Identification of a Novel Dihydrodaidzein Racemase Essential for Biosynthesis of Equol from Daidzein in Lactococcus sp. Strain 20-92

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Equol is metabolized from daidzein, a soy isoflavone, by the gut microflora. In this study, we identified a novel dihydrodaidzein racemase (t-DDRC) that is involved in equol biosynthesis in a lactic acid bacterium, Lactococcus sp. strain 20-92, and confirmed that histidine-tagged recombinant t-DDRC (t-DDRC-His) was able to convert both the (R)- and (S)-enantiomers of dihydrodaidzein to the racemate. Moreover, we showed that recombinant t-DDRC-His was essential for in vitro equol production from daidzein by a recombinant enzyme mixture and that efficient in vitro equol production from daidzein was possible using at least four enzymes, including t-DDRC. We also proposed a model of the metabolic pathway from daidzein to equol in Lactococcus strain 20-92.

T he intake of isoflavones through soy foods (e.g., miso, tofu, and natto) has many reported health benefits. Because isoflavones are structurally similar to the female hormone estrogen, the associated health benefits are thought to be due to their abilities to bind to estrogen receptors (9, 10, 13). Among the isoflavonoids, equol, a metabolite of daidzein produced by members of the gut microflora (4), is thought to be the primary soy isoflavone derivative that is responsible for the prevention of several sex hormone-dependent diseases because of its potent estrogenic activity (13). However, individual differences exist in the ability of the enteric microflora to produce equol, and equol cannot be produced in more than half of the individuals who consume soy isoflavones (2). Therefore, it has been proposed that the development of probiotics using safe bacteria capable of producing equol from daidzein could allow the production of equol in the enteric environments of all individuals.

Lactococcus sp. strain 20-92, isolated from healthy human feces, is an equolproducing lactic acid bacterium that produces equol from daidzein and is classified as Lactococcus garvieae, which is found in several traditional Italian cheeses and in healthy human intestines (6, 16). The application of Lactococcus strain 20-92 to foods is currently being investigated.

Equol is thought to be produced sequentially from daidzein via dihydrodaidzein and tetrahydrodaidzein by intestinal bacteria (7, 17). To elucidate the metabolic pathway from daidzein to equol in Lactococcus strain 20-92, we recently identified three enzymes (daidzein reductase [t-DZNR], dihydrodaidzein reductase [t-DHDR], and tetrahydrodaidzein reductase [t-THDR]) that catalyze the successive conversion steps of the metabolic pathway and showed that the genes for these enzymes form a gene cluster in the bacterium’s genome (14, 15). Dihydrodaidzein, the first metabolite in the production of equol from daidzein, has two enantiomers, as does equol, because of the presence of an asymmetric carbon atom at the C-3 position. We recently showed that although the dihydrodaidzein that was produced by recombinant t-DZNR was largely (S)-dihydrodaidzein, a racemic mixture of dihydrodaidzein was detected when daidzein was converted using a cell extract of Lactococcus strain 20-92, suggesting the existence of a dihydrodaidzein racemase in strain 20-92 (15). Similarly, it has been reported that (R)- and (S)-dihydrodaidzein are rapidly con-

MATERIALS AND METHODS

Chemicals. Daidzein, dihydrodaidzein (racemate), and equol were purchased from Fujico Co. (Kobe, Japan), Toronto Research Chemicals Inc. (North York, Ontario, Canada), and LC Laboratories (Woburn, MA), respectively. cis-Tetrahydrodaidzein and trans-tetrahydrodaidzein were synthesized from daidzein in-house, as previously described (14). The optical resolution of the dihydrodaidzein racemate was performed by Daicel Chemical Industries (Osaka, Japan), and we obtained two enantiomers, (R)-dihydrodaidzein and (S)-dihydrodaidzein.

Bacteria and culture conditions. Escherichia coli strain JM109 (Takara Bio Inc., Otsu, Japan) was used as the host for the standard cloning experiments and was grown in Luria-Bertani (LB) broth containing ampicillin (50 μg/ml) at 37°C. E. coli strain BL21(DE3) (Novagen, Madison, WI) was used as the host for the recombinant protein expression and was grown in LB broth containing ampicillin (50 μg/ml) at 30°C.

Dihydrodaidzein racemase assay. Assays for the dihydrodaidzein racemase activity were performed by chiral high-performance liquid chromatography (HPLC) monitoring of the racemization of each dihy-
Physical map of the genomic region of the predicted gene cluster that is responsible for equol biosynthesis. The coding regions and orientations of the \(\text{L-DZNR}, \text{L-DHDR}, \text{and L-THDR} \) genes are indicated by the closed and open arrows, respectively. The bold line and the two-headed arrows below denote the genomic DNA region containing the \(\text{L-DZNR}, \text{L-DHDR}, \text{and L-THDR} \) genes that was previously reported (GenBank/EMBL/DDBJ accession no. AB599374) (14) and the DNA fragments that were amplified by inverse PCR in this study, respectively.

Dihydrodaidzein Racemase from \textit{Lactococcus Strain 20-92}
tide sequence (total of 24,473 bases) of the genomic region that contains the equol-producing gene cluster was determined by aligning the above-named eight DNA fragments and the previously determined genomic DNA sequence (Fig. 1)(14).

Gene analysis revealed that the genomic sequence contained 10 novel putative genes and four verified genes upstream of orf-US4 (14), denoted orf-US8, -US7, -US6, and -US5, and six genes downstream of orf-DS2 (14), denoted orf-DS3, -DS4, -DS5, -DS6, -DS7, and -DS8. The physical map of this region is shown in Fig. 1. All of these genes are oriented in the same direction in the genome. Homology searches revealed that among the 10 putative genes, one, orf-US6, had a deduced amino acid sequence that was weakly similar to the amino acid sequence of bacterial methylmalonyl-CoA epimerases (EC 5.1.99.1). Figure 2 shows the alignment of the deduced amino acid sequences for orf-US6 and the methylmalonyl-CoA epimerases derived from Chlorobium phaeobacteroides (GenBank/EMBL/DDBJ accession no. ABL65204; 28.5% identity) and Chlorobium parvum (accession no. ACF11246; 30.2% identity). orf-US6 consists of an open reading frame of 477 nucleotides, and the deduced amino acid sequence consists of 158 amino acids (see Fig. S1 in the supplemental material).

Expression and purification of the recombinant US6-His. To investigate the activity of the US6-His protein, we prepared lysate from E. coli BL21(DE) cells harboring pET-US6-His and purified the recombinant US6-His protein. Our SDS-PAGE analysis of the purified recombinant protein showed one major band with a molecular mass of approximately 20 kDa, which is consistent with the expected size (Fig. 3).

Dihydrodaidzein racemase activity of the recombinant US6-His. To confirm the dihydrodaidzein racemase activity of the recombinant US6-His, we performed enzyme assays using the purified recombinant US6-His protein. As shown in Fig. 4, when dihydrodaidzein enantiomers were individually coincubated with the recombinant US6-His, the resulting dihydrodaidzein was detected as the racemate in each case, showing that this recombinant enzyme is able to convert each dihydrodaidzein enantiomer into the other to yield the racemate. Accordingly, we concluded that orf-US6 of Lactococcus strain 20-92 encodes a dihydrodaidzein racemase (L-DDRC).

Production of equol by the recombinant enzyme mixture. We attempted to synthesize equol from daidzein in vitro using an enzyme mixture consisting of recombinant t-DZNR-His, t-DHDR-His, t-THDR-His, and US6-His (t-DDRC-His) and observed efficient equol production (35.7 μM; 89.4% yield) from daidzein (40 μM) (Fig. 5a). In contrast, without the addition of the recombinant US6-His protein, the amount of produced equol was small (6.1 μM; 15.3%). Significant amounts of dihydrodaidzein (7.8 μM; 19.6%) and cis-tetrahydrodaidzein (18.5 μM; 46.3%) were also detected (Fig. 5b).

DISCUSSION

In this study, we have succeeded in the identification of the gene encoding dihydrodaidzein racemase (t-DDRC) in the equol-producing gene cluster of Lactococcus strain 20-92 (15) by the cloning and expression of this gene (Fig. 1).

The deduced amino acid sequence of t-DDRC was weakly similar to the amino acid sequences of bacterial methylmalonyl-CoA epimerases from Chlorobium phaeobacteroides (GenBank/EMBL/DDBJ accession no. ABL65204) and Chlorobium parvum (accession no. ACF11246). The amino acids are numbered on the right side. Identical amino acid residues are indicated in black.
Three enzymes (L-DZNR, L-DHDR, and L-THDR) play an essential role in equol biosynthesis in Lactococcus strain 20-92 and that equol can be efficiently produced from daidzein in a cell-free system using the following four enzymes: L-DZNR, L-DHDR, L-THDR, and L-DDRC.

It is reported that (S)-equol, but not (R)-equol, was exclusively produced from tetrahydrodaidzein in enantiomeric mixtures of dihydrodaidzein in in vitro fermentation experiments using Eggertella sp. strain Julong 372 (17). Furthermore, the hypothesis that the stereoselective production of (S)-equol might be the result of the enantioselective preference of the Julong 372 strain for the starting compound, (S)-dihydrodaidzein, and that the stereochmistry at the C-3 position was subsequently unchanged during the course of the equol biosynthesis from dihydrodaidzein was proposed (8,17).

Therefore, the results described above for the in vitro production of equol by the recombinant enzyme mixture contradict the hypothesis that the stereochemical C-3 configurations of the intermediates are retained throughout the course of equol biosynthesis from (S)-dihydrodaidzein (17), as (S)-dihydrodaidzein is the major product from daidzein by L-DZNR (15).

Kim et al. (7) showed that the DHDR-catalyzed product from dihydrodaidzein is (3R,4S)-tetrahydrodaidzein, which was then converted to (3S)-equol accompanying a stereochemical inversion at C-3; however, this pathway seems unlikely. In fact, the same authors also indisputably reported later that only (3S,4R)-tetrahydrodaidzein is produced from dihydrodaidzein and is then converted into (3S)-equol (8).

When we converted daidzein into dihydrodaidzein using recombinant L-DZNR-His, the stereochemical configuration of dihydrodaidzein was determined by the elution order in the chiral HPLC analysis, using the data from Wang et al. (18) as a reference. Although further study to assign the exact stereochemical configuration of the dihydrodaidzein produced by L-DZNR-His is necessary, the configuration of the dihydrodaidzein produced by L-DZNR-His may not be (S)-dihydrodaidzein but, rather, (R)-dihydrodaidzein. In fact, Park et al. (12) recently reported that the dihydrodaidzein produced from daidzein by an anaerobic human intestinal microorganism, MRG-1, was (R)-dihydrodaidzein and not (S)-dihydrodaidzein and that the elution order of each enantiomer during chiral HPLC was opposite to that of the report of Wang et al. (18). Therefore, in the present study (Fig. 4), the dihydrodaidzein that eluted earlier during chiral HPLC was designated (S)-dihydrodaidzein, and the later-eluting molecule was designated (R)-dihydrodaidzein. Thus, the assignment is different from that in our previous report (15).

If the DZNR-produced dihydrodaidzein is indeed the R form, the biosynthetic pathway from daidzein to (S)-equol could be explained as shown in Fig. 6, based on the following results. We found that the reduction reaction of dihydrodaidzein to tetrahydrodaidzein catalyzed by DHDR-His is a diastereoselective reduction: individual dihydrodaidzein enantiomers, (R)-dihydrodaidzein and (S)-dihydrodaidzein, are converted by DHDR-His into cis-tetrahydrodaidzein and trans-tetrahydrodaidzein, respectively (see Fig. S2 in the supplemental material). Furthermore, L-DDH-His demonstrated a preference for the conversion of (S)-dihydrodaidzein into trans-tetrahydrodaidzein rather than the conversion of (R)-dihydrodaidzein into cis-tetrahydrodaidzein (see Fig. S3 in the supplemental material). These results support the idea that trans-tetrahydrodaidzein, not cis-tetrahydro-
daidzein, was derived from (S)-dihydrodaidzein and was converted into the final product, (S)-equol. Moreover, we have reported that both L-DHDR and L-THDR possess the reverse activity, catalyzing the conversion of tetrahydrodaidzein into dihydrodaidzein (15). Therefore, the cis-tetrahydrodaidzein produced by L-DHDR is likely to be converted back into (R)-dihydrodaidzein and then converted by L-DDRC into (S)-dihydrodaidzein, an L-DHDR-available substrate for (S)-equol production. The facts that although the amount of equol produced from daidzein was small when using the mixture of three recombinant enzymes (no L-DDRC-His), significant amounts of dihydrodaidzein and cis-tetrahydrodaidzein were detected as intermediates (Fig. 5b) and that cis-tetrahydrodaidzein did not appear in the reaction products using the mixture of four recombinant enzymes, which exhibited high equol production, further support the explanations given above.

The reports describing the biosynthetic pathway from daidzein to (S)-equol and the stereochemistry of the intermediates, especially with regard to the C-3 position, are complicated and controversial. However, it is reasonable to assume that daidzein is first converted into (3R)-dihydrodaidzein by L-DZNR and then into (3S)-dihydrodaidzein by L-DDRC. The final product, (3S)-equol, is generated from (3S)-trans-tetrahydrodaidzein by L-THDR following the conversion of (3S)-dihydrodaidzein into (3S)-trans-tetrahydrodaidzein. This biosynthetic pathway is feasible in that the configuration at C-3 is retained through the serial reductive reactions and that this pathway can provide an explanation as to why there is another enzyme, L-DDRC, in the equol biosynthetic

![FIG 5](http://aem.asm.org/)

**FIG 5** Elution profiles of the reaction products using the recombinant enzyme mixture and daidzein in the presence (a) or absence (b) of recombinant L-DDRC-His (US6-His). The elution profiles of the reference standards daidzein (DZN), dihydrodaidzein (DHD), cis-tetrahydrodaidzein (c-THD), trans-tetrahydrodaidzein (t-THD), and equol (EQL) are shown in the “STD” panel.

![FIG 6](http://aem.asm.org/)

**FIG 6** Model of the equol biosynthetic pathway starting from daidzein in *Lactococcus* strain 20-92. The enzyme activities of L-DZNR, L-DHDR, L-THDR, and L-DDRC are indicated by a green, blue, orange, and red arrow(s), respectively. First, daidzein is converted into (R)-dihydrodaidzein by L-DZNR. The (R)-dihydrodaidzein produced is then rapidly racemized by L-DDRC, and the produced (S)-dihydrodaidzein is then preferentially converted into trans-tetrahydrodaidzein by L-DHDR. Subsequently, trans-tetrahydrodaidzein is converted into (S)-equol by L-THDR. Abbreviations are the same as for Fig. 5.
pathway from daidzein to equol. In the equol biosynthetic pathway, 1-DDRC may play the role equivalent to that of methylmalonyl-CoA epimerase in β-oxidation of odd-numbered-chain fatty acids (3).

Thus, the main avenue of the biosynthetic pathway from daidzein to (S)-equol seems to be elucidated; however, uncertainties about several details remain. In fact, in addition to the complexity of the pathway, including the stereochemistry, the functions of most of the genes in the equol-producing gene cluster have not yet been characterized. The functions of these gene products and the mechanisms of equol biosynthesis are expected to be clarified in greater detail in the near future.

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REFERENCES