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Different level of tumor necrosis factor-α expression after administration of silk sericin fraction in RAW264.7 cells

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Abstract

Tumor necrosis factor-α (TNFα) is a representative marker for inflammation. Silk sericin is known as mild TNFα inducer. The purpose of this study was to compare the level of TNFα among different fractions of silk sericin. Silk sericin was extracted from cocoon and separated it by molecular weight. Each fraction was applied to RAW264.7 cells. The level of TNFα was evaluated by western blot and ELISA assay. In results, the level of TNFα was increased as time-dependent manner. Higher molecular weight fraction of sericin induced higher amount of TNFα than lower molecular weight fraction. In conclusion, different molecular weight fraction of sericin induced TNFα differently.

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Introduction

The cocoon of silkworm is mainly composed of silk fibroin and silk sericin (Park and Um, 2018). Silk sericin is removed during degumming process and it is considered as industrial waste (Park and Um, 2016). Recently, beneficial effect of silk sericin has been clarified (Gholipourmalekabadi *et al.,* 2020; Ahsan *et al.,* 2018). Accordingly, silk sericin has been widely used for medical and cosmetic purpose. Silk sericin can be produced by degumming process (Park and Um, 2016, 2018). There are different types of degumming process (Bae *et al.,* 2016). Biological effect of protein is dependent on its conformation and molecular weight. The conformation and molecular weight of silk protein should be different to its degumming process (Bae *et al.,* 2016). There have been several reports about the biological effect of silk sericin according to its degumming process and biological effect of silk sericin is different to its degumming process (Nuchadomrong *et al.,* 20019; Zhang *et al.,* 2006). However, the biological effect of silk sericin according to its molecular weight has not been clarified when it is extracted by the same degumming process.

Silk sericin is glue-like protein. There are 3 types of silk sericin. Among them, sericin 1 and sericin 3 are main component of silkworm cocoon (Kaur *et al*., 2013; Zhang *et al*., 2015). The composition of sericin 1 and sericin 3 are different to layers. Sericin 1 is abundant in the outer layer (Kaur *et al*., 2013; Zhang *et al*., 2015) and sericin 3 is lower in the middle layer (Zhang *et al*., 2015). Main amino acid of silk sericin is serine. Silk sericin is mostly hydrophilic protein. The molecular weight of sericin 1 and sericin 3 from *Bombyx mori* is approximately 120 kDa according to National Center for Biotechnology Information

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(NCBI). In the degumming solution, silk sericin is found from 200 kDa to few kDa because it is found from dimer to fragmented one (Mandal *et al*., 2009).

RAW264.7 cells is mouse macrophage. As RAW264.7 cells expresses most toll-like receptor (TLR), it is appropriate to the study for the cellular response against foreign protein (Dowling and Dellacasagrande, 2016). Most foreign originated materials induce immune reaction in the monocyte/ macrophage lineage via TLR. Silk sericin is also foreign protein to mammals. However, immune reaction against silk sericin is generally mild and known as mild tumor necrosis factor-α (TNF-α) inducer (Jiao *et al*., 2017). As silk sericin induces inflammatory reaction, silk sericin is classified as M1 type macrophage polarizing agent (Kim, 2020). The application of silk sericin to RAW264.7 cells also increases the expression level of vascular endothelial growth factor (VEGF) (Jo *et al*., 2019). As a result, vessels are dilated and vascular permeability is increased (Kim *et al*., 2020).

As silk sericin is easily fragmented during degumming process, its biological response according to its molecular weight would be important for the development of silk sericin based biomaterial. The purpose of this study was to evaluate the level of TNF-α after administration of different molecular weight sericin fraction.

Materials and methods

Cell culture

RAW264.7 murine macrophages (Korean Cell Line Bank No. 40071) were suspended in culture medium. Sericin was extracted by boiling silkworm cocoons kindly gifted by the Rural Development Administration (Wanju, Korea). Silk sericin was extracted from silkworm cocoon of *Bombyx mori*. Ten gram of silkworm cocoon was chopped and placed into sonicator. Sonicator was filled with normal saline (500 cc) and the temperature was set as 37°C. Sonicator was operated at 40 kHz for 48 h. Silk sericin was detached from silkworm cocoon and solubilized in normal saline. Protein concentration of collected solution was measured by spectrophotometer. Collected protein solution was filtered with Microcon-30 (Merck Millipore Ltd., Cork, Ireland). Filtered fraction (M.W.> 30 kDa) and unfiltered fraction (M.W.<30 kDa) were collected separately. RAW264.7 cells were placed in 6-well culture plates and treated with 1, 5, and 10 mg/mL of total sericin, filtered, or unfiltered sericin. The media volume and seeding number were in accord to standard protocol (working volume: 1.8 mL, seeding density: 0.3 x 10^6). After 2, 8, or 24 h of culture, the cells were collected. Cells in the control culture were treated with a volume of solvent equivalent to that required for sericin.

Western blot and enzyme-linked immunosorbent assay (ELISA)

Proteins were collected and mixed with a sodium dodecyl sulfate buffer. After heat denaturation, they were electrophoresed on 10% polyacrylamide gels. The gels were transferred to polyvinylidene difluoride membranes. After blocking, the membranes were probed with primary antibodies for TNF¥á (dilution ratio = 1:500). Blots were imaged and quantified using a ChemiDoc XRS system (Bio-Rad Laboratories).

ELISA for TNF-α was performed for RAW264.7 cells. Silk sericin was applied to RAW264.7 cells at concentrations of 1, 5, and 10 μg/mL, and the supernatant was collected. ELISA was conducted using a commercially available kit (Cat#: ab208348, Abcam, Cambridge, UK) and the detailed protocol supplied by the manufacturer.

Results

The results of western blot demonstrated that the application of silk sericin increased the expression level of TNFα as time- and dose-dependent manner (Fig. 1). When compared between filtered fractions, high molecular fraction of silk sericin (M.W. > 30kDa) showed higher level of TNFα expression compared to low molecular fraction (M.W. < 30 kDa).

The results of ELISA were also in accord to those of western blot (Fig. 2). The application of silk sericin showed increased level of TNFα expression as dose- and time-dependent manner. The application of 10 μg/mL total silk sericin induced 275.82 ± 9.59 pg/mL of TNFα at 24h after administration (Fig. 2a). In case of 1 and 5 μg/mL total silk sericin induced 141.05 ± 8.49 pg/mL and 175.74 ± 14.81 pg/mL of TNFα at 24h after administration, respectively. The difference among groups was statistically significant ($P<0.05$). The application of 10 μg/mL high molecular fraction silk sericin (M.W. > 30 kDa) induced 197.87 ± 9.64 pg/mL of TNFα at 24h after administration (Fig.

2b). In case of 1 and 5 μg/mL high molecular fraction silk sericin induced 119.87 ± 2.53 pg/mL and 160.46 ± 3.34 pg/mL of TNFα at 24h after administration, respectively. The difference among groups was statistically significant ($P<0.05$). The application of 10 μg/mL low molecular fraction silk sericin (M.W. < 30 kDa) induced 86.85 ± 3.22 pg/mL of TNFα at 24h after administration (Fig. 2c). In case of 1 and 5 μg/mL low molecular fraction silk sericin induced 41.17 ± 1.98 pg/mL and 76.16 ± 2.20 pg/mL of TNFα at 24h after administration, respectively. The difference among groups was statistically significant ($P<0.05$). When compared groups of 10 μg/mL at 24 h after administration, they were 275.82 ± 9.59 pg/mL, 197.87 ± 9.64 pg/mL, and 86.85 ± 3.22 pg/mL of TNFα in total silk sericin, high molecular weight fraction of silk sericin (M.W.>30 kDa), and low molecular weight fraction of silk sericin (M.W.<30 kDa), respectively. The difference among groups was statistically significant ($P<0.05$).

Discussion

Silk sericin is mild M1 polarizing agent and induces TNFα in monocyte/ macrophage (Jiao *et al*., 2017). In this study, difference of TNFα inducing ability according to molecular weight was evaluated in RAW264.7 cells. Silk sericin induced different level of TNFα protein expression according to its molecular weight (Fig. 1 and 2). Higher molecular weight fraction of silk sericin (M.W.> 30 kDa) induced significantly higher level of TNFα expression compared to lower molecular weight fraction (M.W.< 30 kDa). The difference of TNFα protein expression level was checked by both western blot and ELISA (Fig. 1 and 2). As our best knowledge, this was first report on the difference of TNFα inducing ability according to silk sericin molecular weight.

Foreign materials such as silk sericin can induce immune

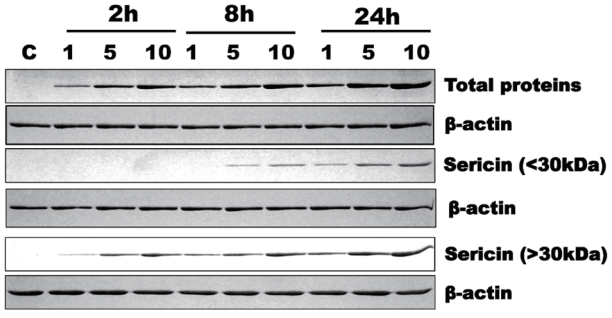

Fig. 1. Western blot results after administration of silk sericin

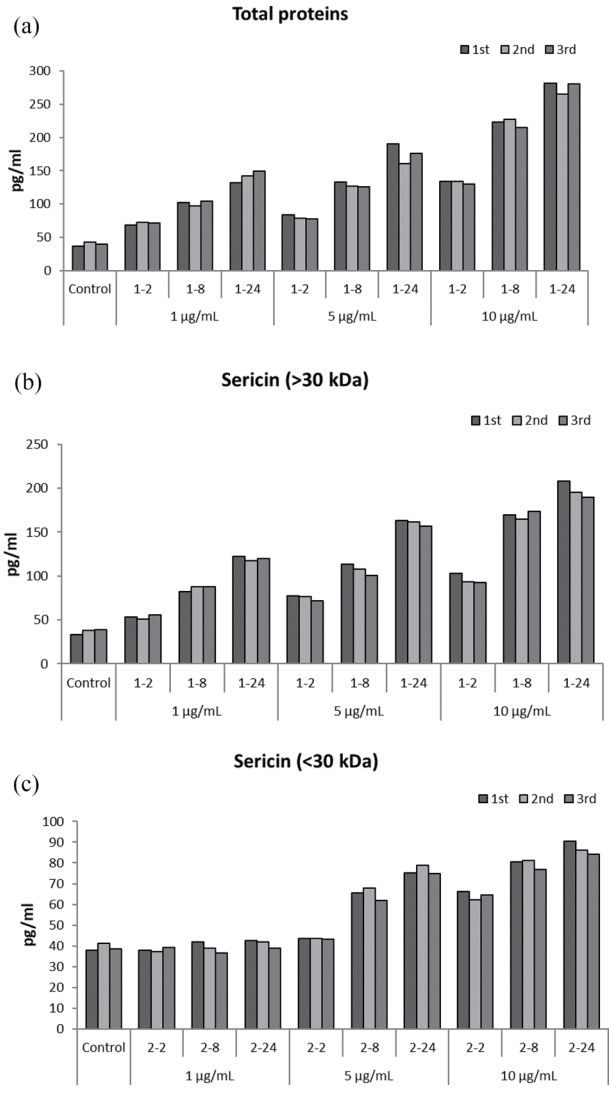

Fig. 2. Enzyme-linked immunosorbent assay (ELISA). Each assay had been repeated 3 times and all raw data were shown. (a) The expression of TNF-α after administration of total silk sericin. (b) The expression of TNF-α after administration of high molecular fraction silk sericin (M.W. > 30 kDa). (c) The expression of TNF-α after administration of low molecular fraction silk sericin (M.W. < 30 kDa).

reactions (Jiao *et al.,* 2017). These immune reactions are mainly mediated by monocytes/ macrophages. Macrophages have pattern recognizing receptors. TLR is representative pattern recognizing receptor (Dowling and Dellacasagrande, 2016). When foreign materials are bound to TLR, conformation of TLR is changed and signal is generated subsequently (Dowling and Dellacasagrande, 2016). The signal transmitted from TLR activates nuclear factor-κB (NF-κB) pathway (Andrade-Oliveira *et al.,* 2019; Padron *et al.,* 2020). As a result, the transcription

of TNFα is increased and TNFα protein level is also increased (Thoma and Lightfoot, 2018). Accordingly, TNFα expression level induced by silk sericin administration might be dependent of signal intensity generated by TLR activation. The intensity of TLR activation is dependent of foreign material conformation, amount, and charge. Therefore, high molecular weight fraction of silk sericin (M.W. > 30 kDa) might activate TLR stronger than low molecular weight fraction of silk sericin (M.W. < 30 kDa).

The silk sericin released from silkworm cocoon is different to layers (Jo *et al.,* 2017). The middle layer of silkworm cocoon shows lowest amount of sericin release and the TNFα expression level is also lowest in the middle layer (Jo *et al.,* 2017). The different profile of released proteins from each silkworm cocoon layer results in different types of cellular response (Kim *et al.,* 2018). Though high molecular weight fraction of silk sericin (M.W. > 30 kDa) increased TNFα expression level more than low molecular weight fraction of silk sericin (M.W. < 30 kDa), the decision between them would be dependent on intended purpose. In terms of tissue engineering, both M1 and M2 type polarizing agent are important for uneventful wound healing (Kim, 2020). In addition, TNFα expression level after silk sericin administration in this study was only picogram level (Fig. 2). Accordingly, the method for the extraction of silk sericin from silkworm cocoon should be tailored to its purpose.

The limitations of this study were as follows. First, there are many kinds of degumming process. However, only sonication in body temperature was used in this study. The effect of other degumming process should be studied in following studies. Second, TLR was mentioned as main target receptor for silk sericin. However, the expression level of TLR or its downstream signaling pathway had not been studied in this manuscript. Though TLR is main receptor for pattern recognition of foreign material (Dowling and Dellacasagrande, 2016), the association between TLR and silk sericin should be clarified. Third, other proteins except for silk sericin are found in the silkworm cocoon (Zhang *et al.,* 2015). Most of these proteins have low molecular weight (<30 kDa) (Zhang *et al.,* 2015). The biological effect of these proteins also should be clarified. Fourth, sericin is hydrophilic, however, is not water soluble in its non-fragmented form (Park and Um, 2018). Sericin molecules and swollen or aggregated particles are dispersed in solution. Accordingly, it was very difficult to divide sericin fragment exactly by the filter on the base of molecular weight. For the precise extraction of sericin according to its molecular weight, more precise instrument will be required.

Conclusion

In this study, silk sericin induced different level of TNFα protein expression according to its molecular weight in RAW264.7 cells. Higher molecular weight fraction of silk sericin (M.W.> 30 kDa) induced significantly higher level of TNFα expression compared to lower molecular weight fraction (M.W.< 30 kDa).

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References

Ahsan F, Ansari TM, Usmani S, Bagga P (2018) An insight on silk protein sericin: From processing to biomedical application. Drug Res (Stuttg) 68(6), 317-327.

Andrade-Oliveira V, Foresto-Neto O, Watanabe IKM, Zatz R, Câmara NOS (2019) Inflammation in renal diseases: New and old players. Front Pharmacol 10, 1192.

Bae YS, Kim CW, Bae DG, Um IC (2016) Effect of degumming on structure and mechanical properties of silk textile made with silk/polyurethane core-spun yarn. Int J Indust Entomol 33(2), 132-137.

Dowling JK, Dellacasagrande J (2016) Toll-like receptors: Ligands, cell-based models, and readouts for receptor action. In: Toll-like receptors. McCoy CE (ed.), pp. 3-28, Humana Press. New York, NY, USA.

Gholipourmalekabadi M, Sapru S, Samadikuchaksaraei A, Reis RL, Kaplan DL, Kundu SC (2020) Silk fibroin for skin injury repair: Where do things stand? Adv Drug Deliv Rev 153, 28-53.

Jiao Z, Song Y, Jin Y, Zhang C, Peng D, Chen Z, *et al*. (2017) In vivo characterizations of the immune properties of sericin: An ancient material with emerging value in biomedical applications. Macromol Biosci 17(12), 1700229.

Jo YY, Kim DW, Choi JY, Kim SG (2019) 4-Hexylresorcinol and silk sericin increase the expression of vascular endothelial growth factor via different pathways. Sci Rep 9, 3448.

Jo YY, Kweon H, Kim DW, Baek K, Kim MK, Kim SG, *et al*. (2017)

Bone regeneration is associated with the concentration of tumour necrosis factor-¥á induced by sericin released from a silk mat. Sci Rep 7, 15589.

Kaur J, Rajkhowa R, Tsuzuki T, Millington K, Zhang J, Wang X (2013) Photoprotection by silk cocoons. Biomacromol 14(10), 3660-3667.

Kim DW, Jo YY, Garagiola U, Choi JY, Kang YJ, Oh JH, *et al*. (2020) Increased level of vascular endothelial growth factors by 4-hexylresorcinol is mediated by transforming growth factor-¥â1 and accelerates capillary regeneration in the burns in diabetic animals. Int J Mol Sci 21(10), 3473.

Kim JW, Jo YY, Kweon HY, Kim DW, Kim SG (2018) The effects of proteins released from silk mat layers on macrophages. Maxillofac Plast Reconstr Surg 40, 10.

Kim SG (2020) Immunomodulation for maxillofacial reconstructive surgery. Maxillofac Plast Reconstr Surg 42(1), 5.

Mandal BB, Priya AS, Kundu S (2009) Novel silk sericin/gelatin 3-D scaffolds and 2-D films: fabrication and characterization for potential tissue engineering applications. Acta Biomater 5, 3007-3020.

Nuchadomrong S, Senakoon W, Sirimungkarart S, Senawong T, Kitikoon P (2009) Antibacterial and antioxidant activities of sericin powder from Eri silkworm cocoons correlating to degumming processes. Int J Wild Silkmoth Silk 13, 69-78.

Padron JG, Saito Reis CA, Kendal-Wright CE (2020) The role of danger associated molecular patterns in human fetal membrane weakening. Front Physiol 11, 602.

Park CJ, Um IC (2016) Effect of centrifugation on the structure and properties of silk sericin. Int J Indust Entomol 33(2), 144-148.

Park CJ, Um IC (2018) Effect of heat treatment on the structural characteristics and properties of silk sericin film. Int J Indust Entomol 37(2), 36-42.

Thoma A, Lightfoot AP (2018) NF-kB and inflammatory cytokine signalling: Role in skeletal muscle atrophy. Adv Exp Med Biol 1088, 267-279.

Zhang YQ, Ma Y, Xia YY, Shen WD, Mao JP, Xue RY (2006) Silk sericin–insulin bioconjugates: Synthesis, characterization and biological activity. J Control Release 115(3), 307-315.

Zhang Y, Zhao P, Dong Z, Wang D, Guo P, Guo X, *et al*. (2015) Comparative proteome analysis of multi-layer cocoon of the silkworm, Bombyx mori. PLoS ONE 10, e0123403.