pISSN 2302-1616, eISSN 2580-2909
Vol 10, No. 1, June 2022, pp. 7-15
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v10i1.24373

Atlas moth (*Attacus atlas* Linnaeus, 1758) silk sericin protein: the effect of extraction methods and storage time on its content

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ABSTRACT. Atlas moth (*Attacus atlas*) cocoons consist of two proteins, fibroin and sericin. Sericin can be extracted using several methods and can be stored for an adjusted time period, but these affect its quality and quantity. Thus, this study aims to elucidate the effect of different extraction methods and storage time to Atlas moth's sericin yield. Atlas moth cocoons were obtained from Entomology Laboratory UGM (old sample) and from Mahogany tree in Manisrenggo, Klaten (new sample). These two different samples were extracted using an alkaline solvent called turkey red oil (TRO) and 8 M urea (AR). The total protein of the extracted sericin was quantified using Bradford Assay and continued by SDS-PAGE. It was found that the two extraction methods (TRO and AR) yielded the same amount of total protein and weight, that is 1.607 mg/mL (TRO) and 1.624 mg/mL (AR) respectively, and the same molecular weight (<10 kDa). The storage time affected the total protein yielded (0.870 mg/mL) and did not show any protein band when SDS-PAGE performed. In conclusion, the two different extraction methods did not affect sericin yield, whilst the storage time greatly affected the sericin yield.

Keywords: *Attacus atlas*; extraction method; silk sericin protein; storage time

Article History: Received 24 October 2021; Received in revised form 30 October 2021; Accepted 14 April 2022; Available online 30 June 2022. Ver: Pre-Press
How to Cite This Article: Alwandri H, Sukirno S, Sumarmi S. 2022. Atlas moth (*Attacus atlas* Linnaeus, 1758) silk sericin protein: the effect of extraction methods and storage time on its content. *Biogenesis: Jurnal Ilmiah Biologi*. vol 10(1): 7–15. doi: https://doi.org/10.24252/bio.v10i1.24373.

INTRODUCTION

Insects have diverse protective mechanisms, which vary from one species to another, even on each of their life stages and one of those mechanisms is using proteins that have certain properties or characteristics. Silkworms are one of the insects that use protein as their protective mechanism during the formation of the cocoon as it is turning into imago on the next step of its life stages. The proteins used by silkworms consist of two main proteins, fibroin, which is a fiber protein that appears as a twin-thread structure linked by disulfide bonds, and sericin, which is a globular protein that acts as glue or adhesive, envelops the fibroin as the core (Wang & Zhang, 2011).

In general, silk fibroin is known to have long been used as fabric-making materials, and discard the silk sericin on its processes (degumming), thus the silk sericin just ends up as waste (Yan *et al*., 2021). Besides, silk fiber processed from silk fibroin is also reported to be used in industrial and medical applications mainly due to its characteristics, such as high permeability to water and oxygen, support cell adhesion and growth, dying affinity, thermo-tolerance, and many more (Amornsudhiwat *et al*., 2013; Xu *et al*., 2018; Puspita *et al*., 2020). However, many studies reported that silk sericin also has its own potentials. Sericin obtained from *Philosamia ricini*, *Bombyx mori*, and *Antheraea assamensis* could protect keratinocytes that had been irradiated by UV-A and UV-B, therefore it showed that sericin act as antioxidant (Kumar *et al*., 2018). Sericin was also shown to have the ability to absorb UV-B when tested using a UV-VIS spectrophotometer (Sukirno *et al*., 2021).

Sericin can be obtained from many silkworms. One of those silkworms is Attacus atlas (Atlas moth), the largest Lepidoptera insect that belongs to the Saturniidae family, has also been researched and reported to produce larger cocoons and properties of silk that is similar to that of Bombyx mori (Reddy *et al*., 2013). However, the atlas moth silk has been less studied than that of B. mori, mainly on factors affecting its quality and quantity (Motta *et al*., 2011; Cao *et al*., 2016; Kumar *et al*., 2018; Kawahara *et al*., 2020).

To extract the silk sericin from the cocoon can be done by many extraction methods. The variety of extraction methods will affect the sericin quantity and quality, such as molecular weight, purity, biochemical activity, and antioxidant activity (Gupta *et al*., 2013; Miguel & Alvarez-Lopez, 2020). Extraction methods that have been used to extract the sericin namely, using sodium oleate (0.3%) and sodium carbonate (0.2%) for 60 minutes (Yang *et al*., 2013), urea (8 M) for 30 minutes (Aramwit *et al*., 2010), neutral soap (Wang *et al*., 2015), novel protease isolated from bacteria Bacillus sp. (Suwannaphan *et al*., 2017), by autoclaving it for 60 minutes (70-65°C) (Prasong, 2011), and several more. Then, in order to effectively use the sericin properties, the extraction methods have to be a concern.

Maintaining the property of sericin must be a priority when wanting to use it as a certain agent. Therefore, how to store it must also be considered. It is reported that when sericin was stored in a decreasing-temperature condition, the strength of the sericin gel increased and the gelation process was prolonged. When sericin was stored for 40 minutes at a temperature higher than 50 °C, the sericin gel was disrupted and the gel strength was also decreased. Moreover, it would be interesting to see if even when the temperature requirements have been met, storage time itself actually affects the silk sericin properties (Jo *et al*., 2015). Therefore, this study aims to compare the effect of extraction methods and storage time on Atlas moth silk sericin content.

MATERIALS AND METHODS

Obtaining the Atlas moth cocoons. Atlas moth cocoons used in this study were obtained from two different sources. The first one was the cocoons that were collected in 2018 (storage time: approx. 3 years) and had been stored in glass case storage at room temperature, were obtained from Entomology Laboratory, Faculty of Biology, Universitas Gadjah Mada. The second one was the freshly collected cocoons in February 2021, the cocoons were obtained by directly collecting them from mahogany trees in Manisrenggo, Klaten, Central Java.

Silk sericin extractions. Silk sericin was extracted by 2 different methods, namely by boiling in TRO (*Turkey Red Oil*) (TRO method) and soaking-heating in 8 M urea (Aramwit *et al.*, 2010) (AR method). Cocoons from both sources were given the same treatment. TRO was only used to extract the new sample (2021 sample) and AR method was used to extract the new and old sample, in consideration that AR method has been widely used in several researches, so the analysis would be more robust. The cocoons were washed thoroughly to remove the remaining dirt. Then, the cocoons were cut into tiny pieces (1 cm^2) and weighted by analytical weighing scale for 7.5 g. For the TRO method, the weighted cocoons were collected and put into 150 mL TRO 0,67% (1 g TRO in 150 mL dH_2O), so it resulted in 5% cocoons in TRO solvent. It was then boiled for 1 hour (100 °C) using the hot plate, and then the solution was filtered by filter paper. The result of this filtering is called sericin and labelled as "TRO". The second method also involved several steps. The sericin sample (7.5 g) was soaked in 8 M urea (187.5 mL) for 30 minutes and then heated in a water bath (85 °C) for another 30 minutes. The result was centrifuged (8000 rpm; 4 °C) and filtered (filter paper) to remove the remaining insoluble residues or debris, and the final result is the silk sericin protein with label "AR_N (new)" and "AR_o (old)".

Total protein determinations. Total protein was determined by Bradford assay. Prior to the assay, the samples were concentrated by adding 5 mL acetone to 5 mL of each sample. The solution then stored in 4 °C for 24 hours, hence that a precipitate was formed. To separate the mixture, centrifugation was performed (4000 rpm; 4 °C; 5 min) and the pellet formed was the sericin. The pellet was air-dried for another 24 hours in 4 °C. It was then resuspended by phosphate buffer (0.1 M; pH 8.0; 100 µL). Then, the Bradford assay was done by collecting each 8 µL of concentrated sample mixed with 200 µL Bradford reagent of each well in 96-well-plate, and then the protein spectrum was read by a microplate reader (596 nm). The reading results were then converted into

total protein value using the standard curve of Bovine Serum Albumin (BSA). Experiments were done in triplicate.

Protein molecular mass determinations. SDS-PAGE was done using Mini-PROTEAN Bio-rad, and the sample used was the concentrated sample. Each sample (92 µL each) was mixed with 92 µL sample buffer, 2 µL ME (2-Mercaptoethanol), sealed, and heated in water bath (95 °C) for 3 min. The sample was immediately cooled in the freezer for 15 min. The lower gel used in this experiment was 12% and made using Ostap laboratory protocol, likewise the upper gel. The marker used was the Smobio PM-5100 ExcelBand™ 3-color Pre-Stained Protein Ladder, High Range (9-245 kDa). The marker and samples were loaded into the upper gel and run for 30 min (50 Volt) and continued for 1 hour (100 Volt). After that, the gel was stained using coomassie blue R-250 for 24 hours by soaking. The stained gel was de-stained by microwaving repeatedly until the protein bands were visible. The molecular size of each sample was compared to each other.

Data analysis. Data was analyzed in a descriptive manner. The dependent variable of total protein determination was total protein (mg/mL), while the independent variables were extraction method (TRO and AR), and sample sources (old and new). The dependent variable of protein molecular weight determination was protein molecular weight (kDa), while its independent variables were extraction method (TRO and AR), and sample sources (old and new).

RESULTS AND DISCUSSION

Silk sericin extractions. In both methods all of the cocoons produce brownish color. The result of both extraction methods and both cocoon sources is shown in the Fig. 1.

A B C

Fig. 1. Sericin extracted by two methods and two different sources: A) AR_N method (AR method using new sample; B) AR_O method (AR method using old sample); and C) TRO method using new sample.

It is assumed this coloration (Fig. 1) was produced due to the pigment of the cocoon itself, which dissolved through the extraction process. Cocoon pigmentation was heavily affected by the diets. Leaf pigments were absorbed by the larva and transported to the middle silk gland (MSG) by the hemolymph and eventually deposited in silk sericin when secreted at late instar (Sakudoh *et al.*, 2012). There are three major genes (*Y*, *I*, and *C*) that orchestrated the pigment transport that expressed yellow coloration in *Bombyx mori* (Tsuchida & Sakudoh, 2015). In another study, green coloration in *B. mori* is expressed by 3 organic compounds (flavonoid) (Napavichayanun *et al.*, 2017). Further exploration needs to be done to elucidate pigment transport in atlas moths. Nevertheless, it means that the brownish pigment of mahogany trees in its leaf was absorbed by the atlas moth larva, deposited in silk sericin, and dissolved in the extraction process of both methods (TRO and AR), thus the brownish colored solution was produced. It also can be assumed that sericin was successfully extracted or separated from fibroin by both methods. The success of extracting sericin has also been estimated because sericin itself is very soluble in hot water. That nature is due to its sol-gel property, which turns into sol at high temperature and returns to gel (β-structure) when the temperature drops. This was in contrast to fibroin which will not dissolve in hot water. It is assumed that it may be caused by their difference in polar amino acids constituent (Kunz *et al.*, 2016).

However, albeit the sericin is easily soluble in hot water, extraction by boiling alone could not separate the whole sericin from fibroin. It happens due to its amino acids near the fibroin consisting

mainly of the non-polar ones. It did not only require boiling but also the addition of Na_2CO_3 to extract more sericin (29.7% in 120 minutes) (Wang & Zhang, 2011). Consequently, additions or agents were used in this study, which was turkey red oil (detergent) and urea.

In the case of TRO, which is sulfated castor oil and also a detergent, it will be readily and completely dispersed in water-producing alkali. The alkali will bind to the sericin to form a soda salt, isolating and separating it from the fibroin better than boiling alone. Actually, this method has been used for a long time but generally only uses ordinary soap (Tsunokaye, 1932). Another study has also tried to use detergent, but TRO is quite new to be used. Compared to soap, TRO is better to handle metal ions Mg^{2+} and Ca^{2+} or acidic conditions, since TRO will not form *scum* (Singh & Singh, 2019). This study result shows that nature since it did not form any deposit. It means TRO is a better agent rather than soap to extract silk sericin.

Urea has been widely used as an agent to extract *sericin* in many studies (Yun *et al.,* 2013; Aramwit *et al.,* 2018; Abdullah *et al.,* 2021). Urea was used due to its nature as a hydrogen bond-breaking reagent. It acts to solubilize the sericin because sericin forms a β-structure and becomes a gel when the temperature drops. Hence, the 8 M urea used in this study serves to ensure the sericin was solubilized and also to extract the sericin near to the fibroin. Lithium thiocyanate solution (LiSCN) and lithium bromide solution (LiBr) also has been used in several studies, but instead of only solubilizing the sericin, it also solubilizes the fibroin. Therefore, 8 M urea is considerably a better option (Kundu *et al.,* 2014; Volkov *et al.,* 2015).

The result of extraction in this study also showed that the silk sericin from two different sources of different storage time (old and new) (Fig 1. (A) and (B)) produced brownish color with different intensity. It showed that AR_O produced a weaker brownish color compared to AR_N. It suggests that the natural color of the cocoons were fading through storation. This could happen due to several factors, one of which is physical factors such as temperature and humidity during storage which could cause cocoons to be susceptible to being damaged by microbes such as fungi. In a particular study using cocoons of *B. mori,* it is showed that protein, carotenoid (pigment) and phenol were affected during storage by several fungi at various intervals, showing that the total protein of cocoons of *B. mori* was reduced sharply by several fungi, namely *F. moniliforme, A. flavus, A. niger* and *P. citrinum* at all incubation periods (20, 40 and 60 days). The maximum loss was observed due to *F. moniliforme* and followed by *P. citrinum, A. flavus* and *A. niger.* The loss of carotenoid was also observed to be loss at maximum level by *F. moniliform* (79.81 %) (Prasad & Singh, 1995). It indicates that the result that was shown by AR_O in this study can be assumed due to the same reason, and it indicates that cocoon storage needs to consider the physical factor that is affecting them.

Colorless cocoons of *B. mori* could happen because of a gene defect. The *Y-gene* recessive mutant, one of the 3 major genes that the products regulating carotenoid transport from diet to hemolymph and cocoons, was unable to perform cellular uptake of lutein. The total carotenoid of a mutant $+^{Y}+^{I}$ was the least (0.4 ± 0.2 µg/g), followed by $+^{Y}I$ (1.3 ± 0.2 µg/g), *YI* (10.5 ± 2.6 µg/g), and $Y+^{I}$ (65.0 ± 7.8 µg/g) (Tsuchida & Sakudoh, 2015). However, AR_O only showed a different intensity of brownish color and it suggests that the main reason for this phenomenon was due to mainly the physical factors. Nevertheless, it can also be assumed that there is a need to confirm the genetic stability of the silkworm used.

Silk sericin characterization: total protein and molecular weight determinations. Both methods (TRO and AR) showed promising results in the extraction process, but it needs to be confirmed by quantifying its total protein content and molecular mass. Hence, the Bradford assay and SDS-PAGE were performed.

Bradford assay was used to quantify the total sericin protein in the 5% sample from the extraction of each method and each source. This technique is relatively simple, easy, fast and quite sensitive. The Bradford reagent that contains *coomassie blue* R-250 will bind to the protein and produce blue coloration that indicates a certain amount of protein (Dong *et al.,* 2011). The intensity of the coloration was read by a microplate reader at 595 nm and the absorbance value or optical density

(OD) was shown (Pal *et al.*, 2013). Eventually, the OD was converted to the total amount of protein by using formula obtained from the previously constructed bovine serum albumin (BSA) standard curve (Kumar *et al.*, 2016). Silk sericin total protein is shown in Table 1. below.

Table 1. Bradford assay result for 3 extraction methods on *Atlas* moth cocoons.

Extraction methods	Mean of Optical Density (OD)	Total protein (mg/mL)
TRO	0.681	1.434
AR_N	0.731	1.550
AR_O	0.437	0.870

These results (Table 1.) indicate a quite high amount of protein in each sample when compared to other research that uses the same method. One of those researches showed that the total protein of *B. mori* was 879 µg/mL when extracted using protease for 1 hour (Sangwong *et al.*, 2016). Hence, it can be assumed that the method that was used in this study was relatively better to extract or separate the sericin from fibroin, or it can be due to its higher amount of sericin since Atlas moth cocoons are bigger in size. In another study, *Samia ricini* silk sericin cocoon extraction yields 1.02 mg/g by boiling at 100 °C and significantly increase by increasing the boiling period (Bungthong & Siriamornpun, 2021). Thus, boiling duration during extraction needs to be considered. However, it cannot be exponentially increasing, since the properties of the silk sericin could be altered completely, then the desired function cannot be achieved (Silva *et al.*, 2012).

Bradford assay was only able to measure the total protein, but is not specific for the type of protein-based on the constituent amino acids or its molecular weight, unless if the protein is already pure (Metsamuuronen *et al.*, 2011). Consequently, SDS-PAGE was performed on each sample and compared to the marker (*smobio pm-5100*) to estimate its molecular weight.

The figure below shows the SDS-PAGE result on each sample or methods used in this study (Fig. 2).

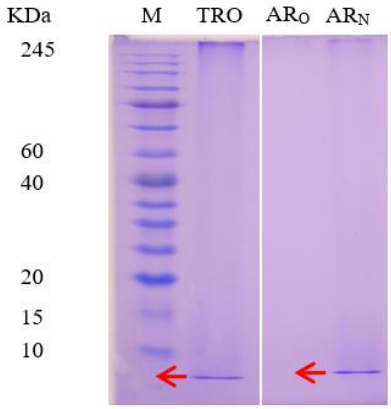

Fig. 2. SDS-PAGE results on several extraction methods: (M) marker smobio pm-5100; (TRO) turkey red oil using new cocoon sample; (AR_O) soaking and heating in 8 M urea using old cocoon sample; (AR_N) soaking and heating in 8 M urea using new cocoon sample. The protein band is only shown in TRO and AR_N, both at < 10 kDa.

The SDS-PAGE result (Fig. 2) shows that the protein band was produced on the TRO and AR_N, while AR_O does not show any protein band. It indicates that only the freshly obtained sample (new) produced the protein band when SDS-PAGE was performed, despite the positive result on the Bradford assay (Table 1.) and it can be assumed due to several reasons. There are few substances that can cause interference to the Bradford assay and show a false positive result, such as sucrose, glycerol, Tris, detergent, strong alkaline buffer, and urea (Bradford, 1976). Urea (8 M) has indeed been used in this study (AR methods) extraction. It was found that 0.16 M urea could significantly increase the assay by 14% by assuming that urea interacts with some specific protein using its electro-neutral properties and perturbing effect (Friedenauer & Berlet, 1989). However, the 8 M urea used in AR

method was air-dried prior to the Bradford assay, but it is still probable to happen if the urea still remains. It also applies to the TRO method, since it uses turkey red oil, a detergent, as the extraction agent. Thus, it makes it also possible to get a false positive result when performing Bradford assay. Therefore, based on this finding, it is highly recommended to dilute or dialyze the sample before carrying out the Bradford assay.

The absent of the protein band at any molecular size on the SDS-PAGE result of AR_O (Fig. 2) can be assumed that silk sericin protein on the old sample has indeed been greatly reduced due to the storage process, and the reason can be assumed to be fungi infestation. The reduction of cocoon sericin protein was probably a result of enzymatic processes by the fungi to use it for growing (Prasad & Singh, 1995). However it needs further experimentation to get to that conclusion.

Another probability to that absent of protein band, and also the only <10 kDa protein band produced on the other method was due to the affinity of the coomassie blue R-250 staining. There is a high correlation to the intensity of the response to coomassie blue staining to the basicity of the protein, which directly correlates to the number of lysine, arginine, and histidine amino acids (Tal *et al.,* 1985). It was revealed that through several extraction methods, silk sericin consists of a high amount of serine, and only counts for 2.35, 3.14, 3.48, and 2.89 for lysine, it counts 2.87, 5.41, 4.92, and 4.92 for arginine, and it counts 1.06, 3.26, 2.47, and 1.72 for histidine, by heat, urea, acid, and alkaline extraction respectively for each amino acid (Aramwit *et al.,* 2010). It suggests that there is a need for consideration when choosing the right stain so that the protein can be stained with more precision.

The estimation of the protein weight on the TRO and AR_N sericin sample was only around <10 kDa. However, it is still in the range of silk sericin protein based on references. Sericin has a fairly large range of molecular weight, which is *Ser1* (>250 kDa), *Ser3* (250 kDa), *Ser2-large* (<250 kDa), and *Ser2-small* (<130 kDa) (Takasu *et al.,* 2010). Hence, the molecular weight of protein of TRO and AR_N can be classified to be *Ser2-small* sericin protein. This result was also reported by another study, showed that the crude protein of sericin from atlas moth and *B. mori* results in protein band ranging from 8.24 to 10.25 kDa for atlas moth, and 8.24 to 8.99 kDa for *B. mori.* In the same study, the middle silk gland (MSG) of both silkworm species was also used, which resulted in a larger range of sericin molecular weight (Endrawati, 2012). Therefore, it is suspected that direct extraction from sericin-secreting glands is able to isolate sericin that are more diverse in weight, but it is not really practical to do for a large scale.

However, it was counterintuitive to a study that found *Ser2* is actually not a cocoon protein. *The Ser2* gene which expresses highly on the anterior portion of MSG, only secrete its protein shortly before each instar transition and prior to cocoon formation. Cocoon silk sericin protein is predominantly occupied by *Ser1* and *Ser3* (Takasu *et al.,* 2010). Even so, in contrast, a study could prove that *Ser2* is actually really expressed in cocoons and it in fact acts as an important factor in silk reelability (Du *et al.,* 2011). Those studies were done using *B. mori* cocoons, therefore it is interesting to look into *Ser2* gene expression in atlas moth cocoons.

The kinds of weights and the range of sericin proteins that appear on the SDS-PAGE gel can also be influenced by alternative splicing of mRNA transcripts (Dong *et al.,* 2019). It is stated that the 3' end of its gene expressed 4 mRNA that indicates a unique alternative splicing. This finding strongly supports that despite the fact that SDS-PAGE result (Fig. 2) only shows 1 band of protein, it can still be assumed to be silk sericin protein.

Another factor that could affect the sericin protein migration on SDS-PAGE is protein glycosylation. It is because when glycosylation exists, SDS that has a negative charge cannot bind to the denatured protein sample, resulting in inconsistency of protein-sds-coated shape, when the estimated shape should be spherical or beads. Study showed that glycosylation exists on *ser1* and *ser2* genes (Zurovec *et al.,* 2013). Glycosylation might make the band appear to be a smear (Lin *et al.,* 2018).

In addition to the factors already mentioned, there is another factor that highly affects quality and quantity of extracted sericin, which is the main problem in question in this study that is the extraction method. In two different studies that using several methods to extract the sericin from *B. mori,* it was found that using urea and alkaline produced an SDS-PAGE band that ranging from 10-120 kDa (Kumar & Mandal, 2017) for urea method and band that ranging from 15-75 kDa (Wang *et al.,* 2018) using an alkaline solution. Thus, this study agrees with or is in line with those studies, that is using urea and alkaline solvent might produce a low molecular weight sericin. Hence, there is no difference in sericin yield between the two methods used in this study (TRO and AR_N). Therefore, it is assumed that the difference might be related to the relative number of its constituent amino acids or its antioxidant activity. Jena *et al*. (2018) also found the same result, which is when using alkaline solvent and boiling produced sericin with molecular weight ranging from 11 to 245 kDa, even when it was from a different type of silkworm that is *Antheraea mylita*. Nonetheless, it indicates that further studies are needed.

CONCLUSION

The extraction method used in this study did not show any difference when a comparison was made between the two, based on total protein using Bradford assay and determination of molecular weight using SDS-PAGE. The TRO and ARN produced a total silk sericin protein of 1.607 mg protein/mL and 1.624 mg protein/mL, respectively. The molecular weight determination of TRO and ARN showed the same weight of protein, that is <10 kDA. However, there is a difference in the molecular weight determination result between the old and the new cocoon sample. The old cocoon sample did not show any protein band when SDS-PAGE was performed. Therefore, based on the results, cocoon storage time needs to be an important concern to meet the quality or quantity desired.

ACKNOWLEDGEMENTS

The gratitude is conveyed to the Faculty of Biology UGM for the Kolaborasi Dosen dan Mahasiswa Grant which funded this study. Entomology Laboratory and Fasilitas Laboratorium Bersama (FALITMA) for the research facilities.

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