# Enzymatic Cleavage of the *C*-Glucosidic Bond of Puerarin by Three Proteins, $Mn^{2+}$, and Oxidized Form of Nicotinamide Adenine Dinucleotide

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**We previously isolated the human intestinal bacterium, strain PUE, which can cleave the *C*-glucosidic bond of puerarin to yield its aglycone daidzein and glucose. In this study, we partially purified puerarin *C*-glucosidic bond cleaving enzyme from the cell-free extract of strain PUE and demonstrated that the reaction was catalyzed by at least three proteins, $Mn^{2+}$, and oxidized form of nicotinamide adenine dinucleotide ($NAD^+$). We completely purified one of the proteins, called protein C, by chromatographic separation in three steps. The molecular mass of protein C was approximately 40 kDa and the amino acid sequence of its N-terminal region shows high homology to those of two putative proteins which belong to Gfo/Idh/MocA family oxidoreductase. Protein C catalyzed hydrogen-deuterium exchange reaction of puerarin to 2″-deuterated puerarin in $D_2O$ condition, which closely resembles those of glycoside hydrolase family 4 and 109.**

**Key words** puerarin; *C*-glucoside; C–C bond cleavage; glycosidase; enzyme purification

Glycosidic bonds between an aglycone and a sugar are generally classified into four groups including *O*-, *N*-, *S*-, and *C*-glycosidic bonds. Glycoside hydrolases that catalyze one of the *O*, *N*, *S*-glycosidic bond hydrolysis are categorized into about 130 families based on the similarities of amino acid sequences (Carbohydrate-Active enZYmes database at http://www.cazy.org/). Most glycoside hydrolases do not require any cofactors, however the glycoside hydrolase family 4 and 109 cleave glycosidic bonds through redox and elimination steps under the specific reaction conditions involving oxidized form of nicotinamide adenine dinucleotide ($NAD^+$) as cofactor.[1,2]

Quite a unique characteristic of *C*-glycosidic bond in *C*-glycosides is found in the acidic hydrolysis or glycosidase treatments because their anomeric carbon in the sugar is directly attached to the aglycone by C−C bonding. Despite of the chemical stability of *C*-glycosidic bond, biotransformation of *C*-glycosides to its aglycone by the human intestinal bacteria were reported.[3–12] A few papers described the partial purification of *C*-glycosidic bond cleaving enzymes.[13,14] According to the paper of Sanugul *et al.*,[14] one of the enzymes for *C*-glycosidic bond cleavage reaction of mangiferin (xanthone *C*-glucoside) consisted of two proteins which required $Mn^{2+}$ and undetermined low molecular cofactor(s).

*C*-Glycosides are distributed in a lot of medicinal plants. Among them, puerarin, isoflavone *C*-glucoside, is a major flavonoid contained in the roots of *Pueraria lobata* Ohwi (Leguminosae), which is a well-known herbal drug Puerariae Radix, so called "Kakkon" in Japanese and has been used as a diaphoretic, antifebrile and antispasmodic[15] in Japan and East Asia.

After oral administration of puerarin in the case of rats, two metabolic pathways were reported by Prasain *et al.*[16] One showed that puerarin was first transferred to the portal circulation by the sodium-dependent glucose transporter, and second entered itself into the bloodstream as an unmetabolized form. Another depicted that puerarin was metabolized to its aglycone daidzein, equol and related compounds by the intestinal bacteria. The latter pathway suggested that the key reaction determining the metabolic fate of puerarin was *C*-glycosidic bond cleavage catalyzed by the intestinal bacteria. To our best knowledge, a dozen of Pueraria isoflavones secondary metabolized from puerarin show various and significant bioactivities, however, the enzyme(s) performed the *C*-glycosidic bond cleavage in puerarin as the initial key reaction has not been characterized, yet.

We previously isolated the human intestinal bacterium, strain PUE, which can cleave the *C*-glucosidic bond of puerarin to yield daidzein and glucose[17,18] (Fig. 1). In the course of our investigation for the mechanistic study of biotransformation of *C*-glycosides, the purification and characterization of the novel *C*-glycosidic bond cleaving enzyme from strain PUE were partially accomplished. In this paper we describes an elucidation of the new enzyme composed of at least three proteins, and an existence of the cofactors associated with this cleaving reaction. Furthermore, we determined the amino acid sequence of N-terminal region of one purified protein.

## MATERIALS AND METHODS

**General** A bacterium was incubated in anaerobic incubator EAN-140 (Tabai Co., Osaka, Japan). UV spectra were measured by a UV-2200 UV/VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan).

**Chemicals and Materials** Strain PUE was isolated from human feces as previously described.[17] Puerarin was isolated from the roots of *Pueraria lobata* (Willd.) Ohwi. General anaerobic medium (GAM) broth was purchased from Nissui Seiyaku Co. (Tokyo, Japan). $NAD^+$ was purchased from Oriental Yeast Co. (Tokyo, Japan). Manganese(II) chloride tetrahydrate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Preparation of Cell-Free Extract** Strain PUE was cultured under anaerobic conditions at 37°C for 14 h in 1 L of GAM broth containing 0.3 mM puerarin as enzyme inducer.

The authors declare no conflict of interest.

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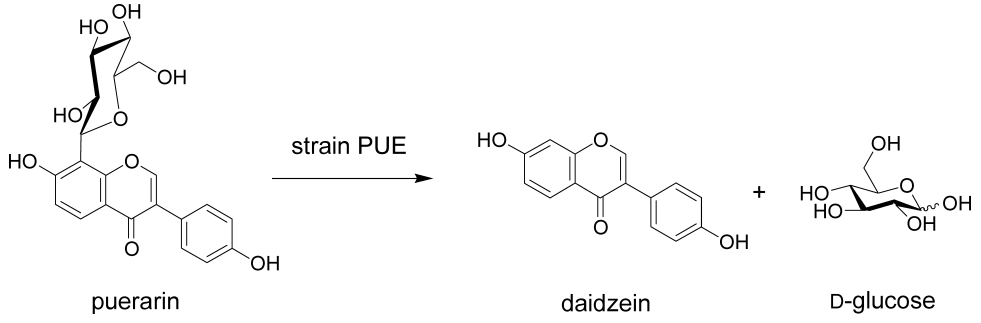


Fig. 1. Cleavage of the *C*-Glucosidic Bond of Puerarin by Strain PUE

The bacterial cells were collected by centrifugation and suspended in 50 mL of 50 mM potassium phosphate buffer (pH 7.4). Cells were disrupted by sonication on ice and centrifuged at 10000×***g*** for 60 min at 4°C to obtain a supernatant as cell-free extract.

**Purification of Puerarin *C*-Glucosidic Bond Cleaving Enzyme (Proteins A, B, and C)** All purification procedures were carried out at 0–4°C. All column chromatography steps for enzyme purification were performed using ÄKTA purifier HPLC system (GE Healthcare, Buckinghamshire, U.K.). Prepared cell-free extract was adjusted to 25% saturation of $(NH_4)_2SO_4$ followed by centrifugation at 10000×***g*** for 10 min. The supernatant was applied to a HiPrep Butyl FF 16/10 column (GE Healthcare) at a flow rate of 5 mL/min. Bound proteins were eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in 100 mM potassium phosphate buffer (pH 7.3). Two protein fractions (Fr. I and Fr. II) essential for the activity were pooled and further enzyme purification were performed as follows.

Partial Purification of Proteins A and B from Fr. I: Fraction I was concentrated and buffer exchanged into 50 mM potassium phosphate buffer (pH 7.4) using Amicon Ultra-15 centrifugal filter devices (10K MWCO, Millipore). It was applied to a Mono Q 4.6/100 PE column (GE Healthcare) at a flow rate of 1 mL/min. Bound proteins were eluted with a linear gradient of 100–400 mM NaCl in 50 mM potassium phosphate buffer (pH 7.4). Two protein fractions (proteins A and B) essential for the activity were pooled.

Complete Purification of Protein C from Fr. II: Fraction II was concentrated and buffer exchanged into 10 mM potassium phosphate buffer (pH 7.4) using Amicon Ultra-15 centrifugal filter devices. It was applied to a Hydroxyapatite-MP column (Kanto Chemical, Tokyo, Japan, 8×100 mm) at a flow rate of 1 mL/min. Bound proteins were eluted with a linear gradient of 10–300 mM potassium phosphate buffer (pH 7.4). The active fractions were pooled and concentrated. It was applied to a Mono Q 4.6/100 PE column at a flow rate of 1 mL/min. Bound proteins were eluted with a linear gradient of 0–400 mM NaCl in 50 mM potassium phosphate buffer (pH 7.4). The active fractions were pooled and used as a purified protein C in the following experiments.

**Measurement of Enzyme Activity** Three protein fractions (proteins A, B and C), $Mn^{2+}$ and $NAD^+$ were essential for puerarin *C*-glucosidic bond cleaving reaction in our result. In the case of determining the active fraction of protein C, beforehand prepared proteins A and B were added to the reaction mixture. The reaction mixture consisted of enzyme solution, puerarin (0.5 mM), $MnCl_2$ (1 mM) and $NAD^+$ (1 mM) in a final volume of 100 $\mu$L. It was incubated at 37°C for several hours, and extracted twice with 200 $\mu$L of 1-butanol (saturated with water and acidified with 0.1% acetic acid). The extract was evaporated to dryness, dissolved in 50% MeOH (100 $\mu$L), and the amount of daidzein (metabolite) was measured by HPLC. HPLC conditions were as follows: column, COSMOSIL $5C_{18}$-MS-II (Nacalai Tesque, Kyoto, Japan, 4.6×150 mm); flow rate, 1 mL/min; detection, 256 nm; mobile phase, 0.1% trifluoroacetic acid in 50% MeOH (isocratic); injection volume, 20 $\mu$L.

**Determination of Cofactors** The reaction mixture consisted of partially purified proteins A (61 $\mu$g), B (69 $\mu$g), and C (21 $\mu$g), puerarin (0.5 mM), in the presence of both $Mn^{2+}$ (1 mM) and cofactor ($NAD^+$, reduced nicotinamide adenine dinucleotide (NADH), NAD phosphate ($NADP^+$) or reduced NAD phosphate (NADPH), 1 mM, respectively) or in the absence of either one, in a final volume of 100 $\mu$L. After two hours incubation at 37°C, the enzyme activity was measured as described above.

**Effects of Three Protein Fractions on Enzyme Activity** The reaction mixture consisted of a couple of partially purified proteins (A: 61 $\mu$g, B: 69 $\mu$g, C: 21 $\mu$g), puerarin (0.5 mM), $Mn^{2+}$ (1 mM) and $NAD^+$ (1 mM) in a final volume of 100 $\mu$L. After two hours incubation at 37°C, the enzyme activity was measured as described above.

**Protein Measurement** The protein content was determined by the method of Bradford, using Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

**Determination of Molecular Weight** The molecular weight of protein C was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using c-Pagel 12.5% gel (ATTO, Tokyo, Japan). Protein Molecular Weight Marker (broad) (TaKaRa Bio Inc., Shiga, Japan) was used as standard. The protein bands were stained with Coomassie brilliant blue R250.

**Determination of Amino Acid Sequence in N-Terminal Region** After the solution of purified protein C was desalted, it was spotted onto a polybrene-coated glass fiber disk. The amino acid sequence of its N-terminal region was determined by the Edman degradation method using the protein sequencer PPSQ-21 (Shimadzu, Kyoto, Japan). Homology searches were performed using the BLASTp search of the NCBI database.

**Hydrogen-Deuterium Exchange Reaction** To the $D_2O$ (870 $\mu$L) was added purified protein C (43 $\mu$g), puerarin (0.5 mM), $Mn^{2+}$ (1 mM) and $NAD^+$ (1 mM) in a final volume of 1 mL (87% $D_2O$ condition). After the reaction mixture was incubated at 37°C for 20 h, it was extracted three times with

500 $\mu$L of 1-butanol (saturated with water and acidified with 0.1% acetic acid). The extract was evaporated to dryness, dissolved in $CD_3OD$ and $^1$H-NMR of the residue was taken on Varian 400-MR (Varian Inc., CA, U.S.A.).

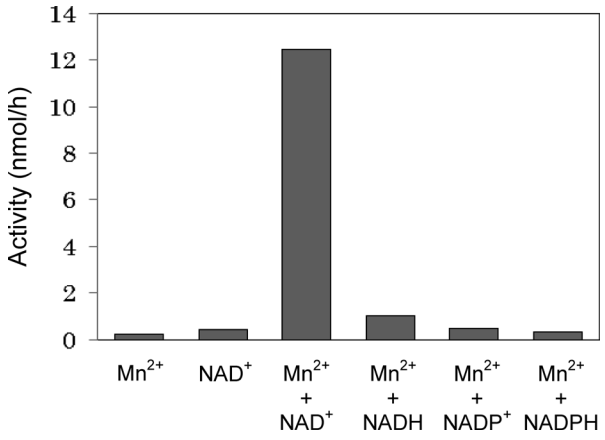


Fig. 2. Effects of Cofactors on Puerarin *C*-Glucosidic Bond Cleaving Activity of Partially Purified Enzyme

The reaction mixture consisted of partially purified proteins A (61 $\mu$g), B (69 $\mu$g), and C (21 $\mu$g), puerarin (0.5 mM), in the presence of both $Mn^{2+}$ (1 mM) and cofactor ($NAD^+$, NADH, $NADP^+$ or NADPH, 1 mM, respectively) or in the absence of either one, in a final volume of 100 $\mu$L. After 2 h incubation at 37°C, the enzyme activity was measured.

## RESULTS

**Effects of Cofactors on Enzymatic Activity** The cell-free extract of strain PUE showed weak puerarin *C*-glucosidic bond cleaving activity, while by addition of $Mn^{2+}$ to the reaction mixture the activity was significantly increased. From the follow-up study, we found that the presence of $Mn^{2+}$ together with some low molecular cofactor(s) furnished more accelerated reaction. In order to identify the cofactor(s), we tried to use combined cocktail of the partially purified enzyme fractions for C–C bond cleaving reaction (Fig. 2). The best condition was found in the presence of both $Mn^{2+}$ and $NAD^+$, but in the absence of either $Mn^{2+}$ or $NAD^+$, the cleavage underwent fairly. The effect of NADH, $NADP^+$, and NADPH were also examined in the same conditions, however, these cofactors showed a slight effect on the cleaving activity.

**Partial Purification of Puerarin *C*-Glucosidic Bond Cleaving Enzyme (Proteins A, B, and C)** We purified puerarin *C*-glucosidic bond cleaving enzyme from the cell-free extract of strain PUE by using column chromatography in several steps (Fig. 3). First step column chromatography was performed using a HiPrep Butyl FF 16/10 column (Fig.

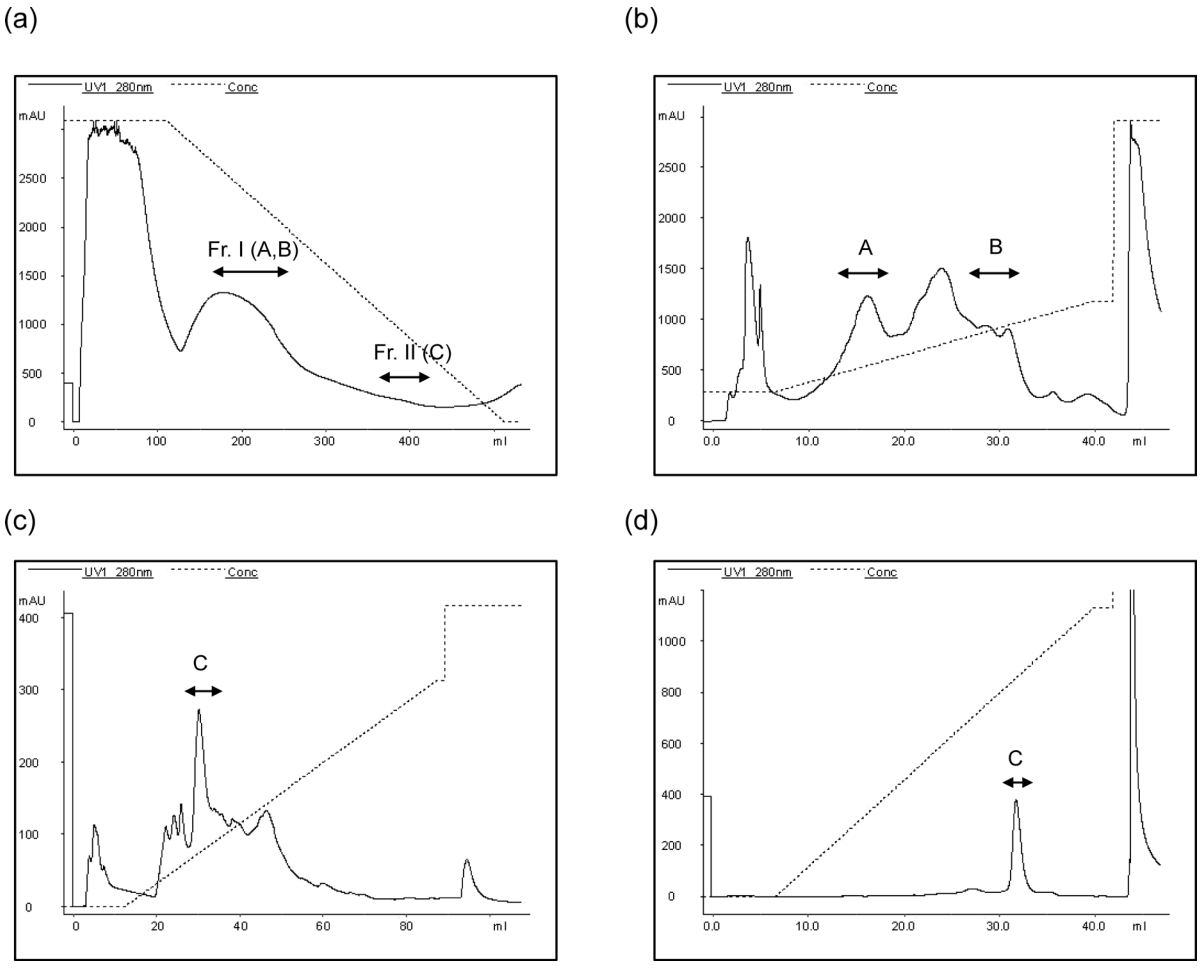


Fig. 3. Purification of Puerarin *C*-Glucosidic Bond Cleaving Enzyme

The arrow represents the active protein fractions (proteins A, B, and C). (a) HiPrep Butyl FF 16/10 column chromatography of cell-free extract, (b) Mono Q 4.6/100 PE column chromatography of Fr. I, (c) Hydroxyapatite-MP column chromatography of Fr. II, (d) Mono Q 4.6/100 PE column chromatography of the active fraction from hydroxyapatite-MP.

3a), to yield in disappearance of the bond cleaving activity in any of one fraction alone. However, the activity was restored in the mixed condition of two fractions (Fr. I and Fr. II), $Mn^{2+}$ and $NAD^+$, indicating that at least two protein fractions were essential for the bond cleaving reaction.

Further enzyme purification of Fr. I was conducted using a Mono Q 4.6/100 PE column (Fig. 3b). We found that the essential protein contained in Fr. I was not of single protein and they were separated again to two protein fractions, which were called proteins A and B, respectively. The other essential protein contained in Fr. II was called protein C. At this stage of enzyme purification, the proteins A, B, and C were partially purified proteins.

Figure 4 shows the requirement of three protein fractions for *C*-glucosidic bond cleaving reaction. We used partially purified proteins A (61 $\mu$g), B (69 $\mu$g), and C (21 $\mu$g). If the three proteins were combined with cofactors, puerarin *C*-glucosidic bond cleaving activity was 12.5 nmol/h. The absence of any proteins resulted in remarkable decrease of the activity, which suggested that all of the three proteins were exactly essential for the bond cleaving reaction. We had tried to identify any reaction intermediate such as daidzin (daidzein

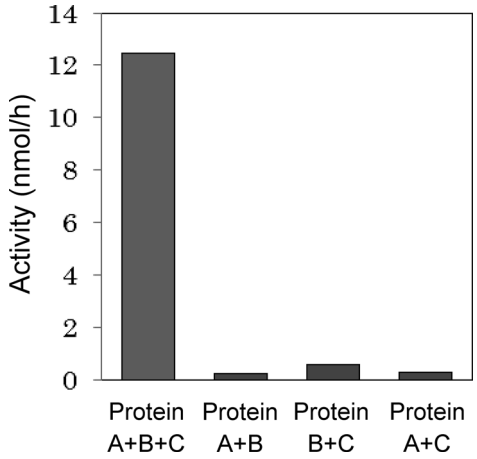


Fig. 4. Requirement of Three Protein Fractions for *C*-Glucosidic Bond Cleaving Reaction

The reaction mixture consisted of a couple of partially purified proteins (A: 61 $\mu$g, B: 69 $\mu$g, C: 21 $\mu$g), puerarin (0.5 mM), $Mn^{2+}$ (1 mM), and $NAD^+$ (1 mM) in a final volume of 100 $\mu$L. After 2 h incubation at 37°C, the enzyme activity was measured.

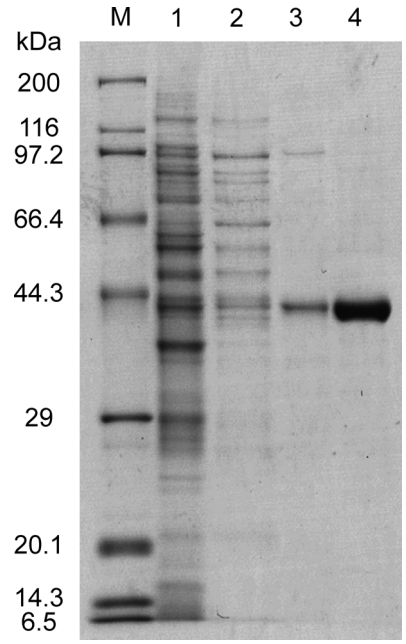


Fig. 5. SDS-PAGE of the Protein C Obtained by Various Steps of Column Chromatography

Lane M, marker proteins; lane 1, cell-free extract; lane 2, the active fraction (Fr. II) obtained from HiPrep Butyl FF 16/10 column chromatography; lane 3, the active fraction from Hydroxyapatite-MP column chromatography; lane 4, the active fraction from Mono Q 4.6/100 PE column chromatography.

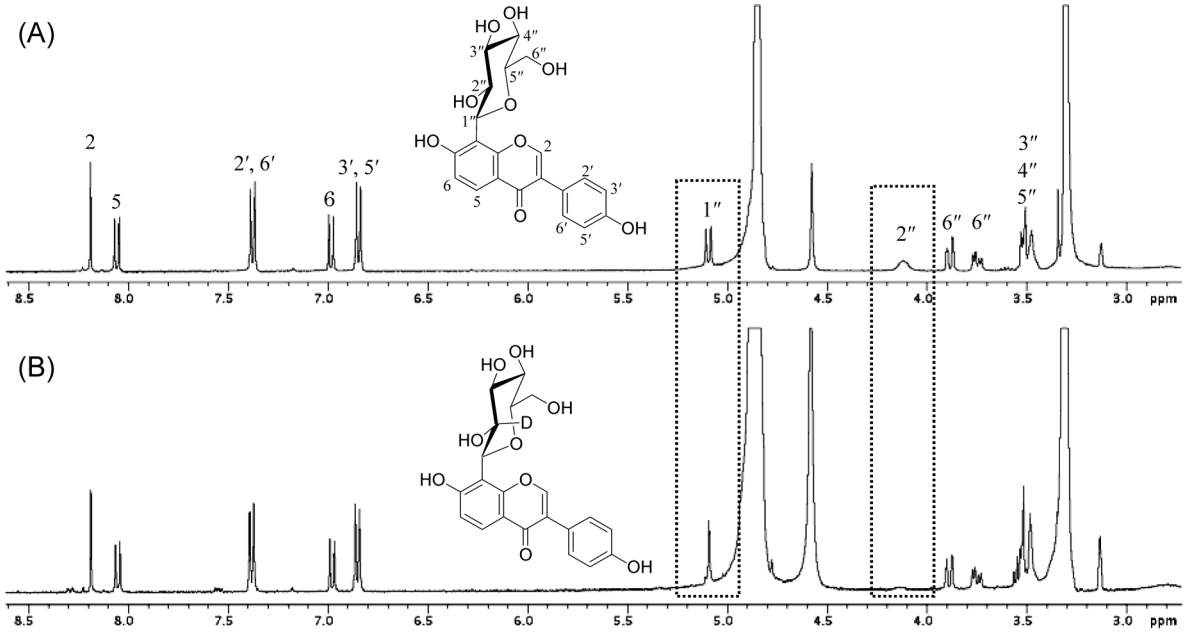


Fig. 6. Hydrogen-Deuterium Exchange Reaction Catalyzed by Protein C

(A) $^1$H-NMR spectrum of puerarin standard, (B) $^1$H-NMR spectrum of puerarin after incubation with protein C, $Mn^{2+}$, and $NAD^+$ in 87% $D_2O$ condition.

7-*O*-*β*-D-glucopyranoside) by HPLC analysis after incubation of puerarin with the partially purified protein(s), but unfortunately we could not detect it yet.

**Complete Purification and Amino Acid Sequence of N-Terminal Region of Protein C** We purified protein C from the cell-free extract of strain PUE by chromatographic separation in three steps, such as a Butyl FF column (Fig. 3a), Hydroxyapatite-MP column (Fig. 3c) and Mono Q column (Fig. 3d). The active fraction obtained from the purification by the Mono Q column showed single band of approximately 40 kDa on SDS-PAGE (Fig. 5) and the amount of obtained protein C was 430 $\mu$g.

The N-terminal amino acid sequence of protein C was determined by the Edman degradation method as SKLKIGIIGXGGIANQKHFPALKNNADLNE. A BLASTp search showed that the amino acid sequence of N-terminal region showed high homology to those of two putative proteins; 93% identity with oxidoreductase, Gfo/Idh/MocA family from *Clostridium* sp. D5 (ZP_08128353.1) and 90% identity with hypothetical protein HMPREF0988_02145 from *Lachnospiraceae* bacterium 1_4_56FAA (ZP_08616560.1). Both putative proteins belong to the Gfo/Idh/MocA family oxidoreductases, in which represented glucose-fructose oxidoreductase,[19] *myo*-inositol 2-dehydrogenase,[20] glycoside hydrolase family 109,[2] and so on.

**Hydrogen-Deuterium Exchange Reaction Catalyzed by Protein C** $^1$H-NMR analysis of puerarin was performed after incubation of puerarin with protein C, $Mn^{2+}$, and $NAD^+$ in 87% $D_2O$ condition (Fig. 6B). The intensity of H-2″ signal ($\delta$ 4.11 ppm) was shortened compared to the $^1$H-NMR spectrum of the standard puerarin (Fig. 6A), indicating hydrogen-deuterium exchange reaction was occurred at the position of H-2″. This result was also confirmed by analyzing the spin-spin coupling pattern of H-1″ signal ($\delta$ 5.09 ppm) which was changed from doublet to singlet. A control experiment was conducted by the same experimental procedure except for the absence of protein C in the reaction mixture, but no reaction was observed. These results suggested that protein C catalyzed hydrogen-deuterium exchange reaction of puerarin to 2″-deuterated puerarin in $D_2O$ condition.

## DISCUSSION

A human intestinal bacterium, strain PUE can cleave the *C*-glucosidic bond of puerarin, however the enzyme involved in this reaction have not been characterized. In this study, we partially purified puerarin *C*-glucosidic bond cleaving enzyme and demonstrated that the reaction was catalyzed by at least three proteins, $Mn^{2+}$, and $NAD^+$ (Figs. 2, 4). Another intestinal bacterium, strain MANG, cleaved the *C*-glucosidic bond of mangiferin and the enzyme involved in this reaction was partially purified.[14] These two *C*-glucosidic bond cleaving reactions have some similarities, such as the requirement of multiple proteins, $Mn^{2+}$ and some cofactors. These results suggested that the *C*-glucosidic bond cleavage in both puerarin and mangiferin might proceed *via* the similar reaction mechanisms.

We purified a single protein, called protein C, from the cell-free extract of strain PUE. The molecular mass of protein C was approximately 40 kDa and it may belong to Gfo/Idh/MocA family, known as oxidoreductases based on the amino acid sequence of its N-terminal region (Fig. 5). Hydrogen-deuterium exchange reaction was observed at the C-2″ of puerarin when it was incubated with protein C in $D_2O$ condition (Fig. 6). The results is closely resembled those of glycoside hydrolase family 4 and 109.[1,2] These glycoside hydrolases involve in redox reaction of carbohydrate at C-3 using $NAD^+$ as cofactor. After the oxidation of hydroxyl at C-3, hydrogen at C-2 was exchanged to deuterium from $D_2O$ because the acidity of H-2 is increased by an adjacent carbonyl group. Finally, the reaction was completed by reduction of ketone at C-3 to hydroxyl using the hydride generated from NADH. The enzymatic function of protein C showed some similarities to the glycoside hydrolase family 4 and 109, reasonably supporting the fact that puerarin is hydrolyzed to daidzein and glucose.[18]

Puerarin *C*-glucosidic bond cleavage reaction still remained some unknown reaction mechanism such as unclear roles of three proteins, together with the interaction of $Mn^{2+}$ and $NAD^+$. Protein C alone could not cleave the *C*-glucosidic bond without proteins A and B. Further studies will be needed to conclude the mechanism of puerarin *C*-glucosidic bond cleavage reaction, and to complete the purification and characterization of proteins A and B.

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