IJIE Int. J. Indust. Entomol. 42(1) 1-6 (2021)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2021.42.1.1

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Toll-like receptor and silk sericin for tissue engineering

Seong-Gon Kim[1,*], HaeYong Kweon[2], and You-Young Jo[2]

[1]*Dept. of Oral and Maxillofacial Surgery, College of Dentistry, Gangneung-Wonju National University, Gangneung 28644, Republic of Korea*
[2]*Sericultural and Apicultural Division, National Institute of Agricultural Science, RDA, Wanju 55365, Republic of Korea*

Abstract

Toll-like receptor (TLR) is responsible for the recognition of foreign protein. Accordingly, TLR is mainly expressed in the immune associated cells. When foreign protein such as silk sericin is considered for the graft, the response of TLR should be considered. TLR is not all or none responsive receptor. TLR can be activated differently by the intensity of the input. Silk sericin is easily fragmented. The protein conformation of silk sericin is different to the degumming method. TLR response to silk sericin may be different to the degumming method. Consequently, objective tailored extraction method should be investigated and developed.

Int. J. Indust. Entomol. 42(1), 1-6 (2021)

Received : 22 Feb 2021
Revised : 12 Mar 2021
Accepted : 22 Mar 2021

Keywords:
silk sericin,
toll-like receptor,
bone morphogenic protein-2,
tissue engineering

Introduction

Graft has been developed to restore the defect in the body (Kang *et al*., 2019). If graft is taken from recipient body, it is called autograft. Autograft has the same genetic background to the recipient. Accordingly, there is no serious immune reaction. If graft is from the outside, it is foreign. When foreign material is implanted into the body, foreign body reaction is unavoidable (Kim *et al.*, 2018). When we differentiate the different kind of materials, each one is characterized by its own features and has a name. This name is a main tool for categorization. However, immune cells don't have sensitizing organs such as eye, ear, or nose. In addition, many types of antigen look similar and there are too many antigens. Accordingly, immune cells recognize the pattern of foreign material. For their recognition, pattern recognition receptors are important (Kulkarni *et al.*, 2020). Pattern recognition receptors can be classified as complement system, formylated peptide receptor, scavenger receptor, and toll-like receptor (TLR) (Kulkarni *et al.*, 2020).

To modulate immune reaction to the graft as favorable, the understanding of these pattern recognition receptors are important (Kim, 2020). Silk sericin is collective term for the degumming product of silkworm cocoon (Park *et al.*, 2019). Except for silk fibroin, silk sericin is bonding protein between fibroin fibers. In silk sericin, there are sericin 1, sericin 3, seroin, and many protease inhibitors (Zhang *et al.*, 2015). Sericin 1 and sericin 3 are approximately 110 kDa each based on protein database (https://www.ncbi.nlm.nih.gov/protein). Inner layer has more sericin 3 and outer layer more sericin 1 (Kaur *et al.*, 2013; Zhang *et al.*, 2015). In boiling condition, small sized proteins are degraded. Only sericn 1 and 3 combination are detected as fragmented forms in this condition (Jo *et al.*, 2020).

When silk sericin is grafted into the body, immune cells will respond to it. As the protein conformation of silk sericin will be

***Corresponding author.**
Seong-Gon Kim
Gangneung-Wonju National University, Gangneung 28644, Republic of Korea
Tel: +82+33+640+2468 / FAX: +82-33-641-2477
E-mail: kimsg@gwnu.ac.kr

different to the degumming condition, the activation of pattern recognition receptors will be different. To modulate host response to silk sericin as favorable, optimization of sericin extraction condition is important. Pattern recognition receptors recognize pattern of protein (Rapsinski *et al.*, 2015). Silk sericin has many β-sheet structures and random coil structures (Oh *et al.*, 2011). The content of β-sheet structures may influence the response of pattern recognition receptors (Tukel *et al.*, 2009).

The purpose of this review was to investigate current knowledge of TLR and protein pattern of sericin from different type of degumming process.

Toll-like receptors (TLR)

Classification

TLR was found in *Drosophila* fliea in 1988, first (Hashimoto *et al.*, 1988). Its association with innate immunity was found later (Belvin and Anderson, 1996). In human, TLR4 was identified in 1997 and its activation produces inflammatory cytokines (Medzhitov *et al.*, 1997). Until now, 11 human TLRs have been reported (Kulkarni *et al.*, 2020). The phylogenetic analysis of TLR is shown in Fig. 1. Different kinds of molecules can bind to TLRs. They can be microbial products, synthetic analogs of natural products, and damage-associated molecules from self. The preference to ligand is different to TLR type. For example, double strand RNA is bound to TLR3 and unmethylated CpG-DNA is bound to TLR9 (Dembic, 2005). TLRs are mainly expressed in immune cells such as macrophages (O'Neill *et al.*, 2013). TLRs are also expressed in non-immune cells such as epithelial cells and fibroblast (Kawasaki and Kawai, 2014).

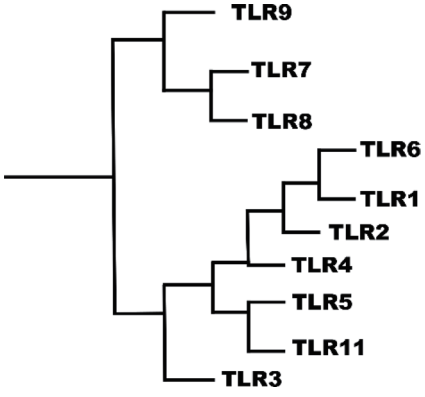

Fig. 1. Phylogenetic analysis of toll-like receptor (TLR). This figure was re-drawn from previous publication (Zhang *et al.*, 2004).

TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are found in cellular surface. However, other TLRs are localized in the endosome (Kawai and Akira, 2010; Celhar *et al.*, 2012).

Signal transmission

All signaling from TLRs except for TLR3 is transmitted through myeloid differentiation primary response 88 (MyD88) (Kulkarni *et al.*, 2020). toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) is another adaptor molecule. TLR3 transmits its signal through TRIF. TLR4 uses both MyD88 and TRIF (Zhao *et al.*, 2014). Schematic drawings for signal transduction is shown in Fig. 2. When TLR is bound to ligand, MyD88 forms complex with interleukin-1 receptor associated kinases (IRAKs). Phosphorylated IRAK1 activates tumor necrosis factor (TNF) receptor associated factor (TRAF6). TRAF6 induces transforming growth factor beta-activated kinase 1(TAK1) activation (Chen, 2012). TAK1 activates mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), subsequently (Ajibade *et al.*, 2012). Among MAPK family, extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) activates activator protein-1 (AP-1), transcription factors (Kawai and Akira, 2010).

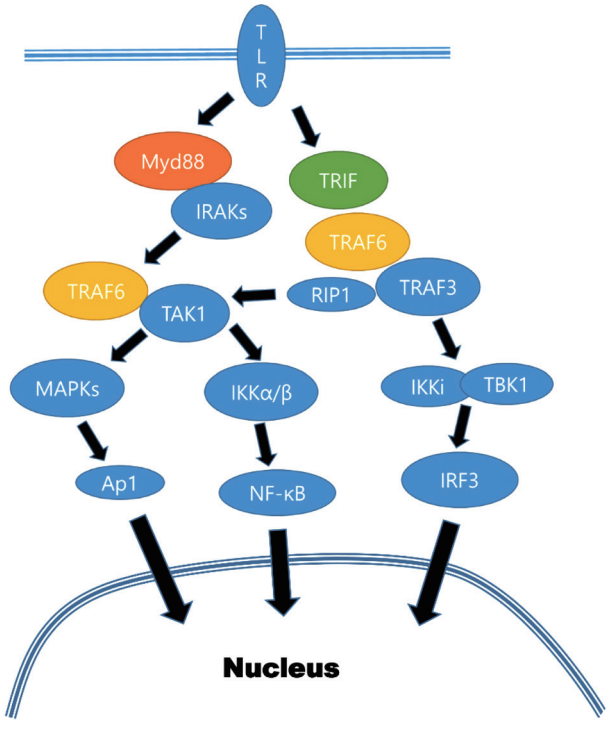

Fig. 2. Summarized signaling pathway of toll-like receptor. The definition of each abbreviation was shown in the main text.

TRIF transduces signal via TRAF3 and TRAF6 (Kawasaki and Kawai, 2014). Receptor-interacting serine/threonine-protein kinase 1 (RIP1) is TRAF6 associated kinase and activates TAK1. RIP1 increases NF-κB transcription and MAPK activation via TAK1 (Kawasaki and Kawai, 2014). However, TRAF3 recruits IκB kinase-i (IKKi) and TRAF family member-associated NF-κB activator (TANK)-binding kinase 1(TBK1). They phosphorylate interferon regulatory transcription factor 3 (IRF3) and it induces interferon genes (Kawai and Akira, 2010).

Protein structures and TLR activation

The consequence of TLRs activation is the production of inflammatory cytokines such as interleukin-1β (IL-1β) and TNFα (Tu *et al.*, 2016). Interestingly, the consequence of TLR2 and TLR4 activation is the increased expression level of bone morphogenic protein-2 (BMP-2) (Yang *et al.*, 2009; Meng *et al.*, 2008, Su *et al.*, 2011). BMP-2 overexpression is pro-inflammatory response in valve interstitial cells and may result in aortic valve calcification (Yang *et al.*, 2009). Peptidoglycan and lipopolysaccharide (LPS) are the ligand for TLR2 and TLR4. The application of peptidoglycan and LPS to human aortic valve interstitial cells can induce BMP-2 (Meng *et al.*, 2008). Oxidized low density lipoprotein also induces similar BMP-2 expression via TLR2 and TLR4 (Su *et al.*, 2011).

Amyloid proteins induce several diseases such as Parkinson's disease and type II diabetes. Amyloid proteins are produced by both human and micro-organisms (Mok *et al.*, 2007). However, there is no amino acid homology between human and micro-organism. Interestingly, all amyloids have cross-β-sheet quaternary structure (Rapsinski *et al.*, 2015). TLR2 recognizes these cross-β-sheet quaternary structure and TLR2 can't recognize amyloids upon the destruction of cross-β-sheet quaternary structure (Tukel *et al.*, 2009). Cross-β-sheet quaternary structure has been interested in the field of vaccine development. Ten amino acid sized self-assembling peptide originated from amyloid activates TLR2/6 (Al-Halifa *et al.*, 2020). This peptide is used for the development of long-lasting and safe vaccine (Al-Halifa *et al.*, 2020). A peptide from β-sheet structure of TIR domain-containing adaptor protein (TIRAP) inhibits TLR4 signaling (Achek *et al.*, 2020). Based on these findings, β-sheet structure of proteins seems to be important in the activation of TLR2/4.

Silk sericin structure from different type of degumming process

Silk sericin is the second large component of silkworm cocoon (Oh *et al.*, 2011). Annually 150,000 tons of sericin are trashed as an industrial waste (Oh *et al.*, 2011). Accordingly, recycling of silk sericin is important for reducing an industrial waste. Silk sericin is degraded during the extraction procedure (Oh *et al.*, 2011). High molecular weight fraction is used for biological and medical application (Zhang, 2002; Aramwit and Sangcakul, 2007). Low molecular weight fraction is used for anti-oxidant and anti-tyrosinase (Chlapanidas *et al.*, 2013).

Main gene is sericin 1 and its product has different sizes by alternative splicing (Okamoto *et al.*, 1982; Miehaille *et al.*, 1990). Based on sericin1 gene splicing information, the protein size will be 76, 123, 284, and 331 kDa (Garel *et al.*, 1997). However, this size is not maintained during extraction because of fragmentation. There have been several methods for the extraction of sericin from silkworm cocoon. Biochemical properties of silk sericin are influenced by its extraction method (Kurioka *et al.*, 2004; Kweon *et al.*, 2009). Using soap in the alkaline condition is not used for the biomedical application because separation of soap from silk sericin is difficult (Oh *et al.*, 2011). Sericin extraction using urea minimizes sericin degradation (Takasu *et al.*, 2002). However, dialysis procedure is required for removing urea and it is expensive and time-consuming (Oh *et al.*, 2011). Accordingly, sericin extraction using boiling water is widely used for its biomedical application.

When sericin is extracted by 100-120ºC distilled water, sericin is degraded in the aspartic acid residue (Teramoto *et al.*, 2006). The spectrum of molecular weight from hot-water extracted sericin is between 17 and 250 kDa (Takeuchi *et al.*, 2005). Silk sericin is precipitated in the ethanol (Tsukada, 1979). When ethanol is mixed with water, much narrow molecular weight spectrum is observed compared to water only extraction (Oh *et al.*, 2011). In terms of silk sericin structure, silk sericin from hot water extraction shows random coil structure, but 75 vol.% ethanol shows β-sheet structure (Oh *et al.*, 2011). Unfortunately, β-sheet structure of sericin is not maintained after removal of residual ethanol (Oh *et al.*, 2011). The development of β-sheet structure can be achieved by adding glycerol to silk sericin (Yun *et al.*, 2013). Glycerol mediated β-sheet structure formation has been explained by protein compaction effect (Vagenende *et al.*, 2009). In the glycerol conjugated silk sericin, water is also

important for the development of β-sheet structure (Yun *et al.*, 2016). When sericin is extracted at 50ºC for 4 weeks, minimum degradation of silk sericin is found (Chirila *et al.*, 2016). When combined with stirring, authors recommend that the duration should be less than 3 weeks (Chirila *et al.*, 2016).

Prospective of silk sericin for bone tissue engineering

Silk protein has been widely studied for bone tissue engineering. Silk fibroin has been studied in advance and it has been considered as biocompatible scaffold. Unlike silk fibroin, silk sericin is brittle and degraded rapidly. Though sericin coated implant shows improved osteogenicity, its findings are stayed in the cellular level. Recently, natural silk mat has been approved for bone regeneration in clinical trials compared to untreated control (Kim *et al.*, 2019). Silk sericin induced osteogenesis is explained by its macrophage activation (Kim, 2020). Sericin increases the gene expression of BMP-2 and BMP-4 in RAW264.7 cells (Kim *et al.*, 2020a). When sericin is separated by its molecular weight, high molecular weight fraction shows higher TNFα expression (Kim *et al.*, 2020b).

For the application of silk sericin for bone tissue engineering, silk sericin should have an osteoinductive property. BMP and vascular endothelial growth factor (VEGF) are representative proteins for osteoinduction. Sericin induces VEGF expression via hypoxia inducible factor (HIF)-dependent pathway (Jo *et al.*, 2019). Sericin looks like inducing BMP-2 and BMP-4 expression in macrophages (Kim *et al.*, 2020a). However, its detailed mechanism is unclear until now (Fig. 3). As sericin is a foreign protein for human, its pattern can be detected by TLR2/4. Thus, sericin based graft should induce immune reaction via TLR. TLR mediated cytokines are TNFα and BMP-2. All of them can be produced by sericin administration (Kim *et al.*, 2020a; Kim *et al.*, 2020b). For safe and long activation of TLR2/4, β-sheet structured protein is required. There have been introduced several extraction methods for collecting more β-sheet structured silk sericin. Among these methods, the method requires toxic chemicals should be avoided for biomedical application. As a consequence, limited number of extraction method can be considered for sericin extraction. This field is just beginning and should have much more investigation for reliable mass production of silk sericin in the bone tissue engineering.

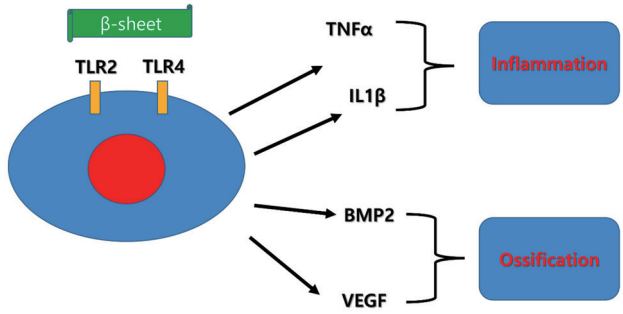

Fig. 3. Protein having β-sheet structure activates toll-like receptor-2/4 (TLR2/4). As a consequence, the expression level of tumor necrosis factor-α (TNFα), interleukin-1β (IL1β), bone morphogenic protein-2 (BMP2), and vascular endothelial growth factor (VEGF) can be increased. Detailed pattern of their expression can be different to the type of antigen and recipient cells.

Conclusion

Silk sericin is biocompatible and can be used for biomedical application. As silk sericin is foreign protein, its activation is mediated by TLR pathway. To optimize the quality of silk sericin for bone tissue engineering, the fraction of β-sheet structure in silk sericin should be increased. As the extraction method is important for the structure of silk sericin, it should be developed and optimized through in-depth investigation.

Acknowledgments

This study was conducted with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project no. PJ01562601)" Rural Development Administration, Republic of Korea.

References

Achek A, Kwon HK, Patra MC, Shah M, Hong R, Lee WH, *et al.* (2020) A peptide derived from the core β-sheet region of TIRAP decoys TLR4 and reduces inflammatory and autoimmune symptoms in murine models. EBioMedicine 52 ,102645.

Ajibade AA, Wang Q, Cui J, Zou J, Xia X, Wang M, *et al.* (2012) TAK1 negatively regulates NF-kappaB and p38 MAP kinase activation in Gr-1+CD11b+ neutrophils. Immunity 36, 43–54.

Al-Halifa S, Zottig X, Babych M, Côté-Cyr M, Bourgault S, Archambault D (2020) Harnessing the activation of Toll-like receptor 2/6 by

self-assembled cross-β fibrils to design adjuvanted nanovaccines. Nanomaterials (Basel) 10(10), 1981.

Aramwit P, Sangcakul A (2007) The effects of sericin cream on wound healing in rats. Biosci Biotechnol Biochem 71(10), 2473–2477.

Belvin MP, Anderson KV (1996) A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol 12, 393–416.

Celhar T, Magalhaes R, Fairhurst AM (2012) TLR7 and TLR9 in SLE: when sensing self goes wrong. Immunol Res 53, 58–77.

Chen ZJ (2012) Ubiquitination in signaling to and activation of IKK. Immunol Rev 246, 95–106.

Chirila TV, Suzuki S, McKirdy NC (2016) Further development of silk sericin as a biomaterial: comparative investigation of the procedures for its isolation from *Bombyx mori* silk cocoons. Prog Biomater 5, 135-145.

Chlapanidas T, Farago S, Lucconi G, Perteghella S, Galuzzi M, Mantelli M, *et al.* (2013) Sericins exhibit ROS-scavenging, anti-tyrosinase, anti-elastase and in vitro immunomodulatory activities, Int J Biol Macromol 58, 47–56.

Dembic Z (2005) The function of toll-like receptors; in Toll and toll-like receptors: an immunologic perspective. Rich T (ed), p18-55, Kluwer Academics, New York, NY, USA.

Garel A, Deleage G, Prudhomme JC (1997) Structure and organization of the Bombyx mori sericin 1 gene and of the sericins 1 deduced from the sequence of the Ser 1B cDNA. Insect Biochem Mol Biol 27, 469–477.

Hashimoto C, Hudson KL, Anderson KV (1988) The Toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52, 269–279.

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 HY, Oh JH (2020) Sericin for tissue engineering. Appl Sci 10(23), 8457.

Kang DW, Yun PY, Choi YH, Kim YK (2019) Sinus bone graft and simultaneous vertical ridge augmentation: case series study. Maxillofac Plast Reconstr Surg 41, 36.

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

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat Immunol 11, 373–384.

Kawasaki T, Kawai T (2014) Toll-like receptor signaling pathways, Front Immunol 5, 461.

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

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 JW, Jo YY, Kim JY, Oh J, Yang BE, Kim SG (2019) Clinical study for silk mat application into extraction socket: a split-mouth, randomized clinical trial. Maxillofac Plast Reconstr Surg 41, 16.

Kim SG, Jo YY, Kweon HY (2020a) Soluble fraction from silk mat induced bone morphogenic protein in RAW264.7 cells. Int J Indust Entomol 41, 51-55.

Kim DW, Jo YY, Kweon HY, Kim SG (2020b) Different level of tumor necrosis factor-a expression after administration of silk sericin fraction in RAW264.7 cells. Int J Indust Entomol 41, 1-5.

Kulkarni HS, Scozzi D, Gelman AE (2020) Recent advances into the role of pattern recognition receptors in transplantation. Cell Immunol 351, 104088.

Kurioka A, Kurioka F, Yamazaki M (2004) Characterization of sericin powder prepared from citric acid-degraded sericin polypeptides of the silkworm, Bombyx mori. Biosci Biotechnol Biochem 68:774–780

Kweon H, Yeo J, Kim K, Kim Y, Song H, Kim S, *et al.* (2009) Characteristics of silk sericin extracted from sericinjam. Int J Indust Entomol 18(2), 121-124.

Medzhitov R, Preston-Hurlburt P, Janeway CAJ (1997) A human homologue of the Drosophila activation of adaptive immunity. Nature 388, 394–397.

Meng X, Ao L, Song Y, Babu A, Yang X, Wang M, *et al.* (2008) Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis. Am J Physiol Cell Physiol 294(1), C29-35.

Miehaille JJ, Garel A, Prudhomme JC (1990) Cloning and characterization of the highly polymorphic Ser2 gene of Bombyx mori. Gene 86, 177–184.

Mok KH, Pettersson J, Orrenius S, Svanborg C (2007) HAMLET, protein folding, and tumor cell death. Biochem Biophys Res Commun 354, 1–7.

O'Neill LA, Golenbock D, Bowie AG (2013) The history of Toll-like receptors – redefining innate immunity. Nat Rev Immunol 13, 453–460.

Oh H, Lee JY, Kim MK, Um IC, Lee KH (2011) Refining hot-water extracted silk sericin by ethanol-induced precipitation. Int J Biol Macromol 48(1), 32-37

Okamoto H, Ishikawa E, Suzuki Y (1982) Structural analysis of sericin genes. Homologies with fibroin gene in the 5' flanking nucleotide

sequences. J Biol Chem 257, 15192–15199.

Park BK, Nho SK, Um IC (2019) Crystallinity of yellow colored silkworm variety cocoons. Int J Indust Entomol 38, 51-55.

Rapsinski GJ, Wynosky-Dolfi MA, Oppong GO, Tursi SA, Wilson RP, Brodsky IE, *et al.* (2015) Toll-like receptor 2 and NLRP3 cooperate to recognize a functional bacterial amyloid, curli. Infect Immun 83(2) ,693-701.

Su X, Ao L, Shi Y, Johnson TR, Fullerton DA, Meng X (2011) Oxidized low density lipoprotein induces bone morphogenetic protein-2 in coronary artery endothelial cells via Toll-like receptors 2 and 4. J Biol Chem 286(14), 12213-12220.

Takasu Y, Yamada H, Tsubouchi K (2002) Extraction and chromatographic analysis of cocoon sericin of the silkworm, Bombyx mori. J Insect Biotechnol Sericol 71, 151–156.

Takeuchi A, Ohtsuki C, Miyazaki T, Kamitakahara M, Ogata S, Yamazaki M, *et al.* (2005) Heterogeneous nucleation of hydroxyapatite on protein: structural effect of silk sericin. J R Soc Interface 2, 373–378.

Teramoto H, Kakazu A, Asakura T (2006) Native structure and degradation pattern of silk sericin studied by ^{13}C NMR spectroscopy. Macromolecules 39, 6–8.

Tsukada M (1979) Structural properties of silk sericin extracted by Mosher's method. J Sericult Sci Japan 48, 301–306.

Tu S, Zhong D, Xie W, Huang W, Jiang Y, Li Y (2016) Role of toll-like receptor signaling in the pathogenesis of graft-versus-host diseases. Int J Mol Sci 17, E1288.

Tukel C, Wilson RP, Nishimori JH, Pezeshki M, Chromy BA, Baumler AJ (2009) Responses to amyloids of microbial and host origin are mediated through Toll-like receptor 2. Cell Host Microbe 6, 45–53.

Yang X, Fullerton DA, Su X, Ao L, Cleveland JC Jr, Meng X (2009) Pro-osteogenic phenotype of human aortic valve interstitial cells is associated with higher levels of Toll-like receptors 2 and 4 and enhanced expression of bone morphogenetic protein 2. J Am Coll Cardiol 53(6), 491-500.

Yun H, Kim MK, Kwak HW, Lee JY, Kim MH, Kim EH, *et al.* (2013) Preparation and characterization of silk seiricn/glycerol/grapheme oxidenanocomposite film. Fiber Polym 14(12), 2111–2116.

Yun H, Kim MK, Kwak HW, Lee JY, Kim MH, Lee KH (2016) The role of glycerol and water in flexible silk sericin film. Int J Biol Macromol 82, 945-951.

Vagenende V, Yap MGS, Trout BL (2009) Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. Biochemistry 48, 11084–11096.

Zhang YQ (2002) Applications of natural silk protein sericin in biomaterials. Biotechnol Adv 20, 91–100.

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, *et al.* (2004) A toll-like receptor that prevents infection by uropathogenic bacteria. Science 303, 1522-1526.

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(4), e0123403.

Zhao H, Perez JS, Lu K, George AJ, Ma D (2014) Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. Am J Physiol Renal Physiol 306(8), F801–811.