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Full Length Research Paper

Hematological assessments of sericin-derived oligopeptides in BALB/c mice

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Sericin, a protein removed from the silk cocoons, possesses various biological activities. Due to less solubility, utilization of the sericin protein may be limited. Enzymatic hydrolysates of this protein would thus, provide an alternative. Since safety assessment is required before any protein hydrolysates being used, the effects of sericin-derived oligopeptides (SP) on some hematological parameters were evaluated. Female BALB/c mice were fed orally with SP (50, 100 and 500 mg/kg body weight daily) or vehicle for 28 days. The total red blood cells (RBCs), percent (%) hematocrit, differential white blood cells (WBCs) and lymphocyte subpopulations were determined. SP, either 50, 100 and 500 mg/kg did not significantly influenced counts of the RBCs and % hematocrit. In addition, SP did not affect the number of types of WBCs. No changes in morphology of RBCs and WBCs were observed. These suggested that SP were not harmful to both RBCs and WBCs. Preliminary immune modulation study demonstrated a significant increased population of the CD8+ (T cytotoxic) cells in mice fed with SP 50 and 500 mg/kg compared to the controls, suggesting immunomodulatory activity of such oligopeptides on the cell-mediated immunity. Our results indicate intoxic significance and warrant further investigations of such SP.

Key words: Hematological assessments, sericin-derived oligopeptides, BALB/c mice.

INTRODUCTION

Proteins are an essential part of the diet as sources of energy and amino acids. Generally, proteins are required to brush-border hydrolysis before their hydrolysis products can be effectively absorbed (Grimble, 1994; Poullain et al., 1989). Protein hydrolysates especially those containing di- and tripeptides have been reported to be more rapidly absorbed into human jejunum than both intact proteins and free amino acids (Grimble et al., 1987, 1994; Raimundo et al., 1988). As such, protein hydrolysates have been extensively used in special formulations to improve nutritional and functional properties, including hypoallogenic formulas, high-energy supplements, weight-control and therapeutic diets (Mahmoud, 1994; Cordle, 1994; Frokjaer, 1994).

Sericin is a glue-like protein that is mostly removed from the cocoons and disposed of as waste in silk processing (Vaithanomsat and Kitpreechavanich, 2008). Sericin-containing water also poses serious impact on environment if there is lack of proper treatment (Fabiani et al., 1996). Studies in the past decades however, reported various biological activities of sericin. These include antioxidation (Kato et al., 1998; Fan et al., 2010), inhibition of tyrosinase (Kato et al., 1998), suppression of colon tumorigenesis (Sasaki et al., 2000; Zhaorigetu et al., 2001) and reduction of cholesterol (Limpeanchob et

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al., 2010). Therefore, sericin has been widely used in cosmetics products as well as some medical applications. Nevertheless, broad ranges, in particular large molecular mass produce less solubility of the sericin protein, and may subsequently limit uses of such protein. Enzymatic hydrolysates of the sericin protein would thus, provide an alternative.

It is of significant importance that safety assessment is required before any protein hydrolysates being used. Body and organ weights and hematological indices indirectly reflect in the physiological responsiveness of the animals. Therefore, the effects of sericin-derived oligopeptides (SP) on hematological parameters as well as body and vital organ weights were evaluated in this study. The results obtained will be of significant value for future considerations when such oligopeptides are to be utilized especially for health benefits.

MATERIALS AND METHODS

Materials

SP with molecular weight of approximately 5 kDa were prepared by proteolytic digestion of the sericin protein from the yellow cocoons of Thai silkworm, *Bombyx mori*. These were kindly provided by the Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Animals and treatments

Female BALB/c mice (6 to 8 weeks old) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were housed in a temperature-controlled environment (25 ± 1 °C) with a 12-h dark: light cycle and had *ad libitum* access to sterile water and standard rodent chow. The animals were treated in compliance with Guidelines in the Care and Use of animals. All experimental protocols were reviewed and approved by the Animal Research Ethics Committee, Naresuan University.

Mice were randomly divided into 4 groups of five each. Treated groups were orally administered with 50, 100 or 500 mg/kg body weight (BW) of SP once daily for 28 days. Vehicle control received only sterile phosphate buffered saline (PBS), pH 7.4. BWs were recorded weekly.

On the completion of feeding, mice were sacrificed by receiving an overdose of 50 mg/kg BW (i.p.) Thiopental sodium (THIOPENTAL, Bigpharma, Thailand). Blood were immediately collected by cardiac puncture, subsequently spleen, thymus, liver, lung and kidney were aseptically removed and weighed.

Hematological assessments

Blood samples collected in 1% ethylenediaminetetraacetic acid were analyzed for total red blood cell (RBC) counts, percent (%) hematocrit and differential white blood cells (WBCs) as follows;

Determination of total RBCs counts

Blood samples were diluted with PBS, pH 7.4 and RBCs were counted using a hemocytometer. The total RBCs was expressed as cells/µL.

Determination of hematocrit values

Na^+ -heparinized microhematocrit tubes (Biomed Co., Ltd., Bangkok, Thailand) were filled with blood collected from each animal and centrifuged at 12,000 × g for 5 min using a Hematokrit 24 Hettich Zentrifugen and % hematocrit read.

Determination of differential WBCs

Blood samples smeared onto microscopic slides were fixed with absolute methanol to stabilize cellular components. Staining of WBCs was performed with Dip-quick solutions (Clinag Co., Ltd., Bangkok, Thailand) according to the manufacturer's instruction. Proportion of different types of WBCs (neutrophils, lymphocyte, monocytes, eosinophils and basophils) was determined under the microscope and expressed in percentage.

Determination of splenic lymphocyte subpopulations

Spleens were aseptically collected in PCM buffer prepared with sterile phosphate buffer saline (PBS) pH 7.4, 7×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$ and 5% (v/v) fetal bovine serum (FBS; Gibco, South America). Splenic single cell suspension was mechanically isolated through a cell strainer (BD Falcon, USA) and RBCs were lyzed by hypotonic solution. After being washed with PCM buffer, cell pellets were resuspended with complete RPMI-1640 (PAA, Pasching, Austria) containing 10% FBS, 0.01 M HEPES (Hyclone), 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine (Hyclone), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA). Viability of splenic cells was examined by trypan blue exclusion (Strober, 2001). A total of 4×10^5 splenic single cells were stained for 30 min on ice with antibody specific for mouse lymphocyte surface markers as follows:

Fluorescein isothiocyanate conjugated anti-CD3 (total T cells), Phycoerythrin (PE) conjugated anti-CD4 (T helper cells), PE conjugated anti-CD8 (T cytotoxic cells) and PE conjugated anti-CD21/35 (mature B cells). Stained cells were washed twice with PBS pH 7.4 containing 2% heat-inactivated fetal bovine serum and subsequently fixed with 1% paraformaldehyde. The percentages of lymphocyte subpopulations were subjected to flow cytometric analysis using a FACScalibur and CellQuest Pro software (BD Biosciences).

Statistical analysis

The data are expressed as mean ± standard error of the means (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS program. Differences among treatment means within a sampling period were compared using Tukey's post hoc test. P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effects of SP on body and organ weights

Oral administration of SP either 50, 100 or 500 mg/kg BW for a period of 28 days did not result in any mortality. There were no significant differences in the BWs between SP-treated groups and vehicle control; no significant differences in the vital internal organ weights were

Table 1. Body and organ weights in mice orally administered with SP for 28 days.

Treatment group[1]	Body weight[2] (g)	Organ weights[2] (g/100 g BW)				
		Spleen	Thymus	Liver	Lung	Kidney
Control	21.09 ± 026	0.51 ± 0.06	0.14 ± 0.02	5.32 ± 0.04	0.71 ± 0.03	1.38 ± 0.03
SP50	20.97 ± 0.60	0.47 ± 0.04	0.16 ± 0.01	5.21 ± 0.15	0.67 ± 0.04	1.44 ± 0.03
SP100	21.35 ± 0.66	0.47 ± 0.03	0.16 ± 0.01	4.92 ± 0.19	0.68 ± 0.01	1.33 ± 0.04
SP500	21.38 ± 0.29	0.44 ± 0.02	0.16 ± 0.01	5.16 ± 0.16	0.71 ± 0.02	1.41 ± 0.03

[1]SP50, SP100 and SP500, Sericin-derived oligopeptides 50, 100 and 50 mg/kg BW treated groups, respectively; [2]Values are mean ± SEM (n = 5).

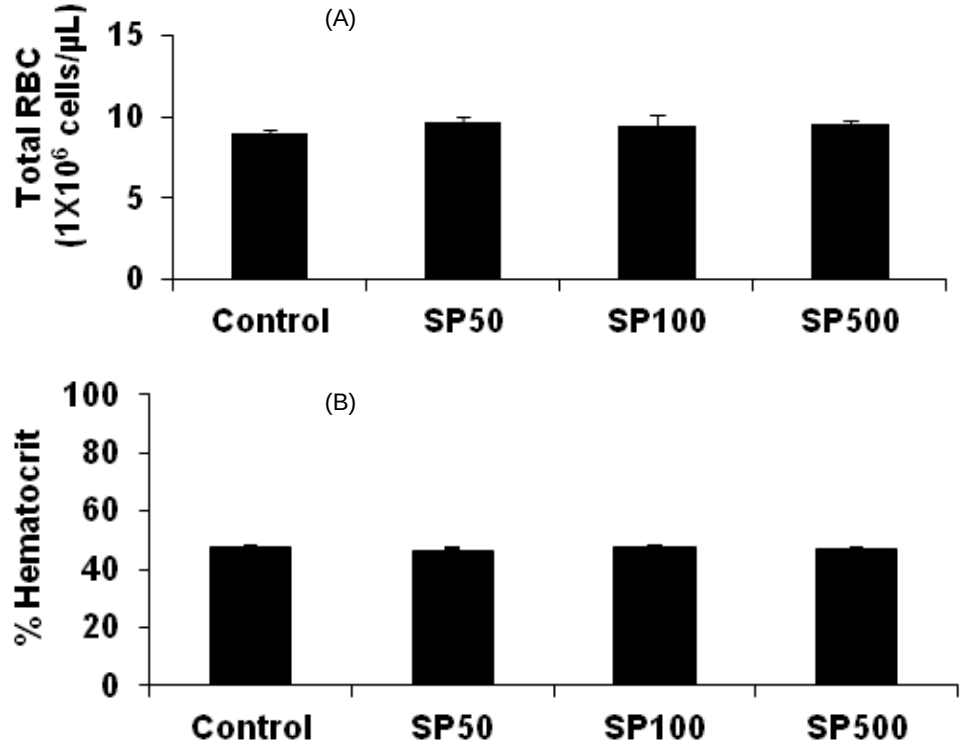

Figure 1. Total RBCs (A) and percent (%) hematocrit (B) in mice orally administered with SP for 28 days. SP50, SP100 and SP500, sericin-derived oligopeptides 50, 100 and 50 mg/kg BW treated groups, respectively. Values are mean ± SEM (n = 5).

observed throughout the study (Table 1), suggesting the SP not to be toxicologically significant.

Effects of SP on RBCs

As known, RBCs are responsible for carrying oxygen to the body' tissues thus, changes in the numbers and/or morphology of the RBCs may indicate abnormalities or some hematological conditions. Effects of SP on the RBCs were therefore examined through the counts of total RBCs and packed cell volume. As shown in Figure 1A, counts of total RBCs in mice fed with SP 50, 100 and 500 mg/kg BW did not significantly differ from those of the control animals throughout 28 days. In addition, packed cell volumes, expressed as % hematocrit in all three SP-treated groups were not different ($P < 0.05$) compared to the controls (Figure 1B). Moreover, morphological examinations of RBCs from mice fed with SP (50, 100 and 500 mg/kg BW) did not differ from those of the control mice (data not shown). The results obtained indicated that the study oligopeptides were not harmful to murine RBCs.

Effects of SP on different types of WBCs

Apart from RBCs, the effects of SP on WBCs were also

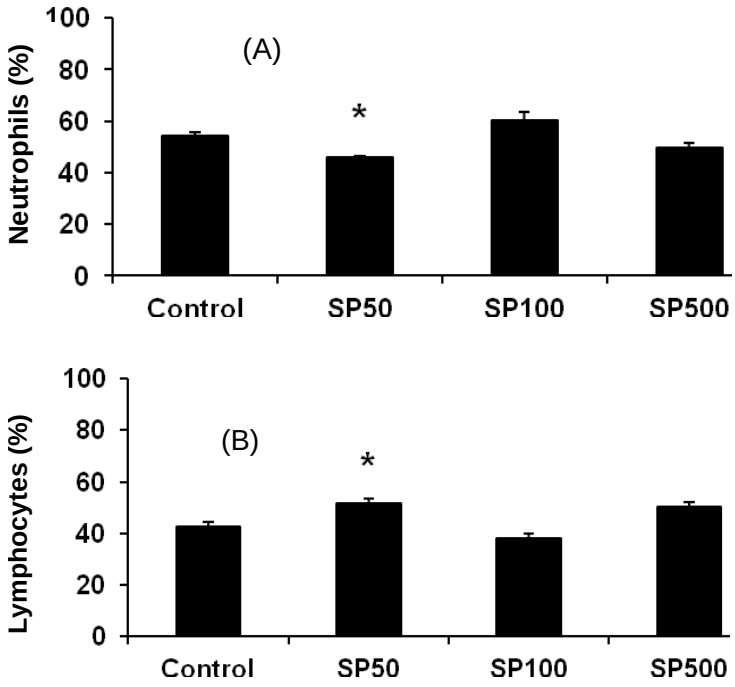

Figure 2. The percentages of neutrophils (A) and lymphocytes (B) in mice orally administered with SP for 28 days. SP50, SP100 and SP500, sericin-derived oligopeptides 50, 100 and 50 mg/kg BW treated groups, respectively. Values are mean ± SEM (n = 5). *, $P < 0.05$ compared to the control.

Table 2. Percentages of splenic lymphocyte subpopulations in mice orally administered with SP for 28 days.

Treatment group[1]	Lymphocyte subsets[2] (%)			
	CD3+ cells	CD4+ cells	CD8+ cells	CD21/35+ cells
Control	17.69 ± 0.68	11.60 ± 0.73	6.92 ± 0.36	72.02 ± 0.58
SP50	19.64 ± 1.53	13.14 ± 0.93	10.37 ± 0.50 *	69.85 ± 1.67
SP100	17.42 ± 1.62	10.68 ± 1.07	7.37 ± 0.85	69.66 ± 1.59
SP500	22.30 ± 0.86	14.64 ± 1.18	12.90 ± 0.58 *	67.43 ± 1.13

[1]SP50, SP100 and SP500, Sericin-derived oligopeptides 50, 100 and 50 mg/kg BW treated groups, respectively. [2]Values are mean ± SEM (n = 5). *, $P < 0.05$ compared to the control.

examined, through differential WBC counts. As shown in Figure 2, changes in proportion of different types of WBCs were observed in mice fed with SP 50 mg/kg. In such animals, there was an increase in the percentages of lymphocytes, while the percentages of neutrophils decreased compared to the controls ($P < 0.05$). Nevertheless, changes in the proportions of these WBC types remained within the normal ranges (Zhang et al., 2011). For other WBC types, no significant differences between SP-treated and control animals could be observed. Moreover, morphological examinations of WBC from mice fed with SP (50, 100 and 500 mg/kg BW) did not differ from those of the control mice (data not shown). This study also indicated the intoxicity of oligopeptides for murine WBCs.

Effect of SP on lymphocyte subpopulations

In addition to safety assessment, we also evaluated the immune modulatory effect of such oligopeptides. The number of lymphocytes, the master cells of the immune system, was preliminarily determined. Upon daily oral administration of SP, the population of CD21/35+ (mature B), CD3+ (total T) and CD4+ (T helper) lymphocytes were similar among treatment groups throughout the 28 days period (Table 2). However, a significant increase in population of the CD8+ (T cytotoxic) cells was observed

in mice fed with SP 50 and 500 mg/kg compared to the controls ($P < 0.05$), suggesting the immunomodulatory activity on the cell-mediated arm of immune system. The study is currently underway to determine effects of the study oligopeptides on such lymphocyte functions.

This study demonstrated that the oligopeptides derived from sericin protein of silkworm *B. mori* were intoxic to all vital organs, in particular lymphoid organs of the immune system. Moreover, these oligopeptides were not harmful to RBCs and WBCs in terms of counts and cell morphology. Preliminary results also suggested the immunomodulatory activity of such oligopeptides on the cell-mediated immunity. Our results warrant further investigations of these SP.

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