

Digestion-resistant fraction from soybean [*Glycine max* (L.) Merrill] induces hepatic LDL receptor and *CYP7A1* expression in apolipoprotein E-deficient mice

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Abstract

Soybean [*Glycine max* (L.) Merrill] is known to have hypocholesterolemic effects; however, the function and mechanism of its digestion-resistant fraction (RF) in cholesterol reduction is not clearly understood. In the present study, we investigated the hypocholesterolemic effects of the RF from soybean in C57BL/6J and apolipoprotein E (apoE)-deficient mice. RFs were prepared either from raw or preheated crops to measure compositional changes in RF during cooking. Preheating reduced the RF yields and the resistant starch (RS) fraction in RF. After 1 week of feeding, the raw soybean RF (5%, w/w) was the most effective in lowering plasma cholesterol concentrations by 27% ($P < .05$) in apoE-deficient (apoE^{-/-}) mice. A smaller but significant reduction was found in C57BL/6J mice. The RF from preheated soybean tended to have lower hypocholesterolemic effects than did the RF from raw soybean in apoE^{-/-} mice. This suggests the RS may be a key hypocholesterolemic component from soybean RF. RF consumption (5%, w/w) dramatically increased hepatic low-density lipoprotein receptor and cholesterol 7 α -hydroxylase expression in both apoE^{-/-} and C57BL/6J mice followed by increased bile acid excretion. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase was only marginally altered. Our results show that the RF, especially from raw soybean containing high level of RS, significantly reduces plasma cholesterol concentrations under hyperlipidemic condition. The cholesterol was reduced by multiple mechanisms such as increased hepatic cholesterol uptake, cholesterol degradation into bile acids and bile acid excretion.

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Keywords: *Glycine max* (L.) Merrill; Resistant digestion fraction; Cholesterol; LDL receptor; *CYP7A1*; Bile acids

1. Introduction

The digestion-resistant fraction (RF) is an indigestible portion of plant food that is not absorbed by the gastrointestinal tract. The RF, generally called *dietary fiber*, is mainly composed of resistant starch (RS) and nonstarch polysaccharides that contain soluble and insoluble fibers. Epidemiological data showed that RF consumption was associated with reduced risk of colon cancer [1,2], coronary heart disease [3–5], diabetes [6–8] and obesity [9]. Potential mechanisms for these health benefits include the promotion of beneficial microflora in the colon that use the RF as a substrate [10–14] and hypocholesterolemic [15–17] and

hypoglycemic [16,18,19] effects of the RS fraction of the RF.

The RS fraction of the RF is divided into four types. RS1 is physically trapped starch in milled grains and is inaccessible to digestive enzymes for 18 h of incubation in vitro. RS2 is ungelatinized starch that is abundant in raw potato, banana and high-amylose maize starch that is not degraded by digestive enzymes for 4–5 h of incubation in vitro. RS3 is retrograded starch that is produced after quick cooling of heat-treated starches. Finally, RS4 is a chemically modified starch that does not occur naturally and is found in commercially manufactured starch products.

Biological effects of RS have been investigated mainly using RS2 and RS3. Reduction of plasma glucose and lipid levels by RS2 and RS3 from corn and rice in diabetic rats [20,21] inhibited cholesterol biosynthesis by RS2-using

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intestinal microflora [22]. One study reported that RS1 was more effective in diabetes and cardiovascular disease risk compared to other RS fractions and nonstarch polysaccharides [23]. Therefore, RS1 may have more health benefits compared with other RS fractions, however, the function of RS1 on the level of plasma lipids is not well known.

In the present study, we investigated the hypocholesterolemic effects of the RF from soybean [*Glycine max* (L.) Merrill]. RF was isolated after 18 h of digestive enzyme incubation; thus, RS fraction in RF was mainly RS1. RF from rice, the major grain food in Asian diets, was used as a reference. We found that the RF, especially from soybean, significantly lowered plasma cholesterol levels, along with the reduction in triglyceride and glucose concentrations. Preheating before RF preparation reduced these effects. The hypocholesterolemic effect of the RF was achieved by increasing hepatic low-density lipoprotein (LDL) receptor level and bile salt production and excretion.

2. Methods and materials

2.1. Preparation of RFs from rice and *G. max* (L.) Merrill

RFs were prepared using a modification of the method of Goni et al. [24]. To investigate the physiological effects of heat treatment on the RF in vivo, two types of RF were prepared from rice and soybean and added to the test diets. Samples were finely ground before heat treatment and autoclaved under standard conditions (121°C, 15 min, 1 atm). Heat-treated and raw samples were then processed further. First, proteins of 10 g of samples were digested by incubation with pepsin in KCl–HCl buffer (1% w/v, pH 1.5) at 40°C for 60 min. After protein digestion, carbohydrates were digested by adding 900 ml of 0.004% (w/v) α -amylase solution in Tris–maleate buffer (100 mM, pH 6.9) and incubating the suspension at 40°C for 18 h. After two digestion reactions, the samples were centrifuged (3000×g, 15 min), the supernatant was discarded, and the pellet was washed three times with distilled water. The pellet was then incubated in a series of alkaline and acidic solutions for 30 min at room temperature [4 M KOH (300 ml), followed by 2 M HCl (550 ml)]. The solution was further incubated with 0.4 M sodium acetate buffer (300 ml, pH 4.75). Finally, the residuals were digested with amyloglucosidase at 60°C for 1 h. Each digestion reaction was performed with appropriate shaking. The final sample was washed and centrifuged (3000×g, 15 min), and the lyophilized pellet was used as an RF in the experiments. Four types of RFs were prepared: a raw and unheated RF (RRF) and a heated RF (HRF) from either rice (RIC) or soybean, *G. max* (L.) Merrill (GLY). The RFs were designated as RRF-RIC, HRF-RIC, RRF-GLY and HRF-GLY, respectively.

2.2. Animals, feeding studies and measurements

The apolipoprotein E-deficient (apoE^{-/-}) and C57BL/6J mice were purchased from The Jackson Laboratory (Bar

Harbor, ME) and maintained in the animal facilities at Hae-Eun Biotech (Seoul, South Korea). The animals were kept in a specific-pathogen-free room at 21–25°C with a 12-h day/night cycle; water and standard chow were provided ad libitum. All experiments were performed under protocols approved by the Committee on Animal Experimentation of the Hae-Eun Biotech Research Committee. In the feeding study, mice aged 10–14 weeks were fed normal chow or chow containing 5% RF for 1 week. After feeding, the mice were fasted overnight (16–19 h), and blood samples were collected in purple-topped EDTA tubes for further analysis.

Plasma was separated from the blood by centrifugation, and then total cholesterol, total triglyceride and glucose levels were measured using enzymatic methods (Asan-pharm, South Korea; Sigma, St. Louis, MO). The total protein concentration was determined using a BioRad protein kit (BioRad, Hercules, CA) with bovine serum albumin as a standard.

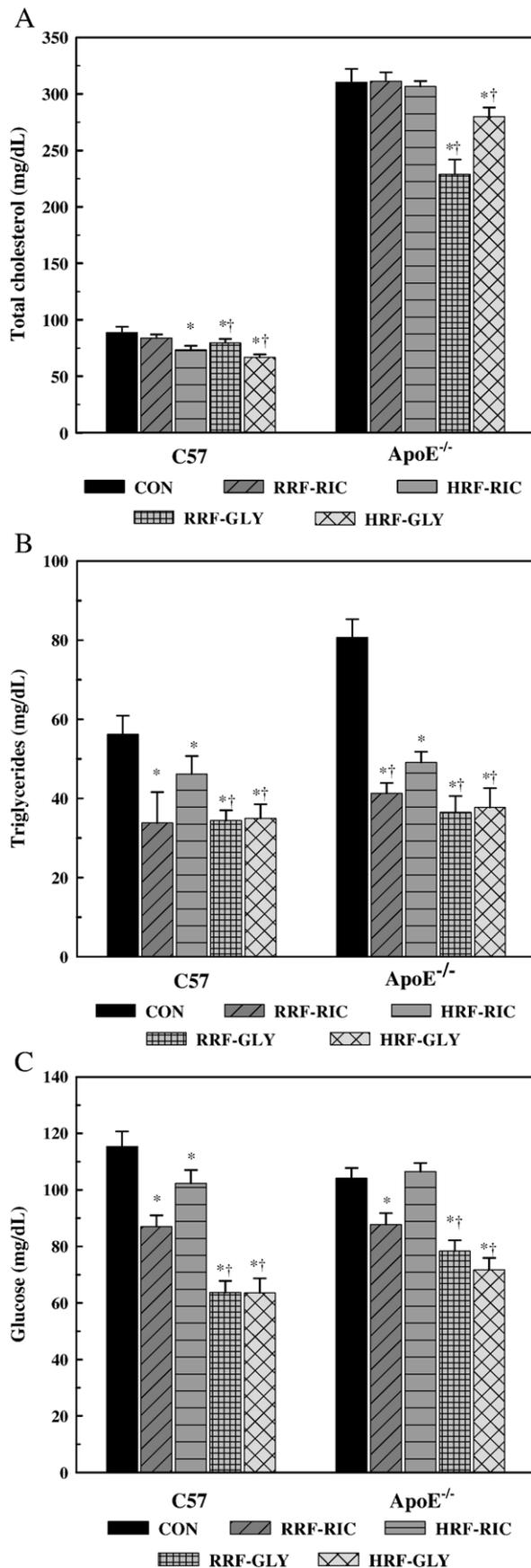
2.3. Western blot analysis

Tissue membrane proteins were prepared as previously described [25], and aliquots were stored at –80°C. For protein analysis, 10 μ g of each sample was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, and then transferred on to a nitrocellulose membrane. Successful transfer was confirmed by staining the membrane with Ponceau S. The blocking solution and washing buffer were 5% nonfat dried milk and 0.1% (v/v) PBS-T, respectively.

The antimouse LDL receptor antibody and antimouse LDL receptor-related protein (LRP) antibody were gifts from Dr. Allen Cooper; horseradish peroxidase-conjugated secondary antibody was purchased from Merck-Korea (Seoul, South Korea). Membrane proteins (20- μ g samples) were loaded onto SDS–PAGE gels, electrophoresed and then transferred onto nitrocellulose membranes. The transferred proteins were transiently stained with Ponceau dye (Sigma). Protein bands on the membranes were detected on X-ray film using the standard enzyme chemiluminescent method (Amersham-Pharmacia Korea, Seoul, South Korea). The density of each band was quantified using Alpha Imager 2200 software (Alpha Innotech, San Leandro, CA).

Table 1
Composition of RFs from rice and soybean *G. max* (L.) Merrill (% w/w)

Components	RF (RS and fibers)			
	Rice		Soybean	
	Raw	Heat-treated	Raw	Heat-treated
Moisture	3.70	1.60	11.10	9.00
Carbohydrates	0.00	0.00	0.00	0.00
Protein	1.10	0.19	0.99	0.66
Ash	0.03	0.01	2.10	2.03
Lipids	0.03	0.02	0.22	0.28
Resistant fraction	95.14	98.18	85.59	88.03
Nonstarch soluble fiber	37.76	46.43	30.17	31.91
Nonstarch insoluble fiber	38.98	44.75	31.32	39.32
RS1	18.40	7.00	24.10	16.80



2.4. RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from tissue using the QIAGEN RNase mini kit (Qiagen, GmbH, Germany) according to the manufacturer's protocol. Total RNA (2 µg) was reverse-transcribed into cDNA using AMV reverse transcriptase (Clontech; Promega Co, Madison, WI) in the presence of RNase inhibitor according to the manufacturer's protocol, using an oligo(dT)15 primer and random hexamers provided by Promega. The resulting volume of cDNA was 20 µL. PCR was performed using 1 U of Taq DNA polymerase (Promega; Promega Co) with 1 µL of cDNA as template.

PCR primers were designed based on published nucleotide sequences. The PCR primer sequences used were as follows: β -actin: 5'-CCCAACTTGATGTATGAAGG-3', 5'-TTTGTGGAAGGTAAGGTGTGC-3'; cholesterol 7 α -hydroxylase (*CYP7A1*): 5'-CCTTGGACGTTTCTCGCT-3', 5'-GCGCTCTTTGATTTAGGAAG-3'; 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase: 5'-GT-TCTTTCCGTGCTGTGTTCTGGA-3', 5'-CTGATATCTT-TAGTGCAGAGTGTGGCAC-3'. PCR using templates for *CYP7A1* and HMG-CoA reductase began with incubation for 10 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. Final extension was carried out for 5 min at 72°C. PCR using the β -actin templates was performed similarly. PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide under UV light. The intensities of the bands were analyzed and quantified using Alpha Imager 2200 software (Alpha Innotech). The intensities of the β -actin transcripts were used for normalization. The identities of the PCR products were verified by DNA sequencing.

2.5. Measurement of fecal bile salt excretion

Stool samples were collected during 1 week of the diet feeding, and bile acids were extracted as previously described [26]. In brief, 200 mg of lyophilized stool samples were extracted with methanol then concentrated to 0.5 ml. Ten microliters of sample was incubated with the reaction solution containing 60 mU of 3 α -hydroxysteroid dehydrogenase for 40 min at room temperature. The OD of the samples were measured at 340 nm for calculating the bile acids concentrations. Total bile acid excretion was calculated with multiplying fecal weight (g) by bile acid concentrations (mg/g).

2.6. Statistical analysis

The results are expressed as mean \pm S.D. Two-group comparisons were performed using Student's *t* test. If

Fig. 1. Effects of the RFs on plasma (A) lipid, (B) triglyceride and (C) glucose levels in C57BL/6J ($n=10$ /group) and apoE^{-/-} mice ($n=7$ /group). CON = control; RRF-RIC = RF from raw rice; HRF-RIC = RF from heated rice; RRF-GLY = RF from raw soybean *G. max* (L.) Merrill; HRF-GLY = RF from heated soybean. * $P<.05$ compared with control; † $P<.05$ between RRF-RIC and RRF-GLY or $P<.05$ between HRF-GLY and HRF-RIC.

necessary, analysis of variance (ANOVA) was performed for comparisons among groups, and differences among samples were examined using Duncan's multiple range tests using SAS (version 9.13, SAS Institute, Cary, NC). $P < .05$ were considered significant.

3. Results

3.1. Chemical composition of RFs

We prepared two types of RFs according to heat treatment status; RRF and HRF were prepared from both rice and soybean. RF was prepared after 18 h of amylase incubation, and HRF was obtained by lyophilization after preheating, thus starch fraction in the RF samples was mostly RS1. The RF from rice, which is a major grain food in Asian diets, was used as a reference in this study. The RF yield from raw crops was approximately 5 g per 100 g raw materials, however, the yield was markedly reduced after the heat treatment (0.4 and 2.4 g for 100 g of rice and soybean, respectively). The composition of the four types of RFs is shown in Table 1. Protein, carbohydrate and lipid contents in RFs were nearly zero. The RF from unheated raw samples tended to have high levels of RS and low levels of total fiber (nonstarch polysaccharides) for both rice and soybean. Preheating reduced the RS more

significantly than it did the fiber content; therefore, the percentage of fiber in both rice and soybean increased after heat treatment. The percentage of both soluble and insoluble fiber increased in rice after heat treatment, whereas that of insoluble fiber increased in soybean after heat treatment. The RS content in soybean was more than twice that in rice in the heat-treated samples.

3.2. Plasma cholesterol, triglyceride and glucose levels in vivo

To determine the effects of RFs on the plasma lipid and glucose levels in vivo, mice were fed standard chow or chow containing 5% of each RF for 1 week. The apoE^{-/-} mice (model mice for hyperlipidemia) and C57BL/6J mice (control black mice) were fed the test diets.

The RFs from soybean reduced plasma cholesterol more efficiently than did the RFs from rice. In the control mice, the HRF from rice and soybean and the RRF from soybean significantly reduced total cholesterol levels. In apoE^{-/-} mice, the RFs from soybean, but not from rice, showed hypocholesterolemic effects (Fig. 1A). In apoE^{-/-} mice, heat treatment did reduce the hypocholesterolemic effect of RF only from soybean. This may be caused by reductions in RS during heat treatment.

The RFs from both rice and soybean lowered plasma triglyceride and glucose concentrations (Fig. 1B and 1C).

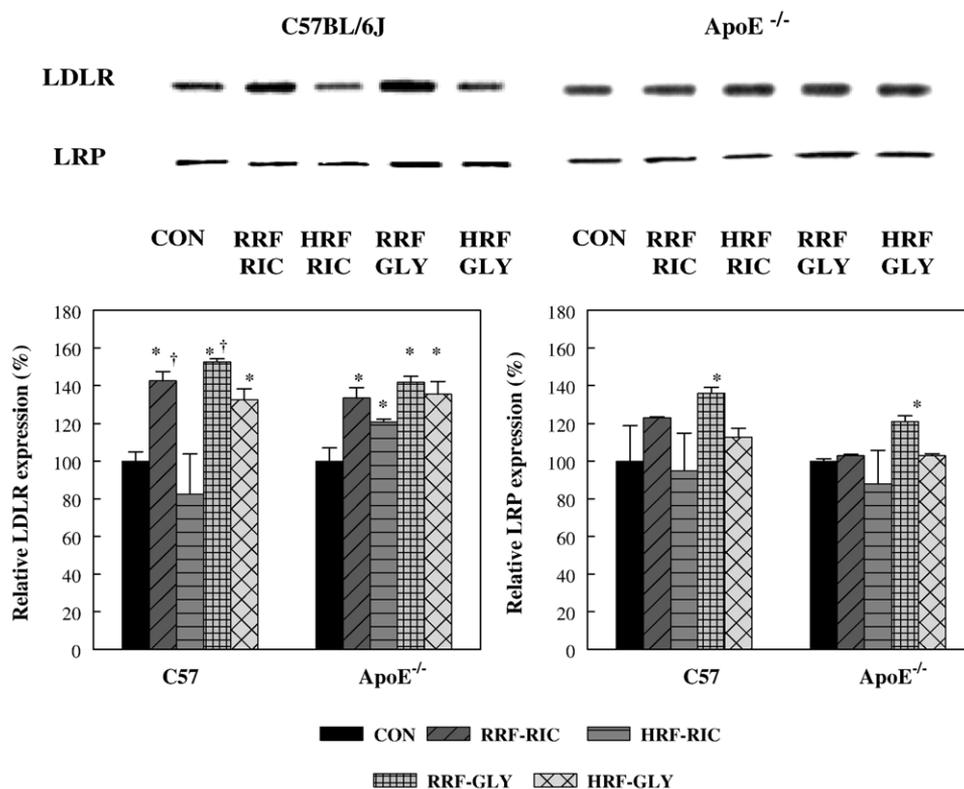


Fig. 2. Western blot of prepared membrane protein from C57BL/6J and apoE^{-/-} mice. Liver membrane proteins were purified from three mice per group, and equal amount of membrane protein was pooled for loading on the gel. LDL receptor (LDLR) and LRP expressions were examined. Treatment names are the same as in Fig. 1. * $P < .05$ compared with control; † $P < .05$ between RRF and HRF of *G. max* (L.) Merrill or $P < .05$ between RRF and HRF of rice.

After RF consumption, triglyceride levels in apoE^{-/-} mice became similar to those in control mice. These dramatic reductions were observed after 1 week of feeding.

RRF-GLY was the most effective in reducing plasma cholesterol, triglyceride and glucose in apoE^{-/-} mice among the four RFs. The reductions by RRF-GLY feeding were 26.3%, 57.8% and 24.8% ($P < .05$) for cholesterol, triglyceride and glucose, respectively. A similar trend was found in C57BL/6 mice; the RRF-GLY feeding significantly reduced plasma cholesterol, triglyceride and glucose by 10.16%, 38.79% and 44.80% ($P < .05$).

3.3. LDL receptor and LRP

We determined the levels of two lipoprotein receptors in the liver: the LDL receptor and the LRP. These two receptors mediate approximately 80–90% of the hepatic removal of triglyceride-rich lipoproteins such as chylomicron remnants [27,28]. The LRP expression was significantly increased by 35.9% and 21.2% in C57BL/6J and apoE^{-/-} (21.2%) mice, respectively, after RRF-GLY consumption. LDL receptor expression increased in C57BL/6J ($n=10$) and apoE^{-/-} ($n=7$) mice after RF consumption (Fig. 2). In C57BL/6J mice, LDL receptor expression was significantly increased by 42.5%, 52.5% and 32.5% after feeding RRF-RIC, RRF-GLY and HRF-GLY, respectively. In apoE^{-/-} mice, LDL receptor expression was significantly increased by 33.3%, 20.8%, 41.7% and 35.4% after feeding RRF-RIC, HRF-RIC, RRF-GLY and HRF-GLY, respectively. RRF-GLY was the most effective both in C57BL/6J and apoE^{-/-} mice (52.5% and 41.7%, $P < .05$).

3.4. HMG-CoA reductase and cholesterol 7 α -hydroxylase (CYP7A1) gene expression

To further examine the mechanism by which RFs lower cholesterol concentrations, the expression of two key genes in cholesterol metabolism, HMG-CoA reductase and CYP7A1 genes, was measured using reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3). The former gene expresses a rate-limiting enzyme in cholesterol biosynthesis, and the latter encodes a rate-limiting enzyme in the classical pathway for bile acid biosynthesis. The expression of HMG-CoA reductase was marginally reduced in C57BL/6J and apoE^{-/-} mice after feeding with RFs. The reduction by HRF-GLY diet was significant (18% reduction, $P < .05$). The CYP7A1 gene expression level was dramatically increased in C57BL/6J and apoE^{-/-} mice after RF feeding. In C57BL/6J mice, CYP7A1 expression was significantly increased by 8.9%, 57.4% and 45.1%, after feeding with RRF-RIC, RRF-GLY and HRF-GLY, respectively. RRF-GLY was markedly effective in both C57BL/6J and apoE^{-/-} mice (48.0% and 73.7%, $P < .05$). RRF-GLY showed the best induction.

3.5. Fecal bile acid excretion

Because CYP7A1 expression was dramatically induced by RF feeding in both strains of mice, plasma cholesterol

may have been excreted as bile acids. Thus, the effect of RF feeding on the bile acid excretion in stool samples was examined in both strains of mice (Fig. 4). Bile acid excretion increased in C57BL/6J ($n=10$) and apoE^{-/-} ($n=7$) mice after feeding with RFs. All RFs significantly increased bile salt excretion in both strains of mice (Fig. 4). RRF-RIC and HRF-RIC increased the bile salt excretion in C57BL/6J by 2.3- and 2.1-fold, respectively; RRF-GLY and HRF-GLY increased the bile salt excretion in C57BL/6J by

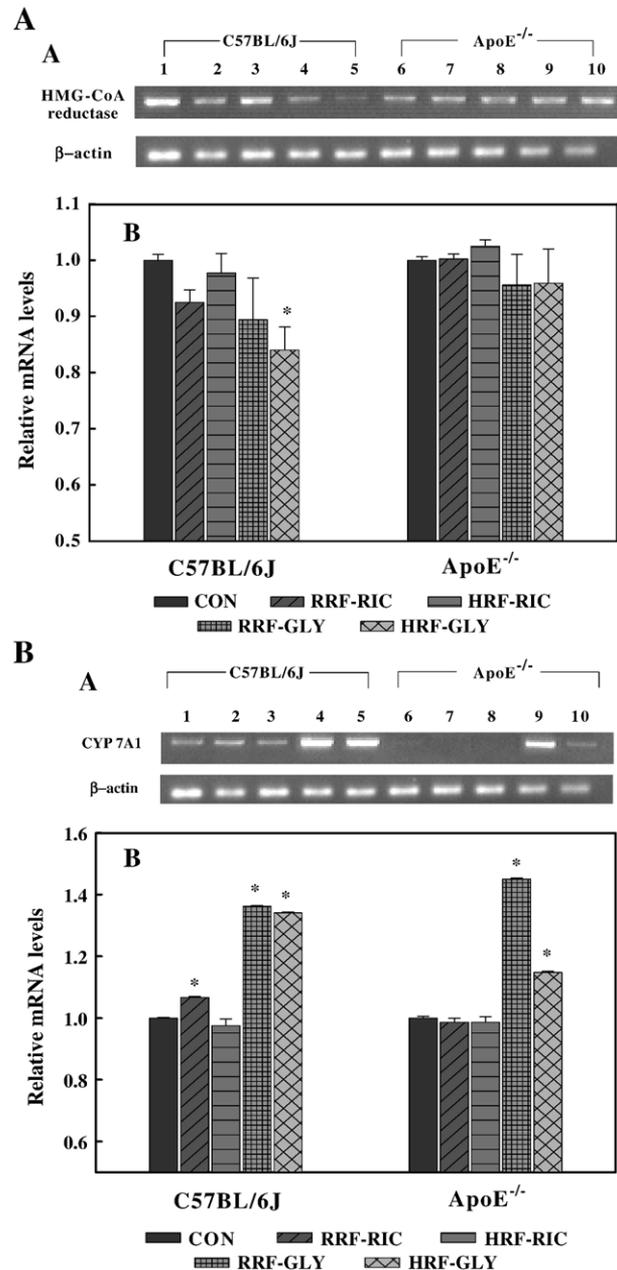


Fig. 3. Effects of the RFs on (A) HMG-CoA reductase and (B) CYP7A1 gene expression. RNA was prepared from three mice, and pooled samples were used for RT-PCR analysis. Lanes 1 and 6 = control diet; lanes 2 and 7 = RRF-RIC; lanes 3 and 8 = HRF-RIC; lanes 4 and 9 = RRF-GLY; lanes 5 and 10 = HRF-GLY. Treatment names are the same as in Fig. 1. * $P < .05$ compared with control.

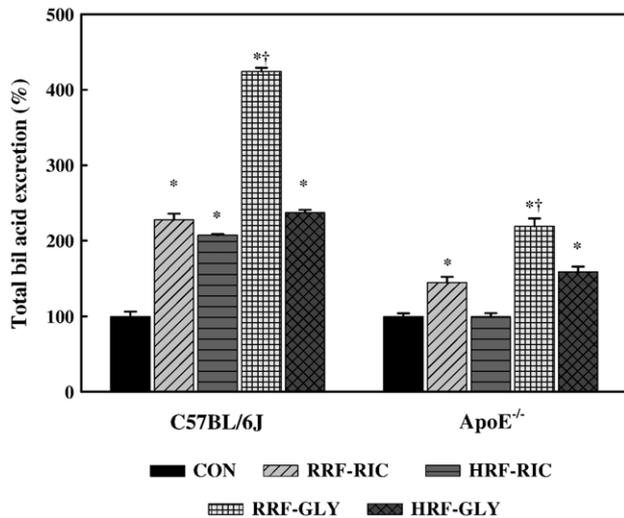


Fig. 4. Effects of the RFs from raw or heated rice and soybean *G. max* (L.) Merrill on the bile acid excretion from C57BL/6J ($n=10$ /group) and apoE^{-/-} mice ($n=7$ /group). Fecal samples were collected during 1-week feeding period and total bile acid was measured as previously described [26]. Total bile acid excretion was calculated with multiplying fecal weight (g) by bile acid concentrations (mg/g). The value was compared among groups using control as a reference. Treatment names are the same as in Fig. 1. * $P<.05$ compared with control; † $P<.05$ between RRF-RIC and RRF-GLY.

4.2- and 2.4-fold, respectively. In apoE^{-/-} mice, RRF-RIC, RRF-GLY and HRF-GLY increased bile salt excretion by 1.5-, 2.2- and 2.6-fold respectively. The HRF-RIC did not significantly change the bile salt excretion. In these mice, RRF-GLY was also the most effective RF for inducing bile acid excretion. Similar results were obtained when bile acid excretion was assessed by TLC method [29–31].

4. Discussion

G. max (L.) Merrill is known to have various health-promoting effects due to its high RF content [32]. In this study, we examined the effects and mechanisms of the RF on plasma cholesterol metabolism in C57BL/6J and apoE^{-/-} mice. RF from the soybean was compared with RF from rice, the major grain food in the Asian diet. The RFs in the current study contained high levels of RS1.

We showed that the RF significantly reduced plasma triglyceride and glucose concentrations as well as cholesterol levels in both C57BL/6J and apoE^{-/-} mice. Our data are in accordance with results of previous experiments [32]. Interestingly, greater hypocholesterolemic effects were seen in apoE^{-/-} mice with the RF from soybean than with that from rice. It appears that the hypocholesterolemic effect of the RF becomes evident under high serum cholesterol levels in vivo.

Our study is one of a few that have investigated the wide spectrum of hypocholesterolemic mechanisms mediated by RF in vivo and suggest that the hypocholesterolemic effects of the RF are achieved by multiple changes. The alterations

observed were increased hepatic cholesterol uptake, bile acid formation and bile acid excretion.

We found that LDL receptor expression was up-regulated with a diet containing the RF. This can contribute to the reduction of both plasma cholesterol and triglyceride because LDL receptors remove plasma lipoproteins such as very-low-density lipoprotein (VLDL) and LDL. The treatment of hyperlipidemia and kintoki bean [33] have shown to induce the expression of LDL receptors, which remove VLDL and LDL particles from the circulation. This induction of LDL receptor expression was most evident with RRF-GLY feeding. We showed that the expression of HMG-CoA reductase was only marginally altered, especially, in apoE^{-/-} mice after RF feeding. This suggests inhibition of cholesterol synthesis was not a major mechanism in cholesterol lowering by the RF consumption in mice. However, the expression of *CYP7A1*, a key gene for bile acid production, dramatically increased in both C57BL/6J and apoE^{-/-} mice after RF feeding. In particular, RRF-GLY was markedly effective in both C57BL/6J and apoE^{-/-} mice (48.01% and 73.65% induction, respectively, $P<.05$). Subsequently, bile acid excretion was increased in C57BL/6J ($n=10$) and apoE^{-/-} ($n=7$) mice after feeding with RF. The transcription rate of *CYP7A1* is linearly correlated with total bile acid excretion in vivo [34,35], accordingly, fecal bile acid excretion was also increased after consumption of an RF-containing diet in accordance with previously reported data [33]. RRF-GLY was the most effective in bile acid excretion in both C57BL/6J and apoE^{-/-} mice.

Although heat treatment during food processing or cooking increases digestibility of foods, heat treatment loses valuable nutrients, including the RF. Accordingly, the RF yield from soybean and rice was reduced by 51% and 92%, respectively, after heating. In RF, RS was more labile to heat treatment than were nonstarch carbohydrate fibers, thus heat treatment selectively reduces RS fraction in RF, which may have biological functions.

In conclusion, our results show that the RF-containing diet improved plasma lipid metabolism. The RF from soybean was more effective than that from rice. In addition, heating tended to reduce the effect. These findings suggest that RS1 may be the key resistant fraction component responsible for cholesterol reduction, and may induce multiple positive effects on cholesterol metabolism by increasing the expression of LDL receptors, *CYP7A1* and the excretion of fecal bile acids.

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