Effect of fibroin gel from Thai silk cocoon on color extract of *Rosa spp*. flowers

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Abstract

Fibroin, a fibrous protein found in silk cocoon was extracted from Thai silkworms (*Bombyx mori* L. var. *Nangnoi Sisaket-1*, family Bombycidae). It is essential to dissolve fibroin into solution prior to use. Comparison of the protein pattern by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) from 2 methods of fibroin solubilization between lithium bromide (LiBr) to obtain FP-1 (fibroin powder from method 1) and the tertiary system of $CaCl_2$:EtOH:H_2O (1:2:8 molar ratio) to obtain FP-1 (fibroin powder from method 2) showed that the heavy chain subunits of FP-1 was mostly degraded whereas FP-2 giving clearly bands of 37 and 52 kDa. The difference of protein patterns between FP-1 and FP-2 was also shown from SDS-PAGE of the ammonium sulfate precipitation. Under the inverted microscope, the fibroin gel which was formed by the SDS sample buffer was able to bind to the color of aqueous extract from *Rosa* spp petals. This binding capability of fibroin gel suggests a complexation between fibroin and anthocyanin. Thus, this finding lead to further investigation for a new method of anthocyanin stabilization.

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Introduction

Silkworm silk is the strongest natural and continuous protein fiber. Native Thai silkworm (*Bombyx mori* L. var. *Nangnoi Sisaket-1*, family Bombycidae) is typical in its cocoons, i.e. intense yellow color, oval in shape and about 4 cm in length. It also highly tolerates to moist heat of the topical conditions, yet giving substantial high yields of premier quality of the famous Thai silk fibers. The development of silk for biomedical applications could be potentially useful as a protective substance because it is non-degradable as categorized by the United States Pharmacopeia, yet, it is digested by proteolytic enzymes or irradiation (Kojthung *et al*, 2008). Normally, a silk fiber consists of two types of proteins, fibroin (about 75%) and sericin (about 25%). Sericin is a water-soluble glue-like protein with 3 major fractions of 150, 250, and 400 kDa although some fraction might be as low as 20 kDa (Takasu *et al*, 2002) with 40% and 16% of glycine (Yamada, 1978). As a skin mois-turizer, it has been used for cosmetic purposes with a precaution on its irritation to the mucous membrane. Sericin is known to stimulate adverse immunological response *in vivo*, hence not applicable for dental products. A fibrous protein like fibroins is potential for development into biomedical materials due to its biocompatibility and mechanical properties, such as fibroin fabricated into structural support for gum regeneration in dental treatment. Unrefined fibroin from silk glands is water insoluble protein, consisted in 2 protein subunits, i.e. heavy chain and light chain. Heavy chain subunits of fibroins are usually composed of 46% of glycine, 30% of alanine, and 12% of serine with the average molecular weight (MW) of about 350 kDa (Zhou *et al* 2000). Light chain subunits, as expected, are smaller than heavy chain ones with MW of 25 kDa and comprises relatively high amount of leucine, isoleucine, valine and acidic amino acids (Shimura, 1983). Fibroin also composes of P25, a 25-kDa poly-peptide (Tanaka *et al,* 1999). When fibroin is dissolved, the regenerated liquid silk, fractions of mixed peptides would usually obtained by peptide bond breakage at different sites or might be termed as degradation, and showed patterns of broad smear bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Conformation of fibroins could be changed from random coil to β–structure, a so-called gelation, which is detectable by Infrared (IR) spectroscopy at 1,630 cm^{-1} (Ayub *et al,* 1993). The application of silk hydrogel has been reported such as in osteoblast cells culture (Motta *et al,* 2004). Fibroin could be solubilized in the presence of certain concentrated salts, such as LiBr (Cebra, 1961) and LiSCN (Schade *et al,* 1970) and the mixture of salt:EtOH:H_2O (Ajisawa 1998). To enable the biomedical applications of fibroin, investigations on extraction and separation of fibroin and its characteristics are essential.

Anthocyanins are the water-soluble pigments. The structure consists of anthocyanidin as aglycone part which is flavylium salt structure and sugar part. Anthocyanins, show a variety of colors range from salmon to pink, through scarlet, magenta and violet to deep blue and possesses strong antioxidant (Bagchi *et al*, 2000). Based on the non-stable properties of anthocyanin, weak interactions with other natural components are usually the natural processes which enable stabilization of natural colors which exist intracellular in flowers, fruits and berries (Brouillard, 1982).

This study intends to evaluate fibroin degradation pattern of 2 solubilization methods: LiBr, and the tertiary system of $CaCl_2$:EtOH:H_2O (1:2:8 mole ratio) by SDS-PAGE for further development to be biomedical material. From the study, gelation could be formed in the present of SDS sample buffer and interestingly, the complexation between the fibroin gels and rose petal pigments was found by the initial characterization using inverted micro-scopy.

Materials and Methods

Chemicals and reagents: All solvents and chemicals used were at least analytical grade and used as received, as follows: LiBr (Riedel-de Haan, Germany), $CaCl_2$ (Merck, Germany), sodium dodecyl sulphate (Merck, Germany), bovine serum albumin (BSA, Sigma-Aldrich, U.S.A.), precision plus protein molecular weight marker (Bio-Rad, U.S.A.) and resin for separation (Sepabeads HP20, Mitsubishi Chemical Corporation, Japan).

Preparation of fibroin from silk cocoon: Fresh cocoons of silkworm were collected in Khon Kaen province, Thailand. These cocoons were cut into small pieces and degummed by boiling twice in 0.02 M Na_2CO_3 for 30 min. The resulting fiber was dissolved by 2 different methods, as follows:

Method 1- The degummed fibroin was dissolved in 9.3 M LiBr and was then desalted by dialysis against water at 4^OC for 3 days through a cellulose membrane. The resultant fibroin solution was subjected to freeze-drying by lyophilizer (Alpha2-4 LD plus, Germany) and dessication at room temperature, resulting in FP-1 (fibroin powder from method 1).

Method 2 - The degummed fibroin was dissolved in a ternary system of $CaCl_2$:EtOH:H_2O (a molar ratio of 1:2:8, respectively) at 70°C for 6 h. The fibroin solutions were subjected to dialysis against H_2O at 4^OC for 3 days through a cellulose membrane, followed by lyophilization and desiccation at room temperature, resulting in FP-2 (fibroin powder from method 2).

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed according to Laemmli (1970). In brief, FP-1 or FP-2 was dissolved in water. The SDS sample buffer was added, and heated at 95°C for 5 min. They were loaded on 4% stacking gel, and separated in 8, 10, and 15% separating gel. After electrophoresis using the SDS-PAGE apparatus (Mini-Protean Tetra Cell, Bio-Rad, U.S.A.), the gels were stained with Coomassie Brilliant Blue R-250 (USB Corporation, U.S.A). The relative protein molecular weight was calculated from the standard plot of protein molecular weight marker (Bio-Rad, U.S.A.) of the individual SDS-PAGE gel.

Ammonium sulfate precipitation of crude fibroin: FP-1 and FP-2 in water (30 mg/ml each) were mixed with $(NH_4)_2SO_4$ at concentration ranges of 0-20%, and 20%-80% saturation, then kept cool (4°C) for 60 min. Protein precipitates were collected after centrifugation at 1,350 rpm for 20 min at room temperature and weighed. The protein precipitates (wet weight) was re-dissolved with water to make the same final concentration, dialyzed to de-salt using the dialysis tubing membrane (MWCO 10 kDa, U.S.A.).

Gelation test of fibroin: FP-1 and FP-2 were dissolved in water or SDS sample buffer without bromophenol

blue. The fibroin in SDS sample buffer were boiled to 95°C for 5 min and cooled to room temperature. The gelation and the viscosity of the gel were recorded.

Extraction of pigments: Petals from fresh flowers of *Rosa* spp. were hot-air dried at 50°C and extracted with deionized water in weight ratio 1:27 at 90°C and cooled to room temperature. The crude extract of the rose pigments was filtered, acidified (pH 1) by 0.2M hydrochloric acid and loaded into the column (Sepabeads HP20, Mitsubishi Chemical Corporation, Japan) which was equilibrated with deionized water. The column was washed with deionized water until the pH became neutral. It was then eluted by methanol. The fractions of pigments were collected, concentrated, and subjected to freeze-drying (Flexi-Dry MP, U.S.A.). The obtained rose pigment powder was kept in -20°C until used.

Total Anthocyanin content: Total anthocyanin content was measured by using the method from AOAC (Lee *et al*, 2005). In brief, the pigment solution was diluted by 25 mM potassium chloride buffer pH 1.0 and 400 mM acetate buffer pH 4.5. Both solutions were measured for UV absorbance at 520 and 700 nm. The total anthocyanin content can be calculated by the following equations;

$$\text{total anthocyanin content}\left(\frac{mg}{L}\right) = \frac{A_{corrected} \times MW \times DF \times 1000}{\varepsilon \times L}$$

where

$$A_{corrected} = (A_{520} - A_{700})_{pH1.0}$$
$$- (A_{520}$$
$$- A_{700})_{pH4.5}$$

A_{520} and A_{700} representing UV absorbance at 520 and 700 nm, respectively, MW molecular weight

of anthocyanin (449.2 g/mol as cyaniding-3-glucoside), DF dilution factors, L path length (1 cm), ε molar extinction coefficient (26,900 L/mol/ cm).

Complex formation: An equal volume of pigment solution in water (0.3% w/v) was mixed with fibroin gel solution (0.5% w/v) which was prepared by dissolving FP-2 in SDS sample buffer without bromophenol blue. The mixture was thoroughly mixed by hand-shaking for 5 min. The complex formation was observed under the inverted microscope (Axiovert25, Carl Zeiss Microscopy, USA).

Results

Comparision of protein patterns using SDS-PAGE : Broad smear of proteins of FP-1 and FP-2 were obtained from 8, 10, and 15% separating gels in the range of 25-200 kDa as shown in Fig 1. FP-1 exhibited clear protein bands of 24 and 26 kDa, whereas those of 25, 37, and 52 kDa were observed from FP-2. This indicates the different pattern of fibroin fragmentation by 2 dissolution methods.
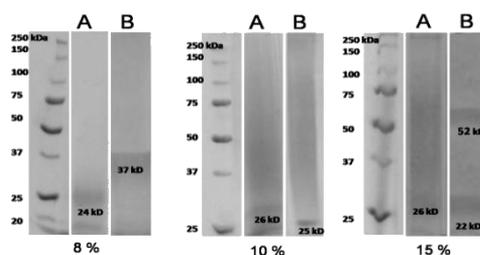

Figure 1 SDS-PAGE of FP-1 and FP-2 on 8, 10, and 15% separating gels

Table 1 Gelation of fibroin FP-1 and FP-2 in water and in SDS sample buffer*

Time (h)	In water		In SDS sample buffer	
	FP-1	FP-2	FP-1	FP-2
0	-	-	-	-
0.5	-	-	-	-
1	-	-	-	-
2	-	-	*g*	*gg*
3	-	-	*gg*	*ggg*
4	*pp*	*p*	*ggg*	*gggg*
24	*ppp*	*pp*	*ggggg*	*ggggg*

*Clear solution; *p* = precipitates from high to low *ppp* > *pp* > *p*; *g* = gelation from high to low viscosity *ggggg* > *gggg* > *ggg* > *gg* > *g*

SDS-PAGE of fibroin precipitates with ammonium sulfate: Most of the FP-1 and FP-2 fibroins in aqueous solutions could not be precipitated in the presence of $(NH_4)_2SO_4$, however most precipitation was obtained in 0-20% $(NH_4)_2SO_4$ saturation whereas much less protein was precipitated in 20-80% $(NH_4)_2SO_4$ saturation. After the precipitates were re-dissolved in SDS sample buffer, they became gel at room temperature. The SDS-PAGE of the remained protein solution from both protein precipitates of FP-1 and FP-2 showed the different patterns. FP-1 gave clear bands of 42 and 46 kDa from both 0-20% and 20-80% $(NH_4)_2SO_4$ saturation while FP-2 gave an intense protein band of 23 kDa from those 2 concentration ranges (Fig 2). The result confirmed the difference between 2 methods of fibroin solubilization.

Gelation: FP-1 and FP-2 in aqueous solutions formed the fine precipitates as shown in Table 1 whereas those in the presence of SDS sample buffer and heated could form gel after placed in room temperature up to 2 h. This result indicated conditions required for gelation included the presence of SDS sample buffer and heat.
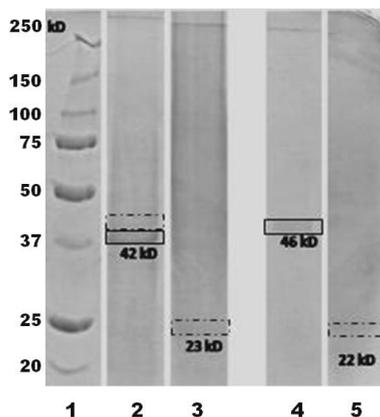

Figure 2 SDS-PAGE of the redissolved precipitates from ammonium sulfate precipitation which was not formed gel: lane 1 - protein MW marker; lane 2 - FP-1, 0-20% $(NH_4)_2SO_4$; lane 3 - FP-2, 0-20% $(NH_4)_2SO_4$; lane 4 - FP-1, 20-80% $(NH_4)_2SO_4$; lane 5 - FP-2, 20-80% $(NH_4)_2SO_4$)
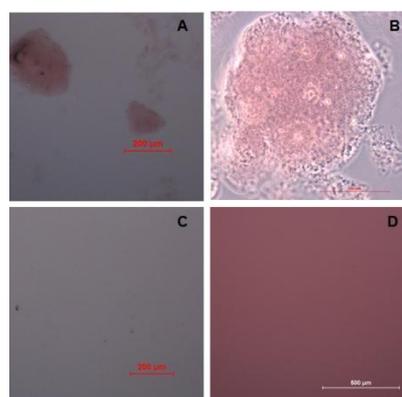

Figure 3 Microscopic photographs of rose pigment: (A) with fibroin (× 400); (B) with fibroin magnified; (C) fibroin alone; (D) rose pigment alone

Complexation between fibroin gel and *Rosa* spp pigment: The pigment from rose petals aqueous extract was in deep red color with the total anthocyanins content of 12.9±1.3% of dried petals. FP-2 gel was able to bind with the rose pigment, resulting in the formation of small gel clusters dispersed in the liquid (Fig 3A and 3B). The color of these clusters was more intense in the presence of fibroin than in the rose pigment solution. It suggests that the fibroin may have dissolved the pigment and separated into small clusters upon hand-shaking. The interaction between fibroin and the rose pigment intensified the color of the pigment and also formed colored gel clusters.

Discussion

The solubilized fibroin solution showed broad smear bands resulting from degradation or the breakage of peptide bonds of the heavy chain of fibroin at various positions leading to peptide fragmentation with broad range of molecular weight. From the present results, the solubilization of fibroin gave smear band ranging from 25-250 kDa and some other protein bands. FP-1 prepared from method 1 gave protein bands of 24 and 26 kDa, whereas FP-2 from method 2 gave the intense protein bands of 25, 37 and 52 kDa. The sharp band at 24-26 kDa indicated fibroin light chain (Shimura *et al,* 1982). Since the degradation of silk proteins was reported that it could occur from the degumming process to the dissolution of fibroins (Yamada *et al*, 2001). Therefore, if the high molecular weight fibroin is needed for biomedical material, it should be aware from the early steps to minimize degradation. For fibroin solubilization by triple solvents of $CaCl_2/EtOH/H_2O$, the use of high temperature for the longer time led to get the mixture between small molecular weight range of peptides and the reduction of high molecular weight peptides. Shang *et al* (2007) reported that boiling of fibroin in $CaCl_2/EtOH/H_2O$ at 70^OC for 6 h yielded 25-200 kDa peptides, while using temperature at 90^OC caused the occurrence of smaller peptides in the range of 8-120 kDa.

Ammonium precipitation at 0-20% saturation of FP-1 and FP-2 yielded small amount of protein precipitate. However, the obtained precipitate was in higher amount than from 20-80% saturation. The SDS-PAGE showed the same protein pattern between 0-20% and 20-80% saturation indicated no protein fractionation based on molecular weight occurrence from protein salting out method. However, there was the difference in the protein patterns between 2 methods of fibroin solubilization as shown in Fig 2. The gelation of the redissoved protein precipitates in SDS buffer indicated that those proteins were fibroin.

The gelation of fibroin was also obtained when the fibroin powders (FP-1 and FP-2) were dissolved in SDS buffer prior to load into SDS gel. Since the gel form is interesting for biomaterial, the gelation of FP-1 and FP-2 were investigated by comparing between using water and SDS buffer as solvent. The result showed that the aqueous solution of FP-1 and FP-2 gave precipitates while adding of SDS buffer and heat at 95^OC for 5 min caused gelation within 2 h at room temperature. It was described that the small precipitates were the early stage of gelation which could become larger aggregates and finally overall gelation (Matsumoto *et al,* 2006). The gelation by SDS buffer and heat

clearly indicate the affecting to fibroin gelation. Ha (2005) reported the gelling time which was less than 2 h in both LiBr and triple solvent as same as the present result. The aforementioned work used X-ray diffraction to describe that the gelation resulted from β-sheet formation. Actually, some β-sheet structures were already formed since the step of dialysis against water after fibroin solubilization, and sodium docecyl sulfate (SDS) acts as a good nucleus for β-sheet formation (Ha, 2005). From Table 1, FP-1 gave less gel amount than FP-2 indicated that fibroin which dissolved in 9M LiBr was more stable against gelation than using triple solvent of $CaCl_2$-H_2O-EtOH. This could be because of trace amount of calcium ion may act as a nucleus for β-sheet formation (Ha, 2005). Although FP-2 was dialyzed against water for 4 days, the calcium ion is still remained. Gelation time decreases with an increasing of fibroin concentration and temperature (Matsumoto *et al*, 2006). Since hydrophobic interaction is the major driving force for fibroin gelation (Ayub *et al,* 1993), therefore SDS, a surface active agent, acts by reducing the interfacial tension between the hydrophobic protein and water. Thus, water molecules could associate with the fibrous proteins at a higher extent and the hydrated fibrous proteins could swell in such condition, and so gel was observed. This process took time and energy from heat which confirms that the protein subunits were fibrous, hence, relaxation of each strain of the proteins by water association requires time and energy.

In nature, the same anthocyanin pigment makes rose red but others blue as a result of pH and complex formation. Blue cornflower pigment was reported to be composed of metal ions in complex with antho-cyanins and flavones, so-called a supermolecular pigment which stabilized anthocyanin (Shiono *et al*, 2005). The use of fibroin protein in the present study has shown to form complex with rose pigment and might be possible to stabilize the anthocyanins.

Conclusion

Solubilization of fibroin fiber by using LiBr (method 1), and triple solvents of $CaCl_2$, ethanol and H_2O (method 2) provided different degrees of peptide bond breakage as shown by broad smear bands of the SDS-polyacrylamide gels with different pattern of intense bands. Both methods caused both heavy chain and light chain fibroin degradation. Most of heavy chains in method 1 were degraded whereas method 2 yielded intense protein bands of 37 and 52 kDa. Ammonium sulfate precipitation could not fractionate fibroin into different molecular weight fractions but there were different protein patterns in SDS –PAGE of these 2 methods of solubilization. Since both methods degraded fibroin into fractions, if the higher molecular weight fibroins were required in undegraded form, it might be further optimized the conditions to dissolve fibroin. Both fibroin solution from method 1 and 2, and the fibroin precipitated from ammonium sulfate could form gel after dissolving with SDS sample buffer which contained SDS at temperature of 95°C. This fibroin gel gave complex with rose pigment, provided more intense color and might be possible to stabilize the anthocyanins.

Acknowledgement

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