CLONING OF SERICIN-LIKE PROTEINS FOR RECOMBINANT PRODUCTION IN BACTERIA

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ABSTRACT

CLONING OF SERICIN-LIKE PROTEINS FOR RECOMBINANT PRODUCTION IN BACTERIA

Silk consists of two main proteins called fibroin and sericin. While fibroin is used in textile production and various biomaterial applications, sericin is considered as waste material in the textile industry. Sericin is a multi-component protein with an indefinite structure and it has been shown to be biocompatible and has biological activity. Because of the positive effects on keratinocytes and fibroblasts have led to the development of sericin-based biomaterials for the repair of skin tissue. Sericin from silkworm cocoons can be obtained by chemical treatment, enzymatic treatment and boiling in water. Although sericin can be separated from fibroin by chemical, enzymatic and boiling in water treatment methods, all these treatment methods are not enough to obtain recovery of high-quality sericin. Moreover, in these treatment methods, the exposure of sericin protein to high temperature causes even sericin protein obtained by the same method to indicate different characteristics. The fact that the obtained sericin demonstrate such major changes in the structure according to treatment methods bring inconsistencies in the quality of sericin produced as a biomaterial. The aim of the study is to produce native sequence of sericin that forms a tetramer contain each containing 38 amino acids with recombinant production in *E.coli* and to characterize structural properties Thus, obtaining sericin protein from the bacteria with recombinant methods will solve these problems in question The results indicate that for the first time, the conformational properties of recombinant sericin were obtained similar to the native sericin structure.

ÖZET

BAKTERİLERDE REKOMBİNANT ÜRETİM İÇİN SERİSİN BENZERİ PROTEİNLERİN KLONLANMASI

Fibroin ile birlikte ipeği oluşturan proteinden biri olan serisin, fibroinlerin birbirine bağlanmasına yardımcı olarak kozanın oluşmasını sağlar. Fibroin tekstil üretiminde ve çeşitli biyomalzeme uygulamaların da kullanılırken, serisin tekstil endüstrisinde bir atık malzeme olarak kabul edilmektedir. Serisin proteinin biyolojik aktiviteye sahip olduğu ve biyouyumlu olduğu yapılan çalışmalarla gösterilmiştir. Serisin değişken aminoasit bileşimi ve çeşitli fonksiyonel grupları ile biyomedikal uygulamalar için ilgi çekici biyoaktif özelliklere sahiptir. Antioksidan karakteri ve memeli hücreleri üzerindeki mitojenik etkisi nedeniyle serisinin hücre kültürü ve doku mühendisliğinde yararlı olduğu son yıllarda yapılan çalışmalarla gösterilmiştir. Ayrıca, keratinositler ve fibroblastlar üzerindeki olumlu etkileri, başta yara bakım malzemeleri olmak üzere deri dokusu onarımı için serisin bazlı biyomalzemelerin gelişmesine yol açmıştır. İpek böceği kozalarından serisin kimyasal muamele, enzimatik muamele ve suda kaynatma yöntemleri ile elde edilebilir. Kimyasal ve enzimatik muamele yöntemleriyle ile serisin fibroinden ayrılabilse de bu yöntemlerle serisin elde edilirken protein yüksek sıcaklıklara maruz kaldığından tutarlı ve özellikleri öngörülebilen serisin elde edilememektedir. Farklı zamanlarda üretilen serisin farklı kalitede ve miktarda elde edilmektedir. Elde edilen serisinin ayrıştırma yöntemine göre yapısında büyük değişiklikler göstermesi, serisinden elde edilen biyomalzemelerin kalitesinde tutarsızlıklar meydana getirmektedir. Çalışmanın amacı, doğal serisin sekansını 4 kez tekrar eden 38 amino asit dizisinden oluşan tetrameri *E. coli'de* rekombinant yöntem ile üretmek ve yapısal özelliklerini karakterize etmektir. Böylelikle, bakterilerden rekombinant yöntemlerle serisin proteini elde edilmesi, söz konusu sorunları çözecektir. Sonuçlar, ilk kez rekombinant yöntem ile üretilen serisin proteinin konformasyon özelliklerinin, doğal serisin yapısına benzer şekilde elde edildiğini gösterdi.

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CHAPTER 1

INTRODUCTION

1.1 Silk

The natural-silk derived proteins are increasingly being used as biomaterials for drug delivery and tissue engineering applications, due to their unique mechanical and chemical properties such as biocompatibility, biodegradability, self-assembly and controllable structure (Lamboni, Gauthier, Yang, & Wang, 2015a).

The silk gland of the silkworm, *Bombyx mori*, synthesizes and secretes two classes of silk proteins, fibroin and sericin (Huang, Valluzzi, Bini, Vernaglia, & Kaplan, 2003). While sericin is synthesized in the middle silk gland (MSG), fibroin is synthesized in the posterior silk gland (PSG). Secreted fibroin moves from the posterior to the middle section of the gland for storage. Fibroin is coated by sericin that acts to glue two silk threads which spun out from both sides of spinneret (Neves & Reis, 2016) (Figure 1.1).

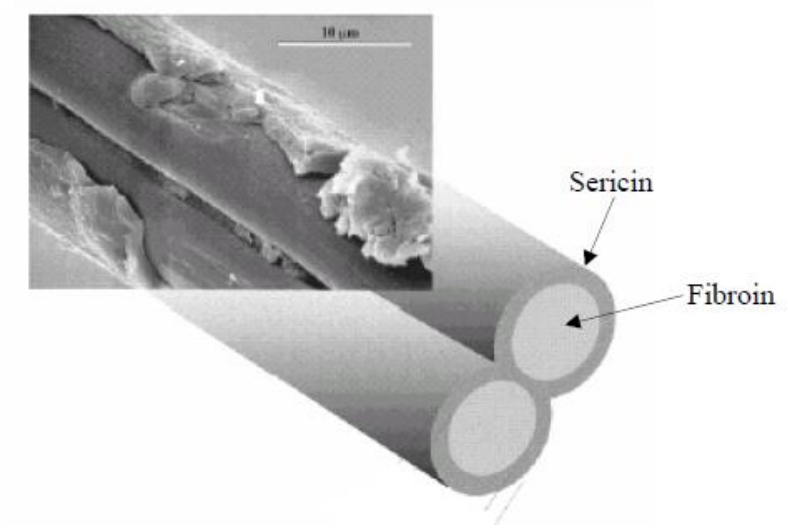

Figure 1.1 Structure of Silk (Source: Pérez-Rigueiro, Elices, Llorca, & Viney, 2001)

Silk fibroin forms over 70% of the cocoon which is a natural fibrous protein and a semi-crystalline structure which provides hardness and strength (Sinohara, Asano, & Fukui, 1971) (Koh et al., 2015). Silk fibroin consists of a heavy (H) chain (~350 kDa) and a light (L) chain (~25 kDa) linked with via a single disulfide bond at the C-terminus of the H-chain, forming an H–L complex (Mori et al., 1995). The main crystal structures of silkworm SF are silk I (water-soluble state) and silk II (crystalline state) (Vepari & Kaplan, 2007) (Figure 1.2). The amino acid composition of SF from *Bombyx mori* consists mainly of Gly (43%), Ala (30%), and Ser (12%) (Koh et al., 2015).

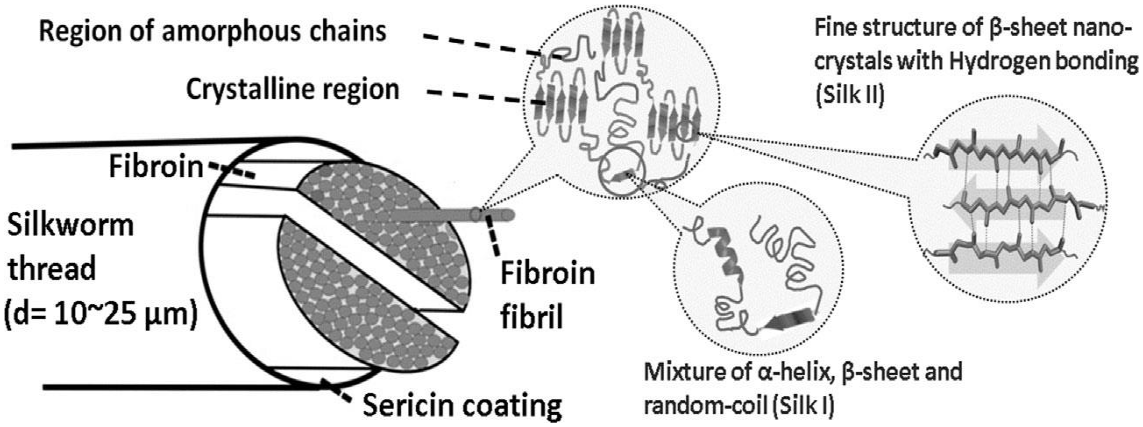

Figure 1.2 Schematic representation of silk fibroin (SF) structure. d. represents the diameter of a single silkworm thread (Source: Volkov, Ferreira, & Cavaco-Paulo, 2015).

1.2 Sericin

Sericin, the second major *B. mori* silk protein, has excellent properties; antibacterial, UV resistant, oxidative resistant and moisturizing properties. It can be applied to many fields such as biomaterials, polymer materials and cosmetics (Pérez-Rigueiro, Elices, Llorca, & Viney, 2001; Shen, Johnson, & Martin, 1998).

To manufacture lustrous silk from the dried cocoons of silkworm, fibroin is separated from sericin that is the other major component of the cocoon by a degumming process and sericin is principally discarded in the wastewater (Y. Q. Zhang, 2002). Approximately 50,000 tons of sericin could be recovered from the waste solution from

one million tons (fresh weight) of cocoon or about 400,000 tons of dry cocoon, (Y. Q. Zhang, 2002).

1.3 Biochemical Properties of Sericin

Sericin constitutes about 20-30 % of the cocoon weight and is hydrophilic glue protein that holds two fibroin fibers together to form a stable composite of the cocoon structure (Huang et al., 2003; Y. Q. Zhang, 2002). The glue-like property of sericin is predicated to be due to the hydrogen bonding between serine residues of sericin in the fibroin structural components of the silk fiber.

Sericin is characterized by unusually high serine content (40%) along with notable amounts of glycine (16%) glutamic acid, aspartic acid, threonine and tyrosine content (Gamo, Inokuchi, & Laufer, 1977; Kato et al., 1998).

Sericin extracted by different treatment methods can even provide different amino acid compositions (Aramwit, Damrongsakkul, Kanokpanont, & Srichana, 2010a). Therefore, sericin has different molecular properties and it exists in a wide range of molecular weights, from 10 to over 400 kDa (Kato et al., 1998; Takasu, Yamada, & Tsubouchi, 2002) depending on the extraction methods, temperature, pH, and processing time (Freddi, Mossotti, & Innocenti, 2003; Vaithanomsat & Kitpreechavanich, 2008; Y. Q. Zhang, 2002).

1.4 Potential Applications of Sericin

Sericin can be characterized by excellent moisture absorption and release, and biological activities such as antioxidation, tyrosinase activity inhibition and pharmacological functions (e.g, anticoagulation, anticancer activities, cryoprotection and promotion of digestion) (Kato et al., 1998). Due to its properties, sericin is particularly useful for improving polymer materials such as polyesters, polyamide, polyolefin and

polyacrylonitrile. Moreover, it can also be applied to degradable biomaterials, biomedical materials, bio-membrane materials fibers and hydrogels (T. T. Cao & Zhang, 2016).

1.4.1 Biodegradability

Biodegradability is one of the important properties of biomaterials used in tissue engineering. The rate of degradation of the biomaterial should correspond to the rate of the healing process or the rate of formation of new tissue. The products which are formed after the degradation of the biomaterial should be non-toxic and should be easily resorbed by the body. Any changes in the mechanical properties of the biomaterial after degradation should be compatible with the process of regeneration of the tissue (Lloyd, 2002). In one process, sericin powder is first dissolved in an organic solvent such as tetrahydrofuran and dioxane. Sericin in the solution is reacted with a polyisocyanate. The polyurethane produced contains biodegradable sericin segments and it is biodegradable. The resulting polyurethane can be made into film and fibers (Hatakeyama, 1996).

1.4.2 Biocompatibility

Biocompatibility refers to the ability of a material to perform with an appropriate host response in tissue engineering (Barrere, Mahmood, De Groot, & Van Blitterswijk, 2008). A significant consideration in the application of any biomaterial is its potential activation of the immune system (Panilaitis et al., 2003), i.e. its biocompatibility. The interplay between implanted biomaterials and the host immune system is one of the most important determinants of the implanted material's biocompatibility (Ratner, 2015). This is, in fact, one of the main determinants of the success or failure of a biomaterial in tissue engineering and regenerative medicine applications (Brown & Badylak, 2012). In the study, new biodegradable poly (L-lactic-co-ε-caprolactone)-sericin (PLCL-SC) copolymer membranes were successfully fabricated by electrospinning (Inthanon et al., 2016).

1.4.3 Antioxidant

Inhibition of lipid peroxidation and tyrosinase activity of sericin was observed in the previous studies. (Kato et al., 1998). Additionally, some studies demonstrate that sericin suppresses against both chemical and UV radiation-induced mouse skin and colon tumorigenesis. The mechanism shows that sericin can protect against oxidative stress (Zhaorigetu, Sasaki, Watanabe, & KATo, 2001; Zhaorigetu, Yanaka, Sasaki, Watanabe, & Kato, 2003). Silk protein can be made into biomaterials with anticoagulant properties by sulfonation of sericin and fibroin (Tamada, 1997).

Another study evaluated the antioxidative activity and free–radical scavenging activity of silk sericin concluding that silk sericin has effective antioxidant activities. Thus, it demonstrates that silk sericin from silkworm *B. mori* is a natural antioxidant (T. T. Cao & Zhang, 2016).

1.4.4 Effect of Sericin on Cell Proliferation and Culture Media

Cell culture has been more and more widely used in the biological industry. Bovine serum albumin (BSA) or fetal bovine serum (FBS) is generally added to the culture medium. The replacement of FBS/BSA with a cheaper, safer and more effective material is important (Terada et al., 2007).

A previous study indicated that sericin can be used as a supplement used to accelerate cell proliferation (Terada, Nishimura, Sasaki, Yamada, & Miki, 2002). The following study demonstrated that sericin successfully induced the proliferation of T lymphocyte cell line. Thus, Terada and co-workers concluded that sericin is a beneficial factor for the development of serum-free cell culture (Terada et al., 2007).

Medium containing 0.5% sericin can protect cells against oxidative stress, improve the early development of bovine embryo culture, evolve the quality of the bovine embryos cultured individually and promote cell survival and collagen production (Isobe et al., 2012; Mahesh N Padamwar, Pawar, Daithankar, & Mahadik, 2005). In another study, adding small molecules of sericin (10–20 kDa) to the medium can induce dermal

fibroblasts formation *in vitro* and increase collagen production by 10% (Terada et al., 2002).

The beneficial effects of sericin on cell proliferation have been broadly observed in several reports, especially on serum-free cell culture in vitro. Serum-free media was supplemented with sericin to culture different cells including human epithelial cells (HeLa), human embryonic kidney transformed cells (293), human hepatoblastoma cells (HepG2), and murine hybridoma (2E3-O). Sericin significantly promoted the growth of these cell lines by shortening the lag phase of the cell cycle and by inducing early entrance of the cultures in their logarithmic phase (Terada et al., 2002) .

1.4.5 Effect of Sericin on Immunological Response

The innate immune system is the first barrier to any foreign body in the host and it plays an important role in determining potential immune response, such as; macrophage response through the expression and release of first response pro-inflammatory cytokines, like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Franz, Rammelt, Scharnweber, & Simon, 2011).In a study, sericin promoted wound healing in rats by inducing collagen production. The levels of inflammatory mediators, IL-1β and TNF-α, found significantly lower in sericin-treated wounds of rat (Aramwit, Kanokpanont, Punyarit, & Srichana, 2009). *In vivo* studies indicate no immune responses by sericin peptides (Y. Zhang, Tao, Shen, Mao, & Chen, 2006). The results demonstrate that sericin can have *in vivo* applications as sericin-based biomaterials. The *in vivo* applications of sericin-PVA scaffolds demonstrated better wound healing effects with less scar formation and a lower level of inflammatory reactions in full-thickness skin wounds (Aramwit, Siritienthong, Srichana, & Ratanavaraporn, 2013). Soluble sericin was also found to promote the growth rather than the death of L929 mouse fibroblasts in a short-term study (Lamboni, Gauthier, Yang, & Wang, 2015b). Sericin has been successfully added to the culture media of several cell lines and it does not promote any cytotoxicity, which indicates the safety of sericin to cells (Kunz, Brancalhão, Ribeiro, & Natali, 2016).

1.4.6 Cryoprotective Effects of Sericin

Cryopreservation of cells adds great flexibility to clinical transplant programs. Currently, 10% DMSO mixed with FBS is extensively used as a cryoprotectant solution (Tsujimoto, Takagi, Takahashi, Yamada, & Nakamori, 2001).

A study concluded that silk sericin peptide which is a repeat of 38 amino acid residues expressed in *Escherichia coli* has a cryoprotective effect using freeze-thaw *in vitro* experiment because of the serine-rich repetitive sequence in the protein (Tsujimoto et al., 2001).

Previous studies obtained that sericin accelerated the proliferation of mammalian cells (Terada et al., 2002) and an insect cell line. Additionally, a study found that sericin successfully protected cells from the toxicity of dimethyl sulfoxide (DMSO). (Takahashi, Tsujimoto, Yamada, Takagi, & Nakamori, 2003). Therefore, sericin could be used as an alternative to FBS and could be used to develop serum-free cryopreservative solutions by preventing cell death induced DMSO dependent cell death.

1.4.7 Biomaterial Applications of Sericin

Sericin is considered as a waste product in the textile industry. Recent studies have indicated that this waste product can be used as a biomaterial in many fields such as cosmetics to pharmaceuticals, drug delivery systems and regenerative medicine production (Figure 1.3) (Lamboni et al., 2015a).

Several different material forms can be created from aqueous or solvent formulations of the natural sericin to use as biomaterials for tissue engineering and other biomedical applications.

Sericin must be extracted followed by reprocessing into desired material formats, for example, electrospun fibers, films, hydrogels, porous 3D sponges, surface modifications and nanoparticles (Figure 1.4). (Lamboni et al., 2015a)

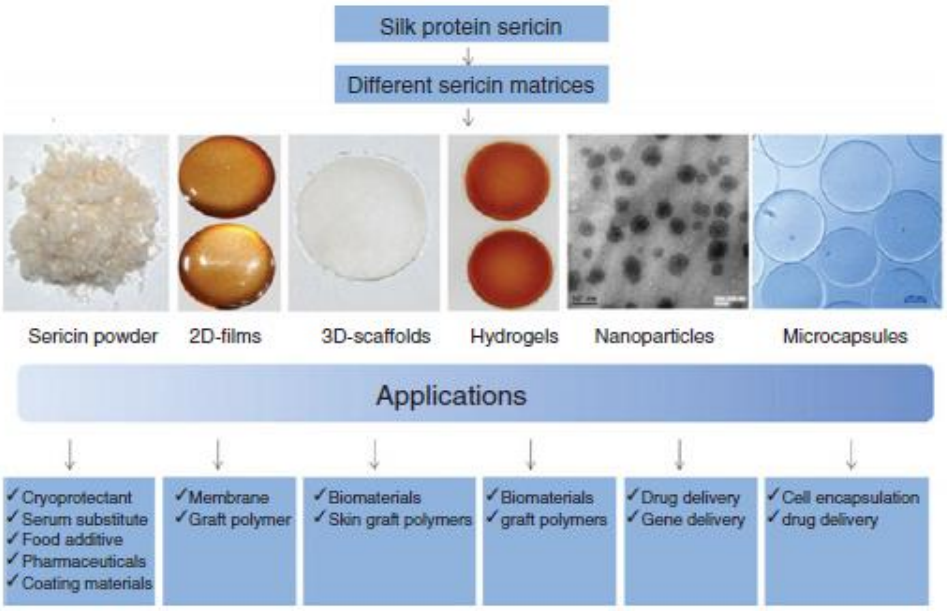

Figure 1.3 The potential applications of sericin in biomedicine (1: coating with SS; 2:cell scaffolding; 3: SS-induced hydroxyapatite nucleation; 4:drug loading and controlled release; 5: stabilization of bioactive molecules by conjugation; 6:SS particles for targeted cell delivery; 7: SS particles or gels for targeted drug delivery.) (Source: Lamboni et al., 2015)

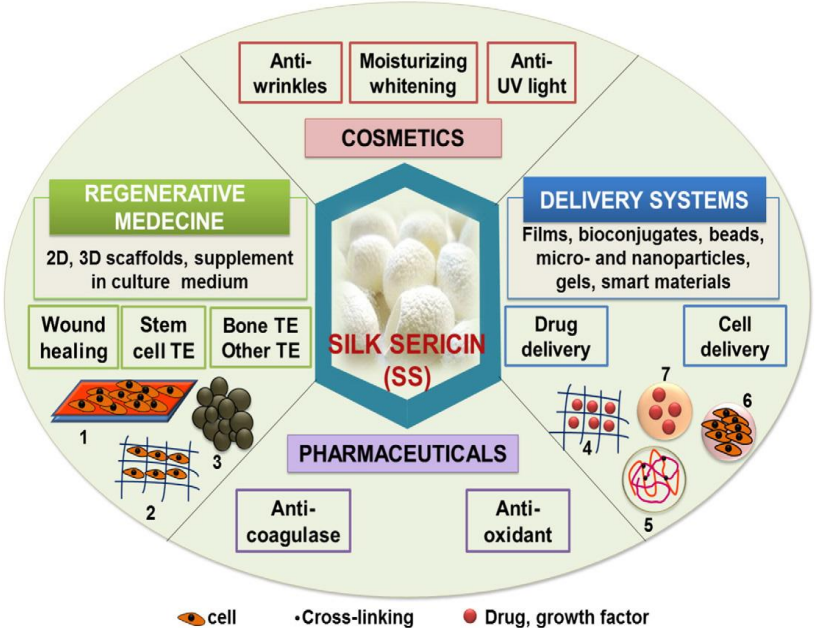

Figure 1.4 Different applications of sericin from different sources. (Source: Neves & Reis, 2016)

1.5 Sericin Extraction and Processing

Various methods can perform for extraction of silk sericin from cocoons of *Bomboxy mori*. The removal of sericin from silk fibroin is accomplished by a process called "degumming". Degumming means the removal of gum from a material, especially the removal of sericin from silk. The degumming process can be done by several methods (Takasu et al., 2002) such as; chemical treatment, boiling with water or enzymatic treatment.

1.5.1 Chemical Treatment of Sericin

This treatment method is used to separate sericin from fibroin by acid or alkali degradation.

1.5.2 Acid Degradation of Sericin

Acids, such as; sulphuric, hydrochloric, tartaric and citric acids can be used as degumming agents (Jiang et al., 2006). As an example, cocoons are cut and added to a 1.25% citric acid solution for acid degradation process, then boiled for 30 min. After removing insoluble fibers by paper filtration, the clear filtrate is dialyzed against distilled water using cellulose tubing for complete removal of citric acid (Aramwit et al., 2009).

1.5.3 Alkali Degradation of Sericin

In this method, sericin is removed from cocoon pieces using sodium carbonate (Na_2CO_3) solution with high heat. The supernatant is collected, filtered and dialyzed using

a 3 kDa dialysis membrane several times to remove alkali solution (Nayak, Talukdar, & Kundu, 2012).

1.5.4 Boiling in Water of Sericin

Boiling in water is the easiest extraction method. To obtain sericin solution the cut cocoons are autoclaved in water using a high temperature (100°C) and high pressure. The aqueous solution obtained from autoclaving the silk cocoons is collected, filtered, frozen and lyophilized to obtain sericin powder (M N Padamwar & Pawar, 2004).

1.5.5 Enzymatic Extraction of Sericin

In this extraction method, sericin is treated with various enzymes (trypsin, papain, alkylase, alkaline protease) at various concentrations and different temperatures (Freddi et al., 2003; M N Padamwar & Pawar, 2004). Enzymatic procedures and degumming in acidic solutions have also been developed, but these methods are likewise limited in application (Freddi et al., 2003).

1.5.6 Problems of Sericin Extraction and Processing of Sericin

Sericin from silkworm cocoon can be obtained by enzymatic treatment, chemical treatment and boiling in water. However, these treatment methods have drastic effects on the structure and composition of obtained sericin in terms of the quality as a biomaterial.

After the degumming treatments using boiling water or an alkaline solution, the size of isolated sericin can range from 10 kDa to over 300 kDa in molecular weight, depending on operating temperature, pH and the processing time. Each method provides sericin products with different amino acid composition and molecular weight (Aramwit,

Damrongsakkul, Kanokpanont, & Srichana, 2010b). For example, while heat and acid degradation give sericin product having a molecular weight of 35-150 kDa, degradation with an alkali solution yields products with a molecular weight of 15-75 kDa (Aramwit, Siritientong, & Srichana, 2012).

Chemical treatments make it difficult to recover high quality sericin for further studies, because additional purification steps needed to remove the chemical impurities (Aramwit et al., 2012). The degumming solution contains a high level of Na_2CO_3 that is difficult to separate from sericin protein. (T.-T. Cao, Wang, & Zhang, 2013). Enzymatic degumming can be effective to remove sericin from the silk cocoon. However, this treatment is an expensive process and promotes specific proteolytic hydrolysis of the primary sericin chain structure, which causes a molecular weight reduction (Freddi et al., 2003).

Consequently, treatment degumming methods do not yield high quality sericin. Sericin obtained shows changes in the structure, amino acid composition and molecular weight (10-400 kDa). These treatment methods bring inconsistencies in the quality of sericin produced as a biomaterial which limits its applications.

1.6 Recombinant Production of Sericin

The genes encoding the silk proteins are very large (up to 15 Mbp) and contain highly repetitive DNA sequence. The biggest obstacle to the production of silk proteins in bacteria (*E.coli*) is that bacteria have difficulty in expression of large genes and codon usage of insects differ from bacteria (Altman et al., 2003).

Therefore, two different methods have been developed to produce silk proteins: First method is construction a cDNA library from mRNA isolated from the glands of silk-producing insects, and the second method is the use of synthetic oligonucleotides designed from the sequence of silk protein optimized for codon usage in bacteria (Vendrely & Scheibel, 2007).

Bacteria are easier to use because of the low cost and a high amount of sericin that can be obtained for industrial use. Thus, the production of silk proteins in bacteria is a

preferred method (S R Fahnestock & Bedzyk, 1997; Lazaris et al., 2002; Lewis, Hinman, Kothakota, & Fournier, 1996).

Sericin gene includes repeated sequences that coding 38 amino acids and has high amounts of serine (Altman et al.,2003). Previous studies have investigated a repetitive motif and designed a 38 amino acid motif representing repetitive regions. (SSTGSSSNTDSNSNSVGSSTSGGSSTYGYSSNSRDGSV.). Designed sericin-like protein did not show the desired stability and structural properties when it was produced. In the production of sericin protein, the use of repeated sequences is an obstacle for cloning and protein production in desired length and function (Huang et al., 2003).

1.7 Aim of the Study

The aim of the study is to produce a sericin like protein (Ser-4mer) in *E.coli* with recombinant production methods. Ser-4mer protein consists of four repeats of 38 amino acid coding repetitive sequence of native sericin. In this study; the expression conditions of the Ser-4mer protein was optimized in *E.coli*. In addition, the secondary structure of the recombinant Ser-4mer protein obtained was determined by using Fourier Transform Infrared Spectroscopy (FTIR), ATR-FTIR (Attenuated total reflection-Fourier transform infrared) and Circular Dichroism (CD). Fibrils structure in the Ser-4mer protein was examined by staining with Congo Red dye and was visualized by Environment Scanning Electron Microscopy (ESEM) and Light microscopy. Furthermore, differential scanning calorimetry (DSC) was used to determine the stability of Ser-4mer protein.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Geneaid Gel/PCR DNA Fragments Extraction clean up kit was used to purify ligated oligonucleotides and digested plasmids. Geneaid Presto ™ Mini Plasmid kit was used to purify plasmid from transformed cells. Geneaid Gel/PCR DNA Fragments Extraction kit was used to purify DNA fragments from agarose gels. The Ni-nitrilotriacetic acid (Ni-NTA) agarose resin was purchased from Thermo Scientific (His Pur ™ Ni-NTA resin). Disposable 5 mL polypropylene Ni-NTA column was purchased to isolate protein from Thermo Scientific. Pierce ™ BCA (Bicinchoninic Acid) Protein Assay kit was used to determine protein concentration from Thermo Scientific. SoluLyse™ Bacterial Protein Extraction Reagent was purchased from Genlantis. isopropyl β-D-1-thiogalactopyranoside (IPTG) and tris base were purchased from VWR Chemicals, imidazole 99 % was purchased from Alfa Aesar. Ampicillin sodium salt was purchased from Fisher Scientific. LB Broth Low Salt was purchased from Duchefa Biochemie Agarose, sodium chloride (NaCl) ≥99.5 %, Congo Red dye content ≥35 % and ethanol ≥99.9 were purchased from Sigma Aldrich. urea ≥99.5 and acetone ≥99.5 were purchased from Isolab. Syringe Filter 0.2 μm Cellulose Acetate Membrane was purchased from VWR, Spectra/Por 1 Dialysis membrane Standard RC Tubing MWCO:6-8 kDa was used to dialyze protein.

2.1.1 Ser-4mer, Vectors, Primers and Restriction Enzymes

Ser-4mer sequence which includes 38 amino acid repetitive motifs was synthesized as Ser1 Oligomer and Ser2 Oligomer by SENTEGEN. pUC19 vector, M13

forward and M13 reverse primers were purchased from New England BioLabs (NEB). The Ser-4mer sequence with pTZ57R/T plasmid which contains the repeating motif of the native sericin sequence was obtained from SENTEGEN. T7 reverse and T7 forward primers were purchased from New England Biolab (NEB). pET21a reverse and pET21a forward primers were synthesized by SENTEGEN. All restriction enzymes (*BamHI*, *EcoRI* and *HindIII*), T4 polynucleotide kinase buffer, T4 DNA ligase, T4 polynucleotide kinase enzyme, Cut smart buffer, T4 buffer, 2X Taq master (mix), NEB 3.1 buffer were purchased from New England Biolab (NEB).

2.1.2 Cells

E. coli DH5α competent cells were used to clone Ser-4mer gene sequence. *E. coli* BL21 (DE3) competent cells were used to express Ser-4mer gene.

2.1.3 Instruments

Incubator and shaker (N-Biotek Shaking Incubator Cooling (NB-205LF)), µDrop Spectrophotometer (Thermo Scientific Multiskan GO), Spectrophotometer (VWR UV-1600 PC), Centrifuge (Isolab and Gyrozen Multipurpose High Speed Centrifugate 1580 R), SDS-PAGE, Agarose gel cassette (Cleaver Scientific, Bio-Rad Power Pac Basic).

FTIR, FTIR-ATR (Perkin Elmer Spectrum ^{TM} 10 Spectrometer), Freeze dryer (CHRIST alpha 1-2/LD plus), DSC (Q10 9.4 Build 287 (T. A. Instruments, USA)).

2.2 Cloning of Ser-4mer into DH5α *E. coli*

For cloning of a sericin-like protein containing four repeats of the 38-amino acid motif (Ser-4mer) protein, two different strategies were attempted. First strategy was

ligating sense and antisense oligonucleotides coding for the consensus sequence of 38 amino acid repeat and cloning the product to pUC19 vector. Second strategy was obtaining a synthetic vector that includes codon optimized native sericin repetitive sequence. (pTZ57R/T vector – sericin tetra repeat sequence). This vector was synthesized by SENTEGEN

2.2.1 Cloning of Sericin 38 Amino Acid Repetitive Motif

Ser 1 oligomer and Ser 2 oligomer were phosphorylated at 37 °C for 45 minutes and 65 °C for 20 minutes (Table 2.1) and were isolated with clean up kit. After phosphorylation, the complementary oligonucleotides which Ser1 oligomer and Ser2 oligomer were annealed by heating at 95 °C for 10 minutes, 96 ºC for 5 minutes and 25 ºC for 5 minutes with by increasing 1 °C from 97 °C and 25 °C. The annealed oligonucleotides were *BamHI*-digested at 37 °C for 1 hours .and was purified with clean up kit. (Table 2.2). Two sets of annealed oligonucleotides were ligated to form one oligonucleotide monomer and was cleaned up using kit. (Table 2.3) Five microliters of the ligation reaction was transformed into 50 µL *E. coli* DH5α competent cells. The obtained clone was named pUC19-SRC4.

Four colonies were chosen to test the presence of insert pUC19-SRC4 in transformed colonies for Colony PCR (cPCR). Selected colonies were dissolved in 0.1 mL deionized distilled water. cPCR was performed initial denaturation at 95 °C for 10 minutes, denaturation at 95 °C for 30 seconds (30 cycle), annealing at 45 °C for 40 seconds (30 cycle), extension at 72 °C for 30 seconds (30 cycle), final extension at 72 °C for 5 minutes with forward and reverse M13 primers. (Table 2.4).

cPCR products which were transformed and loaded on 1% agarose gel. Selected colonies were grown in 7 mL LB medium with 100 µg/mL concentration of ampicillin at 37 °C 220 rpm by shaking incubator for overnight.

The plasmid DNAs were isolated with Presto ^{TM} Mini Plasmid kit from overnight cultures to verify correct plasmid DNA's sequence. Concentration of isolated plasmid DNA was measured in µDrop spectrophotometer. Plasmid DNA was sequenced by

TRIOGEN. The results of sequence analysis were evaluated by using GENEIOUS R11 software.

Table 2.1. Components of Ser1 oligomer and Ser2 oligomer phosphorylation.

Components	**Volume**
DNA	1 µL (2 ng)
T4 PNK Buffer	5 µL
T4 PNK enzyme	1 µL
ddH_2O	13 µL
Total volume	20 µL

Table 2.2. Components of *BamHI* mixture.

Components	**Volume**
DNA	20 µL (1000 ng)
Cut Smart Buffer	5 µL
BamHI	1 µL
ddH_2O	24 µL
Total volume	50 µL

Table 2.3. Component of ligation SRC4 of DNA.

Components	**Volume**
SRC4 DNA (insert DNA)	3.71 µL (6.42 ng)
pUC19 (Vector DNA)	4 µL (25 ng)
T4 Buffer	13 µL
T4 Ligase	1 µL
Total Volume	20 µL

Table 2.4. The components of SRC4 cPCR mix.

Components	**Volume**
Cell mixture (colony)	5 µL
2X Taq master (mix)	5 µL
M13 Forward	0.5 µL
M13 Reverse	0.5 µL
Total volume	11 µL

2.2.2 Cloning Repetitive Sequence of Native Sericin

The designed Ser-4mer sequence with pTZ57R/T plasmid which contains the repetitive sequence of native sericin sequence was synthesized by SENTEGEN. 1 µL pTZ57R/T (pRT_SRC4) was transformed into *E. coli* DH5α competent cells at 59.1 ng concentration.

For sequence analysis, one colony of transformed *E. coli* DH5α was selected from LB-agar plate and grown into 7 mL LB medium (20 g/L) with 100 µg/mL ampicillin at 37 °C 220 rpm by shaking incubator for overnight. Growing cells were pelleted by centrifuge and plasmids were isolated with Presto ™ Mini Plasmid kit. The concentration of purified plasmid DNA was measured in µDrop spectrophotometer Plasmid DNA was sequenced by TRIOGEN. The results of sequence analysis were evaluated by using GENEIOUS R11 software.

The recombinant PRT_SRC4 was digested with *BamHI* and *EcoRI* restriction enzymes at 37 °C for 6 hours (Table 2.5) in PCR. The digested Ser-4mer gene was loaded on a 1% of agarose gel and was isolated with Gel/PCR DNA Fragments Extraction kit.

pET21a could not be digested with two enzymes at the same time due to the small distance between recognition sites. Therefore, instead of double digestion sequential digestion was done. In first digestion, pET21a plasmid was digested with *EcoRI* restriction enzyme (Table 2.6) and *EcoRI* was inactivated at 65 °C for 20 minutes in Dry Bath by heat inactivation. In second digestion , *HindIII* restriction enzyme was added at 37 °C for 6 hours. Clean up kit was applied to digested pET21a to eliminate enzymes.

Digested pET21a was dephosphorylated at 37 °C for 3 hours and followed by cleaned up kit (Table 2.7).

NEB ligation calculation program was used to calculate appropriate DNA concentrations. SRC-4 DNA and pET21a DNA were ligated with T4 DNA ligase enzyme at 16 °C for overnight (Table 2.8). 5 µl of the ligation reaction mix was transformed into 50 µL *E. coli* DH5α competent cells.

Four colonies were chosen to verify the presence of insert pET21a-SRC4 in transformed colonies with cPCR. Selected Colonies was dissolved in 0.1 mL deionized distilled water.

cPCR was performed with initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 30 sec (30 cycle), annealing at 45 °C for 40 sec (30 cycle), extension at 72 °C for 30 sec (30 cycle), final extension at 72 °C for 5 min (Table 2.9) with a primer designed as forward and reverse T7 primers (Table 2.10). cPCR products which were transformed were loaded on 1% agarose gel. Amplified cells were grown in 7 mL LB medium with 100 µg/mL concentration of ampicillin at 37 °C 220 rpm by shaking incubator for overnight. The plasmid DNAs were isolated with Presto ™ Mini Plasmid kit from overnight cultures to confirm correct plasmid DNA's sequence. Plasmid DNA which was isolated of concentration was measured in µDrop spectrophotometer.

Isolated plasmid DNA was sequenced by TRIOGEN. The correct clones of recombinant expression plasmid pET21a_SRC-4 were determinated by DNA sequencing with a primer designed as forward and reverse T7 primers. The results of sequence analysis were evaluated by using GENEIOUS R11 software.

Table 2.5. The components of PRT_SRC4 double digestion mixture.

Components	**Volume**
PRT_SRC4 DNA	40 µL (20.32 µg)
NEB Buffer 3.1 (10X)	5 µL
EcoRI	1 µL
HindIII	1 µL
ddH_2O	3 µL
Total volume	50 µL

Table 2.6. The components of pET21a digestion mixtures.

First Digestion		**Second Digestion**	
Components	**Volume**	**Components**	**Volume**
pET21a DNA	25 µL (9.97 µg)	pET21a DNA	25 µL (9.97 µg)
NEB Buffer 3.1 (10X)	3 µL	NEB Buffer 3.1 (10X)	3 µL
EcoRI	1 µL	*HindIII*	1 µL
ddH_2O	1 µL	ddH_2O	1 µL
Total volume	30 µL	Total volume	30 µL

Table 2.7. The components of dephosphorylation of digested pET21a.

Components	**Volume**
pET21a	20 µL (4.22 µg)
Cut Smart Buffer (10X)	2.5 µL
CIP enzyme	1 µL
ddH_2O	1.5 µL
Total volume	25 µL

Table 2.8. The component of ligation of digested PRT_SRC4 and digested pET21a.

Components	**Volume**
SRC4	1.7 µL (77.08 ng)
pET21a	6.3 µL (304 ng)
T4 Ligase Buffer(10X)	1 µL
T4 Ligase enzyme	1 µL
Total volume	10 µL

Table 2.9. The components of pET21a-SRC4 cPCR mix.

Components	**Volume**
Cell mixture (colony)	5 µL
2X Taq master(Mix)	6 µL
pET21a Forward (10 µM)	1 µL
pET21aReverse (10 µM)	1 µL

Table 2.10. The designed primers to amplify pET21a-SRC4.

Name (F/R)	**Target-specific primer**	**Length**	**GC%**	**T_m**	**T_a**
pET21a Forward primer	CTT TCG GGC TTT GTT AGC AG	20 bp	50 %	52 °C	65.3 °C
pET21a Reverse primer	TCC CGC GAA ATT AAT ACG AC	20 bp	45 %	50 °C	65.3 °C

2.3 Optimization of Expression of Ser-4mer in *E. coli* BL21 (DE3)

Ser-4mer was transformed in *E. coli* BL21 (DE3) cells. The colonies containing pET21a_SRC4 plasmid, were selected from LB-agar plate for expression test. Three colonies which selected were grown with 0.5 mM IPTG at 37 °C for 1, 2 and 3 hours and at 25 °C for overnight For expression test, selected three colonies were grown separately in 2 mL LB medium with 100 µg/mL concentration of ampicillin at 37 °C 220 rpm by shaking incubator for overnight. 100 µL of growing colonies were transferred in 10 mL LB medium with 100 µg/mL concentration of ampicillin at 37°C 220 rpm by shaking incubator.

Upon reaching OD600 ~ 0.5 value, protein expression was induced with 0,5 mM IPTG concentration and samples were taken as normalized so that final OD value was 0.5 without IPTG and with IPTG for 1 hour, 2 hours and 3 hours. Samples were centrifugated

at1500 rpm for 30 minutes. Remaining cell culture was induced at 25 °C with 220 rpm for overnight. Likewise, samples were centrifugated at 1500 rpm for 30 minutes. All collected samples were kept at -80 °C until 5% SDS-PAGE analysis.

2.4 Purification of Ser-4mer Protein

The solubility of expressed Ser-4mer protein was determined by SoluLyse™ method. Histidine-tagged Ser-4mer protein at the C terminal was tested in small scale protein isolation with Ni-NTA column. Ser-4mer protein was also isolated in large scale. The concentration of isolated Ser-4mer was determined by UV-visible spectrum at wavelength 280 nm and Bicinchoninic (BCA) Assay.

2.4.1 Testing the Solubility of Ser-4mer Protein

Expression was performed with IPTG induction. At the end of expression, 1.5 mL samples were taken to test solubility and pelleted by centrifugation at 10,000 rpm for 10 minutes and were stored at -80 °C. 150 µL of SoluLyse™ Reagent was added to dissolve these pellets by pipetting. Centrifugation was performed for 5 minutes at 14,000 rpm to separate soluble and insoluble part. The supernatant part was separated. The pellet was resolubilized with 300 µL of SoluLyse ™ Reagent. Collected samples were visualized by 5% SDS-PAGE analysis. This method is referred as the SoluLyse™ Method.

2.4.2 Small Scale Isolation of Ser-4mer protein

Chemical agents such as urea or guanidine hydrochloride (GuHCl) can be used for making proteins soluble as a result of denaturation. During isolation, Ser-4-mer protein is not soluble in bacteria so it can be isolated in urea in the denatured state. Ser-

4mer which included histidine tag at the C-terminal was tested by small scale protein isolation.

For this purpose, 10 mL scale of expression was performed with expressed two colonies that were previously expressed by IPTG induction as described SoluLyse™ method. Cells obtained were pelleted by centrifugation and SoluLyse™ method was applied to samples. Supernatant and pellet that was obtained by SoluLyse™ method was applied to Ni-NTA column.

Firstly, SoluLyse ™ Reagent (50 mM phosphate buffer, pH 7.4) was used for Ni-NTA (2:1) and was washed by centrifugation at 800 ×g for 2 minutes. After centrifugation, the supernatant was discarded, and the pellet was solved to 400 µl SoluLyse™ reagent and was performed by centrifugation at 800 ×g for 2 minutes. This procedure was repeated once more. 270 µL Ni-NTA resin was added to Ser-4mer protein and incubated in the rotator for 1 hour at +4 °C. After the rotator, Ser-4mer-Resin were centrifuged at 800 ×g for 2 minutes. The pellets were solved to 400 µL SoluLyse™ reagent and were performed by centrifugation at 800 ×g for 2 minutes. The wash step was repeated 3 times. In elution step, 100 mM imidazole was added to SoluLyse™ reagent. The pellets were solved to 400 µL SoluLyse™ reagent and 100 mM imidazole and followed by centrifugation at 800 ×g for 3 minutes. The elution step was repeated 3 times. All samples during the washing and elution steps with resin added to supernatant and pellet were collected and stored at -80 °C. During the washing and elution in Ni-NTA, acetone precipitation (4:1) method was applied to concentrate collected samples. All collected samples were examined by 5% SDS-PAGE analysis.

2.4.3 Large Scale Isolation of Ser-4mer Protein

Ser-4-mer was expressed in3,5 L *E. coli* BL21 (DE3) culture. For purification of protein, cells were collected by centrifugation and resuspended in lysis buffer (100 mM $Na_2H_2PO_4$, 10 mM Tris base, 8 M urea, 20 mM imidazole, pH 8.0), at 5 mL per gram wet weight followed by sonication and centrifugation. Ni-NTA column was used to purify protein .After centrifugation Ser-4mer protein was obtained in supernatant in the presence of urea. Ni-NTA resin was incubated with supernatant for 1 hour at room temperature.

Ni-NTA column was washed with 20 mL wash buffer (8 M urea, 300 mM NaCl, 20 mM imidazole, 50 mM $Na_2H_2PO_4$, pH 8.0) by binding to the Ni-NTA resin column.

The UV-Visible spectra of protein bound to the Ni-NTA column was followed at wavelength of 280 nm. After washing step, 10 mL elution buffer (8 M urea, 300 mM NaCl, 250 mM imidazole, 50 mM $Na_2H_2PO_4$, pH 8.0) was loaded in Ni-NTA column Thus, Ser-4mer protein was obtained in the elution buffer. Ser-4mer protein which was isolated was visualized by 15% SDS-PAGE analysis.

Matrix-assisted laser desorption/ionization flight time mass spectroscopy (MALDI-TOF) analysis was performed for 15 μM dialyzed Ser-4mer protein.

Native gel analysis was performed 7 μg Ser-4mer protein, 7 μg commercial sericin, 7 μg lysozyme and 7 μg albumin. In addition, dialyzed commercial sericin and Ser-4mer protein were visualized by 12 % SDS-PAGE analysis.

2.4.4 The Determination of the Concentration Ser-4mer Protein

The concentration of Ser-4mer protein was analyzed by Bicinchoninic (BCA) Assay against a bovine serum albumin (BSA) standard and was determinated with UV-Visible spectra at wavelength of 280 nm. Firstly, the UV-Visible spectra of isolated Ser-4mer protein was determined at between wavelengths of 280-350 nm. According to Expasy Prot Param, the extinction coefficient of the obtained Ser-4mer protein at 280 nm was calculated as 11.920 $mM^{-1}cm^{-1}$. For BCA assay, the determined volume of BSA (2 mg/mL) standards was prepared by making serial dilutions with a range of 20-2,000 μg/mL in protocol. The total volume of BCA working reagent (WR) was calculated to prepare by mixing 50 parts of BSA reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 10 μL of each BSA standards, isolated Ser-4mer protein and control elution buffer were pipetted as duplicate into a microplate well (working range = 20-2000 μg/mL) (sample to WR ratio = 1:20) 200 μL of the WR to each well were added and were mixed by pipetting plate thoroughly on a plate for 30 seconds. Plate was covered and incubated at 37 °C for 30 minutes in incubator. Plate was cooled to room temperature; absorbance was measured at 562 nm on a plate reader. The average absorbance reading of the blank standard 562 nm was subtracted from the 562 nm reading of average of

absorbance duplicates from all other standards and isolated sericin proteins. For each BSA standard, a standard curve was prepared by plotting the average Blank-corrected 562 nm reading, concentration versus μg/mL. Standard curve was used to determine the concentration of each diluted Ser-4mer protein.

2.4.5 The Determination of Extinction Coefficient

The extinction coefficient of Ser-4mer protein was determined in different concentrations in elution buffer (8 M urea,50 mM Na_2HPO_4, 250 mM imidazole, pH 8). The UV-visible spectrum of extinction coefficient Ser-4mer protein was taken at between wavelengths of 250-350 nm.

2.5 Characterization of Ser-4mer Protein

Initially, the isolated Ser-4mer protein was dialyzed against water to examine its solubility and structure in water. The dialyzed Ser-4-mer protein was lyophilizated to determined secondary structure by Fourier Transform Infrared Spectroscopy (FTIR) analysis for solid and solution state. Congo red assay was used to determine the fibril structure of Ser-4mer proteins. Lastly, stability of Ser-4mer protein was investigated Differential Scanning Calorimetry (DSC) analysis by compare with commercial sericin.

2.5.1 The Dialysis of Ser-4mer Protein against Deionized Water and Phosphate Buffer

Membrane (MW:6-8 kDa) was used for dialysis. Ser-4mer protein was dialyzed against in deionized water at + 4 °C. The following day, the UV-visible spectrum of dialyzed Ser-4mer protein was taken at between wavelengths of 250-350 nm.

Furthermore, the concentration of dialyzed Ser-4mer protein was determined by BCA assay.

Besides, Ser-4mer protein was dialyzed in dialyzer tube at 2.5 mg/mL concentration in 2 mL volume against 1mM phosphate buffer at pH 6 and pH 8 at + 4 °C. To determine the amount of dissolved protein, before dialysis and after dialysis centrifugation was performed at 14000 ×g for 10 minutes. The UV-visible spectrum of dialyzed Ser-4mer protein was taken at between wavelengths 250-400 nm.

2.5.2 The Determination of Secondary Structure of Ser-4mer Protein

500 µL of dialyzed Ser-4mer protein was lyophilized for one day at Freeze dryer. Both lyophilized Ser-4mer protein and commercial sericin were examined for solid state with the Fourier Transform Infrared Spectroscopy (FTIR) in the transmission mode.
For comparison, 20 µL dialyzed Ser-4mer protein (2 mg/mL) and 20 µL Commercial sericin (2 mg/mL) were analyzed with ATR-FTIR (Attenuated Total Reflection- Fourier-transform infrared) spectroscopy in the transmittance mode on background ddH_2O for solution state. Also, Circular Dichroism (CD) was performed to determine the secondary structure of dialyzed Ser-4mer protein and Commercial sericin. 400 µL Dialyzed Ser-4mer protein (0.2 mg/mL) and 400 µL Commercial sericin (0.2 mg/mL) were prepared for CD analysis and ddH_2O was used as buffer.

2.5.3 The Determination of Fibril Structure Ser-4mer Protein

200 µM stock of Congo red (CR) was prepared in phosphate-buffered saline (1X phosphate buffered saline (PBS), 0.01 M phosphate buffer, 0.0027 M potassium chloride (KCl) and 0.137 M sodium chloride (NaCl), pH 7.4) and 10% ethanol. Ethanol was added to the stock solution to prevent CR micelle formation.

CR stock solution was diluted with 1 mM Na_2HPO_4 pH 7 and 40% ethanol.CR solution was filtered twice using Syringe Filter 0.2 µm Cellulose Acetate Membrane. The

concentration of diluted CR solution (7 μM) was determined by measuring the absorbance of an aliquot of the filtrate at 505 nm.

10 μM dialyzed recombinant sericin,10 μM commercial sericin,7 μM CR and 10μM dialyzed recombinant sericin, 7 μM CR and 10μM commercial sericin and as control 10 μM BSA,7 μM CR and 10 μM albumin were prepared for Congo red staining. Mixtures of all samples were incubated at room temperature for spectral analysis and all measurements were taken in wavelength-scanning mode from 300 to 700 nm at different time range (0, 5, 10, 15, 20 mins). The graphs formed according to the UV-visible spectra measurements were analyzed at specific time range.

Besides, 100 μL dialyzed Ser-4mer protein and 500 μL CR solution (100 μM in PBS solution, pH 7,4) were incubated at room temperature overnight. After centrifuge at 14, 000 rpm for 5 min, the pellet was resuspended with 1 mL PBS solution pH 7.4 and centrifuged again to pellet the sample. Unbound Congo red was removed by repeating the process twice. 100 μL PBS, pH 7.4, was added to pellet and 20 μL sample was pipetted onto a glass slide. The slide was then dried for a 1 hour. Fibrils in the structure of Ser-4mer protein were determined with Light Microscopy.

To remove PBS from the remaining of Ser-4mer protein pellets, pellets were resuspended with ddH_2O and this process was repeated 5 times. The pellets could dry in hood for 3 days and fibril morphology was characterized with Environment Scanning Electron Microscopy (ESEM).

2.5.4 The Determination of the Stability of Ser-4mer Protein

Ser-4mer protein and commercial sericin were prepared by adjusting 2 mg/mL concentration. 5.7 mg dialyzed Ser-4mer protein and 5 mg commercial sericin were encapsulated into Al pans.Ser-4mer protein and commercial sericin were analyzed by differential scanning calorimetry (DSC). Standard mode DSC was performed at heating rate of 10 °C/min start temperature to 25 °C and final temperature 230 °C. Therefore, the melting temperature of dialyzed Ser-4mer protein and commercial sericin were determined.

2.6 Electrophoresis

Gel electrophoresis is used to separate charged molecules like DNA and proteins according to their size. Smaller molecules migrate through the gel more quickly and therefore travel further than larger fragments that migrate more. As a result, the molecules are separated by size (Koontz, 2013).

2.6.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was utilized to show the success of plasmid construction (pTZ57R/T-ser gene and pET21a-ser gene). 1% agarose gel (0.3 g Agarose, 333 µL 50X TAE, 30 mL ddH_2O and 3 µL SYBR Safe DNA gel strain dye) was prepared. Colony PCR samples and 1 kb DNA Ladder were stained (6:1) with gel loading dye purple (6X). Colony PCR samples were followed by separation at 100 V for 30 minutes.

2.6.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to observe in the presence of Ser-4mer protein band, to compare expression of protein and to follow isolation and purification steps. For SDS-PAGE analysis, separating gel (450 µL ddH_2O, 2.5 mL 30% acrylamide, 1.950 mL 1M tris (pH 8.8), 50 µL 10% SDS, 50 µL 10% ammonium persulfate (APS) and 4 µL tetramethylethylenediamine (TEMED)) was prepared and 400 µL butanol was added to prevent oxidation of the gel. Then the gel incubated to polymerize for 30 minutes. Stacking gel (2.100 mL ddH_2O, 500 µL 30% acrylamide, 380µL 1M tris (pH 6.8), 30 µL 10% SDS, 30 µL 10% APS and 3 µL TEMED) was prepared gel and incubated to polymerize for around 40 minutes. Protein samples were prepared with 450 µL 6X loading dye solution (375 mM tris.HCl,

9% SDS, 50% glycerol, 0.03% bromophenol blue) and 50 µL 1 M DTT solution by heating 90 °C for 2 minutes. 10 µL (approximately 10 µg) protein samples were loaded the gel. Protein samples were run at 30 Volt for 1 hour and were followed 100 Volt for 2 hours.

2.6.3 Native Gel Electrophoresis

Native gel electrophoresis is run in the absence of SDS. In Native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. For Native PAGE analysis, separating gel (2 mL 40% acrylamide, 2.5 mL tris HCl (pH 8.8), 5.5 mL ddH_2O, 50 µL 10% APS and 10µL TEMED) was prepared and 400 µL butanol was added to prevent oxidation of the gel. Then the gel was incubated to polymerize for 1 hour. Stacking gel (0.4 mL 40% acrylamide, 1 mL tris HCl (pH 6.8), 2.6 mL ddH_2O, 20 µL 10% APS and 5 µL TEMED) was prepared gel and incubated to polymerize for around 1 hour. Protein samples were dissolved in a same volume of 2 X Sample Buffer (2.5 mL 2 M tris-HCl (pH 6.8), 2 mL 20% glycerol, 40 µL 0,02% bromophenol blue, 5.5 mL ddH_2O). 10 µL (7 µg) protein samples were loaded the gel. Protein samples were run at 30 V from the stacking gel to the separating gel. Then, it was increased to 60 V and was run for about 4 hours.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Construction of Synthetic Sericin Motif DNA

To clone a sericin-like protein containing four repeats of the 38-amino acid motif (Ser-4mer) protein, two different strategies were attempted. First strategy was ligating sense and antisense oligonucleotides coding for the consensus sequence of the 38-amino acid repeat and cloning the product to pUC19 vector. Second strategy was obtaining a synthetic vector that includes codon optimized native sericin repetitive sequence. (pTZ57R/T vector - sericin repeat sequence). This vector was synthesized by SENTEGEN.

3.1.1 Encoding Ser-4mer

Previously, the consensus sequence of 38-amino acid repetitive motif of sericin was recombinantly produced by Huang et al (Huang et al., 2003). A similar procedure to Huang et. al was followed to obtain the sericin-like protein. In this context, a DNA sequence encoding 38 amino acid repeats is shown in Figure 3.1. Based on this sequence Ser1 Oligomer and Ser 2 Oligomer were synthesized (Table 3.1.).

Ser1 and Ser 2 oligomers were phosphorylated and ligated with pUC19 vector. The ligated DNA product was transformed into *E.coli* DH5α. Colony PCR was performed as described in the methods section by selecting colonies from transformed cells. The PCR product were visualized on 1% agarose gel electrophoresis (Figure 3.2).

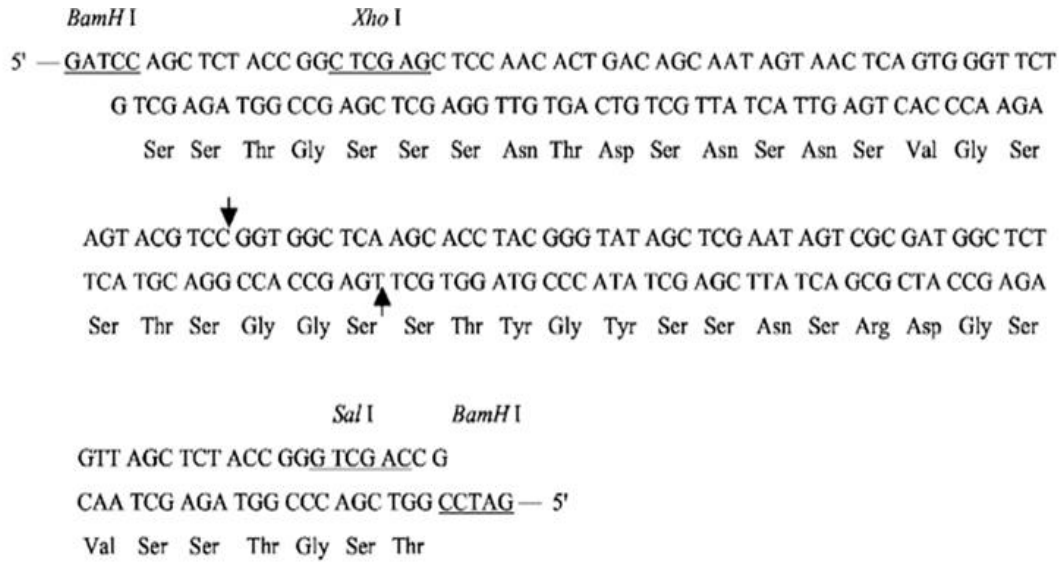

Figure 3.1 Oligonucleotide constituting the gene encoding the 38 amino acid motif repeating in the sequence of sericin (Source: Huang et al., 2003).

Table 3.1. Ser1 and Ser2 oligomer sequences.

Oligomer	**Sequence**
Ser1	5'-GATCC AGC TCT ACC GGC TCG AGC TCC AAC ACT GAC AGC AAT AGT AAC TCA GTG GGT TCT AGT ACG TCC GGT GGC TCA AGC ACC TAC GGG TAT AGC TCG AAT AGT CGC GAT GGC TCT GTT AGC TCT ACC GGG TCG ACC G-3'
Ser2	5'- GATCC GGT CGA CCC GGT AGA GCT AAC AGA GCC ATC GCG ACT ATT CGA GCT ATA CCC GTA GGT GCT TGA GCC ACC GGA CGT ACT AGA ACC CAC TGA GTT ACT ATT GCT GTC AGT GTT GGA GCT CGA GCC GGT AGA GCT G-3'

Sericin repeat sequence as shown in Figure 3.1. corresponds to 138 bp. Bands obtained from PCR of colonies 2,3,4 corresponded to approximately 138 bp compared to the marker on 1% agarose gel electrophoresis. Plasmid isolation of colony 3 was performed and sample was sent to sequence analysis. According to the sequence results, when the obtained sequence was compared with expected sericin sequence using

GENEIOUS R11, no match was found. Plasmid sequence did not contain the inserted sequences.

Ligation and cloning of Ser1 and Ser 2 oligomers into the pUC19 vector were attempted. However, the designed sequence could not be obtained. Therefore, the sericin gene was obtained by gene synthesis.

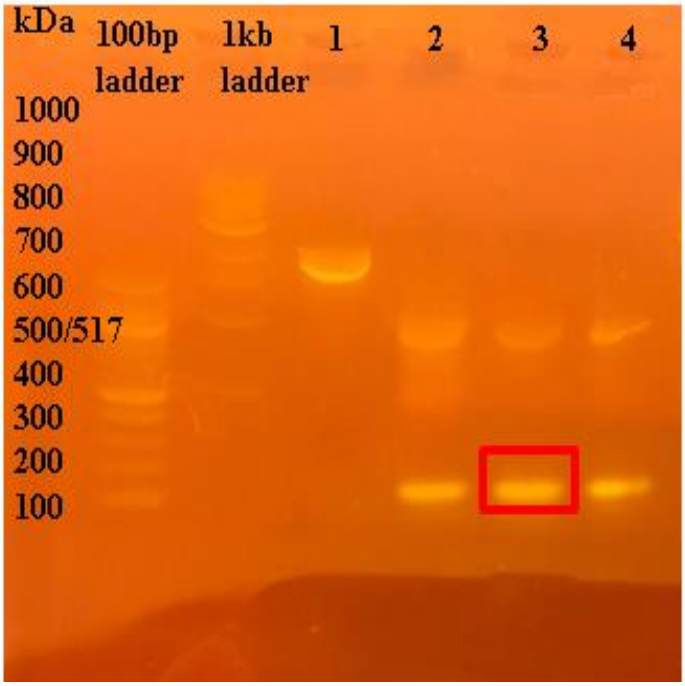

Figure 3.2 1% Agarose gel electrophoresis image of different colonies (1, 2, 3 and 4 bands) containing sericin DNA in pUC19 plasmid (100bp and 1 kb DNA Ladder).

Repetitive motifs were determined by examining the sequences of native silk proteins. Based on the analysis the sequence of the four repeats of the 38-amino acid motif of *Bombyx mori* Sericin 1A protein was obtained (Sericin 1A ID K7WYH1). This sequence is shown in Table 3.2. The amino acid sequence shown in Table 3.2. was codon optimized for *E. coli*, the nucleotide sequence obtained is shown in Table 3.3. The codon optimized sequence shown in Table 3.3. inserted in the pTZ57R/T plasmid was synthesized by SENTEGEN.

Table 3.2. The amino acid sequence containing four repeats of the 38-amino acid motif of *Bombyx mori* Sericin 1A protein (Ser-4mer).

Sequence	VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGSVSSTGSSS NTDSSTKNAGSSTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKS AGSSTSGGSSTYGYSSRHRGGRVSSTGSSSTTDASSNSVGSSTSGG SSTYGYSSNSRDGS

Table 3.3. The codon optimized DNA sequence of Ser-4mer protein.

Sequence	GTG AGT AGT ACC GGA TCA ACG AGT AAT ACA GAT TCT TCT TCT AAA TCT GCT GGT TCA CGC ACT TCG GGA GGG AGT TCG ACT TAT GGG TAT TCG AGT TCG CAT CGT GGG GGT TCC GTT AGT TCC ACG GGG TCC TCA TCG AAC ACG GAC TCG TCG ACT AAA AAT GCG GGG AGC TCT

pTZ57R/T plasmid (pRT_SRC4) containing the Ser-4mer sequence was transformed into *E. coli* DH5α. Plasmid isolation was performed on transformed colonies. The plasmids obtained were sent for sequence analysis. The sequence of pRT_SRC4 plasmid samples and the sequence of Ser-4mer gene were compared with Geneious program, a match was observed (Figure C.1). Thus, pTZ57R/T plasmid (pRT_SRC4) containing Ser-4mer sequence was transformed to *E. coli*.

3.2 Cloning the Ser-4mer Gene Obtained to Expression Plasmid

In order to express Ser-4mer gene, it must be transferred into an expression vector. The pET21a expression vector contains a histidine tag at the C-terminal and the expression is controlled by the T7 gene 10/lac promoter. Therefore, both a high amount of protein expression and a tighter expression control exist in pET21a expression vector.

Also transcription and translation of the entire gene is ensured by the T7 polymerase because there is no Rho termination (Stephen R Fahnestock, Yao, & Bedzyk, 2000). In addition, isolation of the obtained protein is easier with affinity chromatography using the histidine tag. To this end, pRT_SCR4 plasmid containing the Ser-4mer gene and pET21a expression vector were cut with *EcoRI* and *HindIII* restriction enzymes. Double digestion was performed on pRT_SRC4 and the insert can be observed on 1% Agarose gel electrophoresis in Figure 3.3A.Ser-4mer gene and empty pTZ57R/T vector are seen about 0.5 kb and 2.8 kb respectively. Ser-4mer gene is isolated as described in the materials and methods section.

The recognition sites of *EcoRI* and *HindIII* restriction enzymes are very close to each other in pET21a expression vector. Therefore, instead of double digestion each digestion was made separately. pET21a vector, which was cut by the two enzymes, was dephosphorylated to ensure that the vector does not re-circularize during ligation. pET21a vector which was cut with by *EcoRI* and *HindIII* restriction enzymes and Ser-4mer gene were ligated with T4 ligase enzyme. pET21a_SRC4 plasmid obtained by ligation was transformed into *E. coli* DH5α. Colony PCR was used to test the presence of Ser4-mer gene from transformed pET21a_SRC4 colonies and visualized on 1% agarose gel electrophoresis in Figure 3.4 (Bergkessel & Guthrie, 2013).

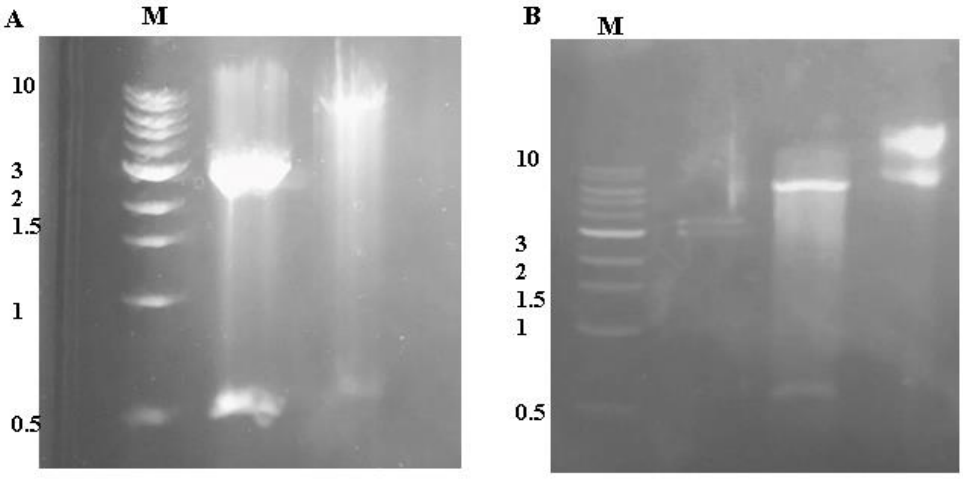

Figure 3.3 1% Agarose gel image of bands including Ser-4mer gene obtained from double digestion. (M: Marker, 1 kb DNA Ladder) A, double digestion of pRT_SRC4 (1), pET21a_SRC4 (2).B, double digestion pET21a_SRC4 (2) and empty pET21a (3).

Approximately 0.5 kb of band is expected to be observed in colony PCR if Ser-4mer gene is inserted, a band of about 0.1 kb was expected in empty pET21a. As shown in Figure 3.4. in empty pET21a was observed about 0.1 kb and pET21a_SRC4 was observed about 0.5 kb. Apart from these, two more unexpected bands were observed. Since the binding temperature of the T7 primers was 50° C, non-specific binding may have taken place. Therefore, non-specific bands were observed around 1.5 kb and 0.7 kb. Plasmid samples from colony D and E were sent to sequence analysis. Colony D was selected according to sequence analysis result that show it contains Sericin-4mer gene sequence in pET21a_SRC4 (Figure C.2). However, colony E did not contain Sericin-4mer gene sequence in pET21a_SRC4 (Figure C.3).

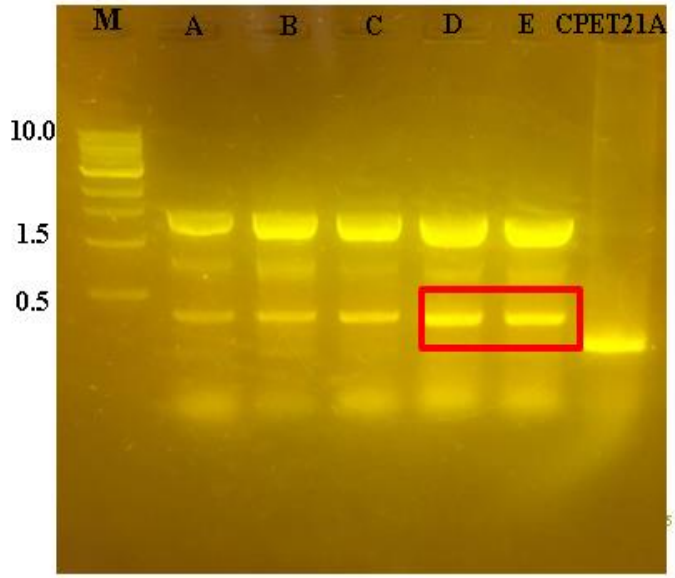

Figure 3.4 Colonies which include pET21a_SRC4 plasmid after ligation (A, B, C, D and E band) and as control empty pET21a (CPET21A) in 1% agarose gel (M:Marker,1 kb DNA Ladder).

3.3 Expression of Ser-4mer Protein

Expression of his-tagged Ser-4-mer protein was optimized for time and temperature.

3.3.1 Optimization of The Expression of Ser-4-mer Protein

pET21a_SRC4 plasmid was transformed into *E. coli* BL21 (DE3). The protein sequence obtained by the addition of Ser-4mer gene to plasmid pET21a is shown in Table 3.4.

Table 3.4. Ser-4mer protein sequence.

Sequence	MASMTGGQQMGRGSEFELGTSRMHLDSVSSTGSTSNTDSSS KSAGSRTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKNAGS STSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKSAGSSTSGGS STYGYSSRHRGGRVSSTGSSSTTDASSNSVGSSTSGGSSTYGYS SNSRDGSSIGSRARRLQRPACKLAAALEHHHHHH

When Ser-4mer amino acid sequence is examined with Expasy protParam tool, the calculated molecular weight is 20 kDa, pI value is 10 and extinction coefficient is calculated as 11920 $cm^{-1}M^{-1}$. The amino acid content of Ser-4mer is shown in Table 3.5.

Table 3.5. Ser-4mer amino acid composition.

Ala (A) 4,9%	Arg (R) 6,3%	Asn (N) 2,9%	Asp (D) 2,9%
Cys (C) 0,5%	Gln (Q) 1,5%	Glu (E) 1,5%	Gly (G) 16,0%
His (H) 4,9%	Ile (I) 0,5%	Leu (L) 2,4%	Lys (K) 1,9%
Met (M) 1,9%	Phe (F) 0,5%	Pro (P) 0,5%	Ser (S) 34,0%
Thr (T) 10,7%	Trp (W) 0,0,%	Tyr (Y) 3,9%	Val (V) 2,4%

As shown in Table 3.5, the hydrophilic amino acid composition of Ser 4-mer contains 34% serine, 11% threonine and 4% tyrosine. These values are consistent with

the average amino acid composition of native sericin protein (38% serine, 7,5% threonine and 4,7% tyrosine (Huang et al., 2003)).

After transformation of pET21a_SRC4 plasmid, colonies were selected for expression test. Expression was followed by 5% SDS PAGE analysis as shown in Figure 3.5. As seen in Figure 3.5 Colony1 and Colony2 resulted in maximum expression with induction time 2 hour and 0.5 mM IPTG at 37 °C.

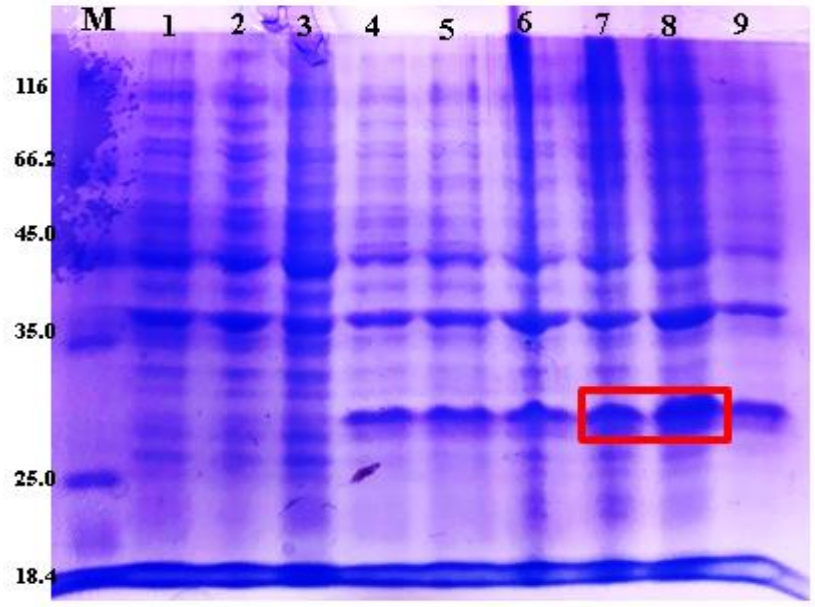

Figure 3.5 SDS-PAGE gel analysis of the expression of Ser-4mer protein (5% SDS-PAGE) M: Protein Ladder. Lane 1,2,3: cell lysate before IPTG Lane 4,5,6: cell lysate after 1 hour of IPTG (0,5 mM) Lane 7,8,9: cell lysate after 2 hour of IPTG (0,5 mM) induction at 37 ° C.5,6: cell lysate after 1 hour of IPTG (0,5 mM) Lane 7,8,9: cell lysate after 2 hour of IPTG (0,5 mM) induction at 37 ° C.

The molecular weight of the expressed protein was observed at 30 kDa which is 10 kDa higher than expected. A similar result was observed in previous studies when proteins containing repetitive amino acids of sericin was produced, the expects molecular weight was 16 kDa, while it was observed at around 40 kDa in SDS-PAGE analysis (Huang et al., 2003). This situation is thought to be due to reduced electrophoretic mobility which is also observed in other proteins (Huang et al., 2003).

To eliminate this confusion, the molecular weight of the Ser-4mer protein was also determined by Matrix-assisted laser desorption/ionization flight time mass spectroscopy (MALDI-TOF) analysis. MALDI-TOF analysis was performed for Ser-4mer protein which was dialyzed against ddH_2O at a concentration of 15 µM.

MALDI-TOF analysis of Ser-4mer protein is shown in Figure 3.6. As seen in Figure 3.6, the molecular weights observed are 20 kDa and 40 kDa. Ser-4mer protein monomer can be observed at 20 kDa. It can form a dimer by forming disulfide bonds which would be observed at 40 kDa. Sericin amino acid sequence contain a cysteine. Since this disulfide bond was not reduced during dialysis, a portion of the Ser-4mer protein formed dimer.

In this case, the m / z values observed in the MALDI-TOF analysis were: 40100, +1 charged dimer; 20150, +1 charged monomer; 12370, +3 charged dimer; 10008, +2 charged monomer; 6700 can be interpreted as +3 charged monomer. The molecular weight of the Ser-4mer protein by MALDI-TOF analysis is consistent with to the value obtained based on the protein sequence.

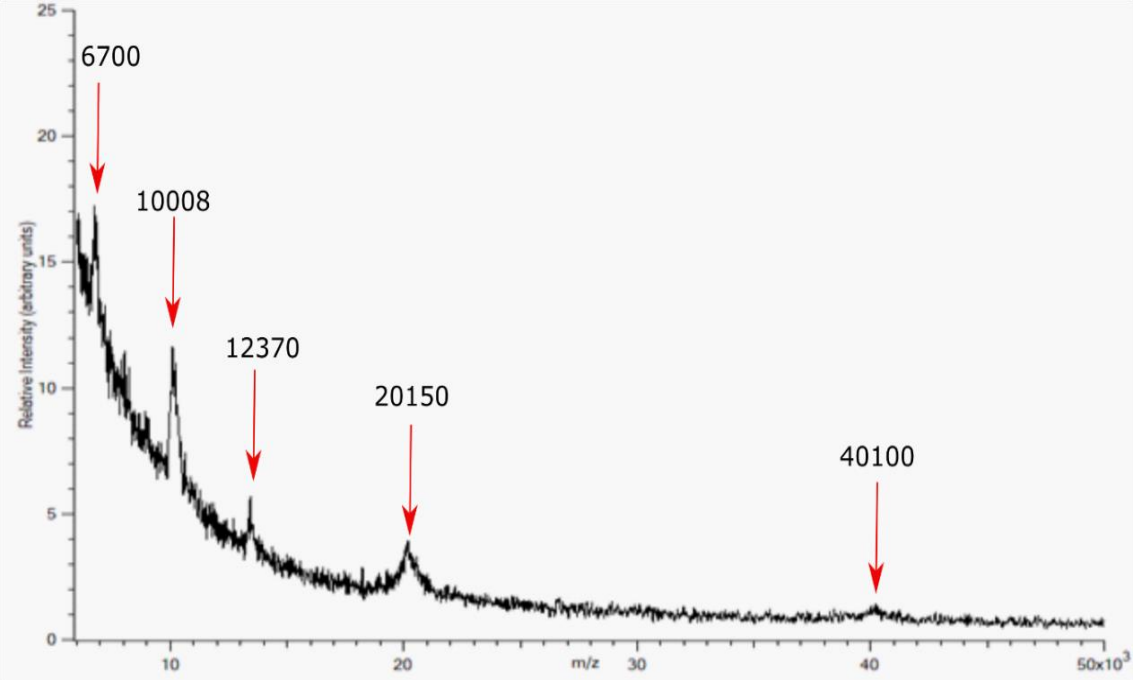

Figure 3.6 Analysis of Ser-4mer protein by mass spectroscopy (MALDI-TOF) is indicated the approximate m / z values by arrows.

3.4 Purification of Ser-4mer Protein

Since silk proteins form insoluble aggregates the solubility of Ser-4mer protein was tested to optimize protein isolation. SoluLyse™ cell lysis reagent was used to determine whether Ser-4mer protein was soluble. After solubility studies small scale protein isolation was carried out for Ser-4mer which contains a histidine tag at C-terminal. Histidine-tagged Ser-4mer was isolated by binding to the Ni-NTA resin. The isolation of Ser-4mer was followed by SDS-PAGE analysis.

3.4.1 Testing the Solubility of Ser-4mer Protein

Two samples of Ser-4mer expression were analyzed as described in SoluLyse™ method. During this analysis, collected supernatant and pellet samples were visualized by 5% SDS-PAGE analysis (Figure 3.7).

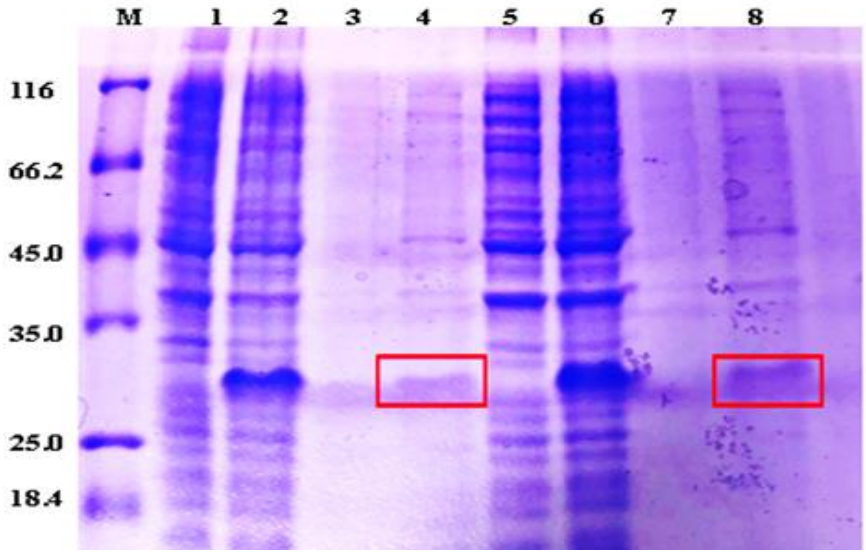

Figure 3.7 SDS-PAGE gel analysis of the supernatant and pellet samples from the SoluLyse method of Ser-4mer protein (5% SDS-PAGE) M: Protein Ladder. *Lane 1 and 5:* Cell pellet before IPTG. *Lane 2 and 6:* Cell pellet after of 2-hours IPTG. *.Lane3 and 7:* Solulyse supernatant. *Lane4 and 8:* Solulyse pellet.

The samples were obtained after expression of Ser-4mer for 2 hours. The presence of Ser-4mer protein band at 30 kDa in supernatant samples indicates that protein is soluble, while the presence the band in pellets indicates that protein is insoluble in water.

As seen in Figure 3.7, supernatant samples did not contain Ser-4mer bands. On the other hand, pellet samples contain Ser-4mer. Therefore, it is concluded that Ser-4-mer protein is not a water-soluble.

3.4.2 The Small-Scale Isolation of Ser-4mer Protein

For small scale isolation of Ser-4mer protein, 10 mL expression was performed with colonies described. The samples were obtained after expression of Ser-4mer for 2 hours. The obtained samples were examined by SDS-PAGE analysis Figure 3.8

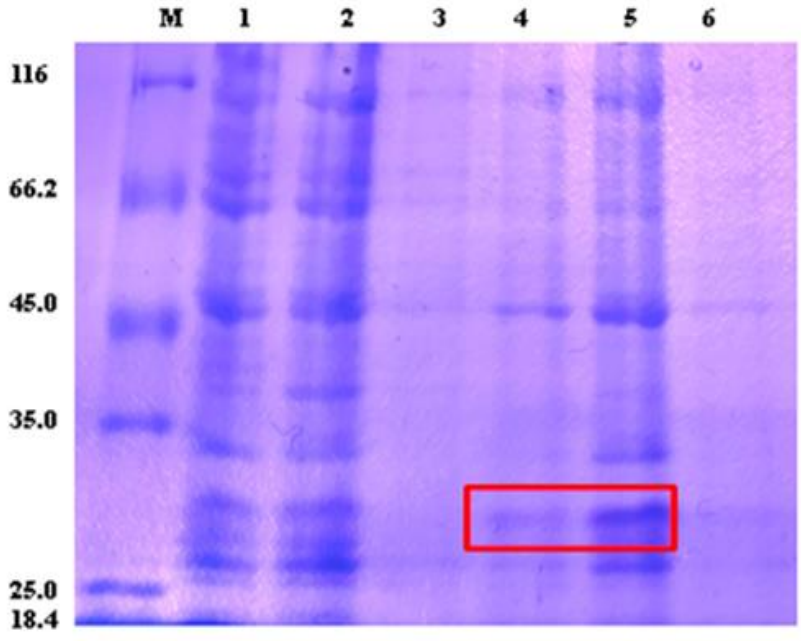

Figure 3.8 SDS PAGE gel analysis of samples that were obtained from Ni-NTA in washing and elution steps (5% SDS-PAGE) M: Protein Ladder. *Lane 1:* Wash step 1. *Lane 2:* Wash step 2.*Lane 3:* Wash step 3.*Lane 4:* Elution step 1 *Lane5:* Elution step 2.*Lane 6:*Elution step3.(SoluLyse™ Bacterial Protein Extraction Reagent.

As show in Figure 3.8, in elution steps lanes 4, 5 and 6 Ser-4mer protein was observed. Thanks to histidine (6XHis) tag, Ser-4mer protein was bound to Ni-NTA column Thus, the presence of 6XHis tag was confirmed in Ser-4mer protein.

3.4.3 The Large-Scale Isolation of Ser-4mer Protein

Sericin protein was observed insoluble in water. Thus, denaturants such as urea or guanidine hydrochloride (GuHCl) were used to solublize Ser-4mer protein.

Ser-4-mer protein was expressed in 3.5 L in *E. coli* BL21 (DE3) culture. After protein expression, approximately 5.45 g cells were harvested. Isolation of Ser-4mer protein was followed by SDS-PAGE analysis in Figure 3.9. Ser-4mer protein was obtained at approximately 30 kDa as seen in SDSS-PAGE and demonstrated 90% purity.

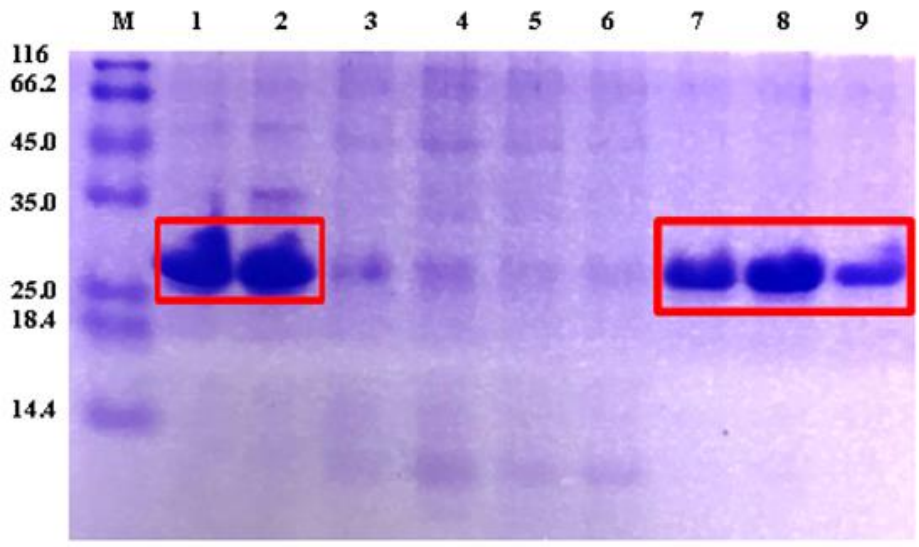

Figure 3.9 SDS PAGE gel analysis of samples obtained from the isolation of Ser-4mer protein (Ser-4mer molecular weight approximately 30 kDa) (15 % SDS-PAGE) M: Protein Ladder.*Lane1:* Elution sampe1. *Lane 2:* Elution sample 2 *Lane 3:* Wash sample 1 *Lane 4* :Wash sample 2. *Lane 5:* Wash sample 3 *Lane 6*: Wash sample 4. *Lane 7:*Elution sample 3. *Lane 8:* Elution sample 4 *Lane 9:* Elution sample 5.

3.4.4 The Determination of the Concentration of Ser-4mer Protein

The UV-visible spectrum of Ser-4mer protein obtained is shown in Figure 3.10. The extinction coefficient of the obtained Ser-4mer protein at 280 nm was calculated as 11.920 $mM^{-1}cm^{-1}$ by the Expasy protParam tool. However, the extinction coefficient of Ser-4mer protein was determined 5.000 $mM^{-1}cm^{-1}$ in different concentration at 280 nm (Figure 3.11). Concurrently, the protein concentration of isolated Ser-4mer was also determined with BCA protein assay as described in the materials and methods section. The measurement curve obtained with BCA method is shown in Figure 3.12.

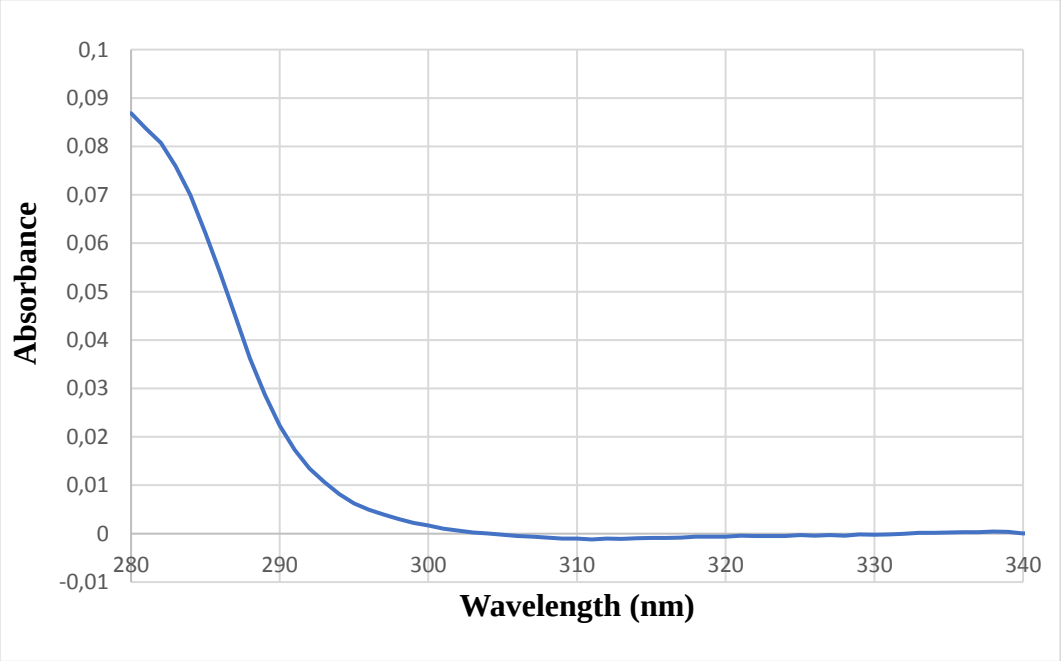

Figure 3.10 Absorbance spectrum of Ser-4mer protein in elution buffer at 280-350 nm.

The concentration of isolated Ser-4-mer protein was determined to be 7.7 mg/mL with BCA in Figure 3.12. Also, the concentration Ser-4mer protein was obtained at 7 mg/mL from UV-visible spectrum. In total, 42 mg of Ser-4mer protein were obtained from a total of 3.5 L.

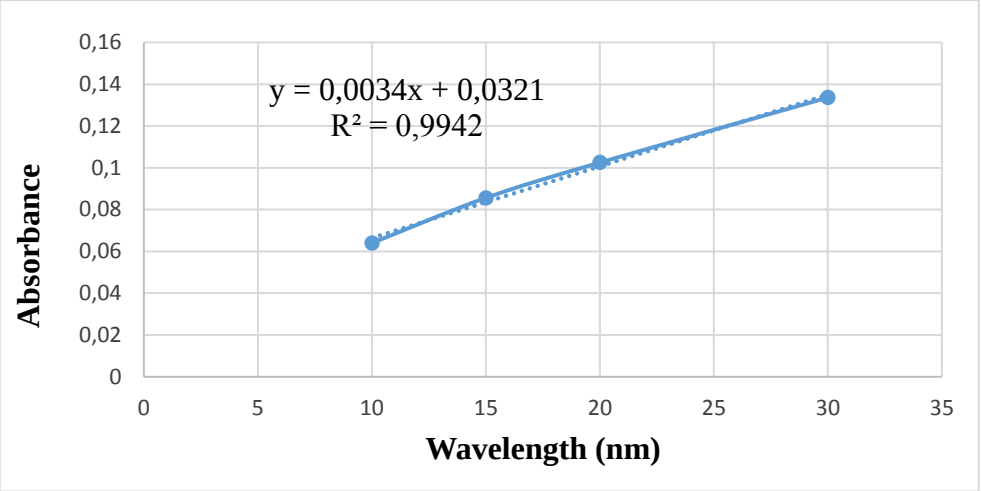

Figure 3.11 The coefficient of Ser-4mer protein at 280 nm.

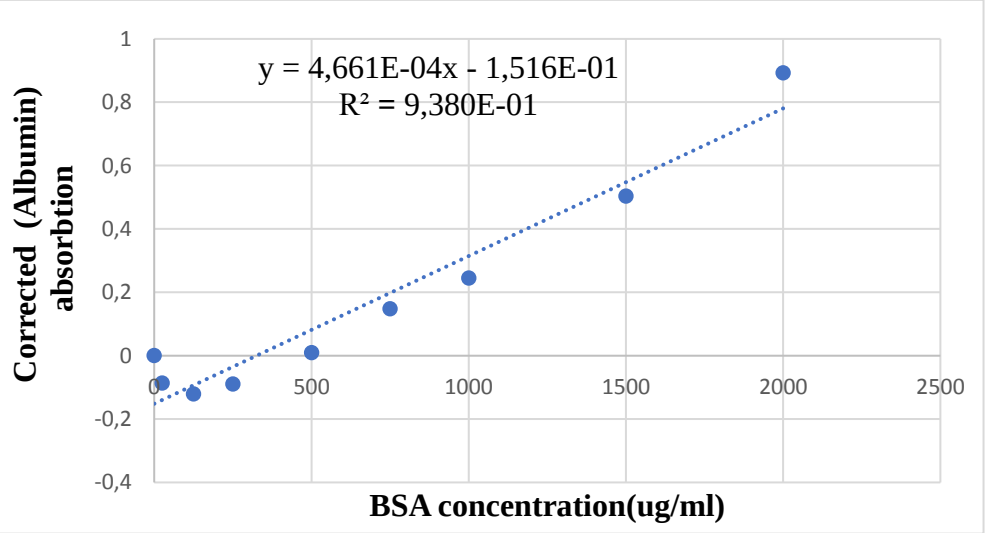

Figure 3.12 Graph of absorbance values obtained against the concentration of Albumin (BSA) standards at 562 nm.

3.5 Characterization of Produced Ser-4mer Protein

Ser-4mer protein was dialyzed against ddH_2O and phosphate buffer to examine its solubility and structure in ddH_2O and phosphate buffer. The secondary structure of

dialyzed Ser-4-mer protein was determined by Fourier-transform infrared spectroscopy (FTIR) and Circular Dichroism (CD) analysis. The dialyzed Ser-4mer protein was stained with Congo red to determine the structure of fibril by Light Microscopy and Environmental Scanning Electron Microscope (ESEM). Differential Scanning Calorimetry (DSC) analysis was used to determine the stability of Ser-4 mer protein.

3.5.1 The Comparison of Ser-4mer Protein and Commercial Sericin

The molecular weight of recombinant Ser-4mer protein and commercial sericin were compared with SDS-PAGE analysis.

The molecular weight of the commercial sericin was observed 14.4 kDa and lower than 14.4 kDa. The molecular weight of Ser-4mer protein was observed approximately 30 kDa (Figure 3.13).

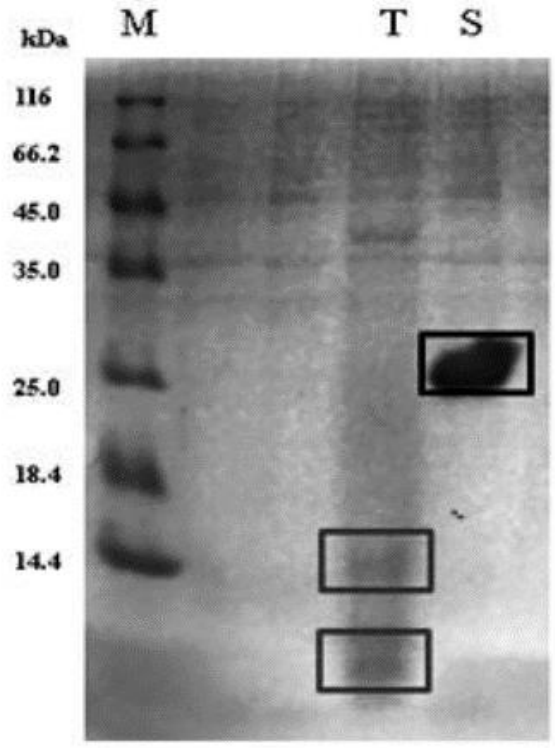

Figure 3.13 SDS-PAGE image of dialyzed commercial sericin (T) and Ser-4mer protein (S) (M: Protein Ladder) (12% SDS PAGE).

Native gel analysis was also performed to understand the structure of Ser-4mer protein. Electrophoretic mobility in native gel depends not only on the charge/mass ratio, but also on the physical shape and size of the protein (Arndt, Koristka, Bartsch, & Bachmann, 2012). Samples were analyzed in native gel at 8% acrylamide concentration: protein marker,7 µM lysozyme (MW 14.3 kDa, pI 11.35), 7 µM BSA(Bovine serum albumin) (MW 66.4 kDa, pI: 4), 7µM Ser-4mer protein (MW 20 kDa, pI 10) and 7µM Commercial sericin (MW 14.4 kDa and smaller) were shown in Figure 3.14.

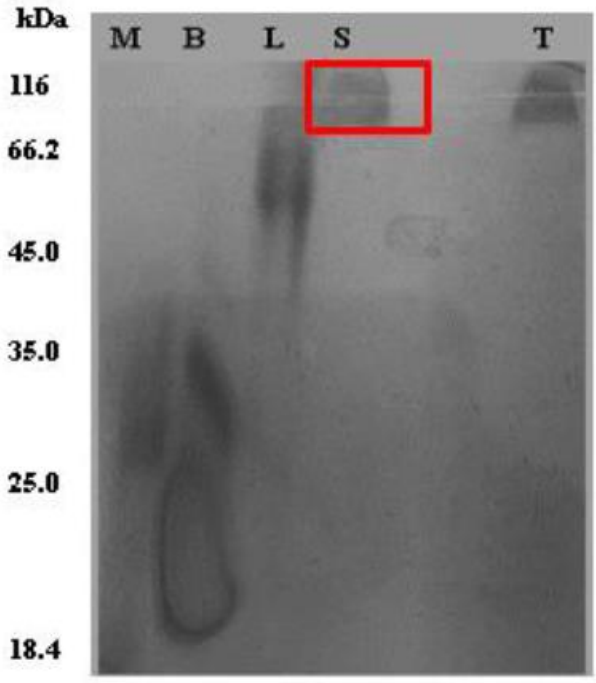

Figure 3.14. Natural gel image of Ser-4mer protein (8 % Native gel) (M: Protein marker, B: Bovine serum albumin, L: lysozyme, S: Ser-4mer protein, T: Commercial sericin).

As shown in Figure 3.14, Ser-4mer protein and commercial sericin were seen at the top of the gel. The reason for this is that native gel was prepared at pH 8.3 below the pI value (pI 10 value for Ser-4mer) of these proteins. Therefore, the proteins are most likely positively charged. A similar situation was observed in lysozyme. Another reason for this may be that Ser-4mer protein forms fibers by aggregation in water. Under these conditions, Ser-4mer protein and commercial sericin showed similar properties.

3.5.2 The Examining the Solubility of Ser-4mer Protein

Ser-4mer protein is obtained in elution buffer that contains 8 M urea as denaturant. Therefore, samples must be buffer exchanged to examine its solubility and structure in water. For this purpose, Ser-4mer was dialyzed against water. The dialyzed Ser-4mer protein was followed by UV-Visible spectroscopy. The UV absorbance at 280 nm at indicated intervals during dialysis are as shown before centrifugation and after centrifugation in Figure 3.15 and Figure 3.16.

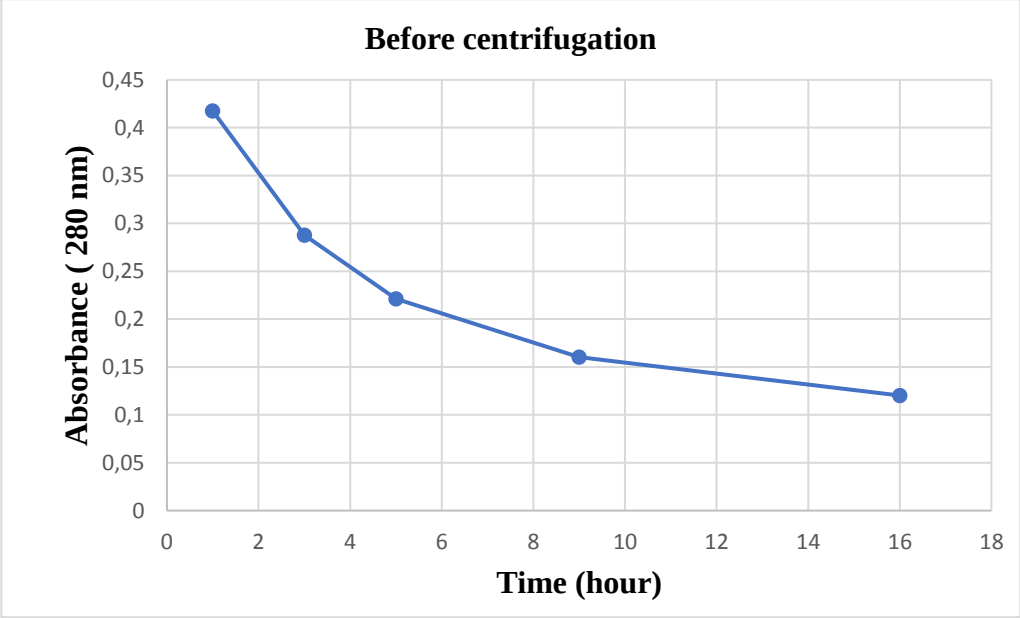

Figure 3.15 Before centrifugation, during dialysis of Ser-4mer protein (2.5 mg/ml), the amount of purified water-soluble Ser-4mer protein was followed at 280 nm.

In previous studies, repetitive region of sericin was observed precipitate within 10 hours (Huang et al., 2003). Ser-4mer protein showed higher solubility in water than previous sericin-like proteins studied. In addition, Ser-4mer protein was dialyzed against buffer solutions with different pH and its solubility was examined. For this purpose, Ser-4mer (2.5 mg/mL) in the elution buffer was dialyzed against 1 mM phosphate buffer at pH 6 and 1mM phosphate buffer at pH 8 at +4°C. UV Visible spectra (250-400 nm) was measured before and after dialysis. (Figure 3.17 and 3.18).

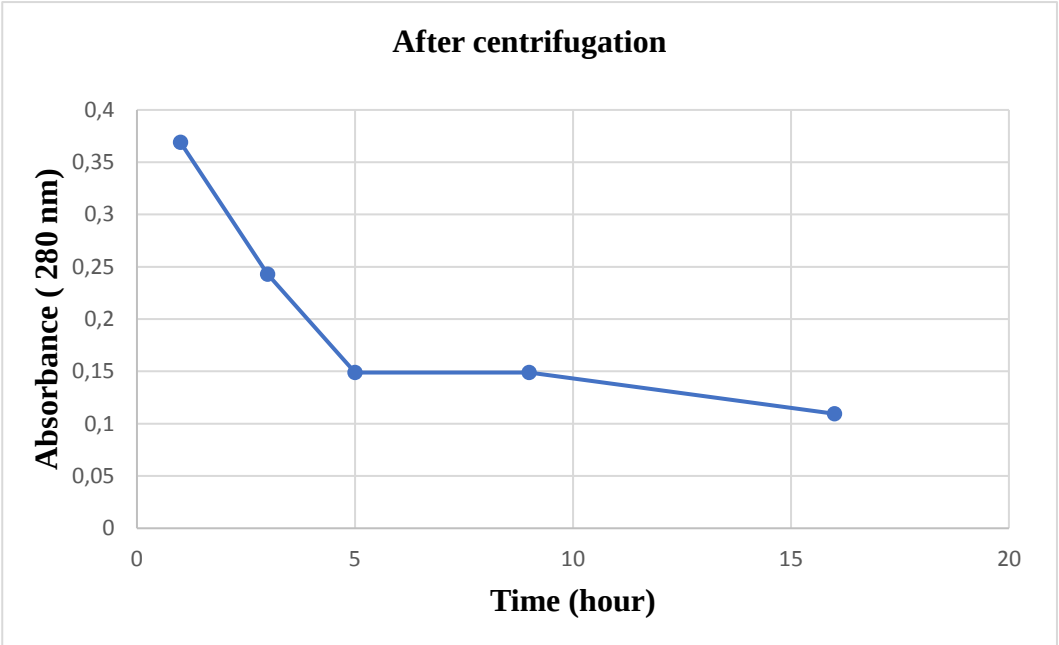

Figure 3.16 After centrifugation, during dialysis of Ser-4mer protein (2.5 mg/mL), the amount of purified water-soluble Ser-4mer protein was followed at 280 nm.

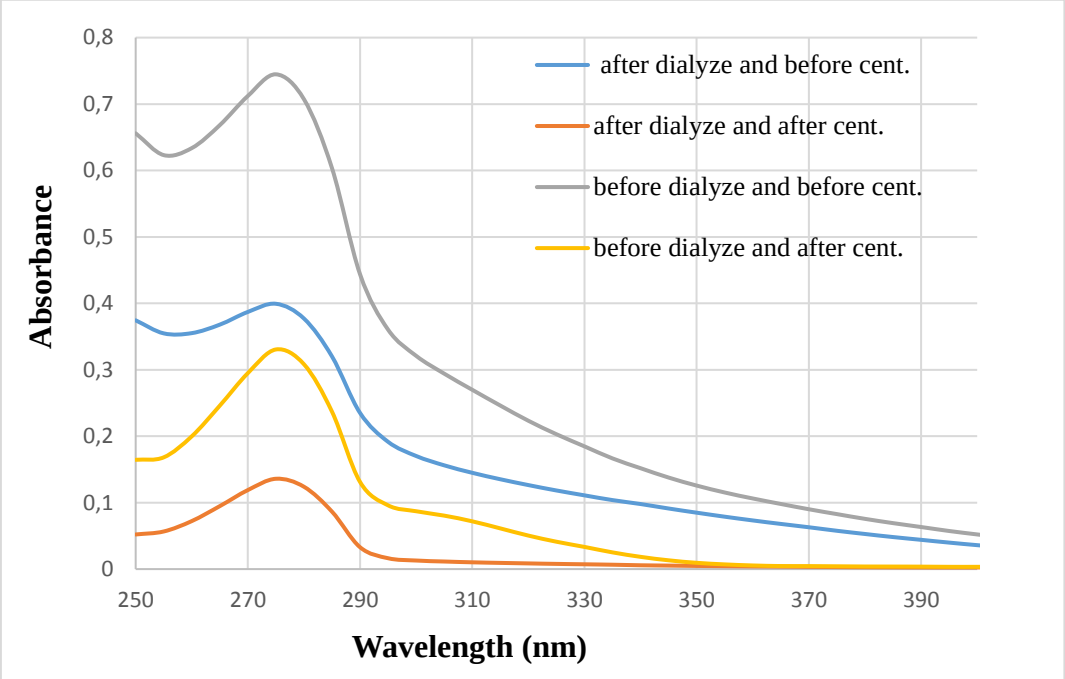

Figure 3.17 The graph obtained by before centrifugation and after centrifugation the of UV absorption of 250-400 nm of Ser-4mer (2.5 mg/mL) protein dialyzed against 1 mM phosphate buffer solution at pH 6.

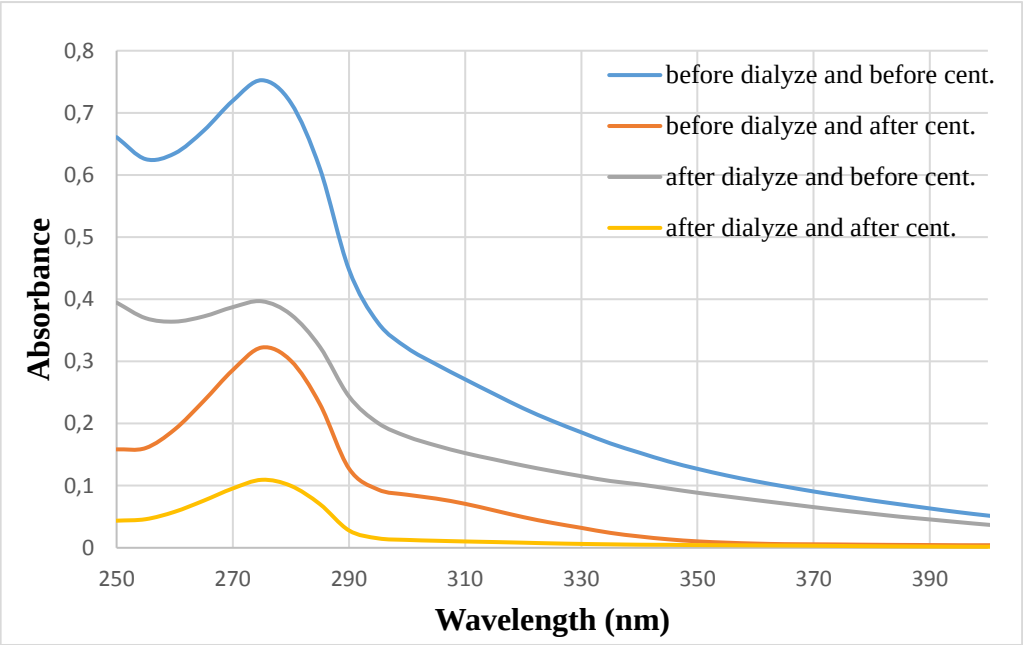

Figure 3.18 The graph obtained by before centrifugation and after centrifugation the of UV absorption of 250-400 nm of Ser-4mer (2.5 mg/mL) protein dialyzed against 1 mM phosphate buffer solution at pH 8.

Insoluble protein was obtained as pellets during dialysis against phosphate buffer at different pH values via centrifugation. The absorbance value of soluble Ser-4mer protein at 280 nm was followed to determine how much of Ser-4mer protein remained soluble after dialysis. The concentration of soluble Ser-4mer protein was determined to be 3 mg/mL. Ser-4mer protein was dialyzed against 1mM phosphate buffer with pH 6 and pH 8 the solubility was examined. After 24 hours, 33% of Ser-4mer protein was soluble in pH 6. After 24 hours, 40 % of Ser-4mer protein was soluble in pH 8. Also, 20% of soluble Ser-4mer protein in water. When the percentages of soluble Ser-4mer protein were compared, it was observed that Ser-4mer protein was more soluble in pH 8 phosphate buffer.

3.5.3 The Determination of Secondary Structure of Ser-4mer Protein

Fourier-transform infrared spectroscopy (FTIR) detects the vibrations of peptides in the structure of proteins. It is used to analyze the secondary structures of the proteins.

FTIR analysis of protein was performed in solution and solid state (Huang et al., 2003). The dialyzed Ser-4-mer protein was lyophilizated for solid state analysis. The FTIR spectra of solid-state Ser-4mer and commercial sericin is shown in Figure 3.19.

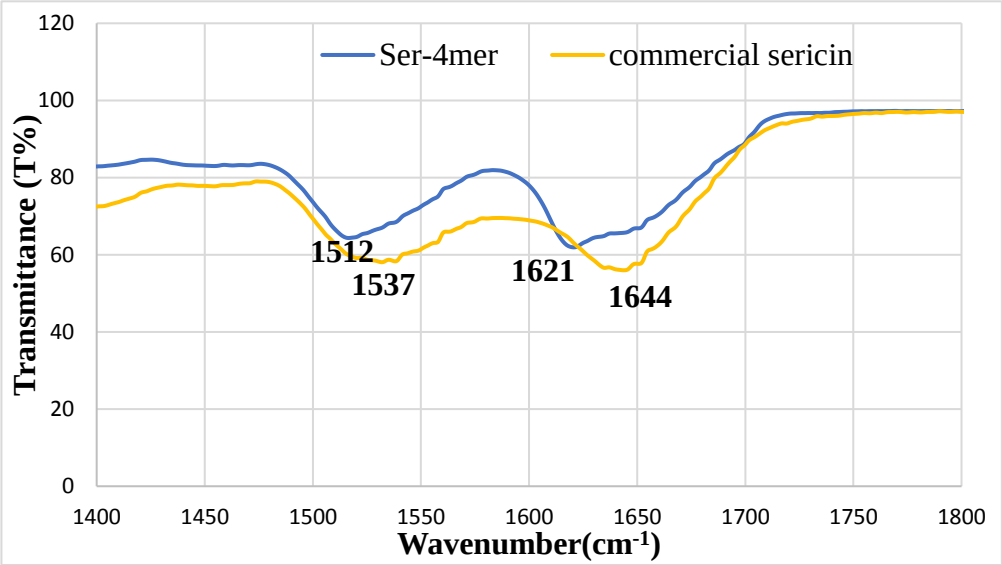

Figure 3.19 Solid state FTIR Spectra of lyophilizated Ser-4mer protein and dried commercial sericin.

The absorption of the peptide group has 9 characteristic bands in the infrared region, named Amide A, B, I-VII, with wavenumbers (cm^{-1}) (Dong, Prestrelski, Allison, & Carpenter, 1995). FTIR spectra of lyophilizated Ser-4mer which has broad peaks around 1621 cm^{-1} (amide I), and 1512 cm^{-1} (amide II), indicating β-sheet conformation (Dong et al., 1995) and dried commercial sericin broad peaks around 1644 cm^{-1} and 1537 cm^{-1} indicating random coil (Dong et al., 1995) in Figure 3.19.

The dialyzed Ser-4mer protein and commercial sericin dissolved ddH_2O were analyzed by ATR-FTIR (Attenuated Total Reflection- Fourier-Transform Infrared) in solution state in Figure 3.20.

ATR-FTIR spectra of dialyzed Ser-4mer has broad peaks around 1618 cm^{-1} (amide I), and 1527 cm^{-1} (amide II) on ddH_2O background. On the other hand, commercial sericin shows peaks around 1642 cm^{-1} (amide I), and 1547 cm^{-1} (amide II) on ddH_2O background (Figure 3.20). Taken together, FTIR and ATR-FTIR analysis of Ser-

4mer protein is consistent with β-sheet structure and commercial sericin with random coil structure.

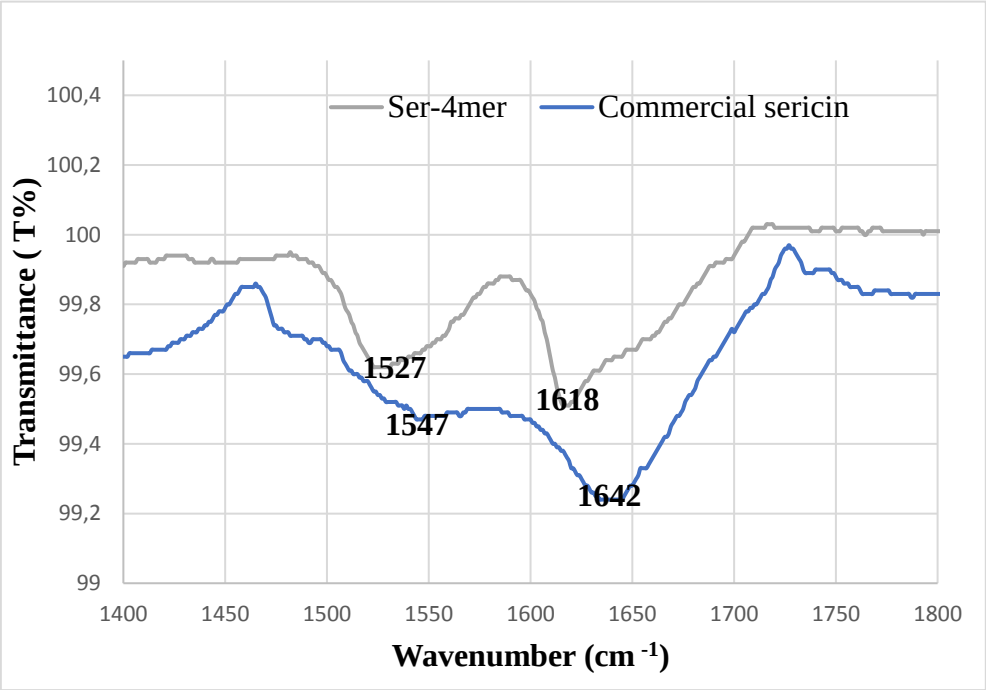

Figure 3.20 Solution state ATR-FTIR Spectra of dialyzed Ser-4mer protein and dissolved ddH_2O commercial sericin.

Circular Dichroism (CD) is a more delicate and more precise method, that was used to determine the secondary structure of the Ser-4mer protein. The most widely used applications of protein CD are to determine whether an expressed, purified protein is folded, or if a mutation affects its conformation or stability.

Dialyzed Ser-4mer and commercial sericin were prepared for CD analysis at concentrations of 0.2 mg/mL and ddH_2O was used as buffer (Figure 3.21).

As can see in Figure 3.21, CD spectra of commercial sericin (0.2 mg/mL) showed negative band at 200 nm, suggesting random coil structure.CD spectra of Ser-4mer protein (0.2 mg/mL) showed a characteristic negative band in β sheet structure at 210-220 nm (Corrêa & Ramos, 2009).

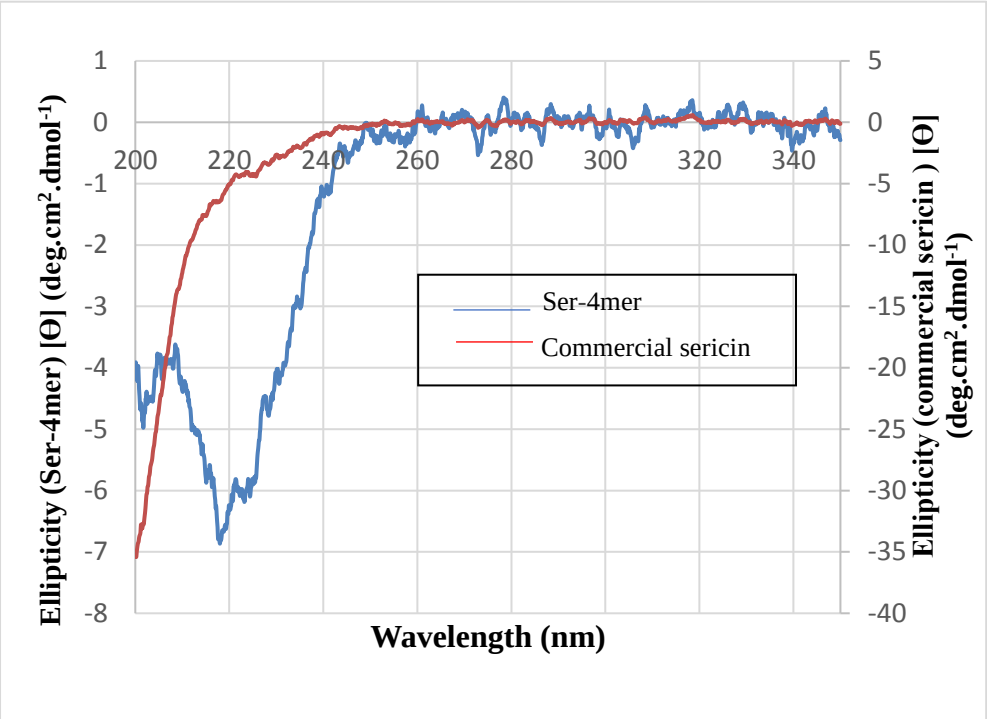

Figure 3.21 CD spectra of the dialyzed Ser-4mer protein and Commercial sericin in ddH_2O.

3.5.4 The Determination of Fibril Structure of Ser-4mer Protein

Congo red staining is used determine the amyloid fibrils structure in amyloidosis, and for the cell walls of plants and fungi, and for the outer membrane of Gram-negative bacteria (Klunk, Jacob, & Mason, 1999).

Identification of the structure of Ser-4mer protein fibrils can be determined by following the changes in absorbance by binding of Congo red (CR) to fibrils. The association of CR with fibril structure causes the characteristic absorbance spectrum to shift from a maximum of ~ 490 nm to ~ 540 nm (Girych et al., 2016).

Therefore, congo red assay was used to determine the presence of fibril structure of Ser-4 mer proteins

The UV-visible spectra were taken at between 300-700 nm at different time point (0, 5, 10, 15, 20 minutes) as shown in Figure 3.22, Figure 3.23, Figure 3.24 and Figure 3.25.

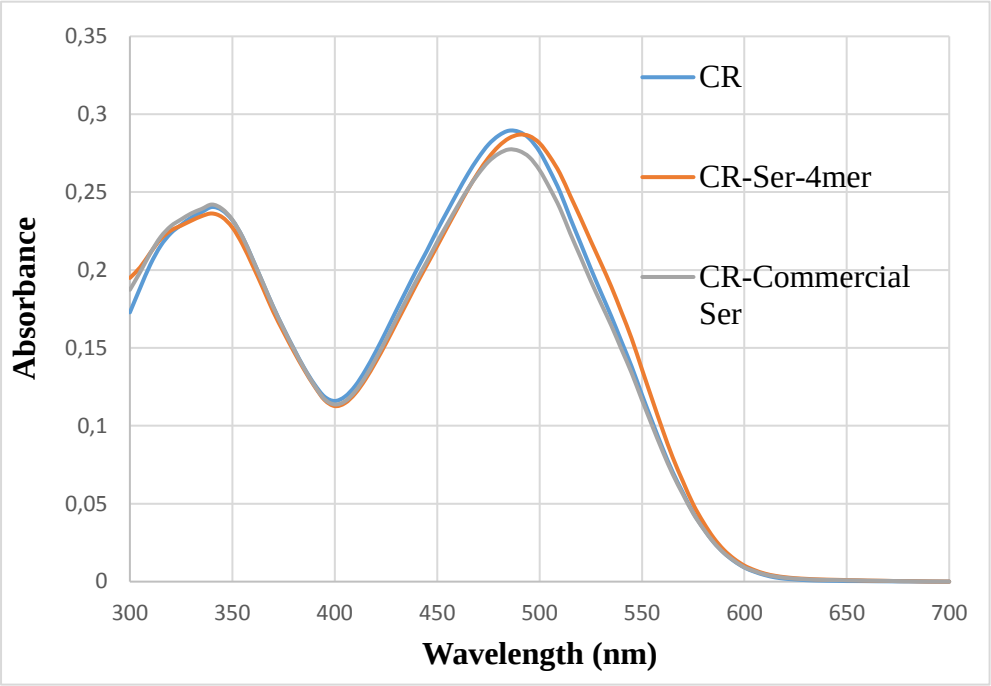

Figure 3.22 UV-Vis spectra of CR, CR-Ser-4mer and CR-Commercial sericin at 495 nm, T=0.

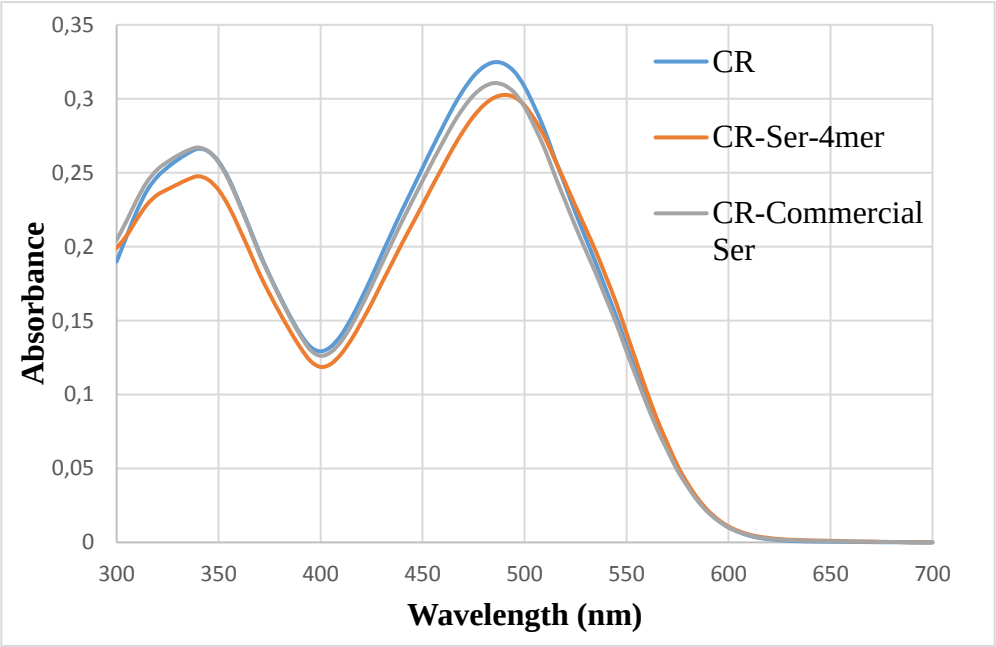

Figure 3.23 UV-Vis spectra of CR, CR-Ser-4mer and CR-Commercial sericin at 495 nm, T=5.

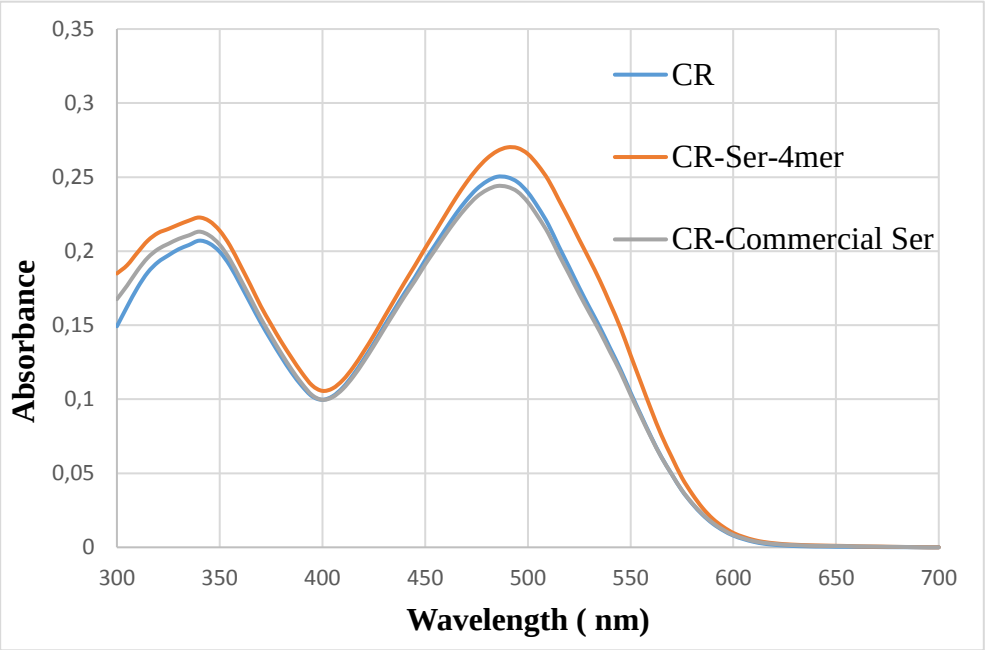

Figure 3.24 UV-Vis spectra of CR, CR-Ser-4mer and CR-Commercial sericin at 495 nm, T=10.

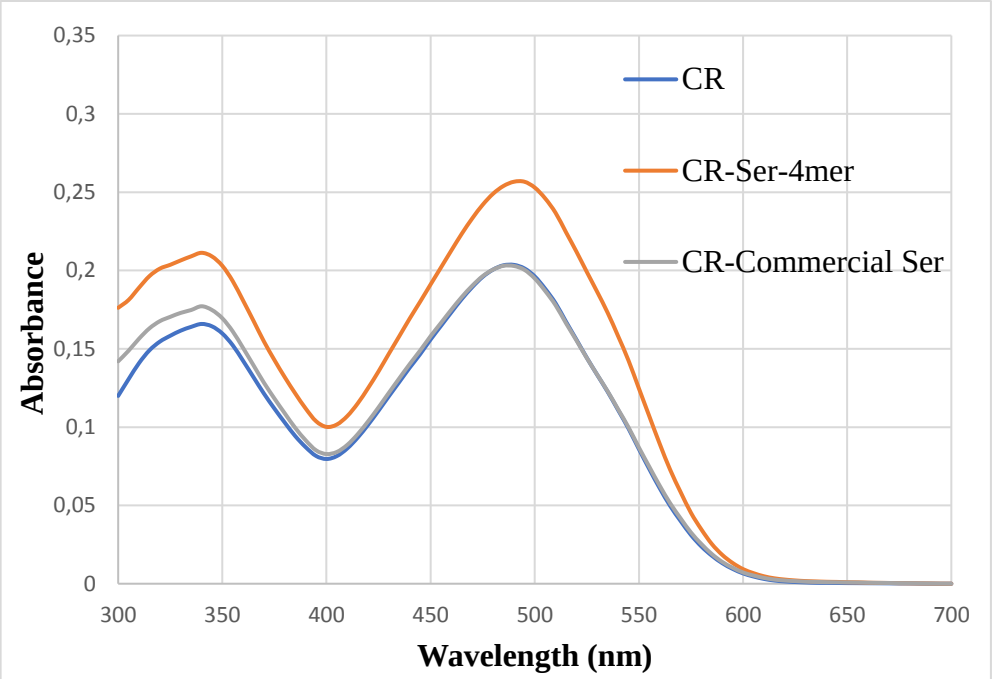

Figure 3.25 UV-Vis spectra of CR, CR-Ser-4mer and CR-Commercial sericin at 495 nm, T=15.

In samples containing Ser-4mer and CR a shift in the maximum UV-Vis absorbance from 345 nm to 495 nm is observed. This shift in the UV-Vis absorbance indicates that Ser-4mer protein exhibits a small amount fibril structure.

In order to observe the fibril structure of Ser-4mer protein in solid state, Ser-4mer was obtained from pellets before and after dialysis were stained with Congo red dye. Congo red-stained Ser-4mer protein shows β-sheet fiber structure. Non-congo red stained Ser-4mer protein indicates non- β-sheet fiber agregate. Therefore, the fibril structure of Ser-4mer protein was observed under light microscope (Figure 3.26).

Furthermore, Ser-4mer pellets stained with Congo red dye were dried and their morphology was determined by Environmental Scanning Electron Microscope (ESEM) (Figure 3.27).

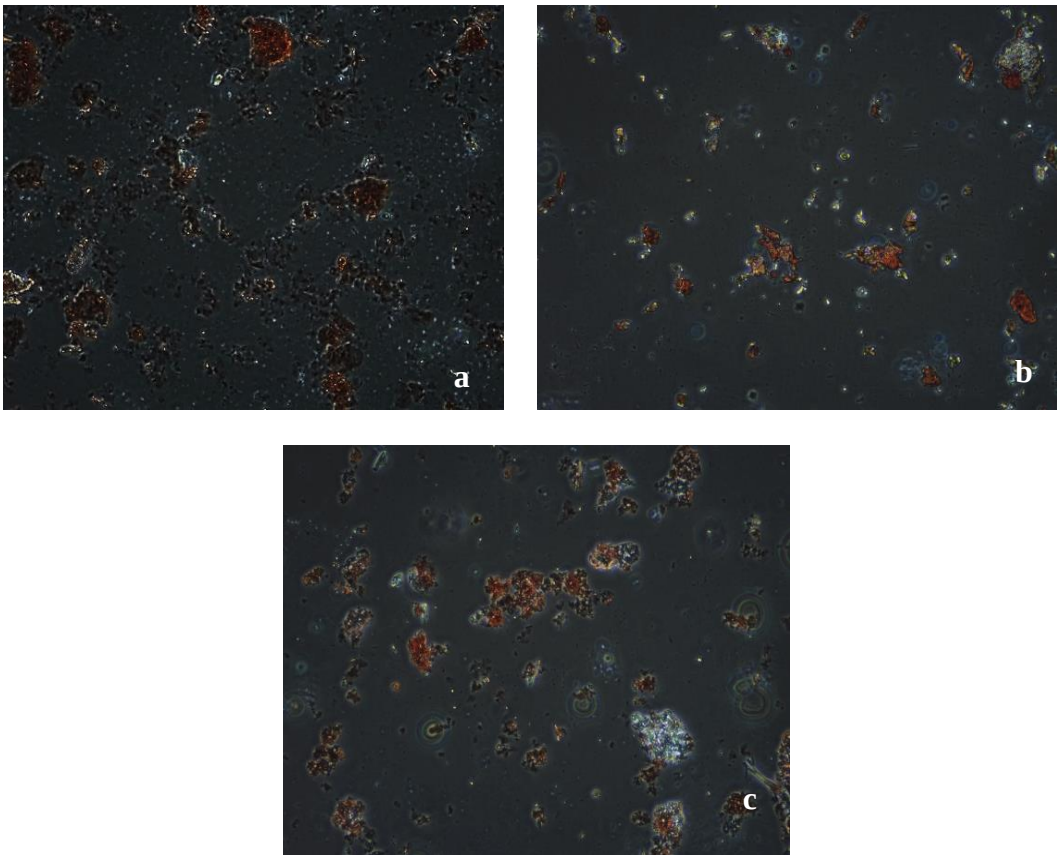

Figure 3.26 Image of Congo red-stained Ser-4mer fibrils formed by dialyzing against a.ddH2O b. pH 6 ,1mM phosphate buffer c. pH 8 ,1mM phosphate buffer (x10 Light microscopy).

As seen in Figure 3.27, the fiber structure of Ser-4mer protein pellets were observed with ESEM. Therefore, ESEM and Congo red staining results suggested that the fiber structure of insoluble state Ser-4mer formed from β-sheet.

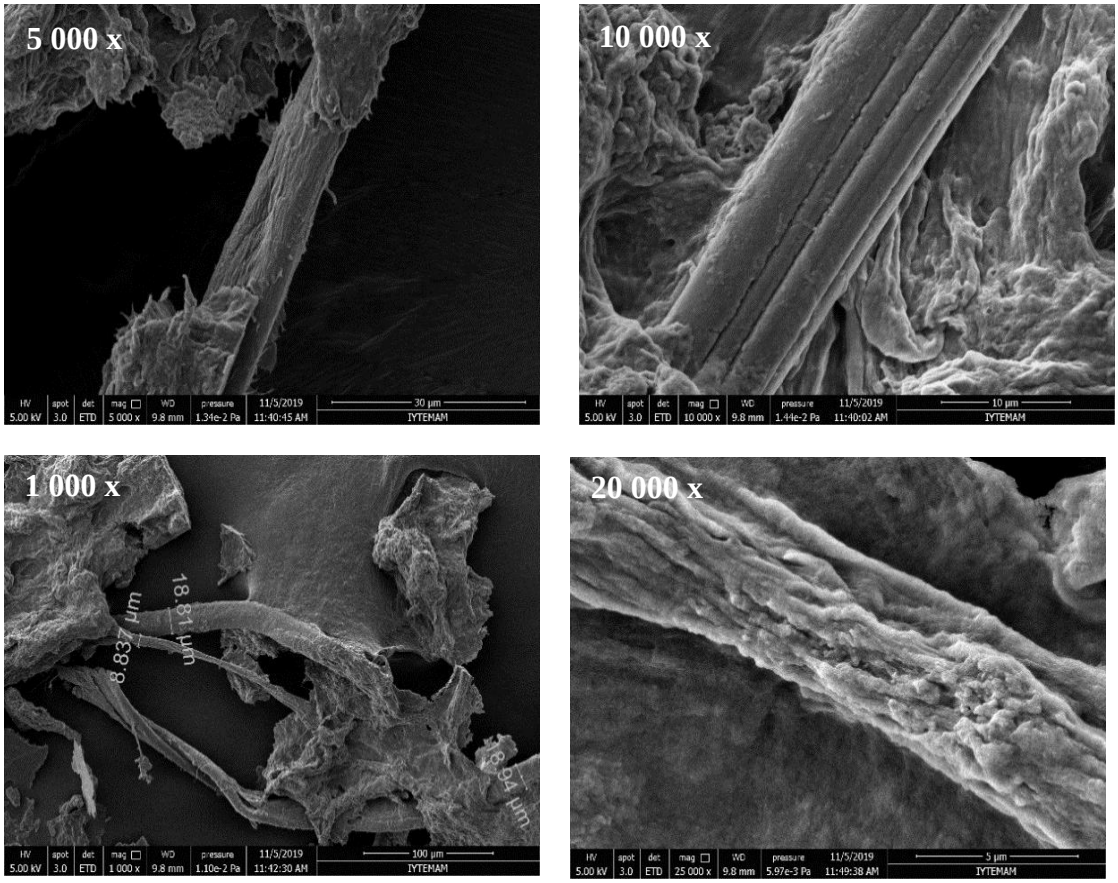

Figure 3.27 Morphology of Ser-4mer dialyzed against 5 000x, 10 000 x and 1 000 x: 1 mM phosphate buffer pH 6, 20 000 x: 1 mM phosphate buffer pH 8.

3.5.5 The Determination of the Stability Ser-4mer Protein

Stability of recombinant Ser-4mer protein was compared with commercial sericin. Ser-4mer (2 mg/mL) dialyzed against ddH_2O and commercial sericin (2 mg/mL) were analyzed with Differential Scanning Calorimetry (DSC) analysis. The graph obtained according to DSC analysis result is as shown in Figure 3.28.

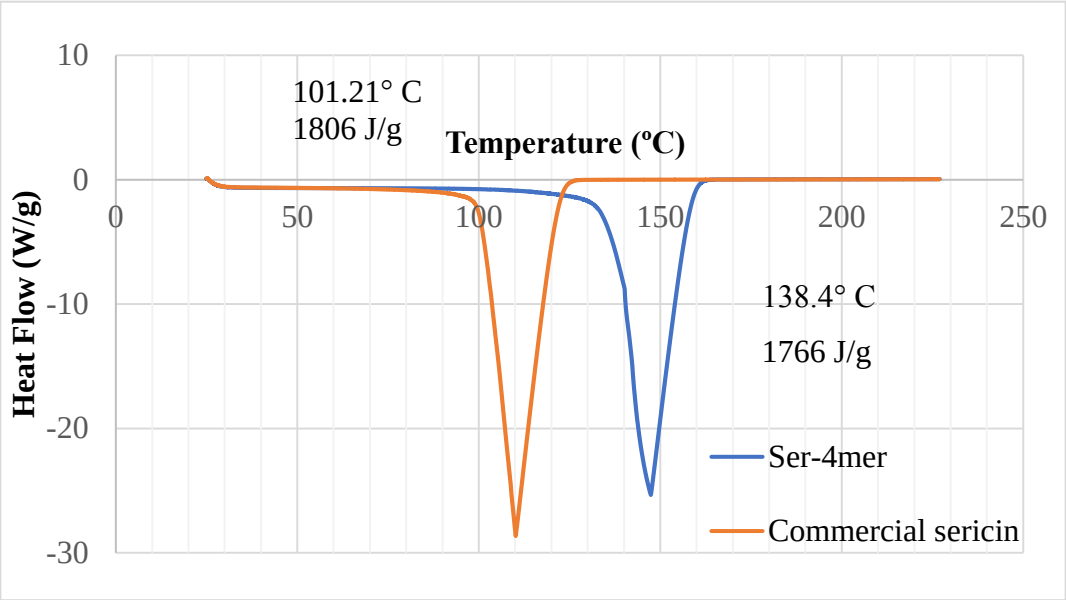

Figure 3.28 DSC analysis of dialyzed Ser-4mer protein and commercial sericin.

DSC widely is used in characterizing the stability of proteins in their native form by measuring the amount of heat required to denature a protein. Generally, proteins with higher thermal transition midpoint (T_m) are considered more stable than those with lower transition midpoints (Chiu & Prenner, 2011).

The melting temperature of dialyzed Ser-4mer and commercial sericin are 138.4°C and 101.2°C respectively as determined by DSC analysis (Figure 3.28). When the melting temperatures of Ser-4mer and commercial sericin are compared, it is concluded that Ser-4mer protein is more stable.

CHAPTER 4

CONCLUSION

The aim of this thesis is to recombinantly produce native sequence of sericin that contains 4 repeat sequences each containing 38 amino acids *E.coli*. and to characterize structural properties for explorations of the potential use of as a biomaterial in biomedicine applications

For this purpose, Sericin like tetra-repeat protein (Ser-4mer) was recombinantly produced from *E. coli*. Fourier Transform Infrared Spectroscopy (FTIR) and Circular Dichroism (CD) results suggested that the conformation of soluble sericin was β-sheet. When the morphology of Ser-4mer protein was viewed with Light Microscopy and Environmental Scanning Electron Microscope (ESEM), fibrils were observed. This research led to production of Ser-4mer protein with structural properties and amino acid composition similar to native sericin and high quality with consistent molecular weight, amino acid composition. This thesis opened the way for designing sericin-like proteins with desired molecular weight and composition. This will promote the application of sericin-based biomaterials for biomedical purposes such as wound dressings, drug delivery systems and tissue culture scaffolds.

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APPENDIX A

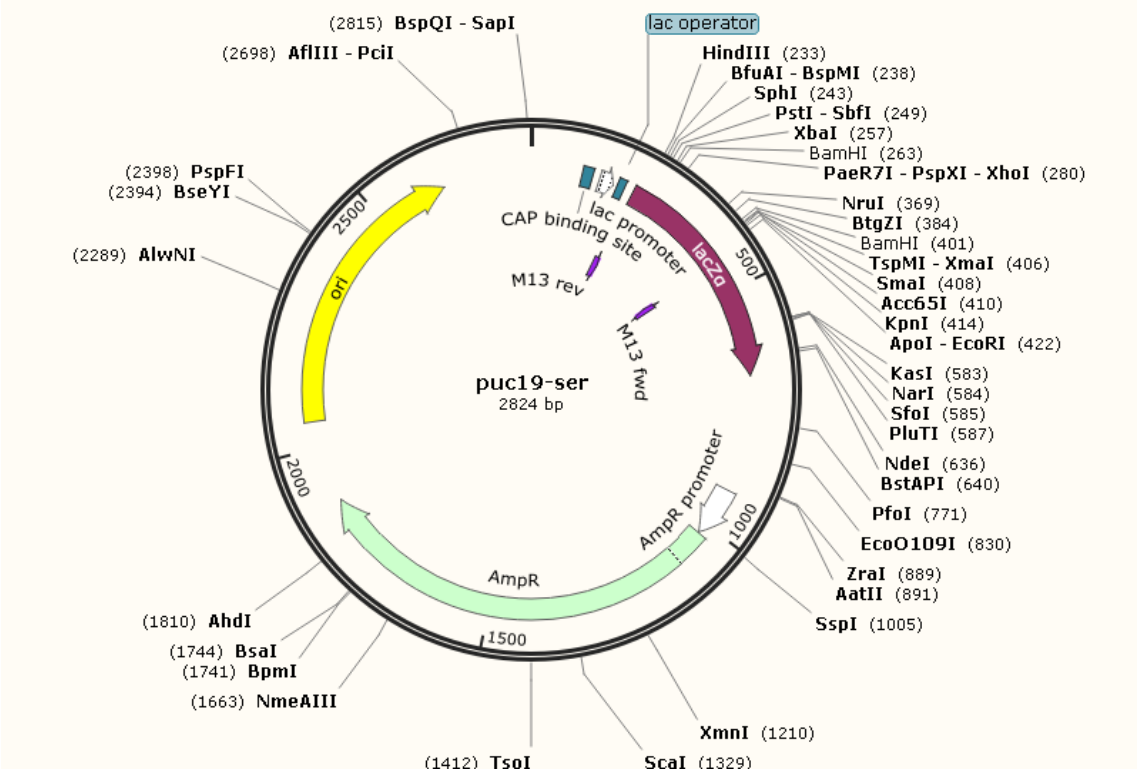

Figure A.1 pUC19-Ser-4mer vector map (This illustration was created with Snap Gene Viewer 3.2.1).

APPENDIX B

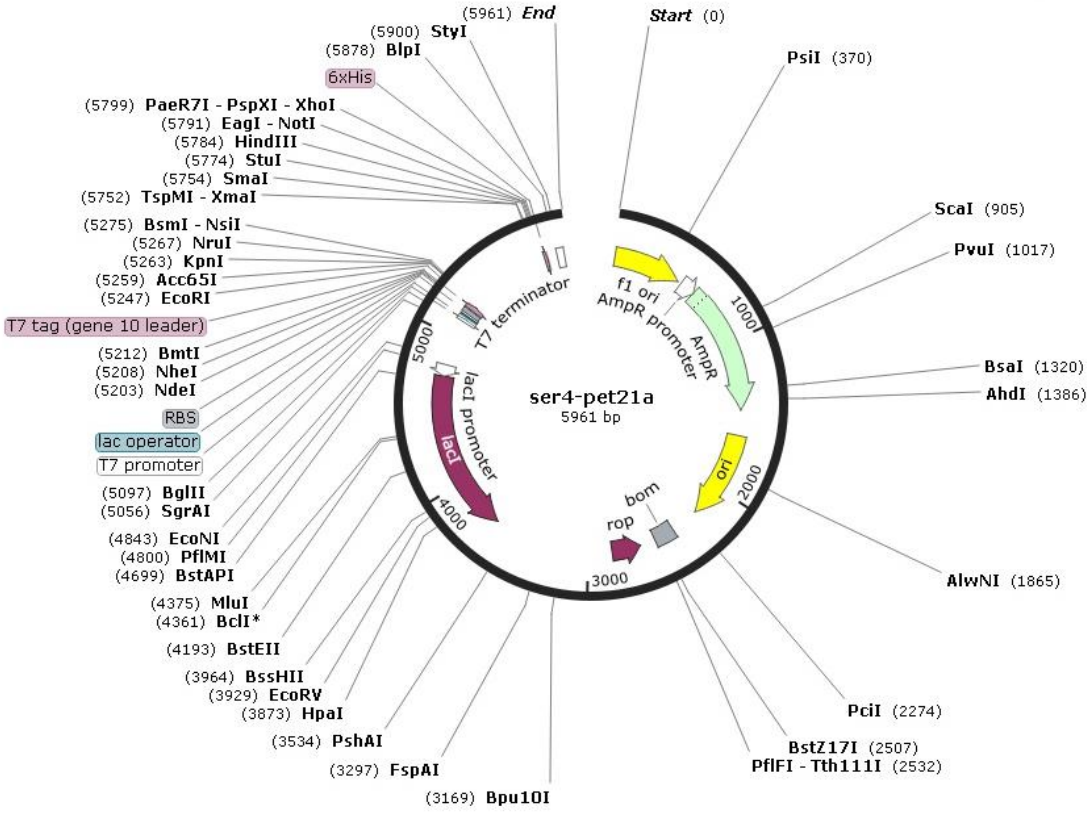

Figure B.1 pET21a-Ser-4mer vector map (This illustration was created with Snap Gene Viewer 3.2.1).

APPENDIX C

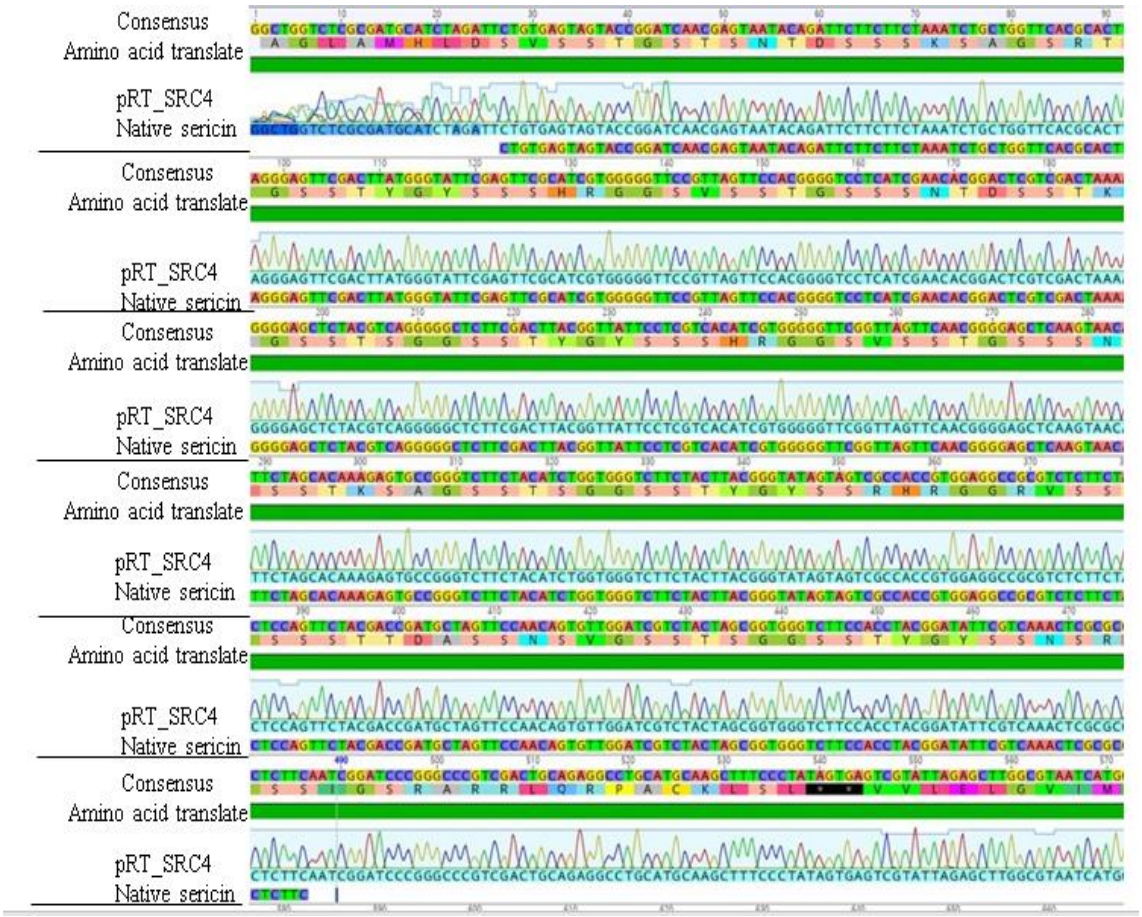

Figure C.1 Comparison of the obtained pRT_SRC4 plasmid with native sericin sequence (by using GENEIOUS R11).

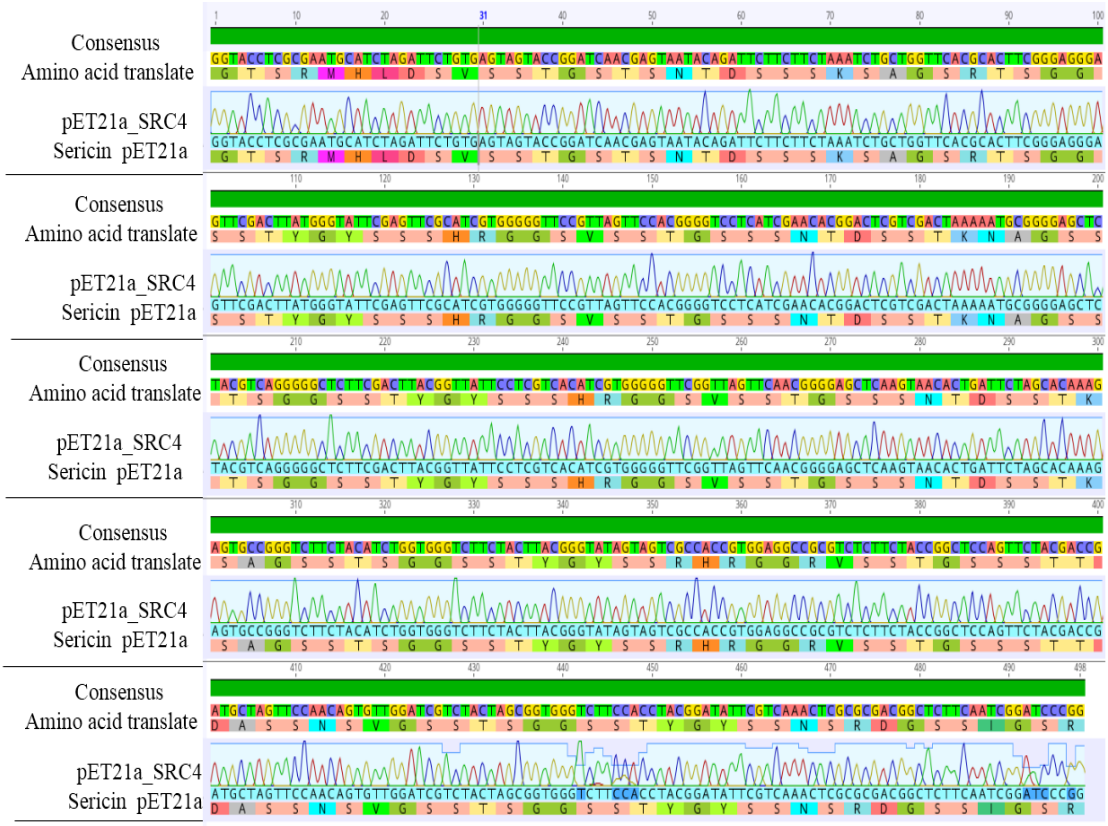

Figure C.2 Comparison of the obtained pET21a_SRC4 plasmid with native sericin-containing pET21A (Sericin pET21a) sequences (by using GENEIOUS R11).

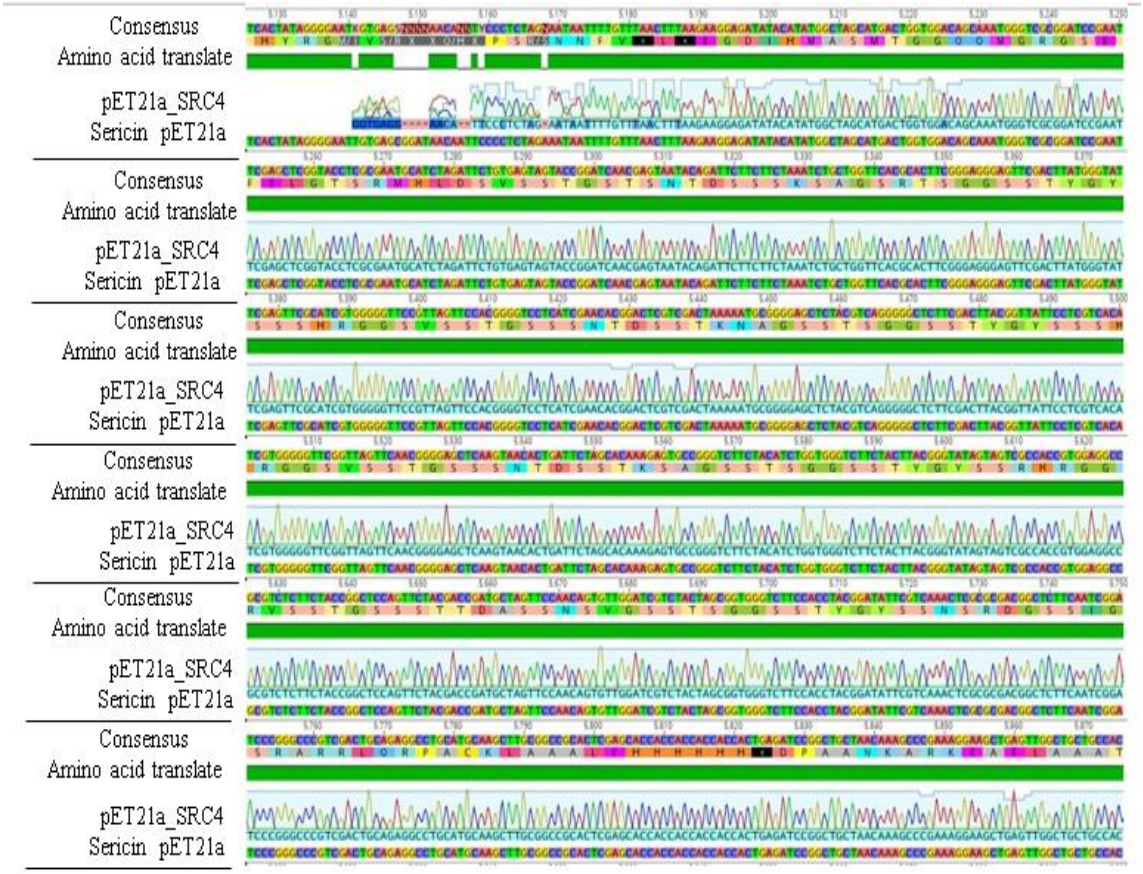

Figure C.3 Comparison of the obtained pET21a_SRC4 plasmid with native sericin-containing pET21a (Sericin pET21a) sequences (by using GENEIOUS R11).

APPENDIX D

The bold amino acids show that repeat Ser-4mer amino acids sequence. The beginning of repeated amino acid sequence is N terminal. The end of repeated amino acid sequence is C terminal. Yellow marked amino acids can form a salt bridge.

MRFVLCCTLIALAALSVKAFGHHPGNRDTVEVKNRKYNAASSESSYLN
KDNDSISAGAHRAKSVEQSQDKSKYTSGPEGVSYSGRSQNYKDSKQAYADYH
SDPNGGSASAGQSRDSSLRERNVHYVSDGEAVAASSDARDENRSAQQNAQAN
WNADGSYGVSADRSGSASSRRRQANYYSDKDITAASKDDSRADSSRRSNAYY
NRDSDGSESAGLSDRSASSSKNDNVFVYRTKDSIGGQAKSSRSSHSQESDAYYN
SSPDGSYNAGTRDSSISNKKKASSTIYADKDQIRAANDRSSSKQLKQSSAQISSG
PEGTSVSSKDRQYSNDKRSKSDAYVGRDGTVAYSNKDSEKTSRQSNTNYADQ
NSVRSDSAASDQTSKSYDRGYSDKNIVAHSSGSRGSQNQKSSSYRADKDGFSSS
TNTEKSKFSSSNSVVETSDGASASRESSAEDTKSSNSNVQSDEKSASQSSSSRSS
QESASYSSSSSSSTLSEDSSEVDIDLGNLGWWWNSDNKVQRAAGGATKSGASSSTQA
TTVSGADDSADSYTWWWNPRRSSSSSSSASSSSSGSNVGGSSQSSGSSTSGSNARGHLG
T**VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS**VSSTGSSSNTDSSTK
NAGSSTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKSAGSSTSGGSSTYGY
SRHRGGRVSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGSVSSTGSSSN
TDSNSNSAGSSTSGGSSTYGYSSNSRDGSVSSTGSSSNTDSNSNSAGSSTSGGS
STYGYSSNSRDGSVSSTGSSSNTDASTDLTGSSTSGGSSTYGYSSDSRDGSVSS
TGSSSNTDASTDLAGSSTSGGSSTYGYSSDCGDGSVSSTGSSSNTDASTDLAG
SSTSGGSSTYGYSSDSRDGSVSSTGSSSNTDASTDLAGSSTSGGSSTYGYSSNS
RDGSVSSTGSSSNTDASTDLTGSSTSGGSSTYGYSSSNRDGSVLATGSSSNTD
AST*TEESTTSAGSSTEGYSSSSHDGSVTSTDGSSTSGGASSSSASTAKSDAASSEDGFW*
WWNRRKSGSGHKSATVQSSTTDKTSTDSASSTDSTSSTSGASTTTSGSSSTSGGSSTSD
ASSTSSSVSRSHHSGVNRLLHK PGQGKICLCFKNIFDIPYHLRKNIGV

The amino acid sequence of the natural sericin planned to be produced:

VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGRVSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS

The amino acid sequence of the produced Ser-4mer protein. The bold amino acids which were planned Ser-4mer and Histidine tag at C terminal.

MASMTGGQQMGRGSEFELGTSRMHLDSVSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGSVSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGRVSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGSSIGSRARRLQRPACKLAAALEHHHHHH.