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Daidzein reductase of *Eggerthella* sp. YY7918, its octameric subunit structure containing FMN/FAD/4Fe-4S, and its enantioselective production of *R*-dihydroisoflavones

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S-Equol is a metabolite of daidzein, a type of soy isoflavone, and three reductases are involved in the conversion of daidzein by specific intestinal bacteria. *S*-Equol is thought to prevent hormone-dependent diseases. We previously identified the equol producing gene cluster (*eqlABC*) of *Eggerthella* sp. YY7918. Daidzein reductase (DZNR), encoded by *eqlA*, catalyzes the reduction of daidzein to dihydrodaidzein (the first step of equol synthesis), which was confirmed using a recombinant enzyme produced in *Escherichia coli*. Here, we purified recombinant DZNR to homogeneity and analyzed its enzymological properties. DZNR contained FMN, FAD, and one 4Fe-4S cluster per 70-kDa subunit as enzymatic cofactors. DZNR reduced the C═C bond between the C-2 and C-3 positions of daidzein, genistein, glycitein, and formononetin in the presence of NADPH. *R*-Dihydrodaidzein and *R*-dihydrogenistein were highly stereo-selectively produced from daidzein and genistein. The K_m and k_{cat} for daidzein were 11.9 μM and 6.7 s^{-1}, and these values for genistein were 74.1 μM and 28.3 s^{-1}, respectively. This enzyme showed similar kinetic parameters and wide substrate specificity for isoflavone molecules. Thus, this enzyme appears to be an isoflavone reductase. Gel filtration chromatography and chemical cross-linking analysis of the active form of DZNR suggested that the enzyme consists of an octameric subunit structure. We confirmed this by small-angle X-ray scattering and transmission electron microscopy at a magnification of ×200,000. DZNR formed a globular four-petal cloverleaf structure with a central vertical hole. The maximum particle size was 173 Å.

[**Key words:** Isoflavone; Equol; Gut microflora; Small-angle X-ray scattering; Reductase; NADPH; FAD; FMN; Old yellow enzyme]

Isoflavone is a type of polyphenol, and its derivatives are produced by Fabaceae, such as soybean and clovers. They bind to estrogen receptor beta because of its structural similarities to estradiol. Studies have focused on their potential beneficial effects against various health problems, such as menopausal disorders and osteoporosis related to diminished estrogen production (1,2). Soybean and soy sprouts contain several isoflavones including daidzein, genistein, glycitein and their glycosides. After ingestion of soy foods, isoflavone-glycosides are digested to form aglycones by glycosylases produced by intestinal bacteria and are absorbed into the consumer's bloodstream. In 30–50% of the human population, daidzein is converted to *S*-equol by specific intestinal bacteria (3). *S*-Equol has 100-fold higher potency in stimulating an estrogenic response than its precursor daidzein (4). Thus, *S*-equol-producing bacteria play a key role in the metabolism and human health effects of isoflavones.

Over the past decade, certain *S*-equol-producing bacteria have been isolated from human and animal feces (5,6) and food (7). Most *S*-equol-producing bacteria belong to the family Coriobacteriaceae (8), including *Slackia* (9,10), *Eggerthella* (11), *Adlercreutzia* (12), and *Asaccharobacter* (13), with the exception of *Lactococcus* sp. 20–92 (14), whose activity may have been caused by lateral gene transfer. We reported the isolation of the equol-producing bacterium *Eggerthella* sp. YY7918 from the feces of healthy humans and analyzed its whole-genome sequence (15).

The biochemical conversion pathway from daidzein to equol by bacteria is composed of at least three steps and proceeds *via* dihydrodaidzein and tetrahydrodaidzein as intermediates (Fig. S1). These steps are catalyzed by three reductases, daidzein reductase (DZNR), dihydrodaidzein reductase (DHDR), and tetrahydrodaidzein reductase (THDR). Corresponding genes and enzymes

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have been identified in four bacterial strains from the human intestine, *Lactococcus* sp. 20–92 (16,17), *Slackia* sp. NATTS (18), and *Eggerthella* sp. YY7918 (19), and in *Slackia isoflavoniconvertens* (20).

In our previous study, we identified a gene cluster in the *Eggerthella* sp. YY7918 genome responsible for converting daidzein to equol (19). This cluster includes three genes (*eqlA*, *eqlB*, and *eqlC*) encoding DZNR, DHDR, and THDR, respectively. Enzymatic conversion at each step was demonstrated using recombinant proteins expressed in *Escherichia coli*. In addition, the enzyme catalyzing the third step (THDR) used tetrahydrodaidzein as a substrate and produced both equol and dihydrodaidzein in the absence of the reducing coenzyme, NAD(P)H. We proposed that this enzyme is a novel dismutase.

According to motif analyses of amino acid sequences (21), DZNR is classified as a member of the old yellow enzyme (OYE) family (Fig. S2). Over 11,000 OYE homologs have been identified in yeasts, fungi, plants, and bacteria by genome analyses. Common characteristic features of all OYEs include NAD(P)H-dependent oxidoreductase activity, and catalysis of the reduction of the C=C bond in a stereo-selective manner (22). Some OYEs have been crystallized and their structures were shown to fold into a $(\beta/\alpha)_8$ barrel, known as a TIM barrel (23), with the FMN-binding site within the barrel near the carboxy-terminus of the β-sheet (24).

The stereo-specific characterization of DZNR remains unclear. The stereospecific bioconversion mechanism of dihydrodaidzein to equol *via* tetrahydrodaidzein has been evaluated previously (25–29). However, the assignment of a stereochemical configuration for dihydrodaidzein, based on circular dichroism spectra (25), was incorrect and led to misunderstanding regarding the stereo-selectivity of DZNR in numerous reports (28,29). A recent report suggested that only (3*S*,4*R*)-tetrahydrodaidzein can be converted to 3*S*-equol *via* a pathway proceeding from only *S*-dihydrodaidzein (27).

The mechanisms of enantioselectivity of DZNR-catalyzed reactions also remained obscure. In general, OYEs catalyze redox reactions in a stereospecific manner. Shimada et al. (30) found that DZNR converted daidzein to *R*-dihydrodaidzein, whereas dihydrodaidzein produced by *Lactococcus*-DZNR, as described by Shimada et al. (16), contained non-negligible quantities of the *S*-form. In our previous report, DZNR of *Eggerthella* sp. YY7918, which was partially purified by 6× His-Tag chromatography, yielded racemized dihydrodaidzein (19). Until recently, all reports of DZNR used only partially purified enzymes but showed clear stereo-selectivity. Analysis of the elementary reaction event using highly purified enzyme preparations is necessary to fully understand the complete pathway from daidzein to equol in bacteria.

Shimada et al. (30) reported that dihydrodaidzein racemase (DDRC) interconverted *R*- and *S*-dihydrodaidzein, thus increasing equol production. However, Schröder et al. (20) reported that no other enzymes or proteins were needed for daidzein conversion by recombinant DZNR, DHDR or THDR in *S. isoflavoniconvertens*.

Certain OYEs use NADPH, while others employ NADH; some of these enzymes contain FMN, while others have FAD, and still more possess Fe-S clusters. These cofactors likely play essential roles in electron transfer during redox reactions involving OYEs as described previously. In motif analyses, DNZR was predicted to contain possible FMN, FAD, and 4Fe-4S-cluster binding sites in the amino acid sequence (Fig. S2). We found that biochemical confirmations were necessary to clarify the composition of these cofactors. In some OYE family enzymes, certain enzymes have been analyzed to determine their subunit configurations; various types have been observed as monomers, dimers, and larger multimers (22).

In this study, we analyzed the enzymatic properties, including enzyme kinetics, cofactor composition, substrate specificity, and enantio-specificity, of the product using highly purified enzyme preparations. Moreover, the subunit configuration and coarse three-dimensional (3D) structure of DZNR was also determined by small angle X-ray scattering (SAXS) analysis and further confirmed by electron microscopy. This is the first report of the detailed enzymatic characterization and structural analysis of DZNR.

MATERIALS AND METHODS

Chemicals Daidzein, formononetin, FMN sodium salt hydrate, and FAD disodium salt hydrate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). β-NADPH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). *R*-dihydrodaidzein, *S*-dihydrodaidzein, and racemic-dihydrogenistein were a kind gift from Daicel Co. (Tokyo, Japan). Genistein and glycitein were provided by Nagara Science Co., Ltd. (Gifu, Japan). Isoflavones and their derivatives were dissolved in DMSO (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and stored at −28 °C. The iron standard stock solution (JCSS grade, 1000 mg/l) was purchased from Wako Pure Chemical Industries, Ltd.

Bacterial strains and culture media *Eggerthella* sp. YY7918 (Gifu Type Culture Collection, GTC16517) was cultured under anaerobic conditions at 37 °C in Gifu anaerobic medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for three days using the AnaeroPack Kenki culture system (disposable O_2 absorbing and CO_2 generating agent; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The culture broth was supplemented with 50 μM daidzein or other isoflavonoids. *E. coli* BL21(DE3) cells harboring pColdII-15730 plasmid, carrying e*qlA* tagged with 6× His, were used to express recombinant daidzein reductase, as described previously (19). Recombinant bacteria were grown in Miller's LB broth (10 g Bacto tryptone, 5 g Bacto yeast extract, and 10 g NaCl/1000 ml) supplemented with 100 μg/ml ampicillin (LB-Amp).

Cultivation of recombinant *E. coli* cells Transformed cells were cultivated at 37 °C in 6 l LB-Amp broth until reaching an optical density at 660 nm (OD_{660}) reached 0.3. Next, the temperature was lowered to 15 °C, and cultivation was continued for 24 h. The cells were harvested by centrifugation (6000 ×*g*, 10 min, 4 °C). The cells (approximately 20 g wet cell mass) were resuspended in 120 ml of sample buffer [20 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.1 mM PMSF], disrupted using a Microson XL-2000 ultrasonic homogenizer (Misonix, Inc., Newtown, CT, USA) at level 10, 10 s for 3 times on an ice bath, and centrifuged (8000 ×*g*, 30 min, 4 °C). The supernatant was used as a crude enzyme preparation and subjected to the following purification steps.

Enzyme purification The supernatant (approximately 160 ml) from centrifugation was loaded onto a Ni-NTA agarose column (5 ml bed volume; Qiagen, Hilden, Germany) and pre-equilibrated with buffer A [20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl]. The column was washed with 25 ml of buffer A containing 20 mM of imidazole. The DZNR active fraction was eluted with 10 ml of buffer A containing 100 mM of imidazole. The eluate was loaded onto a HiTrap desalting column (5 ml, GE Healthcare, Little Chalfont, UK), and the buffer was exchanged with buffer B [20 mM Tris–HCl (pH 8.0), 150 mM NaCl]. The desalted fraction was then loaded onto a HiTrap Q HP column (5 ml, GE Healthcare) that had been equilibrated with 20 mM Tris–HCl (pH 8.0). The column was washed with the same buffer containing NaCl, and then washed with 100 mM NaCl for 2 min and 240 mM for 3 min at a flow rate of 5 ml/min. The active fraction was then eluted for 3 min with the same buffer containing 1.0 M NaCl. Two milliliters of the eluate were then loaded onto a gel filtration column (HiLoad 16/600 Superdex 200 pg, GE Healthcare) in buffer B at a flow rate of 1 ml/min. The active fractions were applied to a Mono Q 5/50 GL column (GE Healthcare). Elution was performed using a 20-min linear gradient of 0–1.0 M of NaCl in 20 mM Tris–HCl (pH 8.0) at a flow rate of 1 ml/min. All purification steps using column chromatography were performed on an HPLC instrument (ÄKTA purifier, GE Healthcare), and UV absorbance was monitored at 215, 280, and 368 nm. After purification, the enzyme was concentrated, and the buffer was exchanged with 20 mM HEPES (pH 7.0) using an ultrafiltration spin column (Amicon Ultra-0.5 30K; Merck Millipore Co., Darmstadt, Germany).

Enzyme activity assays DZNR activity was assayed by monitoring the conversion of daidzein to dihydrodaidzein using HPLC. The enzyme assay mixture (500 μl) contained 0.1 mM daidzein, 1 mM NADPH, and 1 μg of the enzyme in 20 mM citrate-phosphate buffer (pH 6.2). After incubation at 37 °C for 60 s, 500 μl ethyl acetate was added to stop the reaction and extract the product, followed by drying under a vacuum. The extracted sample was dissolved in 100 μl methanol and analyzed on a C18 reversed phase HPLC column (ODS100V, 5 μm, 4.6 mm ID × 250 mm L; Tosoh, Tokyo, Japan) to quantify daidzein and its metabolites as described previously (19).

To determine the optimal pH, the reaction buffer was exchanged with 20 mM citrate-phosphate buffer (pH 5.0–6.2), sodium phosphate buffer (pH 5.8–7.8), and Tris–HCl buffer (pH 7.4–8.2).

HPLC conditions for analysis of other isoflavonoids To quantify the conversion of genistein, an isocratic mobile phase comprising 2% acetic acid with 52.5% acetonitrile at a flow rate of 1 ml/min at 40 °C was used. To detect the conversion of

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