

1 Regular Articles

2 Biodegradation

3

4

5 Deglycosylation of isoflavone C–glucoside puerarin by combination of
6 two recombinant bacterial enzymes and 3–oxo–glucose.

7

8

9

10 Kenichi NAKAMURA,^a Shu ZHU,^b Katsuko KOMATSU,^b Masao HATTORI,^b and Makoto IWASHIMA ^{*,a}

11

12 ^a Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science; 3500–3
13 Minamitamagaki, Suzuka, Mie, 513–8670, Japan: and ^b Institute of Natural Medicine, University
14 of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.

15

16 *To whom correspondence should be addressed. e–mail: iwashima@suzuka-u.ac.jp

17

18 **Abstract**

19 C-Glucosides are resistant to glycoside hydrolase activity because the anomeric carbon of
20 glucose is directly connected to aglycone via carbon-carbon bonding. A human intestinal
21 bacterium strain PUE related to *Dorea* species can metabolize the isoflavone C-glycoside puerarin
22 (daidzein 8-C-glycoside) to daidzein and glucose by more than three bacterial enzymes which
23 have not been well-characterized. We previously reported that 3"-oxo-puerarin is an essential
24 reaction intermediate in enzymatic puerarin degradation and characterized a bacterial enzyme of
25 DgpB-C complex which cleaved the C-glycosidic bond in 3"-oxo-puerarin. However, the exact
26 enzyme catalyzing the oxidation of C-3" hydroxyl in puerarin has not been identified, and the
27 other metabolite corresponding to the precursor of D-glucose, derived from the sugar moiety in
28 3"-oxo-puerarin in the cleaving reaction catalyzed by the DgpB-C complex, remains unknown.

29 In this study, we demonstrated that recombinant DgpA, a Gfo/Idh/MocA family oxidoreductase,
30 catalyzed puerarin oxidation in the presence of 3-oxo-glucose as the hydride acceptor. In addition,
31 enzymatic C-deglycosylation of puerarin was achieved by a combination of recombinant DgpA,
32 DgpB-C complex, and 3-oxo-glucose. Furthermore, the metabolite derived from the sugar
33 moiety in 3"-oxo-puerarin cleaving reaction catalyzed by DgpB-C complex was characterized as
34 1,5-anhydro-D-*erythro* -hex-1-en-3-ulose, suggesting that the C-glycosidic linkage is cleaved
35 through a β -elimination like mechanism.

36

37 **Importance**

38 One important role of the gut microbiota is to metabolize dietary nutrients and supplements such
39 as flavonoid glycosides. Ingested glycosides are metabolized by intestinal bacteria to more
40 absorbable aglycones and further degradation products which show beneficial effects in humans.
41 Although numerous glycoside hydrolases that catalyze O-deglycosylation have been reported,
42 enzymes responsible for C-deglycosylation are still limited. In this study, we characterized
43 enzymes involved in C-deglycosylation of puerarin from a human intestinal bacterium PUE. To
44 our knowledge, this is the first report of the expression, purification and characterization of an

45 oxidoreductase involved in *C*-glucoside degradation. This study provides new insights for the
46 elucidation of mechanisms of enzymatic *C*-deglycosylation.

47

48 **Key words:**

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

50

51

52 Introduction

53 More than 1000 species of bacteria colonize the human gut and affect host health and diseases
54 (1, 2). One important role of the gut microbiota is to metabolize dietary nutrients and supplements
55 such as flavonoids (3, 4). Many natural flavonoids in plants are stored in the form of glycosides.
56 In general, ingested glycosides are poorly absorbed in the human small intestine because of their
57 hydrophilicity but are reported to be metabolized by intestinal bacteria to more absorbable
58 aglycones and further degradation products (3, 4). For example, the isoflavone *O*-glucoside
59 daidzin (daidzein 7-*O*-glucoside) is hydrolyzed to aglycone and the resulting daidzein is reduced
60 to (*S*)-equol, which shows beneficial effects in humans by preventing hormone-related diseases
61 (4-6). From this perspective, naturally occurring glycosides are considered to be a type of prodrug
62 activated by intestinal bacterial metabolism (7).

63 *C*-Glucoside is a naturally occurring glycoside in which the anomeric carbon of glucose is
64 directly connected to the aglycone via carbon-carbon bonding. Because of the stability of
65 *C*-glucosyl bonds, *C*-glucosides are resistant to glycoside hydrolase and acid treatments, in
66 contrast to *O*-glucosides. Although the catalytic mechanisms of enzymatic *C*-deglycosylation
67 have not been well-characterized, some intestinal bacteria were reported to metabolize
68 *C*-glucosides to the corresponding aglycones (8-14). Braune *et al.* reported that heterologous
69 expression of five *Eubacterium cellulosolvens* genes (*dfgABCDE*) in *Escherichia coli* led to
70 metabolization of flavone *C*-glucosides to aglycone (15). This was the first study in which the
71 genes involved in *C*-deglycosylation were cloned; however, the roles of these 5 gene products in
72 the reaction remain unclear.

73 We previously isolated a human intestinal bacterium PUE (92% similarity in 16S rRNA gene
74 sequence with *Dorea longicatena*), which metabolizes the isoflavone *C*-glucoside puerarin
75 (daidzein 8-*C*-glucoside) to daidzein and glucose (10, 16). Enzymatic studies revealed that more
76 than three bacterial enzymes involved in multi-step reaction of *C*-deglycosylation (17). Moreover,
77 a putative puerarin-metabolizing-operon composed of 8 genes (*dgpA-H*) from strain PUE was
78 identified (accession number LC422372), and recombinant DgpB-*C* complex was shown to cleave

79 the C-glycosidic bond in 3"-oxo-puerarin **but not puerarin** (Fig. 1) (18). These results indicated
80 that 3"-oxo-puerarin is an essential reaction intermediate in puerarin degradation reaction, and an
81 unidentified oxidoreductase that catalyzes oxidation at the C-3" hydroxyl of puerarin was
82 predicted to be encoded in the operon.

83 In this study, we demonstrated that recombinant DgpA catalyzed puerarin oxidation in the
84 presence of 3-oxo-glucose as the hydride acceptor (Fig. 1). In addition, enzymatic
85 C-deglycosylation of puerarin was achieved by a combination of DgpA, DgpB-C complex and
86 3-oxo-glucose. Furthermore, the real metabolite derived from the sugar moiety in
87 3"-oxo-puerarin catalyzed by DgpB-C complex was characterized as 1,5-anhydro-D-*erythro*
88 -hex-1-en-3-ulose (**1**).

89 [Fig. 1]

90

91 **Materials and methods**

92 **Chemicals and materials**

93 Puerarin was purchased from Carbosynth Limited.
94 1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose was obtained from TCI. NAD⁺, NADH,
95 NADP⁺, and NADPH were purchased from Oriental Yeast Co., Ltd. 3"-oxo-puerarin was
96 prepared as previously described (18). Genomic DNA of strain PUE was obtained according to
97 the literature (18). Recombinant DgpB-C complex was prepared as previously reported (18).

98

99 **Preparation of 3-oxo-glucose**

100 3-oxo-glucose was synthesized according to the literature procedures (19, 20). Briefly, C-3
101 hydroxyl of 1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose was oxidized using NaOCl,
102 nor-AZADO and KBr in CH₂Cl₂/aq NaHCO₃ (19). The obtained
103 1,2:5,6-di-*O*-isopropylidene-3-oxo- α -D-glucofuranose was treated with trifluoroacetic
104 acid:H₂O (9:1) to give 3-oxo-glucose (20).

105

106 **Construction of recombinant DgpA expression vector.**

107 A DNA fragment encoding *dgpA* gene was amplified from genomic DNA of strain PUE by
108 PCR using forward primer (5'-AAAGAATTCATGAGTAAATTAATAATTGG-3', EcoR I site is
109 underlined) and reverse primer (5'-AAACTCGAGTTAGAATTTAATTGTCTCAT-3', Xho I site
110 is underlined). The amplified fragment was cloned into the EcoR I/ Xho I site of the pET-21a (+)
111 vector. A nucleotide sequence encoding N-terminus T7 tag of the constructed vector was removed
112 by deletion PCR using forward primer (5'-TATACATATGAGTAAATTAATAATT-3') and
113 reverse primer (5'-TTACTCATATGTATATCTCCTTCTTA-3') according to the manufacturer's
114 instructions of a PrimeSTAR Mutagenesis Basal kit (Takara Bio Inc.).

115

116 **Expression and purification of recombinant DgpA.**

117 The constructed vector was transformed into *E. coli* BL21 (DE3) and the transformant was
118 cultured at 37°C in LB broth containing 100 μ g/mL ampicillin. A recombinant DgpA was induced

119 with 1 mM isopropyl β -D-thiogalactopyranoside and the culture was continued at 25°C for 15 h.
120 The cells were disrupted by sonication and centrifuged to obtain a supernatant containing crude
121 recombinant DgpA.

122

123 **Purification of recombinant DgpA.**

124 Recombinant DgpA was purified by two-step column chromatography of an anion exchange
125 column chromatography (HiPrep Q FF 16/10 column, GE Healthcare) and a hydrophobic column
126 chromatography (HiPrep Butyl FF 16/10 column, GE Healthcare). The purified protein was
127 dialyzed against 50 mM potassium phosphate buffer (pH 7.4).

128

129 **Measurement of UV-vis absorption spectrum of the purified DgpA**

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

132

133 **Determination of DgpA-bound NAD(H).**

134 Determination of DgpA-bound NAD(H) was performed according to the literature procedure
135 with minor modification (21). To the 1 mg of purified DgpA in 0.1 mL 50 mM potassium phosphate
136 buffer (pH 7.4) was added 0.9 mL methanol, which was stored at 0°C for 15 min. The solution was
137 passed through 0.22 μ m membrane and the filtrate was concentrated in vacuo to approximately
138 0.1 mL to remove methanol. To the concentrated solution was added H₂O (0.1 mL) and passed
139 through 0.22 μ m membrane. The filtrate containing dissociated NAD(H) from DgpA was analyzed
140 by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: column,
141 COSMOSIL 5C₁₈-MS-II (Nacalai Tesque) 4.6×150 mm; flow rate, 1 mL/min; detection, 260 nm;
142 mobile phase, (A) 20 mM sodium dihydrogen phosphate and (B) acetonitrile (linear gradient from
143 0% to 10% B concentration over 30 min); injection volume, 10 μ L.

144

145 **Enzyme assay.**

146 A reaction mixture (100 μ L) consisting of an enzyme (DgpA with or without DgpB–C complex,
147 1 μ g each), a substrate (puerarin or 3''-oxo-puerarin, 0.5 mM), and an additive (glucose or
148 3-oxo-glucose, 5 mM) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for
149 30 min. Methanol (300 μ L) was added to the reaction solution and metabolites were analyzed by
150 ODS–HPLC. HPLC conditions were the same as previously described (18).

151

152 **Purification and structure determination of a reductive metabolite of 3''-oxo-puerarin**
153 **catalyzed by DgpA.**

154 A reaction mixture (10 mL) including DgpA (100 μ g), 3''-oxo-puerarin (1 mM), and glucose
155 (50 mM) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 60 min. The
156 reaction solution was passed through Amicon Ultra–15 10K centrifugal filter devices (Merck
157 Millipore Ltd.) and the obtained low molecular fraction was acidified with 1 mol/L HCl. The
158 acidified solution was applied to inertSep C18 column (GL Sciences) and washed with H₂O, and
159 then eluted with methanol. The methanol fraction was concentrated in vacuo to give a reductive
160 metabolite. ¹H and ¹³C NMR spectra were identical to that of puerarin standard.

161

162 **Structure determination of a metabolite derived from the sugar moiety of 3''-oxo-puerarin**
163 **catalyzed by DgpB–C complex.**

164 A reaction mixture (30 mL) containing DgpB–C complex (1.8 mg) and 3''-oxo-puerarin (18.6
165 mg) in H₂O was incubated at 37°C for 30 min. The resulting precipitate (daidzein) was removed
166 by filtration. To the filtrate was added 10 mL of water saturated butan–1–ol (containing 0.1%
167 AcOH) and then liquid–liquid partition was carried out. The water layer was concentrated to
168 approximately 3 mL and applied to inertSep C18 column eluting with H₂O. The eluent was
169 concentrated in vacuo to give 1,5-anhydro-D-*erythro*-hex–1–en–3–ulose (**1**, 1.7 mg).

170 ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded with Varian NMR
171 system 600 and the residual solvent of CD₃CN was used as an internal standard (¹H, 1.93 ppm;
172 ¹³C, 1.3 ppm). ¹H NMR of **1** (600 MHz, CD₃CN) δ : 3.75 (1H, dd, J =4.3, 12.7 Hz, one of H-6),
173 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),

174 4.33 (1H, d, $J=13.3$ Hz, H-4), 7.36 (1H, s, H-1). ^{13}C NMR of **1** (150 MHz, CD_3CN) δ : 61.4 (C-6),
175 68.6 (C-4), 84.5 (C-5), 135.2 (C-2), 148.0 (C-1), 191.5 (C-3).
176

177 **Results**

178 **Expression and purification of DgpA, Gfo/Idh/MocA family oxidoreductase.**

179 3"-oxo-puerarin is a key intermediate in the enzymatic C-deglycosylation of puerarin (Fig. 1);
180 however, the exact enzyme catalyzing the oxidation of C-3" hydroxyl in puerarin has not been
181 identified. We previously reported the putative puerarin-metabolizing-operon composed of 8
182 genes (*dgpA-H*) from intestinal bacterium strain PUE (18). DgpA (BBG22493.1) and DgpF
183 (BBG22498.1), both regarded as gene products of the operon suggested as closely related to
184 oxidoreductase in the Gfo (glucose-fructose oxidoreductase) / Idh (inositol 2-dehydrogenase) /
185 MocA (rhizopine catabolism protein MocA) protein family (22). Particularly, DgpA was
186 implicated in puerarin oxidation because the *dgpA* gene deduced amino acid sequence at the N-
187 terminus was identical to that of a previously reported protein involved in puerarin metabolism
188 (17).

189 To characterize the enzymatic activity of DgpA, the encoding gene *dgpA* was heterologously
190 expressed in *E. coli*, and the recombinant protein was purified by two-step column chromatography.
191 In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the purified
192 DgpA appeared as a single band with an apparent molecular mass of 42 kDa, showing good
193 agreement with the calculated molecular mass of 40,161 Da (Fig. 2, lane 1). The recombinant
194 DgpB-C complex which catalyzes the deglycosylation of 3"-oxo-puerarin was also analyzed by
195 SDS-PAGE (Fig. 2, lane 2).

196 [Fig.2]

197 **Determination of DgpA-bound NAD(H).**

198 In the UV-vis spectrum of purified DgpA, a broad shoulder peak at approximately 340 nm was
199 observed, suggesting that nicotinamide cofactors such as NAD(H) or NADP(H) were bound to the
200 enzyme (Fig. 3). To characterize the cofactors, HPLC analysis was performed after the protein was
201 treated with cold methanol to dissociate the cofactors. As shown in Fig. 4b, two major peaks were
202 observed at 10.9 and 13.5 min in HPLC analysis of DgpA-bound cofactors. These two peaks were
203 characterized as NAD⁺ and NADH by comparing the retention times to authentic nicotinamide
204 cofactors (Fig. 4a). These results indicate that NAD(H) functioned as the cofactor which binds

205 tightly but non-covalently to DgpA.

206 [Fig.3] [Fig.4]

207 **Oxidation of puerarin by DgpA and 3-oxo-glucose.**

208 To confirm the oxidation of puerarin catalyzed by DgpA, recombinant DgpA was incubated with
209 0.5 mM puerarin in potassium phosphate buffer (pH 7.4) at 37°C for 30 min. According to HPLC
210 analysis, no metabolites were detected under this condition (Fig. 5a). The same result was obtained
211 when 1 mM NAD⁺ and 1 mM MnCl₂ were added to the reaction mixture, despite these two
212 cofactors have been reported to increase the enzymatic activity during puerarin C-deglycosylation
213 (17). As shown in Fig. 1, DgpA may require 3-oxo-glucose for oxidation of puerarin, as the
214 ultimate sugar metabolite in puerarin degradation should be glucose rather than an oxo-sugar
215 derivative (16). Based on this assumption, 3-oxo-glucose was added to the reaction mixture
216 including DgpA and puerarin, resulting in the detection of two metabolite peaks at 8.1 and 8.6 min
217 in HPLC analysis (Fig. 5b). The retention times and elution profiles of the metabolites were
218 identical to those of 3"-oxo-puerarin in the buffer, which easily isomerized to a mixture of the
219 3"-oxo form (a peak at 8.1 min), 2"-oxo form, and its intramolecular-cyclic acetal (a peak at 8.6
220 min, overlapping) as previously reported (18). These results demonstrate that DgpA catalyzed
221 oxidation at the 3"-hydroxyl of puerarin by using 3-oxo-glucose as the hydride acceptor (Fig. 1).

222

223 **Reduction of 3"-oxo-puerarin by DgpA and glucose.**

224 To identify the actual metabolites in the oxidation reaction by DgpA, an enzymatic
225 counterreaction was proposed. 3"-Oxo-puerarin standard and DgpA were incubated with or
226 without D-glucose at 37°C for 30 min, followed by HPLC analysis (Fig. 5c, d). In the reaction of
227 3"-oxo-puerarin standard and DgpA with glucose, one conspicuous metabolite peak was detected
228 at 7.7 min by HPLC (Fig. 5d). After chromatographic isolation, the metabolite structure was
229 confirmed as puerarin, but not 3"-axial-hydroxyl epimer (D-allose type C-glycoside), based on
230 NMR analysis. These findings indicate that the reaction catalyzed by DgpA was reversible and the
231 metabolites in the puerarin oxidation reaction were verified as 3"-oxo-puerarin and D-glucose, as
232 shown in Fig. 1.

233

234 **C–Deglycosylation of puerarin by a combination of DgpA, DgpB–C complex, and**
235 **3–oxo–glucose.**

236 DgpB–C complex was reported to metabolize 3"–oxo–puerarin to daidzein (18). To achieve
237 enzymatic C–deglycosylation of puerarin, two recombinant bacterial enzymes (DgpA and
238 DgpB–C complex) and 3–oxo–glucose were incubated with puerarin at 37°C for 30 min, which
239 was analyzed by HPLC. The peak detected as daidzein was observed at 15.0 min in the HPLC
240 chromatogram (Fig. 5e), indicating that C–deglycosylation of puerarin was accomplished by the
241 recombinant enzymes.

242 [Fig. 5]

243

244 **Structure determination of 1,5–anhydro–D–erythro–hex–1–en–3–ulose (1) as a metabolite of**
245 **3"–oxo–puerarin catalyzed by DgpB–C complex**

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

256 [Fig. 6]

257

258

259

260 Discussion

261 The human intestinal bacterium strain PUE can metabolize the isoflavone *C*-glucoside puerarin
262 to daidzein; however, the metabolic enzymes have not been well-characterized. In this study, the
263 bacterial DgpA protein was identified as the enzyme responsible for puerarin oxidation, as shown
264 in Fig. 1. Additionally, enzymatic *C*-deglycosylation of puerarin was accomplished by a
265 combination of recombinant DgpA, DgpB-C complex, and 3-oxo-glucose, yielding daidzein and
266 **1**.

267 We previously purified a 40 kDa protein involved in puerarin metabolism from strain PUE and
268 sequenced the 30 N-terminal amino acids (17). The sequence was identical to that of DgpA,
269 indicating that the purified protein, previously designated as protein C, was DgpA. DgpA protein
270 is a member of the Gfo/Idh/MocA oxidoreductase family. These proteins typically utilize
271 NAD⁺/NADP⁺ and are related to the redox reactions of pyranoses (22). Gfo/Idh/MocA family
272 oxidoreductases have a two-domain structure, N-terminal NAD(P)-binding Rossmann fold
273 domain, and C-terminal α/β domain involved in substrate binding (22). Our results revealed that
274 DgpA was an NAD(H)-binding enzyme and used 3-oxo-glucose as the hydride acceptor for
275 puerarin oxidation. Therefore, DgpA-bound NAD(H) at the N-terminal Rossmann fold domain
276 likely plays an important role in the redox reaction between the two substrates.

277 The DgpB-C complex cleaved the *C*-glycosidic bond in 3"-oxo-puerarin, affording daidzein
278 and **1** (Fig. 1). **1** was previously reported as a spontaneous decomposition product of β -elimination
279 of 3-ketocarbohydrates, such as 3-ketosucrose (25), ginsenoside oxidized compound K (26), and
280 3-keto-levoglucosan (23) under alkaline conditions. Similar β -elimination-like cleavage has been
281 observed in glycoside hydrolase families 4 and 109 (27, 28). These protein families show a unique
282 reaction mechanism involving NAD⁺ for glycosyl bond cleavage. The first step of the reaction is
283 oxidation at the C-3 hydroxyl of glycosides to yield 3-keto-glycosides and NADH. Consequently,
284 the acidified C-2 proton adjacent to the C-3 ketone is abstracted, and then a glycosidic linkage is
285 cleaved by β -elimination to give α,β -unsaturated carbonyl intermediate such as **1**. The hydrolase
286 reaction is completed by Michael-type 1,4-addition of H₂O to the intermediate and subsequent

287 reduction of C-3 ketone to hydroxyl assisted by NADH. The glycoside hydrolase family 4 protein
288 was reported to cleave not only *O*-glycosides but also more stable *S*-glycosides (29). In contrast,
289 cleavage of *C*-glycosides by these enzymes have not been observed.

290 A proposed puerarin deglycosylation pathway as shown in Fig. 1 based on the above-mentioned
291 mechanism of glycoside hydrolases 4 and 109. The other enzyme encoded in the putative puerarin-
292 metabolizing-operon from strain PUE was likely involved in the reaction, as more than three
293 enzymes were reported to participate in puerarin *C*-deglycosylation (17). To clarify the enzymatic
294 puerarin *C*-deglycosylation, further studies are needed to characterize the unidentified enzyme
295 responsible for the enantioselective Michael addition of H₂O to **1** to provide 3-oxo-glucose and
296 identify the other gene products DgpD-H.

297

298 **Acknowledgement**

299 This research was supported by JSPS KAKENHI Grant Number 18K14940.

300

301 **Conflict of Interest**

302 The authors declare no conflict of interest.

303

304

305 REFERENCES

- 306 1. Sekirov I, Russell SL, Antunes LCM, Finlay BB. 2010. Gut microbiota in health and disease.
307 *Physiol Rev* 90:859–904.
- 308 2. Rajilić-Stojanović M, de vos WM. 2014. The first 1000 cultured species of the human
309 gastrointestinal microbiota. *FEMS Microbiol Rev* 38:996–1047.
- 310 3. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2018. Gut microbiota
311 functions: metabolism of nutrients and other food components. *Eur J Nutr* 57:1–24.
- 312 4. Braune A, Blaut M. 2016. Bacterial species involved in the conversion of dietary flavonoids
313 in the human gut. *Gut Microbes* 7:216–34.
- 314 5. Setchell KD, Brown NM, Lydeking–Olsen E. 2002. The clinical importance of the metabolite
315 equol – a clue to the effectiveness of soy and its isoflavones. *J Nutr* 132:3577–3584.
- 316 6. Setchell KD, Clerici C. 2010. Equol: history, chemistry, and formation. *J Nutr*
317 140:1355S–1362S.
- 318 7. Kobashi K, Akao T. 1997. Relation of intestinal bacteria to pharmacological effects of
319 glycosides. *Bioscience Microflora* 16:1–7.
- 320 8. Che QM, Akao T, Hattori M, Kobashi K, Namba T. 1991. Isolation of a human intestinal
321 bacterium capable of transforming barbaloin to aloe-emodin anthrone. *Planta Med* 57:15–19.
- 322 9. Sanugul K, Akao T, Li Y, Kakiuchi N, Nakamura N, Hattori M. 2005. Isolation of a human
323 intestinal bacterium that transforms mangiferin to norathyriol and inducibility of the enzyme
324 that cleaves a C–glucosyl bond. *Biol Pharm Bull* 28:1672–1678.
- 325 10. Jin JS, Nishihata T, Kakiuchi N, Hattori M. 2008. Biotransformation of C–glucosylisoflavone
326 puerarin to estrogenic (3S)–equol in co–culture of two human intestinal bacteria. *Biol Pharm*
327 *Bull* 31:1621–1625.
- 328 11. Braune A, Blaut M. 2011. Deglycosylation of puerarin and other aromatic C–glucosides by a
329 newly isolated human intestinal bacterium. *Environ Microbiol* 13:482–494.
- 330 12. Braune A, Blaut M. 2012. Intestinal bacterium *Eubacterium cellulosolvens* deglycosylates
331 flavonoid C–and O–glucosides. *Appl Environ Microbiol* 78:8151–8153.

- 332 13. Kim M, Lee J, Han J. 2015. Deglycosylation of isoflavone C-glycosides by newly isolated
333 human intestinal bacteria. *J Sci Food Agric* 95:1925–1931.
- 334 14. Zheng S, Geng D, Liu S, Wang Q, Liu S, Wang R. 2019. A newly isolated human intestinal
335 bacterium strain capable of deglycosylating flavone C-glycosides and its functional properties.
336 *Microb Cell Fact* 18:94.
- 337 15. Braune A, Engst W, Blaut M. 2016. Identification and functional expression of genes encoding
338 flavonoid O- and C-glycosidases in intestinal bacteria. *Environ Microbiol* 18:2117–2129.
- 339 16. Nakamura K, Nishihata T, Jin JS, Ma CM, Komatsu K, Iwashima M, Hattori M. 2011. The
340 C-glucosyl bond of puerarin was cleaved hydrolytically by a human intestinal bacterium strain
341 PUE to yield its aglycone daidzein and an intact glucose. *Chem Pharm Bull* 59:23–27.
- 342 17. Nakamura K, Komatsu K, Hattori M, Iwashima M. 2013. Enzymatic cleavage of the
343 C-glucosidic bond of puerarin by three proteins, Mn²⁺, and oxidized form of nicotinamide
344 adenine dinucleotide. *Biol Pharm Bull* 36:635–640.
- 345 18. Nakamura K, Zhu S, Komatsu K, Hattori M, Iwashima M. 2019. Expression and
346 characterization of the human intestinal bacterial enzyme which cleaves the C-glycosidic bond
347 in 3"-oxo-puerarin. *Biol Pharm Bull* 42:417–423.
- 348 19. Fukuyama K, Ohru H, Kuwahara S. 2015. Synthesis of EFdA via a diastereoselective aldol
349 reaction of a protected 3-keto furanose. *Org Lett* 17:828–831.
- 350 20. Vetter ND, Langill DM, Anjum S, Boisvert-Martel J, Jagdhane RC, Omene E, Zheng H, van
351 Straaten KE, Asiamah I, Krol ES, Sanders DA, Palmer DR. 2013. A previously unrecognized
352 kanosamine biosynthesis pathway in *Bacillus subtilis*. *J Am Chem Soc* 135:5970–5973.
- 353 21. Dausmann T, Aivasidis A, Wandrey C. 1997. Purification and characterization of an
354 alcohol:N,N-dimethyl-4-nitrosoaniline oxidoreductase from the methanogen *Methanosarcina*
355 *barkeri* DSM 804 strain Fusaro. *Eur J Biochem* 248:889–896.
- 356 22. Taberman H, Parkkinen T, Rouvinen J. 2016. Structural and functional features of the NAD(P)
357 dependent Gfo/Idh/MocA protein family oxidoreductases. *Protein Sci* 25:778–786.
- 358 23. Kitaoka M. 2017. Synthesis of 3-keto-levoglucosan using pyranose oxidase and its

- 359 spontaneous decomposition via β -elimination. *J appl glycosci* 64:99–107.
- 360 24. Chung K, Waymouth RM. 2016. Selective catalytic oxidation of unprotected carbohydrates.
- 361 *ACS Catal* 6:4653–4659.
- 362 25. Pietsch M, Walter M, Buchholz K. 1994. Regioselective synthesis of new sucrose derivatives
- 363 via 3-ketosucrose. *Carbohydr Res* 254:183–194.
- 364 26. Kim EM, Seo JH, Baek K, Kim BG. 2015. Characterization of two-step deglycosylation via
- 365 oxidation by glycoside oxidoreductase and defining their subfamily. *Sci Rep* 5:10877.
- 366 27. Yip VL, Varrot A, Davies GJ, Rajan SS, Yang X, Thompson J, Anderson WF, Withers SG.
- 367 2004. An unusual mechanism of glycoside hydrolysis involving redox and elimination steps
- 368 by a family 4 beta-glycosidase from *Thermotoga maritima*. *J Am Chem Soc* 126:8354–8355.
- 369 28. Jongkees SA, Withers SG. 2014. Unusual enzymatic glycoside cleavage mechanisms. *Acc*
- 370 *Chem Res* 47:226–235.
- 371 29. Yip VL, Withers SG. 2006. Family 4 glycosidases carry out efficient hydrolysis of
- 372 thioglycosides by an α,β -elimination mechanism. *Angew Chem Int Ed* 45:6179–6182.
- 373
- 374
- 375
- 376
- 377
- 378
- 379
- 380

381

382

383

384

385

386

387

388

389

390

391

392

393

394

395

396

397

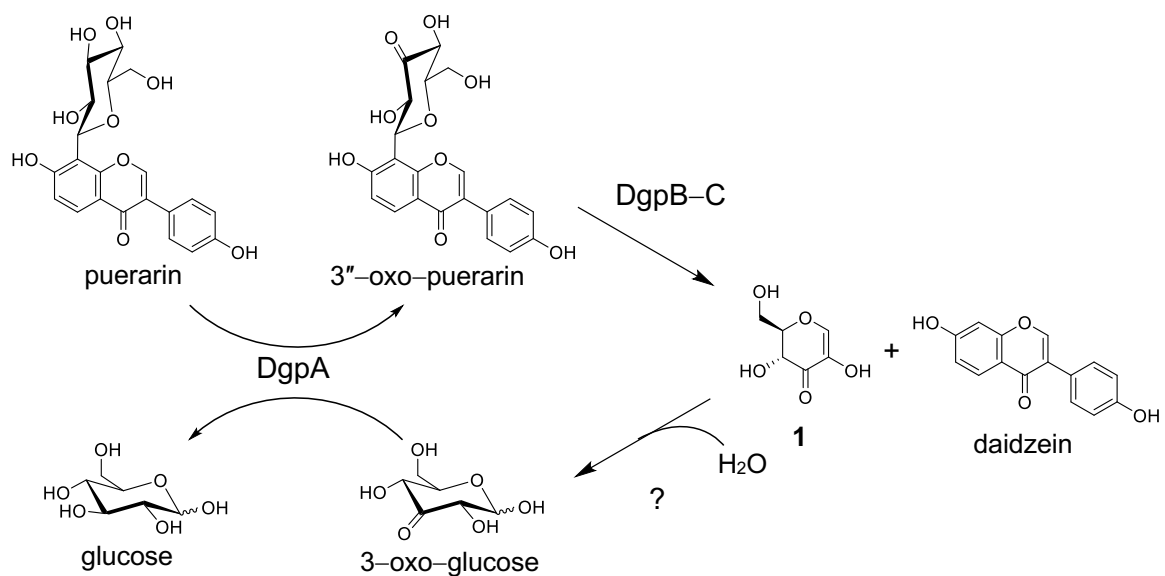
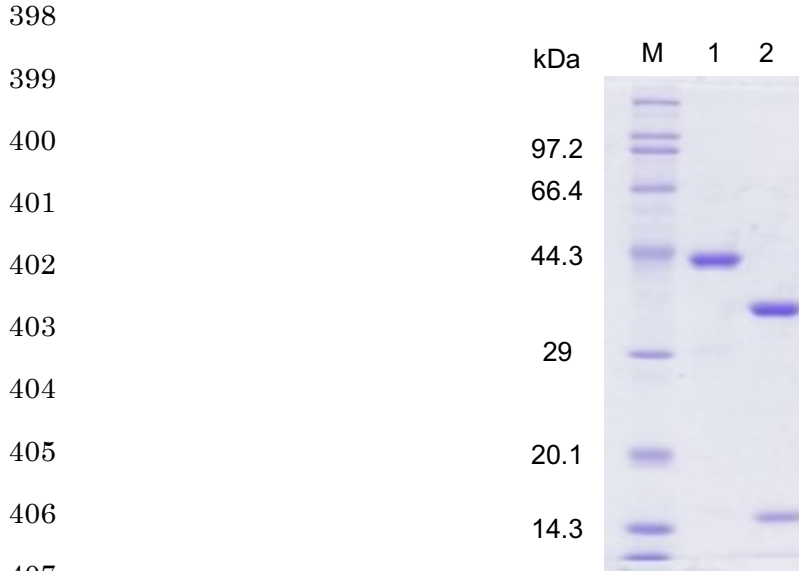


Fig. 1. Proposed puerarin degradation pathway catalyzed by DgpA and DgpB-C complex.



409 Fig. 2. SDS-PAGE analysis of recombinant proteins used in this study.
410 Lane M, marker proteins; lane 1, recombinant DgpA (40,161 Da); lane 2, recombinant DgpB-C
411 complex (DgpB: 16,047 Da, DgpC: 36,883 Da). SDS-PAGE was carried out with 12.5%
412 polyacrylamide gel.

413

414

415

416

417

418

419

420

421

422

423

424

425

426

427

428

429

430

431

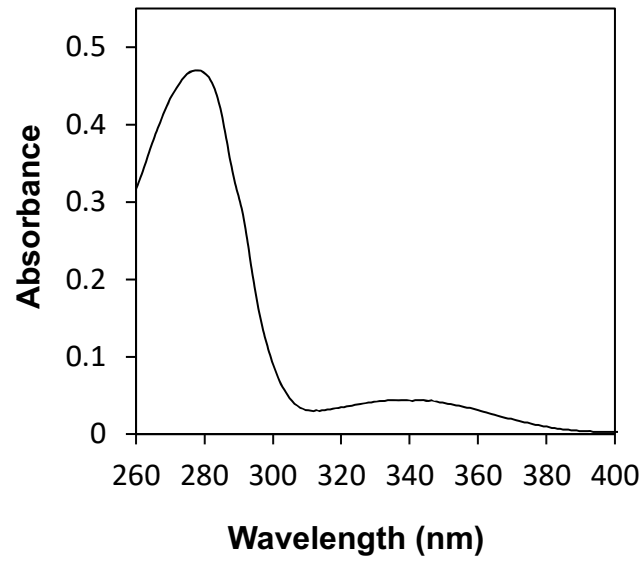


Fig. 3. UV-vis absorption spectrum of the purified DgpA.

432

433

434

435

436

437

438

439

440

441

442

443

444

445 Purified DgpA was denatured with cold methanol and the dissociated cofactors were analyzed by

446 HPLC. (a) Authentic mixture of cofactors, NAD^+ , NADH , NADP^+ , and NADPH , (b) DgpA-

447 bound cofactors.

448

449

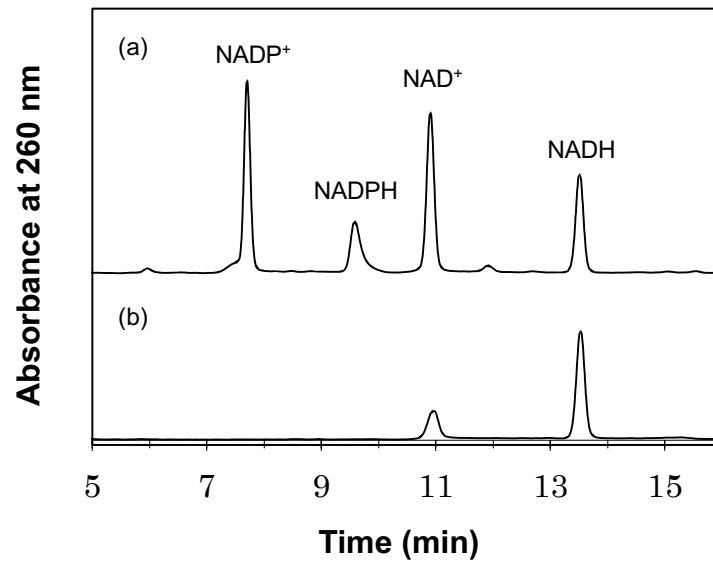


Fig. 4. HPLC analysis of DgpA-bound NAD(H).

450

451

452

453

454

455

456

457

458

459

460

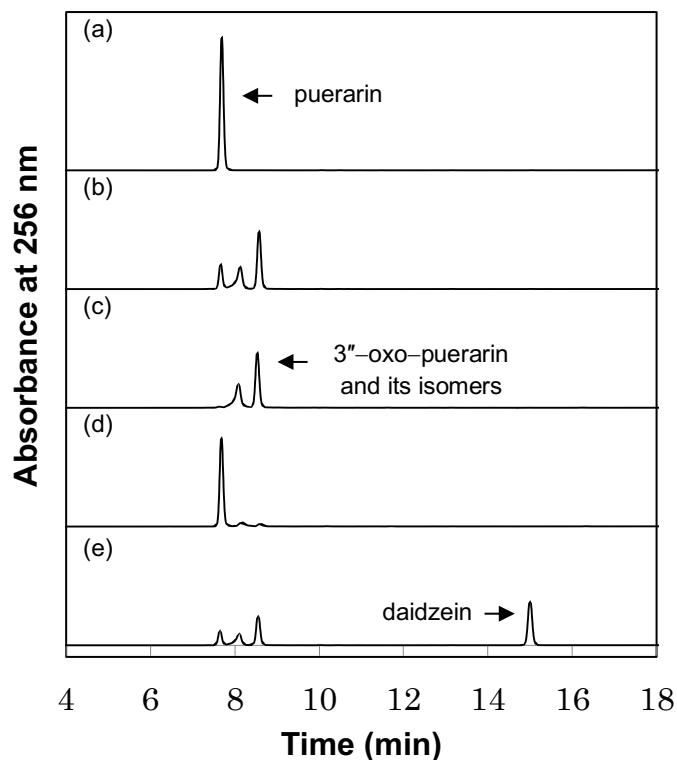
461

462

463

464

465



464

465

466 Fig. 5. HPLC analysis of enzymatic reaction metabolites catalyzed by DgpA and DgpB-C
467 complex.

468 Enzymatic reaction mixtures were incubated at 37°C for 30 min and the reaction solution were
469 analyzed by HPLC. The composition of the reaction mixtures were as follows, (a) puerarin and
470 DgpA, (b) puerarin, DgpA, and 3-oxo-glucose, (c) 3"-oxo-puerarin and DgpA, (d)
471 3"-oxo-puerarin, DgpA, and glucose, (e) puerarin, DgpA, DgpB-C complex, and
472 3-oxo-glucose.

473

474

475

476

477

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492

493

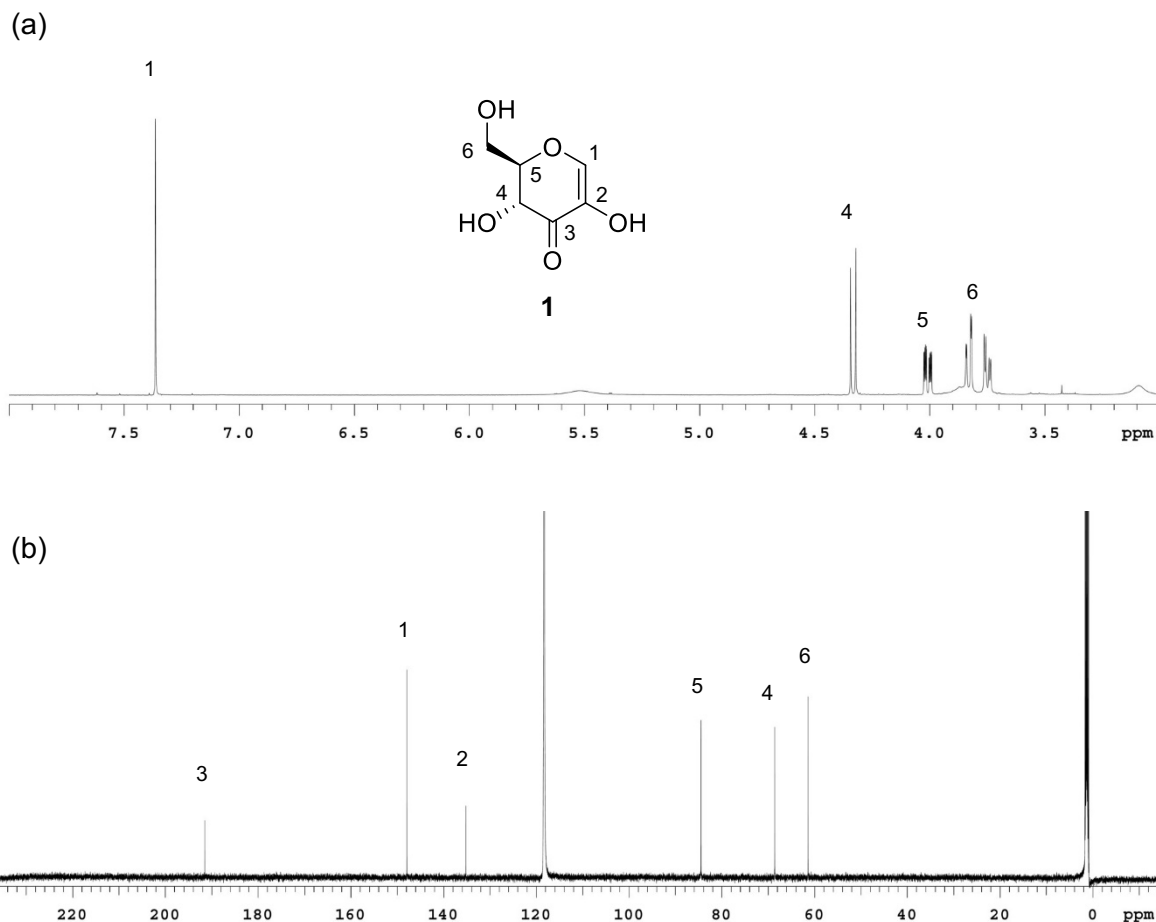


Fig. 6. ^1H - and ^{13}C -NMR spectra of **1**, a metabolite derived from 3''-oxo-puerarin sugar part catalyzed by DgpB-C complex.