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Regenerated Silk Fibroin from B. mori Silk Cocoon for Tissue Engineering Applications

M. K. Sah and K. Pramanik

Abstract—**The present study aims at the preparation of silk fibroin solution for possible applications in tissue engineering. Pure silk fibroin protein was extracted from *Bombyx mori* silk cocoon by degumming method using aqueous Na_2CO_3 solution followed by solubilizing in LiBr aqueous solution. The fibroin protein solution was purified and studied for the protein content. The degumming & solubility were significantly dependent on the salt concentration, treatment temperature, and time. A salt concentration of 0.02 M Na_2CO_3, temperature of 80°C and time more than 40 min were found to be favorable degumming conditions. The proper conditions of dissolution were found as 9.3 M LiBr, 70 °C temperature, and 3 h dissolving time. The morphology of degummed silk were investigated by SEM at different magnification. SEM revealed the absence of glue like sericine over the silk fibroin surface at optimal degumming condition. The fibroin protein content of the solution was measured by Bradford protein assay. The results indicate that the regenerated silk fibroin (RSF) can be used for fabrication of porous silk fibroin scaffolds for various tissue engineering applications.**

Index Terms—**Tissue engineering, scaffold, fibroin, SEM.**

I. Introduction

Silk fibroin (SF), a natural macromolecule spun by silkworm (*Bombyx mori*), has attracted the interest of scientists of various disciplines for a long time. It has a long history of use in medicine, for example, as sutures and artificial ligaments. Silk is a mechanically robust biomaterial that offers a wide range of mechanical and functional properties for biomedical applications in the viewpoint of mechanical properties, environmental stability, biocompatibility and biodegradability (Ning, *et al.* 1999; Zhengyu *et al*., 2000; Altman *et al.*, 2003). Silk fibroin (SF) fibers are one kind of protein which consists of 18 amino acids, such as glycine, alanine and sericine. Application of three-dimensional scaffolds as an important part of tissue engineering, are beneficial because they provide a place for attachment, increased surface area, support to large cell mass, and capable of shaping particular structures. (Minoura *et al.*, 1998)**. S**low degradation in vivo, lack of active groups and the use of organic solvent which prohibits the addition of cell growth factors, limit the use of synthetic polymers. Recent research indicated that similar to collagen, SF found ideal for attaching animal cells cultured *in vitro*, and was also important for maintaining cell function (Kim *et al.*, 2005; Wu *et al,* 2000). For example, Wu *et al* randomly wound SF fibers to form net where animal chondrocytes were three-dimensionally cultured, and their results showed that SF could be used as good scaffolds for chondrocytes in three-dimensional culture. The silk fibroin fibers produced from silkworms at room temperature and from an aqueous solution are strong and stiff fibers, whereas synthetic materials with comparable properties must be processed at higher temperatures and from less benign solvents.(Shao *et al.*, 2002) Silk must be regenerated into a desirable form to meet a specific biomedical application. In general, aqueous SF solution is obtained by dissolving SF in the concentrated neutral salts, such as calcium chloride, lithium bromide, and so on (Yuji *et al*., 2005).To date, it is known that SF is soluble in certain high ionic-strength aqueous solutions of chaotropic salts, which destabilize proteins in solution and increase their solubility (Lizukar *et al*., 1985; Kweon *et al.,* 2001) Many researchers have attempted to find suitable solvents for preparing SF solutions, which may be subsequently spun into fibers , however, very few literatures are available regarding the complete processing of silk ,optimization of process conditions and the characterization of silk solution. In this paper, the regenerated form of SF solution were prepared and characterized for tissue engineering application.

Mahesh Sah and K. Pramanik are with Tissue Engineering Group, Department of Biotechnology & Medical Engineering, National Institute of Technology Rourkela, Rourkela-769008, Orissa, INDIA. (Corresponding Author: Email- kpr@nitrkl.ac.in; Ph-+91-661-2472280(O)/3280(R); Fax-+91-661-2472926)

II. Materials & Methods

A. Materials

Bombyx mori Silk cocoon were purchased from mulberry *farms* in Chittoor district, Hyderabad while analytical grade Lithium Bromide, Sodium Carbonate and chemicals for Bradford Assay were purchased from sigma company.

B. Preparation of aqueous silk fibroin solution

Dried *Bombyx mori* silk cocoons were cut into small pieces and treated with boiling aqueous solution of 0.02M sodium carbonate for 20 minutes with stirring. The whole mass was repeatedly washed with distilled water (Milli-Q water) to remove the glue-like sericine protein and dried in hot air oven. Silk fibroin solution was prepared by dissolving 10gms of degummed silk in9.3M LiBr solution at 70°C for 2½ hrs. The fibroin solution was dialyzed in a cellulose membrane based dialysis cassette (molecular cutoff 12,400.) against deionized water for 3 days, changing water every 6 hrs. in order to remove LiBr. After dialysis, silk fibroin solution was centrifuged at 5-10˚C and 9000 rpm for 20 min. The concentrated solutions were stored at 4 °C for further study.

The protein concentration was then measured using Bradford standard assay procedure (Bradford, 1976).

C. Degumming loss

Degumming loss, which represents a quantitative evaluation of the degumming efficiency, indicates the weight loss of the fabric (expressed as a percentage of the initial weight) after degumming. Before weight measurement, samples were kept at 37◦C in hot air oven for 24 h.
Degumming loss (%) = [(Initial Wt. of silk cocoon-Wt. of silk fibroin recovered) / Initial Wt. of silk cocoon] x100

D. Measurement of protein concentration

The fibroin protein concentration was measured by the Bradford protein assay procedure (Bradford et al., 1976). The fibroin solution was added to the Bradford reagent and incubated at 30°C for 5 min. and the absorbance at 595nm was measured .The Bradford assay relies on the binding of the dye Coomassie Blue G-250 to protein. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form , usually achieved by measuring the absorbance of the solution at 595 nm. Bovine serum albumin was used as a standard protein.

E. Scanning Electron Microscopy

The morphology of degummed silk fibers were determined by SEM at different magnification. Assembled silk fibers were air-dried overnight and affixed via carbon tape to the SEM sample holders and vacuum-coated with a 20-nm layer of platinum. Specimens were observed on a JEOL JSM -6480LV SEM and photographed at a voltage of 15 kV and room temperature.

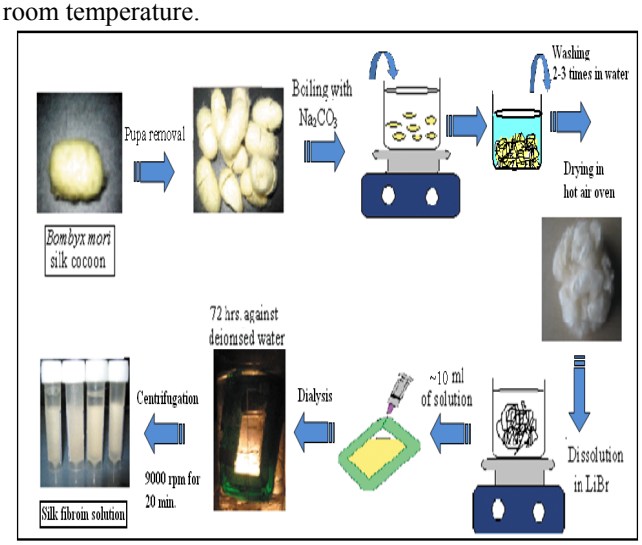

Fig. 1. Extraction of fibroin protein from *Bombyx mori* silk cocoon.

III. Results & Discussion

A. Degumming of B. mori silk cocoon

The mechanism of sericin removal in chemical degumming is a combination of various effects such as: dispersion/solubilization and hydrolysis of the different sericin polypeptides (Freddi et al., 1996). Hydrolysis prevails when strong alkaline compounds are added to the degumming bath. Therefore, suitable procedures for controlling process parameters, such as temperature, time, and salt concentration performed in order to attain effective sericin removal without triggering the hydrolytic degradation of fibres, which can be easily induced by the presence of harsh chemicals in the treatment bath. Initial sample of 10 gm of silk cocoon fibre ware taken for each study.

1) Effect of Na2Co3 salt concentration

To study the effect of the aqueous sodium carbonate on the extent of sericin removal, the treatment time 60 min and temperature 70 C were kept constant, while the salt concentration was changed in the range 0.01-0.04 M. The effect of salt concentration on the preparation of silk fibers is shown in Figure1. From the experimental results it is observed that the amount of silk fiber sharply decreased with increase in salt concentration from 0.01 M to 0.02 M this is due to loss of most of the sericine from the silk fibre into the solution. The degumming loss increases from 19.9 % to 24.4 % in the concentration range of 0.01-0.02 M. The amount recovered is either decrease or shown no effect with further increase in salt concentration. However the maximum degumming loss of 25.4 % was achieved at concentration of 0.04 M when silk fibre of 7.46 gms was obtained.Concentration of 0.02 M when degumming loss approaches the complete loss of sericine from silk fibre was taken for further optimization of the process. The salt concentration was taken minimum to get the silk fibre in intact form since the more amount of harse chemical cause hydrolytic degradation of silk fibroin into solution.Also at the concentration of 0.02 M , the silk surface was found smoother as compared to higher concentration as revealed by SEM.

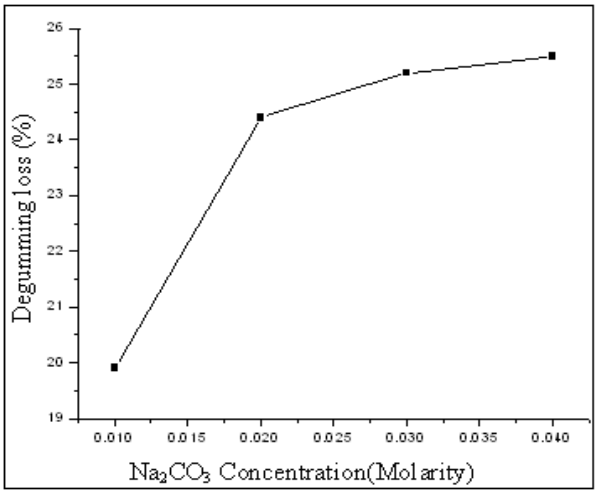

Fig. 2. Effect of Na_2Co_3 salt concentration in degumming at 70 ºC for 60 minutes.

2) Effect of degumming time

To study the effect of the treatment time on the extent of sericin removal, the salt concentration of 0.02 M and degumming temperature of 70 C were kept constant, while the time was changed in the range 20-80 min. At 20 min , degumming loss was only 11% with recovery of 8.9 gm of silk fibre. At 40 min. the loss increases to 18.8 % and the silk recovered was 8.12 gm., while after 60 min. the loss of degumming was 25.1 % . The maximum degumming loss (25.6 %) was after 80 min with recovery of 7.44 gm silk fibroin. The result so obtained is due to more the time silk fibre is treated at higher temperature, more the sericine is removed due to hydrolysis of interlinking bond between

sericine and fibroin and more solubility of sericine into the solution. Moreover, the extension of the treatment time probably exposed sericin to the action of hydrolysis by salt.

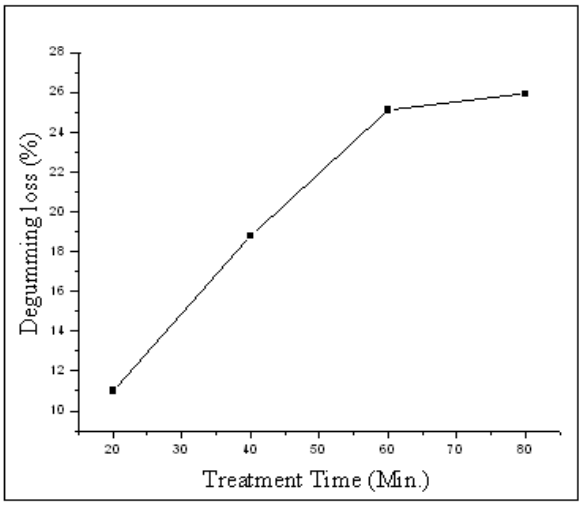

Fig. 3. Effect of degumming time at 0.02 M Na_2Co_3 & 70 ºC.

3) Effect of degumming temperature

To study the effect of the degumming temperature on the extent of sericin removal, the salt concentration of 0.02 M and treatment time of 60 min. were kept constant, while the temperature was changed in the range 60-90ºC. In fact, it is well known that sericin can be removed by using water alone, but high temperature is needed to attain complete degumming (110–120 ºC, under pressure). The temperature rise upto 60 ºC probably contributed to enhance the solubility of partially hydrolyzed sericin fractions adhering to the fibrous core of the silk. The rise in temperature from 60 to 70 C results into loss of most of the sericine from the sercicine with degumming loss of 24.4 %. Rise in temperature by 10 C results into degumming loss of 25.1 % followed by 25.5% at 90 C. The rise in temperature not only provide the activation energy for the breakdown of interlinking bonds but also increases the solubility of sericine into solution.

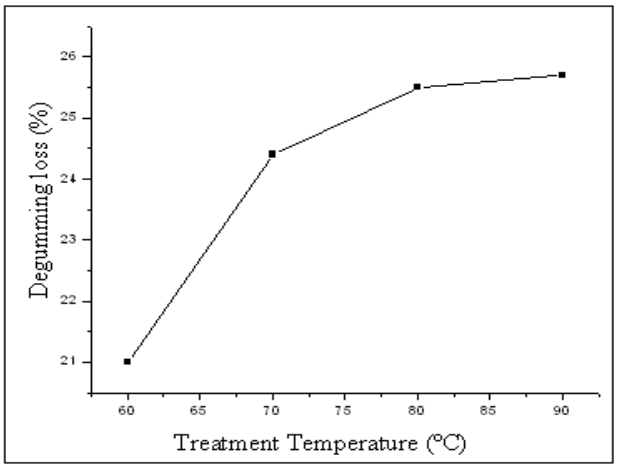

Fig. 4. Effect of treatment temperature at 0.02 M Na_2Co_3 for 60 min.

B. Scanning electron microscopy of degummed silk fiber

Removal of sericin resulted in the separation of the individual silk filaments, which were glued together by sericin and sizing agents in the raw fabric. This conferred on the fabric the denser texture and the rough surface typical of light weight silk fibres. The dull appearance and stiff handle of the raw silk fibre disappeared, and the degummed fabric became shiny, soft, and scroopy.

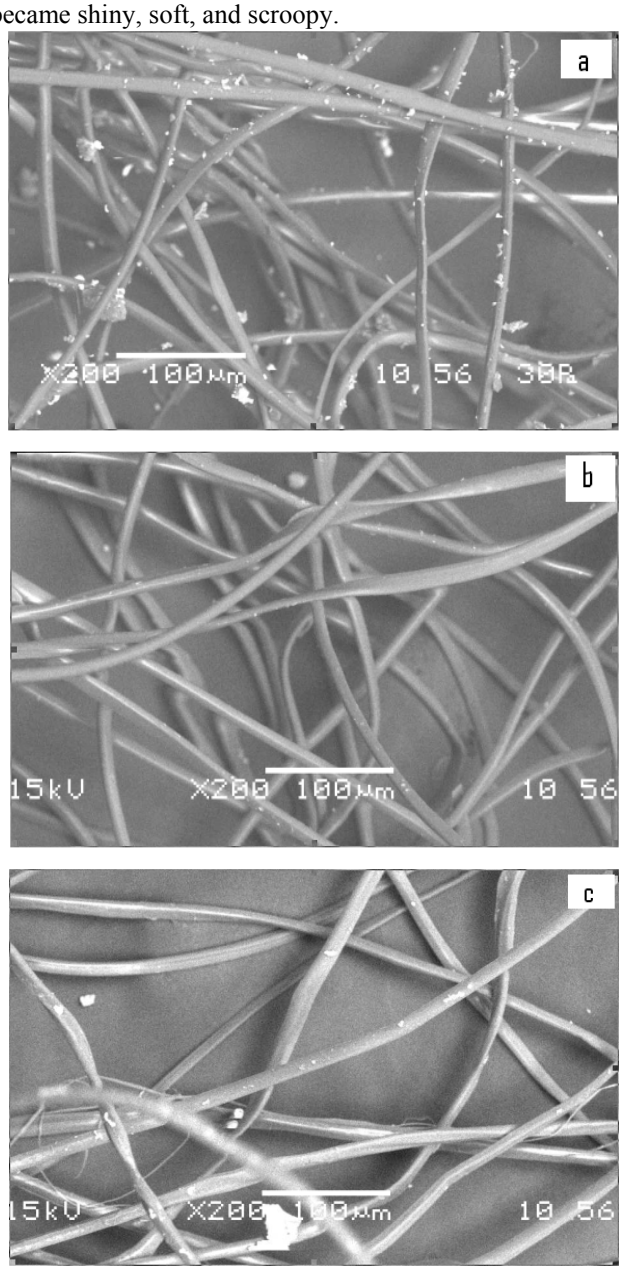

Fig. 5. Scanning electron micrographs illustrating differently degummed cocoon silk morphologies. A) Degumming by 0.01 M Na2CO3 degumming silk, showing comparatively incomplete removal of gum. B) Degumming by 0.02 M Na2CO3 , appearing relatively smooth, and individual longitudinal strands were also clearly visible. C) Degumming by 0.03 M Na2CO3 , individual longitudinal strands were clearly visible.

The SEM observation of silk fibers obtained by different concentration of Na_2Co_3 salt is shown in figure 3. It clearly confirms the presence of sericin stick as white spots over the surface of silk fiber. The presence of sericin in silk fiber is more in 0.01 M salt concentration showing non-uniform removal of gum from interlacing regions of fibers compared to that in 0.03 M salt concentration. So it can be concluded that increase in concentration of Na_2Co_3 decreases the sericin content of silk fiber. The closer SEM examination of the silk fibroin fibre showed that the individual silk filaments split off and their surface was clean and free of sericin (Fig.). On the other hand, silk degummed with lower concentration still showed the presence of sericin residues. It is interesting to note that the deposits were mainly located at the cross over points between silk fibers making it stick together. Silk fibre

diameter so obtained is 10-12 µm.

C. Preparation of regenerated silk fibroin aqueous solution

The solubility of silk fibroin in ionic liquids depends on the identity of both the cation and anion, with the anion having a much larger effect. The more the cation and anion are able to participate in hydrogen bonding, the greater the solubility of the silk fibroin. (Dyme,1989) Presumably, the ionic liquid disrupts the hydrogen bonding present in the β-sheets.

1) Effect of temperature on dissolution of silk fiber

The effect of temperature on solubility of protein is shown in figure 2. When treatment temperature increases from 50 °C to 60 °C , the protein concentration of solution increases appreciably. Dissolving the silk fiber in LiBr solution gave the better result at 70 °C rather than others one. At 70°C and 80°C all the silk fiber were melted while at 60°C silk fibers were not completely melted and it was very difficult to separate the solution through filtration.

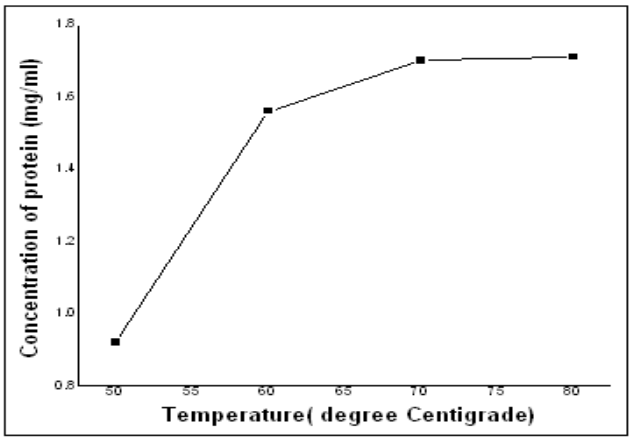

Fig. 6. Effect of temperature on solubility of protein

2) Effect of LiBr salt concentration

The effect of LiBr salt concentration on solubility of protein is shown in figure 3.The amount of protein present is more at 9.3 M than others. So as the concentration of LiBr increases the solubility of protein also increases. At concentration of 9.1 M, protein concentration is 1.4 mg/ml that increases to 1.7 mg/ml at 9.3 M concentration. The increase in solubility is due to enhanced breaking of disulfide bonds between the heavy and light chains of silk fibroin.

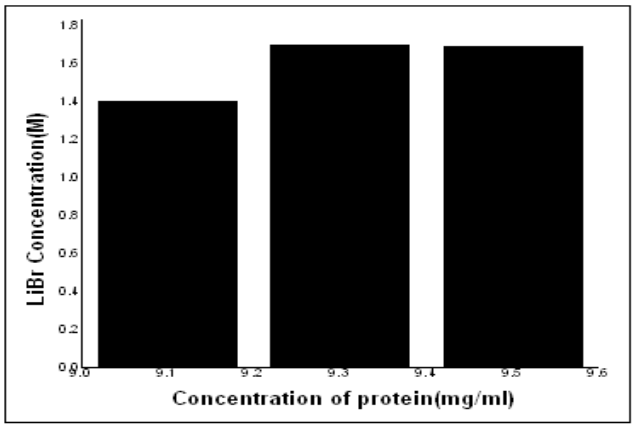

Fig. 7. Effect of concentration of LiBr on solubility of protein at constant temperature at 70° C. (Protein concentration estimation by **Bradford Assay)**

3) Effect of treatment time for solubilization

Effect of treatment time on solubilization of degummed silk fiber was studied at LiBr concentration of 9.3 M at 70 °C. The amount of silk fiber remained after the process was recovered, air-dried and weighed. The amount of protein concentration in silk fibroin solution was also measured using Bradford assay.The concentration is doesn't change appreciable after 3 hrs. as shown in figure 8.

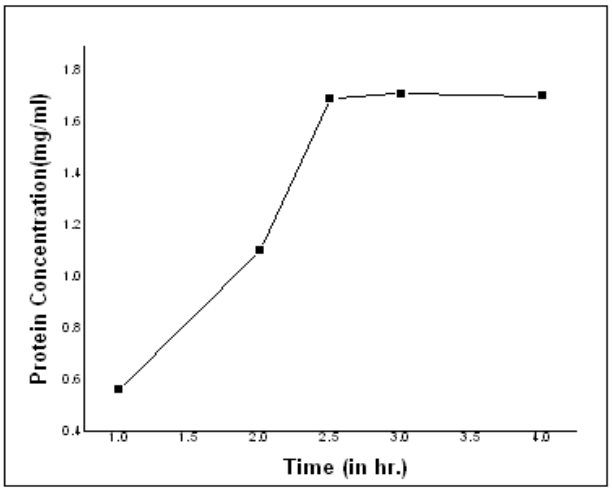

Fig. 8. Effect of treatment time on dissolution of silk fibroin.

The optimum conditions for degumming were found to be at 3 hrs. in 9.3 M concentration of LiBr at 70 °C. The variation in concentration of protein in solution upon dissolution of fibroin fibre has been shown in fig.7.The exponential increase in concentration of protein was obtained at treatment time of 2-2 ½ hrs. The protein concentration at 3 hrs. was obtained maximum (1.71 mg/ml).

IV. Conclusion

The production amount of silk fiber increased sharply at 0.02 M of Na_2CO_3, then amount decreases. From SEM observation of silk fiber it can be deduced that the presence of sericin in silk fiber is more in 0.01 M salt concentration in comparison to0.02 M & 0.03 M Na_2CO_3 . Increase in the concentration of Na_2CO_3 also makes loss of fibroin in to the boiling solution. The quality of silk fiber obtain is better at 40 min. rather than 20, 60 or 80 min.. The solubility of silk fibroin is more in 9.3 M LiBr. The optimum temperature for dissolving the degummed silk fiber was found to be 70 °C. Dissolution of degummed silk fiber in LiBr aqueous solution was better when treated for 3 hrs. at the optimized temperature and salt concentration .The regenerated fibroin solution can be used for thin films or 3D scaffold fabrication. An improved understanding of the in vivo environment and their role in the degradation of silk fibroin will provide the next logical step in modeling an appropriate long-term degradable scaffold for various tissue engineering applications.

References

[1] L. Ning, L. Xue and H. N. Huang.(1999). Explorations on correlation of biological tests of skin reproductive membrane, China J. Mod. Med. 9 (5).

[2] G.H. Altman, F. Diaz, C. Jakuba, T. Calabro, R.H. Horan, J. Chen, H. Lu, J. Richmond and D.L. Kaplan.(2003). Silk-based biomaterials. Biomaterials. 24. PP. 401–416. Available: www.ncbi.nlm.nih.gov/pubmed/12423595

[3] N. Minoura, S. Aiba, Y. Higuchi, M. Tsukada and Y. Imai.(1995) Attachment and growth of fibroblast cells on silk fibroin. Biochem. Biophys. Res. Commun. 208. PP. 511-516. Available:www.ncbi.nlm.nih.gov/pubmed/7695601

[4] U.J. Kim, J. Park, H.J. Kim, M. Wada and D.L. Kaplan. (2005). Three-dimensional aqueous-derive biomaterial scaffolds from silk fibroin. Biomaterials. 26. PP. 2775-2785. Available:www.ncbi.nlm.nih.gov/pubmed/15585282

[5] H. Wu, C. Cui and Y. Gu. (2000). Chinese J. Reparativ. Reconstr. Surg. 14(5). PP. 301.

[6] Z. Z. Shao and F. Vollrath. (2002). Surprising strength of silkworm silk. Nature (London). 418. PP. 741. Available:www.nature.com/uidfinder/10.1038/418741a

[7] Y. Miyaguchi and J. Hu. (2005). Physicochemical Properties of Silk Fibroin after Solubilization Using Calcium Chloride with or without Ethanol. Food Sci. Technol. Res. 11(1). PP. 3742. Available:joi.jlc.jst.go.jp/JST.JSTAGE/fstr/11.37?from=Google

[8] H. Kweon and Y. H. Park.(2001). Dissolution and Characterization of Regenerated Antheraea pernyi Silk Fibroin. J. Appl. Polym. Sci., 82, PP. 750–758. Available:onlinelibrary.wiley.com/doi/10.1002/app.1901/pd

[9] M. Bradford. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72. PP. 248. Available:*www*.ncbi.nlm.nih.gov/pubmed/942051

[10] U.K. Laemmli (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, Londo. 227. PP. 680-685. Available:

[11] *www*.molbio.unige.ch/laemmli/pdf/NatureVol227.pdf