Resistant Starch Fraction Prepared from Kintoki Bean Affects Gene Expression of Genes Associated with Cholesterol Metabolism in Rats

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Feeding rats beans with resistant starch reduces the serum cholesterol concentration; however, the mechanism is not fully understood. We examined the effects of resistant starch of kintoki (*Phaseolus vulgaris*, variety) bean on serum cholesterol and hepatic mRNAs in rats. Male F344/Du Crj rats were fed a cholesterol-free diet either with 5 g of cellulose powder (control)/100 g or 5 g of pancreatin-resistant fraction prepared from kintoki bean (kintoki)/100 g diet for 4 weeks. There were no differences in the body weight gain, food intake, liver weight, and mass of cecum contents between the groups. Serum total cholesterol, very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL)-cholesterol, and high density lipoprotein (HDL)-cholesterol levels in the kintoki group were significantly (at least $P < 0.05$) lower than in the control group throughout the feeding period. There was no difference in the serum triglyceride concentration between two groups throughout the feeding period. Total hepatic cholesterol in the control group was significantly ($P < 0.01$) lower than in the kintoki groups. Fecal bile acid, cecal acetate, propionate and *n*-butyrate concentrations in the kintoki group all were significantly ($P < 0.05$) higher than in the control group. Likewise, hepatic cholesterol 7α-hydroxylase, LDL receptor, and SR-B1 mRNA levels in the kintoki group were significantly ($P < 0.05$) higher than in the control group. The results suggest that resistant starch of kintoki bean reduces serum cholesterol level by increasing hepatic LDL receptor, SR-B1, and cholesterol 7α-hydroxylase mRNAs. Exp Biol Med 229:787–792, 2004

Key words: pancreatin-resistant fraction prepared from kintoki bean; serum cholesterol; short-chain fatty acid; bile acid; hepatic mRNAs

Starch is one of the major components in human and animal diets and originates mainly from cereals and beans. Some starch in the normal diet escapes digestion and absorption in the human small intestine, which is defined as resistant starch (RS) by EURESTA (1). Like dietary fiber, RS is also known to be fermented in the large intestine to short-chain fatty acids (SCFAs), which may be involved in lowering serum cholesterol concentrations (2). Starch appears to be a food source of butyric acid in human fecal inocula (3, 4). Recently, it has been reported that *n*-butyrate induces an increase in the peroxisome proliferator-activated receptor gamma (PPARγ) mRNA level in Caco-2 cells (5) and reduces colonic paracellular permeability by enhancing PPARγ activation (6).

Beans are unique foods, rich in complex carbohydrates, proteins, dietary fiber, starch, and minerals. Relatively few studies have been conducted to investigate the digestibility of starch from beans in the small intestine of humans (7), the content of SCFAs in the hindgut of rats fed processed bean flours (8), and the effects of bean starch on lipid metabolism (9). Most such studies have used retrograded bean starch, which was prepared by a thermal treatment. It is estimated that 8–40 g of resistant starch is consumed per day in Western diets (3), which is similar to the amount of nonstarch polysaccharides ingested daily (8–18 g). Several studies have shown that RS lowers blood lipids in rats (10, 11), whereas some types of RS do not decrease blood lipids in humans (12, 13). This may result from the failure of some types of RS to increase fecal bile acid excretion in humans (14).

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Recently, many researchers have proposed methods for physiologic approaches for *in vitro* determination of RS in foods (15–17). These methods include incubation with pepsin followed by further incubation with a mixture of porcine pancreatic enzymes and amylglucosidase. In this study, we investigated the effect of pancreatin-resistant fraction prepared from kintoki (*Phaseolus vulgaris*, variety) bean on serum and hepatic lipids and hepatic mRNAs. This food is used in Japanese sweets in the form of bean jam (called *An* in Japanese), which is by incubation with pepsin and pancreatic enzymes.

Materials and Methods

Preparing for Pancreatin-Resistant Fraction. The procedure of enzyme-resistant fraction was prepared following the method of Åkerberg *et al.* (15), as modified in our laboratory. First, raw kintoki beans were washed and boiled for 2 hrs. The boiled beans were allowed to reach room temperature by adding cool water. Then, the product was filtrated to separate the bean hulls. The yield of dried filtrate was approximately 79% based on weight. Next, the filtrate fraction was incubated at 37°C for 2 hrs 30 mins with 0.1 *N* HCl buffer containing 0.5% pepsin. After the incubation, 1 *N* NaOH solution was added to terminate the reaction. After centrifugation (13,000 *g* for 15 mins), the residue was incubated at 37°C for 20 hrs with 0.1 *M* phosphate buffer containing 0.8% pancreatin and 2.1% potassium sorbate. The reaction was terminated by adjusting the pH to 4.0 by adding HCl. The sample was centrifuged (13,000 *g* for 15 mins), and the indigestible residue in the crucible was washed with ethanol and acetone to remove residual water. The crucible was dried in an oven at 50°C for 15 hrs and then cooled in a desiccator. Thereafter, the RS was analyzed by the modified Prosky method (18), and the protein, lipid, carbohydrate, moisture, and ash were determined by the Association of Official Analytical Chemists (AOAC) procedure (19). The composition of pancreatin-resistant fraction prepared from kintoki bean was as follows (grams per 100 grams): moisture, 5.6; protein (N × 6.25), 11.5; lipid, 1.2; RS, 64.3 (insoluble RS, 43.6; water-soluble RS, 20.7); carbohydrate, 78.9; and ash, 2.8, respectively.

Animals and Diets. Male F344/DuCrj rats (8 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Rats were housed individually in cages with *ad libitum* access to food and water. The animal facility was maintained on a 12:12-h light:dark cycle, and the temperature was at 23°C ± 1°C with 60% ± 5% relative humidity. Rats were randomly assigned to two groups of five each. There were no significant differences in body weight and serum total cholesterol concentration at the beginning of the experiment. Body weight and food consumption were recorded weekly and daily, respectively. This experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine. All animal procedures described conformed to National Institutes of Health guidelines (20). The composition of each diet was based on the AIN-76 semi-purified rodent diet (21) which contained the following (by dry weight): 60.3% sucrose, 25% casein, 5% corn oil, 3.5% mineral mixture, 1% vitamin mixture, and 0.2% choline chloride. The kintoki group of rats was fed the kintoki bean diet that contained 5% pancreatin-resistant fraction prepared from kintoki bean for 4 weeks, whereas the control group of rats was fed the basal diet with 5% cellulose powder. The Hokkaido Tokachi Area Regional Food Processing Technology Center (Obihiro, Hokkaido, Japan) kindly provided the kintoki beans.

Analytical Procedures. Blood samples (1 ml) were collected weekly into tubes without an anticoagulant between 0800 and 1000 hrs from the jugular veins of fasting rats. After 2 hrs at room temperature, serum was prepared by centrifugation at 1500 *g* for 20 mins. Fecal pellets were collected during the final 2 days of the experiment. Fecal dry weights did not significantly differ among groups. The rats were euthanized by ether inhalation, and the liver and cecum were quickly removed, washed with cold saline, blotted dry on filter paper, and weighed before freezing (–80°C).

Chemical Analysis. Total cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX). The very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL)-cholesterol concentration was calculated as follows:

$$VLDL+IDL+LDL\text{-}cholesterol = total\ cholesterol - HDL\text{-}cholesterol$$

Total lipids were extracted from liver and feces with chloroform-methanol (2:1, v/v) (22). The neutral sterol, in each total lipid, obtained by saponification was acetylated (23) and analyzed by gas liquid chromatography (GLC) using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) with nitrogen as a carrier gas. Acidic sterols in feces were measured by GLC following the method of Grundy *et al.* (24). A part of the cecum was transferred to a vial containing deionized water without exposure to air and suspended. The suspension was deproteinized with perchloric acid (final concentration 50 g/l), cooled in ice, and NaOH was added to the supernatant to precipitate perchloric acid and to form sodium salts of the SCFAs. Individual SCFA was measured by GLC with a glass column (2000 × 3 mm) packed with 80–100 mesh Chromosorb W-AW DMCS with H_3PO_4 (100 ml/l) as the liquid phase after adding H_3PO_4 by the procedure of Hara *et al.* (25).

Table 1. Serum Total Cholesterol, VLDL + IDL + LDL-Cholesterol, HDL-Cholesterol and Triglyceride Concentrations in Rats Fed Control Diet and Kintoki Diet for 4 Weeks[a]

Dietary Group	0 week (mmol/l)	1 weeks (mmol/l)	2 weeks (mmol/l)	3 weeks (mmol/l)	4 weeks (mmol/l)
Total Cholesterol					
Control	1.70 ± 0.08	2.98 ± 0.48	3.05 ± 0.28	2.90 ± 0.18	2.92 ± 0.35
Kintoki	1.76 ± 0.11	2.23 ± 0.13**	2.25 ± 0.19***	2.27 ± 0.13 ***	2.29 ± 0.13 **
VLDL + IDL +LDL-cholesterol					
Control	0.52 ± 0.06	0.98 ± 0.25	1.03 ± 0.18	0.95 ± 0.12	0.88 ± 0.14
Kintoki	0.50 ± 0.06	0.62 ± 0.07*	0.76 ± 0.12*	0.71 ± 0.07**	0.67 ± 0.08**
HDL-cholesterol					
Control	1.18 ± 0.09	2.00 ± 0.26	2.02 ± 0.16	1.94 ± 0.26	2.03 ± 0.22
Kintoki	1.26 ± 0.10	1.61 ± 0.08*	1.49 ± 0.13***	1.55 ± 0.07**	1.62 ± 0.10*
Triglyceride					
Control	0.50 ± 0.17	1.14 ± 0.44	1.79 ± 0.23	1.77 ± 0.29	1.35 ± 0.18
Kintoki	0.66 ± 0.15	1.31 ± 0.25	1.83 ± 0.41	1.66 ± 0.17	1.30 ± 0.33

[a] Control, cellulose powder; Kintoki, pancreatin-resistant fraction prepared from kintoki bean. Values are expressed as means ± SD for five rats. VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. * $P < 0.05$,** $P < 0.01$ and *** $P < 0.001$ versus Control.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from liver by the acid guanidium/phenol/chloroform method using Isogen (Nippon Gene, Tokyo, Japan) (26). Messenger RNAs encoding LDL receptor, cholesterol 7α-hydroxylase, scavenger receptor type B1 (SR-B1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) were analyzed by semiquantitative RT-PCR and subsequent Southern hybridization of PCR products with each inner oligonucleotide probe. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI) to remove genomic DNA before initiating RT-PCR by using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and EX-Taq polymerase (Takara, Tokyo, Japan) with LDL receptor primers of oligonucleotides (upstream primer, 5′-ATTTTGGAGGATGAGAAGCAG-3′; downstream primer, 5′-CAGGGCGGGGAGTGTGAGAA-3′), cholesterol 7α-hydroxylase primers of oligonucleotides (upstream primer, 5′-GCCGTCCAAGAAATCAAGCAGT-3′; downstream primer, 5′-TGTGGGCAGCGAGAACAAAGT-3′), SR-B1 primers of oligonucleotides (upstream primer, 5′-GTAGGGCCCAGAAGACACCAC-3′; downstream primer, 5′-CCTGCCACCGCTGCCACTTAC-3′), and GAPDH primers of oligonucleotides (upstream primer, 5′-GCCATCAACGACCCCTTCATT-3′; downstream primer, 5′-CGCCTGCTTCACCACCTTCTT-3′). The reaction mixtures for the PCR contained 25 pmol of each primer, 1.25 U EX-Taq polymerase, 1× PCR buffer (Takara), and 200 μmol/l dNTP in a 50-μl reaction volume. The expected sizes of DNA fragments amplified with these primers were 931 bp for LDL receptor, 306 bp for cholesterol 7α-hydroxylase, 539 bp for SR-B1, and 702 bp for GAPDH. Temperature cycling was as follows: first cycle, denaturation at 94°C for 3 mins, annealing at 60°C for 1 min, and extension at 72°C for 2 mins. Subsequent cycles were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 mins. The thermal cycling was completed by terminal extension at 72°C for 10 mins. In total, there were 25 cycles for the GAPDH, SR-B1, LDL receptor, and cholesterol 7α-hydroxylase.

Southern Blot Analysis. Amplification products were electrophoresed on 2% agarose gel and transferred to a nylon membrane (Biodyne B; Pall Bio-Support, East Hills, NY). Blots were hybridized with an LDL receptor probe of a 54-base oligonucleotide (5′-GTGAACTTGGGTGAGTGGGCACTGATCTGAGGGGCAGGCAGGCACATGTACTGG-3′), cholesterol 7α-hydroxylase probe of a 54-base oligonucleotide (5′-CCCGAAGGCCTGTTTAAGTGATGACTCTCAGCCGCCAAGTGACATCATCCAGTG-3′), SR-B1 probe of a 54-base oligonucleotide (5′-TGCCGTGTGGACAGTGTGACATCTGGGGGCTCAGGACGTGGCACTGGCGGGTTG-3′), and GAPDH probe of a 54-base oligonucleotide (5′-TGATGACCAGCTTCCCATTCTCAGCCTTGACTGTGCCGTTGAACTTGCCGTGGG-3′). The probe was 3′-tailing labeled with digoxigenin (DIG), using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization, hybridization, and detection were carried out with a DIG luminescent detection kit that contained the alkaline phosphatase–conjugated anti-DIG antibody (Boehringer Mannheim) as recommended by the manufacturer. The relative quantity of mRNA was estimated by densitometry scanning with x-ray film.

Statistical Analysis. Data are presented as means ± SD. The mean and SD for serum total cholesterol, HDL-cholesterol, and VLDL + IDL + LDL-cholesterol for each time point were calculated. The significance of differences between control and kintoki groups was determined by Student's *t* test.

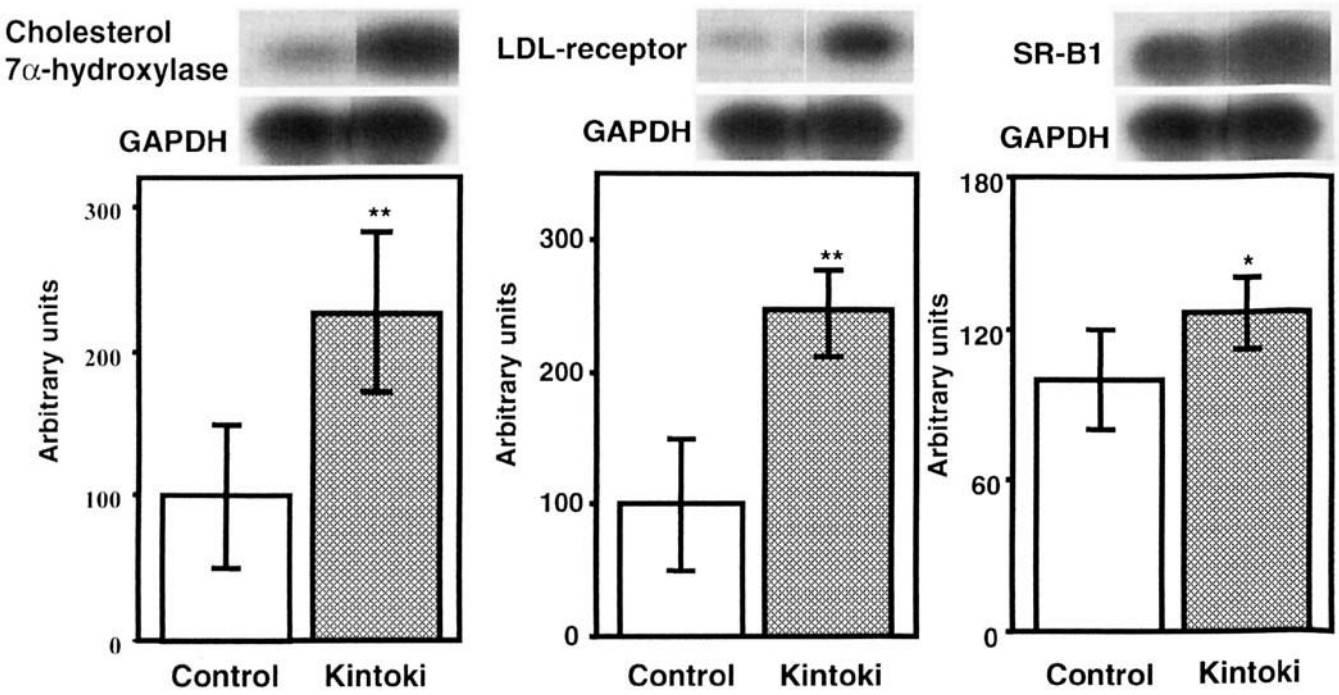

Figure 1. Hepatic cholesterol 7α-hydroxylase mRNA, LDL receptor mRNA, and SR-B1 mRNA levels in rats fed pancreatin-resistant fraction prepared from kintoki bean for 4 weeks. Values are means for five rats, with standard deviations indicated by bars. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* control. Control, cellulose powder; kintoki, pancreatin-resistant fraction prepared from kintoki bean.

Results

Dietary treatment did not significantly affect body weight gain (76 ± 5 and 74 ± 6 for kintoki and control groups, respectively), total food intake (382 ± 18 and 367 ± 23 g for kintoki and control groups, respectively), liver weight (9.1 ± 0.7 and 10.0 ± 0.4 g for kintoki and control groups, respectively), and cecum content (2.9 ± 0.5 and 3.6 ± 0.5 g for kintoki and control groups, respectively). The serum total cholesterol, VLDL + IDL + LDL-cholesterol, and HDL-cholesterol concentrations in the kintoki group were significantly (at least $P < 0.05$) lower than those in the control group throughout the feeding period (Table 1). There was no difference in the serum triglyceride concentration between control and kintoki groups throughout the feeding period. Hepatic cholesterol in the control group was significantly ($P < 0.01$) lower than that in the kintoki group (1.86 ± 0.25 and 2.88 ± 0.46 mg/g wet weight for control and kintoki groups, respectively).

The relative quantities of mRNAs were determined by Southern hybridization of PCR-amplified LDL receptor cDNA, SR-B1 cDNA, and cholesterol 7α-hydroxylase cDNA in the rat liver. The values of LDL receptor, cholesterol 7α-hydroxylase, and SR-B1 mRNAs were normalized to the value of GAPDH mRNA. Values from liver samples from rats fed the pancreatin-resistant fraction from kintoki bean were expressed relative to the average values of the control group, which were normalized to 100. The relative quantities of hepatic LDL receptor mRNA and cholesterol 7α-hydroxylase mRNA in the kintoki group were significantly ($P < 0.01$) higher than in the control group (Fig. 1). Hepatic SR-B1 mRNA level in the kintoki group was significantly ($P < 0.05$) higher than in the control group.

The cecal acetate, propionate, and *n*-butyrate concentrations, as well as total short-chain fatty acid concentrations, in the kintoki group all were significantly higher than in the control group (Table 2). There was no significant difference in the cecal pH level between kintoki and control groups. Although there was no significant difference in the fecal cholesterol excretion between the dietary groups, total

Table 2. Cecal Short-Chain Fatty Acid Concentrations and pH in Rats Fed Control Diet and Kintoki Diet for 4 Weeks[a]

Dietary Group	Acetic Acid	Propionic Acid	Butyric Acid	Total SCFA	pH
Control	16.64 ± 5.34	5.30 ± 4.91	4.08 ± 1.79	26.02 ± 8.89	7.16 ± 0.11
Kintoki	84.92 ± 34.46**	15.16 ± 5.92*	26.16 ± 8.41***	126.24 ± 45.36**	6.96 ± 0.24

[a] SCFA, short-chain fatty acid. Control, cellulose powder; Kintoki, pancreatin-resistant fraction prepared from kintoki bean. Values are expressed as means ± SD (μmol/g cecal content) for five rats.
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus Control.

Table 3. Fecal Cholesterol and Bile Acid Concentrations in Rats Fed Control Diet and Kintoki Diet for 4 Weeks[a]

Component	Dietary group (μmol/100 g body weight/day)	
	Control	Kintoki
Cholesterol	2.54 ± 0.51	2.37 ± 0.55
Cholic acid	<0.01	0.01 ± 0.008
Chenodeoxycholic acid	<0.01	0.05 ± 0.02
Deoxycholic acid	0.02 ± 0.01	0.13 ± 0.04**
Lithocholic acid	0.03 ± 0.01	0.22 ± 0.07**
Total bile acid	0.05 ± 0.03	0.41 ± 0.12**

[a] Control, cellulose powder; Kintoki, pancreatin-resistant fraction prepared from kintoki bean. Values are expressed as means ± SD for five rats.
* $P < 0.05$ and ** $P < 0.01$ versus Control.

fecal bile acid content in the kintoki group was significantly ($P < 0.01$) higher than in the control group (Table 3).

Discussion

In the current study, we examined the effects of pancreatin-resistant fraction prepared from kintoki bean on serum cholesterol and hepatic mRNA levels in rats. The serum total cholesterol concentration in the kintoki group was significantly lower than in the control group. This decrease in total cholesterol concentration in the kintoki group was due to lowering of VLDL + IDL + LDL-cholesterol and HDL-cholesterol concentrations. It is suggested that lowering the LDL-cholesterol and HDL-cholesterol concentrations might be an important factor in lowering the serum total cholesterol concentration. It has been reported that most of serum cholesterol in rats are associated with HDL fraction (27). The lowering of HDL-cholesterol concentration in the kintoki group may be associated with accelerated removal *via* the hepatic HDL receptor, as substituting the pancreatin-resistant fraction prepared from kintoki bean for cellulose in the diet promoted SR-B1 mRNA expression and lowered the serum HDL cholesterol concentration. Consistent with our previous report (9), the hepatic LDL receptor mRNA level in the kintoki group was significantly higher than in the control group. We speculate that the concentration of hepatic cholesterol in the kintoki group was due to an enhancing action of LDL receptor and/or SR-B1 activities in the liver.

The hypocholesterolemic effect of dietary fiber has been attributed to its ability to inhibit intestinal absorption of bile acids and neutral steroids, resulting in greater fecal bile acid and total steroid excretions. Moundras *et al.* (28) reported that the plasma cholesterol–lowering effect of dietary fiber was derived from the increased fecal loss of steroids. Buhman *et al.* (29) also demonstrated that feeding psyllium to rats enhanced the hepatic cholesterol 7α-hydroxylase mRNA and fecal excretion of bile acid and total steroids. In the current study, feeding pancreatin-resistant fraction prepared from kintoki beans significantly increased fecal bile acid excretion compared to the cellulose diet. This difference may be due to the unique properties of RS, such as gelling and binding of bile acids, that increase viscosity of intestinal contents and reduce absorption of bile acid from small intestine. We also observed that the cholesterol 7α-hydroxylase mRNA level in the kintoki group was significantly higher than in the control group. Because the pancreatin-resistant fraction prepared from kintoki bean has components similar to those of dietary fiber, it may combine with bile acids (30) to reduce the amount of bile acids absorbed into the liver through the enterohepatic circulation.

It has been reported that SCFAs may be responsible for the plasma cholesterol–lowering effect (10, 11, 31). In this study, there were significant differences in the cecal acetate, propionate, and *n*-butyrate concentrations among the groups. Higher proportions of *n*-butyrate have been reported to be related to shorter cecal transit time. Mathers and Dawson (32) reported a reverse correlation between molar proportion of *n*-butyrate in cecal contents and cecal transit time in rats fed various diets. Yajima (33) also found that the movement of digesta through the colon is stimulated by *n*-butyrate rather than propionate, thereby promoting gastrointestinal transit time as well as normal laxation. The cecal *n*-butyrate concentrations in our rats correlated positively with the fecal total bile acid concentration ($r = 0.874$; $P < 0.01$). Similarly, the cecal total SCFA level was positively correlated with the fecal total bile acid concentration ($r = 0.841$; $P < 0.01$).

It is estimated that 8–40 g of resistant starch is consumed per day in Western diets (3), which is similar to the amount of nonstarch polysaccharides ingested daily (8–18 g). We also found the same hypocholesterolemic effects in rats fed pancreatin-resistant fraction (1 g/81 kcal/day) prepared from retrograded starch of kintoki bean. However, we do not know whether the RS can reduce the serum cholesterol in humans at the level of 33 g/2700 kcal/day. Although several studies have shown that RS can lower blood lipids in rats (9–11), similar findings have not been observed in humans (12, 13).

In conclusion, the effects of the pancreatin-resistant fraction prepared from kintoki bean were evident in rats compared with rats fed a cellulose diet. The pancreatin-resistant fraction prepared from kintoki bean elevated fecal bile acid excretions and reduced the serum total cholesterol, HDL-cholesterol, and VLDL + IDL + LDL-cholesterol concentrations. It appears that the cholesterol-lowering effect of pancreatin-resistant fraction prepared from kintoki bean is dependent on LDL receptor, cholesterol 7α-hydroxylase mRNA levels, and fecal steroid excretion accelerated by *n*-butyrate. These results demonstrate that the resistant starch of kintoki beans positively influences serum and liver lipid metabolism.

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