Metabolism of Quercetin by Human Intestinal Bacteria and Its Relation to Some Biological Activities

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When quercetin was anaerobically incubated with human intestinal bacteria, quercetin, 3,4-dihydroxyphenylacetic acid and 4-hydroxybenzoic acid were found as metabolites. The main metabolite was quercetin. The bacterium transforming quercetin to quercetin was Fusobacterium K-60. However, Bacteroides JY-6, which produced α-1-rhamnosidase, did not transform quercetin to quercetin. Among quercetin and its metabolites, 3,4-dihydroxyphenylacetic acid and 4-hydroxybenzoic acid had more potent activity than quercetin on in vitro anti-platelet aggregation activity, and quercetin and 3,4-dihydroxyphenylacetic acid showed more potent cytotoxicity against tumor cell lines than quercetin and 4-hydroxybenzoic acid.

Key words: quercetin; Fusobacterium K-60; anti-platelet aggregation; cytotoxicity; intestinal bacteria

Most herbal medicines are orally administered as decoctions. The components of these medicines are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Most components may be transformed by the intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of the components by human intestinal microflora are of great importance in an understanding of their biological effects. In Korea, Houttuynia cordata (Saururaceae) has been used for hypertension, arteriosclerosis and cancer. Its main component is quercetin (quercetin-α-1-rhamnopyranoside). In addition, Bokkenheuser et al. isolated Bacteroides spp. producing α-rhamnosidase, β-glucosidase and β-galactosidase from human intestinal microflora, and reported that rutin and robinin were hydrolyzed by these bacteria. Recently, we also isolated Bacteroides JY-6, a human intestinal anaerobic bacterium, which produced α-rhamnosidase and β-glucosidase. JY-6 transformed flavone rhamnogluco-sides, such as rutin, hesperidin, poncirin and naringin, to their aglycones. However, neither these isolated bacteria nor their enzymes could hydrolyze quercetin.

We isolated the α-rhamnosidase-producing bacteria which transform quercetin to quercetin, and the metabolites of quercetin produced by human intestinal bacteria, and measured their biological activity.

MATERIALS AND METHODS

Materials: Quercetin, p-nitrophenyl α-1-rhamnopyranoside (PNR), p-nitrophenyl α-1-glucopyranoside, o-nitrophenyl α-1-glucopyranoside, p-nitrophenyl β-1-glucuronide, p-nitrophenyl β-1-galactopyranoside, p-nitrophenyl ketate, p-nitrophenyl phosphate, p-nitrophenyl sulfate and rutin were purchased from Sigma Chem. Co. (U.S.A.). The trisoyc broth was from Difco Co. (U.S.A.). General anaerobic medium (GAM) and glucose blood liver broth (BL) media were from Nissui Pharm. Co., Ltd. (Japan). All other chemicals were of analytical reagent grade.

Metabolites: To isolate the metabolite of quercetin by human intestinal bacteria, a reaction mixture containing 0.4 mM quercetin and 0.5 g fresh human fecal bacteria in a final volume of 50 ml of an anaerobic dilution medium was incubated at 37°C for 20 h. Fresh fecal bacteria was prepared as follows: fresh feces obtained from a healthy man (20) were immediately placed in a vinyl bag filled with oxygen-free carbon dioxide and then was uniformly mixed. The reaction mixture was extracted three times with 5 ml of ethylacetate. The ethylacetate extract was applied to silica gel column chromatography (1.5×20 cm) with CHCl3-MeOH (5:1). The main metabolite isolated from quercetin was crystallized with MeOH. Other metabolites, phenolic compounds, were identified by TLC according to our previous method.

Quercetin: Yellow amorphous powder. mp 310–312°C. IR νmax (cm⁻¹): 3380 (OH), 1670 (α, β-unsaturated C=O), 1610, 1510 (aromatic C=C), 1240 (aromatic C=O). FAB-MS m/z 302 (M⁺). The 1H-NMR and 13C-NMR data agreed well with that reported by Ternai and Markham.

TLC: TLC for quercetin, quercetin and phenolic metabolites were performed on silica gel plates (Merck, silica gel 60F-254) as follows: developing solvents systems were CHCl3-MeOH (4:1). The quantity of these compounds were assayed with a TLC scanner (Shimadzu CS-920).

Time Course of the Metabolism of Quercetin by Human Intestinal Bacteria: Quercetin metabolizing activity was measured as follows. Five ml of the 10-fold diluted fresh human fecal suspension was added to 45 ml of GAM broth containing 0.4 mM quercetin and then was incubated at 37°C for 1 d, and an aliquot (2 ml) of the reaction mixture was periodically extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

Assay of Transforming Activity of Rutin and Quercetin to Quercetin by Intestinal Bacteria: Quercetin transforming activity was measured as follows. The human feces was suspended with 50 mM phosphate buffer and centrifuged at 500 rpm. The supernatant was used as an enzyme source. Bacteroides JY-6 or Fusobacterium K-60 were cultured in 50 ml of GAM broth and centrifuged at 6000 rpm. Each precipitate was suspended with 50 mM phosphate buffer and then was used as an enzyme source. The reaction mixture containing 5 ml of the human fecal suspension or the culture bacteria (dried weight mg), 45 ml of 50 mM phosphate buffer and 0.4 mM quercetin was incubated at 37°C, and an aliquot (2 ml) of the reaction mixture was periodically extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

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analyzed by TLC.

Isolation of Intestinal Bacteria Metabolizing Quercitrin to Quercetin  
A suspension of the fresh feces of a Korean man was diluted $10^2$ to $10^5$-fold with GAM medium. An aliquot (200 μL) of each diluted human fecal suspension was inoculated into a BL agar plate, and then anaerobically incubated at 37°C for 4 d. Each colony was incubated in 5 mL of GAM broth containing 0.2 mM quercitrin. The cultured media were extracted with 5 mL of ethylacetate. After evaporation, each ethylacetate fraction was analyzed for quercitrin and quercetin by TLC. Among the tested bacteria, K-60 has the activity to metabolize quercitrin to quercetin. Identification of these isolated bacteria was performed according to Bergey’s manual.  

In Vitro Cytotoxicity Assay  
The in vitro cytotoxicity was tested against SNU C4 (human colon cancer cell line), P388 (mouse lymphoid neoplasma cell line), L1210 (mouse lymphocytic leukemia cell line) and MA104 (Macaca rhesus monkey kidney cell line) by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method of Carmichael et al. Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μL volume/10^6 cells/ well for P388 and L1210; 3 × 10^4 cells/ well for SNU C4 and MA104) into 96-well microtiter plates. P388 and L1210 cell lines were then cultured for 2 h and SNU C4 and MA104 cell lines for 24 h. These cells were exposed to the test compounds for 2 d at 37°C. Fifty μL of MTT solution (2 mg/mL in PBS) was added to each well and the plates were incubated for 4 h. After aspiration of the medium, DMSO (100 μL) was added to solubilize the MTT-formazan product. The plates were read on a microplate reader (540 nm). The 50% inhibitory concentration (IC_{50}) of tumor cell growth was defined compared with the control cell culture.

Anti-platelet Aggregation Activity  
The anti-platelet aggregation activity was determined according to the smearing done by Yun-Choi et al. Platelet rich plasma (PRP) was prepared by centrifugation of the citrated blood at 200 × g for 10 min and platelet poor plasma (PPP) was obtained from the residue by centrifugation at 900 × g for 30 min. Platelet number was adjusted to 3 × 10^11/f by mixing PRP and PPP. PRP (20 μL) and 10 μL of 4 mM quercitrin (or its metabolites) were incubated at 37°C for 8 min and then 10 μL of 1 mM ADP (1 mg/mL) was added to induce platelet aggregation. The reaction mixture was smeared onto a slide glass, then stained with Wright-Giemsa and measured under a microscope. The degree of aggregation was graded as described: −, no aggregation as shown with PRP plus saline alone (negative control); ±, slight aggregation of platelets; +, less aggregation than with PRP plus saline and an appropriate aggregating agent (ADP); ++, as much aggregation as with PRP plus saline and an appropriate aggregating agent (positive control).

RESULTS AND DISCUSSION

Metabolites of Quercitrin by Human Intestinal Microflora  
To investigate the metabolites of quercitrin by human intestinal bacteria, quercitrin was anaerobically incubated for 24 h with a bacterial mixture from human feces. Then, the metabolites were extracted with ethylacetate, separated by silica gel column chromatography, and analyzed by TLC, 13C- and 1H-NMR and EI-MS. The main metabolite was observed by TLC, and it exhibited absorption due to hydroxyl (3380 cm⁻¹) and aromatic function (1510 cm⁻¹) in the IR spectra. Its R_f was 0.65 on TLC (developing solvent system in Materials and Methods). FAB-MS showed a molecular ion peak at m/z 302 (M²⁺). TLC chromatogram and 1H- and 13C-NMR spectra of the main metabolite showed that the flavanol skeleton of quercitrin was intact but a rhamnosyl moiety was missing. Compared with an authentic compound, it was quercetin; other phenolic metabolites, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid, were identified by TLC. We screened the bacteria hydrolyzing quercitrin and dissolving the C-ring of quercitrin from human feces. Fusobacterium K-60 was isolated as a quercitrin-glycosidating bacterium, and Pediococcus Q-5, Streptococcus S-3, Bacteroides KY-6 and Bifidobacterium B-9 were isolated as a B ring fission bacteria of quercetin (data not shown).

Time course of the metabolism of rutin and quercetin by human intestinal microflora was investigated (Fig. 1). Quercetin began to be transformed to quercitin and this increased with incubation time. The major metabolite was quercetin 12 h after incubation with the intestinal microflora. However, quercetin was slowly decreased after 18 h incubation with the intestinal microflora, and other phenolic metabolites, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid, were produced.

To compare the metabolism of quercitrin with that of rutin
by intestinal bacteria, quercitin and rutin were anaerobically incubated for 12 h with fresh human feces, *Bacteroides* Y-6 or *Fusobacterium* K-60 (Fig. 2). These fecal intestinal bacteria all transformed rutin to quercetin. *Bacteroides* Y-6, however, could not transform quercetin to quercitin. Among the isolated bacteria, only *Fusobacterium* K-60 was able to make this transformation.

Relating to the metabolism of these compounds, Bokkenheuser *et al.* isolated *Bacteroides* spp. from human intestinal microflora, and reported that rutin and robinin were hydrolyzed to their aglycones by these bacteria. 5,6 We also isolated *Bacteroides* Y-6, an obligate anaerobic bacterium producing α-rhamnosidase, from human intestinal bacteria. 5,6 The best substrates of these bacterial α-rhamnosidase were poncirins, naringin, neohesperidin, hesperidin and rutin. However, *Bacteroides* spp. previously isolated by us and by Bokkenheuser *et al.* could not transformed quercitin, flavonoid rhamnopyranoside, to quercetin. Therefore, *Fusobacterium* K-60 which transformed quercitin to quercetin was isolated here for the first time.

**Biological Activities of Quercitin and Its Metabolites**

**Table 1. Anti-Platelet Aggregation Activity of Quercitin, Rutin and Their Metabolites**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Platelet aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercitin</td>
<td>+</td>
</tr>
<tr>
<td>Rutin</td>
<td>+</td>
</tr>
<tr>
<td>Isoquercetin</td>
<td>+</td>
</tr>
<tr>
<td>Quercetin</td>
<td>+</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>−</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylacetic acid</td>
<td>−</td>
</tr>
<tr>
<td>Negative control</td>
<td>−</td>
</tr>
<tr>
<td>Positive control</td>
<td>+ +</td>
</tr>
</tbody>
</table>

a) The degree of aggregation was graded as follows: −, no aggregation as shown with PRP plus saline alone (negative control); +, slight aggregation of platelets; +, less aggregation than with PRP plus saline and an appropriate aggregating agent (ADP); + +, as much aggregation as with PRP plus saline and an appropriate aggregating agent (positive control).

The anti-platelet aggregation activity and cytotoxic activity against tumor cell lines of the metabolites of quercitin by human intestinal microflora were investigated. Their anti-platelet aggregation activity is shown in Table 1. 3,4-Dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid had the most potent anti-platelet aggregation activity, and that of the main metabolite, quercetin, was lower than those of the phenolic compounds. We also investigated in vitro cytotoxic activity of quercitin and its metabolites on tumor cell lines (Table 2). The metabolites, quercetin and 3,4-dihydroxyphenylacetic acid, showed potent cytotoxicity against tumor cell lines, with ED₅₀ values of 0.12—0.29 and 0.1—0.5 mm, respectively. However, quercitin and the other metabolites were weak. We found that the cytotoxicity was increased when quercitin was metabolized to quercetin and phenolic compounds by human intestinal microflora.

These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal microflora.

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**REFERENCES**