Spider Silk Processing for Spidroin Recovery from *Crossopriza Lyoni* Web

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Abstract. Spider silk is a potential biomaterial that can be used in various applications for its outstanding physicochemical properties attributed by the spidroin composition. Efforts for commercializing spider silks have been mainly focused on the characterization of spidroins from the Entelegyne spiders for exceptional fibre construction. Hence, studies on silk proteins from the Haplogyne species remain neglected. The aim of this study is to isolate spidroin from *Crossopriza lyoni* web. Silk processing involved the pretreatment of fibres for the shell layer removal from the surface. A screening study was conducted to analyze the effect of temperature, incubation time and agitation speed on spidroin extraction using Ajisawa’s reagent by OFAT analysis followed by statistical optimization of the extraction process via RSM for maximal protein recovery. All parameters exerted significant effect on spidroin recovery (*p*<0.05) in which the maximum protein concentration (451.78 ± 0.110 µg/ml) was obtained at optimal condition of 70°C, 350 rpm and 1.25 hours. The discovery of spidroin from this study provides a basic platform for engineering spider silk to meet the demand for a variety of silk-based products in the near future.

1. Introduction

Nature readily contains plethora of beneficial materials in order to fulfill the very needs of humans. With the endowment of intelligence, men have been able to make clothes out of natural fibers from the silkworm, *Bombyx mori*. The legacy has kept flourishing for thousands of years when the silk thread finally found its applications not only in textiles but also in medicals, military, automobile and building construction [1]. However, silkworm-derived fibers often lack the required strength for such purposes. Thus, researches have steered the direction to the use of spider silk due to its superior properties that are twice stronger than that of silks from the silkworms [2] in order to meet the increasing demand for diversified silk-based products [3].

Spider silk is a proteinaceous biopolymer that is composed of two distinct protein layers; the core and the shell layer [4]. The core contains the silk proteins known as spidroins with molecular weight...
between 200 kDa to 350 kDa while the shell layer is composed of lipid, skin and glycoprotein. Spidroin is the only protein of interest and highly demanded for a wide range of applications due to its contribution for tensile strength and elasticity to silk fibers. Moreover, spidroins can be re-constructed into desired products by mechanical means. Spiders are known to spin up to seven types of silk fibres with the constitution of two types of spidroins namely Spidroin-I (for silk strength enhancement) and Spidroin-II (for elasticity and flexibility) [5]. Specialized abdominal glands are the major site of spidroin production in the form of high concentrated fluid (dope) prior to spinning into functional fibres.

Spidroins can be harvested via silks from the spider web [6], silks from forcible silking [7] and from the silk glands [8]. Silk fibres can be dissolved to recover spidroins in mixed solutions or harsh aqueous solutions such as Ajisawa’s solution, lithium bromide and lithium thiocynate [9].

All spiders produce various types of silk fibres that can be classified into two major groups; the Entelegyne and the Haplogyne species, respectively. Entelgynes such as the Nephilids are entirely more advanced in spinning orb-webs using strong threads [10] whereas Haplogynes constitute mostly the basal lineage spiders that build sheet webs with relatively fragile silks [11]. In ancient times, spider silks have been extensively used in South Pacific areas as fishing lures and knitting etiquette frock [12] as they exhibit a variety of outstanding physicomechanical properties such as high tensile strength, elasticity and toughness.

At present, companies such as Kraig Biocraft Lab and Araknitek have successfully commercialized exceptionally synthetic fibres using recombinant spidroins from the Nephilids. However, such effort is likely unknown in the study of spidroin from the Haplogyne species; causing possible innovations to remain unexploited. Previous studies have shown that spidroins from Haplogynes have a unique molecular architecture of the repetitive amino acid motifs; \(A_n, S_n, (G X)_n\), and \((A Q)_n\) (S, serine; Q, glutamine) which differ from the silk proteins found in Entelegynes [11]. This provides the potential wealth of protein designs.

Thus, the aim of this research was to isolate spidroins from Crossopriza lyoni silk web. *C. lyoni* is a haplogyne that is regarded as sedentary web-building spider. It is a cosmopolitan synanthropic cellar spider distributed widely in Malaysia [13]. Individual spiders can be easily found in large numbers on huge layer of webs in close proximity to humans and they are harmless for handling.

2. Materials and methods

2.1. Sampling of spider webs

*Crossopriza lyoni* webs were collected in the presence of the adult spiders using a net in large amount from abandoned houses as well as in construction sites in Perlis. The collected webs were stored in a plastic container regardless the age and stage-specific.

2.2. Pretreatment of spider silk fibres

Spider webs were initially washed under the running tap water and dipped overnight in distilled water to remove debris. They were dried in hot air oven at 60°C for 1 hour. The whole mass was then boiled in aqueous solution of 0.02 M sodium carbonate for 20 minutes at 100 °C with stirring to remove the tightly bound shell layer, rinsed with distilled water and dried in hot air oven. The dried degummed fibres were readily to be used in the extraction process of spidroins. The extract was subjected to Bradford assay to quantify the protein amount. The degumming step was originally adopted from [14] to remove the sericin layer in silkworm fibres.

2.3. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analysis was conducted at UKM-MTDC Smart Technology Centre, UKM, Bangi. SEM SU1510 (Hitachi) was used to perform microstructural study of pretreated silk fibers in order to validate the degumming process of shell layer removal from the fibres. The morphological structures of the fibres were observed by using backscattered electron imaging without
coating. Initially, the sample was mounted on the stub and screwed to the base stand firmly. Upon loading the sample into the chamber, it was slid onto the holder receptacle on the stage and evacuation process ensued. Accelerating voltage was set at 15 kV. With the adjusted electron optical system and the mode of detector opted for backscattered electron, the images were captured and recorded at 15,000 magnification with a scale of 3 µm.

2.4. Preliminary extraction of spidroins
10 mg of degummed silk fibers was dissolved in 20 ml Ajisawa’s reagent (calcium chloride: ethanol: water; 1/2/8 molar ratio) at optimal conditions of 250 rpm, 2.5 hours and 70°C [14-15]. The protein dope was dialyzed in a visking tube with molecular weight cutoff of 12-14 kDa against deionized water for three days with six consecutive buffer exchanges. Following dialysis, the dope was centrifuged at 4°C at 4000 rpm for 20 minutes. The dope was concentrated in vivaspin tube with molecular cutoff membrane of 30 kDa at 4°C at 1500 rpm for 20 minutes to a volume of 2 ml. The extract was subjected to Bradford assay to determine the protein concentration [16]. The protein solution was stored at -20°C until further use.

2.5. Screening of significant factors
The effects of three parameters on the extraction of spidroins were analyzed in this experiment namely the incubation time, temperature and agitation speed. Previously, these parameters have shown significant effect on the extraction of silk proteins from Bombyx mori as reported by [14]. The experiment was carried out by one-factor-at-a-time (OFAT) analysis to determine their significance on the spidroin extraction. The range values for each parameter were initially set at five levels as shown in table 1. However, a slight modification to the value range for agitation speed was performed at values that fixed to the digital hot plate available in the laboratory. The values of each factor that resulted in maximum silk protein concentration were selected for the subsequent OFAT analysis. Each extract was subjected to Bradford assay to determine the protein concentration.

Table 1. The value range of the selected parameters.

| Parameters            | Range          | Reference |
|-----------------------|----------------|-----------|
| Incubation time (hr)  | 0.5, 1.5, 2.5, 3.5, 4.5 | [14]      |
| Temperature (°C)      | 50, 60, 70, 80, 90  | [14]      |
| Agitation speed (rpm) | 60, 125, 350, 700 | -         |

2.6. Statistical optimization of spidroin extraction
From the OFAT analysis, the magnitude of each parameter and the range were determined based on the presence of curvature [17]. The significant parameters were subjected to optimization study. They were optimized using response surface methodology (RSM) via Box-Behnken Design in order to obtain an optimal extraction condition that yields in maximal recovery of spidroins from the web fibres.

The parameters include the incubation time, temperature and agitation speed. In order to predict the coefficients of the model, a total of 17 runs with 5 replicated center points (runs 3, 6, 7, 10 and 11) were provided by the design. The runs were executed in order to contradistinguish the experimental values and actual values obtained from model. Table 2 shows the experimental range and levels of each parameter and table 3 demonstrates the Box-Behnken Design matrix of the three parameters in non-coded values with the protein concentration as the observable response. Equation (1) involves in the Box-Behnken Design is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon$$  (1)
where the input variables were $X_1, X_2, \ldots, X_k, \beta_i (i = 1, 2, \ldots, k)$ and $\beta_{ij} (i = 1, 2, \ldots, k; j = 1, 2, \ldots, k)$ were unknown parameters and the random error was $\epsilon$. Each extract was subjected to Bradford assay to determine the protein concentration.

| Parameter          | Units       | Level |
|--------------------|-------------|-------|
| Incubation time    | (hr)        | (-1)  |
| Temperature        | (°C)        | (+1)  |
| Agitation speed    | (rpm)       |       |

2.6.1. Validation of extraction model. Following optimization process, validation of the model was performed in order to ensure an accurate model could be predicted by solving the regression equation [16]. Verification study was carried out under the conditions of 70°C and 350 rpm for 1.25 hours. The three replicates for protein yield were supplied with the extraction conditions to affirm the prediction of model. The percentage error between theoretical data and experimental data was calculated and a percentage error less than 1% indicates a high reliability of RSM model. Thus, the response model is appropriate to reflect the anticipated optimization.

2.7. Statistical analysis
All experiments were conducted in triplicates and the data was analyzed using one-way ANOVA with post-hoc Duncan Multiple Range Test. ANOVA was performed using SigmaStat (version 3.1) by Systat Software Inc. For optimization study, the experiment was designed by using Box-Behnken Design (BBD) via Design Expert 7.1.5 software.

| Run | $A^a$ (hr) | $B^b$ (°C) | $C^c$ (rpm) | Protein Concentration (µg/ml) |
|-----|------------|------------|------------|-------------------------------|
| 1   | 2.00       | 70         | 205        |                               |
| 2   | 0.50       | 90         | 205        |                               |
| 3   | 1.25       | 80         | 205        |                               |
| 4   | 0.50       | 80         | 350        |                               |
| 5   | 0.50       | 70         | 205        |                               |
| 6   | 1.25       | 80         | 205        |                               |
| 7   | 1.25       | 80         | 205        |                               |
| 8   | 1.25       | 70         | 60         |                               |
| 9   | 0.50       | 80         | 60         |                               |
| 10  | 1.25       | 80         | 205        |                               |
| 11  | 1.25       | 80         | 205        |                               |
| 12  | 2.00       | 80         | 350        |                               |
| 13  | 1.25       | 90         | 60         |                               |
| 14  | 1.25       | 70         | 350        |                               |
| 15  | 2.00       | 80         | 60         |                               |
| 16  | 1.25       | 90         | 350        |                               |
| 17  | 2.00       | 90         | 205        |                               |

Note: $^a$-Incubation time, $^b$-Temperature, $^c$-Agitation speed
3. Results and discussion

The silk pretreatment was aimed to remove the tightly bound shell layer from the fibres. In order to ensure the efficiency of the shell layer removal, the supernatant extract from the pretreated fibres was subjected to Bradford assay to estimate for its protein content. It was mixed with Bradford reagent and a concentration of 53.83 µg/ml of protein was determined. This indicates a small amount of proteins that were unbound from the threads; presumably the shell layer proteins. Sponner et al. reported that spider silk fibre naturally contains two prominent protein layers; the shell layer and the core spidroin [4]. The former contains a number of glycoproteins and skin type proteins that act as a protective layer to the core spidroin.

In addition, SEM analysis was conducted to compare the morphological differences of the silk fibre before and after the degumming process. The microstructure of the degummed fibre was observed on a SEM SU1510 (Hitachi) and photographed at a 15 kV as shown in figure 1. It can be observed that the silk fibre before the degumming process appeared to be covered with a layer of grooving lines (shell layer) whereas for the degummed fibre, it appeared clean and smooth. This qualitative result corroborates the findings that the degumming pretreatment with 0.02 M sodium carbonate was able to remove the shell layer from the silk fibres.

The pretreated fibers were subjected to solvent-salt based extraction using Ajisawa’s reagent encompassing calcium chloride, ethanol and water (1:2:8 molar ratio) according to the optimum condition set by [14-15] at 250 rpm, 70°C for 2.5 hours. It can be seen from the Bradford assay data that only 20 µg/ml of protein concentration was obtained. The dope is believed to only compose of the shell layer void-spidroins as it was removed during the degumming process. From the preliminary study, it is inferred that the spidroin extraction method was successfully established and this method was used throughout the whole experiment.

Meanwhile, the undissolved fibres were centrifuged and dried in the oven and the weight was recorded. Approximately 26% of undissolved fibres were obtained and suspected to belong to various
types of spider silk fibres other than the dragline silk thread of the web. This is in agreement with the fact that spider web is spun with more than one type of silk thread. In addition, many previous studies that used Ajisawa’s solution for spidroin recovery was performed on the dragline silk fibre. Therefore, the incorporation of Ajisawa’s solution in the present study may favour the extraction of spidroins from the dragline silk thread. However, further investigation is required to support the hypothesis.

Due to the small amount of silk proteins recovered, an optimization study of the extraction condition was carried out to overcome the problem in order to yield maximal protein concentration. Initially, a screening study of the effect of significant parameters on spidroin extraction was conducted. Temperature, incubation time and agitation speed were selected and tested in the study. OFAT analysis was conducted in order to screen for the significant factors affecting the spidroin recovery. From figure 2, all of the three parameters exerted statistically significant effect on spidroin extraction from the web fibre ($p<0.05$) by one-way ANOVA. From the post hoc analysis of comparisons using the Duncan Multiple Range test, it is indicated that the mean score of protein concentration at 80°C (27.95 ± 0.147 µg/ml), 125 rpm (383.61 ± 0.4430 µg/ml) and 1.5 hours (117.22 ± 0.11 µg/ml) were significantly higher compared to others ($p < 0.05$) in each experiment, respectively. Thus, they were opted as the best level of treatments for the optimization study as they led to the highest maximal spidroin recovery.

**Figure 2.** A. Effect of temperature [$F(4,10) = 2295.304$, $p = <0.001$], B. Effect of incubation time [$F(4,10) = 2688235.49$, $p = <0.001$] and C. Effect of agitation speed [$F(3,8) = 122566.659$, $p = <0.001$] on spidroin extraction from *C. lyoni* web.
Interestingly, the present finding is in contrast with [14] which 70°C was the best temperature that led to the maximum amount of protein recovered. Nevertheless, it can be comprehended that the higher the exposing temperature, the more the spidroins can be extracted from the fibre. This is because at high temperature, the protein molecules could move faster as they possessed high kinetic energy to overcome the strong intermolecular force imposed on them.

Meanwhile, 1.5 hours was observed to exert significant effect on the extraction of spidroins. This implies that incubation time is one of the important factors that contribute to silk protein extraction. In contrast to [14], 2.5 hours was the best time which produced the maximum concentration of spidroin. This phenomenon may possibly due to the high hydrolysis rate of interlinking bonds between fibroins themselves and more fibroins were able to be dissolved in the extraction solution at 90 minutes compared to others. Moreover, 125 rpm was observed to exert significant effect on the extraction of spidroins. At present, few screening studies have been performed on agitation speed, but most of the experiments divulged that the higher the agitation speed, the more proteins could be recovered [15]. This is because of the high rate of solubility of silk spidroin at higher agitation speed.

The optimization of extraction conditions within the selected range was conducted using Box-Behnken Design (BBD). In statistical modelling, regression analysis is a process performed by RSM to estimate the relationships among parameters shown in table 2. The dependence of spidroin concentration on the extraction condition was shown by the second order regression equation whereas the multiple regression analysis of the experimental data generated the parameters of the equation. These parameters are presented in the second order polynomial equation as indicated in Equation (2).

\[
Y = 1447.27 - 49.31 \times A + 7.70 \times B + 79.93 \times C - 0.11 \times A \times B - 4.44 \times A \times C + 0.14 \times B \times C + 0.44 \times A^2 + 2.83 \times 10^{-3} \times B^2 + 78.72 \times C^2
\]

(2)

where Y (response) represents the spidroin concentration and A, B, C refer to temperature, agitation speed and incubation time, respectively.

From table 4, both actual and predicted values of spidroin concentration are presented. It is obviously shown that the maximum protein concentration is obtained by run number 14 with 451.78 ± 0.110 µg/ml at optimal conditions of 70°C, 1.25 hours and 350 rpm. Meanwhile, run number 16 contributes to the lowest protein concentration (10.11 ± 0.0057 µg/ml) under the conditions of 90°C, 1.25 hours and 350 rpm.

**Table 4.** Box-Behnken Design matrix of the three parameters with the protein concentration in µg/ml.

| Run | A^a (hr) | B^b (°C) | C^c (rpm) | Protein Concentration (µg/ml) |
|-----|----------|----------|-----------|-----------------------------|
|     | Actual   | Predicted|           |                             |
| 1   | 2.00     | 70       | 205       | 220.17                      |
| 2   | 0.50     | 90       | 205       | 166.67                      |
| 3   | 1.25     | 80       | 205       | 20.22                       |
| 4   | 0.50     | 80       | 350       | 250.61                      |
| 5   | 0.50     | 70       | 205       | 219.56                      |
| 6   | 1.25     | 80       | 205       | 84.11                       |
| 7   | 1.25     | 80       | 205       | 105.83                      |
| 8   | 1.25     | 70       | 60        | 26.22                       |
| 9   | 0.50     | 80       | 60        | 85.56                       |
| 10  | 1.25     | 80       | 205       | 52.22                       |
| 11  | 1.25     | 80       | 205       | 95.00                       |
| 12  | 2.00     | 80       | 350       | 195.17                      |
| 13  | 1.25     | 90       | 60        | 213.44                      |
| 14  | 1.25     | 70       | 350       | 451.78                      |
| 15  | 2.00     | 80       | 60        | 70.00                       |
Based on the analysis of variance of the model by Fisher’s F-test, it is noted that the least square regression line (LSRL) is a good model for the data fitting with Model F-value of 30.07 with the probability value of less than 0.0001. Generally, p-value is the probability to verify the impact of coefficients in which a value less or equal to 0.05 denotes statistical significant at 95 % confidence level. The response surface model is deemed adequate due to the insignificance (p = 0.9592) of the lack of fit with F values of 0.094 relative to pure error for all variables. The adequacy of the model is also corroborated by its determination coefficient, R^2 value of 0.9748.

Generally, 3D surface profiler has been used widely in the analysis of the model graph due to the different effects and interactions that can be easily visualized based on the curvature patterns. From the curvatures in figure 3, it can be inferred that the highest spidroin concentration could be obtained if the temperature, incubation time and as well as agitation speed are kept at higher treatment levels. This is possibly because the proteins are less stable at high temperature and can be dissolved more into the extraction solution; the longer the incubation time, the higher the hydrolysis rate of the interlinking bonds between spidroins themselves. Meanwhile, the higher the agitation speed, the more bonds that linked silk proteins together can be broken by strong forces.

Figure 3. 3D surface of spidroin concentration as a function of (A) temperature and agitation speed, (B) temperature and incubation time and (C) agitation speed and incubation time.
Validation experiment was conducted in order to ensure an accurate model could be predicted as well as the predicted value would similar to the experimental value. The optimal values of the selected variables were obtained by solving the regression equation (Equation 2). The optimal condition for protein yield estimated by the model equation was given at 70°C, 350 rpm and 1.25 hours. The theoretical protein yield predicted under the above condition was 445.862 µg/ml. Verification of the model was performed by comparing the percentage error between the theoretical and the experimental spidroin concentrations. The average protein concentration obtained was 447.81 µg/ml with the percentage error of 0.44% (< 1%). Thus, this indicates a figure well within the estimated value of the model equation. RSM model is highly reliable since the predicted value is in the agreement with the experimental value. This also indicates the RSM model is valid and hence, the response model is appropriate to reflect the anticipated optimization. Furthermore, the results also suggested that the model in Equation (2) is satisfactory and accurate.

4. Conclusion
In this study, spider silk processing from Crossopriza lyoni web using Ajisawa’s reagent under optimized condition of 70°C, 1.25 hours and 350 rpm has successfully recovered a maximal spidroin amount of 451.78 ± 0.110 µg/ml. Spider silk is a potential biopolymer that is at least five times as strong as steel and twice as elastic as nylon and Kevlar synthetic fibre. Thus, the extracted silk protein from Crossopriza lyoni from the present study will address future investigations on the purification and characterization of the protein. With the endowment of high biodiversity of Haplogyne spiders in Malaysia, this study may likely become the first attempt at national level towards the development of novel biomaterials from spider silk in order to meet the ever increasing demand for silk-based products in the near future.

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