolites were identical to those of 3''-oxo-puerarin in the buffer, which easily isomerized to a mixture of the 3''-oxo form (a peak at 8.1 min), the 2''-oxo form, and its intramolecular-cyclic acetal (a peak at 8.6 min, overlapping), as reported previously (18). These results demonstrate that DgpA catalyzed oxidation at the 3''-hydroxyl of puerarin by using 3-oxo-glucose as the hydride acceptor (Fig. 1).

Reduction of 3''-oxo-puerarin by DgpA and glucose. To identify the actual metabolites in the oxidation reaction catalyzed by DgpA, an enzymatic counterreaction was proposed. The 3''-oxo-puerarin standard and DgpA were incubated with or without D-glucose at 37°C for 30 min, followed by HPLC analysis (Fig. 3c and d). In the reaction of the 3''-oxo-puerarin standard and DgpA with glucose, one conspicuous metabolite peak was detected, at 7.7 min, by HPLC (Fig. 3d). After chromatographic isolation, the metabolite structure was confirmed as puerarin on the basis of nuclear magnetic resonance (NMR) analysis. The other expected product, 3-oxo-glucose, could not be detected by HPLC analysis due to a lack of UV absorbance. These findings indicate that the reaction catalyzed by DgpA was reversible, and the metabolites in the puerarin oxidation reaction were verified as 3''-oxo-puerarin and D-glucose, as shown in Fig. 1.

C-Deglycosylation of puerarin by a combination of DgpA, the DgpB-DgpC complex, and 3-oxo-glucose. The DgpB-DgpC complex has been reported to metab-

FIG 4 Double-reciprocal kinetic plots for DgpA. The concentration of 3-oxo-glucose was tested at 0.5, 0.25, 0.15, and 0.1 mM. The concentration of puerarin was tested at 0.5 mM (open triangles), 0.25 mM (filled triangles), 0.15 mM (open circles), and 0.1 mM (filled circles). 3-Oxo-glucose exists as an equilibrium mixture of many isomeric forms in aqueous solution; therefore, only limited isomeric forms of 3-oxo-glucose were likely to be involved in the reaction.

olize 3''-oxo-puerarin to daidzein (18). To achieve enzymatic C-deglycosylation of puerarin, two recombinant bacterial enzymes (DgpA and the DgpB-DgpC complex) and 3-oxo-glucose were incubated with puerarin at 37°C for 30 min, and the solution was then analyzed by HPLC. The peak detected as daidzein was observed at 15.0 min in the HPLC chromatogram, and the rate of conversion from puerarin to daidzein was 45% (Fig. 3e), indicating that the C-deglycosylation of puerarin was accomplished by the recombinant enzymes. Besides, 43% of the puerarin was converted to 3''-oxo-puerarin and its isomers, although the resulting intermediates were not further converted to daidzein under this reaction condition.

Enzymatic properties of DgpA. The optimum temperature and pH for the puerarin oxidation activity of DgpA were examined. The optimum temperature was 35°C, and the optimal pH was around 7.5 to 8.0 (Fig. S3). Kinetic analysis of DgpA revealed that the $V_{\max}$ value was 26 $\mu\text{mol/min/mg}$ and the K_m values of DgpA for puerarin and 3-oxo-glucose were 0.33 and 0.25 mM, respectively. As depicted in Fig. 4, double-reciprocal plots show parallel lines depending on the concentration of puerarin, suggesting that the reaction of DgpA proceeded via a ping-pong mechanism. However, 3-oxo-glucose has been reported to exist as an equilibrium mixture of many isomeric forms, such as anomeric, pyranose-type, and furanose-type isomers and their combination forms, in aqueous solution (22), so only limited isomeric forms of 3-oxo-glucose were likely to be involved in the reaction.

To confirm the role of NAD(H) bound to DgpA, the dissociated cofactor was analyzed by HPLC after the enzyme had been incubated with 3-oxo-glucose or puerarin. When DgpA-NAD(H) was incubated with 3-oxo-glucose, 97% of the dissociated cofactor was in the NAD⁺ form, whereas when DgpA-NAD(H) was incubated with puerarin, 97% was in the NADH form. These results indicated that the NAD(H) bound to DgpA was a cofactor directly involved in the redox reaction of puerarin and 3-oxo-glucose. On the basis of these observations, the reaction mechanism of DgpA was proposed as shown in Fig. 5.

FIG 5 Ping-pong mechanism of the reaction catalyzed by DgpA. E_{NADH} , DgpA-NADH; E_{NAD} , DgpA-NAD⁺; 3OG, 3-oxo-glucose; G, glucose; P, puerarin; 3''OP, 3''-oxo-puerarin.

FIG 6 ^1H and ^{13}C NMR spectra of compound 1, a metabolite derived from the 3''-oxo-puerarin sugar moiety in the reaction catalyzed by the DgpB-DgpC complex. (a) ^1H NMR spectrum; (b) ^{13}C NMR spectrum.

Effects of additional NAD(H) on the activity of purified DgpA in the presence of 3-oxo-glucose. As mentioned above, the stoichiometry of NAD(H) per DgpA was approximately 0.58 mol of NAD(H) per mol of DgpA, suggesting that the purified DgpA was a mixture of the apoenzyme (42%) and holoenzyme (58%). Consequently, additional NAD(H) might stimulate catalytic activity by binding to the DgpA apoenzyme. To confirm the effects of additional NAD^+ and NADH on the activity of purified DgpA, an enzymatic puerarin oxidation reaction was performed in the presence of 3-oxo-glucose. As a result, the reaction velocity was increased 1.5-fold by the addition of NAD^+ or NADH. Based on the rate of increase in enzymatic activity, 33% of the purified DgpA was estimated to be an apoenzyme.

Determination of the structure of 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1) as a metabolite of 3''-oxo-puerarin degradation catalyzed by the DgpB-DgpC complex. The DgpB-DgpC complex cleaves the C-glycosidic bond in 3''-oxo-puerarin to produce daidzein, whereas the other metabolite, corresponding to the precursor of D-glucose, derived from the sugar moiety in 3''-oxo-puerarin, remained unknown. To determine the structure of the real metabolite, enzymatic C-deglycosylation of 3''-oxo-puerarin was used. The major metabolite was obtained by chromatographic separation; the ^1H and ^{13}C NMR spectra are shown in Fig. 6. Based on spectral analysis, the signal for H-1 appeared at δ 7.36 ppm in the ^1H NMR spectrum (Fig. 6a), and signals for C-1, C-2, and C-3 were observed at δ 148.0, 135.2, and 191.5 ppm, respectively, in the ^{13}C NMR spectrum (Fig. 6b). These results suggest that the metabolite contained an α,β -unsaturated carbonyl group. Further analysis and comparison of the spectral data with previously reported data (23, 24) revealed that the structure of the real metabolite was 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1).

FIG 7 Proposed C-glycosyl bond-cleaving mechanism of 3''-oxo-puerarin catalyzed by the DgpB-DgpC complex.

DISCUSSION

The human intestinal bacterium strain PUE can metabolize the isoflavone C-glucoside puerarin to daidzein; however, the metabolic enzymes have not been well characterized. In this study, the bacterial DgpA protein was identified as the enzyme responsible for puerarin oxidation, as shown in Fig. 1. Additionally, enzymatic C-deglycosylation of puerarin was accomplished by a combination of recombinant DgpA, the DgpB-DgpC complex, and 3-oxo-glucose, yielding daidzein and 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1).

We previously purified a 40-kDa protein involved in puerarin metabolism from strain PUE and sequenced the 30 N-terminal amino acids (17). The sequence was identical to that of DgpA, indicating that the purified protein, previously designated protein C, was DgpA. The DgpA protein is a member of the Gfo/Ide/MocA oxidoreductase family. These proteins typically utilize NAD⁺/NADP⁺ and are related to the redox reactions of pyranoses (19). Gfo/Ide/MocA family oxidoreductases have a two-domain structure, comprising an N-terminal NAD(P)-binding Rossmann fold domain and a C-terminal α/β domain involved in substrate binding (19). Among the family proteins, Gfo (20) and WlbA (21) have been reported as NAD(P)(H)-binding proteins. Gfo contains bound NAD(P)(H) and catalyzes the redox reaction of glucose and fructose to form gluconolactone and sorbitol. WlbA contains bound NAD(H) and catalyzes the oxidation of UDP-GlcNAc to UDP-3-keto-GlcNAc in parallel with the reduction of α -ketoglutarate. These two proteins have a tetrameric structure and catalyze the reactions via a ping-pong mechanism. In this study, we revealed that DgpA was an NAD(H)-binding enzyme and used 3-oxo-glucose as the hydride acceptor for puerarin oxidation. In addition, the reaction catalyzed by DgpA proceeded via a ping-pong mechanism. These observations indicate that DgpA is similar to Gfo and WlbA.

The DgpB-DgpC complex cleaved the C-glycosidic bond in 3''-oxo-puerarin, yielding daidzein and 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1) (Fig. 1). The proposed C-glycosyl bond-cleaving mechanism catalyzed by the DgpB-DgpC complex is shown in Fig. 7. On the basis of our previous findings that 3''-oxo-puerarin could be easily isomerized to the 2''-oxo form under nonenzymatic conditions (18), 2''-ene-2'',3''-diol as a putative intermediate in this tautomerization process was naturally considered to be formed in a slightly basic buffer. Therefore, the reaction started from a nonenzymatic conversion from 3''-oxo-puerarin to the 2''-ene-2'',3''-diol form (Fig. 7A), and the aglycone moiety was subsequently converted to a good leaving group, such as a keto-enol tautomerization product, at C-7 (Fig. 7B), enabling enzymatic C-glycosyl bond cleavage to proceed. The final step for C-glycosyl bond cleavage was a β -elimination-like reaction to produce daidzein and compound 1. Compound 1 has been reported previously as a spontaneous decomposition product of the β -elimination of 3-ketocarbohydrates, such as 3-ketosucrose (25), oxidized ginsenoside compound K (26), and 3-keto-levoglucosan (23), under alkaline conditions. Similar β -elimination-like cleavage has been observed in glycoside hydrolase families 4 and 109 (27–29). These protein families show a unique reaction mechanism involving NAD⁺ for glycosyl bond cleavage. The first step of the reaction is oxidation at the C-3 hydroxyl of glycosides to yield 3-keto-glycosides and NADH. After oxidation, the proton at C-2 is slightly acidic

because of the inductive effects of the proximal C-3 carbonyl unit. Next, the acidified C-2 proton is abstracted, and then a glycosidic linkage is cleaved by β -elimination to give an α,β -unsaturated carbonyl intermediate such as compound 1. The hydrolase reaction is completed by Michael-type 1,4-addition of H_2O to the intermediate and the subsequent reduction of C-3 ketone to hydroxyl, assisted by NADH. These multistep hydrolase reactions are catalyzed by single enzymes of glycoside hydrolase families 4 and 109. α -N-Acetylgalactosaminidase, the glycoside hydrolase family 109 protein, is also a Gfo/Ildh/MocA family oxidoreductase to which NAD(H) is tightly bound (29). The glycoside hydrolase family 4 protein has been reported to cleave not only O-glycosides but also more-stable S-glycosides (30). In contrast, cleavage of C-glycosides by these enzymes has not been observed.

A proposed puerarin deglycosylation pathway based on the above-mentioned mechanism of glycoside hydrolase families 4 and 109 is shown in Fig. 1. The other enzyme encoded in the putative puerarin-metabolizing operon from strain PUE was likely involved in the reaction, since more than three enzymes have been reported to participate in puerarin C-deglycosylation (17). To clarify enzymatic puerarin C-deglycosylation, further studies are needed to characterize the unidentified enzyme responsible for the enantioselective Michael addition of H_2O to compound 1, yielding 3-oxo-glucose, and to identify the other gene products, DgpD to DgpH.

MATERIALS AND METHODS

Chemicals and materials. Puerarin was purchased from Carbosynth Limited. 1,2:5,6-Di-O-isopropylidene- α -D-glucofuranose was obtained from TCI. NAD⁺, NADH, NADP⁺, and NADPH were purchased from Oriental Yeast Co., Ltd. 3''-Oxo-puerarin was prepared as described previously (18). The purity of 3''-oxo-puerarin was 97%, based on high-performance liquid chromatography (HPLC) analysis with detection by UV absorbance at 256 nm. However, 3''-oxo-puerarin changed into a mixture of isomers in basic buffers (18). The genomic DNA of strain PUE was obtained according to the literature (18). The recombinant DgpB-DgpC complex was prepared as reported previously (18).

Preparation of 3-oxo-glucose. 3-Oxo-glucose was synthesized according to the procedures in the literature (31, 32). Briefly, the C-3 hydroxyl of 1,2:5,6-di-O-isopropylidene- α -D-glucofuranose was oxidized using NaOCl, 9-azanoradamantane N-oxyl (Nor-AZADO), and KBr in CH_2Cl_2 -aqueous $NaHCO_3$ (31). The 1,2:5,6-di-O-isopropylidene-3-oxo- α -D-glucofuranose obtained was treated with trifluoroacetic acid- H_2O (9:1) to produce 3-oxo-glucose (32). 3-Oxo-glucose was reported to exist in at least 10 isomeric forms. Among the isomers, α -D-ribo-hexofuranos-3-ulose (44%), β -D-ribo-hexopyranos-3-ulose (22%), and β -D-ribo-hexopyranos-3-ulose hydrate (12%) were major forms (22). The results of our 1H and ^{13}C NMR experiments also showed the presence of the three predominating isomeric forms (see Fig. S4 and S5 in the supplemental material). The purity of synthesized 3-oxo-glucose, especially the sum of the three major isomeric forms, was estimated to be 90% based on the integral values of the 1H NMR signals.

Construction of a recombinant DgpA expression vector. A DNA fragment carrying the *dgpA* gene was amplified from genomic DNA of strain PUE by PCR using forward primer 5'-AAAGAATTCATGAGTA AATAAAAATTGG-3' (the EcoRI site is underlined) and reverse primer 5'-AAACTCGAGTTAGAATTTAATT GTCTCAT-3' (the XhoI site is underlined). The amplified fragment was cloned into the EcoRI/XhoI site of the pET-21a(+) vector. A nucleotide sequence of the constructed vector encoding an N-terminal T7 tag was removed by deletion PCR using forward primer 5'-TATACATATGAGTAAATTAAAAATT-3' and reverse primer 5'-TTACTCATATGTATATCTCTCTTA-3' according to the manufacturer's instructions for a Prime-STAR mutagenesis basal kit (TaKaRa Bio, Inc.).

Purification of recombinant DgpA. *E. coli* BL21(DE3) was transformed with the constructed vector. The transformant was precultured in LB broth (2 ml) containing 100 μ g/ml ampicillin at 37°C for 6 h. The preculture was inoculated into LB broth (300 ml) containing 100 μ g/ml ampicillin and was further cultured at 37°C for 3 h. Recombinant DgpA was induced with 1 mM isopropyl- β -D-thiogalactopyranoside, and the culture was continued at 25°C for 15 h. The cells, suspended in 50 mM potassium phosphate buffer (pH 7.4), were disrupted by sonication on ice and were centrifuged (10,000 $\times g$, 4°C, 20 min) to yield a supernatant containing crude recombinant DgpA.

Recombinant DgpA was purified by two-step column chromatography consisting of anion exchange chromatography (with a HiPrep Q FF 16/10 column; GE Healthcare) and hydrophobic chromatography (with a HiPrep butyl FF 16/10 column; GE Healthcare). In detail, the cell-free extract containing crude DgpA was applied to a HiPrep Q FF 16/10 column and was eluted with a 0-to-400 mM NaCl gradient (15 column volumes) in 50 mM potassium phosphate buffer (pH 7.4) at a flow rate of 5 ml/min. $(NH_4)_2SO_4$ was added to the fraction containing DgpA (an approximately 230 mM NaCl elution fraction) to 30% saturation [176 g $(NH_4)_2SO_4$ /liter solution] with gentle stirring, and then the solution was applied to a HiPrep butyl FF 16/10 column. The column was eluted with a 1.2-to-0 M $(NH_4)_2SO_4$ gradient (15 column volumes) in 100 mM potassium phosphate buffer (pH 7.0) at a flow rate of 5 ml/min. The fraction containing DgpA [an approximately 0.2 M $(NH_4)_2SO_4$ elution fraction] was dialyzed against 50 mM potassium phosphate buffer (pH 7.4) to yield purified recombinant DgpA (40 mg).

Measurement of the UV-vis absorption spectrum of purified DgpA. The UV-vis absorption spectrum of purified DgpA (0.5 mg/ml in 50 mM potassium phosphate buffer [pH 7.4]) was recorded using a UV-1800 spectrophotometer (Shimadzu, Japan).

Determination of the amount of DgpA-bound NAD(H). The amount of DgpA-bound NAD(H) was determined according to the literature procedure with minor modifications (33). To 1 mg of purified DgpA in 0.1 ml 50 mM potassium phosphate buffer (pH 7.4), 0.9 ml methanol was added and mixed by pipetting; then the mixture was stored at 0°C for 15 min. The solution was concentrated *in vacuo*, and the resulting residue was dissolved with H₂O (0.2 ml). The solution was passed through a 0.22- μ m membrane, and the filtrate containing NAD(H) dissociated from DgpA was analyzed by HPLC. To quantify the dissociated NAD(H), each concentration of the NAD⁺ or NADH standard was treated under the same conditions as DgpA and was analyzed by HPLC. HPLC conditions were as follows: column, Cosmosil 5C₁₈-MS-II (inside diameter, 4.6 mm; length, 150 mm; Nacalai Tesque); flow rate, 1 ml/min; detection, 260 nm; mobile phase, 20 mM sodium dihydrogen phosphate (A) and acetonitrile (B) (with a linear gradient from 0% to 10% B over 30 min); injection volume, 10 μ l.

Analysis of dissociated NAD(H) after incubation of purified DgpA with a substrate. A reaction mixture (10 ml) consisting of purified DgpA (2 mg) and a substrate (puerarin or 3-oxo-glucose [0.5 mM]) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 30 min. The reaction mixture was concentrated to 1 ml using Amicon Ultra-15 10K centrifugal filter devices (Merck Millipore Ltd.) and was applied to a HiTrap desalting column (5 ml; GE Healthcare) eluting with the same buffer to remove a substrate. NAD(H) bound to the substrate-treated DgpA was analyzed by the method described above.

Enzyme assay. A reaction mixture (100 μ l) consisting of an enzyme (DgpA with or without the DgpB-DgpC complex [1 μ g each]), a substrate (puerarin or 3'-oxo-puerarin [0.5 mM]), and an additive (glucose or 3-oxo-glucose [5 mM]) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 30 min. Methanol (300 μ l) was added to the reaction solution, and metabolites were analyzed by octadecyl silane (ODS)-HPLC. Unless otherwise noted, HPLC conditions were the same as those described previously (18).

Purification and structure determination of a reductive metabolite of 3'-oxo-puerarin obtained by a reaction catalyzed by DgpA. A reaction mixture (10 ml) including DgpA (100 μ g), 3'-oxo-puerarin (1 mM), and glucose (50 mM) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 60 min. The reaction solution was passed through Amicon Ultra-15 10K centrifugal filter devices, and the low-molecular-weight fraction obtained was acidified with 1 mol/liter HCl. The acidified solution was applied to an InertSep C₁₈ column (GL Sciences), washed with H₂O, and then eluted with methanol. The methanol fraction was concentrated *in vacuo* to yield a reductive metabolite. The ¹H and ¹³C NMR spectra of the metabolite were identical to those of the puerarin standard.

Effects of temperature on the enzymatic activity of DgpA. Reaction mixtures (100 μ l) consisting of DgpA (0.2 μ g), puerarin (0.5 mM), and 3-oxo-glucose (0.5 mM) in 50 mM potassium phosphate buffer (pH 7.4) were incubated at different temperatures (20, 25, 30, 35, 40, 45, and 50°C) for 5 min. Methanol (300 μ l) was added to the reaction solution, and metabolites were analyzed by ODS-HPLC. The reactions were repeated three times.

Effects of pH on the enzymatic activity of DgpA. Reaction mixtures (100 μ l) consisting of DgpA (0.2 μ g), puerarin (0.5 mM), and 3-oxo-glucose (0.5 mM) in 50 mM sodium acetate buffer (pH 4.0, 5.0, or 6.0), 50 mM potassium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, or 8.0), or Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5, or 9.0) were incubated at 37°C for 5 min. Methanol (300 μ l) was added to the reaction solution, and metabolites were analyzed by ODS-HPLC. The reactions were repeated three times.

Determination of the kinetic parameters of DgpA. Reaction mixtures (500 μ l) consisting of DgpA (1 μ g), puerarin (0.5, 0.25, 0.15, or 0.1 mM), and 3-oxo-glucose (0.5, 0.25, 0.15, or 0.1 mM) in 50 mM potassium phosphate buffer (pH 7.4) were incubated at 37°C for various times. A total of 16 reaction conditions with different concentrations of puerarin and 3-oxo-glucose were tested. At incubation times of 20, 40, 60, and 80 s, an aliquot (100 μ l) of the reaction mixture was taken and added to cold methanol (300 μ l) to stop the reaction. Metabolites of puerarin in the reaction mixture were analyzed by ODS-HPLC. The reactions were repeated three times, and the kinetic parameters of DgpA were determined using SigmaPlot, version 14 (Systat Software, Inc).

Effects of additional NAD(H) on the activity of purified DgpA in the presence of 3-oxo-glucose. Reaction mixtures (500 μ l) consisting of purified DgpA (1 μ g), puerarin (0.5 mM), 3-oxo-glucose (0.5 mM), and NAD⁺ or NADH (1 mM) in 50 mM potassium phosphate buffer (pH 7.4) were incubated at 37°C for various times. At incubation times of 20, 40, 60, and 80 s, the reaction mixture was treated as described in the preceding section ("Determination of the kinetic parameters of DgpA").

Determination of the structure of a metabolite derived from the sugar moiety of 3'-oxo-puerarin by a reaction catalyzed by the DgpB-DgpC complex. A reaction mixture (30 ml) containing the DgpB-DgpC complex (1.8 mg) and 3'-oxo-puerarin (18.6 mg) in H₂O was incubated at 37°C for 30 min. The resulting precipitate (daidzein) was removed by filtration. To the filtrate, 10 ml of water-saturated butan-1-ol (containing 0.1% acetic acid [AcOH]) was added, and then liquid-liquid partition was carried out. The water layer was concentrated to approximately 3 ml and was applied to an InertSep C₁₈ column eluting with H₂O. The eluent was concentrated *in vacuo* to yield 1,5-anhydro-D-erythro-hex-1-en-3-ulose (compound 1) (1.7 mg).

¹H and ¹³C NMR spectra were recorded with a 600-MHz Varian NMR system, and the residual solvent of CD₃CN was used as an internal standard (¹H, 1.93 ppm; ¹³C, 1.3 ppm). ¹H NMR of compound 1 (600 MHz, CD₃CN) δ 3.75 (1H, dd, *J* = 4.3, 12.7 Hz, one of H-6), 3.83 (1H, dd, *J* = 2.1, 12.7 Hz, another one of H-6), 4.01 (1H, dddd, *J* = 0.5, 2.2, 4.3, 13.3 Hz, H-5), 4.33 (1H, d, *J* = 13.3 Hz, H-4), 7.36 (1H, s, H-1). ¹³C NMR of compound 1 (150 MHz, CD₃CN) δ 61.4 (C-6), 68.6 (C-4), 84.5 (C-5), 135.2 (C-2), 148.0 (C-1), 191.5 (C-3).

Data availability. The nucleotide sequence data of the putative puerarin-metabolizing operon, composed of eight genes (*dgpA* to *dgpH*), from strain PUE are available in the DDBJ/EMBL/GenBank databases under accession number [LC422372](https://doi.org/10.1101/157469).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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We declare no conflict of interest.

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