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Augmentation of natural killer cell activity *in vitro* and *in vivo* by sericin-derived oligopeptides

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Abbreviations:
BW, body weight
ConA, Concanavalin A
ELISA, enzyme-linked immunosorbent assay
GC/MS, gas chromatography/mass spectrometry
IFN-γ, interferon-γ
IL-2, interleukin-2

ABSTRACT

This study investigated the effects of sericin-derived oligopeptides on natural killer (NK) activity. *In vitro* exposure of human peripheral blood mononuclear cells with sericin-derived oligopeptides resulted in an augmentation of NK cell activity against K562 target cells and the effects appeared to be dose-related. Experiments designed to examine whether enhanced NK activity was due to direct or indirect activation of NK cells revealed that sericin oligopeptides did not induce activity of purified NK cells, and that sericin oligopeptides augmented NK activity indirectly by inducing the production of IL-2 and IFN-γ cytokines. In *in vivo* experimentation where mice were orally administered with sericin oligopeptides and splenic mononuclear cells tested against YAC-1 target cells, significant increase in NK activity was obtained compared to control mice. Elevated levels of IL-2 were also evident in all oligopeptides-treated groups. As demonstrated both *in vitro* and *in vivo*, these results indicate that sericin-derived oligopeptides have efficient NK-enhancing activity and suggest the potential therapeutic applications of such oligopeptides for functional improvement of NK cells, and possibly for treatment of tumor and infectious diseases in which NK activity contributes to host defense.

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MTT, 3-(4,5-dimethylthiazol-2-yl)-2,
5-diphenyltetrazolium bromide
NK, natural killer
OD, optical density
PBMCs, peripheral blood
mononuclear cells
PBS, phosphate-buffered saline
SDO, sericin-derived oligopeptides
SEM, standard error of the mean

Introduction

Natural killer (NK) cells are innate immune effector cells which are phenotypically characterized as $CD3^{-}CD56^{+}$ large granular lymphocytes (Trinchieri, 1989; Lotzova, 1993). These cells play a critical role in immune surveillance against virus infections (Andrews et al., 2003; Andoniou et al., 2006) and malignant transformation (Smyth et al., 2000; Wu and Lanier, 2003). Unlike cytotoxic T lymphocytes, NK cells destroy target cells by direct cytotoxicity without requirement for prior sensitization (Cooper et al., 2001; Groth et al., 2011), putting them at a forefront of lymphocyte defenses against virus-infected and tumor cells (Lotzova, 1993). While fully functional NK cells are advisable for optimal health, cumulative evidence indicate that advancing age, chronic diseases, physical and mental stresses or even unhealthy lifestyles can result in the decreased NK cell activity (Imai and Nakachi, 2001; Plackett et al., 2004; Lutz and Quinn, 2012; Solana et al., 2012; Berrou et al., 2013; Chan et al., 2014). The immune impairment states presenting with low NK activity are often associated with malignancies and chronic viral infections (Mysliwska et al., 1992; Ogata et al., 1997; Imai et al., 2000). More seriously, the declining NK function toward tumors and severe infections was reported to be correlated with death in the elderly subjects (Ogata et al., 1997; Imai et al., 2000). Usually, those cellular stresses that lead to functional impairment in NK cells are difficult to avoid, and such undesired state poses a significant challenge in the clinical field across the world. Supplementation of the natural host defense with NK-enhancing agents is a promising means for optimizing the immunological state, or delaying the NK function decline.

Bioactive peptides, the specific protein fragments that have regulatory functions in human system, have demonstrated potential for application as health-promoting agents against numerous chronic diseases and certain physiological conditions, including cancer, cardiovascular diseases and inflammation (Korhonen and Pihlanto, 2006; Hartmann and Meisel, 2007; Udenigwe and Aluko, 2012). With low molecular weight, these peptides are easily absorbed into the intestinal tract. Indeed, absorption as short-chain peptides is considered to be more effective than intact protein and free amino acids of equivalent amounts (Grimble et al., 1987; Seimensma et al., 1993). Since efficient intestinal absorption reflects optimal health benefits, the downstream health effects upon administration of the short-chain peptides would be superior to those of intact protein and free amino acids. Bioactive peptides are mostly derived from dietary proteins of animal or plant origins. Although these peptides can theoretically be released from dietary proteins during gastrointestinal digestion process, the amount of peptides generated during digestion is probably too low to induce significant effects on the target health system, especially when a therapeutic effect is expected (Gauthier et al., 2006). Instead, bioactive peptide concentrates can be produced using enzymatic hydrolysis and separation technologies. To date, enzyme hydrolysis appears to be the most appropriate method for bioactive peptide production, not only because of their commercially availability and moderate cost, but also because of high quality of the peptide products (Clemente et al., 1999). As the potential health valuable molecules, bioactive peptides have increasingly received a great deal of attention both in scientific and commercial scales.

Sericin is the main constituent of cocoon proteins from the silkworm *Bombyx mori*, comprising of 25–30% of the total cocoon weight (Mondal et al., 2007). Although sericin is considered as an unutilized by-product of the textile industry, scientific studies have shown that sericin possesses various biological activities, including inhibition of tyrosinase and lipid peroxidation (Kato et al., 1998; Aramwit et al., 2010), suppression of colon carcinogenesis (Sasaki et al., 2000; Zhaorigetu et al., 2001), reduction of serum lipids (Okazaki et al., 2010), and protection against UV-induced keratinocyte apoptosis (Dash et al., 2008) and alcohol-mediated liver damage (Li et al., 2008). Generally, sericin preparation from the silk cocoons is heterogeneous, with molecular weight ranging widely from 10 to over 300 kDa (Zhang, 2002). In terms of efficient intestinal absorption and amounts of the potent peptides required for inducing significant health effects, a wide molecular weight distribution may limit uses of such sericin preparation. Potent peptide concentrates of narrow and defined molecular size would be preferential, providing an alternative to the native sericin. Recently, biological activities including vasorelaxation and blood pressure lowering activities of sericin oligopeptides have been described (Onsa-Ard et al., 2013). Nevertheless, their effects on immune system have not experimentally been explored. The present study therefore investigated the effects of oligopeptides derived from enzymatic hydrolysis of the sericin protein on the immune system with particular emphasis on the NK cell activity. Both *in vitro* and *in vivo* experimentations were performed to ascertain their potentials for therapeutic applications.

Materials and methods

Preparation of sericin-derived oligopeptides

Sericin-derived oligopeptides (SDO) were prepared from cocoons of the silkworm *B. mori* according to a pending patent with international publication number WO 2013/032411 A1. Briefly, silk cocoons cut into small pieces were extracted for sericin protein under high pressure (150 psi) and high temperature (121 °C) for 15 min. The resulting sericin solution was then subjected to protease enzymatic hydrolysis (0.01 U/mL protease enzyme in 0.036 M $CaCl_2$ solution at a 1:1 volumetric ratio) at 37 °C for 1 h. Enzymatic activity was inactivated at 90 °C for 15 min and the mixture was cooled to room temperature prior to separating the solid components by centrifugation at 9500 × *g* for 15 min at 4 °C. Oligopeptides with a molecular weight lower than 5000 Da were separated from larger oligopeptides by hollow fiber membrane technology using a hollow fiber membrane cartridge with molecular weight cut off (MWCO) of 5000 Da (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The obtained oligopeptides of approximately 5000 Da were kept lyophilized until use. The amino acid composition of SDO analyzed by GC/MS is shown in Table 1.

Tumor target cell lines

The human chronic myelogenous leukemic cell line K562 and the murine Moloney virus-transformed lymphoma cell line YAC-1 (American Type Culture Collection, Manassas, VA) were used as target cells to assay NK cell activity. These NK-sensitive cell lines were maintained under 5% CO_2 and 37 °C in RPMI-1640 medium (PAA, Pasching, Austria) supplemented with 10% (v/v) fetal bovine serum (Gibco, South America), 0.01 M HEPES pH 7.4, 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA). This medium was referred to as complete RPMI medium.

Table 1 – Amino acid composition of sericin-derived oligopeptides.

Amino acids	Amounts (%)
Alanine	2.54
Arginine	0.007
Aspartic acid	13.42
Cysteine	0.007
Glutamic acid	3.72
Glycine	4.79
Histidine	7.66
Isoleucine	1.34
Leucine	3.01
Lysine	15.93
Methionine	0.007
Phenylalanine	1.57
Proline	0.82
Serine	14.27
Threonine	3.33
Tryptophan	0.007
Tyrosine	23.86
Valine	3.70

Isolation of human PBMCs and NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy blood donors by Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient centrifugation according to the manufacturer's instructions. The isolated PBMCs were washed three times before resuspending in complete RPMI medium. Human NK cells were isolated from PBMCs by immunomagentic cell separation through CD56 microbeads and MiniMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) as specified by the manufacturer. Purity of the isolated NK cells was approximately 90% as determined by flow cytometry. The experimental protocol was reviewed and approved by the Institutional Human Review Board.

Cell viability

Approximately 1×10^5 PBMCs were cultured in the absence or presence of SDO at concentration ranging from 100 to 500 μg/mL for 120 h at 37 °C in a humidified CO_2 incubator. Total, viable and non-viable cell numbers were counted under microscope with the help of a hemocytometer following staining by trypan blue. The percentage of cell viability was calculated using an equation:

$$\%\,\text{viability} = \left(\frac{\text{viable cell number}}{\text{total cell number}}\right) \times 100$$

Animals and treatment

Female BALB/c mice at 7 weeks of age were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed in well-ventilated cages at a constant temperature (25 ± 1 °C) under a 12-h dark:light cycle with free access to sterile water and standard mouse diet (CP Company, Thailand). The *in vivo* experiments were performed in compliance with Guidelines in the Care and Use of animals and all procedures were approved by the Institutional Animal Research Ethics Committee. Mice were randomly divided into four groups of five each. Treated groups were orally administered with 50, 100 or 500 mg/kg body weight (BW) of SDO once daily for 7 days. Vehicle control received only sterile distilled water. Routine clinical observations and changes in body weights were recorded throughout the study period. Mice were sacrificed 24 h after the last dose by receiving an overdose of 50 mg/kg BW (intraperitoneal) Thiopental sodium (THIOPENTAL, Bigpharma, Thailand). Vital organs (spleen, thymus, liver, lung and kidney) were removed and weighed immediately and their indices were expressed as 100 × organ weight/body weight.

Preparation of splenic single cells

The individual mouse spleen removed aseptically was placed in a PCM buffer prepared with sterile phosphate-buffered saline (PBS) pH 7.4 containing 7×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$, 5% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA). Single cells were isolated by gently pressing the spleen through a cell strainer (BD

Falcon, NJ). Red blood cells were lysed by 0.17 M NH_4Cl, pH 7.65 and the remaining cell suspensions were washed twice with PCM buffer and adjusted to a desired concentration in RPMI-1640 medium (PAA) containing 10% (v/v) fetal bovine serum (Gibco), 0.01 M HEPES pH 7.4, 5×10^{-5} M β-mercaptoethanol (BioRad, Hercules, CA), 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA).

Natural killer cell activity assay

Natural killer (NK) cell activity was assessed based upon the ability of mononuclear cells to lyse tumor target cells. This was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Choi et al., 2005; Yuan et al., 2009) with some modifications. Human PBMCs (effectors) were mixed with SDO at final concentrations of 50, 100 and 500 μg/mL and co-cultured with K562 (targets) in 96-well flat-bottom plates (Nunc™, Roskilde, Denmark) for 20 h at 37 °C in a humidified 5% CO_2 atmosphere. The effector: target (E:T) ratios were set up to 3.13:1, 1.56:1 and 0.78:1 in a total volume of 200 μL in each well. Recombinant human interleukin-2 (IL-2; Roche) and interferon-γ (IFN-γ; Roche) at the concentration of 100 U/mL and 650 U/mL, respectively, were included in the experiment as positive controls (Ballas et al., 1996; Masera et al., 1996). After the incubation, 20 μL of MTT (5 mg/mL; Sigma, St. Louis, MO) was added. The plates were incubated for additional 3 h and subsequently subjected to an MTT assay (Mosmann, 1983). Control wells contained either effector or target cells alone, and all tests were performed in triplicates. The optical density (OD) at 540 nm was determined by using a microplate spectrophotometer (Labsystem iEM Reader MF). The percentage of NK cell cytotoxic activity was calculated using the following equation:

$$\%\,\text{NK cytotoxic activity} = \left\{1 - \left[\frac{\text{OD test} - \text{OD effector cell control}}{\text{OD target cell control}}\right]\right\} \times 100$$

In a separate experiment, purified NK cells were treated with SDO at a final concentration of 500 μg/mL for 20 h followed by cytotoxicity assay against K562 target cells.

To determine NK activity of mice fed SDO, splenic single cells and YAC-1 cells as effector and target cells, respectively, were cultured together in 96-well flat-bottom plates (Nunc™) at E:T ratios of 100:1 and 50:1 in a total volume of 200 μL in each well. After 20-h incubation, an MTT assay was performed and % NK activity was calculated as described above.

Cytokine assay

Human PBMCs of approximately 1×10^5 cells were incubated with SDO at final concentrations of 50, 100 and 500 μg/mL. After incubating the cells at 37 °C in 5% CO_2 atmosphere for 72 h, the culture supernatants were collected and assayed for IL-2 and IFN-γ by sandwich enzyme-linked immunosorbent assay (ELISA) using ELISA MAX™ Deluxe sets for human IL-2 and IFN-γ (BioLegend, San Diego, CA), respectively. All assay procedures were performed according to the manufacturers' instructions and the detection limits for both assays were 7.8 pg/mL.

In the animal experiment, a total of 2×10^5 splenic cells were cultured in flat-bottom 96-well plates (Nunc™) in the presence of Concanavalin A (ConA, 0.5 μg/mL; Sigma) and incubated at 37 °C in 5% CO_2 atmosphere. After 72-h incubation, culture supernatants were collected and amounts of IL-2 secreted were determined by sandwich ELISA using Ready-Set-Go for mouse IL-2 (eBioscience) as described by the manufacturer. The detection limit was 2 pg/mL.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was analyzed using Student's *t*-test at the significance level $2\alpha = 0.05$.

Results

Effect of SDO on viability of human PBMCs

Viability of human PBMCs after incubation with SDO is presented in Fig. 1. As determined by trypan blue dye exclusion, the percentage of viable cells treated with SDO was similar to that of untreated cells. The result indicated that SDO at the concentration up to 500 μg/mL was not toxic to human PBMCs.

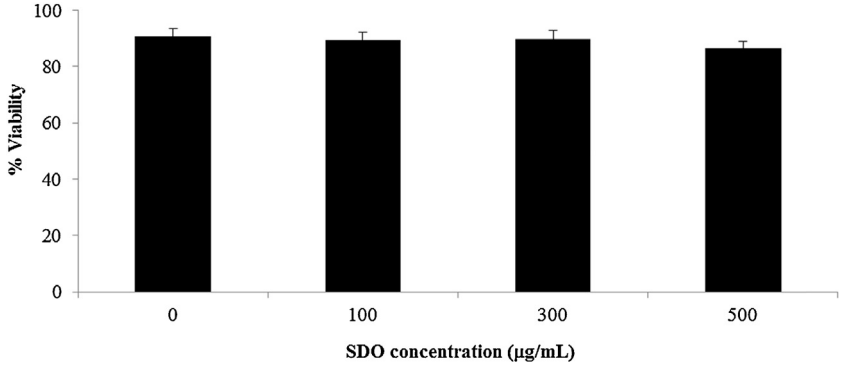

Fig. 1 – Effect of SDO on viability of human PBMCs. Viability of human PBMCs exposed to SDO at concentrations of 100, 300 and 500 μg/mL for 120 h was determined by trypan blue dye exclusion. Values are mean ± SEM, *n* = 5–8.

Table 2 – NK cell activity of human PBMCs after *in vitro* exposure to varying concentrations of SDO at different effector:target (E:T) ratios.[a]

Treatment	Concentration (μg/mL)	% NK cell activity at E:T ratio		
		3.13:1	1.56:1	0.78:1
SDO	0	26.96 ± 0.65	35.61 ± 4.20	33.33 ± 1.52
	50	19.12 ± 3.06	38.18 ± 2.84	38.26 ± 0.98
	100	26.23 ± 4.25	41.52 ± 6.04	36.99 ± 0.61
	500	36.03 ± 2.12*	43.03 ± 4.49	41.21 ± 0.78*
IL-2	100 U/mL	37.82 ± 7.80	39.30 ± 6.59	33.18 ± 6.66
IFN-γ	650 U/mL	39.75 ± 9.70	35.88 ± 5.56	36.35 ± 3.91

[a] Human PBMCs were incubated with SDO at different concentrations and tested for NK cell activity against K562 tumor target cells. Values expressed as mean ± SEM are the representative of four independent experiments with similar results.
* Statistically significant *versus* untreated controls.

Effect of SDO on NK cell activity in vitro

The *in vitro* exposure of PBMCs with SDO resulted in an increased NK cell activity and the effect appeared to be dose-related (Table 2). SDO at the concentration of 500 μg/mL significantly enhanced NK cell activity at most of the effector cell-to-target cell ratios tested, as compared to the untreated control. Interestingly, the percentages of NK activity after exposure to SDO (500 μg/mL; E:T ratios 1.56:1 and 0.78:1) were higher than those of recombinant IL-2 and IFN-γ (Table 2).

To examine further whether the increased NK activity obtained above was due to direct or indirect activation of NK cells, purified NK cells prepared from PBMCs were treated with SDO at a final concentration of 500 μg/mL for 20 h prior to the cytotoxicity assay against K562 target cells. Though detected, % cytotoxicity of SDO-treated NK cells was similar to that of untreated cells (results not shown). However, in an additional experiment where cell-free culture supernatant collected from SDO-treated PBMCs was added to the purified NK cells, significant increase (statistically significant) in NK activity was observed compared to the control supernatant from untreated PBMCs (Fig. 2). The activity of NK cells raised up to 2.5-folds upon the exposure to the SDO-conditioned supernatant. These results suggested an important role of soluble factors in the SDO-conditioned supernatant in augmenting the activity of NK cells. It is particularly to note that ELISA assays to detect cytokine production indicated significant elevation (statistically significant) of IL-2 and IFN-γ levels in the SDO-treated supernatant compared to untreated control (Fig. 3).

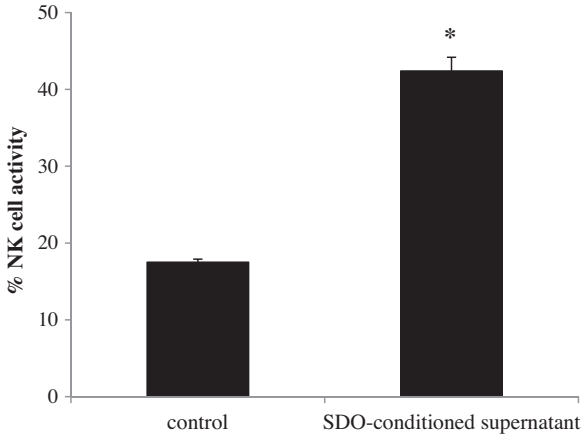

Fig. 2 – Effect of SDO-conditioned culture supernatant on NK activity. Purified NK cells were treated with conditioned supernatant collected from SDO-treated PBMCs for 20 h and the cytotoxicity was assayed against K562 target cells. Values are mean ± SEM from two independent experiments. *Statistically significant as compared with the control (supernatant from untreated PBMCs).

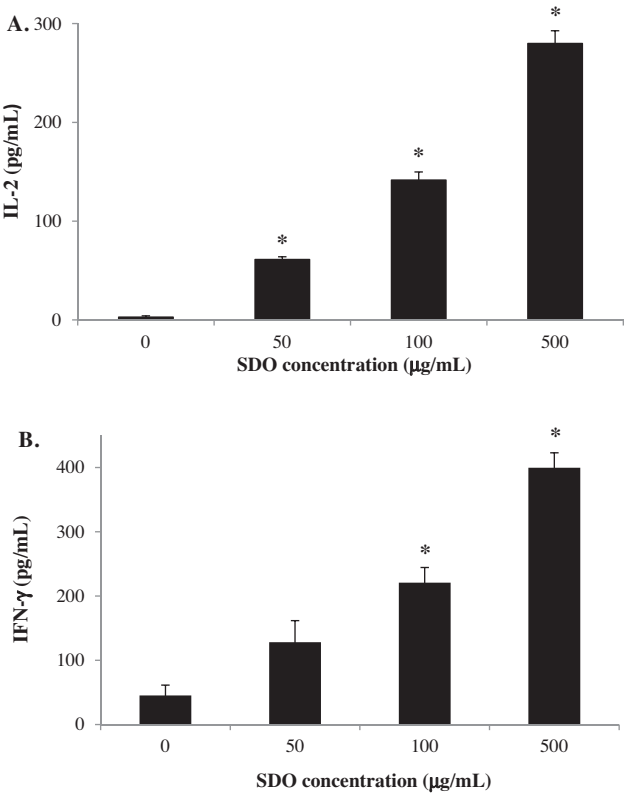

Fig. 3 – Effect of SDO on *in vitro* cytokine production. Human PBMCs were treated with various concentrations of SDO at 37 °C in 5% CO_2 atmosphere. After 72 h, culture supernatants were harvested and measured for IL-2 (A) and IFN-γ (B) by sandwich ELISA. Values are mean ± SEM from two independent experiments. *Statistically significant as compared with the control.

Effect of SDO on body and organ weights

In animal experimentation, daily oral administration of SDO did not produce any signs of illnesses, and no mortality was observed. There were no significant differences in the body weights and vital organs indices between the control and treated mice throughout the study period (results not shown).

Effect of SDO on NK cell activity in vivo

To examine the ability of SDO to induce NK activity *in vivo*, mice were orally administered with SDO consecutively for 7 days and their splenic mononuclear cells tested for the ability to lyse YAC-1 target cells. As shown in Fig. 4, the percentage of NK activity of splenic mononuclear cells from mice fed SDO was significantly higher (statistically significant) than that of control mice. The enhanced NK activity was consistently demonstrated in all SDO-treated groups across the E:T ratios evaluated.

Effect of SDO on cytokine production from mice fed SDO

The production of cytokine measured from the supernatants of ConA-stimulated splenic cultures from mice fed SDO is presented in Fig. 5. Elevated levels of IL-2, though not reached statistically significant, were evident in all SDO-treated groups compared to untreated control.

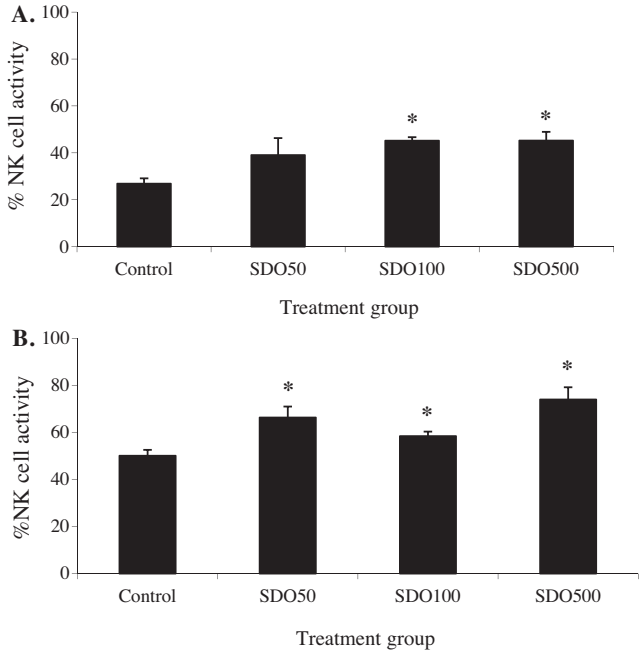

Fig. 4 – Effect of SDO on NK activity *in vivo*. Splenic cells were isolated from BALB/c mice fed SDO daily for 7 days and cultured with YAC-1 target cells at E:T ratios of 25:1 (A) and 50:1 (B). After 20 h in a 37 °C and 5% CO_2 incubator, activity of NK cells was determined by MTT assay. SDO50, SDO100 and SDO500, sericin-derived oligopeptides 50, 100 and 500 mg/kg BW treated groups. Values are mean ± SEM, *n* = 5. *Statistically significant as compared with the control.

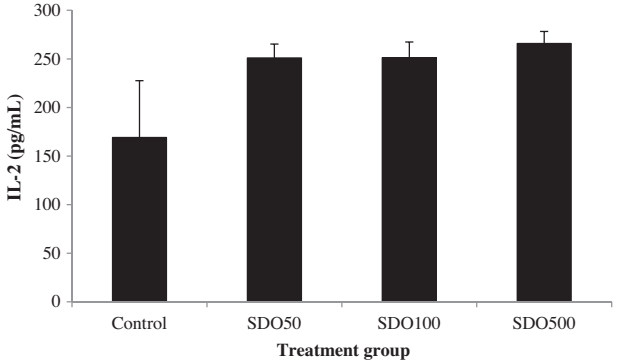

Fig. 5 – Effect of SDO on IL-2 production from mice fed SDO. Splenic cells from mice fed SDO were cultured with ConA at 37 °C in 5% CO_2 atmosphere. After 72 h, supernatants were harvested and analyzed by sandwich ELISA. SDO50, SDO100 and SDO500, sericin-derived oligopeptides 50, 100 and 500 mg/kg BW treated groups. Values are mean ± SEM, *n* = 5.

Discussion

This study reported for the first time the effects of oligopeptides derived from sericin protein on NK cell activity. The results herein clearly demonstrated that sericin-derived oligopeptides efficiently enhanced NK cell activity and the effect was greater than that achieved by recombinant IL-2 and IFN-γ, the known NK stimulators. Significantly, the SDO-enhanced NK activity was consistently observed both in human immune cells, *in vitro* and in mice, *in vivo*. SDO also modulated the immune system by elevating the IL-2 and IFN-γ production, and this effect appeared to be correlated with the augmentation of NK cell activity. Moreover, no signs of harmful effects were detected when SDO were evaluated *in vitro* and *in vivo*, suggesting low toxicity of these sericin oligopeptides.

Up till now, various experimental models and approaches have been used to study immunomodulatory natural health products. While specific effects of the studied product on specific cell types can be seen through *in vitro* studies, systemic effects, however, cannot be taken into consideration by such measurements (Haddad et al., 2005). The *in vivo* animal models would thus additionally be required. Nevertheless, in many cases, investigations of the studied products *in vivo* did not result in the same biological activity as that observed *in vitro* (Otani and Hata, 1995; Cross and Gill, 1999; Gauthier et al., 2006). Undoubtedly, this conflicting result would be a major obstacle for such studied product being developed as potential therapeutics (Eriksen et al., 2008). The fact that significant increases in NK cell activity were noted not only when SDO were added directly to cultures of human PBMCs but also when they were orally administered to the mice, these findings thus indicated the true immunomodulatory potential of these sericin oligopeptides. In this regard, SDO would be unaffected to radical alteration by physiological exposure to gut digestive enzymes in mice. Also, it is likely that SDO were effectively absorbed through the epithelial cells lining the intestinal mucosa, transported to the target immune cells, and ultimately

their significant capability of inducing NK activity retained, as that observed *in vitro*. The sericin-based oligopeptides preparation in this study has proven useful and further development as promising therapeutics is therefore warranted.

It should be noted that our results on NK activity were obtained from mixed mononuclear cells, and not from the purified NK cells. The use of a whole mononuclear cell preparation provides a more physiological system where cell-to-cell regulatory mechanisms are intact and several sources of soluble mediators are present (Masera et al., 1996). With respect to this, SDO may be activating cells which influence NK activity. It is well documented that $CD4^+$ T lymphocytes and macrophages can respectively release IL-2 and IFN-γ, which are potent NK stimulators (Itoh et al., 1985; Robertson and Ritz, 1990; Smyth and Ortaldo, 1991). In our separate experiment, the culture supernatant collected from SDO-treated PBMCs substantially contained IL-2 and IFN-γ cytokines, and the addition of this SDO-conditioned supernatant significantly increased effector function of purified NK cells, suggesting an important role of such cytokines for augmentation of NK activity. Although other soluble mediators and/or additional regulatory mechanisms cannot be excluded, it is conceivable that SDO up-regulated the production of IL-2 and IFN-γ cytokines in $CD4^+$ T cells and macrophages which, in turn, enhanced NK activity. As the increased NK activity and IL-2 production were obviously shown in mice fed SDO, we also believe that the NK increment could be associated with the induction of IL-2 production. Further investigations are however required to explore more fully the mechanisms by which SDO augmented NK cell activity.

In conclusion, this study demonstrated, both *in vitro* and *in vivo*, the efficient capability of the sericin-derived oligopeptides in augmenting natural killer cell activity. These findings suggest the potential therapeutic applications of these sericin oligopeptides for functional improvement of NK cells, and possibly for treatment of tumor and infectious diseases in which NK activity contributes to host defense.

Conflict of interest

The authors declare no conflict of interest.

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