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Hypocholesterolemic Effect of Amaranth Squalene (*Amaranth esculantus*) in Rats Fed a High Cholesterol Diet

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Abstract

In experiment 1, rats (n=6) fed diet containing 10 g/kg cholesterol for 4 wk (control) with either no amaranth (control), amaranth grain (300 g/kg, AG) or amaranth oil (90 g/kg, AO). Both the AG and AO groups had lower concentration of serum and hepatic cholesterol and triglyceride than the controls ($p<0.05$). Fecal excretions of cholesterol and bile acid in AO group increased about 4 fold and 2 fold, respectively, while AG affected only bile acid excretion ($p<0.05$). In experiment 2, rats (n=6) were fed the cholesterol diet for 4 wk and injected intraperitoneally with saline (control) or amaranth squalene (AS) for 7 d. The hypolipidemic effect of AS was evident in both serum and liver. Fecal excretions of cholesterol and bile acid were greater ($p<0.05$) in AS than control. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase activity was reduced in AS group (11.6%, p=0.13). This study suggests that the cholesterol-lowering effect of AS is mediated by greater fecal elimination of steroids through interference with cholesterol absorption.

Key words: amaranth squalene, bile acids, cholesterol, rat

INTRODUCTION

Elevated serum cholesterol concentration is considered a major risk factors for coronary heart disease (CHD) and peripheral arterial disease (1). Increasing dietary fiber is a common dietary manipulations for reducing cholesterol levels (2). Among the different sources of fiber (vegetable, fruit and cereal), cereal fiber has been shown to have the greatest association with a reduced risk of CHD (2). Among the different types of fiber, soluble fiber has been shown to be more beneficial than insoluble fiber (3). Furthermore, the oil fractions of several cereals (barley, oats and rice) have been suggested to contain inhibitors of 3-hydroxy-methylglutaryl coenzyme (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis (4). Among these oil fractions, tocotrienols and squalene have been known to affect cholesterol biosynthesis. Amaranth (*Amaranthus spp.* L) is an old crop from South and Central Asia, and its grain has potential as an important food crop because of its exceptional nutritive value. The protein content of amaranth is 11 to 22%, and is rich in lysine and sulfur amino acids compared to common cereals such as corn, wheat, and rice (5-8). In addition to the unique characteristics of the major components, amaranth grain also contains high levels of calcium, iron, and sodium (9). Amaranth grain contains 10~18% fiber (9,10). It has been suggested that amaranth fiber behaves like soluble fibers in lowering serum cholesterol level (11), suggesting that amaranth grain might be useful as a part of a cholesterol-reducing diet. In addition, amaranth grain contains 2~4 times higher lipid content than other cereals (9,10) and the squalene content of amaranth oil is extremely high (6~8%) compared to other cereals (0.01~0.3%) or olive oil (0.1~0.7%) which is considered a good source of squalene (9,12). It has been suggested that squalene, a precursor of cholesterol, is absorbed in humans to some extent enhancing cholesterol synthesis and serum cholesterol concentration (13). However, the effect of squalene is still controversial. Indeed the increase in cholesterol synthesis is not associated with consistent increase of serum cholesterol levels, possibly as a result of a concomitant increase in fecal elimination (14). Miettinen and Vanhanen (13) supplemented humans with squalene (1 g/day for 9 wk) and found that cholesterol synthesis was not significantly increased even though cholesterol precursors in serum were increased. They suggest that conversion of squalene to cholesterol was inhibited by inhibition of HMG-CoA reductase activity. Inhibition of HMG-CoA reductase activity by squalene feeding has been demonstrated in an experimental animal (15). Hypocholesterolemic effects of amaranth grain and amaranth oil were attributed to the unsaturated fatty acids (10), the

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fraction of fiber other than soluble portion (11), tocotrienols, and squalene (16). The present study was designed to ascertain the effects of amaranth grain and amaranth oil on cholesterol metabolism of rats fed a high-cholesterol diet, and to determine the contribution of squalene from the oil fraction of amaranth grain on hypocholesterolemic effects of amaranth.

MATERIALS AND METHODS

Extraction of amaranth squalene

Amaranth grain was purchased from a market, the oil was extracted with hexane, and then degummed by storing at 0°C for 72 h. Amaranth squalene was obtained by silicagel column chromatography by the method of Lee et al. (17), and the purity was confirmed by TLC (data not shown) with shark liver squalene as a standard. Shark squalene was purchased from Sigma Co. (St. Louis, MO). The structure of the amaranth squalene was analyzed by $^{1}H/^{13}C$-NMR and electron impact mass spectrometry (EI-MS).

Animals and diets

Male Sprague-Dawley rats weighing between 110 and 130 g (Halla Laboratory Animal Center Co., Korea) were maintained at 24°C with a 12 h light-dark cycle. Animals were individually housed and fed a commercial chow diet for 1 wk before being divided into experimental groups (n=6). They were fed the high-cholesterol diet as a control for 4 wk. In experiment 1, amaranth grain (AG, 300 g/kg diet) and oil (AO, 90 g/kg diet) were substituted for a portion of control diet, and other ingredients were modified according to the composition of amaranth grain (9) to establish same percentages of carbohydrate, fat, protein, fiber, and mineral between diet groups. In experiment 2, rats were fed the cholesterol diet for 4 wk and saline (control) and amaranth squalene (AS) was injected (200 mg/kg BW) intraperitoneally for 7 d before sacrifice. Diet and water were given *ad libitum*. The experimental procedures were approved by the guidelines of the Animal Care and Use Committee of Korea University.

Experimental design

Experiment 1 was designed to examine the hypocholesterolemic effects of amaranth grain and oil. Rats were assigned to one of the three experimental diets shown in Table 1. Experiment 2 investigated whether squalene contributes to the hypocholesterolemic effects of amaranth.

Feces were collected for the last 3 d for analysis of steroids excreted. Collected feces were freeze-dried, weighed, ground and stored at -70°C until analyzed. After the 4 wk experimental period, rats were sacrificed by decapitation after withholding food for 12 h. Blood samples were collected from cervical wound for the determination of serum lipids. Livers were removed, rinsed with saline, and stored at -70°C for lipid analysis.

Table 1. Diet composition

Ingredients	Group		
	Control (g/kg)	Amaranth grain	Amaranth oil
Casein	200	160	200
DL-Methionine	3	3	3
Cornstarch	457	240	457
Sucrose	150	150	150
Corn oil	90	64	-
Cholesterol	10	10	10
Cellulose	40	30	40
Mineral mix[1)]	35	28	35
Vitamin mix[1)]	10	10	10
Choline bitartarate	2	2	2
Na-taurocholate	3	3	3
Amaranth	-	300	-
Amaranth oil	-	-	90

[1)]AIN-76 diet (American Institute of Nutrition 1977).

Serum and liver lipids

Serum concentrations of total cholesterol, HDL-cholesterol and triglyceride were measured enzymatically using commercially available kits (Yeongdong Pharm. Corp., Korea). The hepatic lipids were extracted using the procedure developed by Folch et al. (18). The dried lipid residues were dissolved in 1 mL of ethanol for cholesterol and triglyceride determination. Triton X-100 and sodium cholate solutions were added to produce final concentrations of 5 g/L and 3 mmol/L, respectively. Hepatic total cholesterol and triglyceride were measured using the kits described above.

HMG-CoA reductase activity

The liver microsomes were isolated according to the method of Hulcher et al. (19) with a slight modification. HMG-CoA reductase activity was assayed from measurement of released coenzyme A during the reduction of HMG-CoA to mevalonate (19). Microsomal protein concentrations were determined by the method of Lowry et al. (20).

Fecal steroids

Fecal steroids were assessed by cholesterol and bile acid excretion in feces. After extracted with ethyl ether, cholesterol was measured by the same method as the liver samples. Bile acid was assayed by the enzymatic method described by Bruusgaard (21) in ethanol extracts of feces.

Statistics

Data are expressed as mean ± SE. One-way ANOVA was used to determine treatment effects. Differences

among means were inspected using Duncan's multiple range test (22) and were considered to be significant at $p<0.05$.

RESULTS

Hypocholesterolemic effect of amaranth grain and oil (experiment 1)

Consumption of AG significantly decreased body weight gain and food intake ($p<0.05$), but AO affected neither (Table 2). Body weight gain of rats fed AG was decreased 66% with only 16% decrease in food intake resulting in almost a 50% decrease in food efficiency ratio (FER) which indicates the efficiency of the food for gaining body weight. Compared with the control group, consumption of AG for 4 wk significantly ($p<0.05$) reduced the levels of serum cholesterol and triglyceride by 21% and 47%, respectively (Table 2). The effects of these parameters in AO group were greater and reduced by 27% and 58%, respectively, compared to control. The concentration of serum HDL-cholesterol was not affected by AG consumption whereas AO consumption significantly ($p<0.05$) increased (56%) HDL-cholesterol concentrations. The ratios of HDL to total cholesterol were 1.5 and 2 fold greater in the AG and AO groups than the controls, respectively. That is, the greater hypocholesterolemic effect was observed in AO group than in AG group. The activities of HMG- CoA reductase were not significantly altered by AG or AO intake (data not shown). Hepatic cholesterol and triglyceride levels were reduced by 20% and 30% in AG group, and 14% and 22% in AO group compared to control, respectively. Fecal dry weight was greater ($p<0.05$) in AO group compared with AG and control groups (Table 3). Fecal excretions of cholesterol and bile acid were increased with AO consumption, while AG only affected bile acid excretion ($p<0.05$).

Table 3. Effects of amaranth grain and oil on fecal steroid excretion in rats fed cholesterol[1)]

	Group		
	Control	Amaranth grain	Amaranth oil
Food intake, g/3 d	41.6±8.3	38.0±1.7	35.5±1.8
Fecal dry weight, g/3 d	21.7±2.0^{a}	27.3±4.3^{ab}	30.6±2.0^{bc}
Fecal steroid			
Cholesterol, μmol/3 d	180.6±9.4^{a}	225.3±27.9^{a}	424.6±61.5^{b}
Bile acid, μmol/3 d	79.7±5.6^{a}	133.7±11.4^{b}	170.8±19.9^{c}

[1)]Values are means ± SE (n=6); Means in same row with different superscript are significantly different ($p<0.05$).

Effect of amaranth squalene on hypercholesterolemia (experiment 2)

To confirm the structure of AS, $^{1}H/^{13}C$-NMR and electron impact mass spectrometry (EI-MS) were measured. Amaranth squalene had m/z at 410 and major peaks at 69, 81 and 137. From the spectral data, both component was confirmed as 2,6,10,15,19,23-hexamethyl-2,6,10,14, 18,22-tetracosahexane (squalene: $C_{30}H_{50}$) (Fig. 1).

Gains in body weight and food intake were not significantly affected by the injection of AS for 7 days (Table 4). However, AS significantly ($p<0.05$) reduced serum cholesterol and triglyceride by 22% and 14%, respectively, and increased HDL-cholesterol by 36%. The ratio of HDL to total cholesterol was 67% greater in the AS group than control. Liver cholesterol and triglyceride levels were both reduced by 26% in the AS group. The HMG-CoA reductase activity tended to be lowered in the AS group (11.6%) compared with control ($p=0.13$). The HMG-CoA reductase activities were 333.8±46.6 and 295.4±60.7 pmol · min^{-1} · mg $protein^{-1}$ in control and AS, respectively.

Table 2. Effect of amaranth grain and oil on body weight, food intake, serum and liver lipids in rats fed cholesterol[1)]

	Group		
	Control	Amaranth grain	Amaranth oil
Body weight gain, g/4 wk	143±27^{a}	63±10^{b}	130±7^{a}
Food intake, g/4 wk	381.4±17^{a}	322.0±12^{b}	392.6±18^{a}
FER[2)]	0.37±0.01^{a}	0.20±0.01^{b}	0.33±0.02^{a}
Serum			
Total cholesterol, mmol/L	8.40±0.58^{a}	6.63±0.54^{b}	6.10±0.28^{b}
HDL cholesterol, mmol/L	0.98±0.12^{a}	1.03±0.03^{a}	1.53±0.05^{b}
HDL/total cholsterol	0.12±0.02^{a}	0.18±0.01^{b}	0.24±0.03^{c}
Triglyceride, mmol/L	5.64±1.09^{a}	2.98±0.44^{b}	2.38±0.29^{b}
Liver			
Cholesterol, mmol/g	71.6±3.2^{a}	57.6±2.3^{b}	61.9±2.8^{b}
Triglyceride, mmol/g	46.7±1.5^{a}	32.8±2.1^{b}	36.7±0.9^{b}

[1)]Values are means ± SE (n=8); Means in same row with different superscript are significantly different ($p<0.05$).
[2)]body weight gain (g) / food intake (g) × 100.

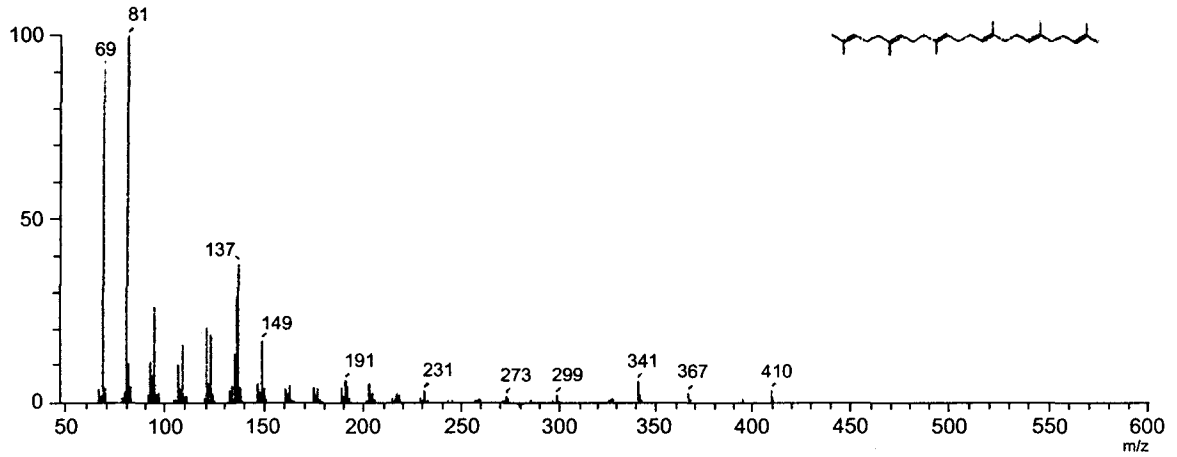

Fig. 1. Electron impact mass spectroscopy (EI-MS) analysis profile of amaranth squalene. Amaranth squalene had m/z at 410 and major peaks were at 69, 81 and 137. From the spectral data, both component was confirmed as 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosa-hexane (squalene: $C_{30}H_{50}$).

Fecal excretions of cholesterol and bile acid were also significantly increased in AS group compared with control group ($p<0.05$) (Table 5).

DISCUSSION

We examined the lipid-lowering potential of amaranth, especially the effect of amaranth squalene in rats. Although hypocholesterolemic effects of amaranth seed and oil have been reported in rats and chickens, the effect of amaranth squalene has not been reported.

In experiment 1, since the body weight gain of the AG group is significantly less than other groups, the effects on serum lipids in AG group could be partly due to a difference in weight gain. However, Jackson et al. (23) reported that the body weight gain varied 2-3 fold by different cereal source in rats fed a cholesterol diet, but serum total cholesterol and HDL-cholesterol levels were not significantly different. When fecal steroid excretions were normalized to body weight, the effects of AG on fecal excretion were greater. Consumption of AG increased cholesterol and bile excretion by 1.8 and 2.4 fold, respectively, per 100 g body weight. These results suggest that the effects of the AG diet on serum lipids were not completely due to a difference in weight gain. Additionally, the effect of AG on serum and hepatic lipid-lowering action could be due, at least in part, to the fiber type of AG. A number of studies have demonstrated that certain fiber such as pectin and guargum increase the excretion of endogenous cholesterol and bile acids compared to cellulose (23,24). Danz and Lupton (11) suggested that the fraction of fiber other than soluble portion in amaranth seeds might play a role in lowering cholesterol. However, consumption of AO had a greater effect on serum lipid profile and fecal steroid excretion than control or AG group, suggesting that fiber is not the only factor contributing to the lipid lowering action. Since the only dietary difference between the control and AO groups was the lipid source (corn oil vs. amaranth oil), this result suggests

Table 4. Effect of amaranth squalene injection on body weight gain, food intake, serum and liver lipids in hypercholesterolemic rats[1)]

	Group	
	Control	Amaranth squalene
Body weight gain, g/ 4 wk	111±6	80±13
Food intake, g/4 wk	413.5±15.2	346.4±36.2
Serum		
Total cholesterol, mmol/L	6.08±0.54[a]	4.72±0.09[b]
HDL cholesterol, mmol/L	0.67±0.08[a]	0.91±0.07[b]
HDL/total cholsterol	0.12±0.02[a]	0.20±0.02[b]
Triglyceride, mmol/L	3.51±0.15[a]	3.03±0.06[b]
Liver		
Cholesterol, mmol/g	35.91±0.56[a]	26.54±1.41[b]
Triglyceride, mmol/g	81.6±1.27[a]	60.27±3.22[b]

[1)]Values are means±SE (n=6); Means in same row with different superscript are significantly different ($p<0.05$).

Table 5. Effects of amaranth squalene injection on fecal cholesterol and bile acid excretion in hypercholesterolemic rats[1)]

	Group	
	Control	Amaranth squalene
Food intake, g/3 d	46.8±1.77[NS]	39.1±2.87
Fecal weight, g/3 d	8.1±0.73[ab]	10.2±0.77[b]
Fecal steroid		
Cholesterol, μmol/3 d	169.7±34.3[a]	283.9±71.1[b]
Bile acid, μmol/3 d	125.4±20.1[a]	182.3±15.9[b]

[1)]Values are means±SE (n=6); Means in same row with different superscript are significantly different ($p<0.05$).

the potential effect of the lipid fraction in amaranth. Dietary fat is a crucial factor in the regulation of serum cholesterol levels, and there is overwhelming evidence to support the hypocholesterolemic effect of vegetable oil that is rich in polyunsaturated fatty acids, mainly linoleic acid (25). The fatty acid composition of AO is similar to that of corn oil, with linoleic acid being the predominant fatty acid (9). Moreover, corn oil contains slightly higher unsaturated fatty acid, especially oleic acid, than AO (9), eliminating unsaturated fatty acid in AO as the cholesterol-lowering element. These results imply that non fatty acid component(s) might be involved in hypocholesterolemic action of AO. Although plant sterols and tocotrienol appear to exert a combined hypocholesterolemic effect, the difference between the sterol and tocotrienol compositions in control and experimental groups is insufficient to cause such a contrasting effect we have seen. Therefore, the possible regulatory role of squalene, which occurs in markedly higher concentrations in AO than corn oil, on hypocholosterolemic action was examined.

Of interest was the finding that AS injection significantly reduced serum and hepatic cholesterol, leading to a 45% decreases in the atherogenic index compared with the control group. Therefore, our data support a clear cholesterol-lowering action of AS. However, results concerning cholesterol-lowering effect of squalene are less conclusive. Oral administration of shark squalene has no effect on serum and hepatic cholesterol and triglyceride concentrations in rat (26). Nine weeks of 1 g squalene administration caused increase in serum cholesterol concentrations in humans (13). Nevertheless, squalene is suggested to reduce serum cholesterol by inhibition HMG CoA reductase (9). Unfortunately, since these reports do not clearly indicate the source of squalene (plant vs. animal), therefore, it is difficult to compare those results directly with ours.

Substantial amount of dietary squalene is absorbed and converted to cholesterol in humans; however, this increase in synthesis is not associated with consistent increase in serum cholesterol concentrations. Several hypotheses concerning the mechanism(s) by which squalene elicits hypocholesterolemic effect have been proposed. The most frequently suggested mechanism is interference with intestinal cholesterol and bile acid absorption, leading to an increase in fecal neutral sterol and bile acid excretion. The excretion of fecal cholesterol and bile acid was significantly greater in the AS group than in control, suggesting reduced intestinal absorption of cholesterol by AS injection. These results coincide with lowered serum and hepatic lipid levels in the AS group. Cholesterol homeostasis is a delicate balance among dietary intake, synthesis, and catabolism. Serum total cholesterol can be also lowered when cholesterol synthesis is inhibited. HMG-CoA reductase mediates the first committed step in the *de novo* synthesis of cholesterol. The inhibition of this rate-limiting enzyme by plant sterols and hypocholesterolemic drugs is one method of reducing serum cholesterol (27). In this study, the activity of HMG-CoA reductase was decreased in the AS group, suggesting that inhibition of this enzyme may be part of the cholesterol lowering mechanism of AS.

In summary, the present study demonstrated that the cholesterol-lowering effect of amaranth is, at least in part, associated with its squalene content. The effect of squalene can be attributed to the enhanced excretion of fecal steroids through interference of cholesterol absorption. Further study is needed to further elucidate the mechanism of the AS modulation of cholesterol metabolism.

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