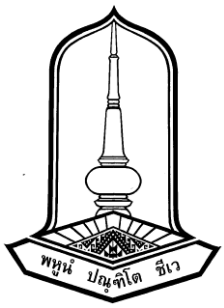

EXTRACTION AND ANTIOXIDANT ACTIVITY OF FAGARA (*Attacus atlas* Linn.) SERICIN PROTEIN

CHONTICHA LOONSUMRONG

A thesis submitted in partial fulfillment of the requirements for
the Master of Science degree in Chemistry
August 2017
at Mahasarakham University

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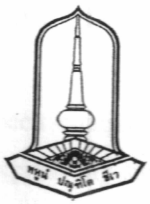

The examining committee has unanimously approved this thesis, submitted by Miss Chonticha Loonsumrong, as a partial fulfillment of the requirements for the Master of Science degree in Chemistry at Mahasarakham University.

Examining Committee

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Chonticha Loonsumrong

TITLE Extraction and antioxidant activity of *Fagara (Attacus atlas* Linn.) sericin protein

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ABSTRACT

The present study investigated relationship between extraction method of sericin protein from *Attacus atlas* (*A.atlas*) cocoons and antioxidant property. The antioxidant activity of sericin protein measured by DPPH and ABTS assay showed the highest antioxidant activity when used water for extraction with water at 98 ◦C for 120 min. For the UV absorption, the sericin extract showed higher UV absorption than sunscreen. The UV absorbtion of sunscreen and sericin mixture was increased in proportion of the increasing of sericin protein. In addition, the relatedness between UV absorption activity and the color of sericin extracts from wild silk of Fagara (*Attacus atlas* Linn.) and Eri (*Philosamia ricini*) silk cocoons together with *Bombyx mori*, bivoltine (W7) and polyvoltine (Nang noi) were investigated. The sericin extracts from Fagara and Eri silk showed higher UV absorption activity (λ_{max} 200 nm) than domestic silks, both from bivoltine and polyvoltine races. The protein precipitated of all cocoon types showed higher UV absorption activity (λ_{max} 200 nm) than proteins remaining in supernatant. The results also indicate that there is unlikely the relatedness between the intensity of yellow or brown color in various races of polyvoltine and Fagara silk cocoon with its UV absorption property.

Keywords : Fagara; Antioxdant ; Sericin ; UV absorption

ชื่อเรื่อง การสกัดและฤทธิ์การต้านอนุมูลอิสระของโปรตีนเซริซินจากไหมกระท้อน (*Attacus atlas* Linn.)
ผู้วิจัย นางสาวชลธิชา ลุนสำโรง
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บทคัดย่อ

งานวิจัยนี้ศึกษาความสัมพันธ์ระหว่างวิธีสกัดโปรตีนเซริซินจากรังไหม ***Attacus atlas*** **(*A.atlas*)** กับฤทธิ์การต้านอนุมูลอิสระของโปรตีนเซริซิน ซึ่งวิเคราะห์โดยวิธี DPPH และ ABTS พบว่าฤทธิ์การต้านอนุมูลอิสระสูงสุด ได้จากการสกัดเซริซินในน้ำที่อุณหภูมิ 98 องศาเซลเซียสเป็นเวลา 120 นาที เมื่อศึกษาความสัมพันธ์ระหว่างการดูดกลืนรังสีอัลตราไวโอเลตของโปรตีนเซริซินเปรียบเทียบกับสารกันแดด พบว่าโปรตีนเซริซินมีค่าการดูดกลืนรังสีอัลตราไวโอเลตสูงกว่าสารกันแดด โดยค่าการดูดกลืนแสงจะเพิ่มขึ้นตามสัดส่วนของสารละลายโปรตีนเซริซินที่ผสมอยู่ นอกจากนี้ความสามารถในการดูดกลืนรังสีอัลตราไวโอเลตกับสีของสารสกัดโปรตีนเซริซินจากไหมกระท้อน ไหมป่า ไหมพันธุ์อีรี่ **(*Philosamia ricini*)** และไหมบ้าน **(*Bombyx mori*)** ชนิดไบโวลไทน์ (W7) และโพลีไวลไทน์ (นางน้อย) พบว่าโปรตีนเซริซินที่สกัดจากไหมกระท้อนและไหมอีรี่มีความสามารถดูดกลืนแสงที่ช่วงความยาวคลื่น 200 นาโนเมตร สูงกว่าไหมบ้าน ทั้งชนิดไบโวลไทน์ และโพลีโวลไทน์ เมื่อแยกส่วนสารสกัดเซริซินโดยวิธีการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตพบว่า ส่วนของตะกอนโปรตีนเซริซินจากรังไหมทุกชนิดแสดงความสามารถดูดกลืนแสงที่ช่วงความยาวคลื่น 200 นาโนเมตร ได้ดีกว่าโปรตีนที่อยู่ในส่วนสารละลายใสของสารสกัดโปรตีนเซริซิน นอกจากนี้ผลการทดลองแสดงให้เห็นว่าค่าการดูดกลืนแสงอัลตราไวโอเลตไม่มีความสัมพันธ์กับความเข้มของสีเหลืองหรือสีน้ำตาลที่มีอยู่ในรังไหมโพลีโวลไทน์ และไหมกระท้อน

คำสำคัญ: ไหมกระท้อน; ฤทธิ์ต้านอนุมูลอิสระ; เซริซิน; การดูดกลืนรังสีอัลตราไวโอเลต

CONTENS

CONTENS (cont.)

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CHAPTER 1

INTRODUCTION

1.1 Background

Sericulture has a long history of more than 5,000 years in China. The well know silkworm, *Bombyx mori* (*B. mori*)., is an economically important animal (Yue, *et al*., 2007) which silk products are utilized in the various field such as textiles, food and cosmetic ingredients along with biomedical materials (Asakura, *et al*., 2007).

Most of silk product materials are from mulberry silk (*B. mori*). This silkworm has been reared and improved as became to domestic silkworm and could not be survived in nature. However, the others were found in nature called wild silk or non-mulberry silk. The mulberry silkworm belongs to Family Bombycidae while the non-mulberry silkworm belongs to Family Satuniidae (Mishra *et al*., 2003). The total production of *B. mori* and wild silk fresh cocoon is about 1 million tons worldwide and this is equivalent to 400,000 tons of dry cocoon (Zhang, 2002; Commodity Research Bureau, 2007). China is the world largest producer and chief supplier of silk to the world markets. India is the second of world production. Ten percent of world silk is produced by Brazil, North Korea, Thailand, Uzbekistan in CIS and Vietnam. In case of wild silk cocoon, almost all races including tasar (Chinese tasar silkworm; *Antheraea pernyi*, Indian tasar silkworm; *Antheraea mylitta*), Eri (*Cynthia samia ricini*) and Muga (*Antheraea assama*) cocoon productions are from China and India (Datta and Nanavaty, 2005). However, non-mulberry silk products (about 18%) is lesser than that of mulberry silk product in the world trade market since the cocoon production does not reach the industrial scale which leading to the limitation of supply.

Attacus atlas Linn. (*A. atlas* or Fagara silk) is one type of non-mulberry silk or wild silk found in the Southeast Asia as well as northeastern Thailand. The *A. atlas* moth has the largest wing of all moths. This is referred to as the giant silkworm moth. The cocoon of this worm is a large brown, oval-round shape and covered with a food leaves (Holloway, 1987). The unique characteristic of *A. atlas* differs from small white

or yellow *B. mori* cocoons. Furthermore, *A. atlas* has some possibility to rear in a larger scale.

While the extraction or recovery process of sericin from the *B.mori* cocoon is well established, the extraction of sericin from the Fagara silk cocoon has not been fully demonstrated.

Kundu, *et al.* (2009) have extensively studied the novel application of the wild silkworm's cocoon or *A. mylitta* sericin (*Am*S). In these studies, two types of solutions for the extraction of *AmS* have been used, namely, sodium chloride solution and 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 temperature (Dash, 2007). Currently, a sodium carbonate solution is widely used for the extraction of *Am*S. 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 technique for extracting the *B.mori* sericin has been extensively known, the extraction of sericin from Fagara silk cocoons has not yet standardized. The aim of this study was to find the optimal conditions for the extraction methods of sericin from Fagara silk cocoon and antioxidant property of its sericin.

The toxic effects of UV from natural sunlight and therapeutic artificial lamps are a major concern for human health. The major acute effects UV radiation on normal human skin comprises sunburn, inflammation, erythema, tanning and local or systemic immunosuppression (Hanson *et al.,* 2006; Sulayman *et al.,* 2008). UV-A, B and C can all damage collagen fibers and therefore accelerate aging of the skin.

UV-A was once thought to be harmless because of its lower energy content, but long-term exposure to UV-A is now known to cause aging of the skin and to act as a promoting agent for skin cancer by stimulating cell proliferation (Elizbeth *et al.,* 2002; Lewis, 2006).

The study by Kaur *et al*. (2013) found that sericin from *B. mori* was primarily responsible for UV-A absorption. When sericin was removed, the photo-induced chemiluminescence intensity increased significantly, indicating higher UV-A induced reactions of cocoons in the absence of sericin.

UV-B is largely responsible for the carcinogenic properties of sunlight. More than 90% of the UV-B radiation emitted by the sun is absorbed by ozone molecules present in the upper atmosphere. UV-B causes sunburn, tanning, aging of the skin, skin cancer (Lewis, 2006) and dependent of lysis of erythrocytes. The immunohistochemical analysis by Zhaorigetu *et al.* (2003) showed that the application of sericin significantly suppressed UV-B induced elevations in 4-hydroxynonenal (4-HNE), expression of cyclooxygenase-2 (COX-2) protein, and proliferating cell nuclear antigen (PCNA)-labeling index in the UV-B exposed epidermis. In experiment 2, HR-1 hairless mice were treated with 200 nmol of 7, 12-dimethylbenz [alpha] anthracence (DMBA) followed 1 week later by irradiation with 180 mJ/cm^2 of UV-B twice weekly for 22 weeks. The protective effect of sericin was evident in terms of significant reduction in tumor incidence and tumor multiplicity at the dose of 5 mg. The results suggest that sericin possesses photoprotective effect against UV-B induced acute damage and tumor promotion by reducing oxidative stress, COX-2 and cell proliferation in mouse skin.

In the past, UV-A is the predominant type of ultraviolet radiation to reach the earth and it can penetrate into the deeper layers of the skin. UV-C radiate the higher energy than UV-B and UV-A, and it is the most damaging type of UV radiation and also causes acute skin burn, DNA damage and skin cancer (Bing *et al.,* 1996; Yun *et al.,* 2011). Normally, UV-C is filtered by ozone layer, however, the ozone hole in the Antarctic or even the artificial UV sources, these would enhance the chance of human health damage from UV-C (Brien, 2001; Lewis, 2006)

Sunscreens are chemicals used to protect the skin from UV radiation, which otherwise may lead to carcinogenesis, actinic keratosis, skin aging and immunosuppression (Hanson *et al.,* 2006; Sulayman *et al.,* 2008). Sunscreen formulations employ a variety of active ingredients (AIs) which are typically strong absorbers, reflectors or scatters of UV radiation to protect against such UV-induced damage (Sulayman *et al.,* 2008; David, 2009). These AIs are usually presented in sunscreen products in varying amounts from 0.1% to 10% (w/w), and they are incorporated into a variety of other cosmetic products (Sulayman *et al.,* 2008). Common physical blockers include zinc oxide (ZnO), titanium dioxide (TiO_2) and more amorphous cerium-titanium phosphates (Ce1-*x*;Ti*x*P_2O_7) (Sulayman *et al,*. 2008; World Health Organization, 2011). Common chemical absorbers include derivatives of

cinnamate, crylene, salicylate, benzophenone, camphor and anthranilate (Sulayman *et al*,. 2008; Lowe *et al.*, 1990).

Besides, the biological substance absorbing UV light occurs when the energy associated with the electromagnetic radiation matches the energy difference between molecular states in a molecule. Chromophores, which are part of these molecules, are very sensitive to their immediate environment including amino acid, fatty acid, carbohydrates (open chain) nucleotides, protein and nucleic acids (David, 2011; Boyer and Rodney, 1990). Amino acids have strong absorbance at around 210 nm and this is the frequently used to detect peptides.

Sericin, a glue protein from silk cocoons, possesses photoprotective effect on UVB-induced sunburn lesions and UVB-induced tumor promotion in mouse skin (Zhaorigetu *et al.*, 2003). The majority of insects that spin silk fiber belong to two families, Bombycidae and Saturniidae. They are divided into two groups: mulberry and non-mulberry silkworms. The non-mulberry silk fibers are higher tensile strength and larger cocoon sizes than that mulberry silk. The cocoons of non-mulberry silkmoths are harder than the cocoons of mulberry silkworms, *B.mori* (Subhas *et al.*, 2008) to protect itself and to endure stress by environmental conditions such as from global warming (Moths of borneo, 2011; Takasu *et al*., 2002).

This study aimed to investigate the UV absorption activity of sericin extracted from wild silk Fagara (*Attacus atlas* Linn.) producing brown cocoon, Eri (*Philosamia ricini*) producing white cocoon and domestic silkworms, bivoltine (W7) strain producing white cocoon and polyvoltine (Nang noi) strain producing yellow cocoon. The UV-VIS at the wavelength from 200-800 nm was used to detect the absorption of sericin extracted from silk cocoons with different color.

1.2 Purpose of the Research

1**.** To investigate the optimal conditions for extraction of sericin protein from *Attacus atlas* Linn (*A. atlas* or Fagara silk) cocoon and its antioxidant property.

2. To investigate the UV absorption activity of sericin extracted from *Attacus atlas* Linn. (*A. atlas* or Fagara silk).

1.3 Significance of the Research

1. Know the optimal conditions for extraction of sericin from *Attacus atlas* Linn (*A. atlas* or Fagara silk).

2. Obtain to investigate the antioxidant activity of the extracted sericin.

3. The UV absorption activity of sericin extracted from *Attacus atlas* Linn (*A. atlas* or Fagara silk).

1.4 The Scope of Research

Sericin extract would be prepared from *A.atlas* silk cocoons. The determination of protein concentration would be performed by Bradford assay and measurement of antioxidant activity by DPPH assay. The sericin molecular weight was determined by SDS-PAGE.

The UV absorption activity of sericin extracted from *A.atlas*. The UV-VIS at the wavelength from 200-800 nm was used to detect the absorption of sericin extracted from these silk cocoons.

1.5 Research Place

Silk Research Unit, SC2 – 402/2 – 3, Department of Chemistry, Faculty of Science, Mahasarakham University

1.6 Definition of Terms

Antioxidant	Molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged**.**
***Attacus atlas* Linn.**	one type of non-mulberry silk or wild silk
Sericin	a protein created by silkworm covering a fibroin
Dialysis	the process of removing small molecules in solution by diffusion through a semi-permeable membrane
Free radical	An atom or group of atoms that has at least one unpaired electron and is therefore unstable and highly reactive. In animal tissues, free radicals that damage cells believed to accelerate the progression of cancer, cardiovascular disease, and age-related diseases.
Bivoltine	a silkworm which yields referring to organisms having two broods or generations per year.
Polyvoltine	a silkworm which yields more than one crop of cocoons a year: usually applied only to those races which have more than four yearly generations.
Protein marker	a mixture of purified proteins with known molecular weight for using as a standard reference to estimate molecular weight of unknown protein
Running buffer	a buffer that is made for SDS-PAGE gels to separate protein
Silver staining	a staining procedure for detecting proteins separated by gel electrophoresis silver nitrate
UV-A	the sun's ultraviolet A (long-wave) ranges from 320-400 nanometers (nm, or billionths of a meter)
UV-B	the sun's ultraviolet B (short-wave) ranges from 280-320 nanometers (nm, or billionths of a meter)

UV-C	ultraviolet C radiate the higher energy than UV-B and UV-A
Sunscreens	chemicals used to protect the skin from UV radiation
Ultraviolet	Ultraviolet are electromagnetic radiation with wavelength longer than that of x-rays but shorter than that of visible light. The visible spectrum in the wavelength range from 10 to 400 nanometers and can break some chemical bonds and cause cell damage.

CHAPTER 2

LITERATURE REVIEW

2.1 Cocoon Characteristics

To form the cocoon, the silkworm draws out the thread of liquid protein and constructed to layer until complete this protective covering (Lee, 1999). Normally, elliptic shape is the most general. The cocoons of the *Attacus atlas* (*A. atlas*) are brown. The pupa stage of the *A. atlas* moth lasts about 4 weeks. Adult of *A. atlas* moth does not eat at all throughout their adult life which lasts for about two weeks. The adults quickly mate, lay eggs, and die shortly thereafter (Sterry, 1995). For wild silkworm cocoon shape possesses peaks on both side and larger than *B. mori* cocoon.

2.2 Sericin Characteristics

Sericin is a hot water soluble glycoprotein and has been partially characterized in the domesticated mulberry silkworm, *Bombyx mori* (*B. mori)* (Garel, *et al*., 1997). The sericin from *B. mori* consists of a group of proteins ranging from 24 to 400 kDa which have an unusually polypeptides of sericin from different middle-gland sections was demonstrated by gel electrophoresis at acidic pH, and had molecular weights of 80-310 kDa. The polypeptides were rich in serine, glycin, and aspartic acid. Three polypeptides of molecular masses 400, 250, and 150 kDa have been detected from the cocoon by SDS-PAGE, and these protein correspond to the sericin present in the middle, anterior, and posterior parts of the middle silk gland (Takasu *et al.,* 2002; Tsubouchi *et al*., 2001). Two genes, *Ser-1* and *Ser-2*, have been identified encoding sericin. *Ser-1* transcribes different mRNAs (10.5, 9.0, 4.0 and 2.8 kb) *Ser-2* priduces 6.4 or 5 and 3.1 kb mRNAs. The secondary structure of native sericin from the middle silk gland (MSG) or cocoon is a largely random coil with some β -sheet structure (Takasu *et al*., 2002; Garel *et al*., 1997).

The sericin protein is made of 18 amino acids most of which have strongly polar side groups. The amount of sericin ranges from 19 to 28% according to the type of

cocoons, usually the sericin content of the cocoon shell is at the maximum level in the middle layers 2 and 3 and the minimum at the inside layer 4. Sericin is insoluble in cold water. However, it is easily hydrolyzed, where by the long protein molecules break down to smaller fractions, which are easily dispersed, or solubilised in hot water (Nakamura *et al*., 2001). Sericin protein is useful because of its special properties viz., resists oxidation, antibacterial, UV resistant and absorbs and releases moisture easily, inhibitory activity of tyrosine and kinase etc. (Kato *et al.*, 1998; Zhu *et al*., 1998).

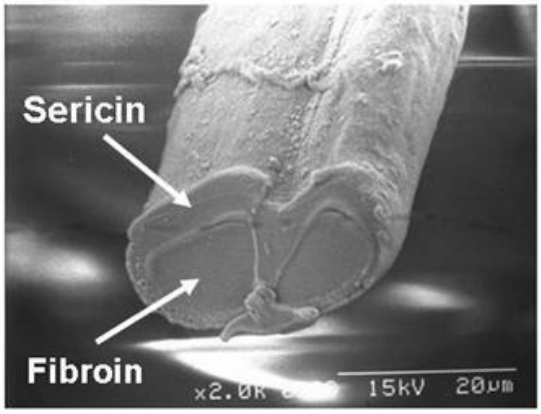

Figure 2.1 Composition of silk fiber
(http://www.silken-soaps.com/silk-in-skin-care-products.htm)

2.2.1 Scientific Classification of Silkworm

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Bombycidae, Saturniidae
Genus: Bombyx, Philosamia, Attacus
Species: mori, ricini, atlas
Binomial name: *Bombyx mori* Linnaeus. (*B. mori*)
Philosamia ricini
Attacus atlas Linnaeus. (*A. atlas*) (Moths of borneo, 2011; Takasu *et al.*, 2002)

2.2.2 Life Cycle of Silkworm

B. mori are characteristic representative of mulberry silkworms, *B. mori* silkworm has very short life cycle of about 6-8 weeks depending on the prevailing climatic conditions. During such developmental period they pass through four stages of complete metamorphosis: egg, larva (worm), pupa, and adult (moths). The larval stage consists of five instars and the mature fifth instars larva spins to cocoon to protect itself during metamorphosis from larva to and pupa to moth. The silkworm life cycle is as follow (Figure 2.2).

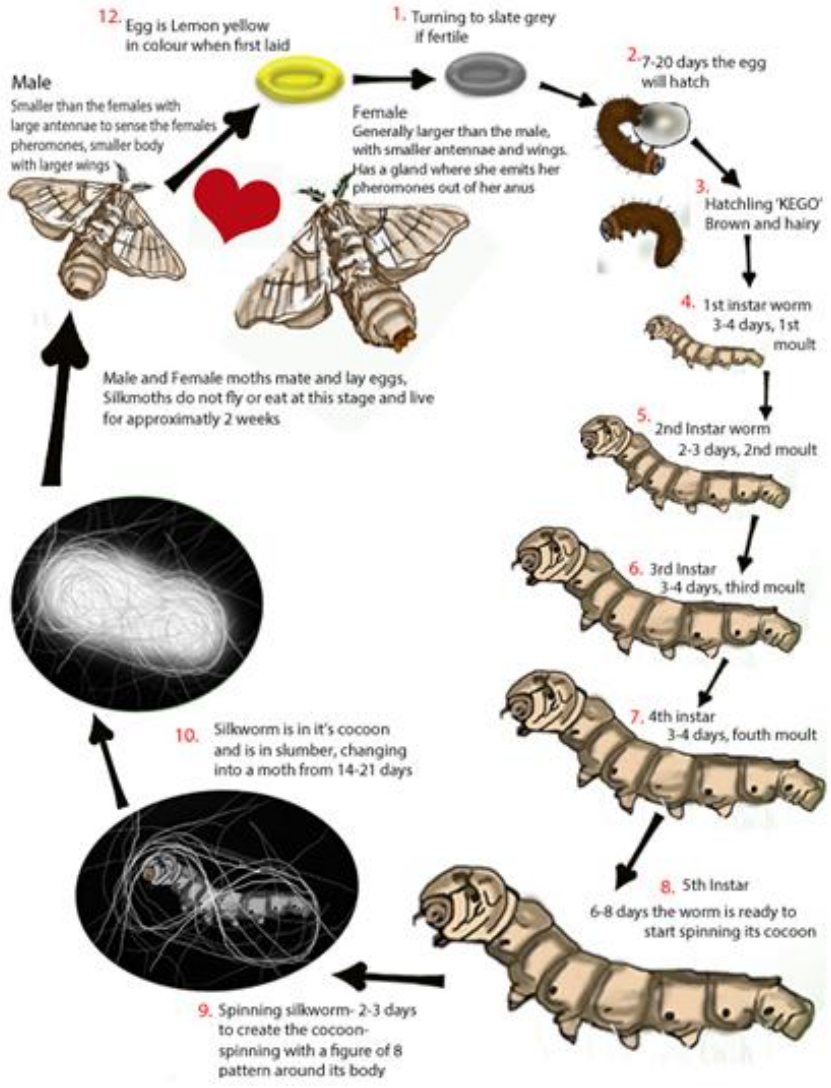

Figure 2.2 Life cycle of mulberry silkworm

(https://joannarosetidey.files.wordpress.com/2012/12/life-cycle-of-bombyx-mori.jpg)

A typical life cycle of mulberry Nang-noi and *B.mori* in this diagram as an example. Non-mulberry silkworm *A. atlas* Linn. have extensively been studied. The life cycles of mulberry silkworm and non-mulberry silkworms are similar, except the durations of their life span. (Moths of borneo, 2011; Takasu *et al.*, 2002)

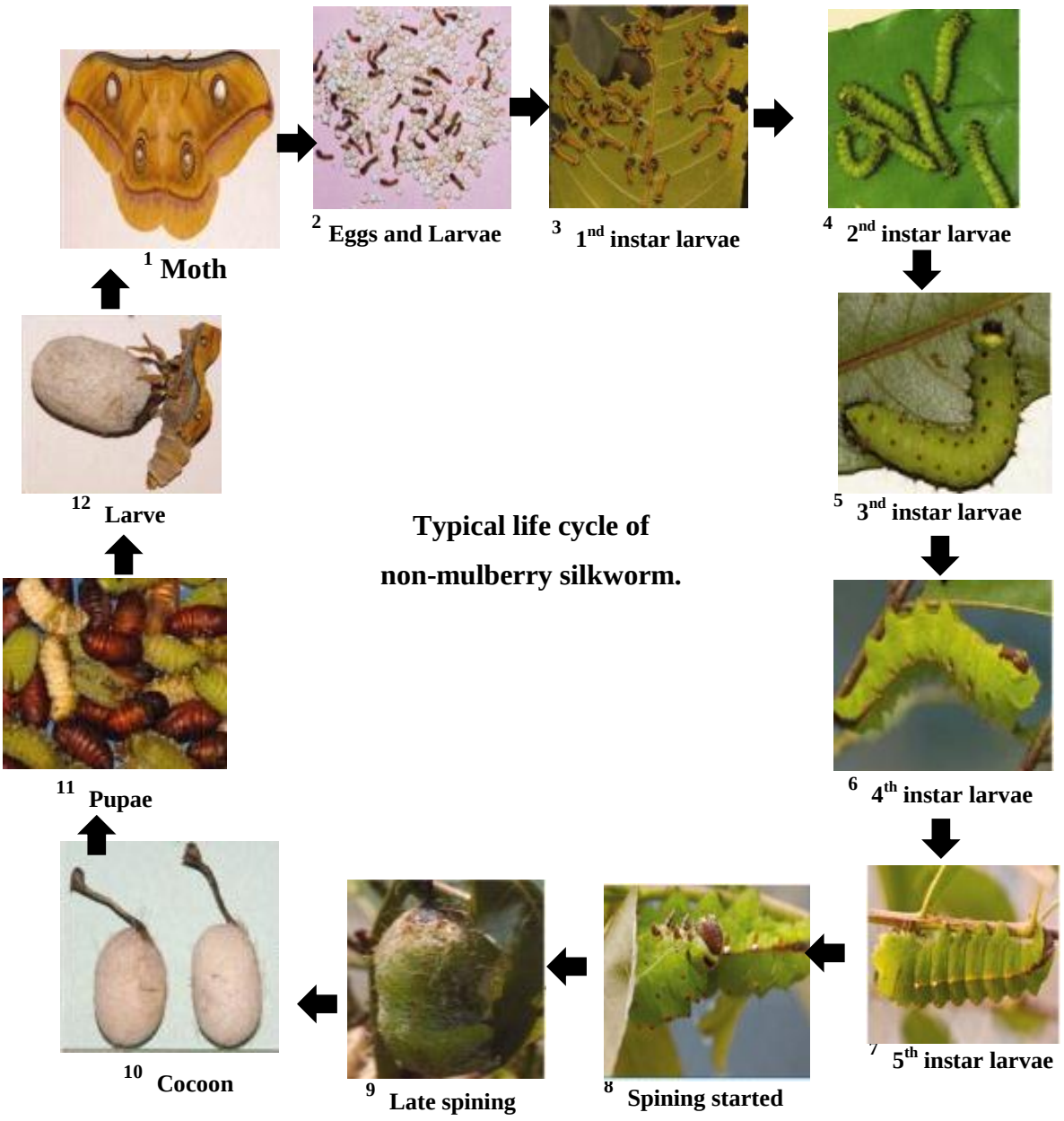

Figure 2.3 Life cycle of non-mulberry silkworm

(https://www.researchgate.net/figure/221743904_fig4_Figure-2-Typical-life-cycledifferent-stages-of-nonmulberry-Indian-tropical-tasar)

2.3 *Attacus atlas*

The *A. atlas* moth is a large Saturniid moth found in the tropical and subtropical forests of Southeast Asia, Southern China, common across the Malay archipelago, Thailand to Indonesia (Holloway, 1987).

A. atlas moths are considered the largest moths in the world (Watson *et al.*, 1983) in terms of total wing surface area upwards of c. 400 cm^2 (62 sq in). Their wingspans are also amongst the largest, reaching over 25 cm (10 in). Females are appreciably larger and heavier.

In India, *A. atlas* moths are cultivated for their silk in a non-commercial capacity; unlike that produced by the related silkworm moth (*Bombyx mori*). *A. atlas* moth silk is secreted as broken strands. This brown, wool-like silk is thought to have greater durability and is known as “Fagara silk” (Jolly, 1979). *A. atlas* moth cocoons have been employed as purses in Taiwan.

A. atlas moths are predominantly tawny to maroon in color with roughly triangular, diaphanous "eyes" on both forewing and hind wing, bordered in black. The purpose of these dramatic, gossamer portals is not clear, but they are thought to play a role in predator avoidance. Their bodies are hairy and disproportionately small compared to their wings. Patterns and colouration vary among the many described subspecies. Male *A. atlas* moths are distinguished from females by their smaller size, more tapered wings, and larger, bushier antennae.

As with other members of the family Saturniidae, neither gender possess fully formed mouthparts and therefore do not feed; throughout their 1–2 week adult life they survive entirely on larval fat reserves that they build up while they are caterpillars (Peigler, 1989).

Larva Once mated, the female lays a number of spherical eggs 2.5 mm in diameter on the undersides of leaves. Dusty-green caterpillars hatch after about two weeks and feed voraciously on the foliage of certain citrus and other evergreen trees (Robinson, 2001). The caterpillars are adorned with fleshy spines along their backs which are covered in a waxy white substance. The pupa after reaching a length of about 115 millimetres (4.5 in), the caterpillars pupate within papery cocoon interwoven into desiccated leaves. The adult moths emerge after about four weeks.

A. atlas moth eggs are laid on the underside of a leaf. They hatch in 8-14 days depending on the temperature. The caterpillars eat a wide variety of food plants such as, the Jamaican Cherry Tree (*Muntingia calabura*), soursop, cinnamon, lime, pomelo, rambutan, guava, citrus fruits. In Thailand this species feeds largely on Santol (*Sandoricum indicum* Cav.).

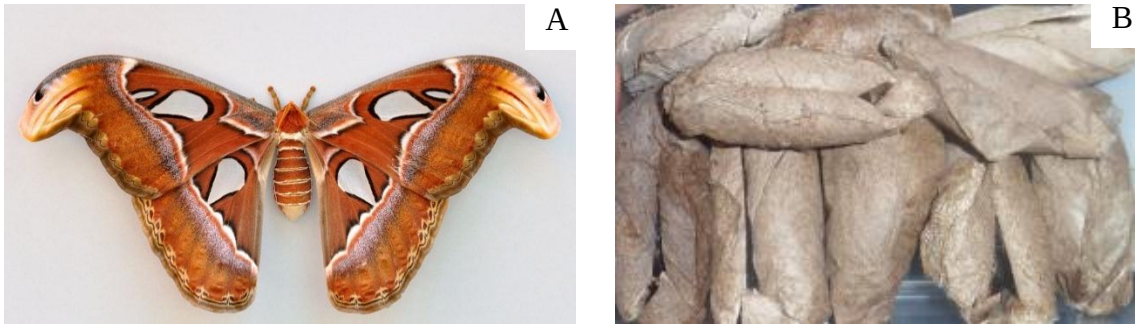

Figure 2.4 *A. atlas* silk moth (A) and *A. atlas* cocoons (B)
(https://breedingbutterflies.com/attacus-atlas/)

2.4 Applications of Sericin

Medical biomaterial about Substrate for the proliferation of adherent animal cells and can be used as a substitute for collagen. The first evidence of antioxidant action of the silk protein by showing that sericin suppressed in vitro lipid peroxidation. Sericin also found to inhibit tyrosinase activity. These results suggested that sericin is the valuable natural ingredient for food and cosmetics Sericin is soluble in hot water and as the time precedes it converts into gel. The conversion of a-random coil to β- sheet structure gives gel. Silk sericin membranes are good bandage materials and the film has adequate flexibility and tensile strength. Due to its good biocompatibility and infection resistant nature, it is a novel wound coagulant material.

Cosmetic Applications about Sericin alone or in combination with silk fibroin has been used in skin, hair, and nail cosmetics. Sericin was used in the form of lotion, cream and ointment shows increased skin elasticity, antiwrinkle, and antiaging effects (Patel, 2011).

2.5 Ultraviolet (UV) Radiation

The ultraviolet rays is electromagnetic radiation with a wavelength shorter than that visible light, but longer than X-rays(10 nm to 400 nm), and energies from 3.10 eV to 124 eV (Table 2.1). It is named because the spectrum consists of electromagnetic waves with frequencies higher than those that humans identify as the color violet. Even if ultraviolet is invisible to the human eye, the effects of UV are aware through the painful condition of sunburn, but the ultraviolet radioactivity has many other effects, both beneficial and damaging, to human health. UV light is found in sunlight and is emitted by electric arcs and specialized lights such as black lights. It can cause chemical reactions, and causes many substances to glow or fluoresce. Most ultraviolet is classified as non-ionizing radiation.

The higher energies of the ultraviolet spectrum from about 150 nm are ionizing, but this type of ultraviolet is not very penetrating and is blocked by air (International standard ISO 21348, 2007).

The sun discharge ultraviolet radiation in the UVA, UVB, and UVC bands. The Earth's ozone layer blocks 97-99% of this UV radiation from penetrating through the atmosphere. The ultraviolet radiation that reaches the Earth's surface, 98.7% is UVA (Lowe *et al*., 1990; David, 2009). (UVC and more energetic radiation is responsible for the generation of the ozone layer, and formation of the ozone there). Ordinary glass is partially transparent to UVA but is opaque to shorter wavelengths, whereas Silica or quartz glass, depending on quality, can be transparent even to vacuum UV wavelengths. Ordinary window glass passes about 90% of the light above 350 nm, but blocks over 90% of the light below 300 nm (International standard ISO 21348, 2007; David, 2009). The electromagnetic spectrum of ultraviolet rays can be subdivided in a number of ways. The draft ISO standard on determining solar irradiances describes the following ranges (International standard ISO 21348, 2007).

Table 2.1 Wavelength range of electromagnetic spectrum of ultraviolet rays

Name	Abbreviation	Wavelength range in nanometers	Energy per photon
Ultraviolet A, long wave, or black light	UV A	400 nm–315 nm	3.10–3.94 eV
Near	NUV	400 nm–300 nm	3.10–4.13 eV
Ultraviolet B or medium wave	UV B	315 nm–280 nm	3.94–4.43 eV
Middle	MUV	300 nm–200 nm	4.13–6.20 eV
Ultraviolet C, short wave, or germicidal	UV C	280 nm–100 nm	4.43–12.4 eV
Far	FUV	200 nm–122 nm	6.20–10.2 eV
Vacuum	VUV	200 nm–100 nm	6.20–12.4 eV
Low	LUV	100 nm–88 nm	12.4–14.1 eV
Super	SUV	150 nm–10 nm	8.28–124 eV
Extreme	EUV	121 nm–10 nm	10.2–124 eV

The effects of UV light in genes or their protein and cells from reported Semagoto *et al*., 2014; Devies *et al*., 2001; Kehoe *et al*., 2008 shows Protein is major cellular targets for direct photo-oxidation because the presence of endogenous chromophores with the protein and their high abundance (certain amino acid side-chains as well as bound chromohores, such as porphyrins and flavins). A major pathway for oxidation of proteins and amino acids by UV light is via the incident radiation by protein. Pattison et al., 2012 reported that the major intrinsinsic chromophoris species present in proteins are tryptophan, tyrosine, phenylalanine, histidine, cysteine and cystine side chains, with all other amino acid side chains and peptide bonds only absorbing radiation with $\lambda<230$ nm. Upon absorption of UV light the chromophoric residues are typically converted to their first excited singlet states that have only short lifetime (Bensasson *et al.,* 1993). These states readily lose energy by intersystem crossing to the triplet state, and energy transfer to other groups through collisional deactivation.

In peptides, the hydrated electron can also add to the carbonyl groups of the peptide backbone to give a radical-anion that can subsequently give rise to backbone cleavage. These reactions have been reported (Hart *et al.,* 1970; Garrison *et al.,* 1987; Von Sonntag *et al.,* 1987; Davies *et al.,* 1997).

2.6 Ultraviolet/Visible Absorption Spectroscopy

The UV absorption and VIS light occurs when the energy associated with the electromagnetic radiation matches the energy difference between molecular states in a molecule. The reverse is also true-by observing the wavelengths of light absorbed by a molecule, it should be possible to speculate on molecular structure (what atom and functional groups are present). Consider the electronic properties of carbonyl group as a functional group present in many types of biomolecules incudding amino acid, fatty acid, carbohydrates (open chain) nucleotides, protein and nucleic acids (David, 2009; Boyer and Rodney, 1990).

2.7 Sunscreen Safety Debate

Medical organizations recommend patients protect themselves from UV radiation by using sunscreen. Five sunscreen ingredients have been shown to protect mice against skin tumors. However, some sunscreen chemicals produce potentially harmful substances if they are illuminated while in contact with living cells (Xu *et al*., 2001; Knowland *et al*., 1993). The amount of sunscreen that penetrates through the stratum corneum may or may not be large enough to cause damage. In one study of sunscreens, the authors write (Damiani *et al*., 1999). The question whether UV filters acts on or in the skin has so far not been fully answered. Despite the fact that an answer would be a key to improve formulations of sun protection products, many publications carefully avoid addressing this question.

Hanson *et al*. (2006) measured amount of harmful reactive oxygen species (ROS) in untreated and in sunscreen treated skin. In the first 20 minutes, the film of sunscreen had a protective effect and the amount of ROS was smaller. After 60 minutes, however, the amount of absorbed sunscreen was so high, the amount of ROS was higher

in the sunscreen treated skin than in the untreated skin (Chatelaine *et al*., 2003). Such effects can be avoided by using newer generations of filter substances or combinations that maintain their UV protective properties even after several hours of solar exposure. Sunscreen products containing photostable filters like drometrizole trisiloxane, bisoctrizole, or bemotrizinol have been available for many years throughout the world, but are not yet available in the U.S., whereas another high-quality filter, ecamsule, has also been available in the U.S. since 2006 (Lewis, 2006).

2.8 Active Ingredients for Sunscreen

Sunscreens are chemicals used to protect the skin from UV radiation, which may lead to carcinogenesis, actinic keratosis, skin aging and immunosuppression (Hanson *et al.,* 2006; Sulayman *et al.,* 2008). Sunscreen formulations employ a variety of active ingredients (AIs) which are typically strong absorbers, reflectors or scatters of UV radiation to protect against such UV-induced damage (Sulayman *et al.,* 2008; David, 2009). These AIs are usually present in sunscreen products in varying amounts from 0.1 % to 10 % (w/w), and they are incorporated into a variety of other cosmetic products. Thus, the AIs and their concentrations must be monitored to ensure regulatory compliance. Sunscreen AIs can be classified as either physical blockers or chemical absorbers. The physical blockers reflect or scatter the harmful UV radiation, while the chemical absorbers absorb it. Common physical blockers include zinc oxide (ZnO), titanium dioxide (TiO_2) and more amorphous cerium-titanium phosphates (Ce1-x;TixP_2O_7) (Sulayman *et al,.* 2008; World Health Organization, 2011). Common chemical absorbers include derivatives of cinnamate, crylene, salicylate, benzophenone, camphor and anthranilate (Sulayman *et al,.* 2008; Lowe *et al.,* 1990).

2.8.1 Zinc Oxide (ZnO)

Zinc oxide is an inorganic compound with the formula ZnO. It usually appears as a white powder, nearly insoluble in water. The powder is widely used as an additive into numerous materials and products including plastics, ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, first aid tapes, etc. ZnO is present in the Earth's crust as the mineral zincite; however, most ZnO used

commercially is produced synthetically (Hughes and McLean, 1988; Nick *et al.*, 2005). The optical and biochemical properties of ZnO and its derivatives impart special features to a variety of cosmetic preparations for care of the hair and skin. In powders and creams it protects the skin by absorbing the ultraviolet sunburn rays; in burn ointments it aids healing. When used as an ingredient in sunscreen, zinc oxide sits on the skin's surface i.e. is not absorbed into the skin, and blocks both UVA (320-400 nm) and UVB (280-320 nm) rays of ultraviolet light. Because zinc oxide (and the other most common physical sunscreen, titanium dioxide) are not absorbed into the skin, they are nonirritating and nonallergenic (Hughes and McLean, 1988; Mu-Hsuan *et al*., 2007).

2.8.2 Titanium Dioxide (TiO_2)

Titanium dioxide (TiO_2) has been an interesting research topic because it could act as a photo-catalyst to decompose various pollutants by absorbing solar energy in the ultraviolet (UV) range. Nevertheless, ultraviolet light only occupies fraction of solar irradiation (Chatelaine *et al*., 2003). In cosmetic and skin care products, titanium dioxide is used as a pigment, sunscreen and a thickener. It is also used as a tattoo pigment and in styptic pencils. Titanium dioxide is produced in varying particle sizes, oil and water dispersible, and with varying coatings for the cosmetic industry. This pigment is used extensively in plastics and other applications for its UV resistant properties where it acts as a UV absorber, efficiently transforming destructive UV light energy into heat. Titanium dioxide is found in almost every sunscreen with a physical blocker because of its high refractive index, its strong UV light absorbing capabilities and its resistance to discoloration under ultraviolet light. This advantage enhances its stability and ability to protect the skin from ultraviolet light. Sunscreens designed for infants or people with sensitive skin are often based on titanium dioxide and/or zinc oxide, as these mineral UV blockers are believed to cause less skin irritation than other UV absorbing chemicals. The titanium dioxide particles used in sunscreens have to be coated with silica or alumina, because titanium dioxide creates radicals in the photocatalytic reaction. These radicals are carcinogenic, and could damage the skin (Hughes and McLean, 1988; Mu-Hsuan *et al*., 2007).

2.8.3 Benzophenones

Benzophenones and all absorb in the UVA region (320-400 nm), while all absorb in the UVB region (280-320 nm); absorption of both regions of UV radiation are required for adequate protection. Structurally, chemical absorbers are generally aromatic compounds conjugated to a carbonyl group usually with an another or paraelectron-donating group (Sulayman *et al,*. 2008; Lowe *et al.,* 1990).

2.9 Protein Precipitation by Salting – Out

Salting out is the method basis of one of most commonly used protein purification procedures. There are hydrophilic amino acid and hydrophobic amino acid in protein molecules. It is used as method of separating proteins based on the principle that proteins are less soluble at high salt concentrations. When the salt concentration is increased, some of the water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the charged part of the protein. The salt concentration needed for the protein to precipitate out of the solution differs from protein to protein. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions and coagulate by forming hydrophobic interactions with each other. (Giraporn,2016)

2.10 Bradford Assay

The Bradford assay is a simple and rapid protein quantification technique compared to the Lowry method. Moreover, this assay is comparatively free from interference by common reagents except detergents. The assay involves the use of Coomassie Brilliant Blue G-250, which reacts primarily to basic (especially arginine) and aromatic amino acid. The assay is based on the immediate absorbance shift 470 nm to 595 nm that occurs when dye binds to protein in acidic solution. The dye is believed to bind to protein via electrostatic attraction of the sulfonic acid groups. The mechanism of dye binding can be explained by the dye existing as three absorbing species, a red cationic species (Amax 470 nm), a green neutral species (Amax 650), and blue ionic species (Amax 595 nm). Color changes are due to successive loss of change. Stepwise addition of sodium hydroxide abolished absorption at 470 nm, but increased absorbance at 650 nm and finally replaced with a new peak at 595 nm. Prior to protein binding, dye molecules exist in doubly protonated (the red cationic dye form). Upon binding of the dye to protein, the blue ionic dye form is stabilized and is detected at 595 nm.(Ahmed, 2005).

2.11 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centred at about 520 nm.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present). Representing the DPPH radical by $Z^{\bullet}$ and the donor molecule by AH, the primary reaction is

$$Z^{\bullet} + AH = ZH + A^{\bullet} \qquad [1]$$

where ZH is the reduced form and $A^{\bullet}$ is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant. The reaction [1] is therefore intended to provide the link with the reactions taking place in an oxidizing system, such as the auto-oxidation of a lipid or other unsaturated substance; the DPPH molecule $Z^{\bullet}$ is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH (Molyneux, P., 2004).

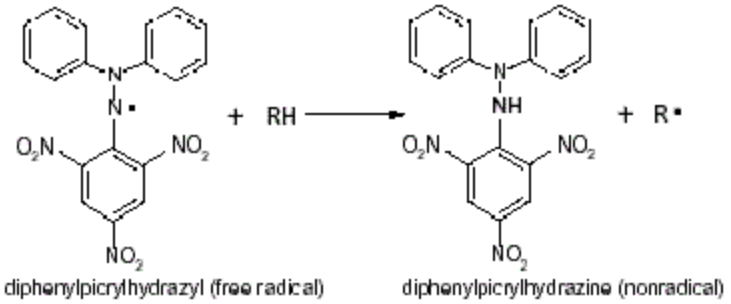

Figure 2.5 Structure of DPPH

2.12 SDS Polyacrylamide Gel Electrophoresis (SDS – PAGE)

SDS – PAGE is an analytical tool for separating protein molecules based on charge and size. Polyacrylamide was used as support matrix provide porous gel, which act as a sieve by retarding and separating molecules by their size. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins that binds to most protein. SDS confers a negative charge to the polypeptide in proportion to its length. The negative charge polypeptide migrates depend on their size towards the anode by applied electric flied. The small size polypeptide rapidly moved than the large size, therefore these polypeptides can be separated by different size. Molecular weight determination can be calculated from relative mobility (R_f) of unknown protein compared to protein marker (Zhao.1996:191-193).

2.13 ABTS Assay

2,2'-azinobis (3-ethylbenzthiazoline-6-acid) (ABTS) is converted into its radical cation ($ABTS^{\bullet+}$) by addition of sodium persulphate.This blue-green radical cation absorbs light at 734 nm. $ABTS^{\bullet+}$ is reactive towards most antioxidants. It is not affected by ionic strength, and it can be used to determine both hydrophilic and hydrophobic antioxidant capacities. During this reaction, the blue-green ABTS radical cation is converted back into its colourless neutral form. The reaction may be monitored spectrophotometrically (Aycicek *et al*. 2006, Cubero *et al*. 2009, Matos *et al*. 2009).

2.14 Related Literatures

Wu and others (2008) found that the antioxidant activity and tyrosinase-inhibitory of sericin peptides from waste water which, produced by using Protease P was examined. The sericin peptides exhibited excellent antioxidant activities with minimum IC_{50} value for the ferrous ion-chelating activity of 0.128 mg/ml, and also exhibited a notable tyrosinase-inhibitory. The result suggested that sericin from waste water can be used as valuable ingredients in cosmetic and pharmaceutical industries.

Haesung Yun and others (2013) found that the extraction of *A.mylitta* sericin (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.

Rupesh Dash and others (2007) found that sericin isolated from the cocoon of the tropical tasar silkmoth *Antheraea mylitta* showed three major bands, with the lowest 70 kDa. This band was purified by anion exchange chromatography. Immunoblotting with concanavalin-A suggests a glycoprotein and CD analysis of secondary structure

includes β-sheet. Amino acid analysis shows that the protein is enriched in glycine and serine while the mole percentages of these two amino acids are different from sericin of mulberry silkworm. An anti *A. mylitta* sericin antibody was able to cross-react with sericin from *A. assamensis* but not the sericin of *Bombyx mori* and *Philosamia ricini*. Immunoblot analysis with proteins isolated from middle silk gland of *A. mylitta* at different developmental stages of larva showed that the 70 kDa sericin is developmentally regulated. These data extend the range of biochemical features found in this unusual family of proteins and may help in developing an improved understanding of their role in forming environmentally stable fibroin fiber–sericin composite structures (cocoons).

Wilaiporn (2010) studied nntioxidant activity by DPPH assay in crude sericin extracted from demestic silkworm (*Bombyx mori*), Thai yellow cocoon (Nang noi) and Japanese white cocoon (WN), compared with wild Eri silkworm (*Philosamia ricini*) demonstrated that sericin extracted from eri silk that of cocoon possessed the highest antioxidant activity (IC_{50} = 0.17 mg/ml) followed by Nang noi (IC_{50} = 0.35 mg/ml) and Japanese (IC_{50} = 1.71 mg/ml), respectively. The crude sericin extract of all races was white precipitated protein by 50%, 70% and 90% $(NH_4)_2SO_4$. The white protein precipitate could be observed in all fractions except the yellow protein from Nang noi could be observed in 50% $(NH_4)_2SO_4$, which was similar to the yellow protein fraction obtained by 70% NaCl precipitation of Nang noi cocoon whereas only white protein could be obtained from Japanese and Eri.

Dash *et al.* (2008) studied antioxidant potential of silk protein sericin from the non – mulberry tropical tasar silkworm *Antheraea mylitta* cocoon and compared with that of the mulberry silkworm, *Bombyx mori*. Skin fibroblast cell line (AH927) challenged with hydrogen peroxide served as the positive control for the experiment. They found that silk protein sericin against hydrogen peroxide induced oxidative stress in skin fibroblasts.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The cocoons are domestic *B. mori* polyvoltine yellow silk cocoons or Nangnoi (NN), Bivoltine white cocoon (W7), wild silk cocoon of Eri (*Philosamia ricini*) and Fagara silk or *A. atlas* (AT) obtained from Center of Excellence for Silk Innovation and The Queen Sirikit Department of Sericulture of Thailand were used in this study.

3.2 The Optimal Condition for the Extraction of Sericin

The four extracting solvent (0.1 M of sodium bicarbonate, sodium carbonate, sodium hydroxide and water) were adopted for extraction of sericin from *A. atlas*. For all of the extraction procedures, 0.2 gram of *A. atlas* cocoon was boiled in 100 ml at 100 ◦C for 30 min. For the water extraction, the cocoon was performed by boiling at 100 ◦C for 30-150 min. After the extraction was fibroin and crude sericin solution, the fibroin were then washed several time with water and dry before measuring their weight. The degumming ratio was calculated using the following equation:

$$\text{Degumming ratio (\%)} = [(\text{Initial weight} - \text{Final weigth})/\text{Initial weight}] \times 100$$

The crude sericin solutions were dialyzed against water to obtain sericin solution. The protein concentration of sericin solution was measured by Bradford assay, antioxidant property was measured by DPPH and ABTS assays. The molecular mass of sericin protein was measured by SDS-PAGE**.**

3.3 Determination of Protein Concentration

Protein concentrations were determined by slightly modified Bradford method (Bradford, 1976) using bovine serum albumin (BSA) (Fluka, Switzerland) as a standard. The protein samples were two-fold diluted and mixed with Bradford reagent (1:2v/v), kept for 5 min at room temperature, and the absorbance was measured with spectrophotometer (Genesys 20, Thermo spectronic, U.S.A.) at 595 nm. The protein concentration was calculated basing on the linear BSA standard curve.

3.4 Antioxidant Activity of Sericin

3.4.1 DPPH Assay

The DPPH radical scavenging activity of samples was measured according to a slightly modified method (Yamaguchi et al., 1998). Samples were subjected to determine the least concentration of radical scavenging. Each sample solution (0.5 ml) was added to 1 ml of a freshly prepared 0.1 mM DPPH solution dissolved in methanol. The sample was mixed and incubated in the dark at room temperature for 30 min. Absorbance of the sample was measured at 517 nm using a spectrophotometer against a control. The radical scavenging of DPPH was calculated by using the formula below:

$$\text{Radical scavenging activity (\%)} = [\ 1 - (\text{Abs. sample}/\text{Abs. control})] \times 100$$

3.4.2 ABTS Assay

Experiments were performed according to Re *et al*. 1999 with small modifications. ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM respectively. These two solutions were mixed and the mixture allowed to stand in the dark at room temperature for 16 h before using order to produce ABTS radical ($ABTS^{\bullet+}$). For the study of antioxidant activity the ABTS radical solution was diluted with distilled water before using. Samples were diluted in distilled water and added into 1 ml of ABTS solution. The sample was mixed and incubated in the dark at room temperature for 6 min. Then the absorbance was measured at 734 nm. The total antioxidant capacity was then expressed as percent inhibition, according to the equation :

$$\text{Percent inhibition} = [\,1 - (\text{Abs. sample}/\text{Abs. control})] \times 100$$

3.5 Sericin Extraction for UV Absorption

0.5 gram of silk cocoon (AT, Eri, NN and W7) were boiled in 100 ml of the distilled water for 60 min to obtain the sericin extract. The crude sericin solutions were determined for protein concentration by Bradford assay and measurement of UV absorption by UV-Vis spectroscopy including determination of molecular mass of sericin protein by SDS-PAGE, respectively.

3.6 Sericin Fractionation by Salting-out with Saturated $(NH_4)_2SO_4$

Sericin protein was precipitated from crude extracts by adding saturated $(NH_4)_2SO_4$ into 10 ml of crude sericin solution until the whole colored-protein are precipitated. The colorless supernatant and protein precipitate could be separated by centrifugation at 8000 g for 20 min. The protein precipitate was redissolved in 10 ml of distilled water and followed with dialyzing (MWcutoff 3.5 kDa) against distilled water for one day. All protein fractions obtained in this step were analyzed for protein concentration by Bradford assay and measurement of UV absorption by UV-Vis spectroscopy including determination of molecular mass of sericin protein by SDS-PAGE, respectively.

3.7 Ultraviolet-visible Wavelength Scaning

The crude sericin, supernatant and precipitate sericin (4.27 µg/ml) from AT, Eri, W7, NN and crude sericin extract from AT, 1% sunscreen and sericin extract mixed with sunscreen. The sericin mixed with sunscreen ratio was 1:3 v/v(SS 25%), 1:1 v/v (SS 50%) and 3:1 v/v (SS 75%) were prepared at room temperature. Three millilitre of all sample was aliquot in to quartz cuvette (3 ml, 1 cm) to expose the light ranged from 200-800 nm using distilled water as a blank. The absorption of crude, supernatant and precipitate of sericin from AT, Eri, W7, NN measured at 200, 284 and 334 nm for UVA, B and C range, respectively. The absorption spectra of samples were recorded with Ultraviolet-visible spectrophotometer (Perkin Elmer Model Lambda 25).

3.8 Determination of Protein Molecular Weight by SDS-PAGE

The molecular mass of sericin protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separating gel and stacking gel were 12.5% and 4.5%, respectively. Pigment from silk cocoons (Fagara, Polyvoltine and samples of yellow silk cocoons) were extracted three times in 70% ethanol (1g of silk cocoon and 30 ml of ethanol) for 24 h at room temperature to remove all flavonoids and caroteniods to silk sericin extraction to confirm that flavonoids or caroteniods would not interfere with molecular mass determination. The sample solution was mixed with 5x buffer and heated in hot water, and then sample was loaded into the well. The electric current of 158 volt, 118 mA was applied to the gel. At the end of electrophoresis, the gel was stained with silver staining technique. Standard molecular mass marker (BenchMarkTM Protein Ladder consists of 15 engineered proteins ranging in molecular weight from 10 to 220 kDa) was applied for estimating the molecular mass.

3.9 Statistical Analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). One-way ANOVA using SPSS software was used to compare the mean values of each treatment. Significant differences ($p< 0.05$) among the means were determined by using Duncan's multiple range Test.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of Sericin

The solution includes sodium bicarbonate; sodium carbonate and sodium hydroxide are alkaline solution for sericin degumming. In this work, the *A. atlas* (AT) cocoons was degummed by different extraction methods. The degumming ratio and protein sericin concentration are presented in Table 4.1. The highest degumming (23.05%) and protein concentration (59.25 µg/ml) were achieved with the 0.1 M sodium hydroxide extraction. The cocoon degummed with 0.1 M sodium carbonate solutions showed degumming ratio of 17% and 35.94 µg/ml of protein concentration which was second high after the sodium hydroxide solution. Although sodium hydroxide has the highest potential for sericin extraction, it is not suitable solution for using as degumming solvent. This was due to it is a strong alkali treatment imposes a relatively harsh irritation to silk fibroins (Yamadaa *et al*., 2001 and Jiang *et al*., 2006).

Generally, a sodium carbonate solution is widely used for extraction of *A. atlas* sericin (Mandal, 2009, 2011; Dash, 2007 and Khire, 2010). This was according to it is gently potential than sodium hydroxide and could be excluded the sericin clearly without erosion of silk fibroin surface (Yun *et al*., 2012)

Table 4.1 The degumming of cocoons from the *A. atlas* subjected to different extraction methods for 30 min and protein concentration of sericin extract.

Method	**Degumming (%)***	**Protein concentration (µg/ml)***
$NaHCO_3$	10.64 ± 2.87^{b}	18.35 ± 6.04^{a}
Na_2CO_3	$17.05 \pm 6.63^{a,b}$	35.94 ± 9.29
NaOH	23.05 ± 8.27^{a}	59.25 ± 4.82
H_2O	5.98 ± 1.20^{b}	8.80 ± 0.68^{a}

* Data are expressed as mean $\pm$ standard deviation (n=3).

[a-b] Values with the same letter in the same column superscript denote no significant difference ($p < 0.05$)

4.2 Antioxidant Activity of Sericin from *A.atlas*

The result of antioxidant activity of sericin extracts from *A. atlas* cocoon with the protein concentration of 2.5 to 40 µg/ml performed by DPPH and ABTS assay was shown in Figure 4.1. The sericin extract obtained from boiling water for 30 min showed the lowest IC_{50} value at 8.474 and 6.955 µg/ml for DPPH and ABTS assay, respectively. It indicated that the antioxidant activity of sericin from water extraction showed effective inhibition on free radicals than other extraction methods. For the extraction with other alkaline solution ($NaHCO_3$, Na_2CO_3 and NaOH), although the protein yield from alkali extractions were higher, however, the antioxidant activity were relatively low. Therefore, the extraction of crude sericin with water was subjected to further study. The obtained results were similar trend with previously reports that water extract has a good potential for antioxidant activity (Plancken *et al.,* 2006; Tang *et al.,* 2009; Raikos, 2010; Rondeau *et al.,* 2007).

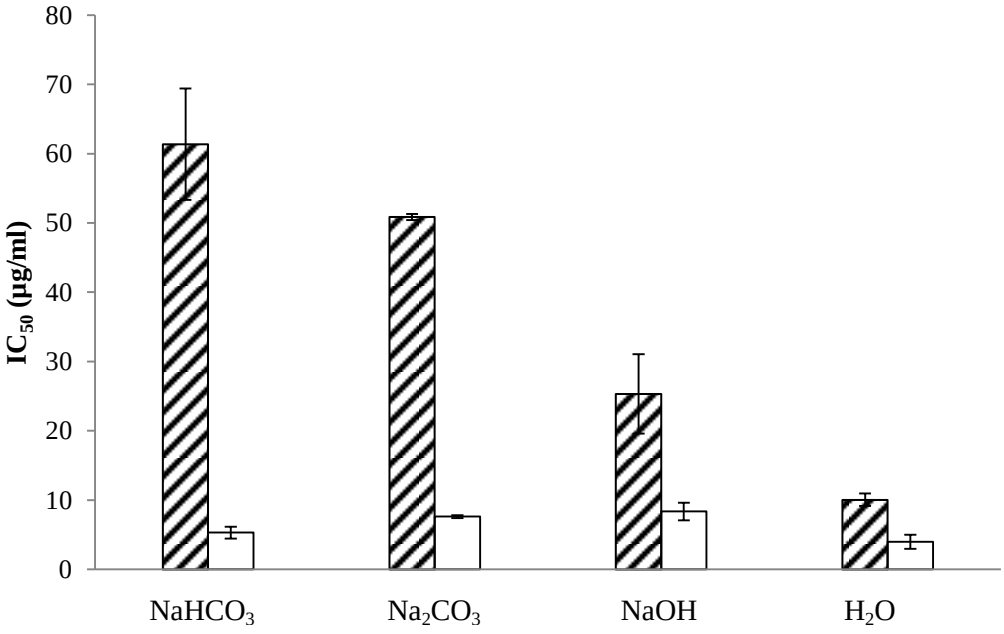

Figure 4.1 The antioxidant activity of sericin extracts from *A.atlas* cocoon at the concentration of 2.5 to 40 µg/ml performed by boiling in various solvent and water at 100 ◦C for 30 min (DPPH assay (▭) and ABTS assay (▨).

4.3 Effect of Extraction Time with Antioxidant Activity

Figure 4.2 shows the increasing of protein concentration and its antioxidant activity according to the increasing of extraction time (30, 60, 90, 120 and 150 min).

The highest antioxidant activity from DPPH assay was shown in the extract at the extraction time 120 min (IC_{50}= 6.75 ± 0.31 µg/ml), followed by 150 min (IC_{50}= 7.25 ± 0.66 µg/ml), 90 min (IC_{50}= 9.46 ± 0.44 µg/ml), 30 min (IC_{50}= 12.60 ± 0.57 µg/ml) and 60 min (IC_{50}= 27.22 ± 3.91 µg/ml). For the antioxidant activity by ABTS assay a similar trend was found with the highest activity found in extraction time at 120 min (IC_{50}= 4.37 ± 0.06 µg/ml). In this study, we have found that antioxidant activity and protein concentration was significantly increased until 150-min of extraction time. However, the antioxidant activity and protein concentration, there was no significant difference between the extraction time at 120 and 150 min.

This result suggested the extraction condition of sericin from *A.atlas* considerable with antioxidant activity when using water should be at 100 ◦C for 120 min.

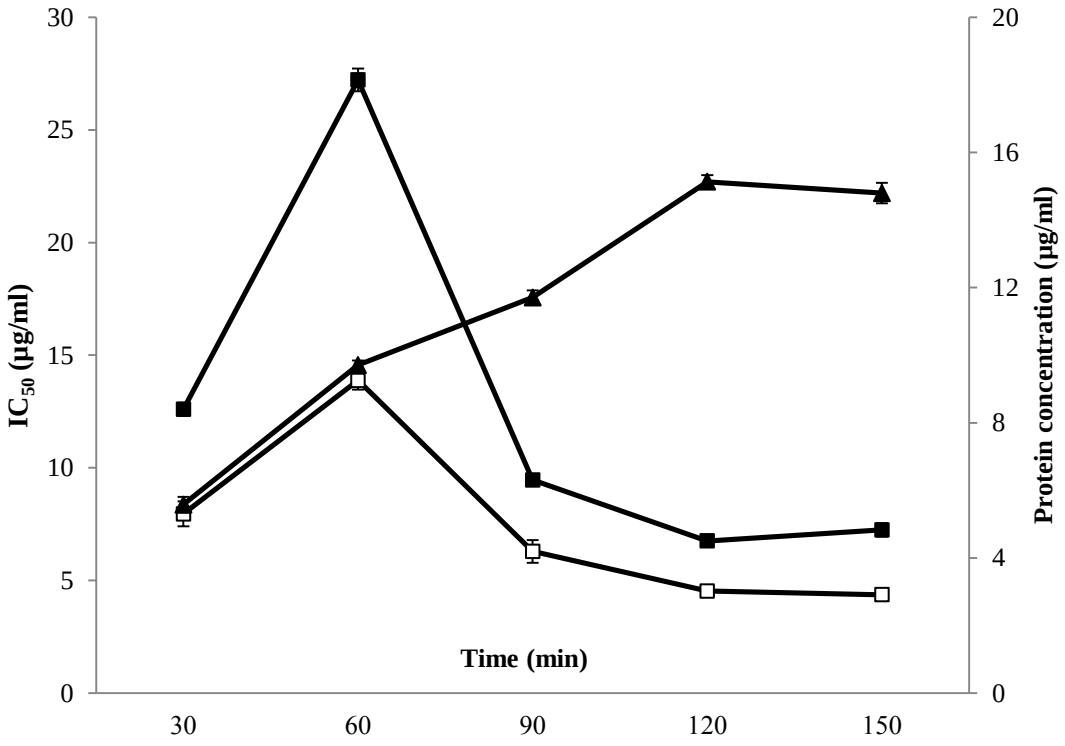

Figure 4.2 The effect of water extraction time on antioxidant activity of the sericin extract from AT cocoon after the water extraction by DPPH assay (-■-) and ABTS assay (-□-), while (-▲-) indicate the protein concentration of sericin extract

4.4 Sericin content of crude sericin extracts

Table 4.2 shows the sericin content in the degumming solutions of polyvoltine (Nang noi), bivoltine (W7) Eri and *A. atlas* (AT) silk cocoon. This result indicates that the *A. atlas* silk cocoon had lower sericin content compared to *B. mori* silk cocoon.

Table 4.2 Sericin content of crude sericin extracts

Cocoon	Subtype	Race	Abbreviation	% Sericin
Non-mulberry silk	Wild silk	*Attacus atlas* Linn.	AT	6.95
		Philosamia ricini	Eri	7.33
Mulberry silk	Bivoltine	*Bombyx mori* (W7)	W7	11.5
	Polyvoltine	*B. mori* (Nang noi)	NN	12.9

4.5 Sericin Fractionation

The crude sericin extract of *A. atlas* (AT), Eri, W7 and NN were fractionated with 50% $(NH_4)_2SO_4$. The supernatant and precipitated protein was obtained. The higher sericin content could be obtained in protein precipitate than in supernatant as shown in Table 4.3. It should be noted that the protein precipitate from *A. atlas* (AT) and yellow silk cocoons, except from Eri and W7, exhibited the brown and yellow protein precipitate, respectively whereas the supernatant from all crude extracts was the colorless protein fraction.

Table 4.3 Protein amount of crude sericin, percipitates and supernatant from AT, Eri, W7 and NN.

Samples	**Protein amount (µg) ($\overline{X}$ ±SD)**			
	AT [a]	**Eri** [b]	**Polyvoltine** [c]	**Bivoltine** [c]
Crude extract (10ml)	31533±11.33	31533±11.33	31533±11.33	31533±11.33
PP50NS (5ml)	1743.35±5.34	1292.2.44±5.00	1540±7.33	1658.9±2.34
SP50NS (5ml)	684.45±1.5	495.00±0.00	643.35±2.00	501.00±0.00

[a] 1.18 g of silk cocoon, [b] 1.0 g of silk cocoon and [c] 0.5 g of silk cocoon

PP50NS, re-dissolved sericin percipitates from crud sericin extracts after addition of $(NH_4)_2SO_4$, followed by centrifugation

SP50NS, supernatant from crude sericin extracts after addition of $(NH_4)_2SO_4$, followed by centrifugation

4.6 The UV Absorption Spectra of Sericin Extracts

The UV absorbance spectra of 4.27 µg/ml of crude extract, re-dissolved precipitate and supernatant fractions of sericin from *A. atlas* (AT), Eri, W7 and NN are shown in Figure 4.3. At 200 nm, the sericin from *A. atlas* (AT) silk cocoons showed the highest UV absorption whereas sericin from Eri, W7 and NN silk cocoons showed the lower absorption at the same wavelength (Figure 4.3). The UV absorption in crude, redissolved precipitate and supernatant of sericin extract from *A. atlas* (AT), Eri, W7 and NN at the wavelength in the range of UVC (200 nm), UVB (284 nm) and UVA (334 nm) are shown in Figure 4.6. It should be noted that in all types of silk cocoons, the UV absorption of protein precipitate was almost similar in crude extract and much higher than that of supernatant fraction. However, when the aspect of colored protein was considered, it is interesting to note that although the brown protein precipitate from *A. atlas* (AT) silk cocoons showed the highest UV absorption, the yellow protein precipitate from NN silk cocoons showed the lowest UV absorption at the same wavelength (Figure 4.3, B and C). In contrast, the white protein precipitate from Eri silkworm cocoons showed much higher absorption potency than of W7 silk cocoons (Figure 4.4). The characteristics of UV absorption of sericin might be independent of color of cocoons.

In the UV absorption at 200, 284 and 334 nm which are the range of UVC, UVB and UVA, respectively of 4.27 µg/ml of sericin extracts from *A. atlas* (AT), Eri, W7 and NN silk cocoons were shown in Figure 4.6. The UV absorption at those three wavelengths of crude, precipitate and supernatant fractions from *A. atlas* (AT) has the highest UV absorption (Figure 4.4).

The results in this study indicate that sericin protein from *A. atlas* (AT) silk showed the highest UV absorption potency, particularly the absorption in the wavelength of UVC by the brown protein precipitate of *A. atlas* (AT). At UVC range (200 nm), activity of *A. atlas* (AT) was similar between crude extract and precipitate. However, at UVB range (284 nm) and at UVA range (334 nm), activity of precipitate dropped appreciably to almost one sixth of the activity of crude extract.

Amino acids show strong absorbance in the wavelength of range and this is frequently used to detect peptides. Some amino acid residues containing aromatic

phenyl groups such as phenylalanine, tyrosine and tryptophan show relatively strong absorbance at 275, 274 and 280 nm, respectively. These wavelengths are therefore widely used in the studies of proteins (Boyer, 2006; David, 2009).

Sericin protein from *B.mori* cocoons was reported for the role of attaching a pair of silk fibers, fibroin, together and embedding crystals for the UV protection property of silk cocoons.

The results in this study indicated that sericin protein from *A. atlas* (AT) silk cocoons shows the highest UV absorption potency, particularly the absorption in the wavelength of UVC by the brown protein precipitate of *A. atlas* (AT) which was different from *B.mori* (NN) sericin that will absorb mostly UVA (Kaur *et al*., 2013)

The result in this study also indicated that crude sericin extract shows higher UV absorption property than that of the protein precipitate or the supernatant of the sericin extract when compared at the same protein concentration (Figure 4.4). This suggested that the alteration of sericin conformation affects UV absorption property. According to Donovan (1968), they indicated that transferring aromatic chromophore from the interior of a protein in to the water solvent upon denaturation of the protein produces an absorption change throughout the UV region, 215 to 320 nm, approximately 6 times the magnitude of that produduced by transfer of the same chromophore from 20% ethylene glycol into water. This number is quite similar to the result in the present experiment. The approximately 6 times decreasing of UV absorption from 0.6 to 0.1 at the wavelength of 284 and 334 nm in the case of *A. atlas* (AT) silk cocoons was observed (Figure 4.4 B and C). It is apparent therefore that, for the efficient UV absortion property, crude extract of sericin should be adopted for its application.

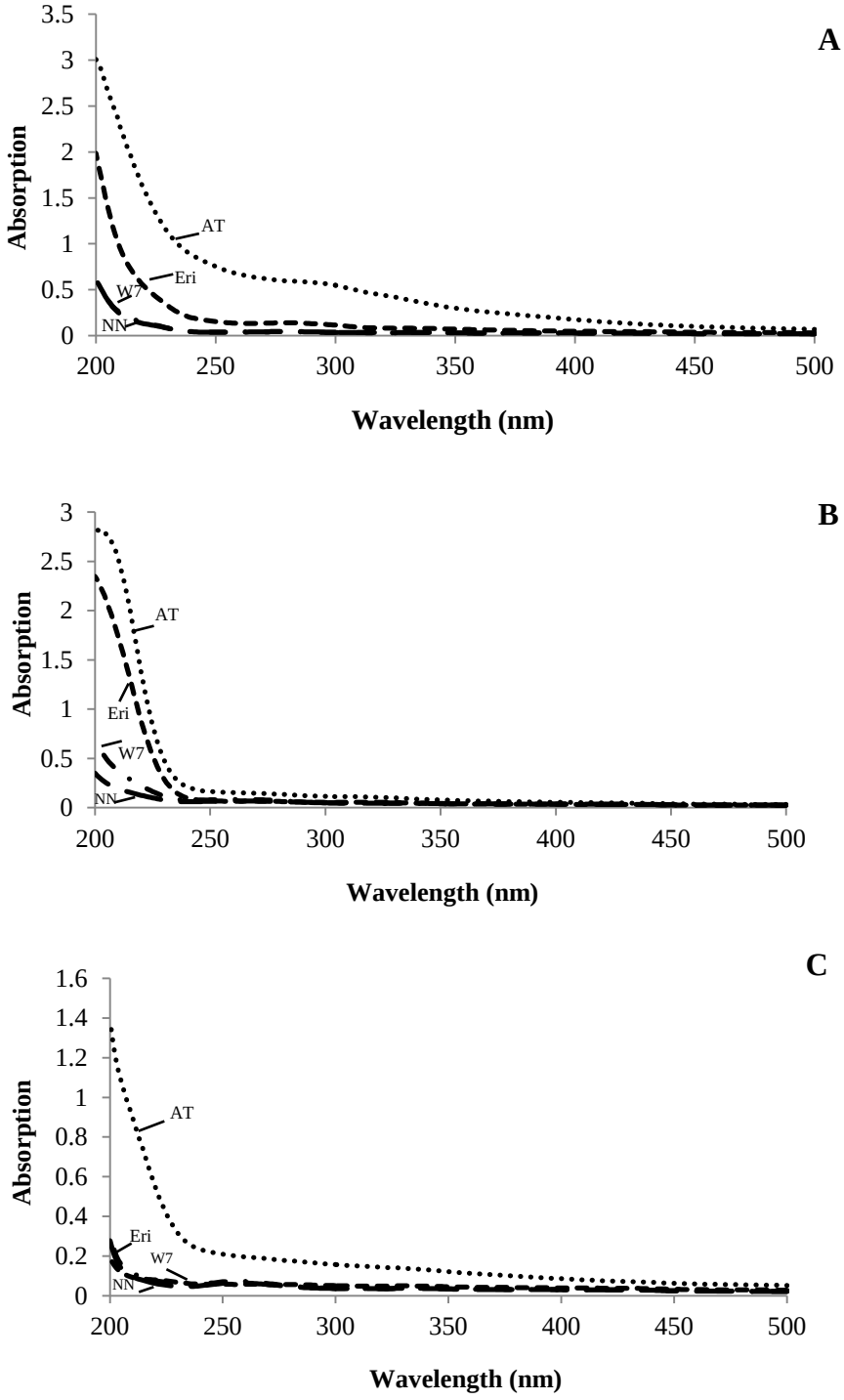

Figure 4.3 The UV absorbance spectra of 4.27 µg/ml of crude sericin extracts from water extraction for 60 min (**A**), re-dissolved sericin percipitates from crud sericin extracts after addition of $(NH_4)_2SO_4$,followed by centrifugation (**B**) and supernatant from crude sericin extracts after addition of $(NH_4)_2SO_4$, followed by centrifugation (**C**) from cocoons of AT, Eri, W7 and NN.

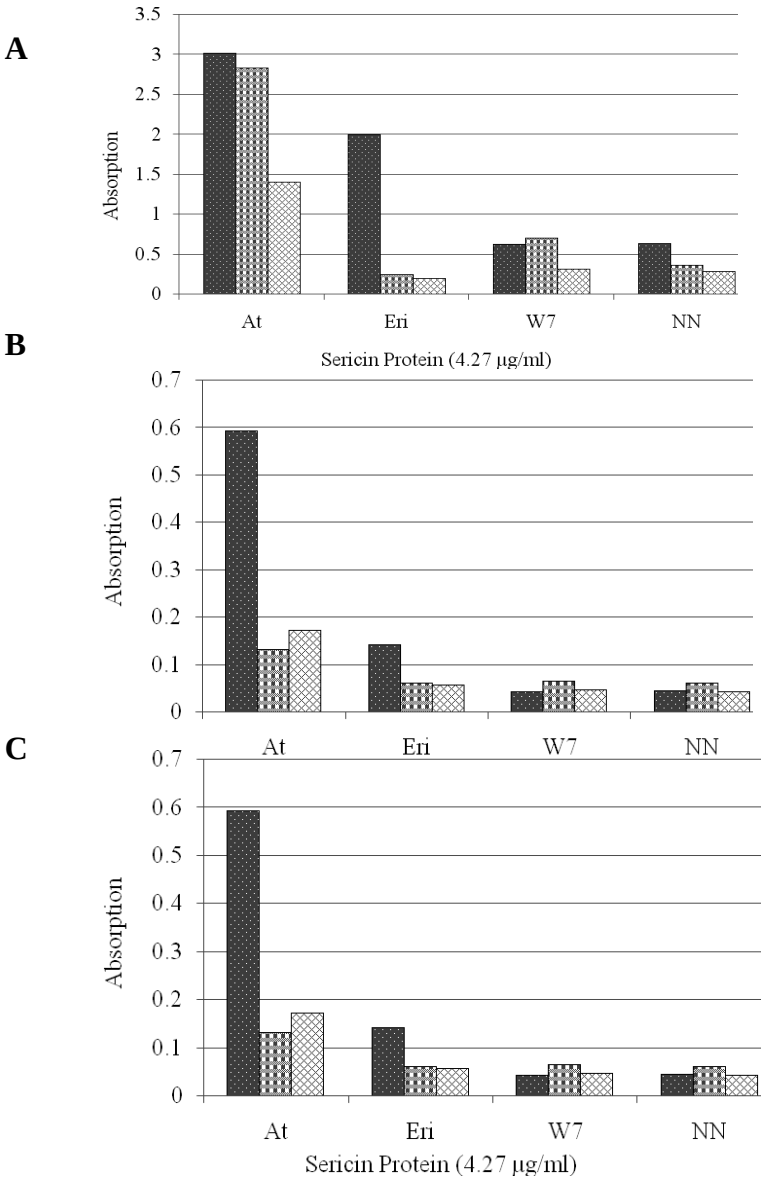

Figure 4.4 The UV absorption at 200 (A), 284 (B) and 334 (C) nm (UVC) of 4.27 µg/ml of sericin extracts was performed by water extraction for 30 min from AT, Eri, W7 and NN silk cocoons, respectively.
(crude sericin extracts, re-dissolved sericin percipitates from crud sericin extracts after addition of $(NH_4)_2SO_4$, followed by centrifugation and supernatant supernatant from crude sericin extracts after addition of $(NH_4)_2SO_4$,followed by centrifugation)

4.7 The UV Absorption of Sericin and Sunscreen

The UV absorbance spectra of 5 µg/ml of crude sericin solution extract (sericin) from *A. atlas* (AT), 1% sunscreen and sericin mixed with sunscreen. The sericin mixed with sunscreen by 1:3 v/v (SS25), 1:1 v/v (SS50) and 3:1 v/v (SS75) are shown in Figure 4.5 (A).

The result at 250 nm is interestingly that the sericin protein showed the highest UV absorption, the 1% sunscreen gave the lowest UV absorption at the same wavelength. Nevertheless, at 350 nm was found the highest UV absorption in SS50 that than sericin protein and sunscreen. The UV absorption at range 250 to 300 nm of crude sericin solution extracts showed the absorption increase.

In this result, we have found that the sericin possesses the highest UV absorption whereas BSA showed lower absorption at the same wavelength. The sericin showed higher UV absorption than sunscreen as well as the UV absorption of sericin mixed with sunscreen had an apparent increase when increasing sericin protein.

Previous studies have reported that the amino acid could absorb UV radiation are tryptophan (Trp), tyrosine (Tyr), histidine (His) and cysteine (Cys) (Mondal., 2007 and Pattison., 2012). Yun *et al*. (2012) found that the amino acid of sericin protein in cocoon from *A. mylitta* contain Tyr is large amino acid. According to Donovan (1968), the amino acid could absorb UV radiation, with regard to *B. mori* sericin, the dominant chromophore in this protein is the amide group.

This result suggested that amino acids contain in the sericin protein in cocoon from *A. atlas* (AT) play a role in the UV absorption potency and its stability.

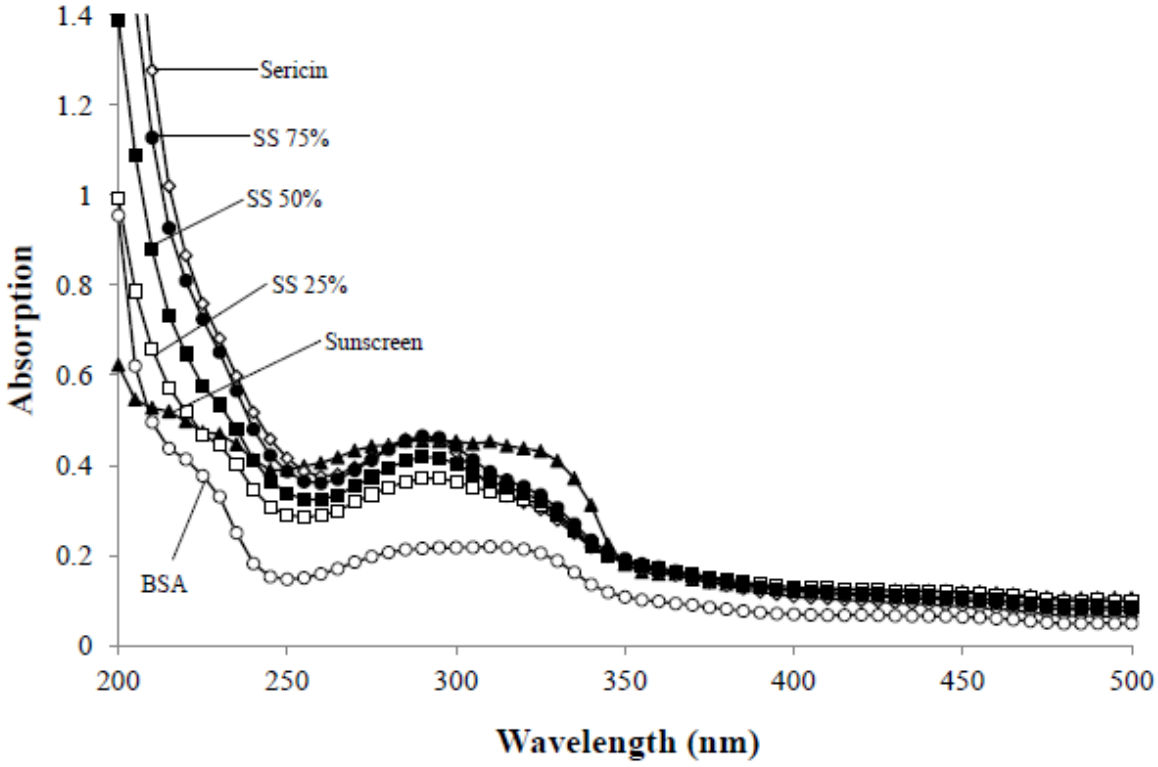

Figure 4.5 The UV absorbance spectra of sericin solution extracted from AT cocoon (-◇-), 1% sunscreen (-▲-), SS25% (-□-), SS50% (-■-), SS75% (-●-) and BSA (-○-).

4.8 Molecular Weight of Sericin Fractions by SDS-PAGE

Molecular weight of crude sericin protein from *A. atlas* (AT), Eri, W7 and NN silk cocoons were measured by SDS-PAGE. As shown in Figure 4.6, the crude sericin extracts from Fagara had the molecular weights in the range between 10 and 50 kDa whereas Eri showed smear protein band with molecular weight ranging from 10 to 220 kDa. SDS-PAGE of crude sericin extracts of NN, W7 showed smear protein pattern with molecular weight ranging from 10 to 220 kDa.

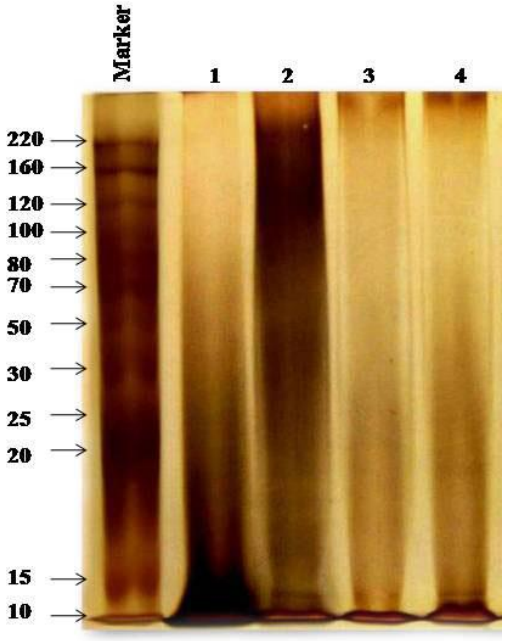

Figure 4.6 SDS-PAGE of sericin protein in crude extracts from AT (1), Eri (2), W7 (3), and NN (4)

CHAPTER 5

CONCLUSION

The relationship between effect of extraction methods on sericin from *A.atlas* silk cocoon with its antioxidant property was investigated. The results revealed that the optimal conditions for the extraction methods of sericin from *A.atlas* silk cocoon with the highest antioxidant property when using water extract at 100 °C for 120 min. For the relationship between UV absortion activity and the sericin extract with sunscreen, the results revealed that the sericin extract from *A.atlas* showed the highest UV absorption than sunscreen. The UV absorption of sericin mix sunscreen solution had an apparent increase when increasing sericin protein at 200-250 nm. The sericin extracts from wild silks, *A.atlas* and Eri silk, showed the higher UV absorption activity than domestic silks, of both bivoltine and polyvoltine races at 200-400 nm. The precipitated protein obtained by salting-out with 50% $(NH_4)_2SO_4$ from crude extracts of all cocoon types showed higher UV absorption potency than that of proteins remaining in the supernatant. SDS-PAGE of crude sericin extract of *A.atlas* had a molecular weights in the range between 10 and 50 kDa.

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APPENDICES

Appendix A

Reagent preparation

1. Preparation of samples

Samples	Dilution (fold)			
	AT	**Eri**	**W7**	**NN**
Crude extract	10	10	10	10
Protein precipitate from 50%$(NH_4)_2SO_4$	10	10	10	10
Supernatant from 50%$(NH_4)_2SO_4$	5	5	5	5

2. Bradford reagent

2.1 0.01% Coomassie brilliant blue G-250

Coomassie Blue G-250 (0.1 g) was dissolved in 50 ml of 95% ethanol, then mixed with 100 ml of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman no. 1 filter paper before use and then stored in an amber bottle at room temperature.

2.2 Protein standard

The stock solution: Bovine serum albumin was prepared at a concentration (200 μg/ml) in distilled water. Protein concentration was by using 2-fold serial dilution as a range of 100, 50, 25, 12,5, 6.25, 3.125 and 1.562 μg/ml, respectively.

3. Reagent for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.1 Tris-HCl (1.5 M), pH 8.8

Tris-(hydroxymethyl)-aminomethane (18.17 g) and SDS (0.4 g) were dissolved in deionized water, after that the solution was adjusted pH to 8.8 by 6 M HCl. Then adjusted the volume to 100 ml and kept at 4 ˚C.

3.2 Tris-HCl (0.5 M), pH 6.8

Tris-(hydroxymethyl)-aminomethene (6.06 g) and SDS (0.6g) were dissolved in deionized water, after that the solution was adjusted pH to 6.8 by 6 M HCl. Then adjusted the volume to 100 ml and kept at 4 ˚C.

3.3 Acrylamide/Bis-acrylamide (30%T)

Acrylamide (30.0 g) and bis-acrylamide (0.8 g) were dissolved in deionized water. Then adjusted the volume to 100 ml after that the solution was filtered through Whatman no. 1 filter paper before using and kept at 4 ˚C.

3.4 10% Ammonium persulphate (APS)

$(NH_4)_2S_2O_8$ (1.0 g) was dissolved in deionized and then adjust the volume to 10.0 ml.

3.5 10X Running buffer

10X Running buffer contains Tris-(hydroxymethyl)-aminomethene (15.0 g), glycine (72.0 g) and SDS (0.5 g) were dissolved in deionized water. Then adjusted the volume to 500 ml and kept at 4 ˚C.

3.6 5X sample buffer

5X sample buffer contains SDS (3.2 g), β-mercaptoethanol (5 ml), Tris-(hydroxymethyl)-aminomethene (0.75 g), glycerol (10.0 g), bromophenol blue (0.005 g) were dissolved in deionized water. Then the solution was adjusted pH to 6.8 by 6 M HCl after that adjusted the volume to 20 ml.

3.7 2X sample buffer

2X sample buffer contains SDS (0.92 g), β-mercaptoethanol (2 ml), Tris-(hydroxymethyl)-aminomethene (0.3 g), glycerol (4.0 g), bromophenol blue (0.002 g) were dissolved in deionized water. Then the solution was adjusted pH to 6.8 by 6 M HCl after that adjusted the volume to 20 ml.

4. Preparation of separating gel (12.5%) and stacking gel (4.5%)

4.1 Separating gel (12.5%)

Reagent	Volume (ml)
1.5 M Tris-HCl, pH 8.8	1.800
Acrylamide/Bis-acrylamide (30% T)	3.000
Deionized water	2.340
10% APS	0.057
TEMED	0.003
Total	**7.200**

4.2 Stacking gel (4.5%)

Reagent	Volume (ml)
0.5 M Tris-HCl, pH 6.8	0.750
Acrylamide/Bis-acrylamide (30% T)	0.450
Deionized water	1.800
10% APS	0.018
TEMED	0.003
Total	**3.021**

5. Reagent for silver staining

5.1 Fixing solution

The fixing solution contains 50 ml of 95% ethanol was mixed with 12.5 ml of glacial acetic acid. Then adjust the volume with deionized water to 125 ml and kept at room temperature.

5.2 Sensitizing solution

The sensitizing solution contains:

- 0.25 g of Sodium thiosulphate ($Na_2S_2O_3$)
- 8.5 g of Sodium acetate
- 37.5 g ml of 95% ethanol

Adjusted the volume with deionized water to 125 ml and kept at room temperature. (note: the solution was added 625 µl of 25% w/v glutaraldehyde before using)

5.3 Staining solution

0.312 g of silver nitrate ($AgNO_3$) was dissolved in deionized water and then adjusted volume with deionized water to 125 ml and kept at room temperature. (note: the solution was added 50 µl of 37% formaldehyde before using)

5.4 Developing solution

Na_2CO_3 (3.125 g) was dissolved in dissolved in deionized water and then adjusted volume with deionized water to 125 ml and kept at room temperature. (note: the solution was added 25 µl of 37% formaldehyde before using)

5.5 Stopping solution

EDTA (1.825 g) is adjusted volume with deionized water to 125 ml and kept at room temperature.

5.6 Preserving solution

95% ethanol (75 ml) was mixed with 98.5% glycerol (10 g) and was adjust the volume with deionized water to 125 ml and kept at room temperature.

6. Saturated salt for protein precipitation

6.1 Saturated $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ (76.5 g) was dissolved in distilled water (100 ml).

Appendix B

Calculation

1. Determination protein concentration

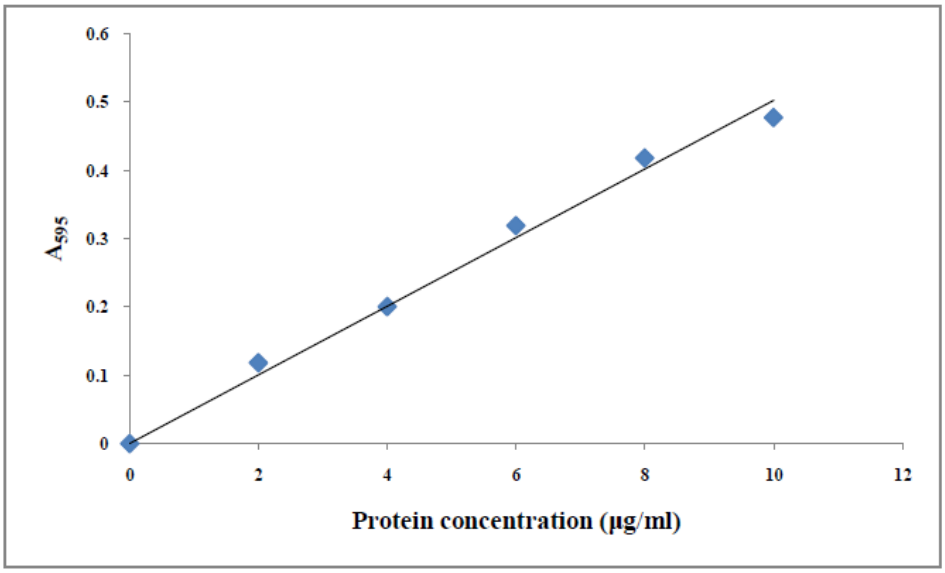

Figure 1 Standard calibration curve for the Bradford protein assay using bovine serum albumin (BSA) as the protein standard ($y = 0.050x$, $R^2 = 0.990$)

The protein concentration was calculated by using linear equation from standard curve.

According to the linear equation, $y = 0.050x$

y = The corresponding absorbance of protein sample at 595 nm

x = The protein sample concentration (µg/ml)

Therefore, the protein sample concentration was calculated by using the detected absorbance of protein sample

Example : Crude sericin extract from 120 min

From equation y	= 0.050x
If corresponding absorbance (y)	= 0.455
The protein sample concentration (x)	= 0.455/0.050
	= 9.12 µg/ml

The protein sample was mixed with Bradford reagent 0.5 : 1 ratio (total volume = 1.5 ml)

So, the total protein	= 9.12 µg/ml x 1.5 ml
	= 13.68 µg

For 0.5 ml in the reaction contained 0.020 ml of protein sample mixed with 0.480 ml of distilled water, therefore 13.68 μg was derived from 0.020 ml of protein sample

So, in 1 ml of protein sample = (13.68 μg x 1 ml)/0.020 ml

Therefore, the protein sample concentration = 684 μg/ml

2. Antioxidant activity determination by ABTS assay

Concentration of sample	**Abs. (λ734)**			**Percent inhibition**		
(μg/ml)	**1**	**2**	**3**	**1**	**2**	**3**
control	0.605	0.608	0.605			
1.875	0.498	0.496	0.499	17.63	18.02	17.58
3.75	0.392	0.395	0.395	35.15	34.66	34.77
7.5	0.235	0.234	0.230	61.16	61.38	62.04
10.5	0.121	0.117	0.118	79.94	80.61	80.44
13.5	0.041	0.040	0.039	93.22	93.44	93.50
IC_{50} (μg/ml)				6.26	6.26	6.25
X					6.2567	

Percent inhibition = [1- (Abs. sample/ Abs. control)] x 100

Percent inhibition = [1- (0.498/ 0.605)] x 100

= 17.7 μg/ml

Percent inhibition plot graph to IC_{50} value

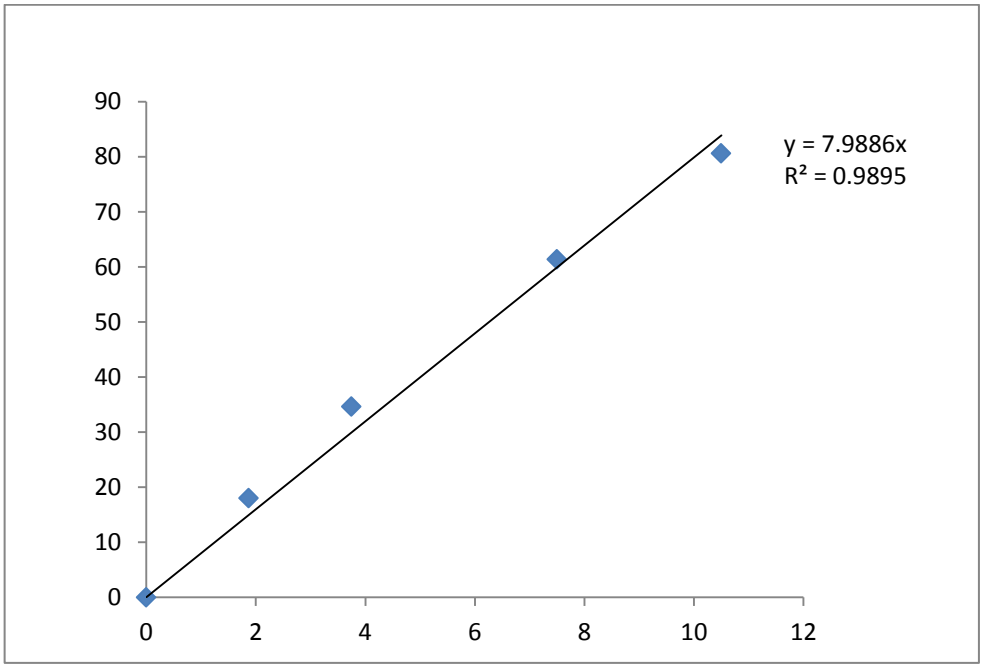

Figure 2 The relation between percent inhibition and protein concentration

y = 0.9895

IC_{50} value = 50/ 0.9895

= 6.26 µg/ml

3. Determination of molecular weight

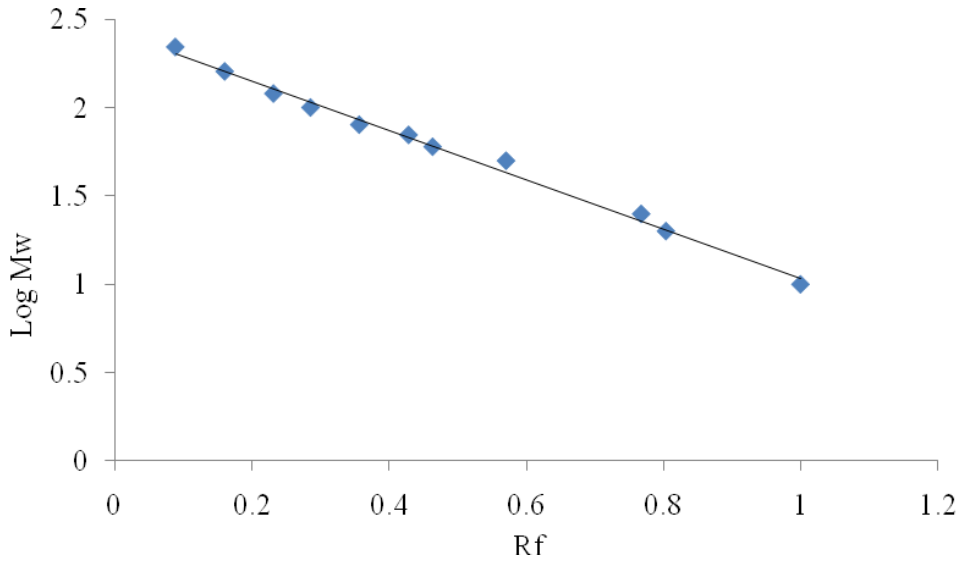

Figure 3 The standard curve of protein molecular weight.

According to the linear equation, $y = -1.371x + 2.412$

y = log molecular weight

x = relative mobility $\left(\frac{\text{distance of protein sample migration}}{\text{distance of dye front}}\right)$

Therefore, the unknown molecular weight of protein sample was calculated by using the relative mobility of protein sample

If relative mobility of sample = 0.43

So, log molecular weight of protein sample = 1.84

Anti log Mw (1.84) = 69.18 kDa

So, The Mw of protein sample = 69.18 kDa

4. The sericin content of silk cocoon

$$\text{Sericin (\%)} = \left(\frac{\text{Total weight of dry cocoon before boil} - \text{Total weight of dry cocoon after boil.}}{\text{Total weight of dry cocoon before boil}}\right) \times 100$$

boil: silk cocoons were boiled in distilled water

5. Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). One-way ANOVA using SPSS software was used to compare the mean values of each treatment. Significant differences ($p < 0.05$) among the means were determined by using Duncan's multiple range Test.

Example :

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	18.1200	.35341	.20404	17.2421	18.9979	17.72	18.39
2	3	21.3567	2.27579	1.31393	15.7033	27.0100	18.84	23.27
3	3	22.7533	3.39665	1.96106	14.3156	31.1911	19.77	26.45
4	3	23.9367	.11676	.06741	23.6466	24.2267	23.81	24.04
5	3	22.8367	.68806	.39725	21.1274	24.5459	22.06	23.37
6	3	25.0300	.79076	.45654	23.0656	26.9944	24.51	25.94
Total	18	22.3389	2.68813	.63360	21.0021	23.6757	17.72	26.45

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
4.405	5	12	.016

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	86.935	5	17.387	5.811	.006
Within Groups	35.907	12	2.992		
Total	122.842	17			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
1	3	18.1200		
2	3		21.3567	
3	3		22.7533	22.7533
5	3		22.8367	22.8367
4	3		23.9367	23.9367
6	3			25.0300
Total		1.000	.116	.161

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

BIOGRAPHY

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