Food Microorganisms that Effectively Hydrolyze O-Glycoside but Not C-Glycoside Isoflavones in *Puerariae Radix*

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ABSTRACT: *Puerariae radix* (PR) is known to contain abundant glucoside conjugates of daidzein isoflavones. This study was performed to determine the daidzin hydrolysis activity of the various strains of probiotic bacteria and edible fungi during fermentation of PR. Among the strains tested, *Bifidobacterium* sp. Int-57 showed the greatest β-glucosidase activity and daidzin hydrolysis activity. When yeast extract (2.5%) was added to the PR medium during fermentation with *Bifidobacterium* sp. Int-57, all of the daidzin was hydrolyzed to near completion. The addition of skim milk and whole milk also improved the conversion of daidzin into daidzein. Puerarin, a C-glucoside of daidzein in PR, was not hydrolyzed into daidzin by any of the experimental strains during fermentation. This study demonstrates that daidzin can be efficiently converted into daidzein by the proper combination of probiotic strains and fermentation conditions.

Keywords: isoflavones, *Puerariae radix*, probiotic bacteria, β-glucosidase activity

Introduction

Phytoestrogens are estrogen-like compounds found in a wide variety of plants. Epidemiological studies provide evidence for a protective role of isoflavones against the development of numerous chronic diseases, including several cancers, cardiovascular disease, and osteoporosis (Alison and others 2003). Studies performed in vitro and in animal models have shown that these substances have an affinity to the estrogen receptor and exert hormonal and antihormonal effects (Kurzer and Xu 1997). There has been tremendous interest in the possibility that dietary phytoestrogens represent an alternative to postmenopausal hormone therapy because of concerns about side effects and long-term health consequences of the hormone therapy (Kurzer 2003), with soybean isoflavones present at concentrations of 100 to 300 mg/100 g dry weight receiving the most attention. The major isoflavones in soybean are daidzin (7,4′-dihydroxyisoflavone 7-glucoside) and genistin (4′,5,7-trihydroxyisoflavone-7-glucoside) as glycosides, and their corresponding aglycone forms, daidzein (4′,7-dihydroxyisoflavone) and genistein (4′,5,7-trihydroxyisoflavone).

The estrogenic activity of various plant food materials that are commonly consumed was recently assessed by a reporter gene assay using MCF-7 breast cancer cell lines transfected with luciferase reporter gene (Kim 2002). Among 47 materials analyzed that included various soybeans, *Puerariae radix* (PR) showed the greatest estrogenic activity, suggestive of a high level of phytoestogen activity. It is known that the isoflavone contents of PR are about 10-fold those of soybean (amounting to 2 g/100 g dry weight). The main isoflavones of PR are purarin and daidzin. In addition to the phytoestrogenic activity, PR daidzin is also known to have inhibitory activity on acetaldehyde dehydrogenase, cAMP phosphodiesterase, and antispasmatic activity (Nikaido and others 1982; Keung and Vallee 1993), whereas puerarin had hypoglycemic effects and increased coronary artery blood flow (Nakamoto and others 1977).

It is reported that the isoflavone aglycones were absorbed faster and in greater amounts than their glucosides in humans and suggested that isoflavone aglycone-rich products may be more effective than glucoside-rich products in preventing chronic disease such as coronary heart disease (Xu and others 1995; Izumi and others 2000). Izumi and others (2000) found that the plasma concentrations after both low and high doses of aglycone (0.11 mmol and 1.7 mmol) were more than 2 and 5 times higher than that after glucoside intake, respectively. In this context, we searched for various microorganisms that have been safely used for the manufacture of food and for the fermentation conditions that would improve the bioconversion of PR isoflavone glycosides into aglycosides. This study is the 1st investigation of the hydrolysis of isoflavone glucosides of PR extracts by probiotic bacteria such as bifidobacteria and lactic acid bacteria.

Materials and Methods

Microbial strains and growth conditions

Five *Bifidobacterium* strains (*Bifidobacterium* sp. Int-57, *Bifidobacterium bifidum* BGN4, *Bifidobacterium* sp. SH5, *Bifidobacterium* sp. SJ32, and *Bifidobacterium* sp. RD54) were isolated as reported previously (Park and others 1999), and *B. bifidum* (ATCC 29521) and *Lactobacillus delbrueckii* subsp. delbrueckii (KCTC 1047) were purchased from ATCC (American Type Culture Collection) and KCTC (Korean Collection for Type Cultures), respectively. Two strains of lactic acid bacteria showing β-glucosidase activities isolated from the PR extract were identified and named *Lactobacillus pentosus* PR and *Leuconostoc paramesenteroides* PR based on 16S rRNA sequences and various biochemical tests and were used in this study. They were grown at 37 °C in de Man Rogosa Sharpe (MRS) (Hardy, Santa Maria, Calif., U.S.A.) medium containing 0.05% (w/v) L-cysteine·HCl and Blood Liver (BL) medium. The bacterial strains

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were cultured anaerobically in a commercial system (Anoxomat WS80, LichteVoorde, The Netherlands) and anaerobic jars (Mart, LichteVoorde, The Netherlands). Growth was quantified by measuring the absorbance at 600 nm.

In addition to the 9 lactic acid bacteria mentioned previously, 2 additional peptostreptococci, 4 yeasts, and 8 molds were purchased from KCTC and KCCM (Korean Culture Center of Microorganisms). Each colony was isolated and incubated with the appropriate media such as MRS (Hardy) for *Bifidobacterium*, *Lactobacillus*, and *Leuconostoc*; Reinforced Clostridial Medium (RCM) for *Peptostreptococcus*; YM agar (Difco, Sparks, Md., U.S.A.) for yeasts; and Potato Dextrose agar (Difco) or Malt Extract agar (Difco) for molds. RCM contained the following (g/L dH_2O): tryptose, 10; beef extract, 10; yeast extract, 3; dextrose, 5; sodium chloride, 5; soluble starch, 1; cysteine hydrochloride, 0.5; sodium acetate, 3; and agar, 15.

Bacteria grown overnight were inoculated to the level of 3 x 10^6 colony-forming units (CFU)/mL into culture media. Bifidobacteria, lactic acid bacteria, and 2 peptostreptococci were anaerobically cultured as described previously. Other yeasts and molds were grown aerobically in the appropriate medium at 20 °C in a shaking incubator (Vision Scientific, Buchon, Korea).

Preparation of PR extract

PR extract (18% solid content) was purchased from Kyoung-Dong Market in Seoul. It was centrifuged (9800 × *g* for 10 min at 4 °C), and then the pH of the supernatant was adjusted to 7.2 and stored at –80 °C until use. For fermentation, PR extract was autoclaved for 15 min at 121 °C.

Assay of β-glucosidase activity

For the preparation of enzyme solution, 1 mL of culture was centrifuged (12000 × *g* for 5 min at 4 °C), and the harvested pellet was washed twice with 0.2 *M* sodium phosphate buffer (pH 6.0). The pellet was resuspended in 500 μL phosphate buffer containing 2% acetone-toluene (9:1) solution and 500 μL of 0.5 m*M* ρ-nitrophenyl-β-D-glucopyranoside (Sigma Chemical, St. Louis, Mo., U.S.A.) and assayed for β-glucosidase activity at 45 °C for 15min (Hernandez and others 2002). The reaction was terminated by adding 100 μL of 0.5 mol/L sodium carbonate solution. ρ-Nitrophenol (PNP) release was quantified at 405 nm in a microplate reader (Bio-Rad Model Benchmark, Tokyo, Japan). One unit of enzyme activity was defined as the amount that released 1 μmol of PNP per min, and specific activity was obtained per milligram dry weight cell (Choi and others 1996).

Analysis of isoflavones by high-performance liquid chromatography

Puerarin (daidzein 8-C-glucoside) and daidzin (daidzein-7-O-glucoside) standards were purchased from Fluka (Buchs, St. Gallen, Switzerland), and daidzein (4′,7-dihydroxyisoflavone) from Sigma Chemical. High-performance liquid chromatography (HPLC)-grade acetonitrile was from Fisher (Fair Lawn, N.J., U.S.A.) and acetic acid from J.T. Baker (Phillipsburg, N.J., U.S.A.). HPLC analyses were conducted using a Young-Lin M930 pump (Young Lin Instrument, Anyang, Korea), a reversed-phase column (Waters μBondapak TM C18), a Young-Lin M720 absorbance detector, and Young-Lin Autochro-win software. Elution was carried out at a flow rate of 1.0 mL/min using a solvent gradient consisting of solution A (0.1% acetic acid in H_2O) and solution B (0.1% acetic acid in acetonitrile)—a gradient of 85% to 65% solvent A to 15% to 35% solvent B over 28 min; and 65% solvent A to 35% solvent B held over 10 min. For the optimum extraction of analysis, 100 μL of cultured PR extract was diluted with 500 μL of 80% methanol for 24 h at 50 °C (Jeon 2002). Samples were subjected to HPLC analysis after centrifugation and filtration (Millex LCR 13 NS, MSCLRO, 0.45 μm; Millipore, Bedford, Mass., U.S.A.). The analysis was monitored by UV detector at 254 nm. Quantitative data were obtained by comparison with known standards such as daidzin, daidzein, and puerarin. Triplicate samples were used throughout the experiments.

Effect of nutrient source on the culture conditions

The effect of various carbon and nitrogen sources on daidzin hydrolysis was assayed. Glucose, maltose, galactose, cellobiose, and xylose (2%) for carbon, and peptone, beef extract, yeast extract, tryptone, and malt extract (2.5%) for nitrogen were added into 500 mL PR extract and the distilled water was added until the final volume was 1 L. The added vitamin mixture (ICN Biomedicals, Ohio, U.S.A.) was tested at concentrations of 0.001%, 0.4%, and 1%, respectively. Skim milk (10% w/v, Hardy) and whole milk (Korea Yakult) were mixed with the same volume of PR broth. The culture medium was heat-treated in a water bath at 95 °C for 20 min. Then *B. adolescentis* Int-57 grown overnight was inoculated to the level of 3 x 10^6 CFU/mL into culture media. After fermentation at 37 °C for 24 h and 48 h, the changes of isoflavone concentration were detected by HPLC analysis.

Results and Discussion

β-Glucosidase activity and isoflavone conversion during fermentation by various bacteria

After a preliminary experiment in which various lactic bacteria were compared for β-glucosidase production, 9 bacterial strains (*Bifidobacterium* sp. Int-57, *B. bifidum* BGN4, *Bifidobacterium* sp. SH5, *Bifidobacterium* sp. SJ32, *Bifidobacterium* sp. RD54, *B. bifidum* ATCC 29521, *L. delbrueckii* subsp. delbrueckii KCTC 1047, *L. pentosus* PR, and *Leu. paramesenteroides* PR) were selected and cultured in MRS broth for 48 h. Their specific activities of β-glucosidase were then compared, from which they were divided into 3 groups: high β-glucosidase producers (*Bifidobacterium* sp. Int-57, 0.50 mU/mg dry wt. cell; *L. delbrueckii* subsp. delbrueckii KCTC 1047, 0.45 mU/mg dry wt. cell; *Bifidobacterium* sp. SJ32, 0.42 mU/mg dry wt. cell), intermediate β-glucosidase producers (*Bifidobacterium* sp. SH5, 0.28 mU/mg dry wt. cell; *Leu. paramesenteroides* PR, 0.21 mU/mg dry wt. cell; *L. pentosus* PR, 0.15 mU/mg dry wt. cell), and low β-glucosidase producers (*B. bifidum* BGN4, 0.04 mU/mg dry wt. cell; *Bifidobacterium* sp. RD54, 0.03 mU/mg dry wt. cell; *B. bifidum* ATCC 29521, 0.01 mU/mg dry wt. cell). When they were grown in the PR extract medium, the bacterial growth and their β-glucosidase activities were relatively weak. To improve the degree of cell growth, a medium comprising 50% PR extract and 50% MRS broth was used. Although there were some differences among the tested bacteria, all of the bacteria except for *B. bifidum* BGN4 showed noticeable daidzin hydrolysis activity (Figure 1). The major metabolite of daidzin during fermentation was daidzein, and the daidzein content gradually increased during fermentation. However, for *L. pentosus* PR and *Leu. paramesenteroides* PR the daidzein concentration decreased suddenly at 48 h. After 48 h of fermentation at 37 °C, the order of the bacteria showing the greater conversion from daidzin to daidzein was *Bifidobacterium* sp. Int-57 (95%) > *Leu. paramesenteroides* PR (85%) > *Bifidobacterium* sp. SJ32 (59%) > *L. delbrueckii* subsp. delbrueckii KCTC 1047 (28%) > *L. pentosus* PR (20%) > *B. bifidum* BGN4 (7%). In contrast to daidzin, puerarin was not hydrolyzed by any of the microbial strains even after extensive fermentation (lasting more than 10 d) in our experiments described previously (data not shown). In soybean, both daidzin and genistin were efficiently hydrolyzed during fermentation (Zubik and Meydani 2003),

whereas only daidzin but not puerarin in PR was hydrolyzed in the present study. Because both daidzin and genistin are O-glycosides and puerarin is a C-glycoside, the studies suggest that few microorganisms can hydrolyze the C-glycoside form of daidzein.

Because *Bifidobacterium* exists as the most predominant intestinal bacteria in human infants and exerts various beneficial effects on human health, it has been considered an important probiotic bacterium (Arunachalam 1999). Consequently, fermentation of PR with *Bifidobacterium* would provide a safe means for the conversion of daidzin into daidzein and could possibly be used for the development of new functional food products.

Effect of various nutrients and growth factors on daidzin hydrolysis in the medium containing PR extract (50%) during fermentation

In the medium containing PR extract (50%) and MRS broth (50%), *Bifidobacterium* sp. Int-57 showed the greatest daidzin hydrolysis activity (Figure 1). Therefore, this strain was used to elucidate the influence of various carbon sources on the daidzin hydrolysis. For the experiment, MRS was replaced with various nutrients as described in the "Materials and Methods" section. The initial levels of daidzin and daidzein were 1560 μg/mL and 300 μg/mL, respectively. As shown in Table 1, carbon sources had less effect than nitrogen sources on daidzin hydrolysis by *Bifidobacterium* sp. Int-57 in the medium containing PR extract (50%). For fermentation with *Bifidobacterium* sp. Int-57, the order of the carbon sources showing greater effect on daidzin hydrolysis was maltose, galactose > glucose, cellobiose > xylose. During 24 h of fermentation with *Bifidobacterium* sp. Int-57, the daidzin hydrolysis rate was 69% in the medium containing 50% PR extract + 50% MRS broth (PR-MRS), but 36% in the medium containing 50% PR extract with 2% maltose. The daidzin hydolysis rate was calculated as [(concentration of daidzin before fermentation – concentration of daidzin after fermentation)/concentration of daidzin before fermentation] x 100. Compared with carbon sources, organic nitrogen sources had substantial effects on daidzin hydrolysis. After 24 h of fermentation with *Bifidobacterium* sp. Int-57, all of the daidzin in the medium with added yeast extract was completely hydrolyzed. However, it is apparent that the daidzin was not stoichiometrically converted to daidzein during PR fermentation. Jeon (2002) also reported that the daidzin was not stoichiometrically converted to daidzein during soybean fermentation and suggested that some of the hydrolyzed daidzein and genestein might have been converted to other metabolites. Additionally, Hur and others (2000) reported that the Gram-positive strain HGH6 reduced daidzein and genestein to dihydrodaidzein and dihydrogenestein, respectively. After 48 h of fermentation, most of the daidzin was hydrolyzed in the media added with yeast extract, peptone, and tryptone. For *Bifidobacterium* sp. Int-57, the order of the effect of nitrogen source on the daidzin hydrolysis was yeast extract > peptone, tryptone > malt extract > beef extract. Overall, among the various organic nitrogen sources, yeast extract showed the greatest improvement on the daidzin hydrolysis by *Bifidobacterium* sp. Int-57. On the other hand, the effects of vitamin mixtures at levels of 0.001%, 0.4%, and 1% were similar (Table

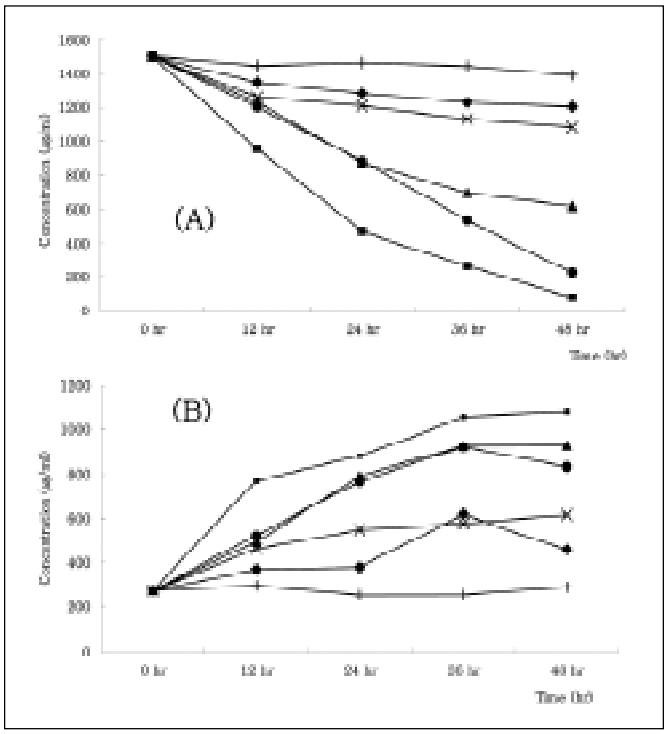

Figure 1—The changes of daidzin (a) and daidzein (b) contents in the medium containing *Puerariae radix* (PR) extract (50%) + MRS broth (50%) with *Bifidobacterium* sp. Int-57 (■), *Bifidobacterium* sp. SJ32 (▲), *Lactobacillus delbrueckii* subsp. delbrueckii KCTC 1047 (□), *Leuconostoc paramesenteroides* PR (●), *Lactobacillus pentosus* PR (◆), and *Bifidobacterium bifidum* BGN4 (□).

Table 1—Effect of various nutrients and growth factors on daidzin content during fermentation with *Bifidobacterium* sp. Int-57

Source		Time	Contents (μg/mL) Daidzin	Daidzein
Carbon source	Control (50% PR extract)	0	1560	300
		24 h	1399	581
		48 h	979	506
	Glucose	24 h	1006	769
		48 h	477	795
	Maltose	24 h	965	816
		48 h	326	877
	Galactose	24 h	971	821
		48 h	294	928
	Cellobiose	24 h	1056	712
		48 h	433	839
	Xylose	24 h	1207	586
		48 h	870	864
Nitrogen source	Peptone	24 h	641	1162
		48 h	90	778
	Beef extract	24 h	1358	625
		48 h	762	875
	Yeast extract	24 h	ND[a]	858
		48 h	ND	787
	Tryptone	24 h	542	1225
		48 h	92	1045
	Malt extract	24 h	1174	723
		48 h	689	1039
Vitamin mixture (0.001%)		24 h	1390	608
		48 h	978	500
Vitamin mixture (0.4%)		24 h	1339	600
		48 h	975	501
Vitamin mixture (1%)		24 h	1316	609
		48 h	924	529
Whole milk		24 h	739	790
		48 h	270	965
Skim milk		24 h	742	842
		48 h	178	1105

[a]ND = not detected; PR = *Puerariae radix*.

1). Skim milk and whole milk are natural food materials that can provide a plentiful and inexpensive supply of various nutrients. In this context, skim milk and whole milk were tested to improve the fermentation condition and hydrolysis of PR isoflavones. Skim milk showed slightly better activity than whole milk, especially in the late stage of fermentation (after 48 h). Compared with the addition of MRS broth (50%), the addition of skim milk and whole milk was less effective. PR extract alone was not sufficient to support the growth of the most of the tested microorganisms, and hence PR extracts are considered to be lacking in nutrients required for microbial growth. Because the vitamin mixture was not very helpful but the yeast extract and various protein digests were effective, we surmise that nitrogen is the primary nutrient lacking in PR extract. Park and others (1992) showed that the production of β-glucosidase in *Bifidobacterium* sp. Int-57 was considerably increased by cellobiose and decreased by glucose and lactose, though the degree of cell growth was similar. In the present study, however, the daidzin hydrolysis was not significantly influenced by 2% cellobiose. Even though the best yield for the hydrolysis of daidzin occurred when the MRS broth was added to PR extract, the addition of skim milk and whole milk had a similar effect. Considering that skim milk and whole milk are common ingredients in food production, it might be appropriate to add them to improve the conversion of PR isoflavones into aglycones during fermentation.

Conclusions

The chemical structures of isoflavones present in food or food supplements can be changed by various types of food processing to alter their bioavailability and thus their biological activity. The conversion of the glucoside isoflavone, dadizin, in PR into the aglycone form, daidzein, was conducted by fermentation with various strains of probiotic bacteria and edible fungi. Selected strains of probiotic bacteria such as *Bifidobacterium* sp. Int-57 can efficiently hydrolyze daidzin under optimized culture medium and fermentation conditions. This finding may advance the use of important phytochemical isoflavones in PR. Fermentation of PR by probiotic bacteria might be a suitable process for improving the level of bioactive structures of PR isoflavones.

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