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Lactic Acid Bacteria Increases Hypolipidemic Effect of Crocin Isolated from Fructus of *Gardenia jasminoides*

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Abstract The fructus of *Gardenia jasminoides Ellis* (GF) has been widely used as a natural colorant in Asian countries, and also as a Chinese traditional medicine for its homeostatic, antiphlogistic, analgesic, and antipyretic effects. In the present study, its main component, crocin, was fermented with lactic acid bacteria (LAB) and their antihyperlipidemic activity was measured. The GF extract, fermented GF (F-GF), crocin, and fermented crocin (F-crocin) significantly inhibited the increase of serum triglyceride (TG) level in corn oil feeding-induced triglyceridemic mice, as well as that of serum TG and total and LDL cholesterol levels in Triton WR-1339-induced hyperlipidemic mice. These agents also showed hypolipidemic activity in hyperlipidemic mice induced by high fat diet for 5 weeks. F-GF and F-crocin exhibited more potent hyperlipidemic effects than GF and crocin. The results suggest that the hypolipidemic effect of GF and crocin can be synergistically activated by LAB, and that F-GF and F-crocin may improve hyperlipidemia in clinic, compared with GF and crocin.

Key words: Gardeniae fructus, crocin, crocetin, lactic acid bacteria, hypolipidemic activity

Most herbal medicines are orally administered, and therefore, their components are inevitably brought into contact with intestinal microflora in the alimentary tract. Most of the 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 a great importance in the understanding of their biological effects [7].

Gardeniae fructus (the fructus of *Gardenia jasminoides* Ellis, GF) is widely used in Asian countries as a natural colorant, and also as a Chinese traditional medicine for its homeostatic, antiphlogistic, analgesic, and antipyretic effects. It contains geniposide and crocin as its main components [15, 20], and these components exhibit antioxidant, cytotoxic, antitumor, and neuroprotective effects [9, 17, 19, 26, 27]. Recently, we reported that crocin isolated from GF and its metabolite crocetin inhibited pancreatic lipase and exhibited antihyperlipidemic effects [14]. In addition, many researchers have reported that lactic acid bacteria (LAB) exhibit biological activities, such as hypocholesterolemic effect [6, 8, 10, 11, 14, 22].

Therefore, we fermented GF and its main component crocin by LAB, and measured their hypolipidemic effects on hypertriglyceridemic and hypercholesterolemic mouse models.

Materials and Methods

Materials

Triton WR-1339 and lovastatin were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Total cholesterol and triglyceride (TG) assay kits were from Asan Pharmaceutical Co. Ltd. (Seoul, Korea), and low-density lipoprotein (LDL) cholesterol assay kits were from BioMerieux (France). Orlistat (Xenical) was kindly donated by Dr. B. W. Song of Kyung Hee Medical Center, Kyung Hee University (Seoul, Korea).

A high fat diet containing 25% beef tallow [American Institute of Nutrition (AiN)-76 fat-diet #180337] was purchased from Dyets, Inc. (Bethlehem, PA, U.S.A.). GF was purchased from Kyung Dong Market (Seoul, Korea) and identified by Dr. Nam-Je Kim, East-West Medical Research Institute, Kyung Hee Medical Center, Kyung Hee University. A voucher specimen (KHUVP-01059) was deposited at the Herbarium of the College of Pharmacy, Kyung Hee University. GF extract, crocin, and crocetin (Fig. 1) were prepared according to the method previously described [10].

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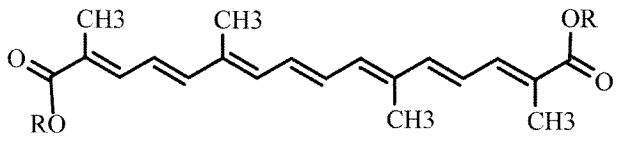

Crocetin R = H

Crocin R = β-Gentiobiosyl

Fig. 1. Structures of crocin isolated from Gardeniae fructus and crocetin.

Assay of Crocin-Hydrolyzing Activity

The reaction mixture, containing 300 μl of 50 mM phosphate buffer, 100 μl of 0.5 mM crocin, and 100 μl of bacteria (0.5 mg as a dried weight), was incubated at 37°C for 1 h, and the amount of hydrolyzed crocin was analyzed by the TLC system (developing solvent, $CHCl_3$-MeOH-water=65:35:10; detector, TLC 9301-PC scanner, Shimadzu, Tokyo, Japan).

Fermentation of GF and Crocin by LAB

The LAB (dry weight, 5 mg) previously cultured in TS broth was incubated with 200 mg of GF water extract or 50 mg of crocin for 24 h at 37°C, and then frozen in liquid nitrogen. Frozen samples were lyophilized using a freeze-drier (Eyela, Tokyo) at 1.5 mmHg for 20 h at −20°C.

Animals

ICR male mice (20–25 g) were purchased from Orient Charles River Co. (Seoul, Korea) and fed a commercial diet (Orient Charles River Co., Korea). These animals were kept for at least 7 days prior to the experiments. To evaluate the hypolipidemic effect, three kinds of hyperlipidemic animal models were established.

First, a hyperlipidemic mouse model, based on corn oil, was established according to the method of Duhault *et al.* [4]. Six mice per group were used. Corn oil (1 g/kg) was orally administered 2 h after each sample was orally administered. Two hours after the administration of corn oil, blood samples of the mice were drawn by cardiac puncture under ether anesthesia.

Second, a hyperlipidemic mouse model based on Triton WR-1339 was established according to the method of Kusama *et al.* [12]. Triton WR-1339 was injected at the end of the regular 16 h fasting period as a 10% solution in saline at a dose of 200 mg/kg body weight into the tail veins of mice under light ether anesthesia. Six mice per group were used. These mice were anesthetized with ether 18 h after Triton WR-1339 injection, and 1–1.5 ml of blood was withdrawn by cardiac puncture. Sera were obtained by centrifugation (1,500 ×*g*, 10 min). Tested samples, lovastatin and orlistat, were orally administered once a day for 3 days. The final administration of the samples was performed 1 h before Triton WR-1339 injection.

Third, a hyperlipidemic mouse model based on high fat diet was established according to the previous method [12]. Mice were divided into 9 groups. Each group contained 6 mice. The high fat control group was fed with high fat diet for 5 weeks. The normal group received a solid normal diet alone. The test agents and xenical were orally administered for 5 weeks. After a 16 h fasting period following the final administration of samples, blood samples were drawn by cardiac puncture under ether anesthesia.

Determination of Serum TG, Total Cholesterol, and LDL Cholesterol

Serum TG was measured by the method designed by Sardesai and Mannig [24], total cholesterol was measured by the enzyme method of Allain *et al.* [2], and LDL cholesterol was measured by the enzyme method of Mainard and Madec [16].

Statistical Analysis

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

RESULTS

Transformation of Crocin by LAB

In the preliminary study in which the crocin-hydrolyzing activity in ten LAB was assayed, all LAB were found to hydrolyze crocin and then produce crocetin (Table 1). Among the LAB, *S. faecium* exhibited the most potent crocin-hydrolyzing activity.

To investigate the metabolic pathway of crocin produced by *S. faecium*, crocin was incubated with *S. faecium* suspension for 4 h, and the metabolites were then extracted

Table 1. Crocin-hydrolyzing activity of some lactic acid bacteria.

Species[a]	Crocin-hydrolyzing activity (μmol/min/mg)
B. breve	1.54
B. bifidum	1.82
B. infantis	1.98
L. bulgaricus	0.36
L. casei	0.68
L. plantarum	0.88
S. faecium	2.54
S. thermophilus	1.87

[a]The tested bacteria were cultured in TS broth (500 ml) for 24 h at 37°C, collected at 5,000 ×*g* for 20 min, and washed twice with saline. The collected cells were incubated at 37°C for 1 h and crocin-hydrolyzing activity was analyzed by a TLC system (developing solvent, $CHCl_3$-MeOH-water=65:35:10; detector, TLC 9301-PC scanner).

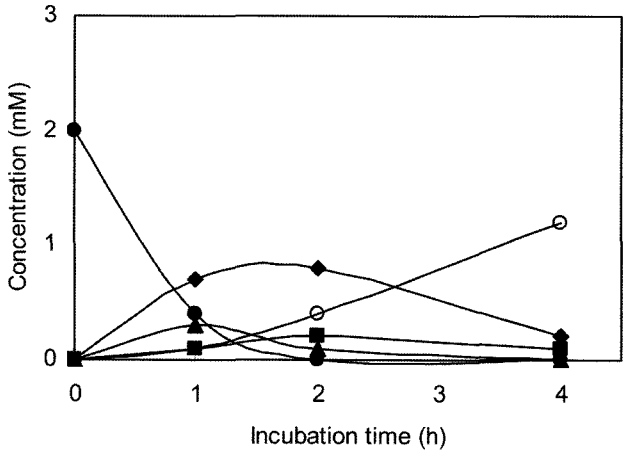

Fig. 2. Time course of metabolism of crocin by *Streptococcus faecium*.
●, Crocin; ▲, β-D-gentiobiosyl crocetin-β-D-glucopyranoside. ◆, crocetin diglycopyranoside; ■, crocetin-β-D-glucopyranoside; ○, crocetin.

with BuOH and analyzed by TLC (Fig. 2). Crocin began to convert to crocetin diglucopyranoside *via* glucopyranosyl crocetin gentiobiose, and subsequently, crocetin was produced *via* crocetin glucopyranoside after 4 h of incubation. Four metabolites, including one major and three minor, were observed. The major metabolite was identified as crocetin, and the three minor metabolites were β-D-glucopyranosyl crocetin-β-D-gentiobiose, crocetin diglucopyranoside, and crocetin-β-D-glucopyranoside. Finally, 62% of the crocin was transformed to crocetin.

In Vivo Hypolipidemic Activity of GF, Crocin, and Their Fermented Products

To evaluate the effect of LAB fermentation of GF and crocin on antihyperlipidemic effect, we fermented GF and crocin with *S. faecium*, shown in Fig. 2 (transformed more than 60% of the crocin to crocetin), and then measured the hypolipidemic activities of GF, crocin, and their fermented products on Triton WR-1339-induced hyperlipidemic mice (Table 2). TG, total cholesterol, and LDL-cholesterol levels in serum were increased by treatment with Triton WR-1339. These results were similar to those in a previous report [10]. Compared with TG, total cholesterol, and LDL cholesterol levels in the Triton WR-1339-alone group, those in GF, fermented GF (F-GF), crocin, fermented crocin (F-crocin), and crocetin-treated groups were all significantly decreased. F-crocin, which showed the most potent hypocholesterolemic effect, decreased total and LDL cholesterol levels by 78% and 93%, respectively, compared with those of control group.

However, HDL-cholesterol levels in the F-GF, F-crocin, crocin, and crocetin-treated groups were increased. In particular, F-GF and F-crocin increased the HDL cholesterol levels more potently than GF and crocin did. The TG level in GF, F-GF, crocin, F-crocin, and crocetin-treated groups was

Table 3. Effect of GF extract, crocin, and crocetin on serum TG and total cholesterol levels on corn oil-induced hyperlipidemic mice.

Group	Dose (mg/kg/day)	Serum level (mg/dl)	
		Triglyceride	Total cholesterol
Normal	-	58.8±6.4^{b}	74.0±3.2^{b}
Control	-	126.5±5.1^{f}	78.7±5.7^{b}
GF extract	200	81.8±7.9^{c,d}	80.0±7.3^{b}
F-GF extract	200 (+ SF 5)	76.3±4.7^{c}	74.0±8.2^{b}
Crocin	50	97.4±1.9^{e}	79.5±5.3^{b}
Crocetin	50	91.0±2.1^{d,e}	76.0±14.0^{b}
F-crocin	50 (+ SF 5)	79.5±5.9^{c,d}	75.5±5.4^{b}
SF[a]	5	96.0±3.2^{e}	76.6±5.0^{b}
Xenical	10	61.1±3.7^{b}	73.5±7.3^{b}

Values of serum levels are mean±SD (n=6).
[a]SF, *Streptococcus faecium*.
[a,b,c,d,e,f]Those with the same letter are not significantly different at $p<0.05$.

Table 2. Effect of GF extract, crocin, and crocetin on serum triglyceride, total cholesterol, high density lipoprotein (HDL) cholesterol, and LDL cholesterol levels in Triton WR-1339-induced hyperlipidemic mice.

Group	Dose (mg/kg/day)	Serum level (mg/dl)			
		Triglyceride	Total cholesterol	HDL cholesterol	LDL cholesterol
Normal	-	98.6±10.8^{b}	71.5±5.9^{b}	24.9±3.2^{c}	26.9±2.4^{b}
Control	-	187.8±14.1^{d}	210.1±6.4^{f}	14.1±4.3^{b}	158.5±3.1^{g}
GF extract	200	117.8±3.8^{b}	128.7±6.1^{d}	34.7±2.9^{d}	63.1±3.9^{e}
F-GF extract	200 (+ SF 5)	114.0±7.9^{b}	111.0±5.9^{c}	42.0±2.9^{e}	46.9±4.5^{d}
Crocin	50	114.1±12.3^{b}	113.3±4.2^{c}	38.1±7.2^{d,e}	52.4±6.1^{d,e}
Crocetin	50	113.4±6.7^{b}	112.4±9.7^{c}	38.0±6.1^{d,e}	51.7±5.4^{d,e}
F-crocin	50 (+ SF 5)	113.7±3.8^{b}	102.0±7.9^{c}	44.5±6.0^{e}	35.7±2.9^{c}
SF[a]	5	150.1±8.8^{c}	150.7±8.4^{e}	25.4±1.8^{c}	92.7±8.4^{f}
Lovastatin	10	111.2±7.5^{b}	85.6±8.1^{b}	32.3±5.4^{d}	31.0±4.1^{b,c}

Values of serum levels are mean±SD (n=6).
[a]SF, *Streptococcus faecium*.
[b,c,d]Those with the same letter in each column are not significantly different at $p<0.05$.

Table 4. Effect of crocin and crocetin on serum TG, total cholesterol, HDL cholesterol and LDL cholesterol levels in high fat diet-induced hyperlipidemic mice.

Group	Dose (mg/kg/day)	Serum level (mg/dl)			
		Triglyceride	Total cholesterol	HDL cholesterol	LDL cholesterol
Normal	-	68.5±4.9[b]	49.5±5.6[a]	32.0±0.9[d]	26.5±1.2[b]
Control	-	193.6±5.0[g]	159.9±10.4[c]	26.8±0.8[c]	143.0±0.9[f]
Crocin	50	91.6±7.4[d]	80.0±6.8[b]	28.3±0.8[c]	47.2±2.4[d]
Crocetin	50	89.5±4.0[d]	87.6±7.0[b]	34.5±0.6[e]	37.4±3.3[c]
F-crocin	50 (+ SF 5)	78.5±7.8[b,c]	72.3±7.4[b]	32.2±0.5[d]	31.8±2.1[b,c]
SF[a]	5	172.5±4.0[f]	140.3±3.7[c]	32.1±0.6[d]	58.6±3.0[e]
Xenical	10	109.6±3.3[e]	67.6±6.8[b]	31.5±2.3[c,d]	64.5±2.2[e]

Values of serum levels are mean±SD (n=6).
[a]SF, *Streptococcus faecium*.
[b,c,d,e,f,g]Those with the same letter are not significantly different at $p<0.05$.

significantly decreased, compared with that in the control group. F-crocin decreased the TG level by 83%, compared with that of the control group.

We also measured the inhibitory effects on corn oil feeding-induced hyperlipidemic mice (Table 3). The serum level of TG, but not cholesterol, was increased by treatment with corn oil. In GF, F-GF, crocin, F-crocin, and crocetin-treated groups, serum TG levels were all significantly decreased, compared with that in the control group. F-GF and F-crocin, which were fermented by LAB, showed more potent hypotriglyceridemic effect than GF and crocin.

Effect of Long-Term Feeding of Crocin and F-Crocin on High Fat Diet-Induced Hyperlipidemic Mice

The hypolipidemic effect of long-term feeding of crocin and crocetin on high fat diet-induced hyperlipidemic mice was measured (Table 4). The TG, total cholesterol, and LDL cholesterol levels in serum were increased by treatment with a high fat diet for 5 weeks. The administration of F-crocin, crocin, and crocetin significantly decreased these levels, with F-crocin being the most potent. Epididymal fat pad mass increased by a high fat diet was also significantly reduced in these groups (Fig. 2). However, the HDL-cholesterol levels in the crocin, F-crocin, and crocetin-treated groups increased.

DISCUSSION

Probiotics are defined as live microbial feed supplements that beneficially affect the host by improving its intestinal balance [5]. Most probiotic microorganisms are LAB such as *S. faecium*, *S. thermophilus*, *Lactobacillus acidophilus*, *L. plantarum*, and *L. casei* [2]. Research has shown that addition of probiotics to food provides several health benefits, including reduction in the level of improved gastrointestinal function, enhanced immune system, and lower risk of colon cancer [3, 10, 11, 18, 21, 23].

In addition, most herbal medicines are orally administered, and their components inevitably come into contact with

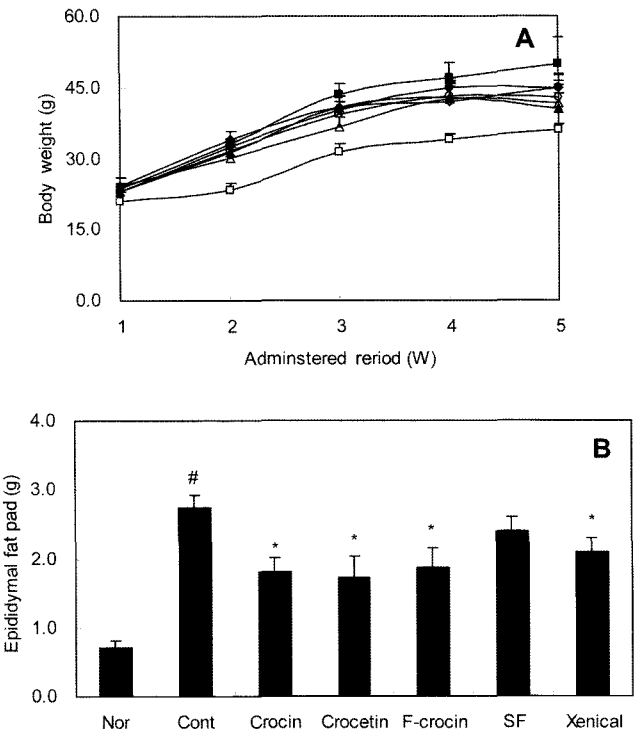

Fig. 3. Effect of crocin, F-crocin, and crocetin on body (**A**) and epididymal fat pad (**B**) weights of hyperlipidemic mice induced by high fat diet.
Mice were divided into 7 groups. Each group contained 6 mice and their body weights were 22.5±1.1 g (mean±SD): □, normal group; ■, control group; ▲, crocin; △, F-crocin; ◇, crocetin; ○, xenical; ●, SF (*S. faecium*). High fat diet control group was fed with high fat diet for 5 weeks. The normal group received a solid normal diet alone. Crocin, F-crocin, or crocetin at a dose of 50 mg/kg/day and Xenical at a dose of 10 mg/kg/day for the high fat diet-treated group were orally administered for 5 weeks. Body weight was measured before the final administration of the samples. The epididymal fat pads were taken under ether anesthesia and its weight was measured. #Significantly different, compared with normal group ($p<0.05$). *Significantly different, compared with control group ($p<0.05$).

intestinal microflora in the alimentary tract. These components may be transformed before they are absorbed from the gastrointestinal tract, and the absorbed metabolites then express their biological activities. Therefore, to transform crocin to crocetin by LAB, we incubated crocin with *S. faecium* and then analyzed the metabolites, and 62% of the crocin was found to be transformed to crocetin after 4 h of fermentation. The biological activity of the fermented crocin (F-crocin) and GF (F-GF) was then evaluated. F-GF and F-crocin had more potent hypolipidemic activity in Triton WR-1339 or corn oil-induced hyperlipidemic mice than those without fermentation, although the fermentation of GF and crocin by LAB increased the hypolipidemic activity. These agents also significantly lowered serum cholesterol and TG levels in hyperlipidemic mice induced by long-term feeding of a high fat diet, and inhibited the increase of body weight in these animal models, compared with that of the control group (Fig. 3). These agents also significantly reduced epididymal fat pad mass increased by a high fat diet. Their potencies at a dose of 50 mg/kg are comparable with that of xenical at a dose of 10 mg/kg. F-GF and F-crocin exhibited not only the hypolipidemic effect, but also reduced the fat pad weight of epididymis. The present results suggest that F-GF and F-crocin can improve hyperlipidemia more potently than GF and crocin. This suggestion is supported by the previous report to show that crocetin exhibits more potent hypolipidemic activity than crocin [14]. These results further suggest that crocin and crocetin can inhibit biosynthesis and absorption of TGs from the intestine into blood, and that LAB inhibit cholesterol absorption. Finally, we propose that F-GF and F-crocin are effective as hypolipidemic agents.

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