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MINI REVIEW

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Functionalization of Isoflavones with Enzymes

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Abstract Considerable progress has been made in functionalization of the soy isoflavones through enzymatic modification of daidzin, genistin, and glycitin. After hydrolysis of β-glucosides into their corresponding aglycones, these compounds were structurally modified via biotransformations such as regioselective hydroxylation, enantioselective reduction, regioselective methylation, and polymerization. These reactions often resulted in an increase of the biological activities (e.g., antioxidative activity, antiproliferative activity) and/or improvement of the physico-chemcial properties (e.g., water solubility, bioavailability). This review briefly summarizes on-going research activities on the biofunctionalization of the soy isoflavones.

Keywords: isoflavone, enzyme, biotransformation, enzymatic modification

Introduction

Isoflavones are polyphenolic compounds which are produced almost exclusively by soybeans. Isoflavones differ from flavones in the position of the phenyl group on the 4*H*-1-benzopyr-4-one skeleton; the phenyl group is in position 3 relative to the oxygen of the ring (Fig. 1), whereas it is in position 2 in flavones.

Isoflavones in soybeans, mostly concentrated in the hypocotyls (1), are found in 4 chemical structures including aglycones of genistein (6,7,4'-trihydroxyisoflavone), daidzein (7,4'-dihydroxyisoflavone), and glycitein (7,4'-dihydroxy-6-methylisoflavone), and their corresponding β-glucosides, 6"-*O*-acetyl-β-glucosides, and 6"-*O*-malonyl-β-glucosides (2) (Fig. 1). Although chemical structures and concentration of isoflavones in soybeans are dependent on many factors including genotypes, crop years, crop locations, and storage period (1,3,4), raw soybeans contain mostly glycoside forms of isoflavone and low percentage of aglycone. They usually consist of about 70-80% of malonyl-β-glucosides, 5% of acetyl-β-glucosides, 25% β-glucoside derivatives, and less than 2% aglycones (5). Conventional thermal treatment under high moisture results in a conversion of malonyl derivatives into β-glucosides via intra-conversion while aglycones have higher heat resistance (6,7). Dry heat treatment such as frying, toasting, or baking also increases the formation of acetyl isoflavones in foods.

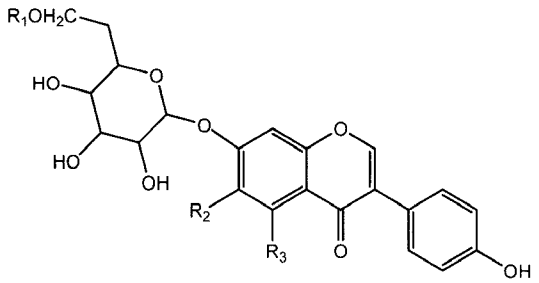

Fig. 1. Structure of isoflavones found in soybean. R_1: -H (β-glucoside), -$CHOCH_3$ (acetyl β-glucoside), -$CHOCH_2COOH$ (malonyl β-glucoside), R_2: -H (daidzin and genistin), -OCH_3 (glycitin), R_3: -H (daidzin and glycitin), -OH (genistin).

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Isoflavones may act as estrogen antagonist in a high estrogen environments and estrogen agonist in a low estrogen environment, which may be due to the structural similarity to human sex hormone, estrogen (8-10). As a result, a broad spectrum of biological activities has been reported in soy isoflavones. Epidemiological studies have revealed that high consumption of isoflavones may lower the risk of cancers in breast, prostate, urinary track, and colon. Positive correlation between isoflavone consumption and coronary heart disease and osteoporosis has been reported (8,9). In addition, isoflavones extracted from a traditional fermented unsalted soybean, *cheonggukjang*, were shown to enhance glucose utilization via activating insulin signaling and to stimulate peroxisome proliferator-activated receptor-γ activity in adipocytes (11).

Hydrolysis of Glycoside Moieties with β-Glucosidases

Aglycones of isoflavone were reported to have higher bioavailability with being absorbed faster and to higher amounts than corresponding glycoside forms, since isoflavone glycosides were not directly transported across the gastrointestinal tract (12).

Hydrolysis of glycoside moieties of isoflavones was often observed in microbial fermentation. The concentration of daidzein, glycitein, and genistein in soybeans increased dramatically during fermentation of cooked soybeans (*cheonggukjang*) by *Bacillus* sp. (13,14). Since *Bacillus* sp. was shown to have β-glucosidase activity, the aglycones were considered to be produced by the fermenting organism. Another example is fermentation of soymilk using *Bifidobacterium animalis* Bb-12, a major natural microflora of the human intestinal tract. The content of aglycone has

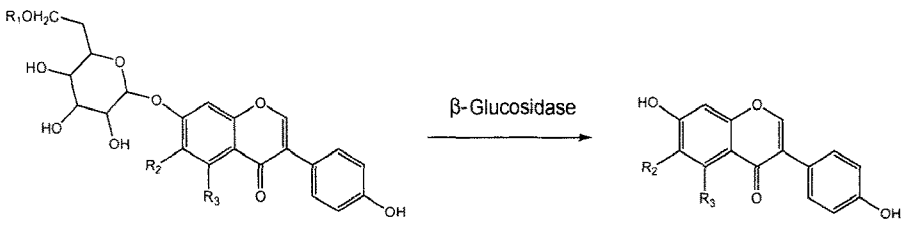

Fig. 2. Hydrolysis of glycoside moieties of soy isoflavones with a β-glucosidase.

increased from 8 to 50% of total isoflavones during fermentation (15).

In addition to microbial fermentation, the glycoside form of isoflavones can be converted into the aglycones by cell-free enzymes β-glucosidase (Fig. 2). For instance, treatment of β-glucosidase isolated from *Escherichia coli* and almonds (16) or of almond powder itself (17) resulted in an increase of the content of aglycones in soybeans. These enzymes showed higher affinity toward β-glucosides as compared to acetyl- or malonyl-β-glucosides.

In summary, it is possible to increase the content of aglycones in soybeans with enzymes and microbes, which may result in an increase of bioavailability of soy isoflavones.

Hydroxylation with Oxygenases

O-Dihydroxyisoflavones (e.g., 8-hydroxydaidzein, 8-hydroxy-genistein) have shown much stronger antioxidative and antiproferative activity compared to their natural forms of daidzein and genistein (18-20). For instance, 8-hydroxy-genistein exhibited 6.9-fold higher antiproferative activity than genistein toward the human promyelocytic leukemia cells (HL-60) (18). In addition, isoflavones gained a new activity with respect to inhibition of melano-genesis by being hydroxylated at the 8-position of A ring (private communication with Prof. Kim BK at Seoul National University). Thus, hydroxylation of the soy isoflavones is considered one of the most effective ways to improve functionality of the isoflavones.

The regiospecific hydroxylation of daidzein and genistein was first observed in soybean fermentation by *Aspergillus saitoi* (21) (Fig. 3A). During submerged cultivation in soybean extract-based medium, 8-hydroxydaidzein and 8-hydroxygenistein have accumulated to a final concentration of 15 and 25 mg/L, respectively, resulting in a decrease of daidzein and genistein concentration in the medium. The hydroxylation was initiated after sporulation of the strain under aerobic condition, indicating that daidzein and

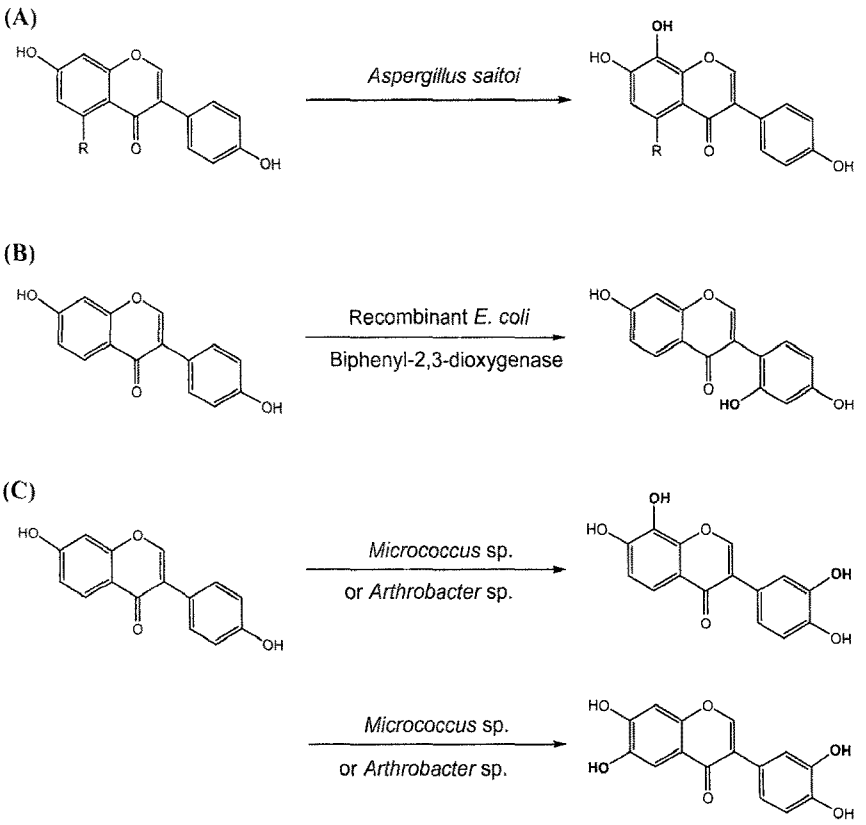

Fig. 3. Hydroxylation of soy isoflavones. (A) Hydroxylation of daidzein and genistein to 8-hydroxydaidzein and 8-hydroxygenistein, respectively, by *A. saitoi*, (B) hydroxylation of daidzein to 2'-hydroxydaidzein by biphenyl-2,3-dioxygenase of *Burkholderia* sp. LG400, (C) hydroxylation of daidzein to 8,3'-dihydroxydaidzein and 6,3'-dihydroxydaidzein by *Micrococcus* sp. and *Arthrobacter* sp.

genistein were oxidized by hydroxylases produced by *A. saitoi* during sporulation.

Another example is the regiospecific oxidation of daidzein by biphenyl-2,3-dioxygenase originated from *Burkholderia* sp. LG400 (22). The C-2' position of B ring of daidzein was hydroxylated by recombinant *E. coli* expressing the gene of biphenyl-2,3-dioxygenase (Fig. 3B). The recombinant *E. coli* was also able to oxidize the synthetic isoflavonoids 7-hydroxy-8-methylisoflavone and 7-hydroxyisoflavone to 7,2',3'-trihydroxy-8-methylisoflavone and 7,3',4'-trihydroxy-isoflavone, respectively. Product yield and product concentration, however, were not reported yet.

The soy isoflavones can also be polyhydroxylated by microorganisms. Five *tempe*-derived bacterial strains identified as *Micrococcus* or *Arthrobacter* species could catalyze (poly)hydroxylation of the isoflavones (23,24). All the strains could convert daidzein into 6-hydroxy-daidzein and 8-hydroxydaidzein. Three strains further hydroxylated them into 6,3'-dihydroxydaidzein and 8,3'-dihydroxydaidzein, respectively (Fig. 3C).

Overall, it is possible to introduce hydroxyl group(s) regiospecifically onto the natural and synthetic isoflavones by using appropriate microbes. However, the product yields and concentrations remained low so far. In order to reach high product yields and concentrations, the enzymes involved in hydroxylation should be characterized including their induction mechanism(s).

Reduction with Reductases and/or Dehydrogenases

Equol, a reducing form of daidzein (Fig. 4), is known to be the most effective in stimulating an estrogenic response among the isoflavone derivatives (25,26). It was, however, detected only in 30-40% of the human population (26) because of lack of the gut microbial floral involved in equol production. Thus, the overproduction of equol is highly required.

(S)-Equol was shown to be produced from daidzein by serial reactions of *Lactobacillus* sp. Niu-O16 and *Eggerthella* sp. Julong 732 (27) (Fig. 4). The first microorganism isolated from bovine rumen catalyzes the reduction of C-2 and C-3 double bonds of daidzein under anaerobic condition yielding dihydrodaidzein as product. The latter organism isolated from human intestine reduced further dihydro-daidzein into (S)-equol also under strictly anaerobic condition. Although the enzymes involved in equol production were not characterized to date, they were postulated to be similar to morphinone reductase and morphine dehydrogenase of *Pseudomonas putida* M10 (28). The morphinone reductase catalyzes reduction of oxo-ene compound (i.e., morphinone) to oxo compound (hydromorphone), which is subject to further reduction by morphine dehydrogenase.

Another report is the production of equol by a single microbe *Eggerthella* sp. isolated from rat intestine (29). The strain, which has 99% similarity in 16S rRNA gene sequences with *Eggerthella* sp. Julong 732, converted daidzein into equol via dihydrodaidzein in an equol-assay medium containing 200 μM daidzein. Equol was produced to a concentration of 30 μM in the culture medium 4 days after anaerobic incubation at 37°C. The conversion yield was increased by 4.7- and 4.5-fold by addition of butyric acid and arginine, respectively. The stimulating mechanism of

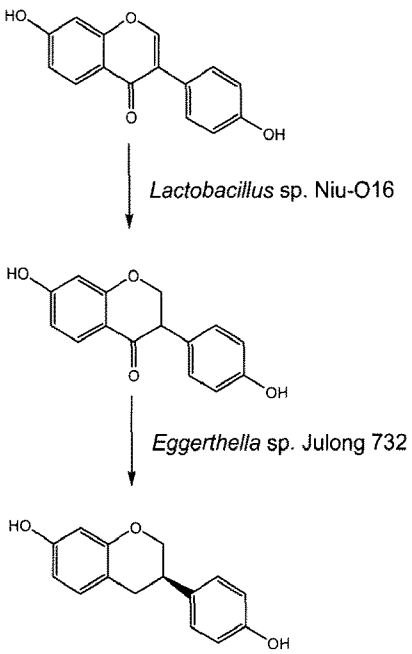

Fig. 4. Proposed metabolic pathway for the production of (S)-equol. (S)-Equol is produced from daidzein through dihydrodaidzein by *Lactobacillus* sp. Niu-O16 and *Eggerthella* sp. Julong 732.

butyric acid has not yet been known, whereas arginine was reported to serve as an energy source for the fermenting microorganism enabling an increase of cell density (30).

In summary, equol can be produced by anaerobic microbial fermentation. However, the product concentrations remained below 1 mM probably due to difficulty in cultivation of the production strains to a high cell density. Further studies to increase equol concentrations may focus on cloning of genes coding for the equol production pathway, functional expression in 'Generally Recognized As Safe' (GRAS) and facultative anaerobic strains, and development of high cell density culture.

Methylation with Methyltransferases

Methylation of flavonoids including isoflavones is considered to play an important role in inactivating the reactive hydroxyl groups of flavonoids as well as altering their solubility and biological activity.

There have been a number of methyltransferase genes cloned and characterized (31-36). Isoflavone 7-*O*-methyl-transferase from alfalfa (*Medicago sativa*) was shown to methylate the isoflavones daidzein and genistein to the corresponding 7-*O*-methylated compounds (31) (Fig. 5A). A methyltransferase from soybean (*Glycine max*), which was expressed in *E. coli*, could convert not only daidzein and genistein, but also apigenin and quercetin into the 4'-*O*-methylated compounds (35) (Fig. 5B).

7-*O*-Methyltransferase from bacteria *Streptomyces avermilitis* showed broad substrate spectrum (33). The isoflavones daidzein and genistein as wells as the flavones kaempferol, apigenin, and quercetin and the flavanone naringenin were transformed into the corresponding 7-*O*-methylated metabolites by recombinant *E. coli* Sa-2 expressing the gene of 7-*O*-methyltransferase.

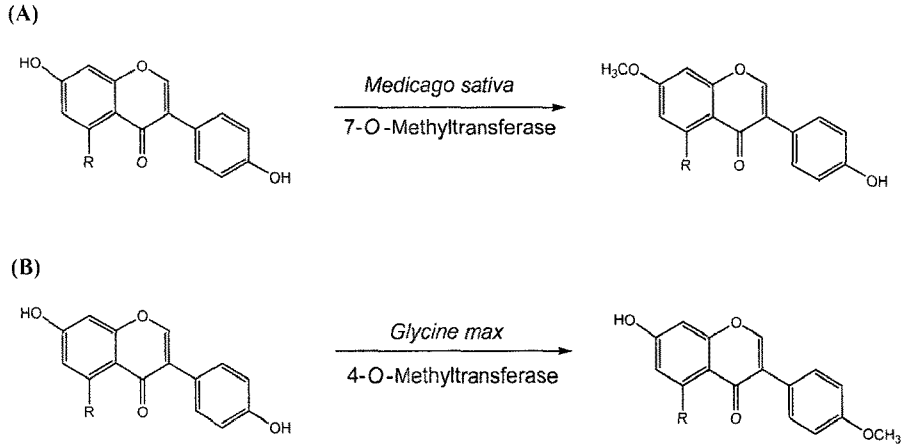

Fig. 5. Regiospecific methylation of isoflavones. (A) Methylation of daidzein and genistein to the corresponding 7-*O*-methylated compounds by isoflavone 7-*O*-methyltransferase from alfalfa, (B) methylation of daidzein and genistein to the corresponding 4'-*O*-methylated compounds by a methyltransferase from soybean.

Overall, it appears that a variety of isoflavones, which differ in degree of methylation, can be produced by various methyltransferases. Functional expression of plant and bacterial methyltransferase genes in *E. coli* implies that overproduction of diverse isoflavones will be possible via whole-cell biocatalysis.

Oligomerization with Oxidases or Peroxidases

Polymerization of flavonoids was shown to influence their stability, solubility, and biological activity (e.g., antioxidant activity) (37). For instance, the laccase- or tyrosinase-catalyzed polymerization of quercetin and kaempferol produced aggregates with higher antioxidant activity than the corresponding monomers. Among the naturally occurring polymers of flavonoids, proanthocyanidins found in red wines show high antioxidant activity and possess preventive actions on diseases such as atherosclerosis, gastric ulcers, cataracts, and diabetes (38-40).

Daidzein and 8-hydroxydaidzein were reported to be oxidatively polymerized via a radical polyrecombination. The reaction is catalyzed by horseradish or soybean peroxidase in the presence of H_2O_2 (41) (Fig. 6). The yield of 8-hydroxydaidzein polymer was low (30%). However, the polymer was very sensitive to radicals, pointing to a possibility of the bioproduction of radical-sensitive polymers. The 8-hydroxydaidzein polymer was soluble in the reaction mixture and therefore purified using dialysis (cut-off 6,000±8,000 Da). On the other hand, the daidzein polymer was precipitated from the reaction medium, while soluble in dimethyl sulfoxide and N,N-dimethylformamide. The gel permeation chromatography analysis (GPC) has shown the presence of macromolecules, oligomers and also a small amount of unmodified monomer. However, biological activities of the polymers remained to be investigated.

Other Isoflavone Derivatives of Interest from Fermented Soyfoods

Daidzein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside, genistein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside, and glycitein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside have been detected in Japanese fermented soybeans *natto* (42,43) (Fig. 7A). Daidzein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside and genistein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside were also identified in *cheonggukjang* (44). Conjugated ethers of tartaric acid with daidzein, genistein, and 8-hydroxygenstein were found in Japanese soy sauces (45,46) (Fig. 7B). Some of the isoflavone derivatives were shown to have reducing bone loss or free radical scavenging activity (43). These reports indicate that possibility to obtain new isoflavone derivatives from fermented soy foods is still large, which may show excellence in biological activity and physico-chemical properties.

Conclusion and Outlook

The soy isoflavones can be functionalized by biotransformations such as hydroxylation, reduction, methylation, and polymerization. The resulting products often showed an increase in their biological activities (e.g., antioxidative

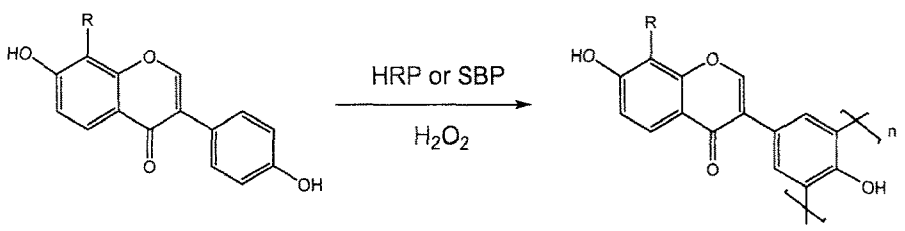

Fig. 6. Proposed pathway for polymerization of daidzein and 8-hydroxydaidzein. The reaction is catalyzed by horseradish or soybean peroxidase in the presence of H_2O_2. R: -H (daidzein), -OH (8-hydroxydaidzein).

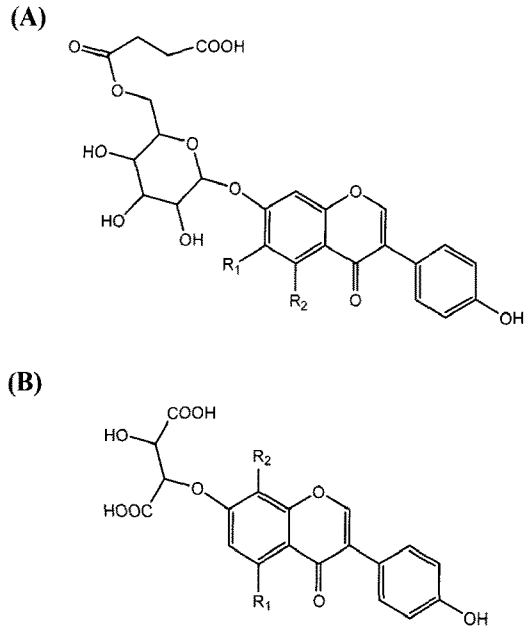

Fig. 7. Chemical structure of isoflavone derivatives found in fermented soybeans. (A) Succinyl daidzin, succinyl genistin, and succinyl glycitin identified from *natto*, (B) tartaric isoflavone derivatives identified in Japanese soy sauce (*shoyu*). R_1: -H (*shoyu*-flavone A), -OH (*shoyu*flavone B and C), R_2: -H (*shoyu*flavone A and B), -OH (*shoyu*flavone B).

activity, antiproliferative activity) and/or improvement in the physico-chemcial properties (e.g., water solubility, bioavailability). However, such functionalization of the isoflavones remained to be on a synthetic scale to date. Characterization and engineering of the enzymes involved in the functionalization as well as process development seem to be necessary to realize implementation of the bioprocesses on industrial scale. Searching new isoflavone derivatives from fermented soyfoods is one of rational ways considering possible toxicity or health beneficial functionality.

Acknowledgments

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