Fibroin Protein Extract from Red Ant Nests for a Production of Electrospun Nanofibers

Arunrat Khamhaengpol[1], Santi Maensiri [2], Sineenat Siri[3]*

ABSTRACT

This research studied fibroin extract from fibers of red ant nests. Four fibroin extraction methods were investigated, including the use of Ajisawa's solution at 80°C, 9 M LiBr at 60°C, 9 M LiSCN at 40°C and 0.5% Na_2CO_3 at 40°C. The result showed that the suitable extraction condition was to use 0.5% Na_2CO_3 at 40°C for 60 minutes, yielding the dried fibroin powder of 26.3% of wet weight of red ant nest fibers. A major protein band of extracted protein was approximately 43 kDa as determined on 12.5% SDS-PAGE. This protein band was further analyzed on 2D-PAGE and 11 protein spots were observed at molecular weight of 39-43 kDa and pI of 4.8-7.16. The result was similar to the decoded fibroin protein of Australian weaver ants. The extracted fibroin solution could be electrospun into continuing fibers when polyethylene oxide (7% PEO) solution was blended into the extracted fibroin (1% EF) solution in a ratio of 1:1 (v/v) to assist the formation of the fibers. The solution was successfully electrospun into continuing fibers under the condition of 0.4 ml/h flow rate, 20 cm. in distance between an injector tip and a collector and 18 kV voltages. Average diameter of fabricated electrospun fibers was 436.62$\pm$106.49 nm.

Keywords: Nanofiber, Electrospinning, Fibroin, Red ant nest

[1] *A graduate student, Department of Biochemistry, Faculty of Science, Khon Kaen University*

[2] *Associate Professor, Department of Physics, Faculty of Science, Khon Kaen University*

[3] *Assistant Professor, Department of Biochemistry, Faculty of Science, Khon Kaen University*

* *Corresponding author, e-mail: ssinee@kku.ac.th*

INTRODUCTION

Electrospinning, widely used in the manufacture of filters, membranes and electronic devices, was one of several techniques used in fiber production. Its popularity was due to its simplicity and cost-effectiveness with high efficiency, yielding fiber production from the level of micrometer to nanometer (Gomes et al., 2007; Jeong et al., 2009). The fiber output was a result of spinning polymer solution with electrostatic caused by the high-voltage electric field. When a sufficiently high voltage was applied to a droplet of polymer solution, the body of the liquid solution became charged. Electrostatic repulsion counteracted the surface tension and droplet was stretched into elongated and non-woven fibers falling onto a collector (Frenot and Chronakis, 2003). Interesting physical properties of the fiber sheets from the experiment were their high surface to volume ratio, low porosity and small diameter with maximum strength. In addition, if the electrospun polymeric fibers had a biocompatibility with cells and degradable by biological processes (Biodegradability), the fiber sheets would then be appropriate for cell adhesion and promote cell growth suitable for medical applications such as artificial tissues, vascular grafts, supporting scaffolds for a repair of broken bones, dental materials, wound dressing materials and so on.

Both synthetic and natural polymers with the mentioned properties were regularly used in the production of electrospun fibers for medical purpose. Taken into account the natural polymer, the silk threads produced from silk worms (*Bombyx mori*) were the most studied polymer. Silk contains two main types of protein: 1) Fibroin was a core protein of silk, 2) Sericin was a glue-like protein surrounding the protein core (Altman et al. ., 2003). The silk threads from silk worms could not only be produced at large number but also had elasticity and be biocompatible with the cells of living organisms and degradable by biological processes. This resulted in an attempt to use bio-polymers in biotechnology and medical purpose such as using bio-polymer as supporting frame for enzyme adhesiveness in the biosensor for diagnosis purpose (Asakura et al., 1992). Fibroin was also used to produce vascular grafts (Sakabe et al., 1989), membrane frames that allowed oxygen flow for contact lens. (Minoura et al., 1990) and artificial skin for use in healing fire burned wound as fibroin had high evaporation rate (Liu et al., 2010). Moreover, fibroin was appropriate for producing artificial ligament tissues as fibroin absorbed calcium ions in a great deal and was very flexible (Fang et al., 2009). Fibroin also helped a drug delivery process which could control drug release (Tsukada et al., 1994) and was regularly used as healing wound pad as it allowed cell adhesion and promoted cell growth (Min et al., 2004).

Besides the silk worms which produced fibroin, several insects were capable of producing fibroin as well. These insects included bees, wasps, hornets, spiders and ants (Holldobler and Wilson, 1990). The studies relating to fibroin production from these insects were limited in relative to fibroin from silk worms or from red ants which were common insects widely founded in every region of Thailand. Red ants or yellow ants had a scientific name as *Oecophylla Smaragdina* Fabricius and were placed in the order of Hymenoptera and the family of Formicidae. Red ants could produce fibers from fibroin used for nest building. The larvae of ants in late stage, the instar larvae, (Gomes et al., 2004) could create fibroin in the labial glands (or salivary gland or the silk gland). The worker ants would have the instar larvaes sprayed fibers to weld the leaves into nests. Although fibroin from ants had similar properties to that from silk worms, such as its strength, waterproof,

and flexibility, amino acids in fibroin were normally different such as having lower glycine and having a highly-acid side chain (Craig et al., 1999). This could result in some different properties of fibroin from ants and that from silkworms. As a consequence, this study would focus on protein extraction from Thai red ants and the properties of the protein extracted while researching the production of electrospun nanofibers from the extracted protein.

METHODOLOGY

1. Samples collection

New ant nest samples were collected from trees that ants frequently nested such as rose-apple trees, mango trees, longan trees, star fruit trees, burmese grape trees, bottle brush oaks, shorea siamensis, neems, and acacias. The white fiber parts from the ant nests were separated and stored at -20 °C.

2. Fibroin extraction

There were four ways in extracting fibroin protein from the ant nests: 1) Extraction with Ajisawa's solution (Ajisawa, 1998) at 80 °C, 2) 9 M LiBr at 60 °C, 3) 9 M LiSCN at 40 °C and 4) 0.5% Na_2CO_3 at 40 °C. Generally, red ant nests were crushed in liquid nitrogen before being extracted with solvent at various temperatures above by using the ratio of the red ant nests to the extract solvent of 1: 30 (weight /volume) and observing the dissolution rate of ant nests every 15 minute for 75 minutes to score a "0, +1, +2, +3, +4" (dissolution rate: low - high) to compare the solubility of ant nest under various environment. After all, the best method would be used to extract ant nests which were later filtered through a thin white cloth and filter paper Whatman No. 1 (Whatman, England). After that, the solution would go through dialysis process and later enhanced the protein concentration by using a cool drier (Lyophilizer, FTS systems, USA). The output at this stage shall be called the extracted fibroin (EF).

3. Study of extracted protein by SDS-PAGE and 2D-PAGE

The extracted protein band would be studied by using 0.5% Na_2CO_3 at 40 °C for 60 minutes with technique Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This technique separated proteins according to the size of molecular weight by using 12.5% SDS-PAGE. Then the proteins would be stained with solution coomassie brilliant blue R-250 while the protein bands of interest would later be cut and immersed with solution elution buffer (20 mM Tris-HCl pH 7.7, 150 mM NaCl, 0.1% SDS, 2 mM EDTA). Protein in the elution buffer would be measured with DC protein assay (Biorad, USA). The protein would be calculated its pI with Two Dimensional Polyacrylamide Gel Electrophoresis technique (2D-PAGE) using 2-D Electrophoresis kit (GE Healthcare, USA) which used 7 cm. IEF strip with a pH between pH. 3-10 cm. The above 50 ml protein solution would be mixed with rehydration buffer 75 ml. Then, protein was extracted in the first dimension under following conditions: Step 1) 300 volts for 40 minutes, Step 2) 1,000 volts for 18 minutes, Step 3) 5,000 volts for 48 minutes, and Step 4) 5,000 volts for 15 minutes. The extracted proteins in the first dimension would be further extracted in the second dimension on 12.5% SDS-PAGE. The gel would be stained by a solution coomassie brilliant blue R-250 for 1 hour and then washing off the gel with destaining solution (10% acetic acid, 40%. methanol) until the protein bands were clearly visible. The final gel was analyzed for size and pI of each protein spot with the program 2D platinum (Amersham Bioscience, USA).

4. Conditions in the production of electrospun nanofibers

Nanofibers manufactured under electrospinning technique could be done by machine KKU ElectroSys I electrospinning unit (manufactured by Dr. Santi Maensiri, Khon Kaen University). To study the optimum condition of the production of fibers from the extracted fibroin solution, following conditions in fiber spinning had been tested: 1) solution concentration of 35%, 2) voltage 10-18 kV, 3) flow rate of 0.3 to 0.2 ml/hr, 4) the distance between the needle tip and the collector of 15 cm. For the production of fiber from the solution, following conditions were set: 7% PEO (MW = 300,000 Da), solution mixture of 1% EF, and 7% PEO in 1:1 ratio by volume. The solvent used is distilled water. Additionally, the conditions in fiber production were voltage of 13-18 kV, flow rate of 3.0 to 0.4 ml/h, and the distance between the needle tip and the collector of 10-20 cm.

5. Fiber qualities from the scanning microscope

Qualities and size of fibers would be studied by using a photo from Scanning Electron Microscope (SEM) by cutting the electropun fiber in a circle form with diameter of 6 mm and coated with gold using a machine Pularon SC500 sputter coater (Fisons, England) for 2 minutes. The samples were prepared for study using the camera SEM (Hitachi S-3000N, Japan) at magnification 1,000, 3,000 and 5,000 times. Then, the diameter of fiber would be measured from the 300 random photo from SEM with magnification of 1,000 times by using program Nano-VB (manufactured by Dr. Santi Maensiri, Khon Kaen University).

RESULT AND DISCUSSION

1. Comparison of different methods in extracting fibroin from red ant nests

Although fibroin was the main protein of silk fiber produced by *B.mori* silk worms and *O. Smaragdina* ants, fibroins from the two types of insect had different amino acid compositions. There were several studies of extracted fibroin from the *B. mori* silk worms with solutions under different extract conditions such as Ajisawa's solution at 80 °C (Yoshimoto et al., 2003), 9 M LiBr at 60 °C (Jin. et al., 2004) and 9 M LiSCN at 40 °C (Yamada et al., 2001). However, no study on the extraction of fibroin from *O. Smaragdina* nest fiber was ever conducted before. In this regard, this study compared the above mentioned methods and included the extraction using 0.5% Na_2CO_3 at 40 °C. The results were shown in Table 1. It was found that the Ajisawa's solution, 9 M LiBr and 9 M LiSCN, which were good solutions to extract silk fibroin from *B. mori*, could not be used for extraction of fibroin from ants. Nevertheless, the solution which could extract ant fibroin was the 0.5% Na_2CO_3 at temperature of 40 °C for 60 minutes. Dissolution of the ant nests in the 0.5% Na_2CO_3 solution and non-biodegradable protein bands which were extracted on the SDS -PAGE could be observed.

The protein dissolution depended on factors including 1) the pH of the solution and the pI of the protein 2) the strength of the ions in the solution, and 3) the concentration of ions in the solution. In the case of silk fibroin, its pI values ranged around 4 (Foo et al., 2006) and its structure consisted mainly of amino acids, glycine and alanine. In addition, the substituent

(R group of amino acids) was short and had no charge. It was found that the Ajisawa's solutions ($CaCl_2$: H_2O: MeOH, pH ~ 7), LiBr (pH ~ 7) and LiSCN (pH ~ 7.7) at pH higher than pI of the proteins, fibroin had a negative net charge and was soluble. Accordingly, the anions and cations in the solution at such concentration would help protein dissolution in a salting manner. The research showed that the strength of the cation in $Li^+ > Ca^{2+}$ and the strength of ion in $SCN^- > Br^- > Cl^-$. As a result, solution LiSCN could extract silk fibroin better than LiBr and $CaCl_2$ could respectively (Sashina et al., 2006).

This experiment discovered that the above solution which was used to extract fibroin of silk worms could not be used to extract fibroin of ants. From the report of Sutherland et al., (2007) which cloned fibroin gene from four species of Australian ants, it was found that fibroin samples from the three species had pI ranging around 5.3 to 6.8. It was, therefore, assumed that fibroin from the studied ants may have pI values close to the pH of the solution Ajisawa's, 9 M LiSCN and 9M. LiBr. As a result, such solution could not be used for extraction of the ant fibroin. Consequently, this study extracted fibroin of ants with a solution, 0.5% Na_2CO_3 (pH ~ 11) which was likely to have pH values higher than pI of the protein. The results revealed that the solution could extract fibroin of ants. However, 0.5% Na_2CO_3 solution yielded low fibroin output, returning fibroin extracted only 26.3%. Therefore, it was suggested that there be a study which could optimize the extracted output which could be done by increasing the concentration of Na_2CO_3 or using higher ionic-strength solutions.

Table 1. The result of red ant nest extraction under different solutions and conditions

Solution	Temperature (°C)	Time (Minutes)	Dissolution Level	Extracted Protein Band
Ajisawa's solution	80	15	0	No Protein band
		30		
		60		
		75		
9 M LiBr	60	15	0	No Protein band
		30		
		60		
		75		
9 M LiSCN	40	15	0	No Protein band
		30		
		60		
		75		
0.5% Na_2CO_3	40	15	+1	Protein band size 43 kDa was clearer in proportion with extracted time
		30	+2	
		60	+3	
		75	+4	Protein band was biodegraded

Remark: "0" The solution could not dissolve ant nests; "+1, +2, +3, +4" The dissolution level of ant nests in the solution from low to high when observing with eyes.

2. Properties of extracted fibroin protein from red ants

The extracted protein from ant nests was analyzed in terms of size and protein components with SDS-PAGE technique using 12.5% SDS-PAGE. The result was shown in Table1 which returned one major protein band with the size of 43 kDa. Although fibroin was the main protein in the ant fibers and the extracted protein had the size similar to that of the *O. Smaragdina* found in Australian continent, there was no study of the fibroin of red ants *O. Smaragdina* found in Thailand. Therefore, this study emphasized the protein band from SDS-PAGE to determine the amino acid sequence. Nevertheless, it was found that the first sequence of the amino acid had changes in the form of alpha amino group since the amino acid sequence analysis reaction could not occur. Therefore, in order to access more information of protein, 2D-PAGE technique was adopted by taking the extracted protein band of 43 kDa from technique SDS-PAGE to analyze with technique 2D SDS-PAGE using 7 cm IEF strip (GE Healthcare, USA) with pH in the range of 3-10. It was found that 11 protein spots were observed, ranging in size 39-43 kDa and the pI 4.8-7.2 (Figurg. 2). The size and pI result were close to the fibroin gene of Australian ants WAF1, WAF2 and WAF4 (Sutherland et al., 2007). However, further studies were required to confirm these conclusions such as finding amino acid sequence by mass spectrometry techniques or sequence of nucleotide from the cloned gene. The result from 2D-PAGE showed several spots of proteins possibly caused by a variety of species and age of the ant nests which affect the size and pI of the protein. However, fibroin of ants extracted in this study would be called the extracted fibroin (EF).

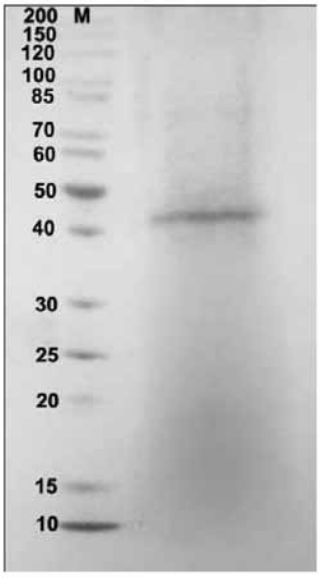

Figure1. Protein extracted from ants nest fibers with 0.5% Na_2CO_3 at 40 ° C for 60 minutes, when studied by using 12.5% SDS-PAGE. M was protein marker.

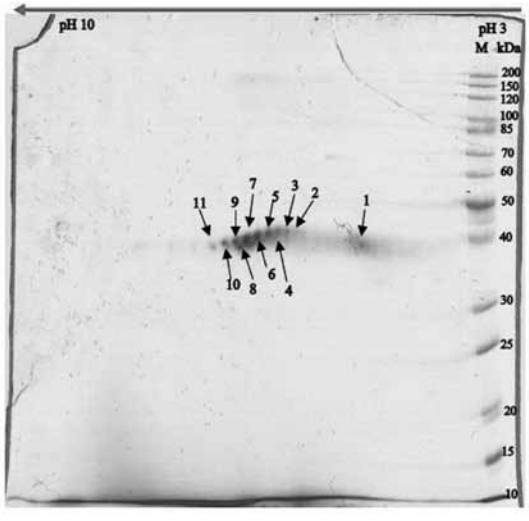

Figure2. Forms of the fibroin protein extracted from red ant nests with 0.5% Na_2CO_3 at 40 ° C for 60 minutes, when studied by using 2D-PAGE. M was a protein marker.

3. The appropriate conditions to produce nano electrospun-fiber from the extracted fibroin

The production of electrospun fiber from the extracted fibroin solution was conducted amid following conditions: solution concentration of 35%, voltage of 10-18 kV, flow rate of 0.3 to 0.2 ml/h, 15 cm distance between the needle tip and the collector (Table 2). This condition was not able to produce a continuous fiber. Therefore, enough polyethylene oxide (PEO) was mixed in order to assist fiber formation. PEO is polymer, a kind of popular use in biomedical materials, such as a scaffold for cell culture, vascular grafts, and materials for wound healing purpose. PEO was soluble, compatible with the cells of living organisms, and non-toxic to cells (Jin et al., 2002; Shin et al., 2007). From the preliminary study in the production of electrospun fiber from the solution 7% PEO only, it was found that the appropriate condition to produce fiber with continuity was the condition in which the distance between the needle tip to the collector was 20 cm and at a flow rate of 3.0 ml/h and with 13 kV voltage. Additionally, electrospun PEO fiber had an average diameter of 232.79 ± 59.94 nm. (Figure 3A). For the production of electrospun fiber from the extracted fibroin solution (1% EF) mixed with 7% PEO under following conditions: voltage 13-18 kV, flow rate 3.0 to 0.4 ml/hr, the distance between the needle tip and the collector of 20 cm (Table 3), The optimum condition to produce a continuous fiber was for voltage 18 kV, flow rate 0.4 ml/h, and 20 cm distance between the needle tip and the collector. Additionally, electrospun fiber had average diameter of 436.62±106.49 nm (Figure. 3B). Morover, electrospun EF-PEO fibers had a diameter larger than the electrospun PEO fiber because the mixed fibroin proteins made an increase in diameters. Additionally, to create smaller EF-PEO fibers to be similar to the properties of PEO fiber, voltage must be increased. However, this experiment could not be done in this research due to limitations of the controllable voltage of electrospinning machine. Additionally, the size of fibers had effect on application of benefits, such as controlling the release of drug .The smaller fibers could control the release of drug at a rate faster than the larger could, etc. (Okuda et al., 2009). Furthermore, this study did not achieve producing fibers from one kind of EF possibly due to inappropriate protein concentration. Further study should focus on a production of electrospun fiber under one kind of EF. The relationship of fibers and different concentrations of EF, appropriate voltage, flow rate, and solutions, etc. should be further explored. (Sill and von Recum, 2008).

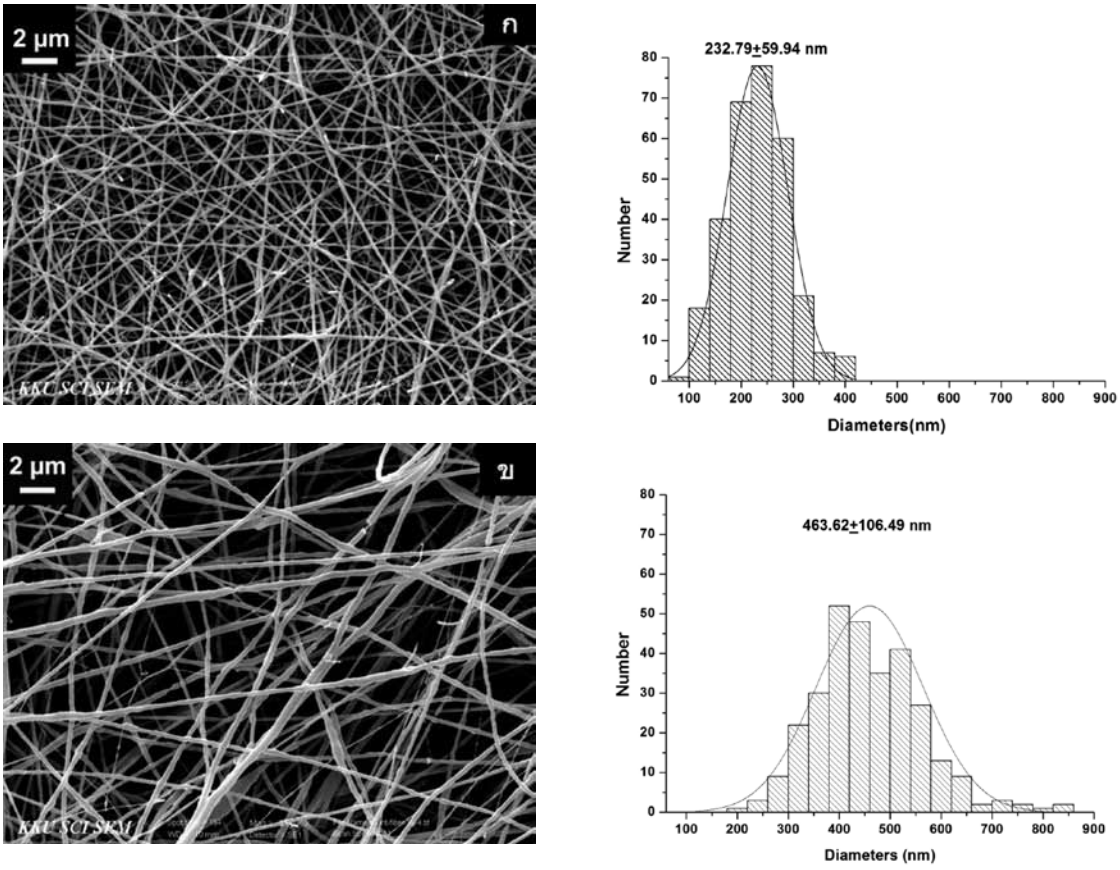

Figure3. SEM photographs and graphs showing the distribution of PEG (A) and EF-PEO (B) electrospun fiber size.

Table 2. Adjusted parameters and conditions in the production of EF electrospun fibers.

EF Concentration	Distance (cm)	Flow rate (ml/hr)	Voltage (kilovolt)	Nanofibers characteristic
35%	15	0.3	10	Large Drop, Slowly dry
			15	Large Drop, Slowly dry
			18	Small Drop, Slowly dry
		0.25	10	Large Drop, Slowly dry
			15	Large Drop, Slowly dry
			18	Small Drop, Slowly dry
		0.2	10	Large Drop, Slowly dry
			15	Large Drop, Slowly dry
			18	Small Drop, Slowly dry

Table 3. Adjusted parameters and conditions in the production of EF-PEO electrospun fibers

PEO Concentration	EF Concentration	Distance (cm)	Flow rate (ml/hr)	Voltage (kv)	Nanofiber characteristic
7%	1%	20	3.0	13	Large Drop, Slowly dry
				15	Large Drop, Slowly dry
				18	Large Drop, Slowly dry
			2.5	13	Large Drop, Slowly dry
				15	Large Drop, Slowly dry
				18	Large Drop, Slowly dry
			2.0	13	Large Drop, Slowly dry
				15	Large Drop, Slowly dry
				18	Small Drop, Slowly dry
			1.5	13	Discontinuing fiber mixed with drop (every minute)
				15	Discontinuing fiber mixed with drop (every minute)
				18	Discontinuing fiber mixed with drop (every minute)
			1.0	13	Discontinuing fiber mixed with drop (every minute)
				15	Discontinuing fiber mixed with drop (every minute)
				18	Discontinuing fiber mixed with drop (every minute)
			0.5	13	Discontinuing fiber mixed with drop (every minute)
				15	Discontinuing fiber mixed with drop (2 minutes)
				18	Discontinuing fiber mixed with drop (5 minutes)
			0.4	13	Discontinuing fiber mixed with drop (every minute)
				15	Discontinuing fiber mixed with drop (10 minutes)
				18	Discontinuing fiber mixed with drop (30 minute)

CONCLUSION

This research was the first showing the extraction and use of the red ant fibroin fibers in the production of electrospun fibers. It was found that the optimum condition for extraction was for a solution of 0.5% Na_2CO_3 at 40 °C for 60 minutes. The further analysis with SDS-PAGE technique revealed the core protein with 43 kDa in size. The 2D-PAGE further separated 11 different spots of protein which had pI values in the range of 4.8 to 7.2 and a molecular weight in the range of 39-42 kDa. This result was similar to the fibroin of Australian ants which had been reported before. After bringing the extracted fibroin (EF) to produce electrospun fibers in various conditions, no fiber was produced except when mixing with the PEO, which could produce a continuous fiber. The optimum condition to produce fibers was for EF-PEO in 1:1 ratio by volume, 20 cm distance between the needle tip and the collector, a flow rate of 0.4 ml of solution per hour, and voltage 18 kV. The extracted fiber had average diameter of 436.62 ±106.49nm.

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REFERENCES

Ajisawa, A. 1998. Dissolution of silk fibroin with calcium chloride/ethanol aqueous solution. **J Sericul Sci Japan** 67: 91-94.

Altman, G.H., Diaz, F., Jakuba, C., Calabro, T., Horan, R.L., Chen, J., Lu, H., Richmond, J. and Kaplan, D.L. 2003. Silk-based biomaterials. **Biomaterials** 24: 401-416.

Asakura, T., Kitaguchi, M., Demura, M., Sakai, H. and Komatsu, K. 1992. Immobilization of glucose oxidase on nonwoven fabrics with *Bombyx mori* silk fibroin gel. **J Appl Polymer Sci** 46: 49-53.

Craig, C.L., Hsu, M., Kaplan, D. and Pierce, N.E. 1999. A comparison of the composition of silk proteins produced by spiders and insects. **Int J Biol Macromol** 24: 109-118.

Fang, Q., Chen, D., Yang, Z. and Li, M. 2009. In vitro and in vivo research on using *Antheraea pernyi* silk fibroin as tissue engineering tendon scaffolds. **Mater Sci Eng** C 29: 1527-1534.

Foo, C.W.P., Bini, E., Hensman, J., Knight, D.P., Lewis, R.V. and Kaplan, D.L. 2006. Role of pH and charge on silk protein assembly in insects and spiders. **Appl Phys A** 82: 223-233.

Frenot, A. and Chronakis, I. S. 2003. Polymer nanofibers assembled by electrospinning. **Curr Opin Colloid Int Sci** 8: 64-75.

Gomes, D.S., Silva, A.N.R.D., Morimoto, N.I.L., Mendes, T.F., Furlan, R. and Ramos, I. 2007. Characterization of an electrospinning process using different PAN/DMF concentrations. **Polímeros** 17: 206-211.

Gomes, G., Elaine C.M. Silva-Zacarin, Fernando J. Zara, Moraes, R.L.M.S.d. and Caetano, a.F.H. 2004. Macromolecular array patterns of silk

gland secretion in social Hymenoptera larvae. **Genet Mol Res** 3: 309-322.

Holldobler, B. and Wilson, E.O. 1990. The ant. Harvard University Press. Cambridge Massachusetts, England.

Jeong, L., Yeo, I.S., Kim, H.N., Yoon, Y.I., Jang, D.H., Jung, S.Y., Min, B.M. and Park, W.H. 2009. Plasma-treated silk fibroin nanofibers for skin regeneration. **Int J Biol Macromol** 44: 222-228.

Jin, H.J., Fridrikh, S.V., Rutledge, G.C. and Kaplan, D.L. 2002. Electrospinning *Bombyx mori* Silk with Poly (ethylene oxide). **Biomacromolecules** 3: 1233-1239.

Jin, H.J., Park, J., Valluzzi, R., Cebe, P. and Kaplan, D.L. 2004. Biomaterial films of *Bombyx mori* silk fibroin with poly(ethylene oxide). **Biomacromolecules** 5: 711-717.

Liu, T.L., Miao, J.C., Sheng, W.H., Xie, Y.F., Huang, Q., Shan, Y.B. and Yang, J.C. 2010. Cytocompatibility of regenerated silk fibroin film: a medical biomaterial applicable to wound healing. **JZUS-B** 11: 10-16.

Min, B.M., Lee, G., Kim, S.H., Nam, Y.S., Lee, T.S. and Park, W.H. 2004. Electrospinning of silk fibroin nanofibers and its effect on the adhesion and spreading of normal human keratinocytes and fibroblasts in vitro. **Biomaterials** 25: 1289-1297.

Minoura, N., Tsukada, M. and Nagura, M. 1990. Physico-chemical properties of silk fibroin membrane as a biomaterial. **Biomaterials** 11: 430-434.

Okuda, T., Tominaga, K. and Kidoaki, S. 2009. Time-programmed dual release formulation by multilayered drug-loaded nanofiber meshes. **J Control Release** 143: 258-264.

Sakabe, H., Ito, H., Miyamoto, T., Noishiki, Y. and Ha, W.S. 1989. In vivo blood compatibility of regenerated silk fibroin. **Sen-i Gakkaishi** 45: 487-490.

Sashina, E., Bochek, A., Novoselov, N. and Kirichenko, D. 2006. Structure and solubility of natural silk fibroin. **Russ J Appl Chem** 79: 869-876.

Shin, J.-W., Shin, H.J., Heo, S.J., Lee, Y.J., Hwang, Y.M., Kim, D.H., Kim, J.H. and Shin, J.W. 2007. Hybrid nanofiber scaffolds of polyurethane and poly(ethylene oxide) using dual-electrospinning for vascular tissue engineering, **3rd Kuala Lumpur International Conference on Biomedical Engineering.** 692-695.

Sill, T.J. and von Recum, H.A. 2008. Electrospinning: Applications in drug delivery and tissue engineering. **Biomaterials** 29: 1989-2006.

Sutherland, T.D., Weisman, S., Trueman, H.E., Sriskantha, A., Trueman, J.W.H., and Haritos, V.S. 2007. Conservation of essential design features in coiled coil silks. **Mol Biol Evol** 24: 2424-2432.

Tsukada, M., Gotoh, Y., Nagura, M., Minoura, N., Kasai, N. and Freddi, G. 1994. Structural changes of silk fibroin membranes induced by immersion in methanol aqueous solutions. **J Polymer Sci B Polymer Phys** 32: 961-968.

Yamada, H., Nakao, H., Takasu, Y. and Tsubouchi, K. 2001. Preparation of undegraded native molecular fibroin solution from silkworm cocoons. **Mater Sci Eng: C** 14: 41-46.

Yoshimoto, H., Shin, Y.M., Terai, H. and Vacanti, J.P. 2003. A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. **Biomaterials** 24: 2077-2082.