Note

Consumption of Sericin Suppresses Colon Oxidative Stress and
Aberrant Crypt Foci in 1,2-Dimethylhydrazine-Treated Rats by
Colon Undigested Sericin

Siquin ZHAREIGETU, Masahiro SASAKI and Norihisa KATO*
Graduate School of Biosphere Science, Hiroshima University, Kagamiyama 1-4-4,
Higashi-Hiroshima 739-8528, Japan
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Summary This study was conducted to examine the mechanisms of the anti-colon tumor
effect of dietary sericin. Dietary supplementation of 3% sericin reduced colon mucosal lipid
peroxide and aberrant crypt foci in 1,2-dimethylhydrazine-treated rats. The colon content
from sericin-fed rats had much stronger antioxidant activity compared to that from control
rats not receiving sericin. The amino acid composition of undigested proteins in the colon
contents from sericin-fed rats was similar to that of sericin ingested. The results suggest that
the strong antioxidant activity of undigested sericin in the colon content causes lower oxidative
stress and tumorigenesis in the colon.

Key Words sericin, resistant protein, colon tumor, aberrant crypt foci, oxidative stress

There is growing evidence that silk protein, sericin (Bombyx mori), is a valuable natural ingredient for cosmetic and food industries (1, 2). Among the physiological functions reported, the strong antioxidant activity of sericin appears to be most important (2). This antioxidant effect appears to be mediated by its chelation with copper because of its high contents of hydroxyl (serine, ~30%) and carboxyl (aspartic acid, ~19%) groups (2). Topical application of sericin markedly suppresses skin oxidative stress and tumorigenesis in dimethylbenz(a)-anthracene-treated mice exposed to UV or 12-O-tetradecanoylphorbol 13-acetate (3, 4). Consumption of sericin inhibited colon tumorigenesis and cell proliferation in mice treated with 1,2-dimethylhydrazine (DMH), a potent colon carcinogen (5, 6). DMH undergoes oxidative metabolism resulting in the electrophilic diazo
ion, which is known to elicit oxidative stress (7). Lower colon tumorigenesis in sericin-fed mice receiving DMH is associated with lower oxidative stress markers in the colon mucosa (6). Interestingly, sericin is a dietary fiber-like protein with very low digestibility (namely, "resistant protein") (8, 9). Such resistance property to proteases might make it beneficial for the health of intestines (9). In view of these facts, we postulated that the antioxidant activity per se of undigested sericin in the colon content is responsible for the reduced colon oxidative stress, leading to lower colon tumorigenesis. The current study was conducted to test this possibility.

Male Wistar rats (3-wk-old) were purchased from Hiroshima Laboratory Animal Center (Hiroshima, Japan) and housed individually in an air-conditioned room (24°C, with a 12-h light cycle; lights on, 0800–
2000 h). They had free access to deionized water. The rats were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. After feeding on commercial stock diet (MF Oriental Yeast Co., Ltd., Tokyo, Japan) for 1 wk, the rats were given a subcutaneous injection of DMH (Nacalai Tesque, Kyoto, Japan, 20 mg/kg body wt). One week after the injection, rats (average 110 g) were divided into two groups of 20 animals each (casein diet and sericin diet groups). The composition of the experimental diets is shown in Table 1. Sericin was added to the diet at the level of 3% (5). The method of preparation of sericin and its amino acid composition were described elsewhere (2). Adjustment of the dietary protein level was made by reducing dietary casein. The feeding experimental period was 28 d. Food intake and body weight were measured every day.

At the end of the feeding period, the colon was removed, slit open longitudinally from cecum to anus and the contents of the large intestine and colonic mucosa from half of the animals of each group were quickly removed, weighed and stored at −85°C for further analysis. The colon from the remaining half of the animals of each group was placed on a paper towel, fixed in 10% neutral formalin for 24 h, and stained with 0.5% methylene blue for 20 min. The number of aberrant crypt foci (ACF) per colon was examined with light microscope at a magnification of ×16 as described elsewhere (5).

An estimate of lipid peroxide (thiobarbituric acid reactive-substances:TBARS) in homogenates of colonic mucosa was obtained by the spectrophotometric determination of Ohkawa et al. (10). The protein content in the homogenates of colonic mucosa was determined

*To whom correspondence should be addressed.
E-mail: nkato@hiroshima-u.ac.jp
Table 1. Composition of experimental diets (%).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>Sericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>Sericin</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Corn oil</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Corn starch</td>
<td>37.2</td>
<td>37.2</td>
</tr>
</tbody>
</table>

1 Casein (nitrogen×6.25), 87.0%.
2 Sericin (nitrogen×6.25), 92.9%.
3 AIN-93.
4 AIN-93G.

using the Bio-Rad protein assay kit (Hercules, CA, USA), with bovine serum albumin as standard.

For determination of amino acid composition of the proteins of the contents of the large intestine, the contents of the large intestine were homogenized with ice-cold deionized water and dialyzed in a 100-fold volume of deionized water at 4°C for 24 h to remove the low-molecular weight fraction, including free amino acids. The amino acid composition was determined by an amino acid analyzer after acid hydrolysis for 22 h with 6 M HCl. Lipid peroxidation in mucosal homogenates was estimated by measuring TBARS. Rat colonic mucosa (male Wistar rats, 200 g) was removed and homogenized with ice-cold 0.1 M phosphate buffer (pH 7.5) to make a 25% homogenate. To 0.125 mL of the homogenate was added 0.5 mL of 0.1 M phosphate buffer (pH 7.5) with the contents of the large intestine (100-fold dilution). The mixture was incubated 37°C for 6 h. After incubation, 0.5 mL of thiobarbituric acid (0.7% in 50 mM KOH) and 0.625 mL of 8% trichloroacetic acid (TCA) were added to 0.125 mL of the incubated samples, and heated at 100°C for 15 min. After centrifugation, the absorbance at 532 nm of the supernatant was measured to estimate TBARS. The protein level of the contents of the large intestine was determined using the Bio-Rad protein assay kit described above.

The Fenton-type reaction system-induced damage to DNA results in the appearance of thiobarbituric acid-reactive chromogens with a maximal absorption at 532 nm (11, 12). In Cu(II)/H₂O₂-induced DNA oxidation system, the reaction system (1.0 mL) contained calf thymus DNA (Sigma-Aldrich, St. Louis, MO, USA) (0.5 mg/mL of reaction mixture), 2.8 mM H₂O₂, 20 μM CuSO₄ solution and the contents of the large intestine (1,000-fold dilution) in 25 mM phosphate buffer, pH 7.4. The mixture was incubated for 60 min at 37°C followed by the addition of 1.0 mL of thiobarbituric acid (0.7% in 50 mM KOH) and 1.0 mL of 2.5% TCA. The mixture was heated at 100°C for 15 min and cooled. The thiobarbituric acid-reactive chromogens were extracted with 3.0 mL of n-butanol/pyridine (15:1, v/v) and measured spectrophotometrically at 532 nm.

The statistical significance of differences between values was analyzed by Student’s t-test. Difference at p<0.05 was considered significant. Some data were analyzed by regression analysis and the correlation coefficient was calculated.

Final body weight and food intake for 28 d were not significantly different between the casein and sericin groups (data not shown). Supplemental sericin caused a 36% reduction in the number of colonic ACF in all areas of the colon compared to the casein diet (p<0.01, Fig. 1A). The sericin diet caused a 34% reduction in the lipid peroxide levels of colon mucosa compared to the control diet (p<0.05, Fig. 1B). There was no difference in the protein concentration of the contents of the large intestine observed between the sericin and control groups (102±2 and 104±2 mg/mL, respectively, p>0.05). The weight of the contents also did not differ between the two groups (data not shown). In our preliminary study with rats not receiving DMH, there was no difference in the level of TBARS of the colon mucosa between the sericin and control groups (5.2±0.3 and 4.8±0.2 nmol/mg protein, respectively, n=5, p>0.05).

Figure 2A shows the effect of the contents of the large intestine on lipid peroxidation in rat mucosal homogenate. The observed inhibitory effect of the contents of the large intestine from the sericin group was 4.1-fold higher than that of the control group (p<0.05, Fig. 2A). As shown in Fig. 2B, the addition of the contents of the large intestine of the sericin diet group caused higher inhibition against Cu/H₂O₂-induced DNA oxidation (58% inhibition) compared to that of the casein diet group (15% inhibition) (p<0.01).
The data in Fig. 3 indicate the similarity of the amino acid pattern between sericin and the colon content (undigested proteins) from rats fed sericin. Actually, there was a significant correlation between the composition of amino acids from the sericin-fed rats and that of sericin ingested (p<0.01). However, the amino acid pattern of casein was quite different from that of the colon content from the rats fed casein (without sericin).

Consistent with our previous studies (5, 6), this study indicated that supplemental sericin suppressed the development of colonic ACE, an early marker of colon tumorigenesis, and colon mucosal level of TBARS in DMH-treated rats. This finding implies that dietary sericin suppresses colon tumorigenesis from the early stage of carcinogenesis and colon oxidative stress.

In this study we tested if the colon content from the sericin group has a strong antioxidant activity. The results indicated that the contents from the sericin group had much higher inhibitory activity against lipid peroxidation of the homogenate of colonic mucosa compared to that from the casein group. Our recent studies have indicated that marked anti-oxidative activity of sericin can be observed especially in the Cu-dependent oxidative stress system, including Cu/H$_2$O$_2$-induced DNA oxidation and Cu-induced LDL oxidation systems (Kato et al. unpublished data). A formation of Cu-sericin complex was also reported previously (13). Accordingly, the antioxidant activity of sericin appears to be mediated by its chelation with copper ion through its hydroxyl (serine) and carboxyl (aspartic acid) groups. Thus, we examined the antioxidant activity of the colon content in the Cu-dependent oxidative stress system. The result shows that the content of the large intestine from the sericin group had much greater inhibitory activity in this Cu-dependent oxidative stress system compared to that from the control group.

We further examined if undigested sericin remains in the contents of the large intestine. The result clearly indicated that the amino acid composition of the undigested proteins in colon content from sericin-fed rats was similar to that of sericin ingested. At present, it seems difficult to exactly determine the relative proportion of the undigested proteins derived from the diets, the proteins of intestinal microflora, and the mucosal proteins removed from the intestinal epithelium in the colon contents. However, the similarity between the amino acid pattern of the colon content from the sericin group and dietary sericin clearly suggests a significant amount of undigested sericin in the colon contents of sericin-fed rats. Previous in vitro study in our laboratory also indicated that sericin is highly resistant to several proteases (8). Thus, undigested sericin or partially digested sericin appears to actually remain to some extent in the colon. Partially hydrolyzed sericin still has some antioxidant activity, although the lower molecular weight of hydrolyzed sericin is associated lower anti-oxidant activity (Kato et al. unpublished data). Taken together, our study provided evidence that undigested sericin in the colon content suppresses colon oxidative stress and development of the ACF in DMH-treated rats.
Recently, we have proposed the hypothesis that a resistant property of dietary proteins to intestinal proteases might be beneficial for the intestinal health through their physiological functions (9). The results obtained in this study support this hypothesis. It has been reported that several dietary proteins, including whey protein, buckwheat protein and lactoferrin, suppress the development of colon tumors (14–16). However, these studies have provided little information about the functions of the colon contents including the undigested dietary proteins. Our study provided the first evidence for the anti-oxidative effect of the undigested dietary protein on colon mucosa, leading to lower development of oxidative-stress related disease.

REFERENCES