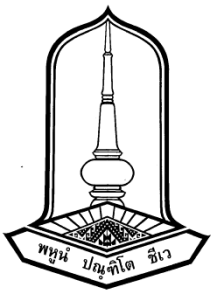

ANTIOXIDANT ACTIVITY OF FRACTIONATED AND MODIFIED SERICIN FROM *Bombyx mori* SILK COCOON

GIRAPORN SANGWONG

A thesis submitted in partial fulfillment of the requirements for

the Master of Science degree in Chemistry

Mahasarakham University

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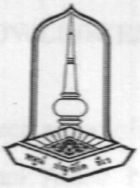

The examining committee has unanimously approved this thesis, submitted by Miss Giraporn Sangwong, as a partial fulfillment of the requirements for the Master Degree of Science in Chemistry, Mahasarakham University.

Examining Committee

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Giraporn Sangwong

TITLE Antioxidant activity of fractionated and modified sericin from *Bombyx mori* silk cocoon

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ABSTRACT

The present study investigated relatedness between sericin protein modified with dithiothreitol, β-mercaptoehanol, UV light and protease and antioxidant activity of result sericin protein. Crude sericin extracted from Thai polyvoltine silkworm cocoons (Nangnoi strain) was extracted by using distilled water at 98 ˚C for 60 min. Then, it was fractionated by salting out with saturated $(NH_4)_2SO_4$ and modified with dithiothreitol, β-mercaptoehanol, UV light and hydrolysis by protease. The antioxidant activity of fractionated and modified sericin was determined by ABTS and DPPH assay. The antioxidant activity of crude sericin extract treated with protease was 5.0 and 3.0-fold higher than untreated sericin determined by ABTS and was DPPH assay, respectively. On the other hands, dithiothreitol, β-mercaptoethanol and UV light treatments were found to decrease antioxidant activity of crude sericin extract significantly. For fractionated sericin, the antioxidant activity of colorless supernatant fraction was higher than yellow-precipitate fraction. The antioxidant activity of fractionated sericin treated with dithiothreitol, β-mercaptoehanol, UV light and hydrolysis by protease were in the same manner of crude sericin extracted. These results indicated that hydrolysis by protease could enhance the antioxidant activity of sericin extract.

Key Words: Sericin; Antioxidant; Modification; Protease, *Bombyx mori*

ชื่อเรื่อง ฤทธิ์การต้านอนุมูลอิสระของเซริซินแยกส่วนและเซริซินดัดแปลงจากรังไหม *Bombyx mori* (นางน้อย)
ผู้วิจัย นางสาวจิราภรณ์ แสงวงศ์
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บทคัดย่อ

งานวิจัยนี้ศึกษาความสัมพันธ์ระหว่างโปรตีนเซริซินที่ดัดแปลงด้วย ไดไธโอธริอิทอล เบตา–เมอแคปโทเอทานอล รังสีอัลตราไวโอเลต และด้วยเอนไซม์โปรติเอส กับฤทธิ์การต้านอนุมูลอิสระของโปรตีนเซริซิน โดยการสกัดเซริซินจากรังไหมไทยสายพันธุ์นางน้อยด้วยน้ำกลั่นที่อุณหภูมิ 98 องศาเซลเซียส เป็นเวลา 60 นาที จากนั้นแยกส่วนเซริซินด้วยเกลือแอมโมเนียมซัลเฟตและดัดแปลงเซริซินด้วย ไดไธโอธริอิทอล เบตา–เมอแคปโทเอทานอล รังสีอัลตราไวโอเลต และย่อยด้วยเอนไซม์โปรติเอส เมื่อวิเคราะห์ฤทธิ์การต้านอนุมูลอิสระของเซริซินแยกส่วนและเซริซินดัดแปลงโดยวิธี ABTS และ DPPH พบว่าสารสกัดหยาบเซริซินที่ย่อยด้วยเอนไซม์โปรติเอสมีฤทธิ์การต้านอนุมูลอิสระเพิ่มขึ้น 5 เท่า และ 3 เท่า เมื่อวิเคราะห์โดยวิธี ABTS และ DPPH ตามลำดับ ในขณะที่สารสกัดหยาบเซริซินดัดแปลงด้วย ไดไธโอธริอิทอล เบตา–เมอร์แคปโทเอทานอล และรังสีอัลตราไวโอเลต มีฤทธิ์การต้านอนุมูลอิสระลดลงอย่างมีนัยสำคัญ สำหรับเซริซินแยกส่วน พบว่าส่วนที่เป็นสารละลายใสไม่มีสี มีฤทธิ์การต้านอนุมูลอิสระสูงกว่าส่วนที่เป็นตะกอนสีเหลือง ฤทธิ์การต้านอนุมูลอิสระของเซริซินแยกส่วนที่ดัดแปลงด้วย ไดไธโอธริอิทอล เบตา –เมอแคปโทเอทานอล รังสีอัลตราไวโอเลต และย่อยด้วยเอนไซม์โปรติเอส เป็นไปในลักษณะเดียวกันกับสารสกัดหยาบเซริซิน จากผลการทดลองชี้ให้เห็นว่าการย่อยด้วยเอนไซม์โปรติเอสสามารถเพิ่มฤทธิ์การต้านอนุมูลอิสระของสารสกัดได้

คำสำคัญ: เซริซิน; ฤทธิ์ต้านอนุมูลอิสระ; โปรตีนไหมเซริซินดัดแปลง; ไหมบอมบิกซ์โมริ; โปรติเอส

CONTENS

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis (3-ethylbenzthiazoline-6-acid)
$(NH_4)_2SO_4$	Ammonium sulphate
BSA	Bovine serum albumin
CSE	Crude sericin extract
DPPH	2,2-diphenyl-1 picrylhydrazyl
DTT	Dithiotheitol
M	Molar
mA	Milliampere
β-ME	β-mercaptoethanol
mM	Milimolar
nm	Nanometer
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylaminde Gel Electrophoresis
SNT	Supernatant
Trp	Trytophan
Tyr	Tyrosine
UV	Ultraviolet
Yellow-PT	Yellow-precipitate

CHAPTER 1

INTRODUTION

1.1 Background

Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. An antioxidant is a molecule that inhibits the oxidation of other molecules. The antioxidant compounds in food play an important role as a health-protecting factor. The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported. (Miller *et al.,* 2000). Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and inhibit the oxidative mechanisms that lead to degenerative diseases.

Silk protein compose of two protein components, fibroin and sericin. Sericin is a family of major cocoon proteins specifically synthesized in the middle silk gland of the silkworm *Bombyx mori*, constitutes about 20-30% of total cocoon weight (Zhang, 2002). Sericin has been found to possess various biological functions, such as antioxidant and antityrosinase activities (Aramwit *et al*., 2010; Kato *et al*., 1998).

There are a large number of polyvoltine silkworm strains which produce Thai silkworm cocoons. The individual strain exhibits different properties such as cocoon shape, color, total cocoon weight, and antioxidant activity. For Thai polyvoltine silkworm cocoons, they are composed of proteins and pigments (carotenoids and flavonoids) (Tabunoki *et al*., 2004; Tamura *et al*., 2002). Pigments are commonly known for their biological properties, including antioxidant activity (Heim *et al*., 2002; Khanam *et al*., 2012; Wu *et al*., 2015). Recently, use of natural protein extract or purified proteins as antioxidant has attracted particular interest. Many food proteins from milk, soy bean, fish and corn were reported to have antioxidant activity

(Chalamaiah *et al*., 2012; Pihlanto, 2006; Ranamukhaarachchi *et al*., 2013; Wang *et al*., 2014). Moreover, there are reports that the chemical and physical treatments (DTT, (*β*-ME), UV light and protease enzyme) increased the antioxidant activity of albumin, whey protein and chickpea protein (Li *et al*., 2008; Medina-Navarro *et al*., 2010; Chen *et al*., 2012). The antioxidant activity of proteins mostly depends on their structure and amino acid compositions. However, no studies are present on effect of some chemical and physical treatments on the structure of sericin protein and on antioxidant activity, particularly from *Bombyx mori* silkworm strain, Nangnoi.

In this study, the sericin from cocoons of polyvoltine silkworm strain, Nangnoi, was extracted and fractionated by salting out, and modified with DTT, *β*-ME, UV light exposure and hydrolysis protease enzyme. In addition, the antioxidant activity of fractionated sericin with respect to some chemicals or physicals treatments was also investigated.

1.2 Research Objectives

1. To determine the antioxidant activity of modified sericin from *Bombyx mori* silk cocoon with DTT, *β*-ME, UV light and protease

2. To identify the antioxidant activity of fractionated sericin from *Bombyx mori* silk cocoons

1.3 Scope of Research

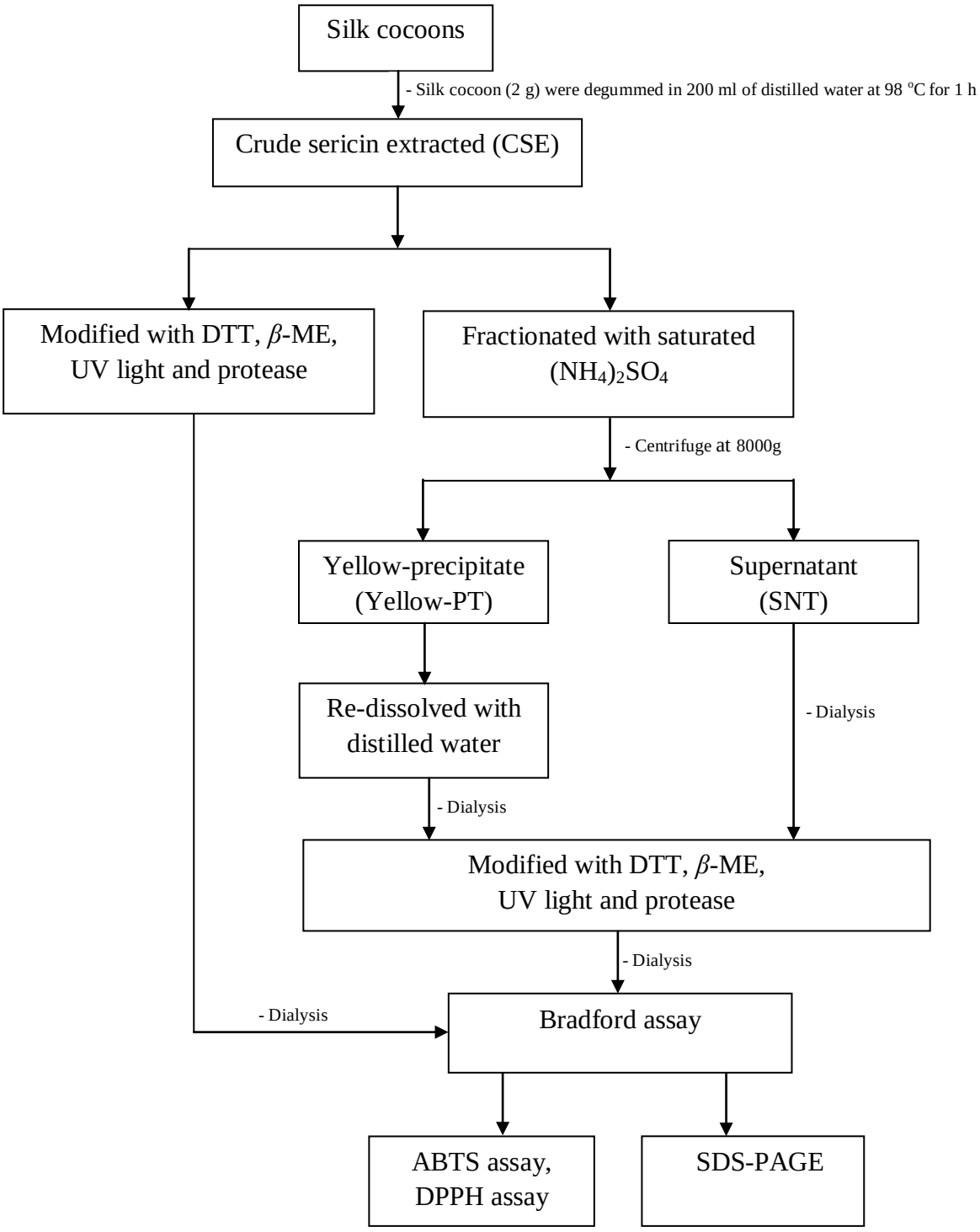

Figure 1.1 Scope of research

1.4 Expected Outcomes

To know antioxidant property of fractionated protein and its mechanism with respect to chemical and physical treatment

1.5 Place of Work

Department of Chemistry and Silk Research Unit, 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**.**
Bombyx mori	a silk moth feeding on mulberry leaves
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.
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
Protease enzyme	any enzyme that catalyzes the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis
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
Salting-out	a method of separating protein based on the principle that proteins are less soluble at high salt concentration
Sericin	a protein created by silkworm covering a fibroin
Silver staining	a staining procedure for detecting proteins separated by gel electrophoresis silver nitrate
Ultraviolet	Invisible solar radiation that lies just beyond the violet end of the visible spectrum in the wavelength range from 10 to 400 nanometers and can harm living tissue.

CHAPTER 2

LITERATURE REVIEW

2.1 Silk characteristics

Silk is a natural protein fiber composed of two kinds of protein (sericin and fibroin) as shown in Figure 1 (Wu *et al*., 2007; Poza *et al*., 2002; Ki *et al*., 2007). Silk is obtained from the cocoons of the larvae of the mulberry silkworm, Bombyx mori. Fibroin is insoluble in hot water while the sericin is a kind of water soluble globular protein. Silk protein similar to other filament proteins namely, collagen, elastin, keratin, fibroin, sporngin etc. (Komatsu., 1975). Silks are produced by several other insects, but generally only the silk of moth caterpillars has been used for textile manufacturing. There has been some research into other silks, which differ at the molecular level (Sutherland *et al*., 2010). Silk derived from silkworm (*Bombyx mori*) is a natural protein that is mainly made of sericin and fibroin protein. Sericin constitutes about 20 – 30% of the total cocoon weight consists of 18 kind of amino acids and it envelops the fibroin fiber with successive sticky layers that help in the formation of a cocoon.

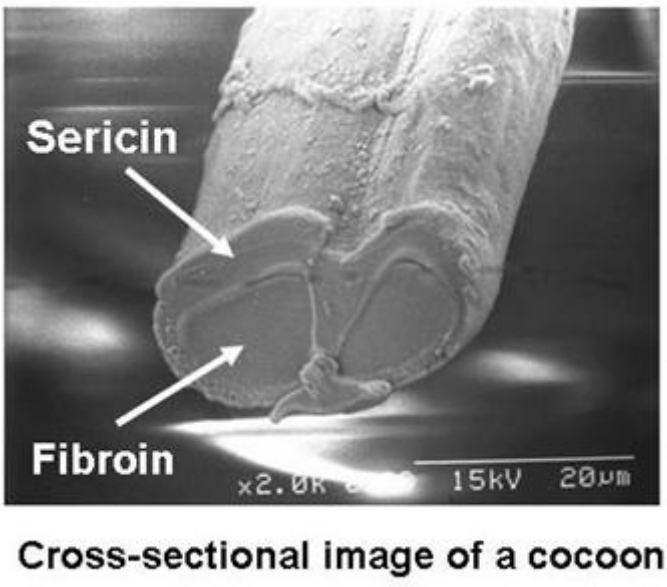

Figure 2.1 Composition of silk fiber

2.2 Thai silk (Nangnoi strain)

Thai silkworms are fed on mulberry leaves. There are several native strains of silkworm with various colors (yellow to green of cocoon shells) in Thailand. Silkworm is produced primarily in the Northeast of Thailand.

Silks exhibit different composition, biochemical properties and structure depending on the specific source and strain. For Thai polyvoltine silk, particularly the yellow Nangnoi silk, contains of protein and pigments (carotenoids and flavonoids). Both protein and pigments are responsible for biological properties, including antioxidant activity of Bombyx mori cocoons (Kato *et al*., 1998; Wu *et al*., 2008; Prommuak *et al*., 2008). In addition, antioxidants represent a significant target in the fields of agriculture, food and medicine, which has led to widespread screening for compounds with potent antioxidant activity.

Figure 2.2 Yellow Nangnoi silk cocoons

2.3 Sericin

The sericin from *Bombyx mori* consists about 20–30% of total cocoon, a group of proteins ranging from 20–400 kDa, three major fraction of sericin have been isolated with molecular weights of 150, 250 and 400 kDa (Sprague, 1975; Takasu *et al*., 2002). The sericin protein is made of 18 amino acids, which have strongly polar side groups like amino groups and hydroxyl carboxyl (Zhang, 2002). Sericin is a natural protein derived from silkworm, *Bombyx mori*. Sericin is a kind of water-soluble globular protein and has been partially characterized in the domesticated mulberry silkworm (*Bombyx mori*). When sericin was hydrolyzed in alkaline or acid solutions, dissolved in a polar solvent, hydrolysis protease enzyme, the size of the sericin molecules depends on factors such as pH, processing time and temperature (Aramwit *et al*., 2010). Sericin remains in a partially unfolded state, with 35% α-sheet and 63% random coil, and with no α-helical content (Tsukada *et al*., 1981). Five polypeptides of sericin (ser-1, ser-2, ser-3, ser-4 and ser -5) have been reported from different sections of the middle silk gland of *Bombyx mori*, which ser-1, ser-2 and ser-3 are the major components (Gamo et *al*., 1977).

Sericin peptides can be applied in cosmetics formulations (Cho *et al*., 2003). Sericin peptides showed functional properties to development of new products such as metal ion-chelating activity and antioxidant property due to the high amount of hydroxyl bonds, derived from some amino acids such as aspartic acid and serine (Wu *et al*., 2007). In addition, the sericin exhibited an pharmacological functions, excellent moisture absorption and released properties antibacterial, and UV resistance (Dash *et al*., 2009).

2.4 Applications of sericin

Silk sericin is susceptible to the action of proteolytic enzymes present in body and hence it is digestible. This property makes it a biodegradable and biocompatible. Due to some additional properties as, moisture retention capacity, skin adhesion and gelling ability, sericin has widely applied in cosmetic fields, medical and pharmaceutical.

2.4.1 Medical and pharmaceutical applications

Sericin is a water soluble-globular protein. It has been found some medical applications such as anticancer drugs, anticoagulant activity and antioxidant activity. Sericin was conversion of α-random coil to β-sheet structure gives gel (Jun *et al*., 1997). Hirabayashi *et al*. (1989) was found that sericin solution (1%) produces gel at pH 6-7 at room temperature and gelation speed increase as the increase concentration of sericin. The sericin solution containing sericin (1.5 and 2 % w/w) obtained from autoclave at 105 ℃ for 30 min does not show good gelling. Sericin gel in the presence of propylene glycol, tween-80 and glycerin shows synerisis, whereas with carbopol and pluronic gives at able gels. In the presence of pluronic sericin gel it shown concentration dependence (Padamwar *et al*., 2003). Study was reported that the concentration of pluronic and temperature on the gel property of sericin (Kenwon *et al*., 2000). The gelling of sericin accelerated with increase ploxamer concentration and increase temperature, whereas the sol-gel transition of sericin becomes irreversible. Blends of sericin and polyvinyl alcohol are cross-linked to give hydrogels. Hydrogels with good water resistance mechanical strength and water resistance are produced by casting aqueous solution containing dimethyl urea and sericin on a plate and heating at 80 and 120 ℃ for 1 and 3 hr, respectively (Nakamura *et al*., 2001).

Sericin and fibroin show antithrombotic effect. One stage condensation of formaldehyde, sericin and salicylic acid creates a copolymer with molecular weight of 6000-8000 kDa. A concentration with 0.01–1.00 mg/ml in blood exhibits anticoagulant, antiaggreagation activity and fibrinolytic toward thrombocytes at 0.5 mg/ml (Khudaiberdiev, 1997). Sericin molecular weight with 1,000,000 shows an inhibitory action for lipid peroxidation and tyrosinase with rat brain homogenates (Kato *et al*., 1998). The addition of sericin (0.1-2.0 mg/mlsericin) into the aqueous solution shows heat resistant DNA polymerase activity (Yamaji, 1998). Sericin has been found to possess wound healing property and can be used as wound healing covering material in the form of film (Wu *et al*., 1996). Sericin also has ashesive propertry due to its chemical composition. It has affinity to keratin (Voegeli *et al*., 1996). Silk threads obtained from mulberry silkworm can be used for making surgical sutures (Gapurova, 1983). Sericin membranes are good bandage material and the film has tensile strength and adequate flexibility. Its good infection resistant nature and biocompatibility, it is a

novel wound coagulant material. Additionally, its flexibility and water absorption properties promote smooth cure for defects in the skin and do not cause any peeling of the akin under regeneration when detached from the skin.

Intake of sericin containing food relives constipation, suppresses development of bowel cancer and accelerates the absorption of minerals. In rats, consumption of sericin elevates the apparent absorption of zine, iron, magnesium and calcium by 41, 41, 21 and 17%, respectively (Sasaki *et al*., 2000). A dietary supplementation of sericin (4%) suppresses induced constripation in rats because of its low digestibility along with water holding capacity (Sasaki *et al*., 2000). Sericin, when given orally, causes a dose dependent decrease in the development of colonic aberrant crypt foci. The incidence and the number of colon tumors are suppressed by consumption of sericin. Sericin has antitumor activity (Kato & Sasaki, 2000).

2.4.2 Cosmetic applications

For the application of pharmaceutical and medical uses purpose, sericin has been applied as component of cosmetic fields. Sericin combination with fibroin or alone has been applied in skin, nail and hair cosmetics. Sericin was used in the form of cream, ointment and lotion had been shown to increase skin elasticity with antigaing and antiwrinkle effects.

Padamwar *et al*. (2002) was reported that the moisturizing property of the sericin gel, investigated by trance epidermal water loss (TEWL), impedance measurement, scanning electron microscopy (SEM) and hydroxyproline assay. Sericin gel decrease skin impedance and increase the hydroxyproline content in stratum corneum, which reveals moisturizing property of sericin. Sericin gels with carbopol and pluronic, as moisturizer by repairing natural moisturizing factor (NMF) as well as prevent water loss from the skin. SEM has shown been the decrease flaking and cracking as compare to dry skin and normal skin replicas.

The powder of sericin (5-30%) with molecular weight 7,000–300,000 and silk fibroin (70–95%) was applied as film shows moisture absorbability. Sericin hydrolysate solution has been proven to control that dermatitis. Sebum and sweat absorbing type of cosmetics containing cellulose fiber impregnated with sericin solution and fibroin dispersion are also reported. Lotion containing D-glucose (4% w/w) and sericin (1% w/w) shows conditioning and moisturizing effect. Creams containing sericin

(0.001–30% w/w) could improve cleansing properties with less skin irritation. Sericin powder in form of sericin hydrolysate coated talc, rion oxide, nylon, titania and mica have been applied to formulate foundation cream and eyeliners. The nanocapsules or microcapsules consisting of polysiloxane gel, UV absorbent core treated titanium dioxide, silicon treated iron oxides, for cosmetic foundation has a SPF value (25.7). Sericin in sunscreen composition enhances the light screening effect of UV filter such as cinnamic acid ester and triazines.

Nail cosmetics, containing sericin (0.02-20%) was reported to prevent nail from, brittleness and imparting the inherent gross to nail (Yamada *et al*., 2001). Chapping bath and hair preparations, containing olive oil (0.01-1%) and sericin (0.02-2%), fatty acid or their salts show reducing of hair surface by binding of sericin to hairs (Hoppe *et al*., 1984). Sericin hydrolysate with molecular weight of 300–3000 kDa was used as conditioner for hair and moisturizer for skin (Hata, 1987). Shampoo containing pelarogenic acid with pH less than six and sericin is useful for the cleaning and care of hairs (Engel & Hoppe, 1988).

2.5 Disulfide bridges in proteins

Disulfide linkages between two cysteine residues are an integral component of the three-dimensional structure of many proteins. The inter conversion between thiols and disulfide groups is a redox reaction: the thiol is the reduced state, and the disulfide is the oxidized state.

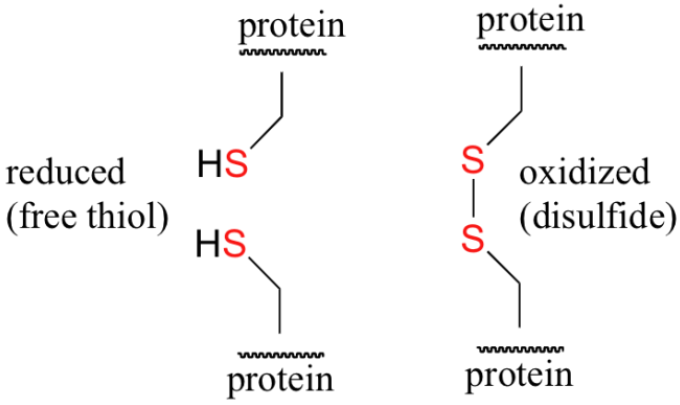

Figure 2.3 The disulfide is the oxidized state and the thiol is the reduced state of disulfide group

Notice that in the oxidized state, each sulfur atom has lost a bond to hydrogen and gained a bond to a sulfur, this is why the disulfide state is considered to be oxidized relative to the thiol state. Disulfide bridges in proteins can also be directly reduced by another flavin-dependent enzyme called "thioredoxin". In both cases, NADPH is the ultimate electron donor, reducing FAD back to $FADH_2$ in each catalytic cycle. In the biochemical lab, proteins are often maintained in their reduced (free thiol) state by incubation in buffer containing an excess concentration of β-mercaptoethanol or dithiothreitol. These reducing agents function in a manner similar to that of GSH, except that DTT, because it has two thiol groups, forms an intramolecular disulfide in its oxidized form.

2.5.1 Dithiothreitol

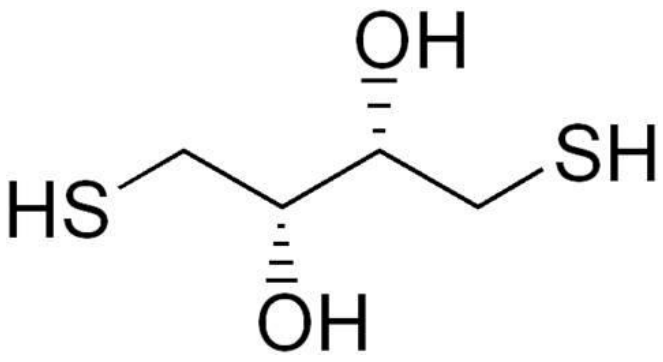

Figure 2.4 Dithiothreitol chemical structure

Dithiothreitol or Cleland's Reagent is a chemical used to reduce disulfide bonds maintain monothiols and quantitatively in the reduced state (Cleland, 1964). For low concentrations, dithiothreitol stabilizes enzymes which possess free sulfhydryl groups and has been shown to restore activity lost by oxidation of these groups *in vitro*. Dithiothreitol can readily permeate cell membranes allowing it to be used in pharmaceutical applications for high yield extraction and isolation of proteins (Hart *et al*., 1970). For higher concentrations, dithiothreitol is an effective protein denaturant which cleaves disulfide linkages between cysteine groups in proteins and peptides. Dithiothreitol is highly soluble in water (clear solution, OD < 0.05 at 0.02 M), but also in chloroform, ethyl acetate, ethanol and ether.

2.5.1.1 Reaction

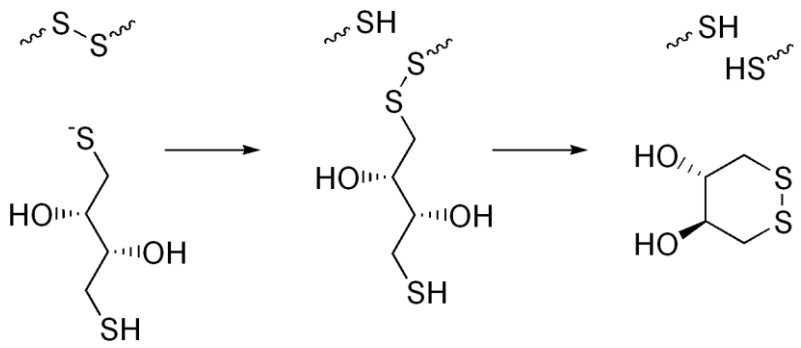

Figure 2.5 Reduction of a typical disulfide bond by dithiothreitol via two sequential thiol-disulfide exchange reactions

Dithiothreitol participates to disulfide exchange reaction that drives its major applications. Dithiothreitol is used typically at 1 to 10 mM for protein S-S reduction. It readily crosses biological membranes. Reducing properties dithiothreitol is an unusually strong reducing agent, with a redox potential at pH 7 for -0.33 V. The reduction of a typical disulfide bond proceeds by two sequential thiol-disulfide exchange reactions and is illustrated below. The reduction usually does not stop at the mixed disulfide species because the second thiol of dithiothreitol has a high propensity to close the ring, leaving behind a reduced disulfide bond and forming oxidized dithiothreitol. The reducing power of dithiothreitol is limited to pH values above 7, since only the negatively charged thiol form - S - is reactive; the pKa of the thiol groups is 9.2 and 10.1.

2.5.2 *β*-mercaptoethanol

Figure 2.6 *β*-mercaptoethanol chemical structure

β-mercaptoethanol is usually included in a sample buffer for SDS-PAGE at a concentration of 5% *β*-mercaptoethanol thiol group is of typical reactivity and ionizes with a pKa of about 9.6 and is suitable for reducing protein disulfide bonds prior to polyacrylamide gel electrophoresis. Cleaving inter-molecular disulfide bonds allows the subunits of a protein to separate independently on SDS-PAGE. Cleaving intra-molecular disulfide bonds allows the subunits to become completely denatured so that each peptide migrates according to its chain length with no influence due to secondary structure. *β*-mercaptoethanol has limitations in this respect that should be recognized. Its thiol group is not a very potent reductant, very similar to that of glutathione. The equilibrium constant for thiol-disulfide exchange between these two molecules, has been measured with glutathione and will be about the same for *β*-mercaptoethanol. Therefore, a large excess of *β*-mercaptoethanol will be required to reduce stable protein disulfide bonds.

2.6 Ultraviolet radiation (UV)

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

and 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 blocked by air and is not very penetrating (International standard ISO 21348, 2007).

There are reported that the effects of UV light in genes or their protein and cells (Semagoto *et al*., 2014; Devies *et al*., 2001; Kehoe *et al*., 2008). Protein is major cellular targets for direct photo-oxidation due to 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. 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 (Pattison *et al*., 2012). 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).

2.6.1 Tryptophan

Flash photolysis studies have reported two non-radiative relaxation channels from the singlet excited state of tryptophan. Electron ejection to the solvent, yielding solvated electrons, e^-_{aq} which has a broad absorption peak centred (~720 nm) and the tryptophan radical cation ($Trp^{\bullet+}$) which has its maximum absorption (~560 nm). Deprotonates rapidly, yielding the neutral radical $Trp^{\bullet+}$ that has its maximum absorption (~510 nm). Intersystem crossing, yield the triplet-state ^3Trp which has its maximum absorption (~450 nm). The triplet state tryptophan can transfer an electron to a nearby disulphide bridge to give $Trp^{\bullet+}$ and the disulphide bridge electron adduct $RSSR^{\bullet-}$, where the latter has its maximum absorption at ~420 nm (Bent & Hayon, 1975a).

$$Trp + h\nu \rightarrow Trp^{\bullet+} + e^-_{aq} \quad (1)$$

$$Trp^{\bullet+} \rightarrow Trp^{\bullet} + H^{+} \quad (2)$$

$$^{1}Trp + h\nu \rightarrow {}^{1}Trp^{*} \quad (3)$$

$$^{1}Trp^{*} \rightarrow {}^{3}Trp \quad (4)$$

$$^{3}Trp + RSSR \rightarrow Trp^{\bullet+} + RSSR^{\bullet-} \quad (5)$$

2.6.2 Tyrosine

Another aromatic residue with non-negligible absorption in the near UV region is tyrosine (Tyr-OH). Photoexcited tyrosine can fluoresce, decay non-radiatively, or undergo intersystem crossing to the triplet state, from which most of the photochemistry proceeds. Alternatively, at neutral pH, tyrosine can be photoionized through a biphotonic process that involves absorption of a second photon from the triplet state. This result in a solvated electron (e^{-}_{aq}) and a radical cation ($Tyr\text{-}OH^{\bullet+}$) that will rapidly deprotonate to create to create the neutral radical ($Tyr\text{-}OH^{\bullet}$). Photoionization of tyrosinate at high pH is monophotonic and results in a neutral ($Tyr\text{-}O^{\bullet}$) and a solvated electron (e^{-}_{aq}). The triplet state tyrosine is rapidly quenched by disulphide bridges or molecular oxygen or nearby residues like tryptophan (Bent & Hayan, 1975b).

$$^{3}Tyr - OH + h\nu \rightarrow Tyr\text{-}OH^{\bullet+} + e^{-}_{aq} \quad (6)$$

$$Tyr\text{-}OH^{\bullet+} \rightarrow Tyr\text{-}OH^{\bullet} + H^{+} \quad (7)$$

$$Tyr\text{-}O^{\bullet-} + h\nu \rightarrow Tyr\text{-}O^{\bullet} + e^{-}_{aq} \quad (8)$$

$$^{3}Tyr - OH + RSSR \rightarrow Tyr\text{-}O^{\bullet} + H^{+} + RSSR^{\bullet-} \quad (9)$$

2.6.3 Cysteine and Cystine groups

A common mechanism of photo-oxidation of disulfide bonds is *via* electron transfer from ^{3}Trp or ^{3}Tyr species to cystine, with this resulting in the formation of disulfide radical anions ($RSSR^{\bullet-}$) (Bensasson *et al.*, 1993; Creed, 2008a; Creed, 1984b; Creed, 1984c; Bent & Hayon, 1975a; Bent & Hayon, 1975b; Vanhooren *et al.*, 2006). The generated solvated electrons can subsequently undergo fast geminate recombination with their parent molecule, or they can be captured by electrophilic species like molecular oxygen, H_3O^{+} (at low pH) and cystines as summarized below:

$$e^{-}_{aq} + O_2 \rightarrow O_2^{\bullet-} \quad (10)$$

$$e^-_{aq} + H_3O^+ \rightarrow H^\bullet + H_2O \quad (11)$$

$$e^-_{aq} + RSSR \rightarrow RSSR^{\bullet-} \quad (12)$$

In the case where the electron is captured by the cystine, the result can also be breakage of the disulphide bridge (Hoffman & Hayon, 1972):

$$e^-_{aq} + RSSR \rightarrow RSSR^{\bullet-} \quad (13)$$

$$RSSR^{\bullet-} \leftrightarrow RS^\bullet + RS^- \quad (14)$$

$$RSSR^{\bullet-} + H^+ \leftrightarrow RS^\bullet + RSH \quad (15)$$

2.7 Protease enzyme

Protease enzymes are produced commercially from plants, animals and microbial sources. Proteases refer to a group of enzymes whose catalytic function is to hydrolyze (breakdown) proteins. They are also called systemic or proteolytic enzymes**.** Proteases are the most important industrial enzymes and widespread in nature. Microbial proteases belong to acid, alkaline or neutral based on their pH optimum for activity, may depend on the composition of the medium.

Protease catabolizes proteins by hydrolysis of peptide bonds. Proteases are inactivated by serine active-site inhibitors such as diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride (PMSF). Protease from *Streptomyces griseus* is a mixture of at least three proteolytic activities including an extracellular serine protease. Serine proteases display a wide range of substrate specificities, which are believed to be mediated by an active site composed of one His, one Asp, and a Ser residue in the molecule. This enzyme prefers to hydrolyze peptide bonds on the carboxyl side of aspartic or glutamic acid. This product is a mixture of at least one aminopeptidase activity and three caseinolytic activities. The caseinolytic enzymes were named as *Streptomyces griseus* Protease A, *Streptomyces griseus* Protease B and *Streptomyces griseus* Trypsin. This product may be used when extensive or complete degradation of protein is required. This protease mixture is highly nonspecific and can digest casein to the extent of >70% as mono-amino acids. Completely inactivated by heating above 80 °C for 15-20 minutes (SIGMA-ALDRICH).

2.8 Protein determination by Bradford assay

The Bradford protein assay is very fast and simple uses about the same amount of protein as the Lowry assay. Moreover, the assay is comparatively free from interference by common reagents except detergents. The method is based on the proportional binding of the dye coomassie to proteins. The assay is based on the observation that the absorbance shift 470-595 nm when binding to protein occurs in an acidic solution of Coomassie Brilliant Blue G-250. The dye is believed to bind to protein via electrostatic attraction of the dye's sulfonic acid groups. The mechanism of dye binding can be explained by the dye existing as three absorbing species, a red cationic species (λ_{max} 470 nm). A green neutral species (λ_{max} 650 nm), and blue anionic species (λ_{max} 595 nm). Color change are due to successive loss of change of charge. 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 from is stabilized and is detected at 595 am (Ahmed, 2005).

2.9 Sodium dodecyl sulfate-polyacrylaminde gel electrophoresis (SDS-PAGE)

The separation of macromolecules in an electric field is called electrophoresis is influenced by both the charge of the size of the protein and the protein at the chosen pH and attendant frictional resistance as they migrate in the electric field. The method is very common for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. SDS confers a negative charge to the polypeptide in proportion to its length. The negative charge polypeptide migration-depend on their size towards the anode by applied electric flied. Furthermore, the charge-to-mass ratio will be essentially identical for different protein because the SDS coating dominates the charge. Thus, the separation of the denatured, detergent-coated polypeptide chains will be due almost exclusively to the size of the polypeptide chain.

2.10 Antioxidant activity determination by 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).

2.11 Antioxidant activity determination by DPPH assay

The molecule of 2,2-diphenyl-1 picrylhydrazyl (DPPH) (α, α-diphenyl-β-picrylhydrazyl; DPPH) is a basis of a common antioxidant assay based on electron transfer that produces a violet solution in ethanol. Absorbance was measured at 517 nm. When DPPH solution reacts with an antioxidant compound, it is can donate hydrogen atom transfer and electron in at least four different sequences (McGowan *et al*., 1959; Hogg *et al*., 1961; Foti *et al*., 2008). Then this gives rise to the reduced form with the loss of this violet colour (from violet to light yellow). The DPPH assay provides an easy to evaluate antioxidant activity by spectrophotometry (Huang *et al*., 2005). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

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

The first step, A• is free radical and ZH is the reduced form. This latter radical will then undergo further reactions which control the overall stoichiometry, the number of molecules of DPPH reduced (decolorized) by a molecule of the reductant. The reaction is therefore intended to provide the link with the reactions taking place in an oxidizing system, such as the autoxidation of a lipid or other unsaturated substance. The Z• is intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH.

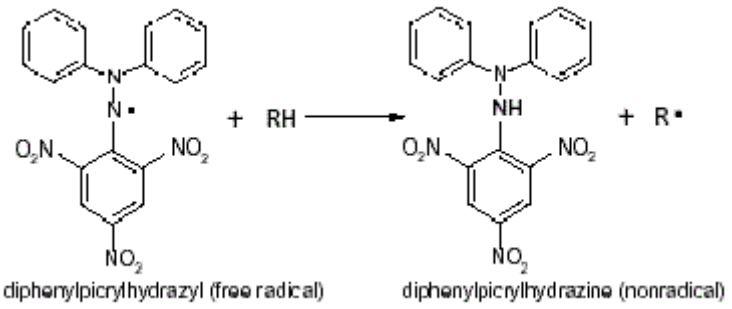

Figure 2.7 Structure of DPPH

2.12 Protein precipitation by Salting – Out

Salting out is the 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.

2.13 Related literature

Kato *et al.* (1998) studied for an antioxidant activity of the silk protein sericin by showing that sericin suppressed *in vitro* lipid peroxidation. The sericin was found to inhibit tyrosinase activity.

Wu *et al.* (2007) studied a new effective technology for the extraction of sericin from silk wastewater. From that study, they found the biological activities of sericin powder, including tyrosinase inhibitory activity and antioxidant activity, which functions are important for potential application of sericin to food manufacturer.

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.

Prommuak *et al.* (2008) studied an extraction of flavonoids and carotenoids from yellow Thai silk waste. The total amount of carotenoids present in the extract was 0.7 mg/g dry weight, of which about 60% was located in fibroin and 40% in sericin. The total flavonoid was found approximately 5.1 mg/g dry silk, and 70% of which was located on fibroin and 30% in sericin. In addition, the extracts of the yellow pigment of Thai silk waste show effective antioxidant activity, with the IC_{50} values lie between 15.5 and 23.3 μg/ml.

Chlapanidas *et al.* (2013) studied different biological properties of sericin were investigated based on the *B.* mori strain. They found that sericin exhibits different activities between strains, while diet only seems to influence antityrosinase activity. Moreover, ROS-scavenging and anti-tyrosinase activities were strongly correlated, while the correlation between anti-elastase and ROS-scavenging activity was low, although significant. No correlation between anti-elastase and anti-tyrosinase activities was evidenced.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

The yellow cocoons of the silkworm, *Bombyx mori*, strain Nangnoi, were obtained from the Center of Excellence for Silk Innovation, Mahasarakham University, Thailand

3.2 Sericin extraction

Silk cocoons (2 g) were added into 200 ml of distilled water with different extraction time for 15, 30, 60, 90 and 120 min at 98 ˚C to obtain crude sericin extracts (CSE). Protein concentration and antioxidant activity of CSE were determined by Bradford assay and DPPH assays, respectively.

3.3 Protein determination by Bradford assay

Sericin extract was diluted with distilled water. Sample (0.5 ml) was added to 1.0 ml of Bradford solution and incubated at room temperature for 5 min. Bovine Serum Albumin (BSA) was used as a standard reference protein. The absorbance of samples was measured at 595 nm with visible spectrophotometer (Thermo Spectronic, U.S.A.).

3.4 Protein fractionation by salting-out with saturated $(NH_4)_2SO_4$

Saturated $(NH_4)_2SO_4$ was added to CSE (10.0 ml) until whole yellow colored-protein precipitated. Then, it was centrifuged at 8000g for 5 min to separate yellow-precipitate (Yellow-PT) and supernatant (SNT). The Yellow-PT was re-dissolved in distilled water and both samples were then dialyzed against distilled water for 5 h. Then, the protein concentration of sample was measured by Bradford assay and antioxidant activity was measured by ABTS and DPPH assays.

3.5 Treatment with dithiothreitol and *β*-mercaptoethanol

CSE (50.0 ml) and SNT were treated with DTT. Samples containing 1,200 µg/ml of protein were incubated with 0.5, 1, 5, 10 and 20 mM of DTT for 10 min at room temperature and then dialyzed against distilled water for 24 h. Protein concentration of the samples was determined by Bradford assay and antioxidant activity of the samples was determined by ABTS and DPPH assays. Treatment with *β*-ME was performed in the same manner of treatment with DTT except concentration of *β*-ME used at 1, 5, 10, 20 and 30 mM.

3.6 Treatment with UV light

CSE, Yellow-PT and SNT of 300 µg/ml were exposed to UV light (254 nm) for 10 minutes at room temperature. Antioxidant activity of the samples was determined by ABTS and DPPH assays.

3.7 Treatment with protease

CSE, Yellow-PT and SNT were treated with protease from *Streptomyces griseus* (Type XIV, ≥3.5 units/mg solid, E.C. 232-90-5) at 37 ˚C, pH 7.5 for 3 h. The enzyme-to-substrate protein ratio was 4:100 (w/w). The pH of the sample solutions was adjusted for enzymatic hydrolysis with 0.1 mM NaOH. The enzymatic reaction was stopped by heating at 90 °C for 20 min. Protein concentration of the samples was determined by Bradford assay and antioxidant activity of the samples was determined by ABTS and DPPH assay. Molecular mass of the samples was estimated by SDS-PAGE.

3.8 Antioxidant activity determination by ABTS assay

Experiments were performed according to Re *et al*. (1999) with slight 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 was allowed to stand in the dark at room temperature for 16 h before using in order to produce ABTS radical cation ($ABTS^{\bullet+}$). For the study of antioxidant activity, ABTS radical cation 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 absorbance was measured at 734 nm. The total antioxidant capacity was expressed as percent inhibition, according to the equation:

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

3.9 Antioxidant activity determination by 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:

Radical scavenging activity (%) = [1- (Abs. sample/Abs. control)] × 100

3.10 Determination of molecular mass of sericin proteins by SDS-PAGE

The molecular mass of sericin proteins was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separating gel and staking gel were 12.5% and 5%, respectively. The sample solution was mixed with 2x buffer and heated in hot water, then sample was loaded into the well. The electric current of 150 volt, 50 mA was applied to the gel. At the end of electrophoresis, the gel was

stained with silver staining technique. Standard molecular mass marker was applied for estimating the molecular mass.

3.11 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 Protein concentration and antioxidant activity of CSE

Protein concentrations of CSE with different extraction time (15, 30, 60, 90 and 120 min) were determined by Bradford assay. The protein concentration showed an obvious increase with increased extraction time as shown in Figure 4.1. The longest time of extraction of 120 min showed the highest protein concentration (879 µg/ml).

Antioxidant activity of CSE with different extraction time (15, 30, 60, 90 and 120 min) was evaluated by ABTS and DPPH assay as shown in Figure 1. Antioxidant activity of CSE with different extraction time was reported as IC_{50} values in which lower IC_{50} value indicates higher antioxidant activity. CSE from 60 min showed the highest antioxidant activity (ABTA assay = 18.27 µg/ml, DPPH assay = 66.56 µg/ml) while CSE from 120 min showed the lowest antioxidant activity. Studies reported that heat treatment induced conformational and functional changes of proteins, including antioxidant activity (Plancken *et al*., 2006; Tang *et al*., 2009; Raikos, 2010; Rondeau *et al*., 2007). In this study we found that CSE lose their activity with increased extraction time. Similar finding was reported by Tang *et al*. (2012), the modification of soy protein using heat treatment decreased antioxidant activity with increased treatment time. This result suggested that antioxidant activity of CSE might be related to its conformational changing according to extraction time.

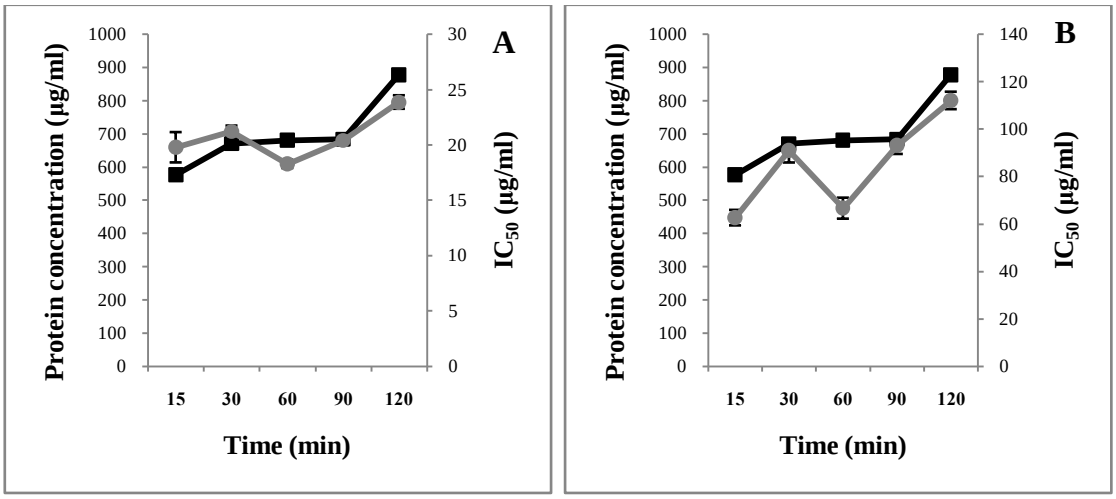

Figure 4.1 Protein concentration (—■—) and Antioxidant activity (—●—) of crude sericin extracted in hot water at 15, 30, 60, 90, and 120 min as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

4.1.1 Effect of treatment with dithiothreitol and *β*-mercaptoethanol

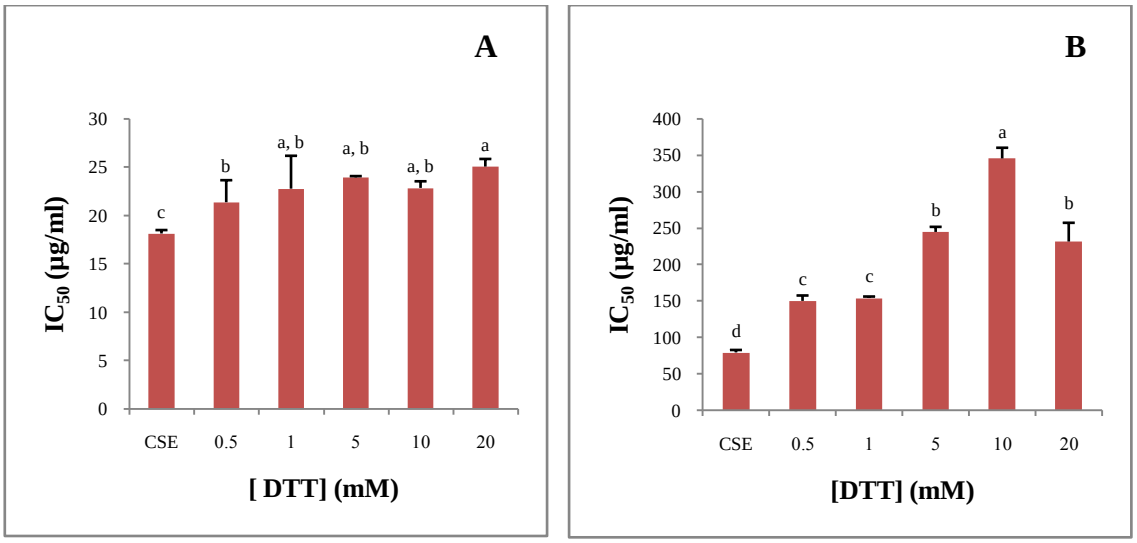

Figure 4.2 Antioxidant activity of crude sericin extracted treated with different concentration of DTT as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

Antioxidant activity of CSE treated with 0.5 to 20 mM of DTT measured by ABTS assay and DPPH assay are shown in Figure 4.2. Antioxidant activities of CSE treated with different concentration of DTT were compared based on the value of IC_{50}. Untreated CSE showed the lowest IC_{50} value (ABTS assay = 11.12 µg/ml, DPPH assay = 79.25 µg/ml) of antioxidant activity when compared to CSE treated with different concentration of DTT, indicating that untreated CSE had the strongest antioxidant activity. Also similarly with the result from DTT treatment, the antioxidant activity of CSE treated with 1 to 30 mM of *β*-ME were lower than untreated CSE as shown in Figure 4.3.

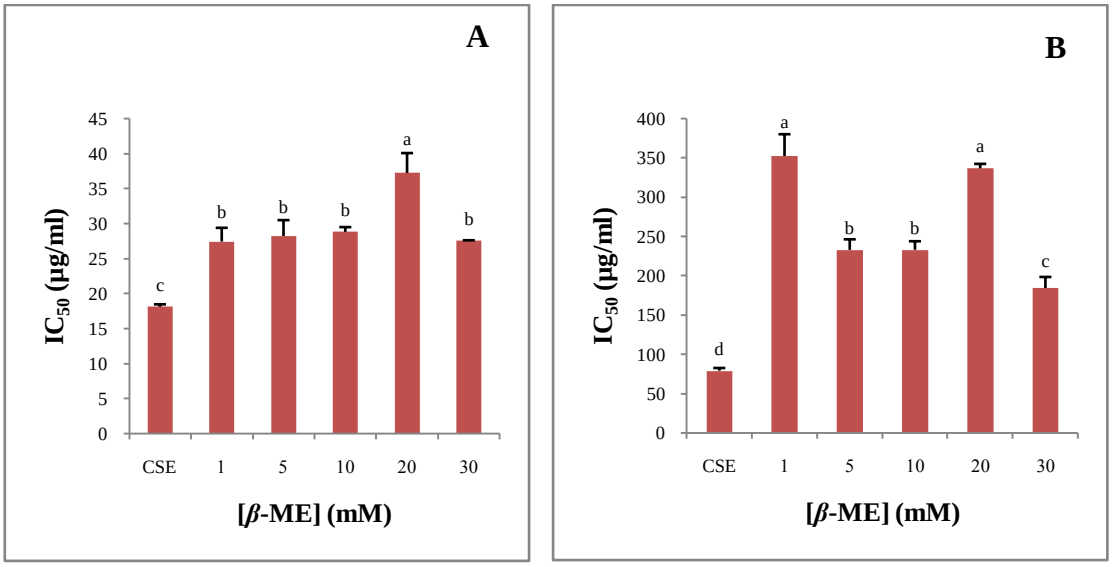

Figure 4.3 Antioxidant activity of crude sericin treated with different concentration of *β*-mercaptoethanol (*β*-ME) as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

DTT and *β*-ME are stressors and introduce instability into the core of a protein that promotes the transition between disulfide and SH groups leading to free sulfhydryl in protein (Cleland, 1994; Weston *et al*., 1995). Medina-Navarro *et al*. (2010) found that DTT treatment increased antioxidant capacity of albumin. Human serum albumin is composed of 35 cysteines, among which 34 are engaged in 17 disulphide bond cysteines, leaving only Cys-34 available for reaction (Peter, 1996). It has been

suggested that Cys-34 is one of the most reactive sulfhydryl groups in serum (Narazaki *et al*., 1997). In contrast, in this study we found that DTT treatment decreased antioxidant activity of CSE. The sericin protein was very low content of Cystiene (~0.53%), while serine, glycine, tyrosine, theonine and hydroxyl groups were higher (Kato *et al*., 1998; Mondal *et al*., 2007; Wu *et al*., 2008). Therefore, it is possible to see firstly, no correlation between antioxidant activity and free sulfhydryl of sericin protein and secondly, the native structure of sericin was important to their activity.

4.1.2 Effect of treatment with UV light

Antioxidant activity by ABTS assay and DPPH assay of CSE after exposure to UV light for 10 min are presented in Figure 4.4. Both methods showed that antioxidant activities of CSE after exposure UV light were lower than CSE before exposure to UV light. Kristo *et al*. (2012) found that UV irradiation could change tertiary and quaternary structure of whey protein. The amino acid side chains affected in UV range are tryptophan, tyrosine, histidine and cysteine (Mondal *et al*., 2007; Pattison *et al*., 2012). UV excitation of aromatic residues can transfer an electron to breakage of intra-molecular disulphide bridges in protein (Hoffman *et al*., 1972). This result suggested that exposure to UV light may cause structural changes that could affect antioxidant activity of sericin protein.

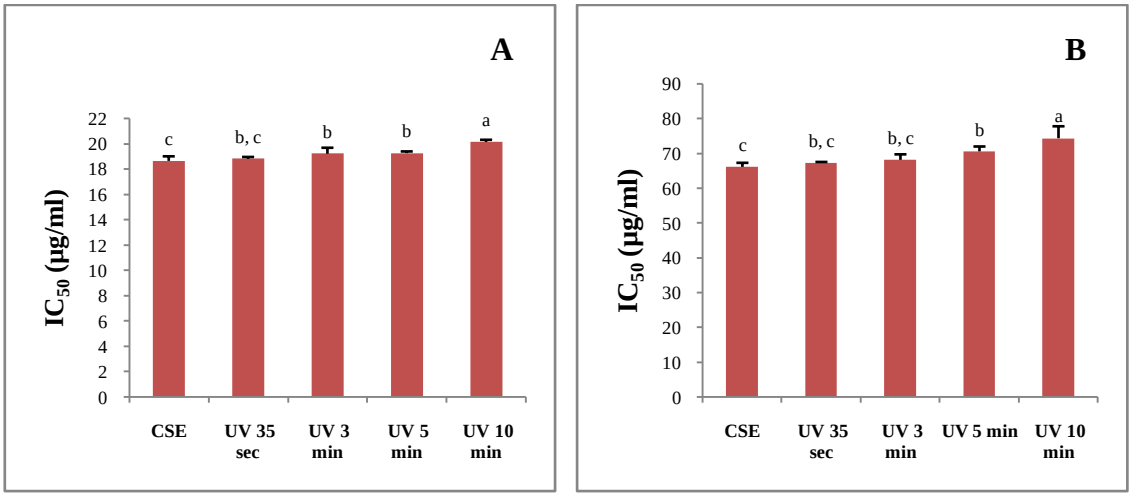

Figure 4.4 Antioxidant activity of crude sericin after exposure to UV light as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

4.1.3 Effect of treatment with protease

As shown in Figure 4.5 antioxidant activity by ABTS assay and DPPH assay of CSE treated with protease was 5.0-fold and 3.0-fold higher than that of untreated CSE, respectively. In this study we found that CSE treated with protease exhibited an excellent antioxidant activity. Similar result was reported by Li *et al*., (2008) a low molecular weight fraction from chickpea protein hydrolysate had the highest activity when compared to higher molecular weight fractions. Je *et al*., (2005) reported that the Alaska Pollack frame protein hydrolysate (APH-V fraction) with molecular weight of below 1 kDa showed the highest antioxidant activity. Chi *et al*., (2015) reported that the peptide isolated from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle with molecular weight of 0.398 kDa showed the highest radical scavenging activity among the hydrolysate. In addition, antioxidant activity of known hydrolysate from other protein sources such as egg white, grass carp, oyster, and whey protein are also introduced (Chen *et al*., 2012; Cai *et al*., 2015; Umayaparvathi *et al*., 2014; Zhang *et al*., 2013). These results suggested that protease from this experiment increased antioxidant activity of CSE

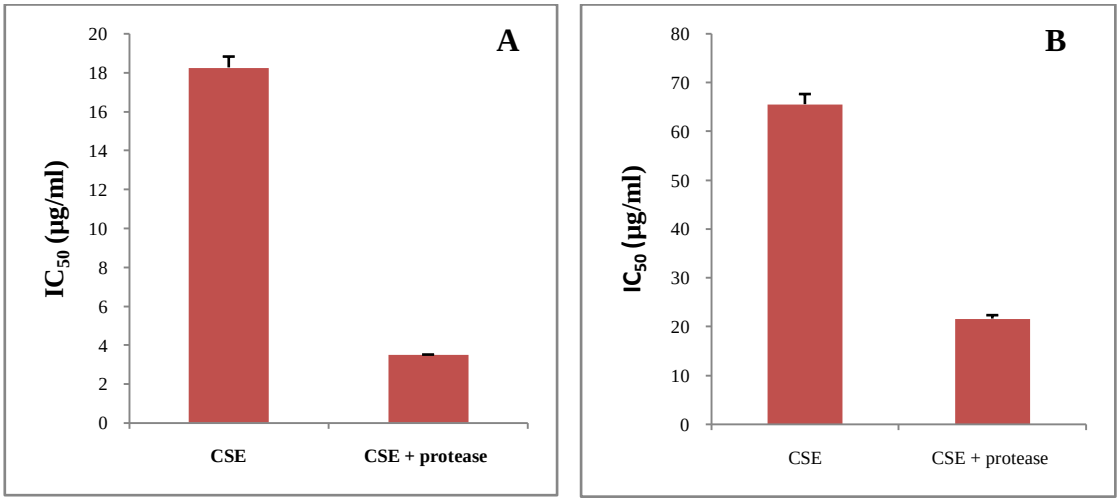

Figure 4.5 Antioxidant activity of crude sericin treated with protease enzyme as determined by ABTS assay (**A**) and DPPH assay (**B**).

Figure 4.6 show that low molecular mass of CSE treated with protease was mostly distributed around 5 kDa when determined according to the linear regression

equation, while the untreated CSE showed a clear band at 70 kDa. SDS–PAGE results are in accordance with the results of antioxidant property of protein.

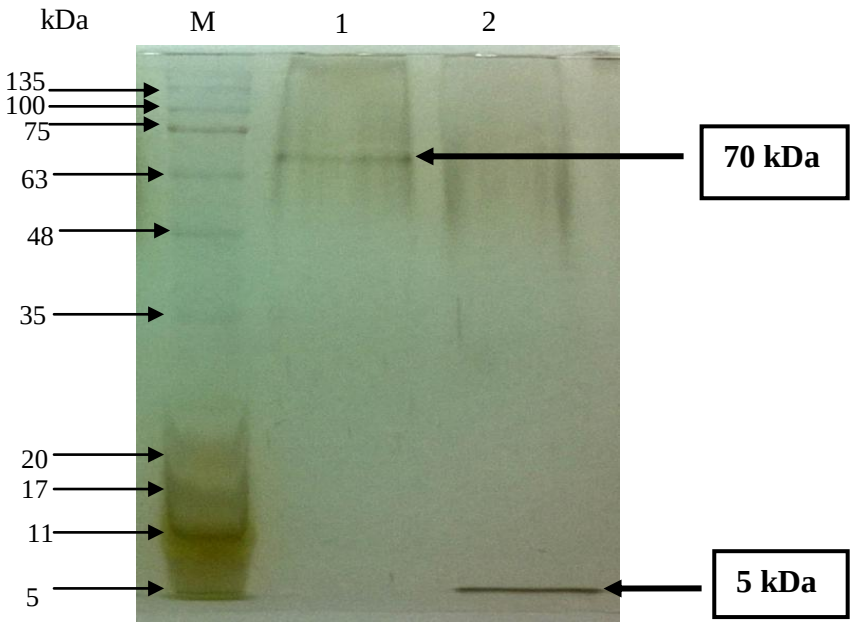

Figure 4.6 The molecular weight of CSE treated with protease enzyme by SDS-PAGE. M: marker protein, Lane 1: untreated CSE and Lane 2: CSE treated with protease enzyme

4.2 Sericin fractions

4.2.1 Antioxidant activity of sericin fractions

CSE was fractionated into 2 fractions, Yellow-PT and SNT by saturated $(NH_4)_2SO_4$. Study was reported that ethanolic extracts of yellow pigment of Thai silk waste show effective antioxidant activity (Prommuak *et al.*, 2008). In this study we found that antioxidant activity of colorless SNT fraction were higher than the Yellow-PT fraction measured by ABTS assay and DPPH assay (Fig 4.7). No correlation between antioxidant activity and yellow pigment was observed, which indicated that pigments are not components in the CSE that could represent antioxidant activity. Hydrophilic amino acids of CSE were up to 76% and it is possible that hydrophilic amino acids could be an electron donor which reacted radical chain reaction (Wu *et al.*, 2008). On the other hand, antioxidant activity of SNT fraction was decreased when

treated with DTT and β-ME. The results suggested that antioxidant activity of CSE was dependent on their amino acid composition in silk protein.

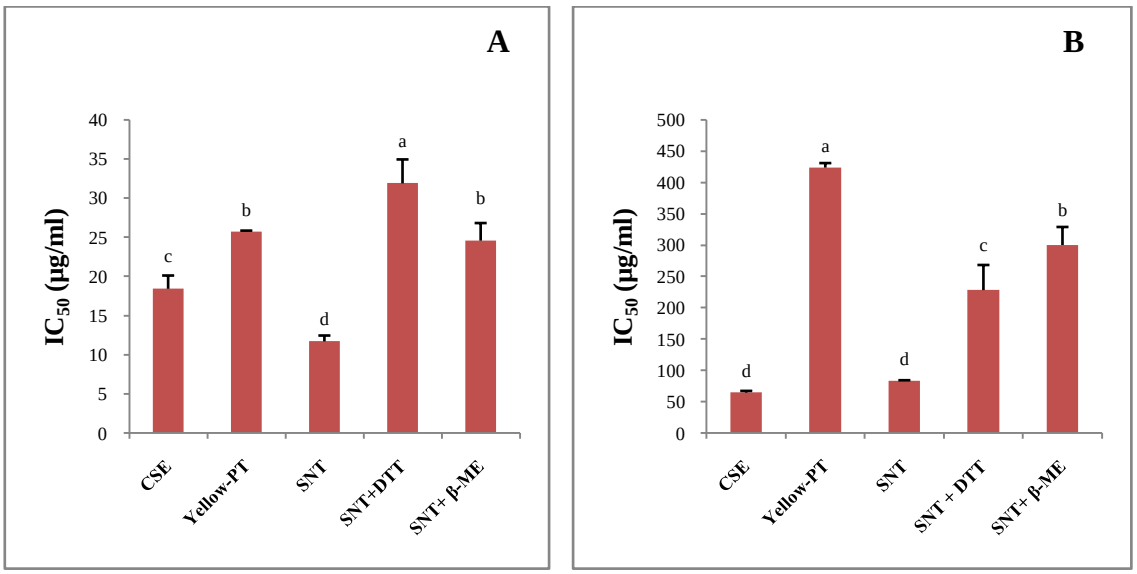

Figure 4.7 Antioxidant activity of sericin fractions as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

4.2.2 Antioxidant activity of sericin fractions after UV exposure

Antioxidant activity of sericin fractions after exposure to UV light were shown in Figure 4.8. The antioxidant activity of sericin fractions after exposure to UV light had lower than the sericin fractions before exposure to UV light. As was expected, results suggested that exposure to UV light might be affected to lower antioxidant activity of protein fractions.

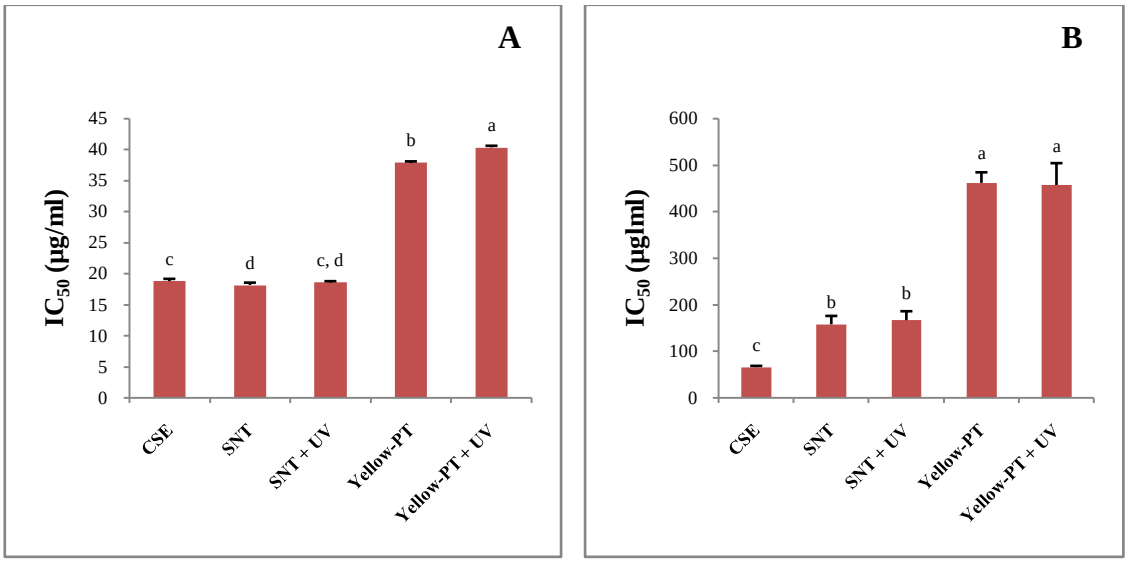

Figure 4.8 Antioxidant activity of sericin fractions after exposure to UV light as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

4.2.3 Antioxidant activity of sericin fractions treated with protease

Antioxidant activity of SNT and Yellow-PT fraction treated with protease determined by ABTS assay and DPPH assay is shown in Figure 4.9. SNT fraction treated with protease showed the highest antioxidant activity when compared to other fractions. Moreover, increased antioxidant activity of Yellow-PT fractions treated with protease was observed. Similar result was reported by Wang *et al*. (2013) that a novel antioxidant peptide from blue mussel (BNH-P7) exhibited good scavenging activity on DPPH radical. Raghavan *et al*. (2009) reported that the tilapia protein hydrolysates with low molecular weights were better antioxidants than those with high molecular weights. The above results demonstrated that the protease used is a key factor in enhancing antioxidant activity of sericin fractions. This result suggested that the treatment with protease enzyme was found to affect antioxidant activity of protein fractions.

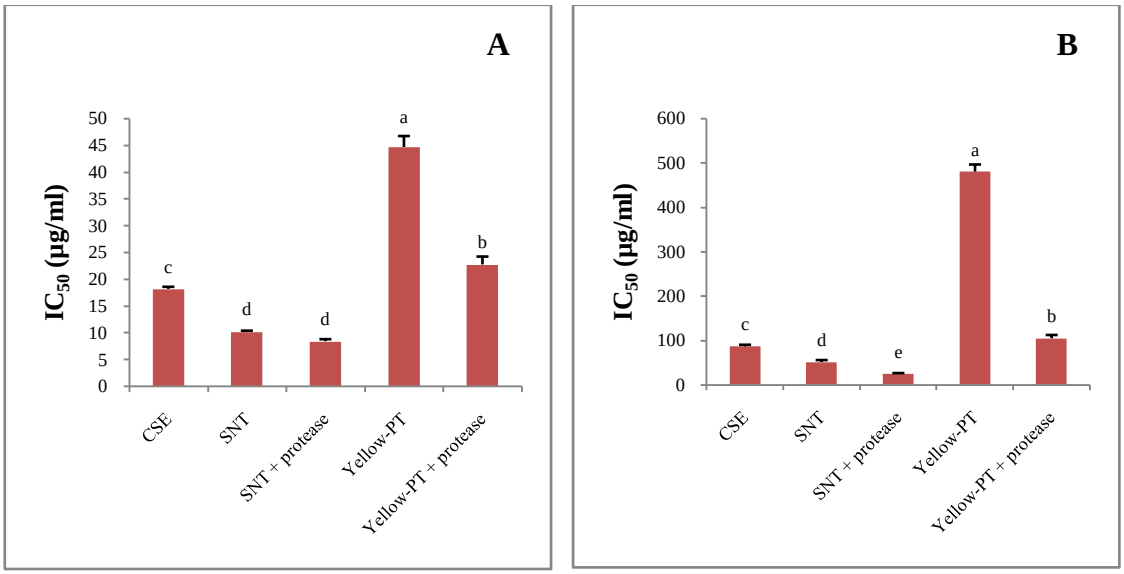

Figure 4.9 Antioxidant activity of sericin fractions treated with protease enzyme as determined by ABTS assay (**A**) and DPPH assay (**B**). Results are expressed as mean ± SD (n = 3). Means with different letters in the same raw represent significant differences at $p < 0.05$.

CHAPTER 5

CONCLUSION

The relationship between effect of DTT, β-ME, UV light treatments and hydrolysis by protease on sericin protein and its antioxidant activity was investigated by ABTS and DPPH assay. It was revealed that treatment with protease enzyme was found to affect antioxidant activity of CSE, suggesting that antioxidant activity could be enhanced by decreasing size of sericin protein. On the other hand, treatments with DTT, β-ME and UV light decreased antioxidant activity of CSE. Moreover, the antioxidant activity of the colorless supernatant protein fraction is higher than that of the yellow-precipitate fraction, suggesting that in addition to the yellow-pigment of flavonoids, colorless protein fraction possesses even stronger antioxidant activity.

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APPENDICES

Appendix A

Chemicals & Instrument

Chemicals	Source
Ammonium sulfate [$(NH_4)_2SO_4$]	Carlo Erba, Italy
Dithiotheitol (DTT)	Sigma-Aldrich Co. (St. Louis, MO, USA)
β-mercaptoethanol (β-ME)	Sigma-Aldrich Co. (St. Louis, MO, USA)
NaOH	Analytical Science (Labscan Asia Co., Ltd)
2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulphonic acid) (ABTS)	Sigma-Aldrich Co. (St. Louis, MO, USA)
2.2-diphenyl-1 picrylhydrazyl (DPPH)	Sigma-Aldrich Co. (St. Louis, MO, USA)
Potassium persulfate	Merck KGaA, AR grade, Germany
Methanol	Promega, Mulecular Biology grade, USA
Ethanol	Ajax Finechem, AR grade, New Zealand
40% Acrylamide	Euroclone, Electrophoresis grade, UK
Ammonium persulphate (APS)	Promega, Mulecular Biology grade, USA
Bis-acrylamide	Euroclone, Electrophoresis grade, UK
Bromophenol blue	Sigma Chemical Co., USA
Citric acid	Ajax Finechem, AR grade, New Zealand
Comassie brilliant blue G-250	Ajax Finechem, AR grade, New Zealand
Glycerol	Sigma-Aldrich Co. (St. Louis, MO, USA)
Glycine	Scharlau, reagent grade, Spain
N, N, N', N'-Tetramethyl ethylene diamine (TEMED)	Scharlau, reagent grade, Spain
Phosphoric acid	Ajax Finechem, AR grade, New Zealand
Sodium acetate	Ajax Finechem, AR grade, New Zealand
Sodium dodecyl sulfate	Sigma Chemical Co., USA
Sodium carbonate	Ajax Finechem, AR grade, New Zealand
Sodium thiosulfate	Carlo Erba, reagent grade, France
Tris-base	Scharlau, reagent grade, Spain
25% w/v Glutaraldehyde	GeneDirex Inc., Taiwan
37% Formaldehyde	CRëc., New Zealand
Protein marker	GeneDirex Inc., Taiwan
Bovine Serum Albumin (BSA)	Sigma-Aldrich Co. (St. Louis, MO, USA

Equipment

Centrifuge

Dialysis bag

Micro pipette size 0.5-10, 10-100 and 100-1000 µl (Brand, Germany)

Gel electrophoresis (Mini-PROTEAN 3 cell, Bio-Rad)

Quartz cuvette (Perkin Elmer)

Shanker water bath (Memmert, Germany)

Vissilble Spectrophotometer (Perkin Elmer, model Lambda Bio 40)

Hotplate stirrer

Appendix B

Reagent preparation

1. Bradford reagent

1.1 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.

1.2 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.

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

2.1 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.

2.2 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.

2.3 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.

2.4 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.

2.5 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.

2.6 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.

2.7 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.

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

3.1 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**

3.2 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**

4. Reagent for silver staining

4.1 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.

4.2 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)

4.3 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)

4.4 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)

4.5 Stopping solution

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

4.6 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.

5. Saturated salt for protein precipitation

5.1 Saturated $(NH_4)_2SO_4$

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

Appendix C
Calculation

1. Determination protein concentration

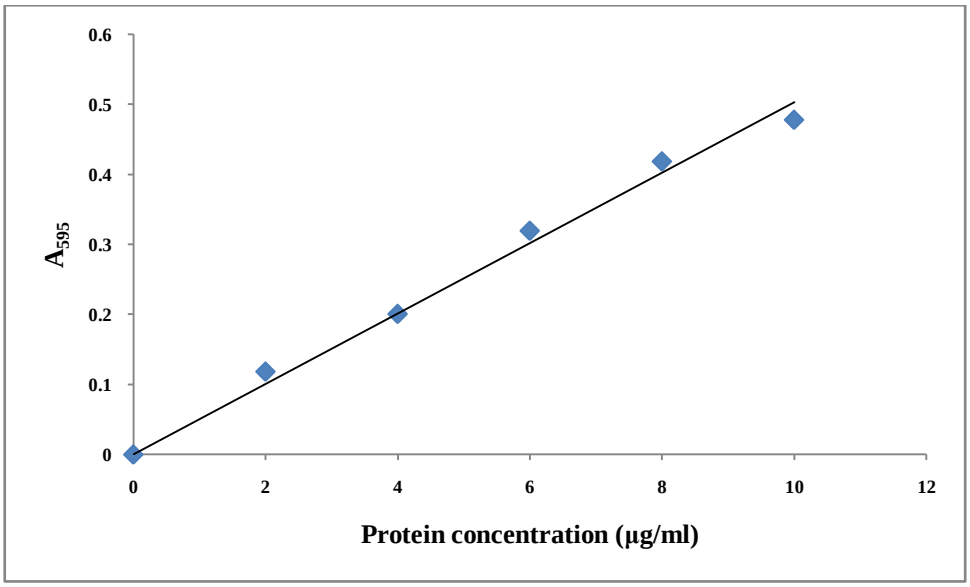


Figure 1 Standard calibration curve for the Bradford protein assay using bovine serum albumin (BSA) as the protein standard ($y = 0.050x$, $R^2 = 0.990$)

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) $= 0.586$

The protein sample concentration (x) $= 0.586/0.050$

$= 11.72$ µg/ml

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

So, the total protein = 11.72 µg/ml x 1.5 ml

= 17.58 µg

For 0.5 ml in the reaction contained 0.020 ml of protein sample mixed with 0.480 ml of distilled water, therefore 17.58 µg was derived from 0.020 ml of protein sample

So, in 1 ml of protein sample = (17.58 µg x 1 ml)/0.020 ml

Therefore, the protein sample concentration = 879 µg/ml

2. Antioxidant activity determination by ABTS assay

Concentration of sample (µg/ml)	**Abs. (λ_{734})**			**Percent inhibition**		
	1	**2**	**3**	**1**	**2**	**3**
Control	0.884	0.852	0.871			
0.52	0.847	0.844	0.852	4.18	0.93	2.18
1.04	0.840	0.825	0.824	4.97	3.16	5.39
2.08	0.796	0.787	0.780	9.95	7.62	10.44
4.16	0.736	0.732	0.726	16.74	14.08	16.64
8.33	0.665	0.611	0.619	24.77	28.28	28.93
16.66	0.507	0.506	0.508	42.64	40.61	41.67
IC_{50} (µg/ml)				**18.23**	**18.67**	**17.90**
$\bar{x}$				**18.27 ± 0.38**		

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

Percent inhibition = [1- (0.844/ 0.852)] ^{x} 100

= 0.93 µg/ml

Percent inhibition plot graph to IC_{50} value

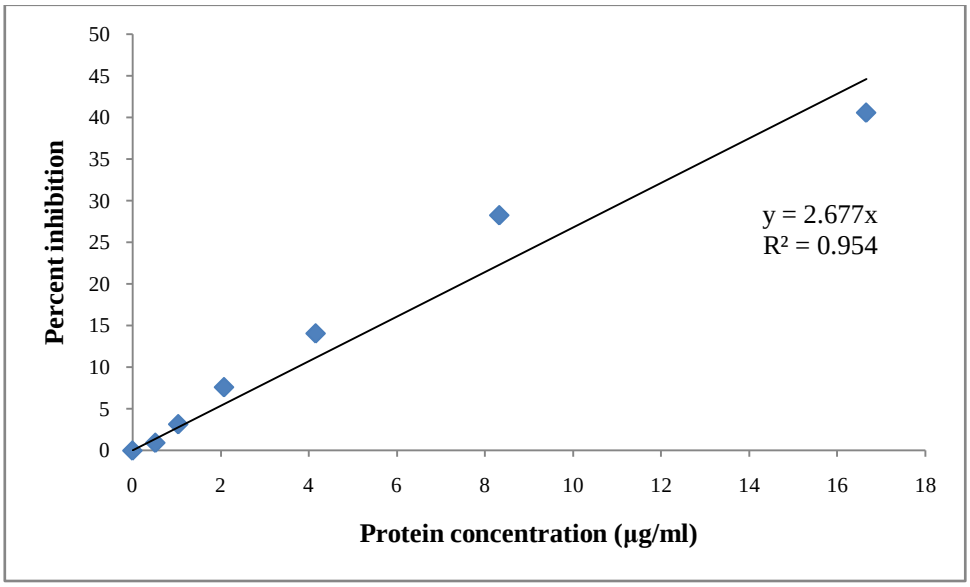


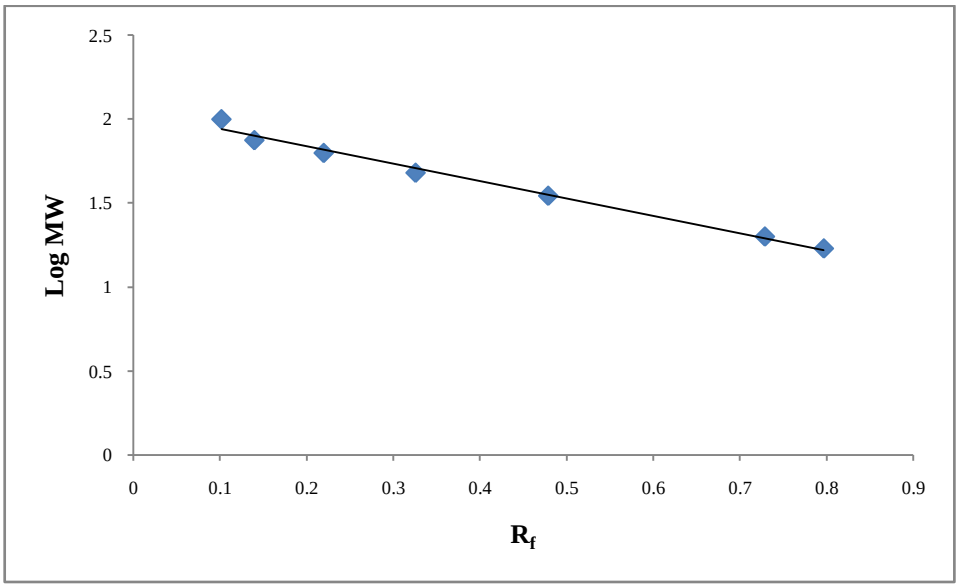
Figure 2 The relation between percent inhibition and protein concentration

y = 0.954

IC_{50} value = 50/ 0.954

= 18.67 µg/ml

3. Determination of molecular weight



According to the linear equation, y = -1.033x + 2.045

y = log MW

R_f = migration distance of the protein / migration distance of the dye front

Migration distance of unknown protein: 2.25 cm

Migration distance of dry front: 11.80 cm

So R_f = 2.25 cm / 11.80 cm = 0.191

y = -1.033x + 2.045

x = R_f = of unknown protein = 0.191

So MW = 10^y = $10^{-1.033(0.191)+2.045}$ = 70.469 kDa **(Lane 1)**

4. 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.

4.1 Statistical analysis of antioxidant activity of CSE by ABTS assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	19.7757	1.37381	.79317	16.3629	23.1884	18.23	20.85
2	3	21.2187	.52201	.30138	19.9219	22.5154	20.70	21.74
3	3	18.2737	.38645	.22312	17.3137	19.2337	17.91	18.68
4	3	20.4060	.43469	.25097	19.3262	21.4858	19.91	20.73
5	3	23.8693	.61615	.35573	22.3387	25.3999	23.16	24.24
Total	15	20.7087	2.02095	.52181	19.5895	21.8278	17.91	24.24

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
2.975	4	10	.074

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	51.424	4	12.856	22.336	.000
Within Groups	5.756	10	.576		
Total	57.179	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

Sample	N	Subset for alpha = 0.05			
		1	2	3	4
3	3	18.2737			
1	3		19.7757		
4	3		20.4060	20.4060	
2	3			21.2187	
5	3				23.8693
Sig.		1.000	.333	.219	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.2 Statistical analysis of antioxidant activity of CSE by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	62.5603	3.27122	1.88864	54.4342	70.6865	58.96	65.35
2	3	90.9300	5.10126	2.94522	78.2578	103.6022	85.61	95.78
3	3	66.6467	4.43064	2.55803	55.6404	77.6530	62.26	71.12
4	3	93.0833	3.65804	2.11197	83.9963	102.1704	89.12	96.33
5	3	112.0167	3.67824	2.12363	102.8794	121.1539	107.99	115.20
Total	15	85.0474	19.23327	4.96601	74.3964	95.6984	58.96	115.20

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
.138	4	10	.965

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	5012.333	4	1253.083	75.247	.000
Within Groups	166.530	10	16.653		
Total	5178.863	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
1	3	62.5603		
3	3	66.6467		
2	3		90.9300	
4	3		93.0833	
5	3			112.0167
Sig.		.248	.533	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.3 Statistical analysis of effect of treatment with dithiothreitol (DTT) by ABTS assay

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
Sig.		1.000	.116	.161

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.4 Statistical analysis of effect of treatment with dithiothreitol (DTT) by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	79.2467	3.37582	1.94903	70.8607	87.6327	75.52	82.10
2	3	149.9433	7.38114	4.26150	131.6076	168.2791	141.64	155.76
3	3	153.8567	2.04640	1.18149	148.7731	158.9402	151.51	155.27
4	3	244.4233	7.00326	4.04333	227.0263	261.8204	240.38	252.51
5	3	311.9100	71.95166	41.54131	133.1722	490.6478	228.94	357.14
6	3	231.3067	25.68270	14.82791	167.5073	295.1060	204.08	255.10
Total	18	195.1144	82.44883	19.43338	154.1136	236.1153	75.52	357.14

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
10.073	5	12	.001

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	103651.262	5	20730.252	20.884	.000
Within Groups	11911.505	12	992.625		
Total	115562.767	17			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

Sample	N	Subset for alpha = 0.05			
		1	2	3	4
1	3	79.2467			
2	3		149.9433		
3	3		153.8567		
6	3			231.3067	
4	3			244.4233	
5	3				311.9100
Sig.		1.000	.882	.619	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.5 Statistical analysis effect of treatment with (β-ME) by ABTS assay

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	27.4233	1.96228	1.13292	22.5488	32.2979	25.21	28.95
3	3	28.2267	2.25638	1.30272	22.6215	33.8318	26.26	30.69
4	3	29.4667	.68311	.39439	27.7697	31.1636	28.68	29.91
5	3	37.2100	2.82936	1.63353	30.1815	44.2385	33.98	39.25
6	3	27.5367	.07506	.04333	27.3502	27.7231	27.46	27.61
Total	18	27.9972	5.89443	1.38933	25.0660	30.9285	17.72	39.25

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
4.745	5	12	.013

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	555.564	5	111.113	38.000	.000
Within Groups	35.088	12	2.924		
Total	590.653	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		27.4233	
6	3		27.5367	
3	3		28.2267	
4	3		29.4667	
5	3			37.2100
Sig.		1.000	.200	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.6 Statistical analysis effect of treatment with *β*-mercaptoethanol (*β*-ME) by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	79.2467	3.37582	1.94903	70.8607	87.6327	75.52	82.10
2	3	351.9833	27.78842	16.04365	282.9531	421.0136	320.51	373.13
3	3	232.7300	13.48417	7.78509	199.2335	266.2265	217.39	242.71
4	3	232.8833	10.87920	6.28111	205.8579	259.9088	224.21	245.09
5	3	336.3833	5.71358	3.29874	322.1900	350.5767	331.12	342.46
6	3	184.3380	14.02254	8.09592	149.5041	219.1719	168.35	194.55
Total	18	236.2608	95.48472	22.50596	188.7773	283.7442	75.52	373.13

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
4.017	5	12	.023

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	152368.546	5	30473.709	139.250	.000
Within Groups	2626.097	12	218.841		
Total	154994.644	17			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
1	3	79.2467			
6	3		184.3380		
3	3			232.7300	
4	3			232.8833	
5	3				336.3833
2	3				351.9833
Sig.		1.000	1.000	.990	.221

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.7 Statistical analysis of effect of treatment with UV light by ABTS assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	22.6333	.94453	.54532	20.2870	24.9797	21.64	23.52
2	3	23.3767	1.01953	.58862	20.8440	25.9093	22.51	24.50
3	3	22.8900	.86122	.49723	20.7506	25.0294	22.12	23.82
4	3	25.8100	.98306	.56757	23.3680	28.2520	24.69	26.53
5	3	26.0733	.48952	.28263	24.8573	27.2894	25.53	26.48
6	3	26.4300	1.53353	.88538	22.6205	30.2395	24.66	27.36
Total	18	24.5356	1.85034	.43613	23.6154	25.4557	21.64	27.36

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
1.235	5	12	.352

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.742	5	9.148	8.809	.001
Within Groups	12.462	12	1.039		
Total	58.204	17			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05	
		1	2
1	3	22.6333	
3	3	22.8900	
2	3	23.3767	
4	3		25.8100
5	3		26.0733
6	3		26.4300
Sig.		.412	.492

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.8 Statistical analysis of effect of treatment with UV light by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	87.8200	2.72108	1.57102	81.0605	94.5795	84.88	90.25
2	3	91.2900	5.07516	2.93014	78.6826	103.8974	86.65	96.71
3	3	100.2433	6.20452	3.58218	84.8305	115.6562	95.60	107.29
4	3	100.0067	7.22912	4.17373	82.0485	117.9648	93.10	107.52
5	3	94.4477	8.97731	5.18305	72.1468	116.7485	84.74	102.45
6	3	96.4400	5.94276	3.43105	81.6774	111.2026	89.76	101.14
Total	18	95.0413	7.03096	1.65721	91.5449	98.5377	84.74	107.52

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
.796	5	12	.573

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	360.733	5	72.147	1.805	.186
Within Groups	479.652	12	39.971		
Total	840.385	17			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05
		1
1	3	87.8200
2	3	91.2900
5	3	94.4477
6	3	96.4400
4	3	100.0067
3	3	100.2433
Sig.		.050

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.9 Statistical analysis of antioxidant activity of sericin fractions by ABTS assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	18.4400	1.69820	.98046	14.2214	22.6586	17.07	20.34
2	3	25.7333	.13429	.07753	25.3997	26.0669	25.58	25.83
3	3	11.4967	1.14897	.66336	8.6425	14.3509	10.17	12.17
4	3	31.8833	3.05706	1.76500	24.2892	39.4775	29.06	35.13
5	3	24.5800	2.24769	1.29770	18.9964	30.1636	22.93	27.14
Total	15	22.4267	7.35970	1.90027	18.3510	26.5023	10.17	35.13

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
2.577	4	10	.102

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	721.074	4	180.268	48.408	.000
Within Groups	37.240	10	3.724		
Total	758.313	14			

Post Hoc Tests

Homogeneous Subsets

IC

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
3	3	11.4967			
1	3		18.4400		
5	3			24.5800	
2	3			25.7333	
4	3				31.8833
Sig.		1.000	1.000	.481	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.10 Statistical analysis of antioxidant activity of sericin fractions by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	65.2233	2.54245	1.46788	58.9075	71.5391	62.42	67.38
2	3	423.8033	7.18536	4.14847	405.9539	441.6528	416.66	431.03
3	3	83.6633	1.12006	.64667	80.8810	86.4457	82.37	84.31
4	3	228.4633	40.06170	23.12963	128.9445	327.9821	204.91	274.72
5	3	300.2897	28.83097	16.64557	228.6696	371.9098	267.73	322.58
Total	15	220.2886	140.60630	36.30439	142.4234	298.1538	62.42	431.03

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
8.493	4	10	.003

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	271790.832	4	67947.708	136.140	.000
Within Groups	4991.026	10	499.103		
Total	276781.858	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
1	3	65.2233			
3	3	83.6633			
4	3		228.4633		
5	3			300.2897	
2	3				423.8033
Sig.		.336	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.11 Statistical analysis of antioxidant activity of sericin fractions after UV exposure by ABTS assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	15.7433	.36638	.21153	14.8332	16.6535	15.36	16.09
2	3	15.1100	.38314	.22121	14.1582	16.0618	14.67	15.37
3	3	15.5427	.18698	.10795	15.0782	16.0071	15.33	15.67
4	3	34.8500	.18520	.10693	34.3899	35.3101	34.67	35.04
5	3	37.3067	.24583	.14193	36.6960	37.9173	37.03	37.50
Total	15	23.7105	10.48852	2.70813	17.9022	29.5189	14.67	37.50

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
.994	4	10	.454

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1539.306	4	384.827	4684.688	.000
Within Groups	.821	10	.082		
Total	1540.128	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
2	3	15.1100			
3	3	15.5427	15.5427		
1	3		15.7433		
4	3			34.8500	
5	3				37.3067
Sig.		.094	.411	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.12 Statistical analysis of antioxidant activity of sericin fractions after UV exposure by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	65.1167	2.63439	1.52097	58.5725	71.6609	62.11	67.02
2	3	157.0633	18.09152	10.44514	112.1215	202.0051	145.77	177.93
3	3	165.8900	19.30702	11.14692	117.9287	213.8513	143.67	178.57
4	3	460.8867	22.72072	13.11781	404.4453	517.3281	434.78	476.19
5	3	456.2333	47.06253	27.17156	339.3235	573.1431	423.72	510.20
Total	15	261.0380	172.32558	44.49427	165.6073	356.4687	62.11	510.20

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
5.394	4	10	.014

ANOVA

IC_{50}

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	408869.249	4	102217.312	148.653	.000
Within Groups	6876.234	10	687.623		
Total	415745.483	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05		
		1	2	3
1	3	65.1167		
2	3		157.0633	
3	3		165.8900	
5	3			456.2333
4	3			460.8867
Sig.		1.000	.689	.832

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.13 Statistical analysis of antioxidant activity of sericin fractions treated with protease enzyme by ABTS assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	15.1733	.37287	.21528	14.2471	16.0996	14.83	15.57
2	3	7.2000	.15716	.09074	6.8096	7.5904	7.02	7.31
3	3	5.4633	.37687	.21759	4.5271	6.3995	5.11	5.86
4	3	41.6233	2.04857	1.18274	36.5344	46.7123	39.30	43.17
5	3	19.1833	1.47507	.85163	15.5191	22.8476	17.50	20.25
Total	15	17.7287	13.45902	3.47510	10.2753	25.1820	5.11	43.17

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
6.287	4	10	.009

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2522.676	4	630.669	472.183	.000
Within Groups	13.356	10	1.336		
Total	2536.033	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
3	3	5.4633			
2	3	7.2000			
1	3		15.1733		
5	3			19.1833	
4	3				41.6233
Sig.		.096	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

4.14 Statistical analysis of antioxidant activity of sericin fractions treated with protease enzyme by DPPH assay

Descriptives

IC_{50}

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	85.8733	3.83126	2.21198	76.3560	95.3907	82.10	89.76
2	3	50.7400	4.44987	2.56913	39.6859	61.7941	46.77	55.55
3	3	24.6200	1.30778	.75505	21.3713	27.8687	23.11	25.39
4	3	479.5900	16.07696	9.28204	439.6526	519.5274	462.96	495.05
5	3	104.3533	7.40017	4.27249	85.9703	122.7364	99.40	112.86
Total	15	149.0353	173.59213	44.82130	52.9032	245.1675	23.11	495.05

Test of Homogeneity of Variances

IC_{50}

Levene Statistic	df1	df2	Sig.
2.591	4	10	.101

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	421180.364	4	105295.091	1506.707	.000
Within Groups	698.843	10	69.884		
Total	421879.207	14			

Post Hoc Tests

Homogeneous Subsets

IC_{50}

Duncan[a]

sample	N	Subset for alpha = 0.05				
		1	2	3	4	5
3	3	24.6200				
2	3		50.7400			
1	3			85.8733		
5	3				104.3533	
4	3					479.5900
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

BIOGRAPHY

BIOGRAPHY

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