
Regular Articles

Expression and Characterization of the Human Intestinal Bacterial Enzyme which Cleaves the C–Glycosidic Bond in 3"–Oxo–puerarin.

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Abstract

Puerarin (daidzein 8–C–glucoside) is an isoflavone C–glucoside contained in the roots of Pueraria lobata Ohwi. We have previously isolated the human intestinal bacterium, strain PUE, which metabolizes puerarin to daidzein, though the enzyme which cleaves C–glycosidic bond has not been clarified. Here, we identified one of the intermediates of enzymatic puerarin C–deglycosylation reaction as 3″–oxo–puerarin (1): C–3 in the glucose moiety connecting to hydroxyl is oxidized to ketone group. 1 was easily isomerized to the mixture of 1, 2″–oxo–puerarin (2a) and cyclic acetal (2b) of 2a in non–enzymatic condition. We identified the putative puerarin–metabolizing operon of strain PUE composed of 8 genes (dgpA–H). Among them, DgpB–C complex was expressed in Escherichia coli, which cleaved the C–glycosidic bond in 1 but not puerarin. These results suggested that the puerarin C–deglycosylation reaction is a two–step enzymatic reaction, including the oxidation reaction at C–3″ in puerarin to give 1, and the subsequent C–deglycosylation of 1 to provide daidzein.

Key words: puerarin, C–glucoside, deglycosylation, human intestinal bacterium, bacterial enzyme, reaction intermediate
Introduction

Puerarin (daidzein 8-β-glucoside) is an isoflavone C-glucoside contained in the roots of Pueraria lobata Ohwi which is used for the treatment of fever and neck stiffness in Asian countries. Although C-glucosides are more stable than the corresponding O-glucosides, puerarin is metabolized to equol by co-culture of two human intestinal bacteria. 1) One bacterium, strain PUE, converts puerarin to daidzein by C-deglycosylation and another bacterium, strain DZE, converts daidzein to equol by reducing isoflavone skeleton. 1) Since equol, the final metabolite of puerarin, has been reported to have various beneficial effects such as estrogenic activity to lower the risks of hormone-dependent diseases, 2,3) studies of bacterial enzymes involved in the metabolic activation of puerarin are of great interest.

The bacterial enzymes that convert daidzein to equol have been studied and precisely characterized. 4-6) By contrast, bacterial enzymes involved in C-deglycosylation of puerarin have not been completely identified, though several human intestinal bacteria that metabolize puerarin to daidzein were reported. 1, 7, 8)

Some papers describe partial features of the intestinal bacterial enzymes involved in the deglycosylation of C-glucosides. In the case of Bacteroides sp. MANG, deglycosylation of mangiferin (xanthone C-glucoside) is catalyzed by at least two partially purified protein fractions, NADH, diaphorase, dithiothreitol, Mn^{2+} and uncharacterized low molecular cofactor. 9,10) In strain CG19–1, the enzymatic activity of puerarin deglycosylation is increased by adding NAD^{+} and Mn^{2+} to the cell-free extract. 11) In addition, two puerarin–induced proteins (DfgC, DfgD) that catalyze O-deglycosylation of daidzin but not C-deglycosylation are determined. 11) In Eubacterium cellulosolvens, the five genes (dfgABCDE) are cloned and expressed in Escherichia coli, which deglycosylates homoorientin and isovitexin (flavone C-glucosides). 11) In these cases, the function of each protein concerning C-glucoside metabolism is unknown, as well as the cleavage mechanism of the C-glucosyl bonding remains unclear.

We have investigated the C-deglycosylation reaction of puerarin by strain PUE, a related species of Dorea longicatena which is demonstrated by analysis of the 16S rRNA gene sequence. 1) We previously reported that first, the C-deglycosylation of puerarin is
catalyzed by at least three partially purified protein fractions, NAD$^+$ and Mn$^{2+}$, 12) second, one of the proteins involved in the reaction is purified, 12) and third, the metabolites of puerarin were determined to be daidzein and glucose by using [6$''$,$6''$−D$_2$]puerarin. 13) However, characterizations of the puerarin–metabolizing enzymes prepared by strain PUE have not been accomplished.

In this paper, we describe the identification of intermediates formed in enzymatic puerarin C−deglycosylation reaction. In addition, the heterologous expression of the key enzyme of strain PUE which cleaves the C−glycosidic bond in the intermediates to yield daidzein is also reported.
Materials and methods

General

$^{1}$H and $^{13}$C NMR were taken on Varian NMR system 600 ($^{1}$H, 600 MHz; $^{13}$C, 125 MHz). Chemical shifts are given on a $\delta$ (ppm) scale relative to the residual solvent signal ($^{1}$H, 3.31 ppm; $^{13}$C, 49.0 ppm for methanol-d$_4$ and $^{1}$H, 2.50 ppm; $^{13}$C, 39.5 ppm for DMSO-d$_6$). High resolution mass spectra (HRMS) were recorded on a bruker daltonics microTOF–Q mass spectrometer.

Chemicals and materials

Strain PUE was previously isolated from human feces. $^{1}$ Puerarin was purchased from Carbosynth Limited. Daidzein was obtained from Acros Organics. 2,6–dichloro–1,4–benzoquinone was purchased from TCI. [(2,9–dimethyl–1,10–phenanthroline)–Pd($\mu$–OAc)]$_2$ (OTf)$_2$ was prepared according to the literature procedures. $^{14}$

Partial purification of the puerarin–metabolizing enzymes from strain PUE

Cell–free extract of strain PUE was prepared as previously reported. $^{12}$ The cell–free extract was applied to an anion exchange column (HiPrep Q FF 16/10 column, GE Healthcare) and eluted with a linear gradient of 0–400 mM NaCl in 50 mM potassium phosphate buffer (pH 7.4). The elution fractions of the 100 mM NaCl and the 200 mM NaCl were used for the reaction described below.

Puerarin metabolic reaction catalyzed by the partially purified enzymes

Enzyme reaction mixtures (100 μL) consisting of the enzymes at the 100 mM NaCl and the 200 mM NaCl eluted fractions, MnCl$_2$ (1 mM), NAD$^+$ (1 mM) and puerarin (0.5 mM) were incubated at 37°C for 0 min, 30 min and 60 min. The reaction mixtures were extracted twice with 200 μL of butan–1–ol (saturated with water and acidified with 0.1% acetic acid). The extracts were evaporated to dryness, dissolved in 50% methanol (200 μL), and then analyzed by HPLC.

HPLC conditions for analyzing enzymatic reaction metabolites.

HPLC conditions for analyzing enzymatic reaction metabolites were as follows: column, COSMOSIL 5C$_{18}$–MS–II (Nacalai Tesque) 4.6×150 mm; flow rate, 1 mL/min; detection, 256
nm; mobile phase, 10–50% acetonitrile (linear gradient, 20 min) with 0.05% trifluoroacetic acid; injection volume, 10 µL. In the following experiments, HPLC analysis was performed under these conditions unless otherwise noted.

**Purification of enzymatic reaction intermediate 1**

The aforementioned puerarin metabolic reaction catalyzed by the partially purified enzymes was scaled up and the reaction mixture was extracted with the acidified butanol. The extract was subjected to silica gel column chromatography [CHCl₃–MeOH–H₂O (8:2.5:0.3)], and ODS column chromatography [COSMOSIL 5C₁₈–PAQ (Nacalai Tesque, 4.6×150 mm; flow rate, 1 mL/min; mobile phase, 25% methanol)], to obtain 1 (The yield of the product was 1 mg or less.).

**Synthesis of 3″-oxo-puerarin (1)**

Puerarin (43 mg, 0.1 mmol), 2,6-dichloro-1,4-benzoquinone (53 mg, 0.3 mmol) and [(2,9-dimethyl-1,10-phenanthroline)-Pd(μ-OAc)]₂(OTf)₂ (5 mg) were dissolved in dioxane/MeOH (1.5 mL, 1:1) and the reaction mixture was stirred at room temperature for 40 min. The reaction mixture was concentrated in vacuo, and the residue was suspended in chloroform. The resulting precipitate was collected by filtration, and applied to silica gel column chromatography [CHCl₃–MeOH–H₂O (8:1.5:0.1)] to yield 3″-oxo-puerarin (1) (30 mg, 70%) as a slightly yellow solid. 

**Whole genome sequencing of strain PUE**

Genomic DNA of strain PUE was isolated using a NucleoSpin Tissue
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(MACHEREY−NAGEL GmbH & Co. KG) and was sequenced using HiSeq2500 (Illumina Inc.). De novo assembly was performed with SPAdes. 15)

Nucleotide sequence accession number

The sequence of the putative puerarin−metabolizing operon encoding 8 genes has been deposited in the DDBJ under accession number LC422372.

Construction of recombinant DgpB−C complex co−expression vector.

The DNA fragment encoding dgpB and dgpC genes was amplified from genomic DNA of strain PUE by PCR using primers A (5′−AAAGGATCCATGGGACTTGCACTTCGATT−3’, BamH I sites are underlined) and B (5′−AAACTCGAGTTAGTCAACAAAAGTTGTGCA−3’, Xho I sites are underlined). The amplified fragment was cloned into the BamH I/ Xho I sites of the pET−21a (+) vector, and then transformed into E. coli BL21 (DE3). The nucleotide sequence encoding N−terminal T7 fusion tag of the constructed vector was deleted by PCR using the PrimeSTAR Mutagenesis Basal kit (Takara Bio Inc.) according to the manufacturer's instructions. The primers used for tag deletion PCR were C (5′−TATACATATGGGACTTGCACTTCG−3’) and D (5′−AGTCCCATATGTATATCTCC TTCTTA−3’).

Expression of recombinant DgpB−C complex.

The constructed DgpB−C complex co−expression vector (without T7−tag) was transformed into E. coli BL21 (DE3). The transformed E. coli was cultured at 37°C in LB broth containing 100 μg/ml ampicillin. The recombinant DgpB−C complex was induced with 1 mM isopropyl β−D−thiogalactopyranoside (IPTG) and cells were further incubated at 25°C for 15 h. The cells were collected by centrifugation and suspended in 40 mL of 50 mM potassium phosphate buffer (pH 7.4). The cells were disrupted by sonication on ice and centrifuged at 10,000 × g for 20 min to obtain a supernatant containing crude recombinant DgpB−C complex.

Purification of recombinant DgpB−C complex.

Recombinant DgpB−C complex was purified by an anion exchange column chromatography (HiPrep Q FF 16/10 column, GE Healthcare) and a hydrophobic column chromatography (HiPrep Butyl FF 16/10 column, GE Healthcare). In detail, the supernatant
containing crude recombinant DgpB–C complex was applied to a HiPrep Q FF 16/10 column and eluted with a linear gradient of 0–400 mM NaCl in 50 mM potassium phosphate buffer (pH 7.4). The fractions containing recombinant DgpB–C complex was adjusted to 25% saturation of (NH₄)₂SO₄, which was applied to a HiPrep Butyl FF 16/10 column and eluted with a linear gradient of 1–0 M (NH₄)₂SO₄ in 100 mM potassium phosphate buffer (pH 7.3). The purified recombinant DgpB–C complex was dialyzed against 50 mM potassium phosphate buffer (pH 7.4).

**Determination of the molecular weight of the purified DgpB–C complex**

The molecular weight of the purified DgpB–C complex was estimated by gel filtration chromatography (Superdex 200 10/300 GL column, GE Healthcare) using Gel Filtration Calibration Kit HMW (GE Healthcare) as standard. The chromatographic conditions were as follows: flow rate, 0.75 mL/min; mobile phase, 150 mM NaCl in 50 mM potassium phosphate buffer (pH 7.4); temperature, 4°C.

**SDS–PAGE analysis**

The molecular weights of the subunits of the purified DgpB–C complex were determined by SDS–PAGE using 15% polyacrylamide gel. The protein bands were stained with coomassie brilliant blue R250. Protein Molecular Weight Marker (broad) (Takara Bio Inc.) was used as standard.

**Protein measurement**

Protein content was determined by the method of Bradford, using Protein Quantification Kit–Rapid (Dojindo Molecular Technologies, Inc.).

**Enzymatic assay**

Enzymatic reaction mixture (100 μL) consisting of recombinant DgpB–C complex (1 μg) and 3″–oxo–puerarin or puerarin (0.5 mM) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for specified time. Methanol (300 μL) was added to the reaction solution, followed by centrifugation, and the supernatant was analyzed by HPLC.
**Results**

**Purification of 1, the intermediate formed in C–deglycosylation of puerarin.**

For the purpose of purification of the puerarin–metabolizing enzymes, cell–free extract of strain PUE, a human intestinal bacterium, was prepared and applied to an anion exchange column chromatography to give partially purified enzyme fractions. Puerarin metabolic reaction was performed by adding puerarin (0.5 mM), MnCl$_2$ (1 mM) and NAD$^+$ (1 mM) to the partially purified enzyme fractions, and the resulting metabolite was analyzed by HPLC (Fig. 1).

![Fig. 1](image1.png)

As a result, 64% of puerarin was converted to daidzein in 30 min incubation and 92% in 60 min incubation. Interestingly, in 30 min reaction condition, two minor peaks (1, 2) that were not detected in 0 min were observed. We presumed that these two peaks were enzymatic reaction intermediates, so tried to determine the structure of them. We purified 1 from the enzymatic reaction mixture, and then recorded its $^1$H NMR (Supplementary Fig. 1S–a). Based on the $^1$H NMR analysis, the structure of 1 was estimated to be 3″–oxo–puerarin: C–3 in the glucose moiety connecting to hydroxyl was oxidized to ketone group.

**Chemical conversion from puerarin to 3″–oxo–puerarin (1)**

In order to confirm the structure of 1, we synthesized 3″–oxo–puerarin through chemical conversion from puerarin by a catalytic regioselective oxidation using 2,6–dichloro–1,4–benzoquinone and [(2,9–dimethyl–1,10–phenanthroline)–Pd(µ–OAc)]$_2$ (OTf)$_2$ (Fig. 2). The $^1$H NMR spectrum of the synthetic 3″–oxo–puerarin (Supplementary Fig. 1S–b) was identical to that of 1 obtained by enzymatic conversion, thus concluding the structure of 1 to be 3″–oxo–puerarin.

![Fig. 2](image2.png)

**Non-enzymatic tautomerization between 1 and 2**

Besides the peak of 1, another peak (2) was observed in the 30 min enzymatic reaction mixture (Fig. 1–b). The same peak was also detected when the synthesized 1 was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and incubated at 37°C for 60 min (Fig. 6–b). After HPLC separation, pure 1 was obtained, however, the NMR data showed 2 was still a...
mixture. A lot of HPLC conditions such as using normal phase silica gel and/or ODS with suitable eluant system were carried out to separate this mixture, but it is not accomplished.

The detailed NMR analysis using DMSO-d$_6$ and methanol-d$_4$ as solvent revealed that 2 was the mixture of 2"−oxo−puerarin (2a) regarding as a tautomeric isomer of 1, and its cyclic acetal (2b) with the ratio 5:1 in DMSO−d$_6$ at 65°C and 1:1 in methanol−d$_4$ at 22°C, respectively (Fig. 3, Supplementary Fig. 2S). Interestingly, incubation of the mixture of 2a and 2b in above mentioned buffer at 37°C for 60 min gave the mixture of 1, 2a and 2b. These results indicated 1 and 2 could be easily isomerized to each other in non−enzymatic condition.

On the basis of spectral analyses of 1D and 2D NMR as well as COSY, NOESY, HSQC, HMBC, and so on, the structures of 2a and 2b were determined as shown in Fig. 3. According to the $^1$H NMR of 2a in DMSO-d$_6$, the signal of H−1" in sugar moiety showed singlet at $\delta$ 5.46 ppm. The $^{13}$C signal at $\delta$ 210.7 ppm is assigned as C−2" carbonyl quaternary carbon. The coupling constant between H−3" and H−4" was 3.4 Hz, and that between H−4" and H−5"was 1.4 Hz. These observations together with HSQC and HMBC analyses suggested the sugar moiety of 2a to be like a boat form rather than a chair form. The cross peaks in NOESY were observed between H−3" at $\delta$ 5.02 ppm and one of H"−6 at $\delta$ 3.60 ppm, and between H−1" at $\delta$ 5.46 ppm and H−5" at $\delta$ 3.88 ppm (Fig. 3, Supplementary Fig. 3S). These NOE correlations concluded the stereochemistry at C−3" to be assigned as 3"R. Occurring the tautomeric isomerization from 1 to 2a, the protonation at C−3" via 2"−ene−2",3"−diol proceeded facial selectively to be converted to (3"R)−2a, which is 2−oxo-allose type configuration.

On the other hand, the $^1$H and $^{13}$C NMR of 2b in methanol-d$_4$ showed some similarities to that of 2a, except for the $^{13}$C signal at $\delta$ 111.0 ppm for C−2" quaternary carbon. The signals in the A ring system of isoflavone at H−5 and H−6 in $^1$H NMR, and C−7 and C−8 in $^{13}$C NMR, respectively, were shifted a little more downfield area compared with those of 2a (Table 1). In addition, a long−range coupling was observed between H−1" in sugar moiety and H−6 in A ring system according to the COSY analysis. These evidences revealed that the structure of 2b had a cyclic acetal functionality formed between C−7 phenolic hydroxyl and C−2" (Fig. 3).
The NOESY and J value analysis in sugar protons suggested the stereochemistry at C-3" was the same as 2a.

[Fig. 3]

**Putative puerarin–metabolizing operon of strain PUE**

We have reported that three enzymes of strain PUE, which were induced by addition of puerarin to the culture medium, are essential for the C–glucosidic bond cleavage or C–deglycosylation of puerarin. One of the enzymes was purified and 30 residues of its N–terminal amino acid sequence were determined. Since puerarin–metabolizing enzymes are induced by puerarin, the genes encoding these enzymes would constitute the puerarin–metabolizing operon. In order to identify the operon, next generation sequencing and de novo assembly of genome DNA of strain PUE were performed.

Fig. 4 shows the putative puerarin–metabolizing operon of strain PUE composed of 8 genes that are all oriented in identical direction.

[Fig. 4]

The designation of the genes and gene products are related to their function (deglycosylation of puerarin, dgp / Dgp). The DNA sequence of the operon was quite similar to that of *Ruminococcus faecis JCM 15917* (approximately 95% sequence similarity with NZ_BBDW01000020.1, location: 3610-13282). The *dgpA* gene of strain PUE encoded a 40 kDa protein and its deduced amino acid sequence showed 98% identity with the *gfo/Idh/MocA* oxidoreductase of *Ruminococcus faecis* (WP_054754277.1). The N–terminal amino acid sequence of the DgpA protein of this enzyme was consistent with that of the previously reported one of the puerarin–metabolizing enzymes. The DgpB protein expressed 98% identity with the hypothetical protein of *Ruminococcus faecis* (WP_054754279.1) and it had no significant sequence similarity with functionally known proteins. The DgpC protein exhibits 98% homology with the sugar phosphate isomerase/epimerase of *Ruminococcus faecis* (WP_054754280.1). The DgpD, DgpE and DgpF proteins showed 64% identity with MFS transporter of *Butyricicoccus pullicaecorum* (WP_087370720.1), 38% identity with AraC family transcriptional regulator of
Faecalibaca orotica (WP_109733629.1) and 89% identity with gfo/Idh/MocA family oxidoreductase of Roseburia faecis (WP_055260959.1), respectively. DgpG and DgpH proteins expressed 98% identity with sugar phosphate isomerase/epimerase of Ruminococcus faecis (WP_054754287.1) and 96% identity with glucokinase of Ruminococcus faecis (WP_054754289.1), respectively.

Expression and purification of recombinant DgpB–C complex

As mentioned above, we isolated and verified 3″-oxo-puerarin (1) in the enzymatic reaction mixture of puerarin degradation as one of the intermediates formed just before C–glucosidic bond cleavage, which suggested that 1 might serve as exact substrate in the following enzymatic reaction. In addition, there would be enzyme(s) which execute C–deglycosylation from 1 to daidzein and D-glucose. To ascertain the enzyme, heterologous expression and characterization of the proteins encoded in the putative puerarin–metabolizing operon was performed. As a result of our various investigations, DgpB and DgpC proteins were found to be crucial for C–deglycosylation of 1, as follows.

Recombinant DgpB and DgpC were co–expressed in E. coli and purified by an anion exchange column chromatography and a hydrophobic column chromatography. In the course of enzyme purification, DgpB and DgpC were always eluted in the same fractions. Fig. 5 shows the SDS–PAGE of recombinant DgpB and DgpC.

[Fig. 5]

The molecular weights of DgpB and DgpC, calculated from the deduced amino acid sequences, were 16 and 37 kDa, respectively, which was in good agreement with the data of SDS–PAGE analysis. In addition, the molecular weights of the DgpB and DgpC were estimated with gel filtration chromatography. These two proteins were eluted as a single peak in a gel filtration and the estimated molecular weight showed 222 kDa. These results predicted DgpB and DgpC proteins to be consisted a complex, such as α₄β₄ heterooctamer (calculated: 212 kDa).

Enzymatic activity of DgpB–C complex which deglycosylates 3″–oxo–puerarin to daidzein

We tested the enzymatic activity of the purified DgpB–C complex (Fig. 6).
Fig. 6–a shows the HPLC chart of a certainly pure 3″-oxo–puerarin (1), which was chemically synthesized from puerarin. The standard 1 was partially isomerized into the mixtures of 1 and 2 in a slightly basic condition, such as in potassium phosphate buffer (pH 7.4) without any enzymes (Fig. 6–b). As shown in Fig. 6–c, when 1 and DgpB–C complex were incubated at 37°C for 60 min, a peak of metabolite was detected in HPLC chart at an elution time of 14 minutes, which was identical with that in the case of using daidzein standard. On the contrary, the incubation of DgpB–C complex together with puerarin instead of 1 was demonstrated at 37°C for 60 min. We did not observe daidzein as well as any enzymatic degradation, except for the intact puerarin (Fig. 6–d). These results indicated that DgpB–C complex worked as a key enzyme to cleave C–glycosidic bond in 1. 3″–Oxo–puerarin (1) is alternatively confirmed as the intermediate for C–deglycosylation, because DgpB–C complex regards 1 as the substrate in enzymatic cleaving reaction, and does not recognize puerarin with quite a little difference at C–3″ as it.

Finally, the time course of C–deglycosylation of 1 by DgpB–C complex was measured (Fig. 7). As shown in Fig. 7, 43% of 3″–oxo–puerarin was converted to daidzein in 20 min incubation, though it was only 46% even in 60 min incubation, suggesting that the reaction has reached the plateau after 20 minutes.
Discussion

We reported that at least three human intestinal bacterial proteins of strain PUE, Mn$^{2+}$ and NAD$^+$ were required for the C–deglycosylation of puerarin. In this study, 3″–oxo–puerarin (1) was identified as one of the intermediates of the enzymatic puerarin metabolizing reaction. Based on the puerarin–metabolizing operon, we characterized the DgpB–C complex, which cleaves the C–glycosidic bond in 1. These results suggested that the puerarin C–deglycosylation reaction is a two–step enzymatic reaction, including the oxidation reaction at C–3" in puerarin to give 1, and the subsequent C–deglycosylation of 1 to provide daidzein (Fig. 8).

From our previous study, the protein involved in the puerarin metabolism was isolated from the cell–free extract of strain PUE. The partial amino acid sequence of the purified protein was completely consistent with DgpA, categorized as gfo/Idh/MocA oxidoreductase. The purified protein catalyzed hydrogen–deuterium exchange reaction of puerarin to 2″–deuterated puerarin in D$_2$O condition. Such a hydrogen–deuterium exchange reaction has been reported in the glycoside hydrolase family 4 and 109, which oxidizes the C–3 hydroxyl group of carbohydrates to ketone group prior to the exchange reaction. Therefore, DgpA was predicted to be responsible for the oxidation from puerarin to 1.

92% of puerarin was deglycosylated in 60 min incubation by partially purified enzymes of strain PUE (Fig. 1). On the other hand, C–deglycosylation of 1 catalyzed by the DgpB–C complex was only 46% in 60 min incubation (Fig. 7), suggesting the deglycosylation reaction of 1 appeared to be inhibited by some factors, such as the sugar metabolite derived from 1. We have previously reported that the metabolite derived from the sugar moiety of puerarin is an intact D–glucose. In contrast, DgpB–C complex cleaved the C–glycosidic bond in 1 without a redox cofactor, indicating the exact metabolite in this reaction would not be D–glucose but some novel oxo–sugar derivative. Additionally, unidentified enzyme(s) should be able to perform the last important step from an oxo–sugar intermediate to D–glucose in puerarin metabolism (Fig. 8). We expect that DgpB–C complex regarding as the enzyme for C–deglycosylation of 1 might reversibly catalyze C–glycosylation of daidzein, which is
under investigation.

In conclusion, we identified the putative puerarin–metabolizing operon of strain PUE and characterized the function of DgpB–C complex, which cleaves the C–glycosidic bond in 1. Further studies are needed to understand the mechanisms of enzymatic C–deglycosylation reaction, such as the functional analysis of DgpA and the other proteins encoded in the puerarin–metabolizing operon.

**Acknowledgement**

This research was supported by JSPS KAKENHI Grant Number 15K18891, 18K14949 and a Grant–in–Aid for the Cooperative Research Project from Institute of Natural Medicine at the University of Toyama.

**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.
REFERENCES


Fig. 1. Puerarin C–deglycosylation reaction catalyzed by the partially purified enzymes of a human intestinal bacterium, strain PUE.

Enzyme reaction mixtures consisting of the partially purified enzymes of strain PUE, MnCl₂ (1 mM), NAD⁺ (1 mM) and puerarin (0.5 mM) were incubated at 37°C for (a) 0 min, (b) 30 min and (c) 60 min, and the samples were analyzed by HPLC. At 30 min incubation condition, two minor peaks (1, 2) to be intermediates of the reaction were detected.
Fig. 2. One step chemical synthesis of 3″-oxo-puerarin (1) from puerarin.
Fig. 3. The structures of 2 (the mixture of 2a and 2b), which are the reaction intermediates of puerarin deglycosylation.
Fig. 4. Putative puerarin−metabolizing operon of strain PUE.

The products of \textit{dgpB} and \textit{dgpC} genes consisted DgpB−C complex, which cleaves the \textit{C}-glycosidic bond in 1. The putative proteins encoded by the respective genes are as follows: \textit{dgpA}, \textit{gfo/Idh/MocA} oxidoreductase; \textit{dgpD}, MFS transporter; \textit{dgpE}, AraC family transcriptional regulator; \textit{dgpF}, \textit{gfo/Idh/MocA} family oxidoreductase; \textit{dgpG}, sugar phosphate isomerase/epimerase; \textit{dgpH}, glucokinase.
Fig. 5. SDS–PAGE analysis of the recombinant DgpB–C complex.

The molecular weights calculated from the deduced amino acid sequence of DgpB and DgpC are 16 and 37 kDa, respectively. Lane M, marker proteins; lane 1, crude recombinant DgpB–C complex expressed in *E. coli*; lane 2, purified recombinant DgpB–C complex.
Enzymatic reaction was performed under the conditions described below, and the reaction solution was analyzed by HPLC. (a) 3″-oxo–puerarin standard prepared from puerarin by catalytic regioselective oxidation, (b) pure 3″-oxo–puerarin (0.5 mM) was incubated in potassium phosphate buffer (pH 7.4) at 37°C for 60 min, (c) 3″-oxo–puerarin (0.5 mM) and DgpB–C complex (10 μg/mL) in potassium phosphate buffer (pH 7.4) were incubated at 37°C for 60 min, (d) puerarin (0.5 mM) and DgpB–C complex (10 μg/mL) in potassium phosphate buffer (pH 7.4) were incubated at 37°C for 60 min.
Fig. 7. The time course of deglycosylation reaction of 1 by DgpB–C complex.

1 (0.5 mM) and DgpB–C complex (10 µg/mL) in potassium phosphate buffer (pH 7.4) were incubated at 37°C for 5 min, 10 min, 20 min, 40 min and 60 min, and the reaction mixture was analyzed by HPLC.
The first step is oxidation of puerarin to afford 1, and the subsequent $C$–glycosidic bond cleavage is catalyzed by DgpB–C complex to give daidzein and a presumable oxo–sugar derivative. The resulting oxo–sugar would be converted to $D$–glucose by unidentified enzymes.
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<td>8.08, 1H, d (8.9)</td>
<td>7.99, 1H, d (8.8)</td>
</tr>
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<td>7.01, 1H, d (8.8)</td>
<td>6.98, 1H, d (8.9)</td>
<td>7.01, 1H, d (8.8)</td>
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<tr>
<td>7</td>
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<td>160.6</td>
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<td>112.4</td>
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<td>158.1</td>
<td>157.7</td>
<td>155.3</td>
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<td>9</td>
<td>124.2</td>
<td>124.1</td>
<td>124.0</td>
<td>122.1</td>
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<tr>
<td>2'</td>
<td>7.37, 2H, d (8.8)</td>
<td>7.37, 2H, d (8.6)</td>
<td>7.36, 2H, m</td>
<td>7.38, 2H, (8.7)</td>
</tr>
<tr>
<td>3',5'</td>
<td>6.84, 2H, d (8.8)</td>
<td>6.84, 2H, d (8.6)</td>
<td>6.85, 2H, m</td>
<td>6.81, 2H, (8.7)</td>
</tr>
<tr>
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<td>158.7</td>
<td>158.8</td>
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</tr>
<tr>
<td>1''</td>
<td>5.10, 1H, d (10.0)</td>
<td>75.6</td>
<td>75.8</td>
<td>73.5</td>
</tr>
<tr>
<td>2''</td>
<td>4.12, 1H, m</td>
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<td>73.5</td>
<td>5.14, 1H, s</td>
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<td>3''</td>
<td>3.53, 1H, m</td>
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<tr>
<td>4''</td>
<td>3.37, 1H, m</td>
<td>74.1</td>
<td>73.3</td>
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<tr>
<td>5''</td>
<td>3.48, 1H, m</td>
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<td>6''</td>
<td>3.90, 1H, dd (12.0, 2.1)</td>
<td>62.7</td>
<td>3.95, 1H, dd (12.3, 2.0)</td>
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