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Sericin Reduces Serum Cholesterol in Rats and Cholesterol Uptake into Caco-2 Cells

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A cholesterol lowering effect of sericin was investigated both in vivo and in vitro. Rats were dosed with cholesterol with and without sericin for 14 days. Non-high-density lipoprotein (HDL) and total serum cholesterols were reduced in rats fed high-cholesterol diet with all three tested doses of sericin (10, 100, and 1000 mg kg^{-1} day^{-1}). The potential mechanism of actions was determined by measuring the uptake of radiolabeled cholesterol into differentiated Caco-2 cells and cholesterol solubility in mixed lipid micelles. Concentration of sericin as low as 25 and 50 μg/mL inhibited 30% of cholesterol uptake into Caco-2 cells whereas no effect was found at higher concentration. Cholesterol micellar solubility was reduced in the presence of sericin. This study suggests the cholesterol lowering effect of sericin results from its inhibition of cholesterol absorption in intestinal cells and its reduction of cholesterol solubility in lipid micelles.

KEYWORDS: Cholesterol lowering effect; cholesterol uptake; sericin; silk protein; Caco-2 cell; cholesterol solubility

INTRODUCTION

There is growing interest not only in functional foods but also in dietary supplements and nutraceutical products to improve human health, especially those that act prophylactically to prevent disease. Elevated levels of blood cholesterol increase risk of vascular disease, and the current treatment of choice for blood cholesterol lowering agents is the statins. These HMG-CoA reductase inhibitors are generally considered safe though a few undesirable effects of these have recently been identified. Thus the search for alternative agents continues. Several reports suggest that various types of dietary proteins could affect serum cholesterol levels (*1*−*5*). The most extensively studied proteins are proteins from soybean and milk. Some other proteins such as those found in rice, buckwheat, potato, and sunflower appear to lower cholesterol (*1*, *2*, *6*, *7*).

Sericin is a protein removed from the silk cocoon, and most of this appears as waste in silk processing, and in Thailand alone tons lost annually represents a potentially valuable product. Silk proteins produced by silkworm are composed of fibroin and sericin (25−30% of total cocoon weight). Sericin has high serine content (30−33%), and in forming a cocoon, it glues the fibroin together. Sericin is a mixture of macromolecule polypeptides of molecular mass varying between 10 and 300 kDa (*8*). In the past decade, various biological activities have been reported including antioxidation (*9*), inhibition of tyrosinase (*9*), and protection against tumorigenesis (*10*−*12*), alcohol-induced liver, gastric injuries (*13*), and UV-induced keratinocyte apoptosis (*14*). Thus sericin is currently employed in some food and cosmetic products.

Sericin is protease-resistant and thus relatively resistant to proteolysis in the gastrointestinal tract (*15*), and this explains why it is capable of protecting against 1,2-dimethylhydrazine-induced colon tumorigenesis in animals (*10*, *11*). Thus this physicochemical characteristic indicates that further biological effects within the gastrointestinal tract might be explored such as cholesterol lowering. It was recently reported that dietary sericin lowered the levels of triglyceride and cholesterol in rats fed a high-fat diet (*16*). However, the mechanism of this lipid lowering effect is still unknown. Thus, the present study was carried out to verify the hypocholesterolemic activity of sericin in rats fed high cholesterol diet and its potential mechanisms such as inhibition of cholesterol absorption in differentiated Caco-2 cells and disruption of cholesterol micellar solubility.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM)/F12, other cell culture materials, cholesterol, phosphatidylcholine, and sodium taurocholate were all purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA). [1α,2α(n)-^{3}H]Cholesterol was purchased from Perkin-Elmer (Wellesley, MA). Sericin was provided by the Institution of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Its amino acid composition is shown in **Table 1**, which is similar to that of previous reports (*11*, *13*).

Animal Experiments. Twenty male Sprague-Dawley rats (200−250 g) were obtained from the National Laboratory Animal Centre, Mahidol University, Thailand. All animal procedures were approved by the Animal

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Table 1. Amino Acid Composition of Sericin

amino acid	% w/w	amino acid	% w/w
serine	33.40	valine	2.80
aspartate	16.70	histidine	1.30
glutamate	4.40	leucine	1.10
glycine	13.50	isoleucine	0.70
threonine	9.70	phenylalanine	0.50
lysine	3.30	tryptophan	0.70
tyrosine	2.60	proline	0.70
arginine	3.10	cystine	0.20
alanine	6.00	methionine	0.04

Research Ethics Committee, Naresuan University, Thailand. Animals were housed in stainless steel cages at 22 ± 2 °C with a 12 h light/dark cycle and had free access to water and food during 1 week of acclimatization. Rats were randomly divided into four groups of five animals each and fed a standard rat diet. A lipid mixture of cholesterol, bile extract, and coconut oil was prepared and daily administered with and without sericin solution by gastric tube for 14 days. Each rat received cholesterol, bile extract, and coconut oil at 1.5, 0.75, and 0.75 g (kg body weight)$^{-1}$ day^{-1}, respectively. The percentage of cholesterol that each animal received was approximately 2% of daily food intake. Three doses of sericin at 10, 100, and 1000 mg kg^{-1} day^{-1} were tested. Fasting blood was collected at day 0 (baseline), 4, 8, 11, and 14 from the tail vein to measure serum lipid levels. During the experimental period, body weight and food intake were recorded every other day.

Measurement of Serum Lipid Levels. Serum total cholesterol, HDL cholesterol, and triglyceride levels were measured using commercial enzymatic assay kits (HUMAN GmbH, Germany). The procedures were conducted as shown in the protocol provided by the manufacturer but adapted for rat samples. Non-HDL cholesterol was calculated by subtracting HDL cholesterol levels from total cholesterol levels.

Cell Culture Preparation. Caco-2 cells were obtained from the American Type Culture Collection (ATCC). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. The cells were maintained at 37 °C in a 5% CO_2 incubator. All cells were grown in culture flasks and then plated out into 24-well plates to assess cholesterol uptake.

Cholesterol Micelle Preparation. Briefly, stock solutions of [1α,2α(n)-^{3}H]cholesterol, cholesterol, and phosphatidylcholine were dissolved in chloroform, and a stock solution of sodium taurocholate was prepared in methanol. These lipid and bile salt solutions were mixed and evaporated under a nitrogen stream. The lipid film was stored under nitrogen gas at −20 °C until use. The micelle solutions were freshly prepared by hydrating the lipid film in serum-free DMEM/F12 to give final concentrations of 1 μM cholesterol, 2 mM sodium taurocholate, 50 μM phosphatidylcholine, and 1 μCi/mL [1α,2α(n)-^{3}H]cholesterol. The micelle solutions were sonicated and passed through a 0.22 μm syringe filter and kept at 37 °C. Subsequently, the solutions were added to the plated Caco-2 cells.

Cholesterol Uptake Determination. Caco-2 cells were seeded on 24-well plates at a cell density 50000 cells/well and cultured for 14 days to allow differentiation with fresh medium given every 2 days. After 14 days, the cells were incubated with serum-free medium overnight and then treated with sericin or ezetimibe (positive control) for 1 h before incubating with [1α,2α(n)-^{3}H]cholesterol micelles. After a 3 h incubation, the cells were washed twice with ice-cold PBS and then disrupted with 0.2 N NaOH and 0.1% Triton X-100. One part of the aliquot was added to a scintillation cocktail (MicroScint-20; PerkinElmer, MA, USA); the other part was taken for protein determination by BCA protein assay kit (Pierce, IL, USA). The radioactivities of cell lysates were measured using a Packard β-counter.

Micellar Cholesterol Solubility Determination. Cholesterol micelles were prepared by ultrasonication of a 7 mL micellar dispersion. This solution contained 10 mM sodium taurocholate, 2 mM cholesterol, 5 mM oleic acid, 132 mM NaCl, and 15 mM sodium phosphate (pH 7.4). Various concentrations of sericin or BSA (0.25−2 mg/mL) were added either before or after micelle preparation and incubated for 24 h at 37 °C. The precipitated cholesterol was separated from the intermicellar cholesterol by filtering through a 0.22 μm syringe filter. The intermicellar cholesterol concentrations in the filtrates were determined using the cholesterol assay kit (HUMAN GmbH, Germany).

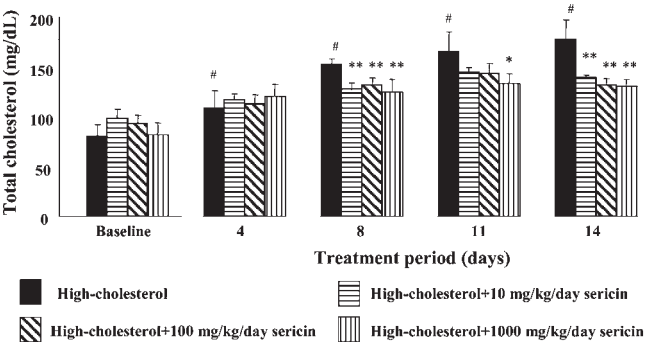

Figure 1. Serum total cholesterol in rats fed high-cholesterol diet. Rats were fed cholesterol (2% cholesterol of total daily diet) with and without sericin at 10, 100, and 1000 mg kg^{-1} day^{-1} for 14 days. The data represent mean ± SD (#, $p \leq 0.05$ compared to baseline; *, $p \leq 0.05$, and **, $p \leq 0.01$, compared to high-cholesterol group, $n = 5$).

Statistical Analysis. All data from animal experiments are expressed as means ± standard deviation (SD), and means ± standard error of means (SEM) were used for the in vitro experiments. The data were analyzed by *t* test, paired *t* test, and one-way analysis of variance (ANOVA). Differences were considered to be significant when the *p* value is equal to or lower than 0.05.

RESULTS

Lipid Profile in Rat Serum. Over the 14 day sampling period, there was a progressive increase in serum cholesterol in the control group (**Figure 1**), and even at 4 days, there was a clear elevation. By day 14, the control rats showed substantial serum cholesterol increases while sericin treatments at all studied doses inhibited partially but significantly such cholesterol enhancement. Thus clear differences in the treatment group emerged by day 8 (**Figure 1**). **Table 2** shows the data for the complete lipid profile expressed as the differences for values at day 14 compared to day 0. Although sericin inhibited total and non-HDL cholesterol increase, the treatments with different doses of sericin did not show a clear dose-dependent fashion (**Figure 2**).

For HDL cholesterol, there was no consistent trend over the 14 days in any of the groups and no difference between the sericin and controls. Serum TG levels were rather erratic over the treatment period, but no noticeable changes due to sericin could be discerned (**Figure 2**). None of the cholesterol effects could be related to food intake since this remained constant during the 14 days. Taken together, sericin supplement effectively decreased the enhancement of serum levels of total cholesterol and non-HDL cholesterol in rats fed high-cholesterol diet.

Cholesterol Uptake in Differentiated Caco-2 Cells. To examine the effect of the sericin on cholesterol absorption in the intestine, differentiated Caco-2 cells were used as an in vitro model. The uptake of [1α2α(n)-^{3}H]cholesterol by Caco-2 cells was expressed as the amount of tritium cholesterol per mg of protein of cell lysates. In this study, ezetimibe, an inhibitor cholesterol across the gut wall, was used as a positive control, and it caused a substantial depression of cholesterol uptake (**Figure 3**). Bovine serum albumin (BSA, 25−1000 μg/mL) was employed as a control protein, and it showed no effect on cholesterol uptake at any of the concentrations tested. With sericin at 25 and 50 μg/mL, there was a reduction in cholesterol uptake (**Figure 3**), but there was no apparent effect at the higher concentrations (100−1000 μg/mL). This result suggests that sericin can reduce the uptake of cholesterol into Caco-2 cells, and this effect is not dose-dependent.

Lipid Micellar Cholesterol Solubility. The effect of sericin on the micellar solubility of cholesterol is shown in **Table 3**. The cholesterol solubility in mixed lipid micelles appeared to be decreased in the

Table 2. Differences for Serum Lipid Levels and Amount of Food Intake after 14 Days of Treatment Compared to Those at Day 0[a]

groups	changes from day 0 (mg/dL) total cholesterol[b]	HDL-C	non-HDL-C[b]	triglyceride	food intake (g day^{-1} (100 g BW)$^{-1}$)
high cholesterol	97.45 ± 31.52	−0.38 ± 1.49	97.83 ± 31.01	56.05 ± 40.70	6.56 ± 1.34
high cholesterol + sericin, 10 mg kg^{-1} day^{-1}	41.06 ± 7.59**	1.80 ± 3.87	39.25 ± 4.26**	30.71 ± 29.00	6.18 ± 0.29
high cholesterol + sericin, 100 mg kg^{-1} day^{-1}	38.61 ± 12.45**	2.64 ± 4.80	35.97 ± 16.84**	72.79 ± 43.02	8.43 ± 1.86
high cholesterol + sericin, 1000 mg kg^{-1} day^{-1}	49.05 ± 13.02*	2.64 ± 4.80	51.75 ± 9.84*	97.87 ± 16.61	7.52 ± 0.51

[a] Values are expressed as mean ± SD (n = 5). [b] Significantly different from high cholesterol group (*, $p \leq 0.05$; **, $p \leq 0.01$).

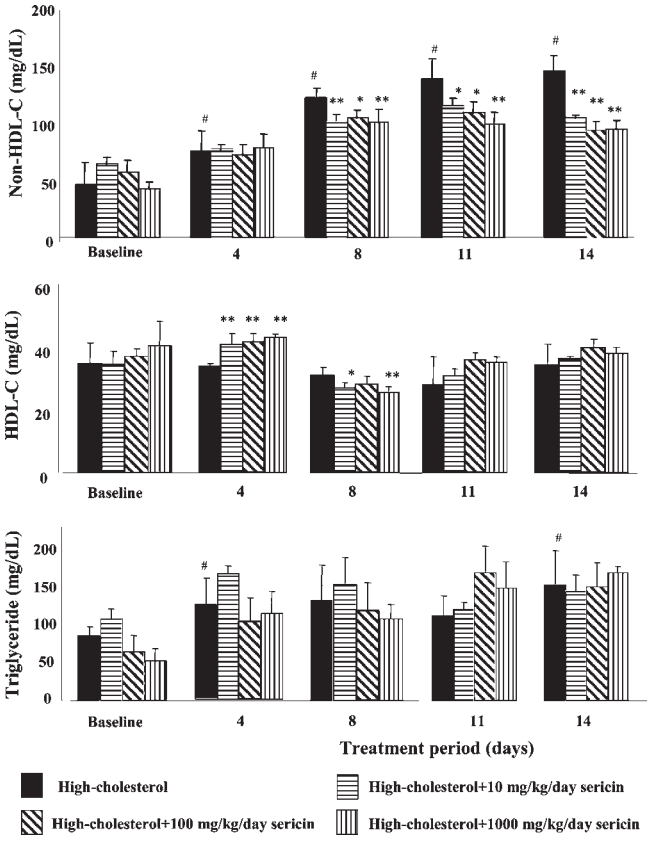

Figure 2. Serum HDL cholesterol (HDL-C), non-HDL cholesterol (non-HDL-C), and triglyceride in rats fed high-cholesterol diet. Rats were fed cholesterol (2% cholesterol of total daily diet) with and without sericin at 10, 100, and 1000 mg kg^{-1} day^{-1} for 14 days. The data represent mean ± SD (#, $p \leq 0.05$ compared to baseline; *, $p \leq 0.05$, and **, $p \leq 0.01$, compared to high-cholesterol group, n = 5).

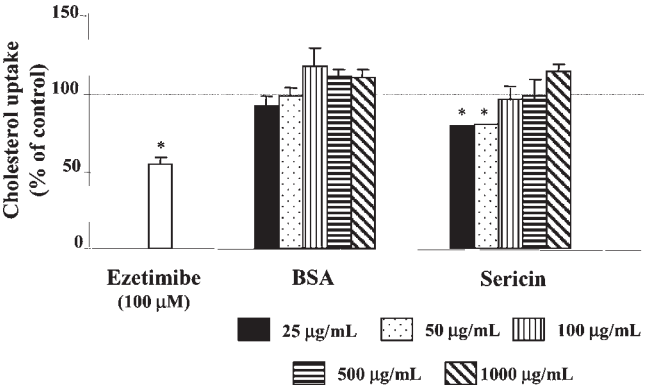

Figure 3. Cholesterol uptake in differentiated Caco-2 cells. Differentiated Caco-2 cells were pretreated with various concentrations of either sericin or bovine serum albumin (BSA) (25−1000 μg/mL) for 1 h before incubating with [1α,2α(n)-^{3}H]cholesterol micelles for 3 h. Ezetimibe was used as positive control. The data represent mean ± SEM from five to six experiments (* indicates significant difference between treatment and control groups with $p \leq 0.05$).

Table 3. Effect of Sericin on the Micellar Solubility of Cholesterol[a]

protein concn (μg/mL)	cholesterol solubility (%) (protein added before micelle formation)[b] sericin	BSA	cholesterol solubility (%) (protein added after micelle formation)[b] sericin	BSA
control	93.55 ± 1.14	93.55 ± 1.14	84.08 ± 3.73	84.49 ± 5.07
0	91.05 ± 3.36	91.05 ± 3.36	84.49 ± 5.07	84.08 ± 3.73
250	80.04 ± 3.59*	90.10 ± 4.15	73.56 ± 2.39	87.13 ± 0.50
500	73.68 ± 4.45**	86.07 ± 2.50	75.18 ± 3.15	83.90 ± 2.64
1000	69.88 ± 4.41**	85.32 ± 0.89	65.70 ± 1.71**	84.01 ± 3.16
2000	68.24 ± 3.36**	85.84 ± 2.93	64.13 ± 0.79**	87.13 ± 2.66

[a] Values are expressed as mean ± SEM (n = 3−4). [b] Significantly different from control (*, $p \leq 0.05$; **, $p \leq 0.01$).

presence of sericin either before or after micelle preparation. This reduced cholesterol solubility by sericin showed a dose-dependent trend for both preparation methods. On the contrary, bovine serum albumin (BSA) as a control protein could not reduce the solubility of cholesterol in the lipid micelles.

DISCUSSION

Our data provide evidence that sericin suppresses serum total cholesterol and non-HDL cholesterol concentrations in animals fed supplemental cholesterol; about half of the augmentation accounted for by cholesterol feeding was reversed by sericin, but no dose dependency could be established. Recent evidence shows the reduction of triglyceride and cholesterol levels in rats given sericin-supplemented high-lipid chow (*16*). In our study, sericin was administered as a solution form, and the cholesterol lowering effect was demonstrated at lower doses compared to the previous report (*16*). In addition, the present study demonstrates potential mechanisms of sericin to inhibit cholesterol absorption and micellar cholesterol solubility which might then lead to the reduction of serum cholesterol levels.

How dietary proteins might reduce serum cholesterol level is not clear, but one suggestion was that low methionine content could be involved. There is a strong positive correlation between serum cholesterol concentration and dietary methionine concentration or methionine:glycine ratio (*6*); the low methionine content in dietary proteins was thought to be involved in their cholesterol lowering effects (*6*, *17*). This accords with the very low methionine content of our sericin (0.04 mol %) and of others (< 0.05 mol %) (*11*, *13*). The amino acid composition of the sericin protein used in the present study is similar to that in previous reports (*11*, *13*). Our sericin contains 0.04% w/w methionine and 13.5% w/w glycine which makes its ratio to about 0.003 which is far lower than those of dietary proteins. Methionine and glycine contents in soy as well as potato proteins are 1−2% and 3−4.5% w/w, respectively (*6*, *18*). Although the exact mechanism was not clearly identified, the decreases in apoprotein A-I and HDL secretion from rat livers

were thought to be partially responsible for cholesterol lowering effect of low methionine protein diet (*19*, *20*). This suggestion may not operate with sericin because its systemic absorption is unlikely. According to sericin result, low-methionine content protein may also possess the intestinal effect.

There is some evidence that dietary proteins can decrease cholesterol absorption in the Caco-2 cell model (*18*, *21*), and our data confirm this idea. One mechanism for this might be disruption of cholesterol incorporation into lipid micelles (*2*, *21*) thereby reducing the efficacy of micelles taken up by Caco-2 cells and hence also by intestinal epithelial cells. This is the case with soy protein derivatives in rats and in Caco-2 cells (*18*). But buckwheat protein was only shown to reduce cholesterol solubility in lipid micelles when added prior to micelle formation (*21*). This is in contrast to our current study where cholesterol solubility in mixed lipid micelles was dose dependently reduced by sericin regardless of the order of micelle formation. These contradictions might arise from differences in physicochemical properties such as hydrophobicity, molecular weight, and/or binding capacity to bile acids (*2*, *18*) where sericin is a hydrophilic protein as well as being heterogeneous in size. Although some of the inhibitory activity of sericin could arise from the interaction to bile acids, other factors create more complex interactions which are harder to predict.

Sericin is a protein with very low digestibility (*15*), and since the undigested protein extends to the colon, it has the potential to influence the gut absorptive function along its entire length. Thus its influence on serum cholesterol is likely to be confined to the gut by either blocking the absorption or sequestering the cholesterol. In differentiated monolayers of Caco-2 cells (*22*), cholesterol uptake was blocked with low concentrations of sericin but not at the high concentrations. This might suggest another competing process which could also explain the apparent dose dependency in the vivo experiments. If so, it implies that sericin could be far more effective than illustrated here. Nevertheless, our data do show that sericin is a potent hypocholestremic agent with the lack of any complications arising from systemic effect. Thus further work could yield a very cheap and effective way of reducing blood cholesterol.

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