International Journal of Biological Macromolecules 52 (2013) 59–65

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Extraction conditions of *Antheraea mylitta* sericin with high yields and minimum molecular weight degradation

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ARTICLE INFO

Article history:
Received 27 July 2012
Received in revised form
11 September 2012
Accepted 24 September 2012
Available online 28 September 2012

Keywords:
Silk
Sericin
Antheraea mylitta
Protein-based material

ABSTRACT

Although the technique for extracting the *Bombyx mori* sericin has been extensively known, the extraction of sericin from wild-silkworm cocoons is not yet standardized. The aim of this study was to find the optimal conditions for the extraction of sericin from *Antheraea mylitta* cocoons, with high yields and minimum degradation. We attempted to apply various protocols for the extraction of the *A. mylitta* sericin (AmS). Among these, we found that the extraction of AmS with a sodium carbonate solution exhibited the highest yield except the conventional soap-alkali extraction. To find the optimal conditions for the AmS extraction with the sodium carbonate, we changed the concentration of sodium carbonate and the treatment time. With an increase in the sodium carbonate concentration and the extraction time, the yield of AmS increased, but the molecular weight (MW) of AmS decreased. Considering the yield, molecular weight distribution (MWD) and amino acid composition of AmS, we suggest that the optimal conditions for the AmS extraction require treatment with 0.02 M sodium carbonate and boiling for 60 min.

1. Introduction

Sericin is the name of the protein that the silkworm secretes from its middle gland. This protein envelops two brins of fibroin and glues them together. The feel and luster of silk fabrics are gained after the removal of sericin by a degumming process. Sericin is usually discarded, but there have been continual efforts to recover and reuse it as a natural polymer in various applications [1–3]. The utilization of waste products such as sericin does not only increase the farmers' incomes but also lessens the environmental impact by reducing waste.

Silkworms are usually domesticated, but there are also silkworms that live in the wild. Five species of wild silkworms are economically important, including *Antheraea mylitta*, *Antheraea pernyi*, *Antheraea yamamai*, *Antheraea assamensis*, and *Samia cynthia ricini*. Traditionally, their silks are called Indian Tasar silk, Chinese Tasar silk, Japanese Oak silk, Muga silk and Eri silk, respectively. Of these wild silkworms, India produces three varieties of wild-silkworm silk: Tasar, Muga and Eri silk. During the year 2010–2011, the production of raw silk from these wild silkworms in India was 1166, 2760 and 123 metric tons, respectively [4]. The production of silk from these wild silkworms has almost doubled during the past 5 years. With the increase of wild-silkworm silk production, the use of silk proteins from these wild silkworms in the biomedical field – similar to the silk proteins from the domestic silkworm (*Bombyx mori*) – have just begun during the past 3 years. Particularly, fibroin and sericin from *A. mylitta* have been studied for biomedical applications [5–10].

Whereas the extraction or recovery process of sericin from the *B. mori* cocoon is well established, the extraction of sericin from the wild silkworm's cocoon has still not been fully demonstrated. Kundu et al. [9–15] have extensively studied the novel application of the *A. mylitta* sericin (AmS). In these studies, two types of solutions for the extraction of AmS have been used, namely, a sodium chloride solution and a sodium carbonate solution. In the case of the sodium chloride solution, the *A. mylitta* cocoons are immersed in a 1% NaCl solution and stirred overnight at room

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http://dx.doi.org/10.1016/j.ijbiomac.2012.09.017

Table 1
Summary of the AmS extraction methods used in this study.

Method	Chemicals	Temperature (°C)	Time
Soap-alkaline	0.02% (w/v) Na_2CO_3, 0.03% (w/v) Marseilles soap	100	1 h
Hot-water	None	120	1 h
Urea	8 M urea	80	10 min
Urea–mercaptoethanol	8 M urea, 5% (v/v) mercaptoethanol	80	10 min
NaCl	1% (w/v) NaCl	25	15 h
Na_2CO_3	0.01–0.06 M Na_2CO_3	100	15–120 min

temperature [11,12]. Currently, a sodium carbonate solution is widely used for the extraction of AmS [9,10,13–15]. Normally, 0.02 M or 0.2% Na_2CO_3 solutions are used, and the cocoons are boiled for 30 or 60 min, either with or without pressure. In the case of boiling under pressure, the temperature rises to 121 °C.

Although the degumming rate, the molecular weight distribution and the amino acid composition analysis have been determined for the NaCl extraction method, there are few data regarding the Na_2CO_3 extraction method. It appears that a common method for the degumming of the *B. mori* sericin has been adopted. However, a comprehensive analysis of the extraction conditions is important because these conditions could affect the final properties of sericin. Particularly, in the case of sericin, the extraction conditions should be carefully controlled because sericin is highly susceptible to heat [16]. Various parameters such as the types and concentrations of chemicals, the temperature, and the treatment time will affect the degumming rate, molecular weight distribution, and even the amino acid composition of sericin [17]. The degumming rate, in other words, the extraction efficiency is important because it will determine the economic value of the sericin extraction method. The molecular weight distribution and the amino acid composition are also important because they affect the physical and chemical properties of sericin [18]. The aim of this study was to provide fundamental data on the extraction of AmS, which could form the basis for future applications of AmS in various fields.

2. Experimental

2.1. Materials

The *A. mylitta* cocoons were collected from the Warangal District of Andhra Pradesh. All of the chemicals in this study were purchased from Sigma–Aldrich (Yongin, Korea).

2.2. Extraction of AmS

We performed six different extraction methods that are currently used to extract sericin from *B. mori* and *A. mylitta* (Table 1). For all of the extraction procedures, the *A. mylitta* cocoons were cut into small pieces before the extraction and 10 g of the cocoon pieces was added to 250 ml of the extraction solutions. The heating was performed under refluxing conditions except for the hot-water extraction. First, the conventional soap-alkaline process was used for the full extraction of sericin. The cocoon pieces were boiled without pressure in a solution containing 0.02% (w/v) of Na_2CO_3 and 0.03% (w/v) of Marseilles soap. A hot-water extraction was performed by boiling the cocoon pieces with distilled water at 120 °C for 1 h. For the urea extraction, the cocoon pieces were immersed in a 8 M urea solution and were heated at 80 °C for 10 min. For the urea–mercaptoethanol extraction, the same condition as the urea extraction was adopted except the addition of 5% (v/v) 2-mercaptoethanol to the 8 M urea solution. For the NaCl extraction, the cocoon pieces were immersed in a 1% (w/v) NaCl solution and stirred at room temperature overnight. Finally, for the Na_2CO_3 extraction, various concentrations of the Na_2CO_3 solution were used. The cocoon pieces were added to the solution and boiled without pressure for various lengths of time.

After the extraction, the solutions were filtered through a non-woven filter to remove the remaining cocoon pieces. The cocoon pieces were then washed several times with warm water, dried in an oven at 50 °C for 3 days, and conditioned at room temperature for 12 h before measuring their weights. The degumming ratio was calculated using the following equation:

$$\text{Degumming ratio}\,(\%) = \frac{W_0 - W_F}{W_0} \times 100$$

where W_0 and W_F are the initial weight and the final weight of the cocoon pieces, respectively. The extraction yield was calculated using the following equation.

The surface of the cocoon pieces was observed using a scanning electron microscope (SEM) (JSM-5410LV, JEOL, Japan). The filtered AmS solutions were dialyzed against distilled water in a dialysis tube (Spectra, USA, MWCO 6000-8000) for 3 days to remove the chemicals. Finally, the AmS powders were obtained by freeze-drying.

2.3. Characterization of the extracted AmS

To measure the molecular weight distribution (MWD) of each sample, the AmS was dissolved in a 4 M urea solution at room temperature and was filtered through a cellulose acetate membrane that had a pore size of 0.2 μm. The MWD of AmS was measured by gel filtration chromatography (GFC) (ÄKTA purifier, GE Healthcare, USA) using a Superdex column (Superdex 200 10/300GL, GE Healthcare, Sweden) at a flow rate of 0.5 ml/min. A 4 M urea solution was used as the eluent. The standard molecular weight markers for the GFC consisted of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

The amino acid compositions of the AmS samples were analyzed using HPLC (HP1100, USA). Each sample was hydrolyzed by 6 N hydrochloric acid with 0.02% (v/v) 2-mercaptoethanol at 110 °C for 24 h in a nitrogen filled vial. The final hydrolysates were dissolved in sodium citrate buffers (pH 2.2) and were analyzed by HPLC using an Inno-C18 column. The column temperature was 40 °C, and the flow rate was 1 ml/min.

3. Results and discussion

3.1. Degumming ratios of the A. mylitta cocoons using different extraction methods

We used the conventional degumming ratio to quantify how much sericin could be removed from the *A. mylitta* cocoons by each extraction method. The degumming ratios of the *A. mylitta* cocoons from each extraction method are presented in Table 2, and the SEM image of the *A. mylitta* cocoon before and after the extraction are shown in Fig. 1. Because we peeled off each of the cocoon layers before the SEM observation, some residual AmS in the upper layer (arrow) could be observed in the intact *A. mylitta* cocoon (Fig. 1A). Furthermore, the two brins of fibroins were not separated.

Table 2
Degumming ratio and extraction efficiency of the *Antheraea mylitta* cocoons subjected to different extraction methods.

Method	Degumming ratio (%)[a]	Extraction efficiency (%)
Soap-alkaline	19.5 ± 1.9[*]	100
Hot-water	8.3 ± 3.1[*]	42.7
Urea	3.7 ± 2.3[*]	18.8
Urea–mercaptoethanol	9.0 ± 4.4	46.2
NaCl	9.0 ± 2.0[*]	46.2
Na_2CO_3 [b]	13.2 ± 3.6	67.5

[*] Indicates significant differences between Na_2CO_3 and other groups (Student's *t*-test, $p < 0.05$).
[a] Values are the mean ± standard deviation ($n = 3$).
[b] The extraction condition was sodium carbonate at a 0.02 M concentration and an extraction time of 30 min at 100 °C.

The highest degumming ratio was achieved with the soap-alkaline extraction. After the extraction of AmS, the surface of the cocoon fiber became clean, and the two brins of fibroin were separated, which indicated a complete degumming (Fig. 1B). It is known that the sericin content of the wild-silkworm cocoon is lower than that of *B. mori* [19]. Generally, the degumming ratio of the *B. mori* cocoon that is treated with a soap-alkaline solution is approximately 25%; in contrast, in this study, the degumming ratio was 19.5 ± 1.9% for the *A. mylitta* cocoon. Although the soap-alkaline extraction exhibits the highest degumming ratio of all the extraction methods, it is not recommended for the extraction of AmS because it is difficult to separate the AmS from the soap. Actually, this process had been developed to remove sericin efficiently from fibroin for further application, so it is inaccurate to call the process "extraction". Nonetheless, we regarded this degumming ratio (19.5 ± 1.9%) as representing a 100% extraction of AmS and

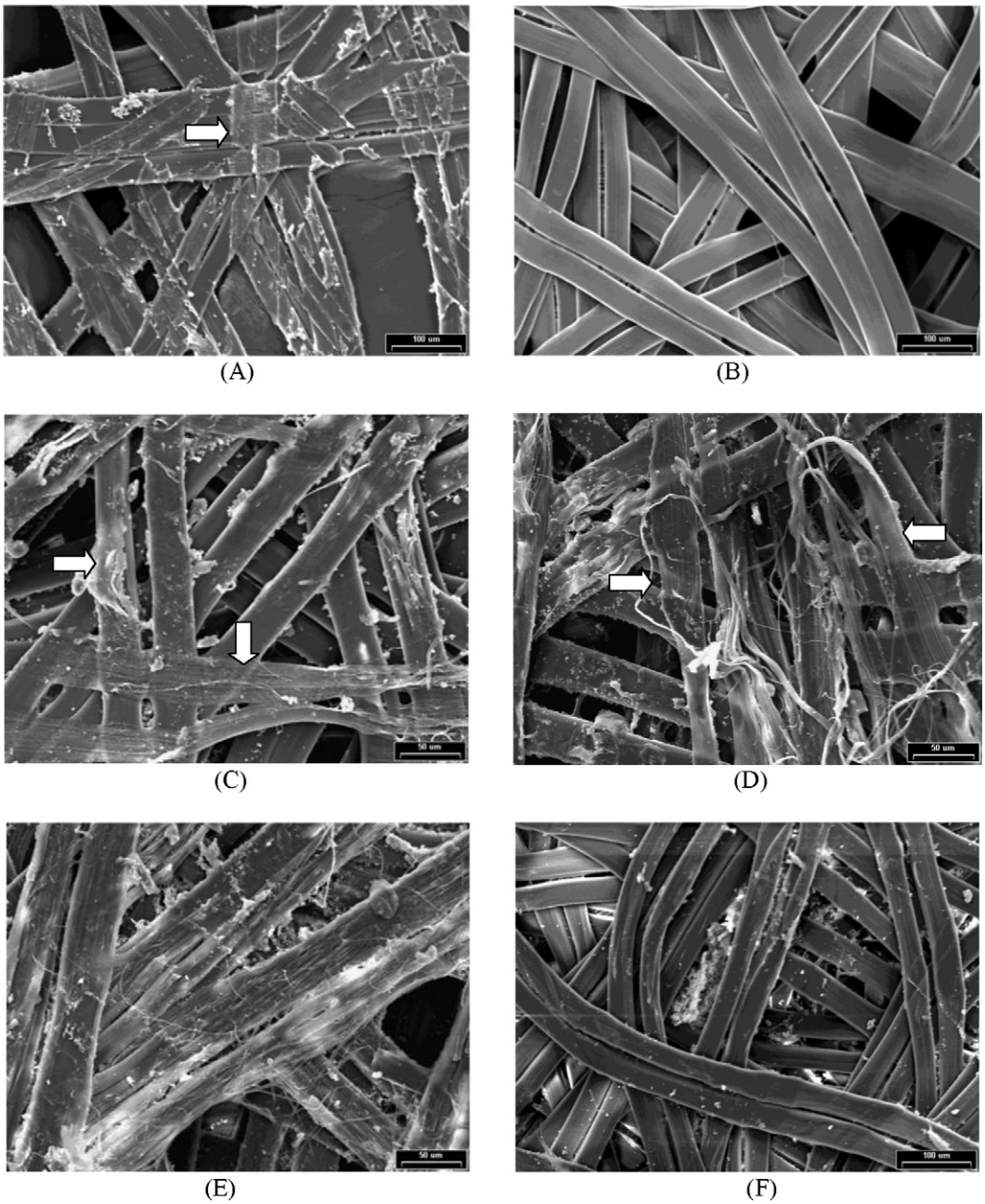

Fig. 1. SEM images of the original *Antheraea mylitta* cocoon fiber (A) and the fiber after different extraction methods, including soap-alkaline (B), hot-water (C), urea–mercaptoethanol (D), NaCl (E) and Na_2CO_3 (F).

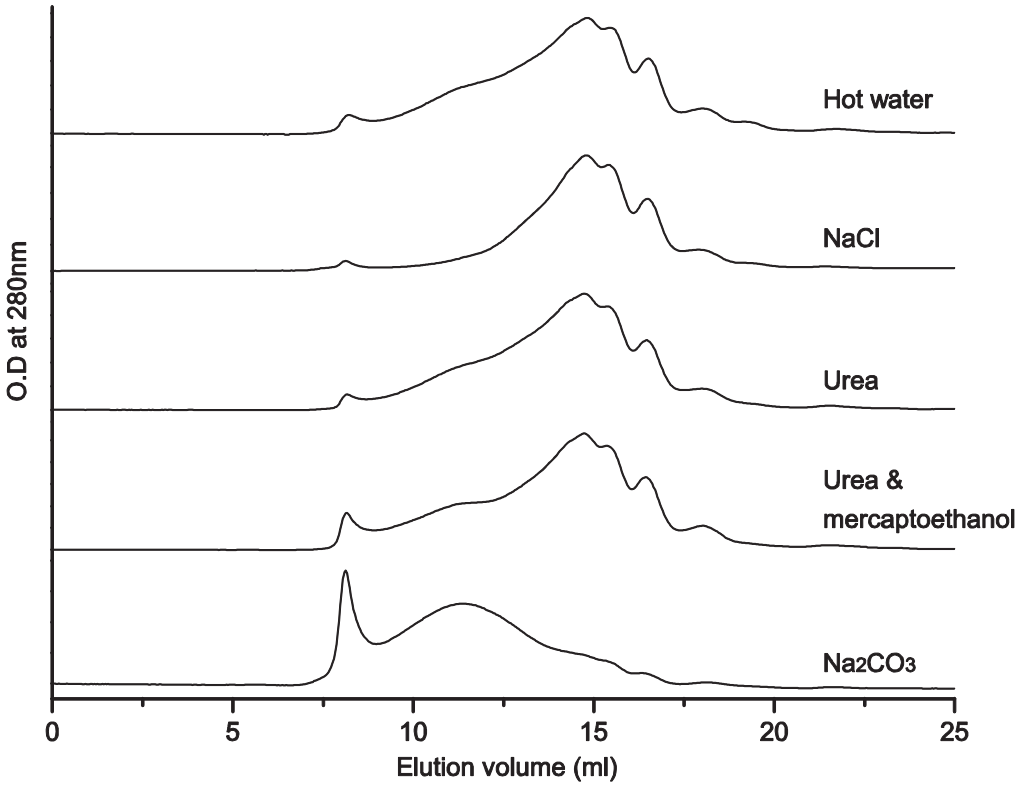

Fig. 2. Molecular weight distributions of the AmS extracted by different extraction methods.

calculated the extraction efficiency for the other extraction methods. Here, the extraction efficiency means how much AmS is extracted from the total AmS that exists in the *A. mylitta* cocoon.

Other extraction methods have been developed for the *B. mori* cocoon, including the hot-water extraction [18] and the urea extraction with or without mercaptoethanol [17]; the degumming ratio is generally greater than 15%. We adopted the same extraction method for the *A. mylitta* cocoons. However, as presented in Table 2, the degumming ratios of the *A. mylitta* cocoons subjected to these methods were less than 9.0%; particularly, the degumming ratio was only $3.7 \pm 2.3\%$ when urea without mercaptoethanol was used as the degumming agent. The extraction efficiency was only 18.8–46.2%, whereas more than 90% of extraction efficiency has been reached for *B. mori* cocoons using these methods [17,18]. Currently, we do not have the direct structural evidence to explain why such low extraction efficiencies were obtained for the *A. mylitta* cocoons compared with the *B. mori* cocoons. However, we can draw assumptions from the harsh environment of the wild silkworms' habitat. The wild-silkworm cocoon is harder than that of *B. mori* because the former has to protect the pupa against its natural enemies and the climate conditions. Therefore, the AmS might have a more compact structure than the *B. mori* sericin. Practically, for the reeling of silk fibers, the cocoons should be softened by a cooking process for both *A. mylitta* and *B. mori*. Whereas, for the *B. mori* cocoon, this procedure involves mild conditions (using only water), for the *A. mylitta* cocoon, harsher conditions (such as the inclusion of ethylenediamine) are adopted [20]. In the SEM images of the *A. mylitta* cocoon after these extraction methods (Fig. 1C and D), the residual sericin (arrows) could be observed and was responsible for the low degumming ratio. Interestingly, the sericin that was located between the two brins of fibroin was clearly removed although the degumming was not complete. This finding might be due to differences in the cross-sectional morphology between the *A. mylitta* and *B. mori* cocoons. In the case of the *B. mori* cocoon, the cross-section of the native cocoon fiber has a triangular shape, and the two brins of fibroin are embedded in the sericin matrix. When the degumming of sericin is not complete, the two brins of fibroin are not separated because some sericin remains between the two brins of fibroin. In contrast, the cross-section of the *A. mylitta* cocoon fiber has a flat and ribbon-like structure, and in some members of the Saturniidae family, the two brins of fibroin are spun in their separated state [21]. As illustrated in Fig. 1A, the two brins of fibroin were also distinguishable even in the intact *A. mylitta* cocoon. Therefore, unlike the *B. mori* cocoon, the two brins of fibroin in the *A. mylitta* cocoon were not fully covered by sericin, and the two brins of fibroin were observed to be separated although the degumming was not complete.

The sodium chloride solution has been previously used for the extraction of AmS from the *A. mylitta* cocoon, and the degumming ratio has been calculated to be 7% according to our formula [11]. Our result was $8.3 \pm 3.1\%$, which is consistent with that previous study. The extraction of AmS with sodium chloride solution can be explained by the solubility of protein. The solubility of protein depends on various parameters including the ionic strength of the solution. The addition of NaCl increased the ionic strength of the solution and thereby some AmS might dissolve into the solution. In the SEM image (Fig. 1E), striations on the surface of the fiber could be observed, indicating an incomplete degumming [21].

Currently, the sodium carbonate solution is the most frequently adopted method for the extraction of sericin from the *A. mylitta* cocoon. Typically, the sodium carbonate method has been adopted to obtain pure fibroin by the removal of sericin from the *B. mori* cocoon [22]. The degumming ratio for the *A. mylitta* cocoon was $13.5 \pm 2.0\%$ when 0.02 M Na_2CO_3 was used for the extraction at 100 °C for 30 min. The SEM image of the cocoon after the sodium carbonate extraction is shown in Fig. 1F. The surface of cocoon fiber was not as clean as the soap-alkaline method, but the two brins of fibroin could be clearly observed, and neither a stratified surface nor residual sericin could be observed, indicating a relatively high degumming efficiency.

Because our goal in this study was to find the optimal extraction conditions to recover sericin for material applications, the following requirements were necessary: first, a high extraction yield must be achieved to ensure economic value. Second, the molecular weight of the sericin should be high enough for material applications. The sodium carbonate extraction met the first criterion.

3.2. Molecular weight distribution of the extracted AmS using different extraction methods

There is no doubt that the molecular weight of a polymer is one of the important factors that affect its physical properties. The molecular weight distribution (MWD) of sericin depends on the extraction method for the *B. mori* sericin and AmS. Therefore, it is important to examine the MWD of the extracted AmS from the above described extraction methods. The MWD of the native AmS collected directly from the silk gland has been reported. Similarly to the *B. mori* sericin, AmS consist of more than 200 kDa, 200 kDa and 70 kDa polypeptides in addition to some low molecular weight polypeptides [23]. Fig. 2 shows the MWDs of the AmS that was extracted using the hot-water, NaCl, urea and urea–mercaptoethanol methods; all of the extracted AmS exhibited similar MWDs. There were two distinct regions: a small peak at the elution volume of approximately 7 ml and a broad band, for which the highest peak was observed at the elution volume of 14 ml. The molecular weight of the former was approximately 200 kDa, and the highest peak of the broad band was approximately 70 kDa. In these cases, the main AmS molecules had molecular weights of between 40 and 100 kDa. These results are consistent with previous studies, in which a 70 kDa sericin has been isolated from NaCl extracts after ethanol precipitation. In the case of the urea–mercaptoethanol extraction, the 200 kDa AmS was extracted in limited quantities from the *A. mylitta* cocoons, although this extraction method is most effective for the extraction of the 200 kDa sericin from *B. mori* cocoons [17]. The hot-water extraction of the *B. mori* sericin also successfully yielded the 200 kDa sericin [18]. It appears that these extraction methods are inefficient for the extraction of the 200 kDa AmS. As described in the previous section, the degumming ratios from these methods are relatively low; therefore, these low degumming ratios might be due to a failure to extract the high-molecular weight sericin. As mentioned previously, these low ratios might be due to the structural differences between the *B. mori* and *A. mylitta* sericin. In contrast, the Na_2CO_3 method was effective for the extraction of the 200 kDa AmS and the mid-range-molecular weight AmS. The relatively high degumming ratio of the Na_2CO_3 extraction was the result of the successful extraction of the 200 kDa AmS. Apparently, Na_2CO_3 is effective for removing sericin from the AmS cocoon. The highest degumming ratio was obtained when the soap-alkaline extraction method was adopted. Here, Na_2CO_3 was used as the alkaline source. Because the role of the soap is limited to the isolation of the removed sericin, thereby preventing the re-adsorption of sericin, it is Na_2CO_3 that separates sericin from the cocoon. Furthermore, the MWD of the AmS extracted by this method is very similar to that of the *B. mori* sericin from the hot-water extraction. From these results, we conclude that the Na_2CO_3 extraction satisfies the requirements regarding the extraction efficiency and the MWD for the polymeric application of AmS – most likely demonstrating the reason Na_2CO_3 is widely used for the extraction of AmS. However, no standard method for the Na_2CO_3 extraction has been previously reported, as in the case of *B. mori*. Various concentrations of Na_2CO_3 are used, and the treatment times and temperatures also differ [9,10,13–15]. Therefore, we more closely considered the extraction conditions when using Na_2CO_3 as the agent to extract sericin from *A. mylitta*.

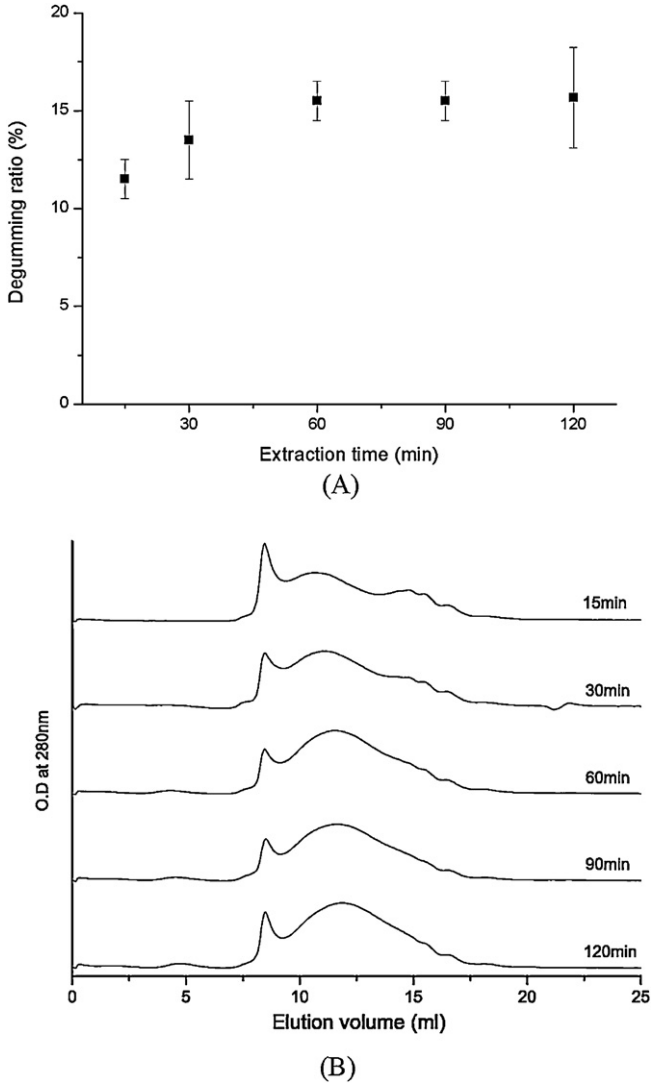

Fig. 3. Effect of the extraction time on the degumming ratio of *Antheraea mylitta* cocoon (A) and on the molecular weight distribution of the extracted AmS (B). The Na_2CO_3 concentration was fixed at 0.02 M.

3.3. Optimum extraction conditions for the sodium carbonate method

Because the sodium carbonate extraction has the best yield among the other examined extraction methods, we attempted to find its optimal conditions. The extraction conditions can be controlled by various parameters, such as the extraction time, temperature and concentration of sodium carbonate. Here, we examined only the effect of the time and the sodium carbonate concentration.

3.3.1. Effect of extraction time and sodium carbonate concentration

Fig. 3A displays the effect of the extraction time on the degumming ratio of the AmS. After 60 min of extraction, the degumming ratio reached a plateau ($15.5 \pm 1.0\%$) and did not increase further significantly. Fig. 3B shows the MWD of the AmS according to the extraction time. There are two distinctive regions in the MWD. First is the sharp peak at 7 ml of elution volume, which corresponds to 200 kDa. Second is the broad band between 9 and 17 ml of elution volume, which corresponds to 150–30 kDa. As the extraction time increased, the intensity of the 200 kDa band decreased,

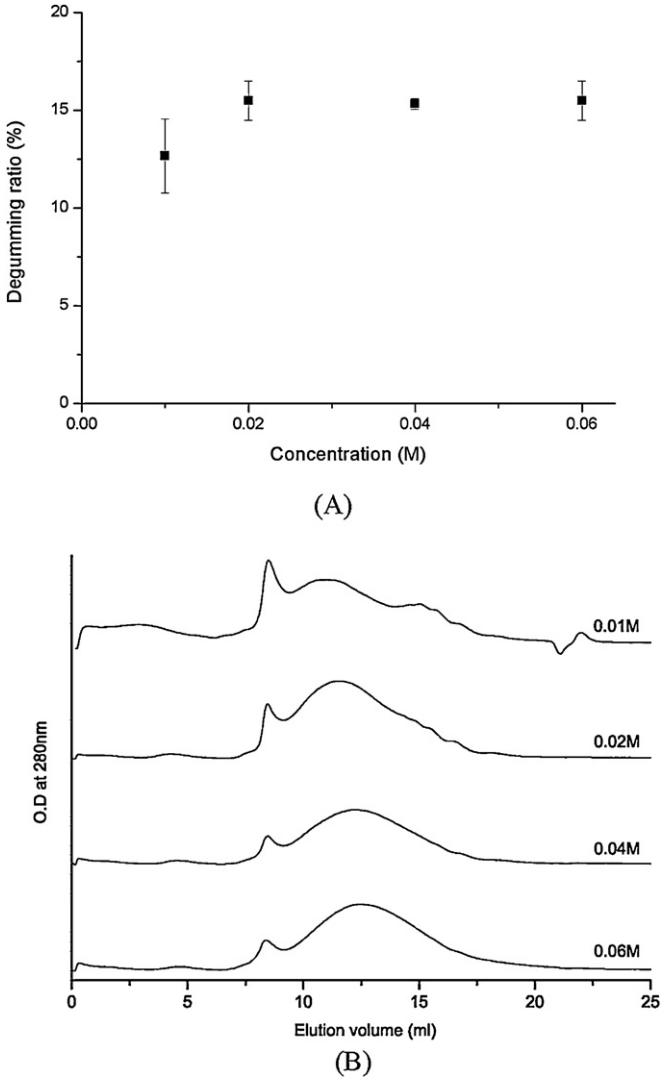

Fig. 4. Effect of the Na_2CO_3 concentration on the degumming ratio of the *Antheraea mylitta* cocoon (A) and on the molecular weight distribution of the extracted AmS (B). The extraction time was fixed at 60 min.

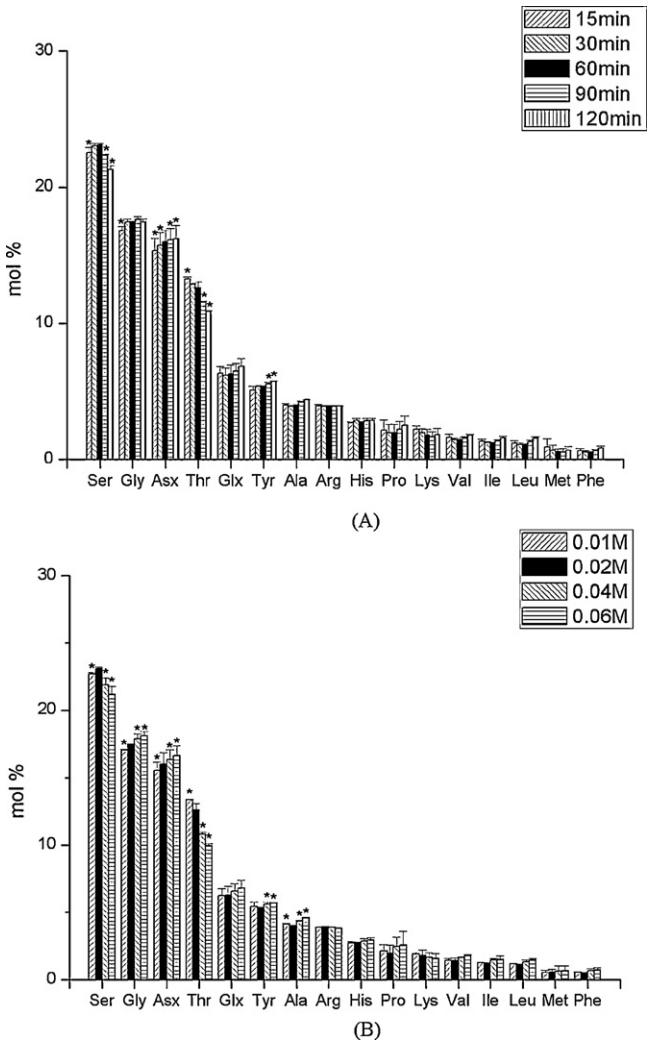

Fig. 5. Effect of extraction time (A) and Na_2CO_3 concentration (B) on the amino acid compositions of extracted AmS. *Indicates significant differences compared to the 60 min extraction time and 0.02 M Na_2CO_3 (Student's *t*-test, $n = 5$, $p < 0.05$).

and the area under the curve of the broad band increased. Since the degumming ratio did not changed significantly after 60 min of extraction, these results indicate that there is some degradation of the AmS by Na_2CO_3 when the extraction time is extended. The maximum peak of the broad band was down-shifted, also indicating the degradation of the AmS.

The effect of the sodium carbonate concentration is illustrated in Fig. 4. The degumming ratio reached a plateau ($15.5 \pm 1.0\%$) at the 0.02 M concentration (Fig. 4A); a further increase of the sodium carbonate concentration did not change the degumming ratio significantly. However, the MWD of the extracted AmS was affected by the concentration of sodium carbonate. Similar to the effect of the extraction time, the increased sodium carbonate concentration led to the degradation of the AmS (Fig. 4B).

From the materials aspect, a higher molecular weight leads to improved mechanical properties. In the case of synthetic polymers, the degree of polymerization should be sufficiently high, whereas for natural polymers, the extraction conditions should be mild enough in order not to degrade the natural molecular weight. Therefore, it might be preferable to extract a higher-MW AmS even though the extraction yield is low. However, this is not a rule of thumb in the case of proteins. Whereas most synthetic polymers are constructed as a linear chain, proteins have their own three-dimensional structures that are not conductive to the chain entanglement as are synthetic polymers. Therefore, it is not always recommendable to use a high-MW polymer for enhancing the mechanical properties. The mechanical properties of any form of the extracted AmS are beyond the scope of this study; however, there are other references that provide this speculation [18,24]. However, if the aim is to extract a relatively high-MW AmS, then it is recommended that a shorter extraction time and/or low sodium carbonate concentration be employed.

3.3.2. Effect of extraction conditions on the amino acid composition

The effect of the extraction conditions on the amino acid composition of AmS was also investigated. As the extraction time and the sodium carbonate concentration increased, the content of Gly, Asx and Tyr increased, while that of Ser and Thr decreased (Fig. 5). This change of amino acid composition might be due to a partial dissolution of fibroin during the degumming process and/or a loss of low molecular weight sericin during the dialysis process. First, a partial dissolution of fibroin can be occurred during the degumming process. If we compare the amino acid composition of *A. mylitta* fibroin and sericin, fibroin has a higher content of Gly and Tyr and a lower content of Ser and Thr than sericin

[20]. Yamada et al. [25] have reported that increasing the heating time during sodium carbonate degumming process can cause the degradation of fibroin. If the degradation of fibroin results in the dissolution of fibroin then the degumming ratio should be increased. But according to their report there was no significant effect on the degumming ratio. The same was observed in this study; the degumming ratio did not change any further after 60 min of extraction time and above 0.02 M sodium carbonate. Therefore, a partial dissolution of fibroin cannot be the reason of the amino acid composition changes. In addition, the increase of Asx content cannot be explained in this manner because Asx content in *A. mylitta* fibroin is lower than AmS. More reasonable explanation on the changes of the amino acid composition would be the loss of low molecular weight of AmS during the dialysis. In order to remove the sodium carbonate, we used a dialysis tube that has a MWCO of 6–8 kDa. If there is a molecule that has lower molecular weight than this range will be lost. In Figs. 3A and 4A, we have shown that the molecular weight of AmS decreased with the increase of extraction time and sodium carbonate concentration. The broad band about 15 ml of elution volume gradually disappeared as the extraction time and the sodium carbonate concentration increased. Therefore, AmS molecules that have relatively low molecular weights will be continuously degraded into more low molecular weights and finally removed from the dialysis tube. This indicates that even though the degumming ratio is the same, extended extraction time and increased sodium carbonate concentration can affect the final yield. Therefore, we suggest the use of sodium carbonate at a 0.02 M concentration and an extraction time of 60 min at 100 °C; these conditions would be optimal condition for the extraction of AmS extraction with high yields and minimal degradation.

4. Conclusion

A detailed study of the extraction conditions of natural polymers should be the first step of the utilization of these polymers. However, despite the increasing interest in AmS, there have been no detailed investigations of the optimal conditions for extracting AmS. In the present study, we confirm that the Na_2CO_3 extraction method is most suitable for the extraction of AmS. Based on the yield and the MWD analysis, the optima conditions for the extraction of AmS require 0.02 M Na_2CO_3 and an extraction time of 60 min.

Acknowledgements

This work was supported by National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2011-00073) and Department of Science and Technology, Indian Government (DST) (13/18 2009). The authors thank National Instrumentation Center for Environmental Management (NICEM) for instrumental analysis.

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