Development of Fecal Microbial Enzyme Mix for Mutagenicity Assay of Natural Products

Yeo, Hee Kyung¹, Yang-Jin Hyun², Se-Eun Jang¹, Myung Joo Han¹*, Yong Sup Lee², and Dong-Hyun Kim²*

¹Department of Food and Nutrition, and ²Department of Life and Nanopharmaceutical Sciences and Department of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

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Orally administered herbal glycosides are metabolized to their hydrophobic compounds by intestinal microflora in the intestine of animals and human, not liver enzymes, and absorbed from the intestine to the blood. Of these metabolites, some, such as quercetin and kaempherol, are mutagenic. The fecal bacterial enzyme fraction (fecalase) of human or animals has been used for measuring the mutagenicity of dietary glycosides. However, the fecalase activity between individuals is significantly different and its preparation is laborious and odious. Therefore, we developed a fecal microbial enzyme mix (FM) usable in the Ames test to remediate the fluctuated reaction system activating natural glycosides to mutagens. We selected, cultured, and mixed 4 bacteria highly producing glycosidase activities based on a cell-free extract of feces (fecalase) from 100 healthy Korean volunteers. When the mutagenicities of rutin and methanol extract of the flos of Sophora japonica L. (SFME), of which the major constituent is rutin, towards Salmonella typhimurium strains TA 98, 100, 102, 1,535, and 1,537 were tested using FM and/or S9 mix, these agents were potently mutagenic. These mutagenicities using FM were not significantly different compared with those using Korean fecalase. SFME and rutin were potently mutagenic in the test when these were treated with fecalase or FM in the presence of S9 mix, followed by those treated with S9 mix alone and those with fecalase or FM. Freeze-dried FM was more stable in storage than fecalase. Based on these findings, FM could be usable instead of human fecalase in the Ames test.

Keywords: Metabolism, mutagenicity, Ames test, fecalase, fecal microbial enzyme mix

Most herbs are orally administered to humans. Therefore, their hydrophilic components are inevitably contact with intestinal microflora in the gastrointestinal tract and metabolized by intestinal microflora, before absorption from the gastrointestinal tract to the blood [13, 14]. For example, when ginsenoside Rb1, which is a major component of ginseng, is administered to humans or rats, it is transformed to 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (compound K) by intestinal microflora and absorbed to the blood [1, 2, 18]. Ginsenoside Rb1 was not detected in blood. The transformed and absorbed compound K exhibits the potent antitumor and antiallergic actions more than ginsenoside Rb1 [5, 9, 27]. Orally administered rutin is also transformed to quercetin by intestinal microflora and then the metabolites will be absorbed from the intestine to the blood [6, 13]. The transformed and absorbed quercetin is regarded as mutagenic in the Ames test, although it possesses a variety of pharmacological activities. Therefore, intestinal bacteria may play the important role in expressing the pharmacological and toxicological activities of herbal medicines.

Damage to DNA by mutagens, including chemical and drugs, is likely to be a major cause of cancer and other diseases. To screen these mutagens, the Salmonella test was developed [3, 4, 7]. Naturally occurring glycosides of mutagenic aglycones are not mutagenic in the Salmonella test. However, when these glycosides are hydrolyzed by intestinal bacteria, their mutagens are liberated from some glycosides. Therefore, Tamura et al. [25] developed a fecalase – a model for activation of dietary glycosides to mutagens by intestinal microflora – for the Ames test.

However, Kobashi et al. [14] reported that some drug-metabolic enzyme activities of intestinal bacteria were significantly different between Jitsu-syo and Kyo-syo Japanese, although the compositions of intestinal bacteria between Jitsu-syo and Kyo-syo Japanesees were not different [15]. We also reported that some fecal microbial enzymatic activities related to the pharmacological actions of herbal medicinal components, including ginsenosides and baicalin,
were variable among individuals [9, 17, 28]. The intra- and inter-individual variations of these intestinal bacteria are not significant, although their enzyme activities are affected by dietary change and physiological factors [20, 22, 24]. Nevertheless, Ikeda et al. [11] reported that these are rebound if diet or supplements were stopped for the short term. To understand the pharmacological and toxicological effects of herbal medicines, intestinal bacterial enzymatic (fecalase) activities are of a great importance. Furthermore, fecalase has been used for evaluating the pharmacological and toxicological effects of herbal medicines and their constituents [7, 19]. However, the fecalase preparation is laborious and odious and it is very unstable in storage.

Therefore, we isolated intestinal bacteria from human stools and developed a fecal microbial enzyme mix (FM) usable instead of human fecalase.

**Materials and Methods**

**Materials**

Glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate, rutin, L-histidine, 4-nitrophenyl-β-D-glucuronide, 4-nitrophenyl-α-L-arabinopyranoside, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-xylopyranoside, 4-nitrophenyl-α-L-arabinopyranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-β-D-fucopyranoside, 4-nitrophenyl-β-D-maltoside, 4-nitrophenyl-α-L-rhamnopyranoside, 4-nitrophenyl-β-D-rhamnopyranoside, 4-nitrophenyl-α-D-acetylgalactosamine, 4-nitrophenyl-N-acetyl-β-D-galactosamine, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, 4-nitrophenyl-N-acetyl-β-D-mannosaminide, 4-nitrophenyl-α-D-arabinofuranoside, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-glucuronoside, 4-nitrophenyl-α-D-glucosamine, 4-nitrophenyl-β-D-glucosaminide, 4-nitrophenyl-α-D-arabinofuranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-sulfate, 4-nitrophenyl-α-L-rhamnopyranoside, and biotin were purchased from Sigma (St. Louis, MO, USA). S9 mix was from Molecular Toxicology Inc. (Boone, NC, USA). Tryptic soy broth and Bacto agar were from Becton Dickinson (Franklin Lakes, NJ, USA). BL (blood-liver) and GAM (Gifu anaerobic medium) broth were from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan).

The flos of **Sophora japonica** L. (family Leguminosae), which was identified by Adjunct Professor Nam-Jae Kim (Kyung Hee University), was extracted from 80% methanol for 2 h and freeze-dried (yield, 23%).

**Subjects**

The subjects were 100 healthy Korean persons (average, 40.74 ± 13.87 years; range, 20–72 years; 54 males, 46 females). Exclusion criteria included smoking and current medication, especially regular or current use of antibiotics. The recruit of subjects and collection of their stools were approved by the Committee for the Care and Use of Clinical Study in the Medical School, Kyung Hee University.

**Fecalase Preparation**

The human fecal specimens (about 1 g) prepared according to a previous method of Lee et al. [17], were collected in plastic cups 9 h after fasting, and then carefully mixed with a spatula and suspended with 9 ml of cold saline. Fecal microbial suspension was centrifuged at 500 × g for 5 min. The resulting supernatant was sonicated for 10 min and then centrifuged at 10,000 × g for 20 min. The resulting supernatant was used for the assay of enzyme activity. The fecal suspension was centrifuged at 500 × g for 5 min. The supernatant was then centrifuged at 10,000 × g for 20 min. The resulting precipitate was suspended in PBS 10-fold, disrupted by an Ultrasonicator (8 min), and centrifuged at 10,000 × g for 20 min. The resulting supernatants were used as a fecalase. The preparation and assay of the enzyme source were performed within 24 h. The specific activities of β-glucuronidase, β-xylosidase, α-rhamnosidase, and β-glucosidase in the fecalase were 0.156 ± 0.018 (mean ± SD), 0.138 ± 0.019, 0.101 ± 0.056, and 0.219 ± 0.042 µmol/min/mg, respectively, by using 4-nitrophenyl-β-D-glucuronide, 4-nitrophenyl-β-D-xylopyranoside, 4-nitrophenyl-α-L-rhamnopyranoside, and 4-nitrophenyl-β-D-glucopyranoside as substrates.

**Isolation of Intestinal Bacteria from Human Feces and Their Identification**

To isolate and identify intestinal bacteria from the stools of healthy humans, 1–10 g of fresh fecal samples were collected and suspended in diluted anaerobic broth, and inoculated into GAM, BL, and TS agar plates. The plates were cultured for 24 h for TS agar plates and for 72 h for the other plates. The grown colonies in these plates were cultured in GAM broth, and the bacteria collected and used for the assay of enzyme activities. The selected bacteria were identified by Gram staining and 16S rDNA sequencing analysis. Thus, chromosomal DNA extracted from selected bacteria, using prokaryotic 16S RNA universal primer sequences, was analyzed with a PCR. Strains were identified by NCBI BLAST search.

**Culture of Isolated Intestinal Bacteria and Preparation of Intestinal Microbial Enzyme Fraction**

To prepare the fecal microbial enzyme mix, the selected bacteria, *Megaphora elsdensii*, *Parabacteroides distasonis*, *Klebsiella pneumoniae*, and *Eubacterium rectale*, were inoculated into a GAM broth. *Megaphora elsdensii* and *Parabacteroides distasonis* were cultured for 12 h at 37°C and *Klebsiella pneumoniae* and *Eubacterium rectale* were for 24 h at 37°C. The cultured bacteria were collected by the centrifugation (8,000 × g, 30 min), suspended in PBS 10-fold, disrupted by an Ultrasonicator (8 min), and centrifuged at 10,000 × g for 20 min. The resulting supernatants were mixed and used as an intestinal microbial enzyme mix. The specific activities of β-glucuronidase, β-xylosidase, α-rhamnosidase, and β-glucosidase in the fecal microbial enzyme mix were 0.220 ± 0.071, 0.193 ± 0.037, 0.181 ± 0.041, and 0.281 ± 0.041 µmol/min/mg, respectively, by using 4-nitrophenyl-β-D-glucuronide, 4-nitrophenyl-β-D-xylopyranoside, 4-nitrophenyl-α-L-rhamnopyranoside, and 4-nitrophenyl-β-D-glucopyranoside as substrates.

**Enzyme Activity Assay**

The enzyme activities were assayed according to the previous reported method of Choi et al. [8]. Briefly, the reaction mixture (total volume of 0.5 ml) containing 0.2 ml of 1 mM p-nitrophenyl-β-D-glucopyranoside for β-glucosidase (or 1 mM p-nitrophenyl-β-D-glucuronide for β-glucuronidase, 1 mM p-nitrophenyl-α-L-rhamnopyranoside for α-rhamnosidase, 1 mM p-nitrophenyl-α-L-arabinopyranoside for α-arabinosidase, 1 mM p-nitrophenyl-β-D-xylopyranoside for β-xylosidase, 1 mM 4-nitrophenyl-α-L-arabinopyranoside for α-arabinofuranosidase, 1 mM 4-nitrophenyl-α-L-arabinofuranoside for α-arabinofuranosidase, 1 mM 4-nitrophenyl-
β-α-galactopyranoside for β-galactosidase, 1 mM 4-nitrophenyl-β-α-arabinopyranoside for β-arabinopyranosidase, 1 mM 4-nitrophenyl-β-α-celllobioside for cellulase, 1 mM 4-nitrophenyl-α-β-fructofuranoside for α-fucosidase; 1 mM 4-nitrophenyl-β-α-fucopyranoside for β-fucosidase, 1 mM 4-nitrophenyl-β-α-maltoside for β-maltosidase; 1 mM 4-nitrophenyl-N-acetyl-α-β-galactosamine for α-galactosaminidase, 1 mM 4-nitrophenyl-N-acetyl-β-α-galactosamine for β-galactosaminidase, 1 mM 4-nitrophenyl-N-acetyl-α-β-glucosaminide for α-glucosaminidase, 1 mM 4-nitrophenyl-α-β-glucopyranoside for α-glucosidase, 1 mM 4-nitrophenyl-α-β-glucopyranoside for α-glucosidase, 1 mM 4-nitrophenyl-β-α-mannopyranoside for β-mannosidase, or 1 mM 4-nitrophenyl-sulfate for sulfatase), 0.2 ml of 0.1 M phosphate buffer, pH 7.4, and 0.1 ml of the fecal suspension (wet weight, 4 mg) were incubated at 37°C for 15 min. Then the reaction mixture was stopped by the addition of 0.5 ml of 0.5 N NaOH, centrifuged at 2,000 × g for 10 min, and the absorbance measured at 405 nm (using a BioTek spectrophotometer; London, England).

Storage Stability Assay
The fecal microbial enzyme mix was suspended in 10% glycerol (9:1 mix) or freeze-dried, and stored at 4°C, −20°C, or −80°C for 120 days.

Ames Test
The Ames test was performed according to the method of Tamura et al. [25]. Salmonella Typhimurium strains TA 98, 100, 102, 1,535, and 1,537 were cultured in nutrient broth at 37°C for 24 h. Each cultured Salmonella suspension (100 μl) and test agents with or without 59 mix, fecalase, and/or fecal microbial enzyme mix [0.5 ml, sterilized by filtration (Millipore 0.22 μm)] were mixed in top agar. Then, the top agar was poured onto a Vogel–Bonner agar plate with top agar before the hardness to tilt it to spread evenly over the medium. The top agar solidified plates were inoculated upside down, and then incubated for 48 h at 37°C and counted for grown colonies.

Statistics
All the data were expressed as the mean ± standard deviation, and statistical significance was analyzed by one-way ANOVA followed by Student t-test.

RESULTS

Properties of Intestinal Microbial Enzymes
To prepare FM usable for evaluating the toxicological effect of herbal medicines and their constituents instead of Korean fecalase, we isolated 200 intestinal bacteria from human fresh feces, and measured their activities of 14 representative sugar-hydrolyzing enzymes, and identified bacterial species potent with sugar hydrolyzing enzymes by the 16S rDNA sequencing method (Fig. 1). Of the identified bacteria, we selected four intestinal bacteria producing each enzyme most potently. Megaspheora elsdonii most potently produced α-β-galactosidase, α-β-rhamnosidase, and β-α-glucosidase. Parabacteroides distasonis most potently produced β-galactosaminidase and α-glucosaminidase. Klebsiella pneumoniae most potently produced α-arabinopyranosidase, β-xylosidase, β-arabinofuranosidase, and α-arabinofuranosidase. Eubacterium rectale most potently produced β-glucuronidase, β-galactosidase, α-galactosidase, cellulase, β-fucosidase, β-maltosidase, and β-mannosidase.

To optimize the optimal culture condition, we anaerobically cultured these bacteria in GAM broth and measured the activities of four representative enzymes, β-glucosidase, β-xylosidase, β-glucuronidase, and α-rhamnosidase periodically (Fig. 2A). Eubacterium rectale strains produced most potently β-glucosidase by cultivating for 24 h. Megaspheora elsdonii and Parabacteroides distasonis strains produced most potently β-glucosidase and α-glucosaminidase by cultivating for 12 h, respectively. Klebsiella pneumoniae and Eubacterium rectale strains produced most potently β-xylosidase and β-glucuronidase by cultivating for 24 h, respectively. Therefore, we prepared FM by mixing these four bacterial suspensions and compared its representative sugar-hydrolyzing enzyme activity for 4 substrates with that of the fecalase (Fig. 2B). These enzyme activities between them were not significantly different.

Next, we measured the temperature stability of FM (Fig. 3). When FM was stored in 4°C (with or without freeze-drying), −20°C, or −80°C (in the presence or absence 10% glycerol), its β-xylosidase and β-glucosidase activities were stable, but its β-glucuronidase and α-glucosaminidase activities were unstable, except for the storage of freeze-dried FM at 4°C. Therefore, we used freeze-dried FM stored in 4°C in the present study.

Effect of FM on the Mutagenicity of SFME and Rutin
To evaluate whether FM can be used in the Ames test instead of human fecalase, we investigated the mutagenicity of SFME and its constituent rutin by using Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and...
TA1537. SFME and rutin showed little impact to revert the mutants of *S. typhimurium* strains. However, when SFME and rutin were treated with FM or fecalase in the absence or presence of S9 mix, these increased the number of their revertants.

When rutin (20 µg/ml) was treated with or without fecalase to *S. typhimurium* TA98, the numbers of the mutants reverted by rutin were 321 ± 19.1 and 46 ± 9.5 CFUs, respectively (Fig. 4). When rutin (20 µg/ml) was treated with FM instead of fecalase, the number of reverted mutants was 162 ± 2.5. Treatment with fecalase or FM increased the number of the reverted mutants. When rutin (20 µg/ml) was treated with fecalase in the absence or presence of S9 mix to *S. typhimurium* TA98, the numbers of reverted mutants were 321 ± 19.1 and 602 ± 15.5 CFUs, respectively. When rutin (20 µg/ml) was treated with FM in the absence or presence of S9 mix, the numbers of reverted mutants were 162 ± 2.5 and 400 ± 39.9 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of fecalase to *S. typhimurium* TA98, the numbers of reverted mutants were 274 ± 18.6 and 602 ± 15.5 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of FM, the numbers of reverted mutants were 274 ± 18.6 and 400 ± 39.9 CFUs, respectively. The number of the mutants reverted by rutin was dose-dependently increased. When SFME (20 µg/ml)

Fig. 2. Periodical enzyme productions of four selected intestinal bacteria for cultivation (A) and comparison of enzyme activities of intestinal bacterial mix with human fecalase (B). The enzyme activities were assayed after incubation for 15 min at 37°C, according to the method indicated in Materials and Methods.

Fig. 3. Stability of fecal microbial enzyme mix in storage of 4°C (open square), -20°C (closed square), -80°C (closed triangle), -80°C in 10% glycerol (open circle), or 4°C/freeze-drying (closed circle). The enzyme activities were assayed after incubation for 15 min at 37°C, according to the method indicated in Materials and Methods.
was treated with or without fecalase in the absence or presence of S9 mix to *S. typhimurium* TA98, the numbers of the mutants reverted by SFME were similar to those by rutin and dose-dependently increased. The mutagenicities of rutin and SFME against *S. typhimurium* TA98 were more potently dose-dependently increased by treatment with fecalase or FM mix in the presence of S9 mix than by treatment with S9 mix alone.

When rutin (20 µg/ml) was treated with or without fecalase to *S. typhimurium* TA100, the numbers of reverted mutants were 156 ± 1.4 and 137 ± 6 CFUs, respectively (Fig. 5). When rutin (20 µg/ml) was treated with FM instead of fecalase, the number of reverted mutants was 183 ± 8.5 CFUs. When rutin (20 µg/ml) was treated with fecalase in the absence or presence of S9 mix to *S. typhimurium* TA100, the numbers of reverted mutants were 156 ± 1.4 and 368 ± 7.1 CFUs, respectively. When rutin (20 µg/ml) was treated with FM in the absence or presence of S9 mix, the numbers of reverted mutants were 183 ± 8.5 and 186 ± 19.5 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of fecalase to *S. typhimurium* TA100, the numbers of reverted mutants were 175 ± 5.0 and 368 ± 7.1 CFUs, respectively, but when rutin (20 µg/ml) was treated with S9 mix in the absence or presence of FM, the numbers of reverted mutants were 175 ± 5.0 and 186 ± 19.5 CFUs, respectively. The number of the mutants reverted by rutin was dose-dependently increased. When SFME (20 µg/ml) was treated with or without fecalase in the

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**Fig. 4.** Effect of Sophorae flos extract and its main constituent rutin on *S. typhimurium* TA98 reverse mutation assay. Each cultured *Salmonella* suspension (100 µl) and test agents with or without S9 mix, fecalase (F), and/or fecal microbial enzyme mix (FM) (0.5 µl) were mixed in top agar; then, the top agar was poured onto Vogel–Bonner agar plate with top agar and incubated for 48 h at 37°C and counted for grown colonies. NC, negative control; M, test agent alone; M+FM, agent + fecal microbial enzyme mix; M+F, agent + fecalase; M+S9, agent + S9 mix; M+FM+S9, agent + fecal microbial enzyme mix + S9 mix; M+F+S9, agent + fecalase + S9 mix. The mutant frequency is expressed as the number of revertant colonies. The mutant potential of the test is assumed if the mutant frequency is 2-fold higher than negative control (expressed by dot lines). *p*<0.05, M vs. M+F or M vs M+FM; *p*<0.05, M vs. M+S9; *p*<0.05, M+S9 vs. M+S9+F or M+S9+FM.
absence or presence of S9 mix to S. Typhimurium TA100, the numbers of reverted mutants were similar to those by rutin and dose-dependently increased. The mutagenicities of rutin and SFME against S. typhimurium TA100 were more potently dose-dependently increased by treatment with fecalase or FM mix in the presence of S9 mix than by treatment with S9 mix alone.

When rutin (20 µg/ml) was treated with or without fecalase to S. typhimurium TA102, the numbers of reverted mutants were 610 ± 0.7 and 547 ± 24 CFUs, respectively (Fig. 6). When rutin (20 µg/ml) was treated with FM instead of fecalase, the number of reverted mutants was 660 ± 17 CFUs. When rutin (20 µg/ml) was treated with fecalase in the absence or presence of S9 mix to S. typhimurium TA102, the numbers of reverted mutants were 610 ± 0.7 and 1,002 ± 180.6 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of fecalase to S. typhimurium TA102, the numbers of reverted mutants were 908 ± 48.6 and 1,002 ± 180 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of FM, the numbers of reverted mutants were 908 ± 48.6 and 1,188 ± 29.7 CFUs, respectively. The number of the mutants reverted by rutin was dose-dependently
increased. When SFME (20 µg/ml) was treated with or without fecalase in the absence or presence of S9 mix to S. typhimurium TA102, the numbers of the mutants reverted by SFME were similar to those by rutin and dose-dependently increased. The mutagenicities of rutin and SFME against S. typhimurium TA102 were more potently dose-dependently increased by treatment with fecalase or FM mix in the presence of S9 mix than by treatment with S9 mix alone.

When rutin (20 µg/ml) was treated with or without fecalase to S. typhimurium TA1535, the numbers of reverted mutants were 23 ± 6.2 and 14 ± 2.1 CFUs, respectively (Fig. 7). When rutin (20 µg/ml) was treated with FM instead of fecalase, the number of reverted mutants was 22 ± 7.0 CFUs. When rutin (20 µg/ml) was treated with fecalase in the absence or presence of S9 mix to S. typhimurium TA1535, the numbers of reverted mutants were 22 ± 8.0 and 28 ± 3.5 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of fecalase to S. typhimurium TA1535, the numbers of reverted mutants were 22 ± 8.0 and 28 ± 3.5 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of FM, the numbers of revertants were 22 ± 8.0 and 40 ± 11.6 CFUs, respectively. The number of revertants by rutin was dose-dependently increased. When SFME (20 µg/ml) was treated with or without fecalase in the absence or presence of S9 mix to S. typhimurium TA1535, the numbers of reverted mutants were 23 ± 6.2 and 28 ± 3.5 CFUs, respectively. When rutin (20 µg/ml) was treated with FM in the absence or presence of S9 mix, the numbers of reverted mutants were 22 ± 7 and 40 ± 11.6 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of fecalase to S. typhimurium TA1535, the numbers of reverted mutants were 22 ± 8.0 and 28 ± 3.5 CFUs, respectively. When rutin (20 µg/ml) was treated with S9 mix in the absence or presence of FM, the numbers of revertants were 22 ± 8.0 and 40 ± 11.6 CFUs, respectively. The number of revertants by rutin was dose-dependently increased. When SFME (20 µg/ml) was treated with or without fecalase in the absence or presence of S9 mix to S. typhimurium TA1535, the number of revertants was 22 ± 8.0 and 40 ± 11.6 CFUs, respectively.
revertant mutants were similar to those of rutin and dose-dependently increased. The mutagenicities of rutin and SFME against \textit{S. typhimurium} TA1535 were more potently dose-dependently increased by treatment with fecalase or FM mix in the presence of S9 mix than by treatment with S9 mix alone.

When rutin (20 µg/ml) was treated with or without fecalase to \textit{S. Typhimurium} TA1537, the numbers of reverted mutants were 30 ± 5.7 and 12 ± 2.3 CFUs, respectively. When rutin (20 µg/ml) was treated with FM instead of fecalase, the number of reverted mutants was 24 ± 4.9 CFUs. When rutin (20 µg/ml) was treated with fecalase in the absence or presence of S9 mix to \textit{S. typhimurium} TA1537, the numbers of reverted mutants were 30 ± 5.7 and 38 ± 3.0 CFUs, respectively (Fig. 8). When rutin (20 µg/ml) was treated with FM in the absence or presence of S9 mix, the numbers of reverted mutants were 24 ± 4.9 and 47 ± 4.9 CFUs, respectively. When SFME (20 µg/ml) was treated with or without fecalase in the absence or presence of S9 mix to \textit{S. typhimurium} TA1537, the numbers of the mutants reverted by SFME were similar to those by rutin and dose-dependently increased. The mutagenicities of rutin and
Discussion

Many herbal medicines orally administered to humans contain hydrophilic constituents, such as glycosides, which are metabolized to their aglycones by intestinal microflora in the intestinal tract, and then their metabolites are absorbed from the intestine to the blood [13, 14]. For example, of glycosides isolated from natural products, glycyrrhizin improves hepatic injury in normal rats, but not in germ-free animals. Cycasin and amygdalin have been shown to be toxic in normal rats, but not in germ-free rats [16, 21]. Baicalin is transformed to baicalein and/or oroxylin A by intestinal microflora, which exhibits anti-pruritic effect in normal mice, but attenuates its effect in antibiotics-treated mice [26]. Among these metabolites of constituents from natural products, some have been shown to be mutagenic in Salmonella tests or to mutate mammalian cells. Therefore, intestinal microflora play an important role in transforming naturally occurring glycosides to pharmacological and toxic forms. These studies suggest that the rat liver homogenate used in the Salmonella test should be complemented by the addition of an intestinal microbial metabolic system, fecalase, to the test to detect potential carcinogens in herbal medicines. Nagao et al. [23] reported that tea treated with glycosidase exhibits the mutagenic activity on Salmonella strain TA98. In the Ames test against S. typhimurium TA1537 were more potently dose-dependently increased by treatment with fecalase or FM mix in the presence of S9 mix than by treatment with S9 mix alone.

SFME Fecal Microbial Enzyme Mix for Ames Test
test for various glycosides, microbial cell-free extracts from rat feces or heperidinase from the mold Aspergillus niger have been developed [23]. Nevertheless, these enzyme fractions could not compensate human fecalase consisted of various intestinal bacteria. To overcome it, Tamura et al. [25] developed human fecalase, an enzyme extract from human feces, which activates the mutagenicity of many naturally occurring glycosides, such as rutin and hydroquinone glycosides. This fecalase is a simple and physiologically relevant model usable in the Salmonella test that considers the metabolism by human intestinal microflora. Nevertheless, its preparation is tedious and its enzyme activity was liable. Furthermore, its metabolic activity was significantly different to that of Korean feces. Therefore, we developed usable FM instead of Korean fecalase.

When the mutagenicities of rutin and SFME towards S. typhimurium stains (TA98, 100, 102, 1535, and 1537) were tested by fecalase or FM in the absence or presence of S9, their mutagenicities were dose-dependently increased by treatment with fecalase, FM, or S9 mix. Furthermore, their mutagenicities were more potently increased by treatment with fecalase or FM in the presence of S9 mix than by treatment with S9 mix. Their mutagenicity caused by treatment with FM was similar to those by fecalase. These results were supported by the report of Tamura et al. [25] that fecalase increases the mutagenicity of the constituents of many natural products. These mutagenicities are originated to quercetin, which may exhibit carcinogenicity [7, 25], metabolized by fecalase or FM. There was no significant difference between these two enzyme systems. Furthermore, FM was more stable in storage than fecalase. In particular, the metabolic enzyme activities of freeze-dried FM stored in −20°C were stable for 3 months. Based on these findings, FM should be usable instead of human fecalase in the Ames test.

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