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RESEARCH NOTE

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Cholesterol Lowering Effect of Enzymatic Hydrolysates of Squid in Rats

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Abstract This study evaluated effects of enzymatic hydrolysates of squid on cholesterol lowering in rats. Thirty male rats were blocked into 3 groups [high cholesterol diet (control), 5% normal squid, and 5% enzymatic hydrolysates of squid] and were raised for 10 weeks. Triglyceride level in enzymatic hydrolysates of squid-fed rats was lower than that in the control. Serum low density lipoprotein-cholesterol level followed in the order of control>normal squid>enzymatic hydrolysates. Serum high density lipoprotein-cholesterol level in enzymatic hydrolysates of squid-fed rats was higher than that in control rats. Liver cholesterol level in enzymatic hydrolysates of squid-fed rats was lower than that in control rats.

Keywords: squid, enzymatic hydrolysis, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol, triglyceride

Introduction

Most consumers are concerned about excessive intake of fats and the accumulation of adipose as major contributing factors as a strong positive correlation exists between increased serum cholesterol concentrations and the risk of coronary heart disease (1). In recent years, many reports have focused on how to decrease serum lipid concentrations and the absorption of fat in the intestinal tract to reduce diet-related chronic disease (2,3).

Squid (*Todarodes pacificus*), one of the most important commercial fishes over the world, is a cephalopod of the Ommastrephes genus. Tanaka *et al*. (4) observed that the serum cholesterol concentration was significantly lower in mice fed squid with 0.1% cholesterol diet when compared with that in mice fed a diet without squid. Furthermore, it has been reported that the nonlipid fraction of squid exerts a hypocholestrolemic effect by increasing the excretion of total steroids in feces, and the fraction induces a triglyceride-lowering activity in the liver by decreasing hepatic lipogenesis (5).

Enzymatic modification of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is generally employed in the food industry. The resulting protein hydrolysates are known to act as potential physiological modulators of metabolism during intestinal digestion of nutrients (6,7). The hydrolysis of protein with proteolytic enzymes can provide more marketable and value-added products (6). The protein hydrolysates have been reported to possess antioxdative, antihypertensive, antimicrobial, and hypocholesterol properties (6-12). In general, Alcalase 2.4 L-assisted reactions have been widely employed for producing fish hydrolysates, because of the high degree of hydrolysis that may be completed in a relatively short time under moderate pH conditions (12).

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Although the hypocholesterolemic action of squid has been explained by depressing cholesterol absorption and by interfering with bile absorption (4,5), the mechanism is still unclear. Moreover, the hypocholesterolemic effects of the enzymatic hydrolysates of squid have not been examined yet. Therefore, the present study was conducted to investigate cholesterol lowering effects in serum and liver of the enzymatic hydrolysates of squid in rats.

Materials and Methods

Materials Squid was purchased from a local fish market (Jumunjin, Korea) and stored at –40°C until needed. Alcalase 2.4 L was purchased from Novo Co. (Bagsvaerd, Denmark). Cholesterol and 5α-cholestane were obtained from Sigma-Aldrich (St. Louis, MO, USA), and all other reagents were of analytical grade.

Preparation of normal squid and enzymatic hydrolysates of squid Normal squid and enzymatic hydrolysates of squid were prepared using the procedure described by Choi (13). Briefly, the frozen squid meat was freeze-dried using a freeze dryer (Type FD-1000; Eyela, Tokyo, Japan) and ground to make dried normal squid. To prepare the enzymatic hydrolysates of squid, 100 g of the normal squid was added with 500 mL of distilled water, and then incubated at pH 7.0, hydrolysis time 5.9 hr, and Alcalase 2.4 L concentration 2.4%. The hydrolysis was terminated by the addition of 500 mL of 20% trichloroacetic acid (TCA) followed by centrifugation (10,000×g, 15 min) to collect the 10% TCA soluble material as the supernatant. The sediments were freeze-dried using a freeze dryer (Type FD-1000; Eyela) and ground to make the enzymatic hydrolysates of squid.

Animals and diets Thirty-five-week-old male rats (175± 10 g) of Sprague Dawley strain were purchased from Jung-Ang Laboratory Animal, Inc. (Seoul, Korea). After an 1-week adaptation period on an *ad libitum* chow diet with distilled water, the animals were divided into 3 dietary

groups fed as follows: 1) control, non-normal squid-enriched high cholesterol diet, 2) normal squid, normal squid-enriched high cholesterol diet, and 3) enzymatic hydrolysates of squid, enzymatic hydrolysates of squid-enriched high cholesterol diet. High cholesterol diets were formulated according to the recommendations of the American Institute of Nutrition (AIN-93G purified rodent diet). During the 10-week experimental period, all rats were allowed access to diets and distilled water *ad libitum*. Rats were housed in individual cages at 22±3°C, 50±10% humidity, and lights on from 19:00 to 07:00 hr.

Sampling At the end of the experimental period and once a week, all rats were fasted for 24 hr, and blood was collected from orbital sinus from eyes. Blood samples were centrifuged at 3,000×g (Centrifuge 5415C; Eppendorf, Hamburg, Germany) for 15 min to extract serum, and all serum samples were preserved in a deep freezer. The liver was collected at the end of experiment and feces were collected for 24 hr during the last 3 days of experiment. The samples were stored at −20°C until analysis.

Serum lipid concentrations Total serum cholesterol, triglyceride (TG), and high density lipoprotein-cholesterol (HDL-C) contents were measured by total cholesterol-PII, TG-PII, and HDL-C-P, respectively, from Fuji Photo Film Co., Ltd. (Kanagawa-ken, Japan). The low density lipoprotein-cholesterol (LDL-C) content was calculated by the following equation described by Friedewald *et al*. (14):

$$\text{LDL-C content} = \text{total cholesterol content} - (\text{HDL-C content} + \text{TG}/5)$$

Liver lipid concentrations Liver lipid total cholesterol and triglyceride were extracted using the procedure described by Folch *et al*. (15). Total liver cholesterol and triglyceride contents were measured by TCHO-PII and TG-PII, respectively, from Fuji Photo Film Co., Ltd.

Fecal excretion of total cholesterol and bile acids The cholesterol and bile acid in feces were extracted using the procedure described by Czubayko *et al*. (16). The bile acid was quantified using the Runpia bile acid kit (Kyokuto Co., Ltd., Tokyo, Japan).

For the quantification of cholesterol, 1 g of a sample was placed in a screw-capped glass tube (15×180 mm), and 1 mL of 5α-cholestane (1 mg/mL) was added as an internal standard. The sample was saponified at 60°C for 30 min with 5 mL of 2 M ethanolic potassium hydroxide solution (17). The process was repeated 4 times. The hexane layers were transferred to a round-bottomed flask and dried under vacuum. The extract was re-dissolved in 1 mL of hexane and was stored at −20°C until analysis.

The cholesterol was determined on a silica fused capillary column (HP-5; 30 m×0.32 mm i.d.×0.25 μm thickness) using a Hewlett-Packard 5890A gas chromatography (Palo Alto, CA, USA) equipped with a flame ionization detector. The injector and detector temperature were 270 and 300°C, respectively. The oven temperatures were programmed from 200 to 300°C at 10°C/min and held for 20 min. Nitrogen was used as a carrier gas at a flow rate of 2 mL/min with a split ratio of 1:50. Quantification of cholesterol was done by comparing the peak areas with the response of an internal standard.

Statistical analysis Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (SAS Institute, Cary, NC, USA, 1985), and differences among treatments were determined by Duncan's multiple test at $p<0.05$, unless otherwise stated.

Results and Discussion

Serum lipids Serum lipid concentrations of rats are shown in Table 1. In blood analysis, after 10 weeks of the high cholesterol and squid feeding, the total serum cholesterol was 185.75, 172.15, and 159.92 mg/dL in the control, normal squid, and enzymatic hydrolysates of squid-fed groups, respectively. The cholesterol-lowering effect in normal squid was consistent with Tanaka *et al*. (4) who observed that the serum cholesterol concentration was significantly lower in mice fed squid with 0.1% cholesterol diet when compared with that in mice fed a diet without squid. In the present study, the enzymatic hydrolysates of squid-fed rats showed a remarkable decrease in the total serum cholesterol level as compared to the control-fed rats. Based on these data, it is indicated in the present study that feeding 5% of the enzymatic hydrolysates of squid can lead to a marked hypocholesterolemic effect after 10 weeks.

The triglyceride concentration (54.36 mg/dL) of the enzymatic hydrolysates of squid-fed rats was considerably lower than that (69.15 mg/dL) of the control group, while feeding the normal squid did not significantly affect the triglyceride concentration as compared to the control group.

Among all the groups, the serum LDL-cholesterol level followed the order: control-fed rats>normal squid-fed rats >enzymatic hydrolysates of squid-fed rats. Especially, the serum LDL concentration in the enzymatic hydrolysates of squid-fed rats was decreased by 18.8% of the control and 11.4% of the normal squid-fed rats. The serum HDL-

Table 1. Effects of experimental diets on the change of blood total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, and triglyceride in rats fed enzymatic hydrolysates of squid for 10 weeks

Group[1)]	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	Triglyceride (mg/dL)
Control*	185.75±21.21[a2)]	21.42±4.92[b]	150.51±20.67[a]	69.15±19.79[a]
Normal squid**	172.15±8.77[ab]	22.32±3.76[ab]	137.91±8.62[b]	59.57±10.83[ab]
Enzymatic hydrolysates of squid***	159.92±15.51[b]	26.83±6.09[a]	122.22±18.10[c]	54.36±10.94[b]

[1)] *Cholesterol diet+no supplemented squid, **cholesterol diet+5% normal squid, ***cholesterol diet+5% enzymatic hydrolysates of squid.
[2)] Values are mean±SD of 10 rats/group; Values within the same column with different superscripts are significantly different at $p<0.05$ by Duncan's multiple test.

Table 2. Effects of experimental diets on the change of liver lipid total cholesterol and triglyceride in rats fed enzymatic hydrolysates of squid for 10 weeks

Group[1)]	Total cholesterol (mg/g)	Triglyceride (mg/g)
Control*	23.39±12.15[a2)]	37.25±13.57[a]
Normal squid**	22.38±12.00[ab]	32.36±14.60[ab]
Enzymatic hydrolysates of squid***	14.21±1.32[b]	24.17±2.65[b]

[1)]*Cholesterol diet+no supplemented squid, **cholesterol diet+5% normal squid, ***cholesterol diet+5% enzymatic hydrolysates of squid.
[2)]Values are mean±SD of 10 rats/group; Values within the same column with different superscripts are significantly different at $p<0.05$ by Duncan's multiple test.

Table 3. Effects of experimental diets on the change of fecal excretion of total cholesterol and bile acids in rats fed enzymatic hydrolysates of squid for 10 weeks

Group[1)]	Total cholesterol (mg/day)	Bile acids (μmol/day)
Control*	131.88±19.76[b2)]	81.56±5.59[b]
Normal squid**	153.77±26.76[ab]	86.93±26.76[b]
Enzymatic hydrolysates of squid***	160.74±26.44[a]	96.66±10.92[a]

[1)]*Cholesterol diet+no supplemented squid, **cholesterol diet+5% normal squid, ***cholesterol diet+5% enzymatic hydrolysates of squid.
[2)]Values are mean±SD of 10 rats/group; Values within the same column with different superscripts are significantly different at $p<0.05$ by Duncan's multiple test.

cholesterol level in the enzymatic hydrolysates of squid-fed rats was markedly higher than that in the control rats, while HDL-cholesterol level in the normal squid-fed rats was not significantly different from that in the control rats. Moreover, feeding the enzymatic hydrolysates of squid showed the considerable decrease in LDL-cholesterol, whereas the slight increase in HDL-cholesterol compared with the normal squid feeding, demonstrating that the enzymatic hydrolysates of squid could influence more on the LDL-cholesterol level. Therefore, it is indicated in the present study that feeding the enzymatic hydrolysates of squid can be effective in reducing the serum total cholesterol, LDL-cholesterol, and triglyceride contents and in increasing the serum HDL-cholesterol content.

Liver lipids The effects of feeding the normal squid or enzymatic hydrolysates of squid on liver lipid in rats are presented in Table 2. The liver cholesterol (14.21 mg/g) and triglyceride (24.17 mg/g) levels in the enzymatic hydrolysates of squid-fed rats were considerably lower than the liver cholesterol (23.39 mg/g) and triglyceride (37.25 mg/g) levels in the control-fed rats. On the other hand, the liver cholesterol and triglyceride levels in the normal squid-fed rats were not significantly different from those in the control-fed rats. Based on the data obtained from the present study, it is plausible that feeding the enzymatic hydrolysates of squid to rats could cause a decrease in the level of the liver cholesterol and triglyceride.

Fecal excretion of total cholesterol and bile acids The lipid contents of dry feces collected for the last 3 days were measured as shown in Table 3. Fecal cholesterol and bile acids in the enzymatic hydrolysates of squid-fed rats were remarkably higher than those in the control rats, while fecal cholesterol and bile acids in the normal-fed rats were not significantly different from those in the control rats. In other words, when fed the supplement of the enzymatic hydrolysates of squid, rats excreted significantly much fat than those fed the control diet. Previous studies reported the effect of squid for combating hypercholesterolemia (4,5). Tanaka *et al.* (4) found that the serum cholesterol concentration was significantly lower in mice fed squid with 0.1% cholesterol diet when compared with that in mice fed a diet without squid. According to them, the hypocholestemic effects of squid can be associated with the non-lipid fraction, possibly a protein fraction, of squid. Furthermore, it was revealed that the non-lipid fraction of the squid would bind to the bile acids in the intestinal lumen, interfere with the micellar formation of cholesterol, and hence, inhibit cholesterol absorption and increase bile acid excretion into the feces (5).

Based on the data obtained from the present study, it is concluded that the enzymatic hydrolysates of squid can be effective in lowering serum and liver lipid levels in rats fed high-fat diets and in increasing excretion of lipids in feces. In addition, all of these results can provide greater insight on the potential of the food application of the enzymatic hydrolysates of squid.

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