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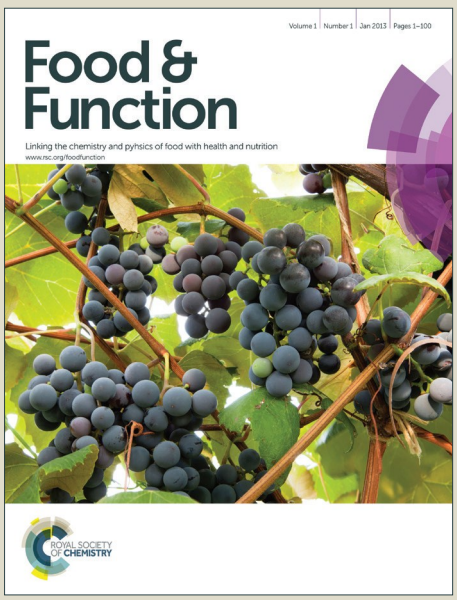

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Sea cucumber (*Codonopsis pilosula*) oligopeptides: Immunomodulatory effects based on stimulating Th cells, cytokines secretion and antibody production

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Abstract: This study was aimed to investigate the immunomodulating activity of small molecule oligopeptides from sea cucumber (*Codonopsis pilosula*) (SOP) in mice. Seven assays were performed to determine immonomodulatory effects which included splenic lymphocyte proliferation and delayed-type hypersensitivity assays (cell-mediated immunity), IgM antibody response of spleen to sheep red blood cells (SRBC) and serum hemolysin level assays (humoral immunity), the carbon clearance assay and phagocytic capacity of peritoneal cavity phagocytes assay (macrophage phagocytosis) and NK cell activity assay. Spleen T lymphocyte subpopulations, multiplex sandwich immunoassays of serum cytokine and immunoglobulin levels and enzyme-linked imunosorbent assay for small intestinal secretory immunoglobulin were determined to study the mechanism of SOP affecting immune system. We found that SOP could improve immune functions in mice, which may be due to the enhancement of the functions of cell-mediated immunity, humoral immunity, macrophage phagocytosis and NK cell activity. From the cellular and molecular assays, we postulated that the immunomodulatory effects most likely attributed to the stimulation of Th cells, cytokines secretion and antibody production.

Keywords: Sea cucumber oligopeptides; adaptive immune; innate immune; T cell subpopulation; cytokine; immunoglobulin subtype; multiplex sandwich immunoassays

1. Introduction

Continued exposure to stress and environmental extremes has been linked to worsened immune system function, leaving a person more liable to infection. Nutritional interventions have become an effective and widely-applied approach to enhance immune responses, innate

and adaptive immune responses, and to resist to disease. As specific protein fragments that in addition to acting as sources of nitrogen and amino acids, food-derived bioactive peptides, which have low molecular weight, and easily absorbable and digestible features, are have numerous potential physiological functions, including antimicrobial, cholesterol-lowering, antioxidant, cyto- or immunomodulatory activities [1, 2]. Moreover, some peptides exhibit multifunctional properties [3]. Therefore, many food-derived bioactive peptides are receiving increasing attention in the fields of therapeutics and nutritional interventions.

Sea cucumber, *Codonopsis pilosula*, traditionally used as a restorative medicine, has been the most widely used in Chinese communities for thousands of years, just as that the detailed medical applications of sea cucumber were officially recorded in the "Compendium of Materia Medica (Bencao Gangmu)" in 1758, in which it was held in high esteem as ginseng". Expect China, sea cucumber is widely consumed as a putative functional food in other countries, particularly Singapore, Korea and Norway. The dried body wall of sea cucumber contains many biologically active substances and nutrients, such as, about 90% of protein (polypeptides), 6% of polysaccharides (oligosaccharide), and 4% of lipids in organics [4], vitamin A, vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (niacin), and minerals, especially calcium, magnesium, iron and zinc [5], which make it have many biological activities including anticancer, antioxidant, anti-inflammatory, antithrombotic, antitumor, antimicrobial, immunoregulatory activity and wound healing [5, 6]. Although there are some functionality studies about sea cucumber, most of them used the whole body, while few studies have reported the immunoregulatory activity of oligopeptides from sea cucumber.

In the present study, we used deionized water and whey protein as controls, and

intragastically administered small molecule oligopeptides from sea cucumber (*Codonopsis pilosula*) to BALB/c mice. The aim was to investigate the immunomodulatory effects of SOP, and to further clarify the mechanism.

2. Experimental Section

2.1 Treatment of mice with SOP

SOP were derived from the root of *Jilin* sea cucumber (*Codonopsis pilosula*) by enzymatic hydrolysis and donated by *Jilin Taigu* Biological Engineering Co., Ltd. (*Jilin*, China). Oligopeptides sample was purified by high performance liquid chromatography (HPLC, Water Corp., Milford, MA, USA) using a Phenomenex C18 column (10 mm×250 mm), and the molecular weight distribution of the oligopeptides sample was determined by LDI-1700 matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Linear Scientific Inc., Reno, NV, USA). Then the amino acid composition was analysed with an automatic amino acid analyser (H835-50, Hitachi, Tokyo, Japan), and the amount of free amino acids was measured by the HPLC. After HPLC purity and MALDI-TOF-MS analysis, we found that the content of relative molecular mass more than 2 000 was 2%, between 1 000 and 2 000 was 8.56%, less than 1 000 (small molecule oligopeptides) in SOP was 89.44%, and the contents of free amino acids were 7.496%.

Female BALB/c mice (Qualified certificate No., SCXK (JING) 2011-0012) aged eight weeks with the weight of 18-22 g were obtained from the Animal Service of Health Science Center, Peking University. The mice were randomly divided into five groups, i.e., vehicle control group, whey protein control group, SOP 0.15, 0.30 and 0.60 g/kg body weight (g/kg BW) groups. SOP was intragastrically administered to the mice in SOP groups, 0.30 g/kg BW

whey protein was intragastically administered to the mice in whey protein control group, and the vehicle control mice were given vehicle only. All of mice were administered of 1 mL/100 g BW.

The mice were group-housed (five mice per cage) under constant conditions (temperature: 23±1 °C, humidity: 40-60%, light and dark cycle:12 h), were fed the AIN-93 diet and allowed free access to deionized water. The mice and food intake were weighed every week. All animals were performed according to the National Institutes of Health guideless for Care and Use of Laboratory Animal (NIH publication No. 85-23, revised 1996) and the protocols were approved by the Institutional Animal Care and Use Committee of Peking University.

After 30 days, blood samples of the mice were collected from ophthalmic venous plexus and sacrificed by cervical dislocation. The spleen and thymus were removed and weighed. Spleen and thymus indices were calculated as the ratio of immune organ weight to body weight.

2.2 Spleen cell suspension preparation

The spleens of the mice (n=10/group) sacrificed under the aseptic condition were washed with Hank's balanced salt solution (HBSS), and crushed gently to isolate the splenocytes, then the splenocytes were passed through a 200-mesh stainless steel sieve. The harvested single cell suspension was washed twice with HBSS and with centrifugation at 180×*g* for 10 min. The recovered splenocytes were resuspended in red blood cell lysis buffer (7 g/L NH_4Cl and 2.6 g/L tris-HCl) for 5 min to remove red blood cells. After centrifugation, the harvested splenocytes were resuspended in 1 mL of RPMI-1640 complete medium containing 10% (v/v) FBS, 100 units/mL penicillin and 100 μg/mL streptomycin, and the cell viability was assessed

with a hemocytometer using trypan blue dye exclusion method.

2.3 Lymphocyte proliferation assay

The splenocyte suspension was adjusted to a concentration of 5×10^6 cells/mL, and seeded in 24-well plates (1 mL/well) with/without (control wells) 75 μL/well Con A as a T cell stimulant. After 68 h incubation at 37 °C under humidified 5% CO_2–95% air condition, 0.7 mL of supernatant per well was removed, then 0.7 mL of RPMI-1640 without FBS and 50 μL of MTT (5 mg/mL) were added into each well, and each tested well was triplicated. Following 4 h incubation at 37 °C under humidified 5% CO_2–95% air condition, 1 mL of 3% (w/w) SDS solution per well was added to the insoluble purple formazan product into a colored solution, and the absorbance at 570 nm was measured using an ELISA reader (Bio-Rad, Hercules, CA). The difference of the absorbance with/without Con A was used to represent the proliferation capacity.

2.4 NK cell activity assay

Splenic NK cell activity was tested using the lactic acid dehydrogenase (LDH) release method[25]. Briefly, mouse splenocytes were used as effector cells at 2×10^6 cells/mL, and YAC-1 cells were used as target cells at 4×10^4 cells/mL resulting in an effector/target cell ratio of 50:1. After 4 h incubation and 5 min centrifugation at 400×*g*, LDH substrate mixture was added to the culture supernatants. After 10 min incubation at 37 °C, the absorbance at 490 nm was determined in an ELISA reader. NK cell activity (%)=$(OD_{experimental}-OD_{spontaneous})\times100/(OD_{maximum}-OD_{spontaneous})$.

2.5 DTH assay, IgM-PFC assay and serum hemolysin level measurement

Defibrinated SRBCs were collected from a normal sheep, then washed three times with

sterile saline. BALB/c mice (n=10/group) were immunized with 0.2 mL of 2% (v/v) SRBCs (about 1×10^8 cells) per mouse by intraperitoneal injection on day 26.

On day 30, the thicknesses of the left rear footpads of mice were determined with a Vernier caliper, then the left rear footpads of these mice were injected with 20 μL of 20% (v/v) SRBCs (about 1×10^8 cells) by subcutaneous injection. After 24 h, the thicknesses of the left rear footpads were measured again. Each measurement was taken three times and the mean calculated. Swelling degree was expressed as the difference in footpad thickness.

On day 31, the mice were sacrificed, the spleen cell suspension was prepared for IgM-PFC assay performed by a modified Jerne's method[26]. In brief, twenty microliters of splenocyte suspension in RPMI-1640 complete medium, 50 μL of 10% (v/v) SRBC and 500 μL of agar solution (0.5 g/mL in HBSS, pH 7.2) were mixed, then poured onto slides covered with 0.5% agarose layer. Following incubation at 37 °C, 5% CO_2 and adequate reaction with guinea pig complement, the number of plaque production was counted, and the results were expressed as the number of PFC per 10^6 viable splenocytes.

Blood samples were collected to measure serum hemolysin level. Half a milliliter of 10% (v/v) SRBC and 1 mL of diluted guinea pig complement (1:10 diluted with buffer solution) were successively added to sample tubes with/without (control) 1 mL of diluted mouse serum (1:200 diluted with SA buffer solution). After 30 min at 37 °C water bath, ice bath terminated the reaction, then centrifugated to collect 1 mL of supernatant, and added into 3 mL of Drabkin's reagent (1.0 g $NaHCO_3$, 0.2 g $K_3Fe(CN)_6$ and 0.05 g KCN, solved in 1 000 mL stilled water). The positive control was set with 0.25 mL of 10% (v/v) SRBC and 3.75 mL of Drabkin's reagent. Following 10 min reaction, the absorbance at 540 nm was determined, and

the serum half hemolysis value (HC_{50}) was calculated. $HC_{50}=(OD_{sample}/OD_{positive\ control})\times200$.

2.6 Macrophage phagocytosis capacity assay

Carbon clearance assay. On day 31, BALB/c mice (n=10/group) were intravenously injected 0.1 mL/10 g BW pre-warmed carbon solution (India ink diluted by four times with sterile saline) via the tail vein. After 2 and 10 min, 20 μL of blood samples were collected from ophthalmic venous plexus into 2 mL of 0.1% (w/v) Na_2CO_3 solution, then the absorbance at 600 nm was measured. At the same time, these mice were sacrificed, then the spleen and liver were removed and weighed, and the carbon clearance capacity was expressed as carbon clearance index (α, ΔOD/time). $\alpha=\text{Body weight}\times\sqrt[3]{k}/(\text{liver weight+spleen weight})$. $k=(lgOD_1-lgOD_2)/(t_2-t_1)$.

CRBCs phagocytosis capacity assay. On day 31, BALB/c mice (n=10/group) were intraperitoneally immunized with 1 mL of 20% (v/v) CRBCs obtained from White Leghorn cocks. After 30 min, the mice were sacrificed and peritoneal cells were harvested by peritoneal lavage with 2 mL of saline, then 1 mL of cell-rich lavage fluid was pipetted and smeared on glass slides. Following 30 min incubation at 37 °C, non-adherent cells were washed off with saline, and the macrophages were fixed with acetone:methanol (1:1, v/v), then stained with 4% (v/v) Giemsa-PBS solution. The percentages of pahgocytes phagocytosed CRBCs per 100 macrophages were expressed as the phagocytic rate, and the number of phagocytosed CRBCs per 100 macrophages was counted and expressed as the phagocytic index.

2.7 Splenic T lymphocyte subpopulations assay by flow cytometry

The splenocyte suspension was adjusted to a concentration of 1×10^6 cells/mL for splenic T

lymphocyte subpopulations assay. The splenocyte surface markers were stained with PerCP-Cy5.5-conjugated anti-mouse CD3 (BioLegend, San Diego, CA, USA) to determine T lymphocyte frequency in spleen, and stained with FITC-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD8a and PE-conjugated anti-mouse CD25 (BioLegend, San Diego, CA, USA) to determine the frequencies of helper T (Th, $CD4^+$), cytotoxic T (Tc, $CD8^+$) and regulatory T lymphocytes (Treg, $CD25^+$). After blocking Fc receptors, the splenocytes were labeled fluorescein-conjugated antibodies or isotype controls for 30 min, followed by two washed with cell staining buffer (BioLegend), then the labelled cells were resuspended in cell staining buffer. FACSCanto instrument settings were adjusted using the control cells stained with isotype-matched antibodies. The fluorescence activated cell sorting (FACS) was performed using a FACSCanto™ flow cytometer (BD Biosciences), and the data were analyzed using FACSDiva and CellQuest software (BD Biosciences).

2.8 Multiplex sandwich immunoassays of cytokine and immunoglobulin

Blood samples were centrifuged at 2 000 r/min for 10 min to collect the serum. Serum samples were stored at −80 °C until cytokine and immunoglobulin analysis using the Luminex xMAP® platform.

Cytokine and immunoglobulin levels were determined from the serum samples using the multiplex sandwich immunoassays with the Milliplex Mouse 11 Cytokine/ 5 Immunoglobulin Premixed Kit (Millipore; Billerica, MA) and established protocols in accordance with manufacturer's instructions. The 11 cytokines included interleukin (IL)-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, IFN-γ, TNF-α and GM-CSF. The 5-immunoglobulin panel comprised IgA, IgM, IgG1, IgG2a, IgG2b and IgG3. The assays were carried out according to

177 the protocols provided by the manufacturer. Finally, the 96-well plate was read on the
178 MAGPIX® instrument (Luminex, Austin, Texas, USA) based on magnetic-bead technology
179 and level of magnetic field to separate the beads. The following cytokines/immunoglobulins
180 were analyzed using the Milliplex Analyst 5.1 software. Serum cytokine and immunoglobulin
181 concentrations were calculated using a standard curve derived from the reference cytokine
182 concentrations supplied by the manufacturer. A five-parameter model was used to calculate
183 final concentrations and values. The lower levels of detection for the 11 cytokines were
184 between 0.74 and 23.11 pg/mL and for the five immunoglobulins were between 0.37 and 3.95
185 ng/mL. Serum aliquots had not undergone any previous freeze/thaws cycle.

186 *2.9 Enzyme-linked imunosorbent assay for small intestinal SIgA*

187 After the visceral organs of mice were excised and weighted, the small intestine was
188 removed and flushed with 2 mL cold calcium- and magnesium-free Hank's balanced salt
189 solution to remove intestinal contents [27]. The washing fluids were collected and stored at
190 −80 °C for the analysis of intestinal SIgA level by ELISA using a polyclonal goat anti-mouse
191 IgA to coat the plate, a purified mouse IgA as standard, and a horseradish
192 peroxidase-conjugated goat anti-mouse IgA [28].

193 *2.10 Statistical analysis*

194 Data were expressed as mean ± standard deviation (SD) and analyzed by one-way analysis
195 of variance (ANOVA) test, then multiple comparison of least-significant difference (equal
196 variances assumed) or Dunnett's T3-test (equal variances not assumed) was used to evaluate
197 the difference of parametric samples among groups. Final analyses of Luminex data for
198 cytokines considered data that fell within the detection limits of the Luminex assay.

Concentrations obtained below the sensitivity limit of detection (LOD) of the method were calculated as LOD/2 for statistical comparisons as reported in previous studies [29-31]. If raw data or log-transformed data did not meet the statistical criteria for the assumption of normality showing equal variance, the nonparametric Kruskal-Wallis test was used to determine statistical differences, and the adjusted significances were calculated and adopted to decrease the probability of type 1 error. Differences were considered to be statistically significant at $P<0.05$. All analyses were carried out using IBM SPSS Statistics version 20.0 and GraphPad Prism 5 Software.

3. Results

3.1 Effects of SOP on body weight and immune organ indices

We found that daily oral administration of SOP did not cause any mortality and no significant changes in final body weight and the indices of spleen and thymus, suggesting that SOP did not have over toxicity in mice (Table 1). Compared with whey protein control group, SOP 0.15 g/kg BW increased final body weight of mice (p=0.055).

Table 1. Effects of SOP on body weight and immune organ indices in mice

Groups	Initial body weight (g)	Final body weight (g)	Spleen index (mg/g)[♯]	Thymus index (mg/g)[♯]
Vehicle control	18.80±1.10	20.66±1.44	4.55±0.49	1.71±0.41
Whey protein control	18.77±0.81	20.06±1.07	4.50±0.64	1.52±0.38
SOP 0.15 g/kg BW	18.81±1.25	21.07±0.58	4.67±0.26	1.79±0.47
SOP 0.30 g/kg BW	18.79±0.95	20.21±1.37	4.35±0.36	1.80±0.55
SOP 0.60 g/kg BW	18.80±1.17	20.32±1.05	4.67±0.33	1.47±0.43

The data were presented as the means ± SD (n=10 per group), which were analysed by one-way analysis of variance (ANONA) test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

[#] Spleen index: Spleen weight/body weight; Thymus index: Thymus weight/body weight.

3.2 Effects of SOP on cell-mediated immunity

To evaluate the cell-mediated immunity in mice, we performed splenic lymphocyte proliferation and delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) (Figure 1). The treatment with SOP 0.15, 0.30 and 0.60 g/kg BW significantly increased the ConA-stimulated proliferation of splenic lymphocyte compared with vehicle control and whey protein control groups (Figure 1A). The increase of footpad thickness of each mouse in 24 h was significantly enhanced by 30%, 44%, 59% and 75% in whey protein control, SOP 0.15, 0.30 and 0.60 g/kg BW groups, respectively, compared with vehicle control group, and the treatment with SOP 0.30 and 0.60 g/kg BW significantly increased by 22% and 35% compared with whey protein control group (Figure 1B).

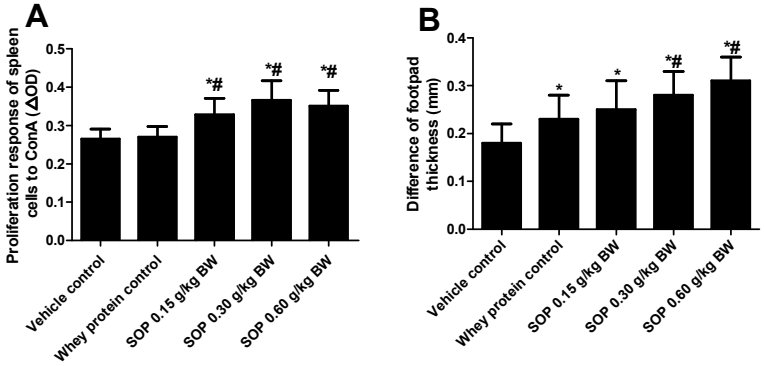

Figure 1. Effects of SOP on cell-mediated immunity in mice. (A) Proliferation response of splenic lymphocyte to ConA; (B) Delayed-type hypersensitivity to SRBC. Values represented the

mean ± SD (n=10 per group), which were analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * p<0.05 versus vehicle control group, and # p<0.05 versus whey protein control group.

3.3 Effects of SOP on humoral immunity

The effects of SOP on the humoral immunity in mice were assessed by IgM antibody response of spleen to SRBC [the plaque-forming cells (IgM-PFCs) assay] and serum hemolysin level (HC_{50}) in mice (Figure 2). The value of PFC per 5×10^6 spleen cells was significantly increased by 34%, 37% and 43% in SOP 0.15, 0.30 and 0.60 g/kg BW groups compared with vehicle control, and by 17%, 19% and 25% compared with whey protein control group, respectively (Figure 2A). Moreover, the mice treated with whey protein had a slight improvement in the value of PFC compared with vehicle control group (p=0.093). As shown in Figure 2B, the treatment with whey protein, SOP 0.15, 0.30 and 0.60 g/kg BW significantly enhanced HC_{50} by 12%, 21%, 24% and 23% compared with vehicle control group, respectively. The treatment with SOP 0.15, 0.30 and 0.60 g/kg BW also caused the increase of HC_{50} by 8% (p=0.077), 11% (p<0.05) and 10% (p<0.05) compared with whey protein control group, respectively.

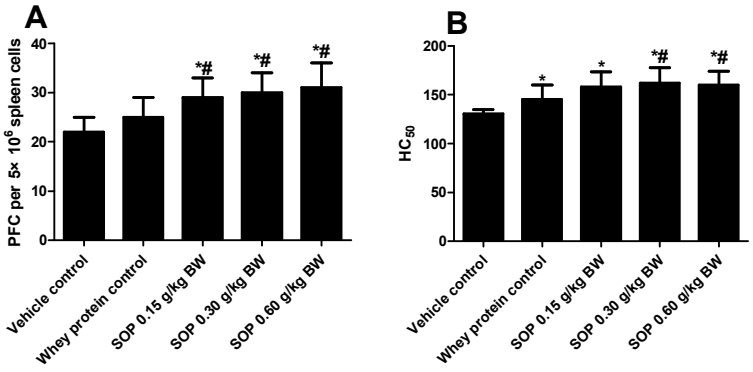

Figure 2. Effects of SOP on humoral immunity in mice. (A) IgM antibody response of spleen to SRBC; (B) Serum hemolysin level (HC_{50}). Values represented the mean ± SD (n=10 per group),

which were analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.4 Effects of SOP on macrophage phagocytosis

The phagocytosis capacity of peritoneal cavity phagocytes was studied through carbon clearance assay and chicken red blood cells (CRBCs) phagocytosis capacity assay (Figure 3). Macrophage phagocytosis in carbon clearance assay was significantly increased by 11%, 35%, 33% and 15% in whey protein control and SOP 0.15, 0.30 and 0.60 g/kg BW groups compared with vehicle control group, and by 22% and 20% in SOP 0.15 and 0.30 g/kg BW groups compared with whey protein control group, respectively (Figure 3A). From phagocytic capacity assay of peritoneal cavity phagocytes (Figure 3B), SOP 0.15, 0.30 and 0.60 g/kg BW significantly increased phagocytic rate by 29%, 24% and 24% compared with vehicle control group, respectively, and the treatment with SOP 0.15 g/kg BW significantly enhanced phagocytic rate by 25% compared with whey protein control groups. In addition, SOP 0.15, 0.30 and 0.60 g/kg BW significantly enhanced phagocytic index by 30%, 23% and 26% compared with vehicle control group, and by 20%, 13% and 17% compared with whey protein control group, respectively.

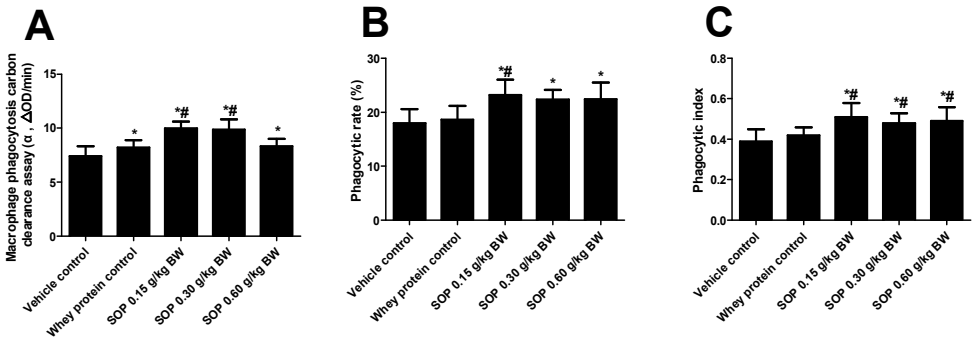

Figure 3. Effects of SOP on macrophage phagocytosis in mice. (A) Macrophage phagocytosis in mice

analyzed by carbon clearance assay; (B) Phagocytic rate of peritoneal cavity phagocytes in mice; (C) Phagocytic index of peritoneal cavity phagocytes in mice. Values represented the mean ± SD (n=10 per group), which were analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.5 Effects of SOP on NK cell activity

Besides macrophage phagocytosis, NK cell activity was another important function for innate immune system. We observed that the mice treated with SOP 0.30 and 0.60 g/kg BW had a significant increase in NK cell activity by 57% and 63% compared with vehicle control group (Figure 4).

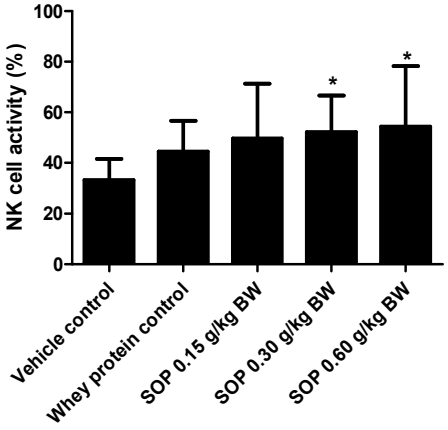

Figure 4. Effects of SOP on NK cell activity in mice. Values represented the mean ± SD (n=10 per group), which were transferred to $\sin^{-1}\sqrt{P}$ (P was NK cell activity) and analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.6 Effects of SOP on splenic T lymphocyte subpopulations

Given that SOP may increase the activities of T cells by altering the quantities of T cells or

285 their subpopulations, we conducted phenotypic analysis of total T cells and T cells subsets (Table
286 2). Compared with vehicle control, the significant increase in the percentage of total T cells ($CD3^+$)
287 was observed in mice treated with SOP 0.30 and 0.60 g/kg BW, and the treatment with SOP 0.15
288 g/kg BW showed a trend toward increasing the percentage of $CD3^+$ (p=0.074). In contrast to whey
289 protein, the treatment with SOP 0.30 (p<0.05) and 0.60 g/kg BW (p=0.075) improved the
290 percentage of $CD3^+$. We also observed a parallel increase in the percentage of $CD3^+CD4^+$ in mice
291 treated with SOP 0.30 and 0.60 g/kg BW compared with vehicle control (p<0.05). A significant
292 change in the percentage of $CD3^+CD8^+$ was absent among all groups. There was a trend toward
293 enhancing the ratio of $CD4^+/CD8^+$ in whey protein control (p=0.070) and SOP 0.30 g/kg BW
294 (p=0.064) groups. The treatment with whey protein, SOP 0.15, 0.30 and 0.60 g/kg BW
295 significantly increased the ratio of $CD4^+CD25^+$ by 87%, 52%, 36% and 74% compared with
296 vehicle control, and whey protein significantly enhanced the ratio of $CD4^+CD25^+$ compared with
297 SOP 0.15 and 0.30 g/kg BW.

298 Table 2. Effects of SOP on splenic T lymphocyte subpopulations

Groups	$CD3^+$ (%)	$CD3^+CD4^+$ (%)	$CD3^+CD8^+$ (%)	$CD4^+/CD8^+$	$CD4^+CD25^+$ (%)
Vehicle control	38.47±4.28	27.23±2.82	13.37±2.51	2.05±0.39	7.98±2.57
Whey protein control	39.26±3.87	28.28±2.71	12.58±1.83	2.26±0.27	14.90±1.54*
SOP 0.15 g/kg BW	41.24±2.94	28.19±2.85	12.79±2.24	2.19±0.27	12.12±2.40*#
SOP 0.30 g/kg BW	42.69±5.49*	29.40±3.51*	12.82±3.06	2.28±0.31	10.83±2.34*#
SOP 0.60 g/kg BW	42.58±3.11*#	29.43±2.76*	13.11±2.33	2.23±0.36	13.90±1.50*

299 The data were presented as the means ± SD (n=10 per group), which were analysed by ANONA

test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.7 Effects of SOP on serum cytokine concentrations

The previous study demonstrated that bioactive peptide enhanced Th cells to produce increased amounts of various cytokines [7]. Therefore, we investigated serum cytokine concentrations in mice. Compared with vehicle control, mice treated with whey protein significantly enhanced granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 concentrations in serum, and the treatment with SOP 0.30 g/kg BW showed an increasing trend in serum IL-6 concentration ($p=0.063$), but the depression were observed in IL-5 in mice treated with SOP 0.30 and 0.60 g/kg BW ($p<0.05$) and in tumor necrosis factor (TNF-α) in mice treated with SOP 0.15 g/kg BW ($p=0.058$) (Figure 5). In contrast to whey protein, the treatment with SOP significantly reduced serum GM-CSF, IL-5 and TNF-α concentrations of mice. There were no significant differences in IL-1α, IL-2, IL-4, IL-10, IL-12p70, IL-17A and interferon (IFN)-γ concentrations among all groups.

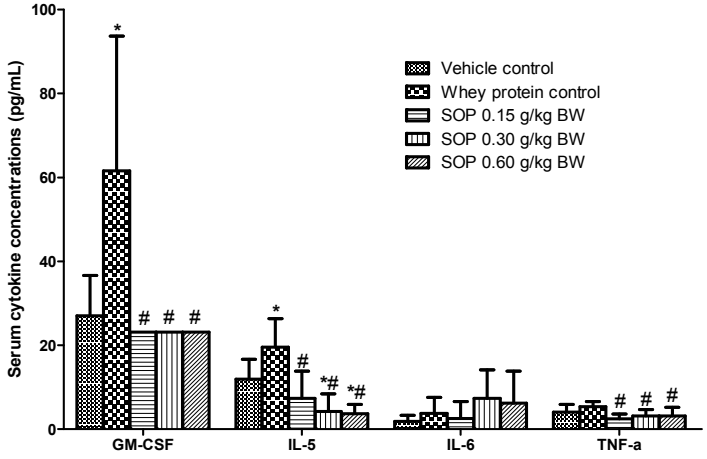

Figure 5. Effects of SOP on serum cytokine concentrations in mice. Values represented the mean ± SD (n=10 per group), which were analysed by ANONA test, and followed by least-significant

difference for post-hoc test between multiple groups. GM-CSF concentration was analysed by the nonparametric Kruskal-Wallis test was used to determine statistical differences, and pairwise comparisons were used for post-hoc test between multiple groups. To decrease the probability of Type 1 error, the adjusted significances were calculated. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.8 Effects of SOP on serum immunoglobulin levels

Immunoglobulin A (IgA) is the most abundant antibody isotype in mucosal secretions and owes its success in frontline immunity to its ability to undergo transcytosis across epithelial cells [8]. Antibodies of the IgG isotype, including IgG1, IgG2a, IgG2b and IgG3, play a crucial role in the cascade of the activation of humoral immune system, resulting in the clearance of invading microbes and the generation of long lasting immunity [9]. IgM has the key properties to eliminate infection in primary immune responses and promote the removal of apoptotic cells [9]. To investigate the pattern of SOP affecting humoral immunity, we conducted the serum immunoglobulin levels. As showed in Figure 6, we observed that mice treated with SOP 0.30 and 0.60 g/kg BW had significant increases in serum IgG1, IgG2b and IgG3 levels, and mice treated with SOP 0.30 g/kg BW also had significant increase in serum IgG2a level. The treatment with SOP 0.15 g/kg BW had significant increases in IgG1 and IgG2a levels and had a slight increase in IgG2b level ($p=0.079$). There were no significant differences in serum immunoglobulin levels between whey protein control group and vehicle control group. In contrast to whey protein, the significant increases in IgA, IgG2a, IgG2b and IgM levels were observed in mice treated with SOP 0.30 g/kg BW, and the treatment of SOP 0.60 g/kg BW showed a trend toward enhancing IgG2b level ($p=0.062$).

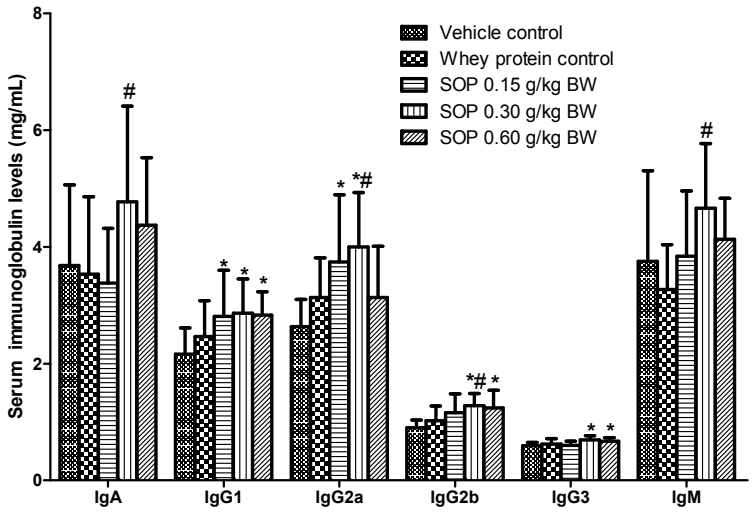

Figure 6. Effects of SOP on serum immunoglobulin levels in mice. The data were presented as the means ± SD (n=10 per group), which were analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * $p<0.05$ versus vehicle control group, and # $p<0.05$ versus whey protein control group.

3.9 Effects of SOP on small intestinal secretory IgA (SIgA) level

SIgA plays an important role in the protection and homeostatic regulation of intestinal, respiratory, and urogenital mucosal epithelia separating the outside environment from the inside of the body; and this primary function of SIgA is referred to as immune exclusion, a process that limits the access of numerous microorganisms and mucosal antigens to these thin and vulnerable mucosal barriers [10]. We investigated small intestinal SIgA level to confirm the effects of SOP on mucosal immunity. The significant increase in SIgA level was observed in mice treated with SOP 0.15, 0.30 and 0.60 g/kg BW compared with vehicle control (Figure 7). In addition, there was an increasing tread in SOP 0.15 (p=0.080) and 0.30 g/kg BW (p=0.067) groups compared with whey protein control.

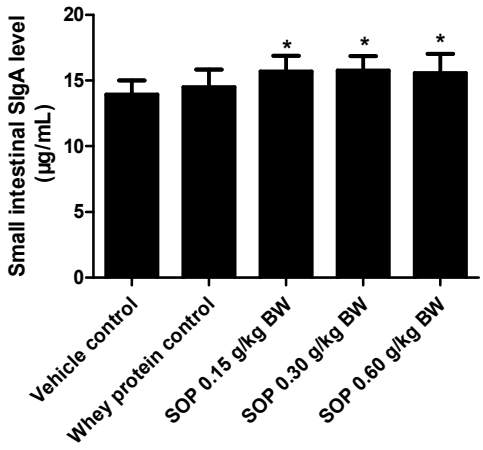

Figure 7. Effects of SOP on small intestinal SIgA level in mice. Values represented the mean ± SD (n=10 per group), which were analysed by ANONA test, and followed by least-significant difference for post-hoc test between multiple groups. * p<0.05 versus vehicle control group, and # p<0.05 versus whey protein control group.

4 Discussion

Derived from enzymatic hydrolysis, SOP, in which major components were small molecule oligopeptides, is potential as immunomodulator. In vertebrates, two types of immunity are used to protect the host from infections: innate and adaptive. The innate immune system is genetically programmed to detect invariant features of invading microbes, and the adaptive immune system, comprised on T and B lymphocytes, employs antigen receptors that are not encoded in the germ-line but generated de novo in each organism [7]. Adaptive immune response included two types: humoral immunity and cell-mediated immunity [11, 12]. Innate immune cells include dendritic cells, macrophages, and neutrophils, among others [13], and we investigated innate immune response via the macrophage phagocytosis and NK cell activity. The present study demonstrated that SOP could improve the innate immunity by enhancing the functions of cell-mediated immunity and humoral immunity, and improve adaptive immune responses by

increasing macrophage phagocytosis and NK cell activity. Furthermore, the immunomodulatory effects of SOP were generally better than those of whey protein, suggesting that SOP would be a potential immunomodulator. Although the mechanism underlying the immunomodulatory effects of food-derived bioactive peptides remains uncertain, there is accumulating evidence that they may enhance lymphocyte proliferation and antibody synthesis [14], macrophages function [15, 16], humoral immune response, $CD4^+$ T helper percentage, cytokines [7] and NK activity [17]. We also observed that the treatment of SOP increased cell-mediated immunity by enhancing ConA-stimulated proliferation of splenic lymphocyte and delayed-type hypersensitivity to SRBC, as well as humoral immunity by increasing IgM antibody response of spleen to SRBC and serum hemolysin level. These results were consistent with previous data, for example, marine oligopeptide preparation from Chum Salmon (*Oncorhynchus keta*) could significantly increase lymphocyte proliferation and the number of plaque-forming cells (antibody production) [7].

T lymphocytes coordinate the immune response and play a central role in cell-mediated immunity [11]. T lymphocyte ($CD3^+$) subsets include two main lymphocyte T subsets - helper ($CD4^+$) T cells and suppressor/cytotoxic ($CD8^+$) T, and their ratio ($CD4^+/CD8^+$) is the critical indicator to evaluate the homeostasis of immune system. $CD4^+$ T cells can be divided to two distinct subsets of effector cells: T helper type 1 (Th1) cells which produces inflammatory cytokines (IL-2, IL-12, IFN-γ, GM-CSF and TNF) inducing cell-mediated immune responses, or T helper type 2 (Th2) cells which secrets cytokines as like IL-4, IL-5, IL-6 and IL-10 inducing humoral or allergic responses [18, 19]. In our study, SOP significantly increased the percentages of $CD3^+$ and $CD4^+$ T cells in agreement with the results in cell-mediated immunity assays. We further performed cytokine profile measurement using new multiplex sandwich immunoassays

technology. We found that the increases in inflammatory cytokines secreted by Th1 were absent, revealing that the elevated function of cell-mediated immunity might be due to the increase of effector cells, as like $CD3^+$ and $CD4^+$ T cells. In addition, although serum IL-5 in mice treated with SOP 0.30 and 0.60 g/kg BW was depressed, serum IL-6, derived from Th2 cells, in mice treated with SOP 0.30 g/kg BW had an increasing trend compared with vehicle control, and that might partly contribute to the improvement of humoral immunity. In contrast, the mice treated with whey protein had an increased GM-CSF and IL-5 concentrations, probably inducing the increment of DTH, HC_{50} and macrophage phagocytosis in carbon clearance assay. Moreover, the previous study demonstrated that human $CD4^+CD25^+$ regulatory T cells (Tregs) could suppress both proliferation and cytokine production, and this suppression was mediated by cell-to-cell contact but not by a humoral factor or the inhibition of antigen-presenting cell [20]. In the present study, we obtained significant enhancement in the percentage of $CD4^+CD25^+$ in whey protein and SOP groups, which might result to the decreased IL-5 concentration in SOP 0.30 and 0.60 g/kg BW groups. Therefore, Tregs were critical in regulating the function of other immune cells and preventing potentially harmful autoimmune responses [21].

Besides secreted cytokines from Th2 cells, the antibody responses of IgM, IgG and IgA were also belonged to the effector classes of humoral immune responses. IgA antibodies play an important role in mucosal immunity. IgG, the smallest but most common antibody, is very critical in promoting the phagocytosis of monocyte macrophages to defense against bacterial and viral infections. IgM, the largest antibody, is the first type of antibody made in response to an early-stage infection, and also causes other immune system cells to destroy foreign substances. When the host is attacked, antimicrobial peptides, secretory IgA and circulatory IgM, as the

low-cost effector mechanisms of defense mechanisms of immune system, are expressed constitutively; when they are insufficient to contain pathogens, the response of next lowest cost, tissue-resident macrophages, is induced; and when this response is insufficient, IgG1 is induced, and so on. The order of next engagement is IFN-γ–activated macrophages, neutrophils, IgG2a and IgG2b, CTL and Th17 [22]. A previous study has shown that oral administration of β-casomorphins, opioid like bioactive peptides released on digestion of β-casein of milk, significantly increased humoral immune response (IgE, IgG and IgG1/IgG2a in intestinal fluid) [23]. We also observed that the treatment of SOP induced major increases in small intestinal SIgA, serum IgG1, IgG2a, IgG2b and IgG3 concentrations, which may be the main effectors contributing to the improvement of humoral immune, macrophage phagocytosis and NK cell activity. This might be attributed to the protection of SIgA on gut immune system, the antibody functions of IgG, and the interaction of innate immune and adaptive immune. The cells of innate immune system, such as mast cells, monocytes, macrophages, eosinophils, and neutrophils, including a small population of NK cells express activating and inhibitory Fcγ receptors simultaneously, thereby setting a threshold for cell activation by IgG, which prevents unwanted initiation of potentially dangerous effector functions such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), degranulation, and release of various immunoregulatory molecules [9]. In addition, cytokines or activated complement proteins released during pro-inflammatory reactions can also modulate this threshold [24, 25].

4. Conclusions

The data presented in the current study clearly demonstrated that oral administration of SOP had the potential to increase the innate immune responses via macrophage phagocytosis and NK

cell activity and enhance the adaptive immune responses via cell-mediated immunity and humoral immunity, which most likely attributed to the stimulation of Th cells, cytokines secretion and antibody production. It is supposed from these results that SOP could be a candidate immunomodulator to improve immune function of immunocompromised persons or cancer patients.

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Conflict of interest

The authors declare no conflict of interest.

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