Estrogenic Effect of Main Components Kakkalide and Tectoridin of Pueraria Flos and Their Metabolites

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To understand the relationship between the metabolism and estrogenic activity of kakkalide and tectoridin, main isoflavones in the flower of Pueraria thunbergiana (family Leguminosae), these isoflavones and their metabolites by human intestinal microflora as well as their estrogenic effects were investigated. All human fecal specimens metabolized kakkalide and tectoridin. All isolated kakkalide-hydrolyzing intestinal bacteria also hydrolyzed kakkalide and tectoridin to irisolidone and tectorigenin, respectively. When the estrogenic effects of kakkalide and tectoridin were compared with those of their metabolites irisolidone and tectorigenin, the metabolites more potently increased proliferation of MCF-7 cells than kakkalide and tectoridin. These metabolites also potently induced estrogen-response c-fos and pS2 mRNA expression. These results suggest that kakkalide and tectoridin may be metabolized mainly to irisolidone and tectorigenin, respectively, by intestinal microflora in the intestines, and which may be subsequently absorbed into the blood where they can express their estrogenic effect.

Key words Pueraria thunbergiana; kakkalide; tectoridin; intestinal microflora; metabolism; estrogenic effect

Most herbal medicines are orally administered as decoctions. The components of these herbal medicines are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Some components may be transformed by intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of such components by human intestinal microflora are of great importance to understanding their biological effects.1,2)

The flower of Pueraria thunbergiana (family Leguminosae), which contains isoflavones such as kakkalide and tectoridin as main components, is frequently used for counteracting symptoms associated with drinking alcohol, liver injury, and menopause in the traditional medicine clinic.3—6)

Lee et al. and Han et al. reported that kakkalide isolated from the flower of P. thunbergiana (Puerariae Flos, PF) is metabolized to irisolidone by human intestinal microflora, and that irisolidone shows hepato-protective activity.7,8) Park et al. reported that tectoridin isolated from PF is metabolized to tectorigenin, which showed more potent antiallergic activity than tectoridin.9) Many researchers have reported that isoflavones exhibit estrogenic effect.10—12) Nevertheless, the estrogenic effects of kakkalide and tectoridin have not been studied.

Therefore we isolated kakkalide and tectoridin from PF as well as their metabolites by human intestinal microflora and measured their estrogenic effects.

MATERIALS AND METHODS

Materials Sulforhodamine B (SRB), NP40, 17β-estradiol, p-nitrophenyl-β-D-xylopyranoside (PNX), Dulbecco’s modified Eagle medium (DME), fetal bovine serum (FBS), and charcoal dextran-stripped FBS (CD-FBS) were purchased from Sigma Chem. Co. (U.S.A.). General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Tokyo, Japan). Protein assay reagent was purchased from Bio-Rad Laboratories (U.S.A.). The enhanced chemiluminescence (ECL) kit was purchased from American sham Co. (U.S.A.). Progesterone receptor (PR) was purchased from Santa Cruz Co. (U.S.A.). Protease inhibitor cocktail was purchased from Roche Co. (Germany). All other chemicals were of analytical reagent grade, and all solutions used after redistillation.

Kakkalide, kakkalidone, and ginsenoside Ra1 were isolated according to our previously reported methods.7,8,13)

Preparation of Specimens Fresh fecal specimens from volunteers (male adults in their twenties) (3 g) were septicly collected in plastic cups, carefully mixed by a spatula, and suspended with 27 ml of cold saline. The fecal suspension was centrifuged at 100×g for 5 min. The supernatant was then centrifuged at 10000×g for 20 min. The resulting precipitates (about 0.3 g) were used as metabolic enzyme source for the assay of kakkalide- or tectoridin-hydrolyzing activity.

Isolation and Culture of Kakkalide-Hydrolyzing Bacteria Bacterial strains previously isolated from feces of healthy men and some lactic acid bacteria were cultured in GAM broth and assayed for kakkalide-transforming activity. Isolated bacteria were cultured in GAM broth then collected by centrifugation at 10000×g for 20 min.

Assay of Metabolizing Activity of Kakkalide and Tectoridin Reaction mixture containing 100 μl of 1 mM kakkalide (or tectoridin), 100 μl of fecal suspension (or isolated bacteria/purified enzyme), and 300 μl of 50 mM phosphate buffer (pH 7.0) was incubated for 1, 5, and 12 h at 37°C. The reaction mixture was extracted with ethyl acetate, evaporated, and finally assayed by TLC: TLC plates, silica gel 60F254 (Merck Co., U.S.A.); developing solvent, CHCl3—MeOH (5 : 1). TLCs were then analyzed by TLC scanner (Shimazu model CS-9301PC, Japan).

Isolation of Kakkalide, Tectoridin, and Their Metabolites by Human Intestinal Bacteria Human feces (3 g) was suspended in 27 ml of saline and centrifuged at 500×g for 10 min. The resulting supernatant was centrifuged at 10000×g for 30 min then washed twice with saline. The resulting precipitate was suspended in 100 ml of 20 mM phos-
phenyl-
were centrifuged at 10000 
for 15 min (100 watts, 60% pulsed mode). Disrupted cells 
buffer, pH 7.0, and the suspended cells ultrasonicated on ice 
buffer (Buffer A) then suspended in 100 ml of the same 
was used as crude enzyme solution. Purification of 
agarose gel electrophoresis.

**RESULTS**

**Metabolites of Kakkalide and Tectoridin by Human Intestinal Bacteria** To investigate the metabolites of kakkalide and tectoridin produced by human intestinal microflora, kakkalide and tectoridin were anaerobically incubated with human fecal suspension for 24 h and their metabolites investigated (Table 1). When kakkalide was incubated with human fecal suspensions, all specimens showed kakkalide-hydrolyzing activity, which varied among individuals and produced both irisoloside and kakkalidol as previously reported. The main metabolite was irisoloside. These specimens also potently hydrolyzed tectoridin to tectorigenin as previously reported. Four kakkalide-hydrolyzing intestinal bacteria (KHIB) were also isolated from human feces and assayed for metabolites of kakkalide. These KHIB hydrolyzed kakkalide and produced irisoloside, and also hydrolyzed tectoridin; the main metabolite was tectorigenin. The commercial probiotics *Streptococcus faecium* and *Lactobacillus acidophilus* potently hydrolyzed tectoridin; however, they only weakly metabolized kakkalide.

The most potent kakkalide-hydrolyzing *B. breve* K-110 was cultured, and β-D-xylosidase purified as previously reported and its substrate specificities were investigated (Table 2). Kakkalide was a good substrate, although PNX and intestinal bacteria (KHIB) were also isolated from human feces and assayed for metabolites of kakkalide. These KHIB hydrolyzed kakkalide and produced irisoloside, and also hydrolyzed tectoridin; the main metabolite was tectorigenin. The commercial probiotics *Streptococcus faecium* and *Lactobacillus acidophilus* potently hydrolyzed tectoridin; however, they only weakly metabolized kakkalide.

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<tr>
<th>Kakkalide</th>
<th>Tectoridin</th>
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<td>Hydrolyzing activity (μmol/h/mg)</td>
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**Kakkalide**

- *B. breve* K-110: 0.82
- *B. breve* K-111: 0.78
- *B. infantis* K-525: 0.08

**Tectoridin**

- *B. breve* K-110: 0.75
- *B. breve* K-111: 0.69
- *B. infantis* K-525: 1.45

**Tectorigenin**

- *L. acidophilus* KCTC3168: 0.01
- *S. faecium* KCTC3077: 0.06
- Human feces 1: 0.01
- Human feces 2: 0.02
- Human feces 3: 0.02

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a) Donated from KCTC (Korean Collection for Type Cultures). b) Not detectable.
substrate as previously reported. However, this enzyme did not transform tectoridin.

**Estrogenic Effect of Kakkalide, Tectoridin, and Their Metabolites** Estrogenic activity of kakkalide and its metabolite irisolidone was investigated (Fig. 1). Irisolidone exhibited more potent estrogenic activity than kakkalide. At a concentration of 10 μM, irisolidone increased proliferation of MCF-7 cells 1.9-fold, which was comparable to that of 17β-estradiol at a concentration of 10 nM. Estrogenic activity of tectoridin and its metabolite tectorigenin was also investigated (Fig. 2). Tectorigenin also potently increased proliferation of MCF-7 cells in comparison with tectoridin. Of these isoflavones, irisolidone showed the most potent estrogenic activity.

We compared the estrogenic effect of PF extract with that of its metabolized product by human intestinal microflora (Fig. 3). Metabolized PF extract more potently increased proliferation of MCF-7 cells than non-metabolized PF extract, although the fraction of human intestinal microflora alone did not show proliferation activity. When PF was incubated with intestinal bacteria isolated from human feces, that metabolized with *B. breve* K-110 showed the most potent increment of MCF-7 cell proliferation. However, samples metabolized with *L. acidophilus* or *S. faecium* showed weak MCF-7 cell proliferation effect compared with that of *B. breve* K-110.

To evaluate the potential of kakkalide, tectoridin, and their metabolites as activators of estrogen-responsive genes, c-fos and pS2 mRNA induction in MCF-7 cells was examined after treatment with these isoflavones (Fig. 4). Steady-state c-fos and pS2 mRNA levels were measured by RT-PCR assay on the total RNA prepared from MCF-7 cells treated with these isoflavones, with constitutively expressed GAPDH mRNA used as internal control. c-fos and pS2 mRNA expression was induced after 17β-estradiol treatment. The isoflavones also activated transcription of c-fos and pS2 genes, although the effects were not as prominent as those of 17β-estradiol. Among these isoflavones, irisolidone most potently activated transcription of these genes.

**DISCUSSION**

Recently, *Purariae Radix* and PF have been used as herbal medicines, against bone loss and climacteric symptoms in Korea, Japan, and China. In Western countries, these herbs are used as alternative therapies in postmenopausal
women. Many researchers have reported that Puerariae Radix and its isoflavones exhibit estrogenic activity. 10,11,17,18) These isoflavones also activate estrogen-responsive genes and regulate growth of human breast cancer cells. Most studies focused on estrogenic effects of daidzein, genistein, and their glycosides. PF contains kakkalide (>3%) and tectoridin (0.3%) as main components. However, their estrogenic effects have not been studied.

When estrogenic activity of kakkalide, tectoridin, and their metabolites irisolidone and tectorigenin were measured by E-Screen assay, irisolidone and tectorigenin showed more potent estrogenic effect than kakkalide and tectoridin. Thus estrogenic effects of isoflavone algycones were more potent than those of their glycosides. PF contains kakkalide (>3%) and tectoridin (>0.3%) as main components. However, their estrogenic effects have not been studied.

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Lee et al.\textsuperscript{7)} and Han et al.\textsuperscript{8)} reported that orally administered kakkalide isolated from PF is metabolized by intestinal microflora, and irisolidone might be absorbed into blood. Tectoridin is readily metabolized to tectorigenin by human intestinal bacteria.\textsuperscript{4)} The metabolite irisolidone exhibited blood alcohol-lowering and hepatoprotective effects.\textsuperscript{7,8)} Tectorigenin also showed more potent hepatoprotective effect than tectoridin. Therefore we also confirmed the metabolites of kakkalide and tectoridin by human intestinal microflora. When kakkalide and tectoridin were incubated with human intestinal microflora for 24 h, they were metabolized mainly to irisolidone and tectorigenin, respectively. These results were supported by the finding that β-D-xylosidase purified from kakkalide-hydrolyzing \textit{B. breve} K-110 hydrolyzed kakkalide to kakkalidone. The previously purified K-110 β-D-glucosidase hydrolyzed kakkalidone to irisolidone and tectoridin to tectorigenin.

Based on these findings, we suggest that if PF extract is orally administered kakkalide and tectoridin of PF may be metabolized mainly to irisolidone and tectorigenin, respectively, by intestinal microflora in the intestines, which can be subsequently absorbed into the blood where these metabolites may express their estrogenic effect.

**Acknowledgement** This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (2004—2006).

**REFERENCES**