CLONING, EXPRESSION AND CHARACTERIZATION OF SERICIN LIKE OCTAMER-REPEAT PROTEIN

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ABSTRACT

CLONING, EXPRESSION AND CHARACTERIZATION OF SERICIN LIKE OCTAMER-REPEAT PROTEIN

Silk proteins are natural polymers which are synthesized by insects like silkworms and spiders. There are two proteins in the silk obtained from the cocoon of *B. mori*, these are silk fibroin and silk sericin. While the silk fibroin is the main part of the cocoon with a high commercial importance, sericin is the "glue" protein that holds fibroins together. Sericin has wide variety of applications from cosmetics to biomaterial research. Because of its high fibrillar structure, it can be used in wound healing or drug delivery studies. It can be obtained from the degumming process of cocoons which includes chemical treatment like alkali treatment and boiling, or biological treatment like enzymatic digestion. However, sericin obtained by these processes are not consistent in structure, and the sequence of the protein obtained depends on the extraction methodology and conditions. However, proteins obtained by recombinant production can be standardized, and repeat-chain length can be adjusted as desired. In this thesis study, a sericin like protein which includes eight repeats of the 38 amino acid sequence of the natural sericin was cloned and expressed in *E. coli*. The resulting protein, Ser-8mer, was analyzed in terms of fibril structure and secondary structure. It was found out that the protein has beta sheet conformation in contrast to commercial sericin with random coils. And as a result of this conformation, it forms insoluble self-assembled fibril structures which shows a promising contribution to the biomaterial research.

ÖZET

SERİSİN BENZERİ OKTOMER TEKRARLI BİR PROTEİNİN KLONLANMASI, EKPRESYONU VE KARAKTERİZASYONU

İpek poteinleri, ipek böcekleri tarafından sentezlenen doğal polimerlerdir. *B. mori* kozasından elde edilen ipek, fibroin ve serisin adı verilen iki temel proteinden oluşur. Fibroin, kozayı oluşturan ve ticari önemi yük- sek ana bileşen iken serisin, fibroinleri bir arada tutan "tutkal" görevi gören bir proteindir. Serisin, kozmetikten biyomalzeme araştırmalarına kadar geniş bir uygulama alanına sahiptir. Yüksek fiber yapısı nedeniyle yara iyileşmesi ve ilaç taşınımı çalışmaları için kullanılabilir. Serisin eldesi için alkali muamele ve kaynatma gibi kimyasal işlemler veya enzimatik sindirim gibi biyolojik işlemler kozalara uygulanabilir, bu işlemlere gam giderme adı verilir. Ancak bu işlemler sonucu elde edilen serisinin yapısı tutarlı değildir, amino asit dizisi ve elde edilen proteinin yapısı ekstraksiyon metodolojisine ve koşullarına bağlıdır. Öte yandan, rekombinant üretim ile elde edilen proteinler standardize edilebilir ve tekrarlayan zincir uzunluğu istendiği gibi ayarlanabilir. Bu çalışmada, doğal serisinin tekrar eden 38 amino asit dizisinin sekiz tekrarını içeren serisin benzeri bir protein klonlanmış ve *E. coli* bakterisinde eksprese edilmiştir. Elde edilen proteine Ser-8mer adı verilmiş, bu proteinin fibril yapısı ve ikincil yapı analizleri yapılmıştır. Rastgele sarmallara sahip ticari serisinin aksine, elde edilen proteinin beta yaprağı konformasyonuna sahip olduğu tespit edilmiştir. Bu konformasyonun bir sonucu olarak, proteininin çözünür olmayan ve kendi kendine birleşen fibril yapıları oluşturduğu da gözlenmiştir. Elde edilen proteinin yoğun fibril yapısından dolayı bu proteinin biyomalzeme çalışmalarına yararlı bir katkı sunacağı düşünülmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

Figure **Page**

LIST OF TABLES

Figure **Page**

CHAPTER 1

INTRODUCTION

1.1 Silk Proteins

Silk is a natural polymer which is produced by insects like spiders and silkworms. Among them, silkworm species *Bombyx mori* produce the most well-known and commercially important type of silk. Silk produced by *B. mori* species has the finest quality and the highest fiber content (Kundu et al. 2008). Silk is produced by silkworms to create protective cocoons against environmental conditions (Cao and Zhang 2016). There are two major proteins in the cocoon, fibroin and sericin.

1.1.1 Fibroin

Fibroin is the major protein which corresponds to 70% of the cocoon. It has a heterodimer structure with a heavy chain around 395 kDa molecular weight. Fibroin is secreted from the posterior region of the silk gland. Fibroin, as the name indicates, is a fibrous protein constituted of beta sheet structures linked by disulfide bonds. Silk fibrils have been used for a very long time in areas like fabrics and surgical sutures (Kundu et al. 2008; Aramwit, Siritientong, and Srichana 2012). Figure 1.1 shows the scanning electron microscope image for silk fibril, including sericin and fibroin.

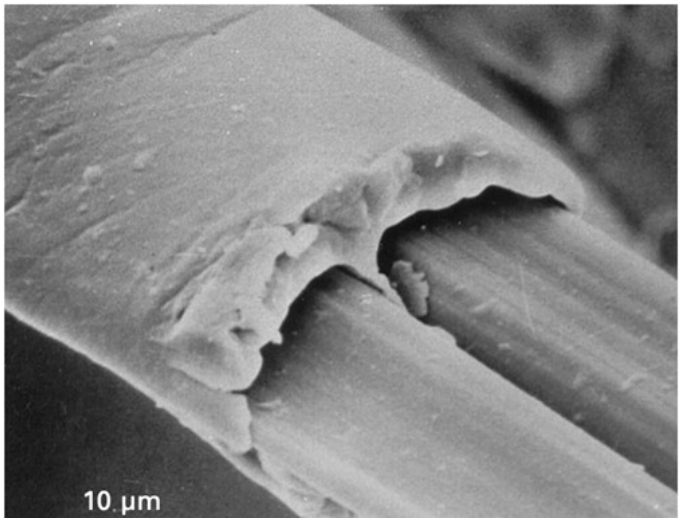

Figure 1.1 Scanning electron microscopy (SEM) Image of silk fibers coated by sericin (Vollrath, Porter, and Dicko 2009)

1.1.2 Sericin

Sericin is secreted by the middle region of the silk gland of *B. mori.* The major role of the sericin in the silk cocoon is being a glue that holds fibroin structures together. Sericin shows fluctuation in molecular weight between 24 kDa to 400 kDa, this obtained molecular weight depends on the extraction methodology. Even though the amino acid structure of the sericin depends on the source organism and extraction method, most of the natural sericin have strong polar residues like serine and aspartic acid (Aramwit, Siritientong, and Srichana 2012; Kundu et al. 2008; Cao and Zhang 2016).

1.2 Application of Sericin

Sericin has many functions from industrial applications to cell proliferation studies in laboratory scale. The application area of sericin protein can be divided into three main categories; Industrial applications which includes industries like textile cosmetic etc.,

Biomaterial application like wound healing hydrogels, and finally, biological applications. The ranging molecular weight of sericin has given it a wide variety of applications based on the molecular weight, for example, lower molecular weight (< 20 kDa) versions of the protein is mostly used in areas like cosmetics and pharmaceuticals, on the other hand, sericin with high molecular weight is used mostly in biomaterial research because of its high fibril structure (Zhang 2002).

1.2.1 Industrial Applications of Sericin

Sericin has a wide variety of industrial applications in areas like food, cosmetics, material, and coatings. Polymer based films, foams, molding resins can be produced by reaction of sericin with other polymers like polyol, these materials show high moisture absorption rates which are two to five times higher than the control group (Nomura, Iwasa, and Araya 1995). Polyurethane which is produced in the presence of waste sericin is used in biodegradable film coatings, fibers and molded objects and shows good thermal and mechanical properties (Hatakeyama 1996).

Sericin is also used in membrane-based separation systems. Because of its molecular weight, sericin is not applicable for membrane production like silk fibroin, however it can be used as an additive by cross linking polymerization. Sericin based membranes have large hydrophilicity because of the sericin's abundant hydrophilic residues, because of this hydrophilicity of the protein, sericin based cross linked membranes have showed efficiency in separating water-alcohol mixtures (Mizoguchi, Iwatsubo, and Aisaka 1991; Zhang 2002). Another sericin based membrane was produced by copolymerization of sericin with acrylonitrile used to separate organics from water (Zhang 2002)

Sericin is also used in some extraordinary areas like functional film coatings. In one study, sericin was coated onto liquid crystals and contribute to production of distortion free high quality liquid crystal displays (LCD) (Nakajima 1994). It was also used in surface coating of refrigerators as an anti-frosting agent (Tanaka 2001). Another interesting area of usage is protection of paintings against warp or drying by sericin coating (Li 1996).

Sericin is a popular compound in the cosmetics, where it is used in skin, and nail products. Due to its high hydrophilicity, sericin is an effective agent in skincare products, it increases the elasticity of skin and has antiaging effects (Padamwar and Pawar 2004). It is also used in moisturizing products (S.V. Ghonmode and ijrbat 2016). Nail products that contain 0.02 to 2% sericin shows anti brittle and gloss effects (Kundu et al. 2008).

1.2.2 Biomedical Applications of Sericin

Sericin also has promising contribution to the biomedical field. One of the major applications of sericin in biomedical area is wound dressings. Sericin can accelerate healing of wounds by increasing the proliferation of adherent cells (Tsukada et al. 1999). One study showed that film made from sericin, and fibroin conjugation can be used as an artificial cornea due to their oxygen permeability similar to the human cornea (Murase 1994). Polymer made from acrylic acid and sericin showed mucoadhesive properties and showed promising effect in transmucosal drug delivery systems (Ahn et al. 2001).

It was also stated that sericin has anticoagulant effect which can be used for surface treatment of biomedical materials and instruments and a promising agent to replace heparin (Zhang 2002). Because of its self-assembled fibrillar structure, sericin is a promising candidate for drug delivery applications. In order to decrease immunogenicity and increase stability, sericin-polymer conjugates have been produced. Asparaginase - sericin conjugates in the presence of glutaraldehyde shows increased substrate affinity and higher stability and can be a successful drug candidate against acute lymphoblastic leukemia (Zhang, Tao, et al. 2006). Another sericin-drug conjugate was used with insulin and increased bioavailability with reduced immunogenicity has been achieved (Zhang, Ma, et al. 2006).

Another important contribution of sericin into biomedical field is hydrogels and porous scaffolds. The reason why sericin is a good candidate for hydrogel formation is that the intermolecular hydrogen bonds between the protein and used polymers. Hydrogels fabricated by sericin, and polyvinyl alcohol (PVA) blend were used in wound healing applications. Sericin - glutaraldehyde or sericin - dimethyl urea cross linked

hydrogels were also created and provided mechanical and thermal durability (Kundu et al. 2008).

1.2.3 Biological Applications of Sericin

Another reason why sericin is a good candidate in biomaterial study is its proliferative effect on animal cells. This activity of sericin, especially on fibroblast and epithelial cells is responsible for wound healing capability of the protein. Compared to collagen, sericin films provides acceleration in attachment and growth of L929 mouse fibroblast cells (Minoura et al. 1995) and good cytocompatibility against rat embryo epithelial cells (Xie et al. 2007). In another study using sericin, rapid proliferation of human skin fibroblast cells has been observed after inoculation, this makes sericin a good candidate for wound healing studies (Tsubouchi et al. 2005). Sericin can also be used in serum-free media as supplement for culture of mammalian cell lines: murine hybridoma 2E3-O, human hepatoblastoma HepG2, human epithelial HeLa and human embryonal kidney cells, and help increase in proliferation of these cells (Terada et al., n.d.).

In another study, toxicity of sericin was investigated by measuring interleukin (IL-1β) and tumor necrosis factor (TNF-α) levels. As a result, while sericin accelerating proliferation rate of cells, it did not induce IL-1β and TNF-α secretion from monocytes and macrophages. This may indicate that the sericin does not induce inflammatory response and not toxic to cells (Aramwit et al. 2009).

In contrast to its proliferative effects against mammalian cells, sericin has shown anti-microbial properties against *E. coli* cells. In a study conducted by Thomas et al. 38 amino acid repeat of natural sericin was recombinantly fused with cecropin B peptide which is known with its anti-microbial activity. According to results, both wild type sericin and sericin-cecropin fusion protein has shown significant anti-microbial effects against both gram negative (*E. coli* JM 109) and gram positive (*S. aureus* ATCC25923) bacteria (Thomas et al. 2020).

Sericin has also shown cryoprotective effects. According to the study, recombinantly produced 38 amino acid repeats of sericin showed cryoprotective effect against *E. coli* cells. It is considered that, free water in the media creates strong hydrogen

bonds with sericin because of its high hydrophilicity and protects cells from damage while freezing and thawing. In addition to cryoprotection, same study stated that lactate dehydrogenase enzyme was mixed with sericin, and it remained its activity after freezing and thawing process, showing that sericin can also be used in cryoprotection of enzymes or other biochemicals (Tsujimoto et al. 2001).

Sericin also has antioxidant effects, because of its high hydrophilic residues, it is considered that it chelates trace elements, and this mechanism is responsible for the antioxidant activity. Sericin shows inhibition against lipid peroxidation and suppress inhibition of tyrosinase. A sericin rich diet was also reported to reduce oxidative stress and therefore inhibits number of colon tumors induced by 1,2-dimethylhydrazine, another study on the colonic oxidative stress markers showed that sericin decrease the level of 8-hydroxy- deoxyguanosine, 4-hydroxynonenal, and nitric oxide synthase protein (Sasaki et al. 2000).

Due to its antioxidant properties, sericin is also used in some dietary foods, it is useful against constipation. Increased bioavailability of minerals like Zn, Mg, Fe and Ca has been also observed after sericin digestion since it increases the intestinal absorption (Kundu et al. 2008).

1.3 Extraction and Obtaining of Sericin

Since the main target of the silk processing has to obtain silk fibroins for textile industry, sericin has been considered as a waste product. Therefore, most of the extraction methods were focused on the silk fibroin and did not consider the standardization of the sericin. This process is also known as degumming. Silk processing techniques can be divided into three major categories which are physical, chemical, and biological treatment (Cao and Zhang 2016).

Chemical treatment of sericin includes alkali treatment, acid treatment and boiling. One of the most frequently used chemical treatment methodology is alkali treatment of silk cocoons by Na_2CO_3 solution. Since this process cause significant amount of sericin degradation and it is hard to separate residual Na_2CO_3 from sericin. Therefore, alkali treatment is not a desirable choice for sericin extraction especially for biomaterial

applications where amino acid chain length and secondary structure is important (Cao and Zhang 2016).

Another widely used method for silk degumming is boiling of cocoons at 120°C for two or more hours, this process is also result in uncontrollable chain length and most of the protein is degraded (Cao and Zhang 2016).

Sericin can also be extracted using 8M urea solution, it was stated that this methodology result in 100% conversion of sericin. However, this process is expensive and only applicable for small scale laboratory application rather than industry.

Enzymatic treatment can be given as an example for biological treatment of silk. In this process, trypsin which is a serine protease is widely used (Gulrajani 1992). Again, this process is relatively expensive than other methods and mostly applicable for extraction of fibroin rather than sericin.

Although all the mentioned processes can be used for sericin extraction, it is not possible to provide standardized sericin as a result. The amino acid chain length and also characterization of the protein is highly depending on the extraction methodology. For example, heat and acid extraction is result in chain length between 35-150 kDa and alkaline extraction may result in between 15-75 kDa. It was also reported that, even in the same extraction method, the resulting protein characteristics may depend on the pH or temperature (Aramwit, Siritientong, and Srichana 2012). While this fluctuation has no effect on some application areas, it is definitely important in terms of biomaterial research where chain length is important. Therefore, recombinant sericin expression has gained attention because of its much more controllable nature and standardized protein yield.

1.4 Recombinant Expression of Sericin

As it was mentioned before, there are three genes that encodes for sericin in *B. mori*, these genes are called Ser1, Ser2 and Ser3. The Ser1 gene has eleven 114 bp conserved and repetitive unit which encodes for a peptide with 38 amino acid length. This repetitive 38 amino acid peptide is the main unit of the sericin. This motif consists of highly hydrophilic amino acid composition, it contains 44.7% serine, 10.5% threonine and 7.9% tyrosine which is very close to natural sericin with 38.1% serine, 7.5% threonine

and 4.7% tyrosine (Huang et al. 2003). Because of this similarity, this motif has been the main building block in the construction of recombinant sericin like proteins.

Huang et al. aiming to produce sericin-like protein in bacteria has performed the expression and isolation of polypeptides with molecular weights of 17, 32, 47 kDa using the repeating 38 amino acid sequence. This recombinant sericin self-assembled during dialysis to form insoluble fibers. It has been observed that the structures of these sericin proteins were transformed from random coils to beta sheets during dialysis. The obtained recombinant sericin contains high amounts of hydrophilic amino acids as in the natural sericin which causes the formation of beta sheets with hydrogen bonds. It was observed that the obtained recombinant sericin showed similar structural properties to the natural sericin. However, the octameric sericin that was expressed by Huang et al. did not show any solubility in water after dialysis. (Huang et al. 2003)

Thomas et al. also used 38 amino acid repeat sequence and created a fusion protein which contains cecropin at the C-terminal of sericin. This sericin-cecropin fusion protein showed significant anti-microbial effects against gram-positive and gram-negative bacteria. They were also discovered that, even without the cecropin fusion, sericin like protein which contains the repeating 38 amino acids was also have antimicrobial effect, however it was lower than the fusion protein (Thomas et al. 2020).

Tsujimoto et al. were another group that used repeating 38 amino acid sequence. They created two, four, six and eight repeats of the 38-amino acid peptide with molecular weights of 9, 18.4, 25.6 and 32.7 kDa respectively. During protein expression studies, they observed significant inhibition in growth of *E. coli* cells that express six and eight repeats of the peptide and proceeded their study with the two repeats of the peptide. This dimer sericin-like protein showed cryoprotective effect against *E. coli* cells during freezing. The dimer sericin-like protein also showed protection for enzymatic activity of lactate dehydrogenase enzyme during freezing and thawing processes (Tsujimoto et al. 2001).

Finally, in a study conducted by Bostan and Surmeli, which was also the precursor of this thesis study, first four repeats of the natural sericin which consist of eleven repeats of 38 amino acid repeat sequence was cloned and expressed in *E. coli*, this protein was named as Ser-4mer. Ser-4mer protein showed structural similarity to native sericin and also higher solubility than the previously designed 4-mer protein by Huang et al.

Ser-4mer protein was also showed self-assembled insoluble fibril structure in water. Fibril composition of the Ser-4mer protein found to be higher and consistent than

the natural sericin. Also, it was found out that the protein transforms from random coil conformation to beta sheet conformation in contrast to commercial sericin which has random coil structure (Bostan and Surmeli 2021).

1.5 Aim of the Thesis Study

The aim of this thesis study is to clone, express, isolate and characterize a sericin like protein Ser-8mer which constitutes the eight repeats of the 38 amino acid repeating sequence to understand the role of number of repeat sequences in sericin structure and function. For cloning of this protein, previously created Ser-4mer gene was taken as a basis and Ser-8mer gene was constructed using recursive directional ligation (RDL) method. Characterization includes, solubility measurement, detection of fibril structure by congo red assay and detection of secondary structure by Fourier Transform Infrared Spectroscopy (FTIR). After the characterization studies, it is expected that the Ser-8mer protein to show higher beta sheet structure and lower solubility compared to Ser-4mer protein and natural sericin. Because of its increased self-assembled fibrillar structure, Ser-8mer protein is expected to be a better candidate for biomaterial applications then previously mentioned proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemically competent *E. coli* DH5α cells and *E. coli* BL21(DE3) pLysS cells were used for cloning and protein expression studies, respectively.

2.1.1 Chemicals, Reagents and Kits

Geneaid PrestoTM plasmid isolation kit was used for plasmid isolation. NucleoSpin gel and PCR extraction kit (Macherey-Nagel) was used for gel extraction and DNA clean-up. SoluLyse™ Bacterial Protein Extraction Reagent was purchased from amsbio. HisPur™ Ni-NTA resin, disposable polystyrene columns, Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Thermo Scientific. Ampicillin and LB broth were purchased from Fisher Scientific. Imidazole 99% was purchased from Alfa Aesar. Urea, ethanol, glycerol, and tris base was purchased from Isolab chemicals. Sodium Dodecyl Sulfate (SDS), Dipotassium hydrogen phosphate (K_2HPO_4) and Potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck. Sodium chloride (NaCl), LB Agar and Tetramethyl Ethylene amide (TEMED) were purchased from Sigma Aldrich. Agarose was purchased from VWR. 3.5 kDa dialysis membrane was purchased from Spectrum labs.

2.1.2 Ser-4mer, Vectors and Primers

The Ser-4mer sequence with pTZ57R/T plasmid which contains the repeating motif of the native sericin sequence was purchased from SENTEGEN. PET-21a (+) vector was obtained from Assist. Prof. Dr. Hümeyra Taşkent Sezgin at İzmir Institute of Technology. M13 forward and M13 reverse primers was purchased from New England Biolabs. Primers pet21a forward and pet21a reverse were purchased from SENTEGEN. Restriction enzymes (*NsiI*, *PstI*, *EcoRI* and *HindIII*), Calf intestine alkaline phosphatase (*CIP*), NEBuffer 3.1 and CutSmartTM buffers, 1 kB DNA ladder and 6X purple gel loading dye was purchased from New England Biolabs. T4 DNA ligase and T4 Ligase buffer was purchased from Thermo Scientific. 2X Taq master mix was purchased from Ampliqon.

2.1.3 Instruments

Shaking incubator (n-biotek), water bath (Nuve), thermal cycler (Thermo Scientific), nanodrop plate (Thermo Scientific), UV spectrophotometer (VWR), centrifuge (Gyrozen), plate reader (Thermo Scientific), vertical and horizontal electrophoresis systems (Cleaver Scientific) were used for this study.

2.2 Method

In the scope of this study, the first step was cloning the gene encoding for the Ser-8mer protein using general molecular biology tools, after that the constructed plasmid was tested to validate sufficient amount of protein was expressed. The final procedure was the large-scale production of the target protein and its characterization studies.

2.2.1 Cloning of Ser-8mer Gene

In order to construct Ser-8mer gene, recursive directional ligation (RDL) method was used. According to this methodology, pRT_SRC4 plasmid which contains Ser-4mer gene was used as a base and all cloning studies for Ser-8mer gene was originated from this plasmid.

2.2.1.1 Recursive Directional Ligation

In this study, previously generated pRT_SRC4 plasmid was double digested using *NsiI* and *PstI* restriction enzymes according to RDL method, the obtained insert Ser_4mer is then ligated into pRT_SRC4 plasmid which was single digested with *NsiI* restriction enzyme, and the new plasmid pRT_SRC8 containing Ser-8mer gene was created. The schematic representation of the cloning methodology can be seen in the Figure 2.1.

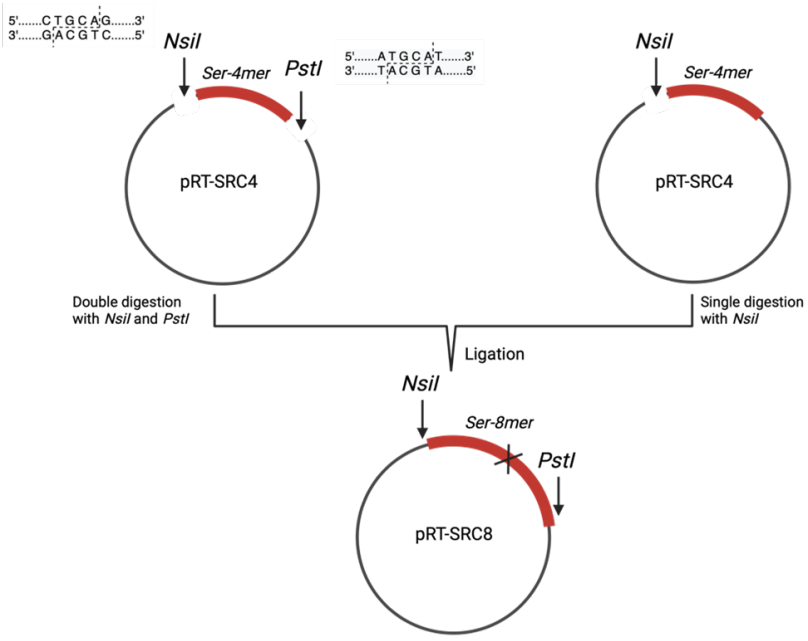

Figure 2.1 Cloning of Ser-8mer using RDL method (Created with BioRender)

2.2.1.2 Double Digestion of pRT_SRC4

The first step of the RDL method is the double digestion of the pRT_SRC4 plasmid containing the gene encoding for Ser-4mer protein. In order to achieve this, firstly, pRT_SRC4 plasmid was isolated from the *E. coli* DH5α host cells using Geneaid PrestoTM plasmid isolation kit according to manufacturer's instruction. For double digestion, 16.5 µg of pRT_SRC4 plasmid was incubated with 2 µL of *PstI* and 3 µL of *NsiI* restriction enzymes for 3 hours at 37°C (Table 2.1).

Table 2.1 Components of pRT_SRC4 double digestion mixture.

Components	Volume
DNA (pRT_SRC4 plasmid)	50 µL (16.5 µg)
PstI	2 µL
NsiI	3 µL
NEBufferTM 3.1 (10X)	7 µL
Ultra-Pure Water	8 µL
Total mixture	70 µL

After the double digestion, gel extraction procedure was used to isolate digested Ser-4mer insert from the mixture. For this purpose, all of the double digestion mixture was loaded into 1% agarose gel. Gel electrophoresis was run at 100 volt for half an hour. After the electrophoresis, the section of the gel containing Ser-4mer insert was cut and the gene was extracted from the gel using NucleoSpin gel and PCR extraction kit (Macherey-Nagel) according to manufacturer's instruction. The insert concentration was measured using Thermo Scientific µDrop plate.

2.2.1.3 Single Digestion of pRT_SRC4

In the single digestion step, 11.5 µg of previously isolated pRT_SRC4 plasmid was incubated with 2 µl of *NsiI* restriction enzyme for 3 hours at 37°C (Table 2.2). Dephosphorylation step was performed by CIP enzyme. Before this step, clean-up procedure was performed using NucleoSpin gel and PCR extraction kit (Macherey-Nagel). After the clean-up, dephosphorylation was performed by incubating 1 µL of CIP enzyme with 2.6 µg single digested plasmid for 3 hours at 37°C (Table 2.3). After dephosphorylation, a second clean-up procedure was performed using the same kit to remove reaction residues.

Table 2.2 Components of pRT_SRC4 single digestion mixture.

Components	Volume
DNA (pRT_SRC4 plasmid)	40 µL (11.5 µg)
NsiI	2 µL
NEBufferTM 3.1 (10X)	5 µL
Ultra-Pure Water	3 µL
Total mixture	50 µL

Table 2.3 Components of dephosphorylation mixture.

Components	Volume
DNA (pRT_SRC4 plasmid)	18 µL (2.6 µg)
CIP	1 µL
CutSmart® Buffer (10X)	2.5 µL
Ultra-Pure Water	3.5 µL
Total mixture	25 µL

2.2.1.4 Ligation of Ser-4mer Insert into Single Digested pRT_SRC4 Plasmid

Double digested and gel extracted Ser-4mer insert was ligated into single digested pRT_SRC4 plasmid according to RDL method. The ligation reaction was performed at 16°C for 16 hours (Table 2.4). For ligation calculation, 5:1 insert to vector ratio was used. A control ligation which includes ultra-pure water instead of an insert was also performed parallel to standard ligation as negative control. After the ligation reaction, obtained plasmids was named as pRT_SRC8 and transformed into chemically competent *E. coli* DHα cells.

Table 2.4 Components of ligation mixture.

Ligation		Control	
Vector (pRT_SRC4)	3.32 µL	Vector (pRT_SRC4)	3.32 µL
Insert (Ser-4mer)	4.68 µL	Insert (Ser-4mer)	-
T4 DNA Ligase	1 µL	T4 DNA Ligase	1 µL
T4 DNA Ligase Buffer	1 µL	T4 DNA Ligase Buffer	1 µL
Ultra-pure water	-	Ultra-pure water	4.68 µl
Total mixture	10 µL	Total mixture	10 µL

2.2.1.5 Colony Polymerase Chain Reaction for pRT_SRC8

Colony polymerase chain reaction (cPCR) was used to quickly amplify the multiple cloning site of the pRT_SRC8 plasmid, using this methodology, ligation step can be verified and presence of the Ser-8mer insert can be easily detected. In order to perform cPCR, transformed colonies of three ligation samples and one control sample were picked from the LB agar plates and dissolved in 100 µL of ultra-pure water, the remaining colonies were stored in +4°C for short period of time.

M13 forward (5'-TGTAAAACGACGGCCAGT- 3') and M13 reverse (3'-CAGGAAACAGCTATGACC-5') primers were selected for the cPCR procedure since they are capable of amplify the multiple cloning site of the plasmid including the Ser-8mer gene. Components and procedure of the cPCR mixture is given in Table 2.5 and 2.6 respectively. After cPCR, samples were analyzed using 1% agarose gel electrophoresis, 50 µL of the colonies that show the presence of successfully cloned pRT_SRC8 plasmids were taken from +4°C and incubated overnight in 2 mL of LB media containing 100 µg/mL ampicillin. After overnight incubation, colonies were stored in -80°C in the presence of 50% glycerol.

Table 2.5 Components of cPCR Mixture for pRT_SRC8 plasmid

Components	Volume
M13 Forward Primer (10 µM)	1 µL
M13 Reverse Primer (10 µM)	1 µL
Dissolved Colonies	5 µL
Taq Master Mix	7 µL
Total mixture	14 µL

Table 2.6 cPCR Procedure for pRT_SRC8 plasmid

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95°C	10 minutes	1X
Denaturation	95°C	30 seconds	30X
Annealing	53°C	40 seconds	30X
Extension	72°C	1 minute	30X
Final Extension	72°C	5 minutes	1X

2.2.2 Cloning of Ser-8mer Gene into Expression Vector

Ser-8mer gene was cloned into pET-21a(+) expression vector. For this purpose, two restriction enzymes were selected: *EcoRI* and *HindIII*.

2.2.2.1 Sequential Digestion of pET-21a(+)

Since restriction sites for *EcoRI* and *HindIII* restriction enzymes located on the pET-21a(+) vector within a small distance, the classical double digestion methodology which includes simultaneous addition of the two enzymes into the mixture could not be proceeded. Instead of double digestion, sequential digestion protocol was followed (Table 2.7). According to this protocol, first 1 µL *EcoRI* enzyme was added to mixture and the digestion was proceeded for 2 hours at 37°C, after this, mixture was incubated for 20 minutes at 65°C to heat inactivate *EcoRI*. Finally, when the heat inactivation was done, *HindIII* enzyme was added, and the mixture was incubated for 2 hours at 37°C. After the sequential digestion, remaining enzymes was cleaned and dephosphorylation procedure was followed using CIP enzyme at 37°C for 3 hours, a second clean-up was performed after this step using the same kit.

Table 2.7 Components of sequential digestion mixture for pET-21a(+) plasmid

Components	Volume
pET-21a(+)	45 µL (6.4 µg)
EcoRI	1 µL
HindIII	1 µL
NEBufferTM 3.1 (10X)	6 µL
Ultra-pure water	8 µL
Total Mixture	61 µL

2.2.2.2 Double Digestion of pRT_SRC8

Double digestion of previously created pRT_SRC8 was performed with *EcoRI* and *HindIII* restriction enzymes for 2 hours at 37°C (Table 2.8). After the double digestion, gel extraction was used in order to isolate digested Ser-8mer insert. For this purpose, all of the double digestion mixture was loaded into 1% agarose gel. After the electrophoresis, separated Ser-8mer insert was extracted from the gel using NucleoSpin gel and PCR extraction kit (Macherey-Nagel) according to manufacturer's instruction.

Table 2.8 Components of double digestion mixture for pRT_SRC8 plasmid

Components	Volume
pRT_SRC8	30 µL (12.1 µg)
EcoRI	1 µL
HindIII	1 µL
NEBufferTM 3.1 (10X)	4 µL
Ultra-pure water	4 µL
Total Mixture	40 µL

2.2.2.3 Ligation of Ser-8mer Insert into Sequentially Digested pET-21(+) Expression Vector

Double digested and gel extracted Ser-8mer insert was ligated into sequentially digested blank pET-21a(+) plasmid. The ligation reaction was performed at 16°C for 16 hours (Table 2.9). For ligation calculation, 5:1 insert to vector ratio was used. A control ligation which includes ultra-pure water instead of an insert was also performed parallel to standard ligation. After the ligation reaction, obtained plasmids was named as pET-21a_Ser8 and transformed into chemically competent *E. coli* DHα cells.

Table 2.9 Components of ligation mixture.

Ligation		Control	
Vector (pET-21a(+))	5.06 µL	Vector (pET-21a(+))	5.06 µL
Insert (Ser-8mer)	2.94 µL	Insert (Ser-8mer)	-
T4 DNA Ligase	1 µL	T4 DNA Ligase	1 µL
T4 DNA Ligase Buffer	1 µL	T4 DNA Ligase Buffer	1 µL
Ultra-pure water	-	Ultra-pure water	2.94 µl
Total mixture	10 µL	Total mixture	10 µL

2.2.2.4 Colony Polymerase Chain Reaction for pET-21a_Ser8

In order to perform cPCR, transformed colonies of three ligation samples and one control sample were picked from the LB agar plates and dissolved in 100 µL of ultra-pure water, the remaining colonies were stored in +4°C for short period of time. Previously designed primers: pet21a forward (5'-CTTTCGGGCTTTGTTAGCAG-3') and pet21a reverse (3'-TCCCGCGAAATTAATACGAC-5') were selected for the cPCR procedure since they are capable of amplify the multiple cloning site of the plasmid including the Ser-8mer gene. Components and procedure of the cPCR mixture is given in Table 2.10 and 2.11 respectively.

Table 2.10 Components of cPCR Mixture for pET-21a_Ser8 plasmid

Components	Volume
pet21a Forward Primer (10 µM)	1 µL
pet21a Reverse Primer (10 µM)	1 µL
Dissolved Colonies	5 µL
Taq Master Mix	7 µL
Total Mixture	14 µL

Table 2.11 cPCR Procedure for pET-21a_Ser8 plasmid

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95°C	10 minutes	1X
Denaturation	95°C	30 seconds	30X
Annealing	48°C	40 seconds	30X
Extension	72°C	1 minute	30X
Final Extension	72°C	5 minutes	1X

After cPCR, samples were analyzed using 1% agarose gel electrophoresis, 50 µL of the colonies that show the presence of successfully cloned pET-21a_Ser8 plasmids were taken from +4°C and incubated overnight in 2 mL of LB media containing 100 µg/mL ampicillin. After overnight incubation, colonies were stored in -80°C in the presence of 50% glycerol.

2.2.3 Expression Test for Ser-8mer Protein

After the cloning step, pET-21a_Ser8 plasmids were isolated from *E. coli* DH5α cells using Geneaid PrestoTM plasmid isolation kit according to manufacturer's instruction. Then, plasmids were transformed into chemically competent BL21(DE)3 pLysS *E. coli* cells. After transformation, four colonies were selected for expression test. Firstly, selected colonies were transferred into 8 mL LB medium containing 100 µg/mL (1:1000) ampicillin and incubated overnight at 37°C and 220 rpm. In the next morning, 80 µL (1:100) of these cultures were transferred into 8 mL of fresh LB medium containing 100 µg/mL ampicillin and incubated 37°C and 220 rpm until optical density at 600 nm (OD_{600}) values reached 0.5. When the OD_{600} values reached 0.5, protein expression was induced using 0.5 mM (IPTG) and incubation was proceeded for additional 2 hours at the same temperature and shaking rate. After the incubation, each culture was centrifugated at 3900 rpm for 30 minutes and pellets were stored ad -45°C for further analysis.

During the expression test, two samples were collected from each colony; before and after IPTG induction, these samples were centrifugated at 15000g for 30 minutes and stored at -20°C for SDS-PAGE analysis.

2.2.3.1 Small Scale Isolation for Ser-8mer

Small scale expression and purification procedure was also performed to investigate protein expression and validation of C-terminal histidine residues.

For small scale expression, four different 8 mL of starter cultures including 100 µg/mL ampicillin were prepared each containing previously selected colonies and incubated overnight at 37°C and 220 rpm. In the next morning, 250 µL of these cultures were transferred into 20 mL of fresh LB medium containing 100 µg/mL ampicillin and incubated at 37°C and 220 rpm until OD_{600} values reached 0.5, when the desired OD_{600} values were reached, protein expression was induced using 0.5 mM IPTG and the incubation was proceeded for additional 2 hours at the same temperature and shaking rate. After the incubation, each culture was centrifugated at 3900 rpm for 30 minutes, pellets were weighted and stored at -45°C for further analysis.

For small scale isolation, one of the stored pellets was selected and cell lysis was performed using SoluLyse™ Bacterial Protein Extraction Reagent according to manufacturer's instructions. This reagent was used as both wash and elution buffers during isolation. HisPur™ Ni-NTA resin was also washed with SoluLyse™ reagent before the procedure, for this purpose 800 µL of slurry resin was mixed with 1.6 mL of SoluLyse™ reagent and centrifugated at 800 rpm for 2 minutes, then supernatant was discarded, and this step was repeated for two more times. For protein binding step, 540 µL washed resin was mixed with all of the lysate obtained from the lysis step and incubated at rotator for 1 hour at room temperature. After the incubation, the mixture was centrifugated at 800 rpm for 2 minutes, then the supernatant was discarded. For the wash step, the pellet was dissolved in 800 µL fresh SoluLyse™ reagent, the mixture was centrifugated again at 800 rpm for 2 minutes, the supernatant was discarded, and the wash step was repeated for two more times. Finally, for the elution step, 100 mM imidazole was added to fresh SoluLyse™ reagent to generate an elution buffer.

Remaining pellets from the wash step was dissolved in 800 µL of elution buffer and centrifugated at 800 rpm for 3 minutes, the supernatant was discarded, and the elution step was repeated for two more times. Wash and elution samples collected during isolation was concentrated for five times using acetone precipitation method and all samples were analyzed using SDS-PAGE analysis.

2.2.4 Large Scale Expression of Ser-8mer

Ser-8mer protein was expressed in 2 L culture of BL21(DE)3 pLysS *E. coli* cells. The incubation was performed as previously described with IPTG induction, after the incubation cell pellet was weighted and stored in -80°C.

2.2.4.1 Large Scale Purification of Ser-8mer Using Ni-NTA Column

For large scale purification, cell pellet was resuspended in lysis buffer (8 M urea, 300 mM NaCl, 0.1 M KPi at pH 8.0), 5 mL of lysis buffer was used per 1 gram of pellet. Ultrasonication was used for cell lysis. After ultrasonication, lysate was centrifugated at 3900 rpm for 1 hour. For 8 mL of supernatant, 1 mL of HisPur™ Ni-NTA resin was added, and the mixture was incubated for one hour using rotator at room temperature.

After incubation, the mixture was placed into disposable gravitational column until the resin was set and the flowthrough was collected. After this, wash buffer (8 M urea, 300 mM NaCl, 20 mM imidazole, 0.1 M KPi at pH 8.0) was loaded into column and samples were collected until UV visible spectra results at 280 nm were stabilized and remained close to zero. Finally, elution buffer (8 M urea, 300 mM NaCl, 250 mM imidazole, 0.1 M KPi at pH 8.0) was loaded into the column and samples were collected until protein signals at 280 nm were stabilized. After the purification step, all samples were analyzed using SDS-PAGE analysis.

After large scale expression, protein concentration was determined via Pierce™ BCA Protein Assay Kit according to manufacturer's instructions.

2.2.5 Characterization of Ser-8mer Protein

Expressed Ser-8mer protein was characterized using basic chemical techniques. The first step of characterization was the determination of the extinction coefficient of the Ser-8mer protein. Solubility of the protein in the water was also examined, for this purpose, the protein was dialyzed against distilled water. For FTIR analysis, insoluble aggregate of dialyzed protein was lyophilized for one day. For SEM and congo red analysis, dialyzed protein in the soluble state was used.

2.2.5.1 Determination of The Extinction Coefficient of The Protein

In order to calculate the extinction coefficient of the Ser-8mer protein, a fraction of protein with a known concentration was serially diluted with elution buffer (Table 2.12) and absorbance values at 280 nm was measured. The slope of the concentration vs absorbance graph will give the value for the extinction coefficient. Extinction coefficient was also calculated theoretically using ExPASy ProtParam tool.

Table 2.12 Serial dilution of Ser-8mer protein for calculation of the extinction coefficient

Dilution	Concentration (mg/ml)	Protein (mL)	Buffer (mL)	Total Volume (mL)
1x	1.58	10	-	10
2x	0.79	5	5	10
4x	0.395	2.5	7.5	10
8x	0.198	3.75	26.25	30
16x	0.099	5 (from 8x)	5	10
32x	0.049	2.5 (from 8x)	7.5	10

2.2.5.2 Determination of Solubility of Ser-8mer Protein

Solubility of Ser-8mer was determined by measuring the concentration of the protein before and after dialysis using the BCA assay. For dialysis, protein dissolved in elution buffer was loaded into 3.5 kDa dialysis membrane and dialyzed against 1 L distilled water, the water was renewed for every two hours and after the second refill, dialysis was continued until overnight. In the next day, dialyzed solution was collected from the membrane and centrifugated at 15000g for 15 minutes, the supernatant was separated and the protein concentration at the soluble state was measured using BCA assay.

2.2.5.3 Determination of Fibril Structure

For congo red assay, 200 µM congo red (CR) was dissolved in 90% 1X phosphate buffered saline (PBS) at pH 7.4 (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4) and 10% Ethanol. The CR solution was filtered three times using 0.22 µM filters to remove any CR micelles left in the solution. In order to measure the concentration of the solution, a dilution buffer (1 mM Na_2HPO_4, 40%) was prepared, the CR solution was diluted in this buffer and its concentration was measured at 505 nm. For the assay, following samples were prepared in a 96-well plate; 2 µM CR solution, 5 µM Ser-8mer solution, 5 µM BSA, 2 µM CR + 5 µM Ser-8mer, 2 µM CR + 5 µM BSA. Absorbance spectra of samples was measured between 350-560 nm for 30 minutes with 5 minutes intervals.

In order to analyze the fibril structure under light microscopy, insoluble aggregate of the protein and 200 µL CR solution was incubated overnight at room temperature. The next day, the mixture was centrifugated at 14000g for 5 min and supernatant which includes unbound CR was discarded. Remaining pellet resuspended in 1 mL of PBS buffer and centrifugated again, this procedure was repeated two more times to wash the remaining pellet and remove unbound CR. Washed pellet was resuspended in 100 µL

PBS buffer and transferred into a glass slide and left to dry. The fibril structure was analyzed using light microscope.

The fibril structure and morphology of the Ser-8mer protein was also analyzed using Scanning Electron Microscopy (SEM). For the analysis, insoluble aggregate of the protein was lyophilized for one day using. The dried aggregate was spliced into different sections and the SEM analysis was conducted.

2.2.5.4 Determination of Secondary Structure of Ser-8mer

In order to determine secondary structure of the protein, insoluble aggregate was lyophilized for one day. The protein was analyzed at solid state using (FTIR) in transmission mode.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Cloning of Ser-8mer Protein into DH5*α E. coli*

In order to construct Ser-8mer, the first step was to clone the gene which encodes for this protein. In the cloning study, Ser-4mer gene which was created in the previous study (Bostan and Surmeli 2021) was used as a basis for the Ser-8mer gene, here, the idea is to double the number of repeating sequences of the Ser-4mer protein and creating the eight repeat Ser-8mer protein.

In this study, Recursive directional ligation (RDL) was used for this purpose, RDL methodology was specifically designed to create plasmid libraries for repetitive polypeptides like sericin. Controlled and stepwise oligomerization of DNA can be achieved by RDL, and desired chain length can be adjusted by copying repetitive number of oligomers from the starting DNA (Meyer and Chilkoti 2002). According to RDL, the donor plasmid should be digested with two different restriction enzymes whose restriction sites are different but result in the same sticky ends, so that the new ligation site cannot be digested with either enzyme in the next cycle of the RDL procedure. Therefore, *PstI* and *NsiI* restriction enzyme are chosen for this study (Figure 3.1).

pRT_SRC4 plasmid which includes gene encoding for the Ser-4mer protein was double digested using *PstI* and *NsiI* restriction enzymes and the was gel extracted to be used as an insert. The same plasmid was also single digested using *NsiI* restriction enzyme in a separate reaction to be used as a vector. The vector and the Ser-4mer insert was ligated using DNA ligase enzyme and the plasmid pRT_SRC8 encoding for Ser-8mer protein was created. Resulting plasmid was transformed into chemically competent *E. coli* DH5*α* cells.

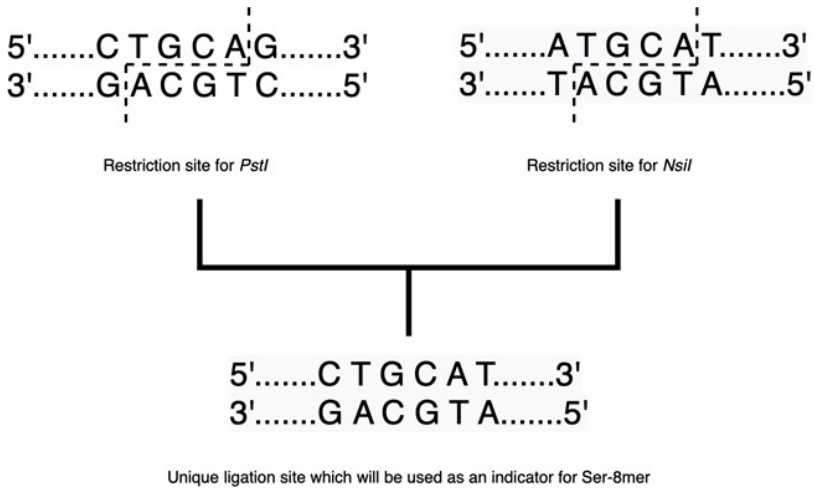

Figure 3.1 Restriction sites for *PstI* and *NsiI* restriction enzymes and the new ligation site

3.1.1 Validation of the pRT_SRC8 Plasmid

The validity of the pRT_SRC8 was proved using colony PCR (cPCR) which is a quick and easy methodology that amplifies a target gene from a single transformed colony. In cPCR, the selected colony first degraded in an initial denaturation step and then the target DNA is amplified using a standard PCR procedure. In this study, M13 forward and reverse primers were selected to amplify the multiple cloning site of the pRT_SRC8 which includes the cloned Ser-8mer gene. Resulting PCR products were analyzed on 1% agarose gel (Figure 3.2).

Since the Ser-4mer gene has a length of 0.5 kb, Ser-8mer gene was expected to be approximately 1 kb. As shown in the Figure 3.2, colonies 1, 3, 4 and 5 show a slight band around 1 kb, since these bands can also be seen in the control colony without any insert, they may be result of the non-specific binding of the M13 primers at 53°C. They also show dense bands around 0.5 kb which may be belong to Ser-4mer gene indicating self-ligated vectors. However, colonies 2 and 6 show an additional band around 1 kb different than the control colony which may show the presence of the Ser-8mer gene. For additional justification, plasmids obtained from colonies 1, 2, 5 and 6 were double digested with *HindIII* and *EcoRI* restriction enzymes whose restriction sites located at the either ends of the Ser-8mer gene and analyzed using 1% agarose gel (Figure 3.3).

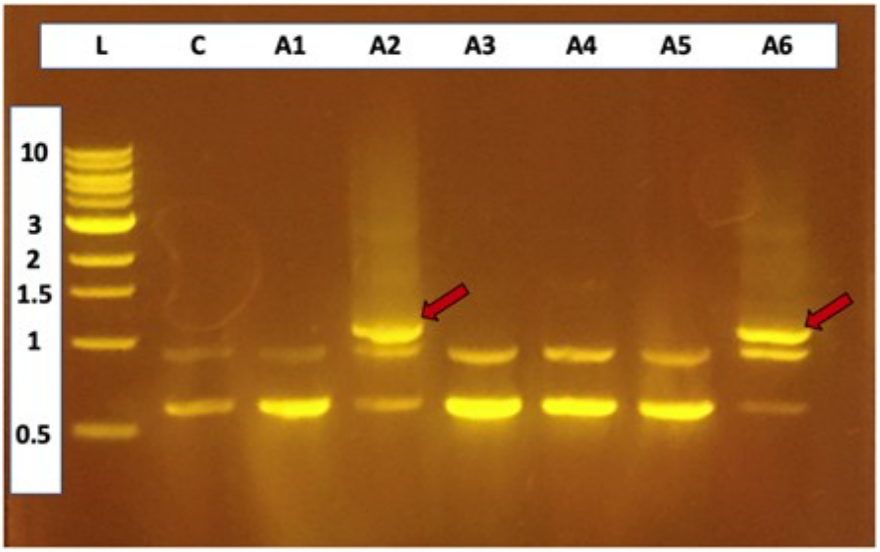

Figure 3.2 1% Agarose gel result for colonies after cPCR. (L: 1 kb DNA ladder, C: Control colony without Ser-8mer insert, A1-6: Colonies after ligation with Ser-8mer insert). Arrows indicate Ser-8mer inserts.

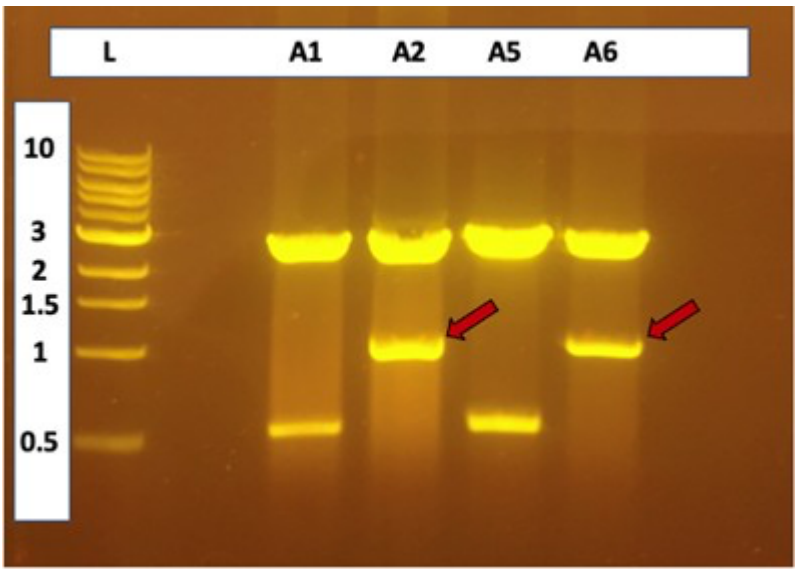

Figure 3.3 1% Agarose gel result after double digestion with *HindIII* and *EcoRI* restriction enzymes (L: 1 kb DNA ladder, A1-6: Colonies after ligation with Ser-8mer insert). Arrows indicate Ser-8mer inserts.

The result which can be seen in the Figure 3.3 justifies the result found in the agarose gel that was made after the cPCR, while colonies 1 and 5 show no indication for the Ser-8mer insert, colonies 2 and 6 show presence of Ser-8mer gene in these colonies. Colonies 2 and 6 were also further analyzed using sequence analysis (Appendix B).

While investigating the sequence analysis results, it is important to detect the ligation site formed by sticky ends of *PstI* and *NsiI* digestion which is unique to Ser-8mer (Figure 3.1). As it can be seen from the results, while both colonies show strong alignment with the original Ser-8mer sequence, only colony 6 shows the unique ligation site. Therefore, it was concluded that the cloning of Ser-8mer gene was successfully achieved, and studies was continued using the colony 6.

3.2 Cloning of Ser-8mer into pET-21a(+)

In order to obtain large scale protein expression, the constructed gene should be transferred into an expression vector. As an expression vector for this study, pET-21a(+) was selected, The selection of pET-21a(+) was based on numerous advantages; the first one was the ability of pET-21a(+) to encode for polyhistidine tag (6xHis-tag) at the C-terminus of the protein, which will be helpful for protein purification via immobilized metal affinity chromatography (IMAC) at later stages. The second advantage of this vector is its ability to encode T7 expression system and lacI repressor which will be relieved by IPTG induction and turn the plasmid into a dedicated expression system for Ser-8mer protein.

Restriction enzymes *HindIII* and *EcoRI* were selected for the cloning study. The first step of the cloning was to double digestion of blank pET-21a(+) with respective enzymes. It was stated in the previous study (Bostan and Surmeli 2021) that due to the close proximity of two restriction sites found on the blank plasmid, it was not possible to double digest the plasmid simultaneously in the same reaction mixture, instead of that a sequential digestion procedure was proceeded and the plasmid was first cut with *EcoRI* and followed with *HindIII*. In the second step, pRT_SRC8 plasmid was cut with same restriction enzymes and obtained Ser-8mer gene was ligated into sequentially digested blank pET-21a(+) plasmid. Obtained plasmids were transformed into chemically

competent *E. coli* DH5α cells. cPCR procedure followed by 1% agarose gel analysis was proceeded with transformed colonies (Figure 3.4).

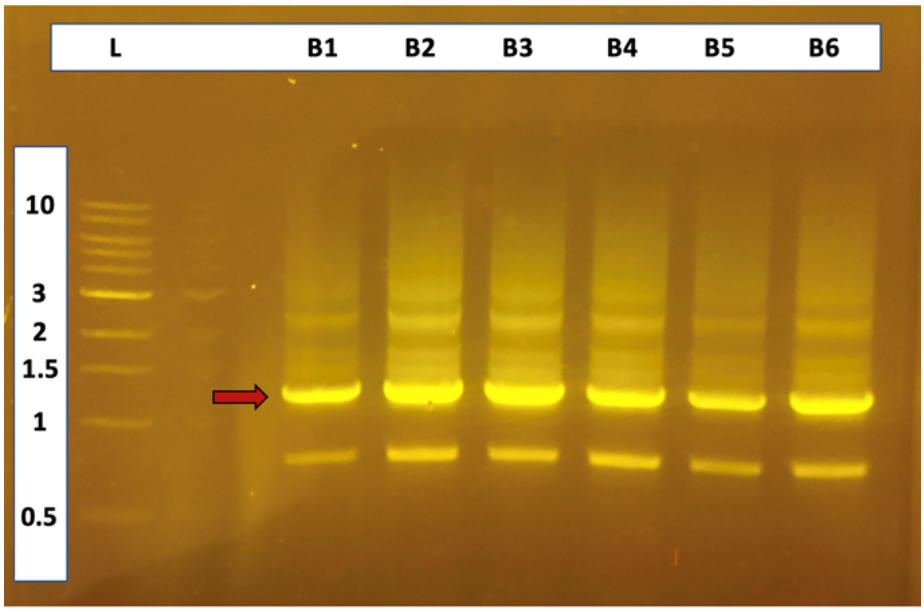

Figure 3.4 1% Agarose gel result after cPCR for pET-21a(+) cloning. (L: 1 kb DNA ladder, B1-6: Colonies after ligation with Ser-8mer insert). Arrows indicate Ser-8mer inserts.

As it can be seen from the Figure 3.4 expected bands for Ser-8mer gene at 1 kb can be clearly detected for every chosen colony. On the other hand, other bands that are slightly seen may be the result of the non-specific binding of the pet21a primers at 53°C. In order to further analyze the results, isolated pET_21a-Ser8 plasmids from chosen colonies were double digested with *HindIII* and *EcoRI* restriction enzymes.

As the Figure 3.5 shows, colony B2 shows the most reliable result at the 1 kb band. Finally, the colony B2 was analyzed using with sequence analysis (Appendix 2) and the results shows the cloning study is successful.

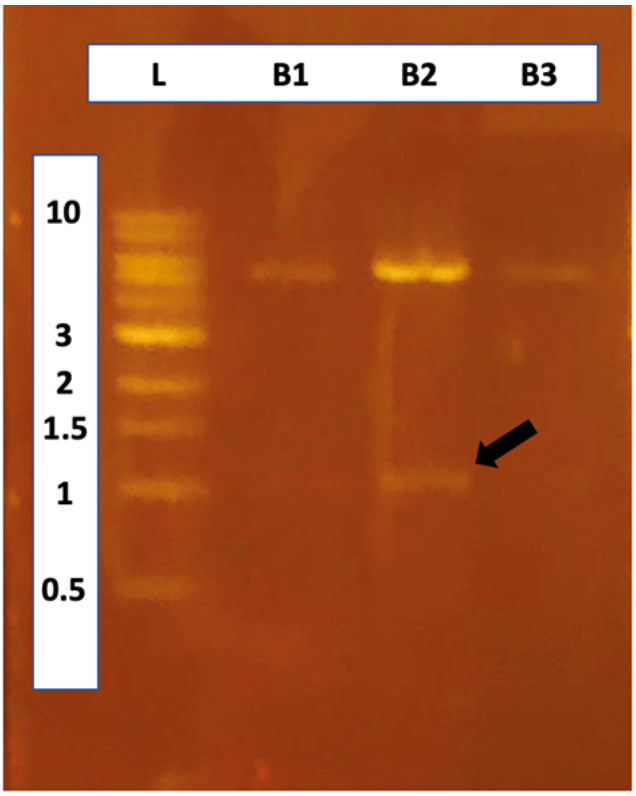

Figure 3.5 1% Agarose gel result after double digestion with *HindIII* and *EcoRI* restriction enzymes (L: 1 kb DNA ladder, A1-3: Colonies after ligation with Ser-8mer insert). Arrow indicates Ser-8mer insert.

3.3 Expression Test for Ser-8mer

After the Ser-8mer gene was constructed and cloned successfully into the expression vector, the gene was tested for expression of the Ser-8mer protein, the expression test consist of small-scale culture followed by SDS-PAGE analysis.

Since *E. coli* DH5α strain is not suitable for protein expression and it was desired to take full advantage of T7 expression system encoded by pET-21a(+) vector, pET-21a_Ser8 plasmid must be transformed into an expression host for the protein expression studies.

BL21(DE3) pLysS cells carry pLysS plasmid which encodes for T7 lysozyme, these gene decrease the basal metabolism level of cells and the background expression of

the target protein, because of that these cells are generally used for expression of toxic proteins. Since it was discovered that the sericin protein has antimicrobial effects on *E. coli* cells and may be considered as toxic (Thomas et al. 2020), BL21(DE3) pLysS cells were chosen for the protein expression host for this study.

For this purpose, pET-21a_Ser8 plasmid were transformed into chemically competent *E. coli* BL21(DE3) pLysS cells. Four of the transformed colonies were chosen from the LB agar plate and 8 mL cultures were prepared per each colony, protein expression was induced with 0.5 mM IPTG. Two samples were collected from each culture; one is before IPTG induction and the other one is IPTG induced colonies after 2 hours of expression. All collected samples were analyzed using SDS PAGE to verify protein expression (Figure 3.6).

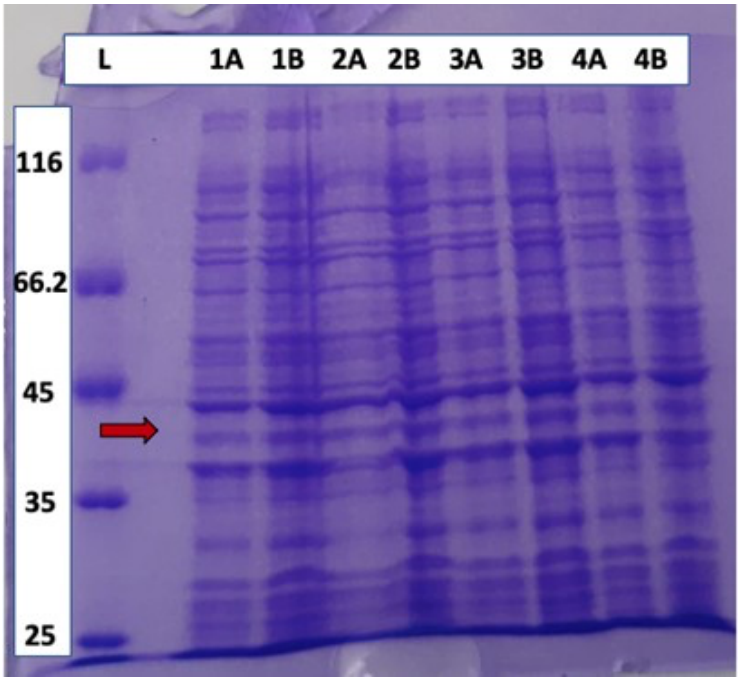

Figure 3.6 15% SDS-PAGE analysis for the expression test of the Ser-8mer protein. (L: Unstained Protein MW Marker (Pierce), Letter A indicates colonies before IPTG induction, Letter B indicates colonies after IPTG induction). Arrow indicates the expected location of the Ser-8mer protein.

In order to investigate the SDS-PAGE analysis, the molecular weight of the protein was estimated using Expasy protParam tool, for this estimation, the amino acid sequence in the Table 3.1 was used. According to this tool, molecular weight of Ser-8mer protein was estimated as 36 kDa. However, according to two previous studies (Bostan and Surmeli 2021; Huang et al. 2003), because of the reduced electrophoretic mobility of the protein, real bands can be observed at higher molecular weights than expected. Therefore, to examine the Ser-8mer expression, bands between 35 to 45 kDa will be investigated.

Table 3.1 Amino acid sequence of Ser-8mer protein (Underlined sequence shows the 38 aa repeat sequence, bold sequences show 8 repeats of repeat sequence of native protein)

Sequence	MASMTGGQQMGRGSEFELGTSRMHLDS **VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS** **VSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGS** **VSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGR** **VSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS** SIGSRARRLHLDS **VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS** **VSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGS** **VSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGR** **VSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS** SIGSRARRLQRPACKLAAALEHHHHHH

When the SDS-PAGE analysis in the Figure 3.6 is investigated, no clear bands was observed around the expected location. Since the protein expression was induced with IPTG, a sharper contrast was expected between induced and uninduced samples around 40 kDa. This result indicates a possible problem in the protein expression. However, when in the previous studies conducted with *E. coli* BL21(DE3) cells were investigated, a continuous dense bands were observed around 40 kDa similar to Figure 3.6. Since this analysis was made as a whole cell assay without any purification step, there can be a possible blocking of Ser-8mer protein by other proteins expressed by cells at the exact location. In order to clarify this confusion and revalidate the protein expression, a small-scale isolation study was performed.

3.3.1 Small Scale Isolation for Ser-8mer

In order to isolate Ser-8mer protein, Ni-NTA affinity chromatography was used. In this technique, Ser-8mer protein is bound to Ni-NTA resin via the 6X histidine tag located at its C-terminal.

Small scale isolation study consists of small-scale expression followed by batch purification with Ni-NTA chromatography. Generally, Ni-NTA chromatography is used in column systems where there is at least one stationary phase which is generally agarose beds loaded with Ni atom and a mobile phase which is the cell lysate and respective buffers. In batch chromatography, both resin and buffers are mixed together, and the separation is made by simple centrifugation and collecting the supernatant. Even though the controlled gravitational column systems are much more effective and precise for protein purification in bulk, they are not the best option for small scale applications where the only purpose of the chromatography is detection of the target protein since the process is time consuming. Therefore, small scale isolation technique was chosen for the detection of Ser-8mer expression. This technique will also be helpful to validate the 6X histidine tag portion of the protein is expressed without any frameshift.

For the analysis, 8 mL culture of Ser-8mer was prepared and the protein expression was induced with 0.5 mM IPTG, expression was performed for 2 hours at 37°C after induction. After the expression, collected pellet was undergo batch isolation using Ni- NTA resin and samples for wash and elution were collected. All samples were investigated using SDS-PAGE analysis (Figure 3.7).

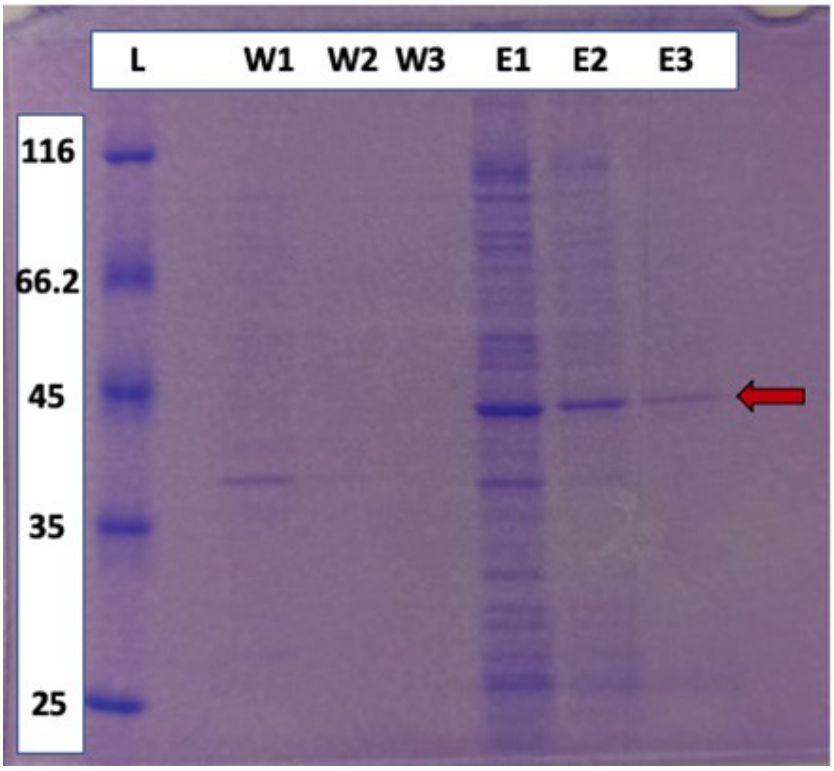

Figure 3.7 15% SDS-PAGE analysis after small scale isolation procedure. (L: Unstained Protein MW Marker (Pierce), W1-3: Wash samples, E1-3: Elution Samples). Arrow indicates the Ser-8mer protein.

When the Figure 3.7 investigated, it can be clearly seen that the Ser-8mer protein was successfully isolated via Ni-NTA affinity chromatography. Results also show that there is no complication during the expression of the protein and the 6X Histidine tag. When the density of bands is examined, it can also be seen that most of the protein is lost without complete isolation during elution steps one and two, however this result was expected since the isolation efficiency of the batch chromatography is low. Finally, when the location of the band is examined, it can be observed at around 45 kDa, which is about 10 kDa higher than the estimated value. However, as it was stated before, this result was expected, in fact, it is correlated with Ser-4mer result which was 10 kDa higher than the estimated value (Bostan 2019).

3.4 Large Scale Expression of Ser-8mer Protein

For the large-scale expression of Ser-8mer, the same procedure as the small- scale expression was followed. The protein was expressed in 2 L culture of *E. coli* BL21(DE3) pLysS cells containing pET-21a_8mer plasmid. After the expression, harvesting and cell lysis, protein was isolated using Ni-NTA gravitational chromatography. The SDS-PAGE analysis after the large-scale expression can be seen from the Figure 3.8. As it can be seen from the figure, the protein was successfully expressed and isolated. After the analysis, first and second elution samples were mixed and aliquoted as 1 mL samples with a total volume of 6 mL. The protein concentration was measured with Bicinchoninic Acid Assay (BCA) and found to be 2.28 mg/mL, the standard curve of the BCA analysis can be seen in the Appendix C. As a conclusion, 13.71 mg Ser-8mer protein was expressed and isolated from 2 L of culture.

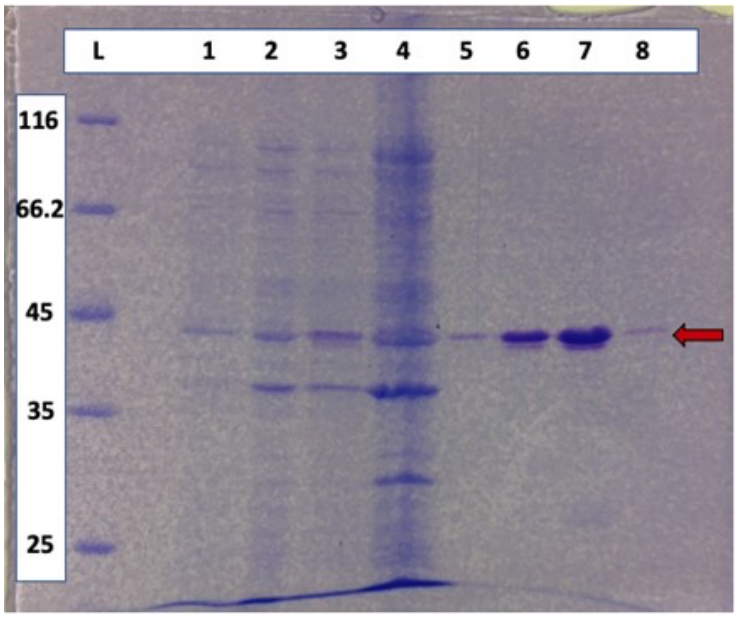

Figure 3.8 15% SDS-PAGE analysis after large-scale expression. (L: Unstained Protein MW Marker (Pierce), 1: Before IPTG induction, 2: After IPTG induction, 3: After sonication-supernatant, 4: After sonication-pellet, 5: Last wash sample, 6: First elution, 7: Second elution, 8: Third elution) Arrow indicates the Ser-8mer protein.

In order to understand protein structure properly, a series of analysis was done using ExPASy ProtParam tool, first of them was the analysis of protein sequence (Table 3.2) and alignment of the protein sequence with Ser-4mer and in-silico Ser-8mer (Appendix B). Also, amino acid composition of the Ser-8mer is shown in the Table 3.2. Finally, extinction coefficient of the protein was estimated as 23840 M^{-1} cm^{-1}.

When the Table 3.2 is examined, hydrophilic amino acid composition of the Ser-8mer protein is found to be 37% serine, 11% threonine and 4.3% tyrosine, these results are consistent with Ser-4mer protein which has 34% serine, 11% threonine and 4% tyrosine (Bostan 2019) and native sericin which has 38% serine, 7.5% threonine and 4.7% tyrosine (Huang et al. 2003).

Table 3.2 Amino acid composition of Ser-8mer protein

Amino acid		Number	%	Amino acid		Number	%
Ala	(A)	15	4.0%	Gln	(Q)	3	0.8%
Arg	(R)	23	6.2%	Glu	(E)	3	0.8%
Asn	(N)	12	3.2%	Gly	(G)	61	16.4%
Asp	(D)	12	3.2%	His	(H)	14	3.8%
Cys	(C)	1	0.3%	Ile	(I)	2	0.5%
Leu	(L)	7	1.9%	Ser	(S)	137	36.9%
Lys	(K)	7	1.9%	Thr	(T)	42	11.3%
Met	(M)	4	1.1%	Trp	(W)	0	0.0%
Phe	(F)	1	0.3%	Tyr	(Y)	16	4.3%
Pro	(P)	1	0.3%	Val	(V)	10	2.7%

3.5 Characterization of Ser-8mer Protein

Characterization studies was performed to understand the important aspects of the protein like solubility, secondary structure, and fibril structure. Before these studies, the protein was dialyzed against distilled water and insoluble aggregates are separated from

the soluble fraction. The soluble fraction was used to determine the solubility and the extinction coefficient of the protein. Insoluble fraction was lyophilized and used to determine the secondary structure of the protein by FTIR) and fibril structure by congo red staining.

3.5.1 Determination of Solubility

According to previous study, almost all of the Ser-8mer protein was self-assembled into fibrillar structure in the presence of water and aggregated (Huang et al. 2003), that's why it was important to use a denaturant like urea during isolation studies. After isolation, to investigate the fibril structure of the protein, urea was removed by dialysis against distilled water. 2.35 mg/mL Ser-8mer protein was dialyzed overnight using a dialysis membrane with a molecular weight cut off of 3.5 kDa. After dialysis, the protein was self-assembled into fibrillar structure as expected. (Figure 3.9).

Figure 3.9 Self-assembled insoluble fibrillar structure of Ser-8mer protein.

The soluble and insoluble fractions are separated by centrifugation at 15000g. In order to determine the solubility of the protein, BCA assay was performed to soluble fraction. According to this analysis, protein concentration in the soluble fraction was found as 0.19 mg/mL which is equal to 8.25% of the initial protein concentration. Therefore, the solubility of Ser-8mer protein in water was found as 0.19 mg/mL with a percent solubility of 8.25%. Although the value is low, this result show that there is still soluble fraction of Ser-8mer left in water after dialysis on the contrary to Huang et al.

Re-solubilization of the protein was also investigated, for this purpose, insoluble fraction of the protein was resuspended in the elution buffer containing 8M urea and centrifugated again at 15000g. The protein concentration after resuspension was found as 0.38 mg/mL which is a two-fold increase than the insoluble fraction, however it was not possible to re-solubilize entire aggregate using the elution buffer.

3.5.2 Determination of Extinction Coefficient

The extinction coefficient of the Ser-8mer protein was theoretically estimated as 23840 M^{-1} cm^{-1} using ExPASy ProtParam tool. The extinction coefficient was determined experimentally by measuring UV-visible spectra of different concentration of protein in the presence of urea at 280 nm. Extinction coefficient can be calculated from the slope of the concentration vs. absorbance at 280 nm graph. When the Figure 3.10 is examined, it can be seen that the slope of the graph is 1306.6 M^{-1}, with a nanodrop cuvette pathlength of 0.052 cm, the extinction coefficient can be calculated as 25127 M^{-1} cm^{-1}. The calculated value is found to be consistent with the estimated value with %error of 5%

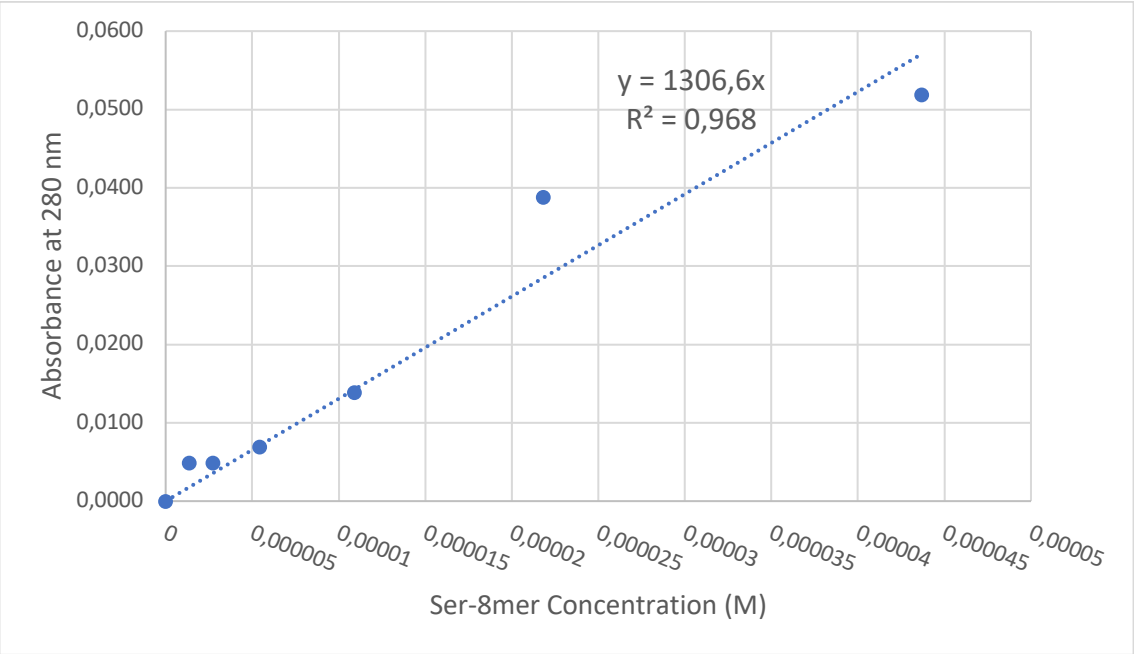

Figure 3.10 Concentration vs. Absorbance at 280 nm graph for Ser-8mer

3.5.3 Determination of Fibril Structure of Ser-8mer

Recombinant sericin proteins are highly capable of self-assemble into fibrillar structure which are formed by beta turns and beta sheets. Polar amino acids which are abundant in the structure of the protein create interactions called “polar zippers” by linking beta sheets together through hydrogen bonding between polar side chains (Huang et al. 2003). In this study, aggregates created by the self-assembled fibril structures were also observed visually, so further analytical techniques were applied to confirm that the aggregates formed are indeed fibril structures created by beta sheets.

Congo red (CR) is a dye that is widely used in histological and pathological studies to observe the fibril structures created by amyloid beta proteins. Amyloid beta protein aggregation is thought to be the main player in Alzheimer’s disease. Previous study showed that the fibrils created by sericin proteins are structurally similar to those created by amyloid proteins (Huang et al. 2003), therefore assays created for these proteins can also be applied to Ser-8mer. Congo red can selectively bind to beta sheet

structures of proteins and is detectable by eye and also cause a spectral shift caused by beta sheet-CR interaction (Klunk, Jacob, and Mason 1999).

For the CR assay, the soluble fraction of the protein was incubated with CR and absorbance spectrum was measured. As it can be seen from the Figure 3.11 and 3.12, a small spectral shift can be observed at 495 nm. The magnitude of the CR shift is observed for Ser-8mer is less than for Ser-4mer. This may be due to low protein concentration in the soluble state of the Ser-8mer, which is caused by the high amount of aggregation, making the protein detection below the limit for the assay.

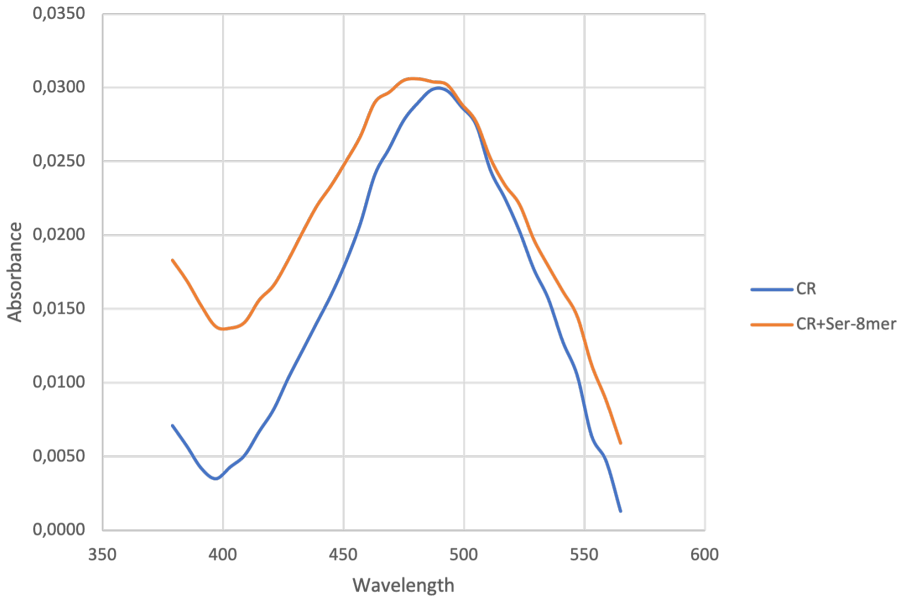

Figure 3.11 UV-Vis spectra for CR assay at the 0th minute.

The fibril structure of the protein was also analyzed visually using light microscopy. For the analysis, the insoluble fraction of the protein was incubated with CR solution overnight and the unbound CR was washed away using PBS. As it was stated before, because of the interaction between beta sheets of the protein and CR, fibril structures of the protein can easily be detected under light microscopy if there is any. Results of the analysis can be seen in the Figure 3.13. These results are the proof that the Ser-8mer protein can form fibril structure.

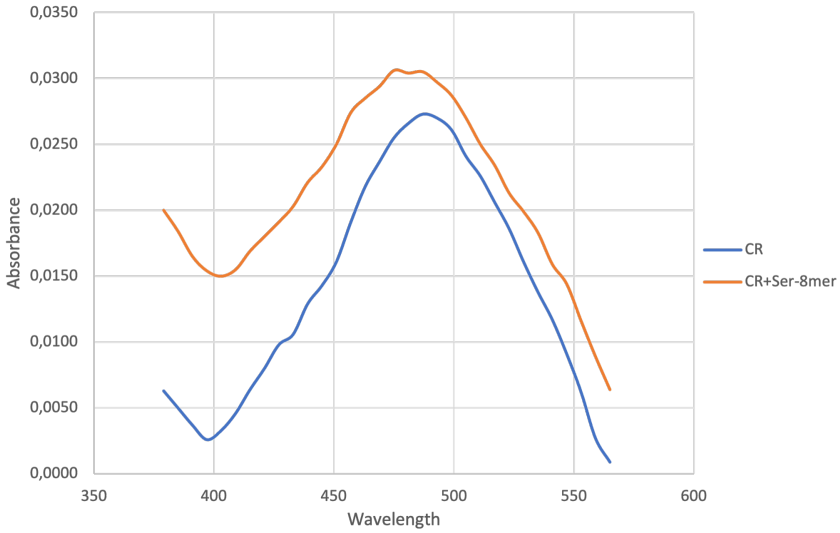

Figure 3.12 UV-Vis spectra for CR assay at the 15th minute.

The morphology of the fibril structure was also analyzed using Scanning Electron Microscope (SEM) whose results are given in the Figure 3.14. Fibril structure of the Ser-8mer protein was also observed with SEM analysis (Figure 3.14 c,d).

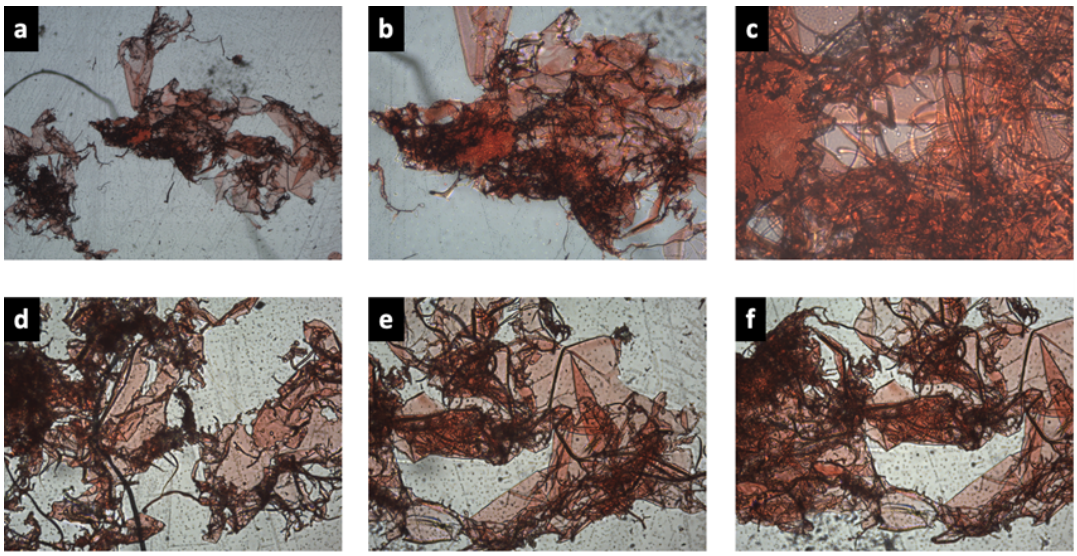

Figure 3.13 Image of Ser-8mer fibrils stained with congo red under light microscope. a: 4X Magnitude, b: 10X magnitude, c: 40X Magnitude, d-f: 10X Magnitude

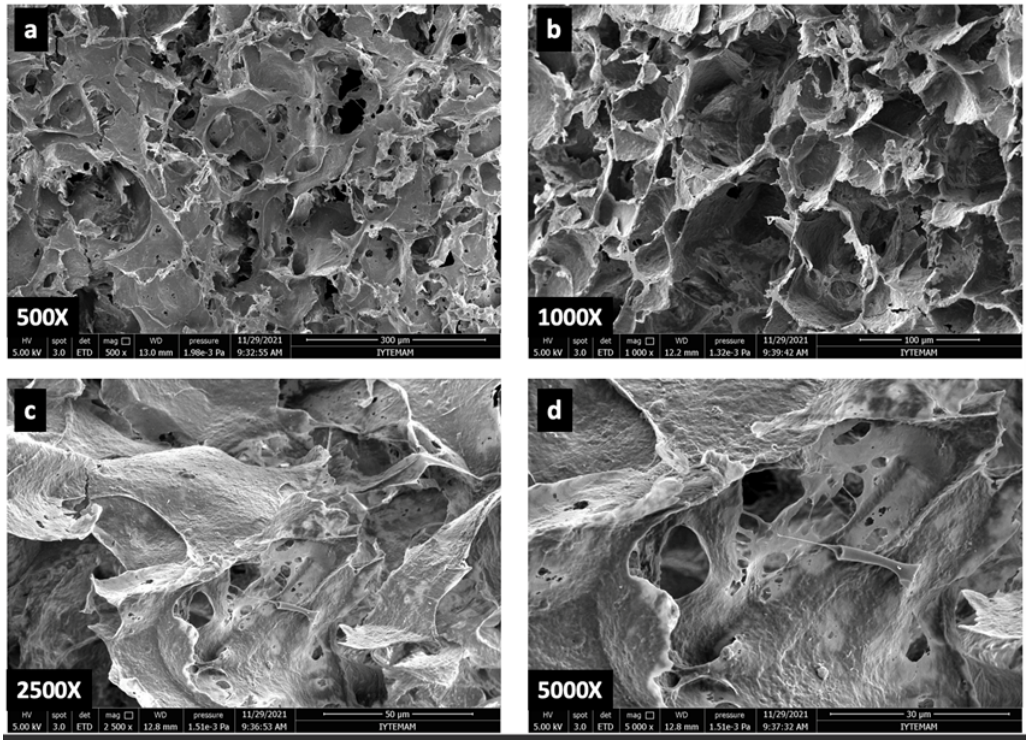

Figure 3.14 Scanning Electron Microscope Image of Ser-8mer fibrils

3.5.4 Determination of Secondary Structure of Ser-8mer

As it was mentioned before, the fibril structure of the Ser-8mer is due to the abundance of beta sheet conformation in the secondary structure of the protein. In order to analyze the secondary structure, Fourier Infrared Transform Spectroscopy (FTIR) analysis was conducted with lyophilized insoluble fraction of the protein. According to literature for FTIR, characteristic peaks for beta sheet conformation can be found in wavelengths around 1621 cm^{-1} (amide I), 1624 cm^{-1} (amide I), and 1512 cm^{-1} (amide II), while the peaks for random coils can be found around 1642 cm^{-1} (amide I), 1644 cm^{-1}, and 1537 cm^{-1} (amide II) (Huang et al. 2003; Dong et al. 1995).

When the results are analyzed (Figure 3.15), characteristic peaks for beta sheet conformation at the 1621 cm^{-1} and 1512 cm^{-1} can be seen for the Ser-8mer protein, the results are clearly different then the commercial sericin which has characteristic peaks for random coil conformation at the wavelengths of 1537 cm^{-1} and 1644 cm^{-1}

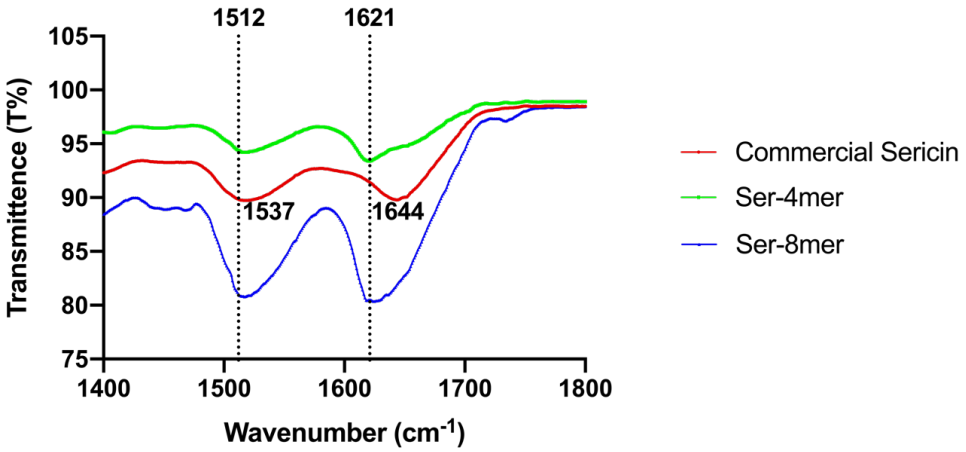

Figure 3.15 FTIR Spectra for Commercial sericin, Ser-4mer and Ser-8mer proteins

CHAPTER 4

CONCLUSION

Sericin is the one of the major proteins which make up natural silk, it has a broad range of application areas from industrial to biomaterial research, it is a good candidate for the wound healing studies due to its fibrillar structure. Since natural extraction of the sericin results in unstandardized end product, recombinant production has become one of the major sericin production method. Sericin can have up to eleven 38-amino acid repeats which are highly conserved. Previous studies have characterized recombinant Ser-4mer protein, which contains first four repeats of the natural sericin sequence pattern. The purpose of this thesis study was to clone, express and characterize Ser-8mer protein which includes the eight repeats of the 38 amino acid repeating pattern. For this purpose, previously constructed gene for Ser-4mer protein was taken as basis and the new gene was constructed successfully using recursive directional ligation methodology. The cloned Ser-8mer protein was successfully expressed in *E. coli* BL21(DE3) cells and isolated using immobilized metal affinity chromatography. In this study, 2.28 mg/mL Ser-8mer protein was expressed with a yield of 6.86 mg protein/L media. Characterization studies were applied to isolated protein. The solubility of the protein in the water was found as 0.19 mg/mL which is two-fold lower than the Ser-4mer protein. It was found out that the Ser-8mer protein is capable of creating fibril structures which was later analyzed with Congo red assay and scanning electron microscopy. According to FTIR spectroscopy, these fibril structures are result of beta sheets formed by polar amino acids located in the eight 38 amino acid repeats of the protein. The fibril structure and beta sheet amount of the protein was found to be larger than the Ser-4mer protein. Also, the decreased solubility of the 8mer protein may be the reason of the increased fibrillar structure. As a conclusion, the Ser-8mer protein will contribute to application of sericin based biomaterial studies which were initiated by the Ser-4mer protein and thanks to its increased fibril structure, this contribution is expected to be more effective than the Ser-4mer protein.

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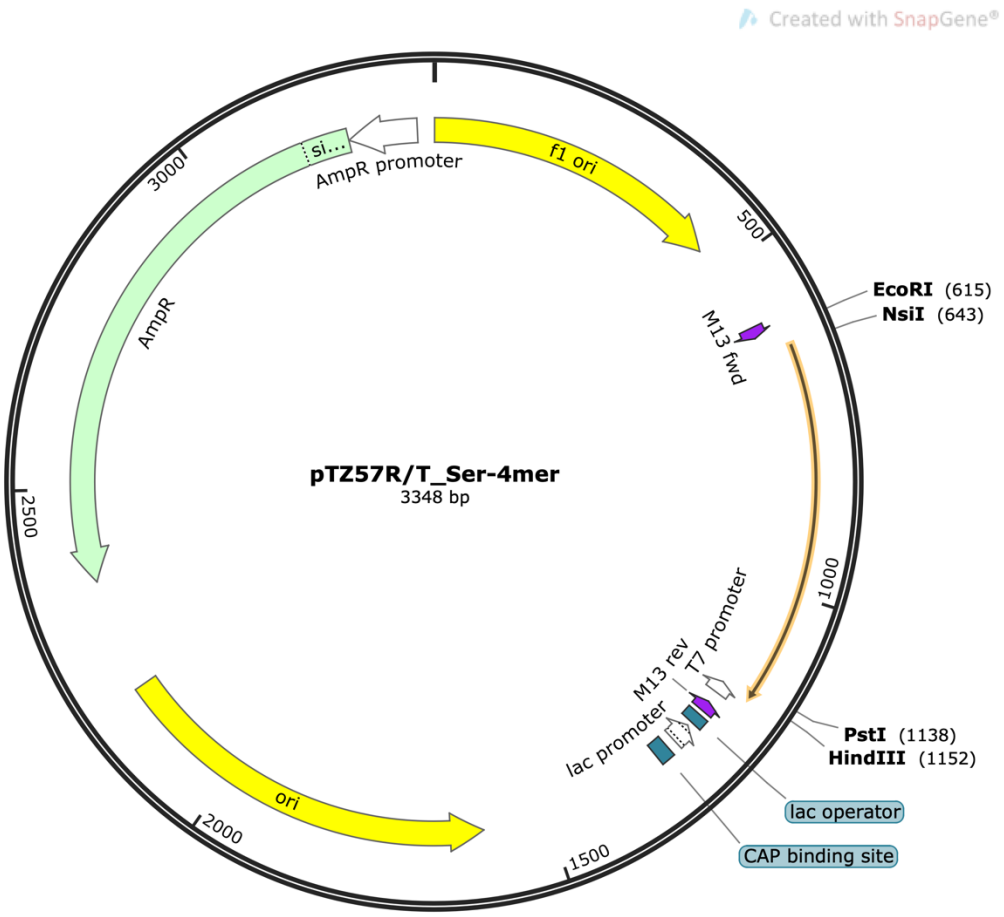
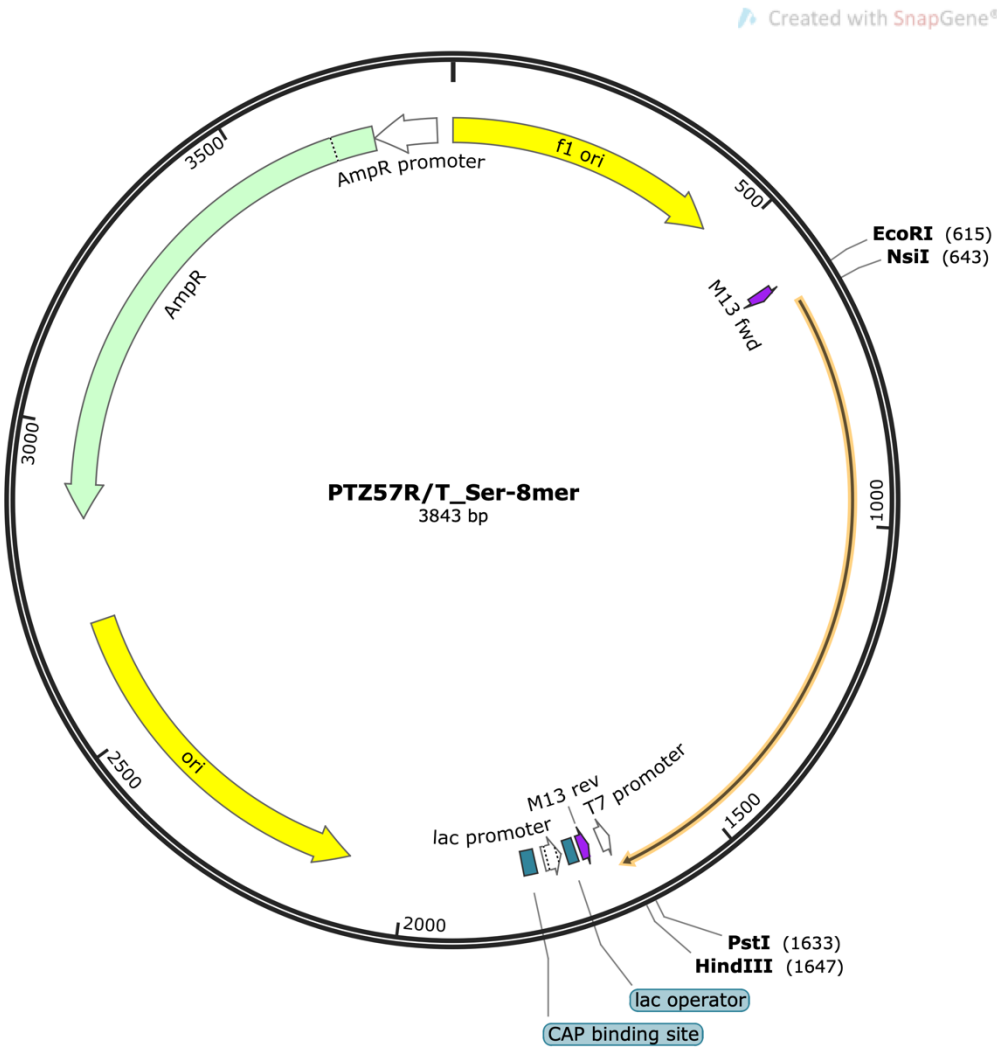
Zhang, Yu-Qing, Mei-Lin Tao, Wei-De Shen, Jian-Ping Mao, and Yu-hua Chen. 2006. "Synthesis of Silk Sericin Peptides–L-asparaginase Bioconjugates and Their Characterization." Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 81 (2): 136–45.

APPENDIX

APPENDIX A - Plasmid Maps



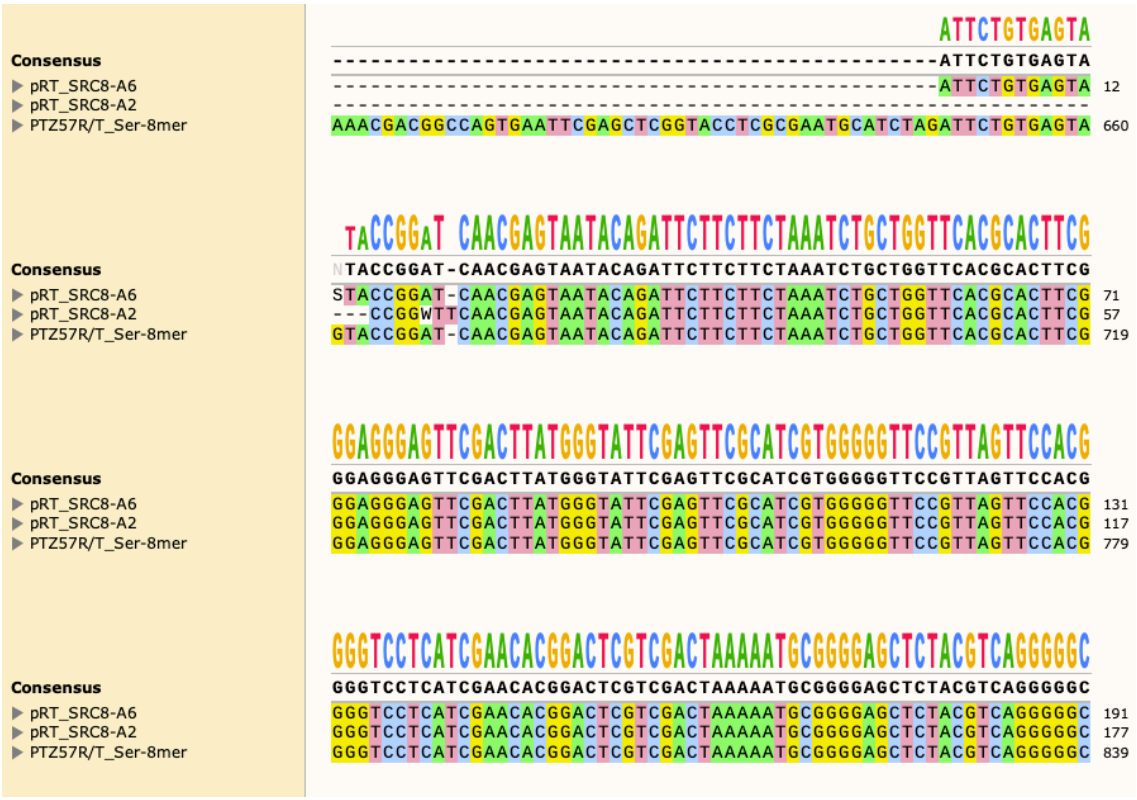
Plasmid map for pRT-SRC4 which encodes for Ser-4mer (Created with SnapGene 6.0)

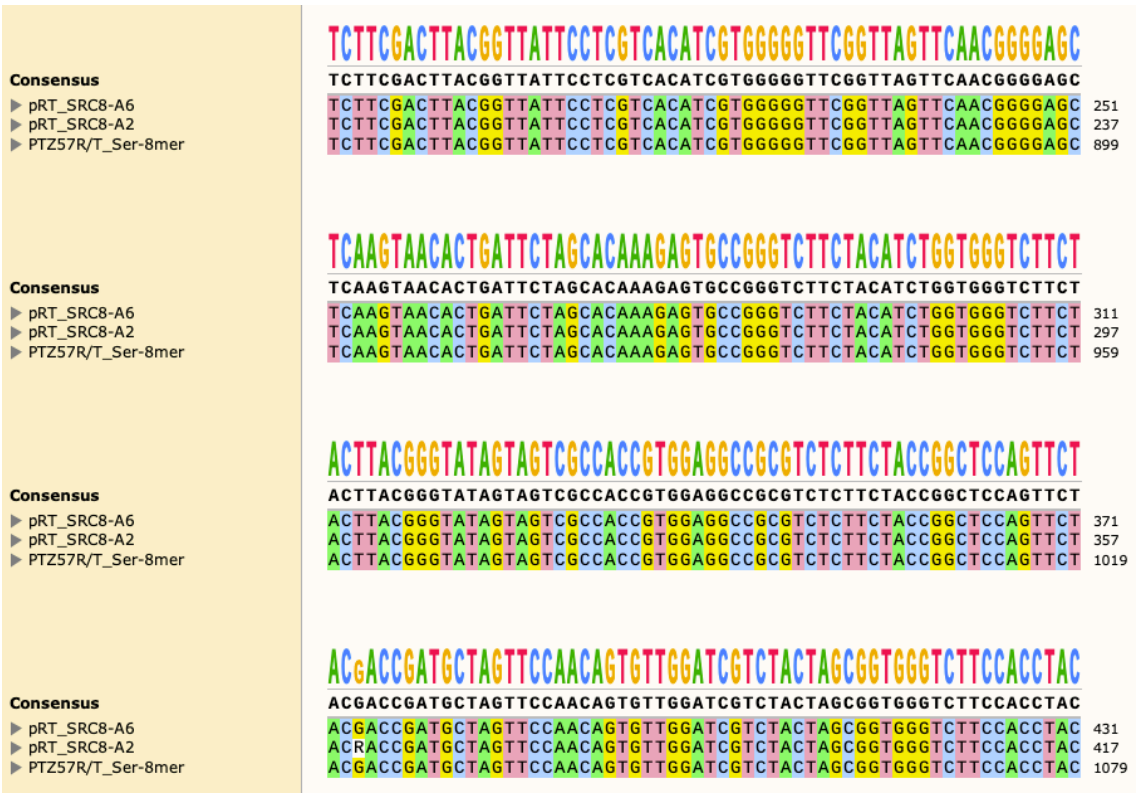


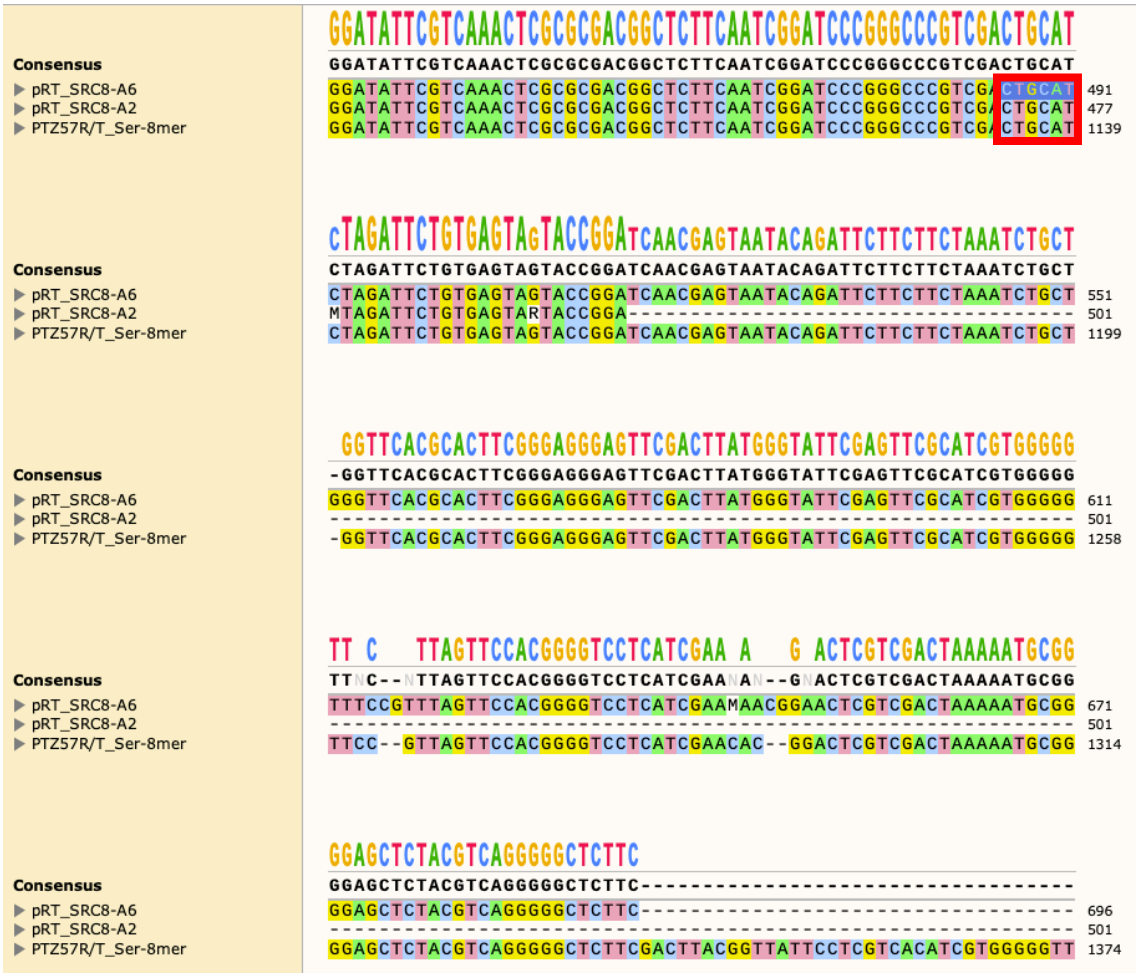
Plasmid map for pRT-SRC8 which encodes for Ser-8mer (Created with SnapGene 6.0)

Plasmid map for pER-21a_Ser-8mer which encodes for Ser-8mer (Red) (Created with SnapGene 6.0)

APPENDIX B - Sequence Analysis For pRT-SRC8



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pRT_SRC8-A2
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477
1139
Consensus
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pRT_SRC8-A2
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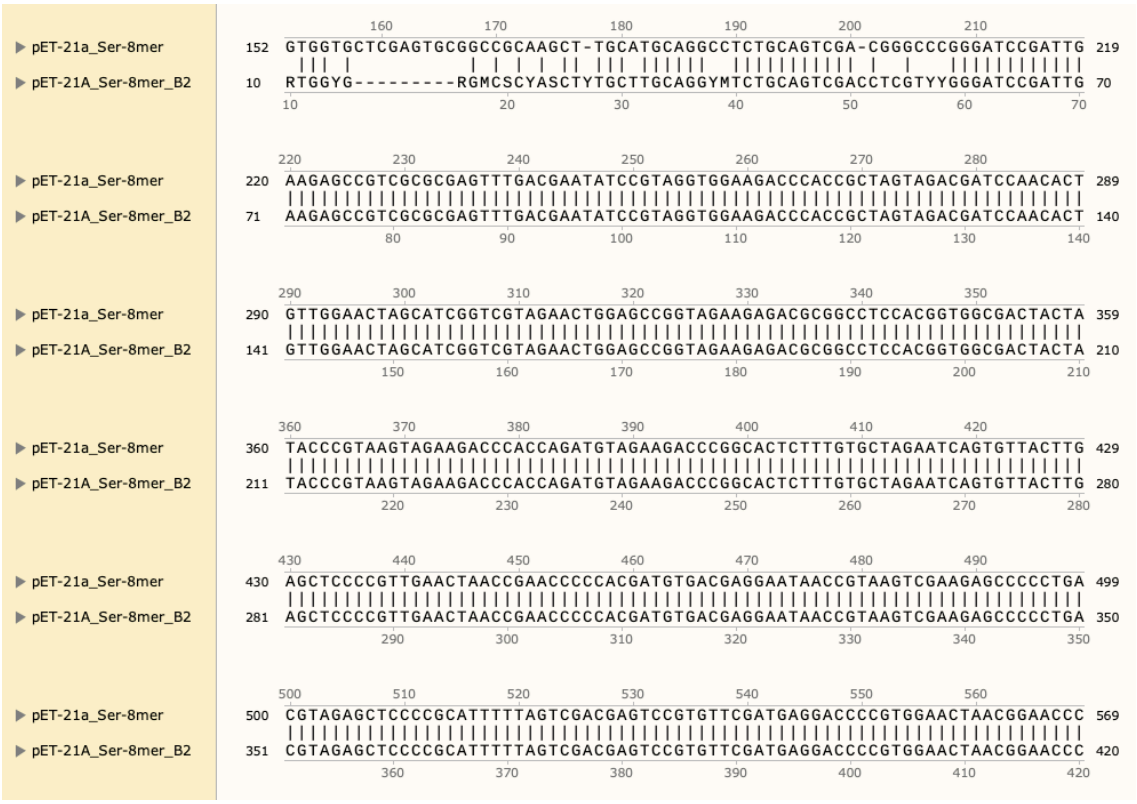
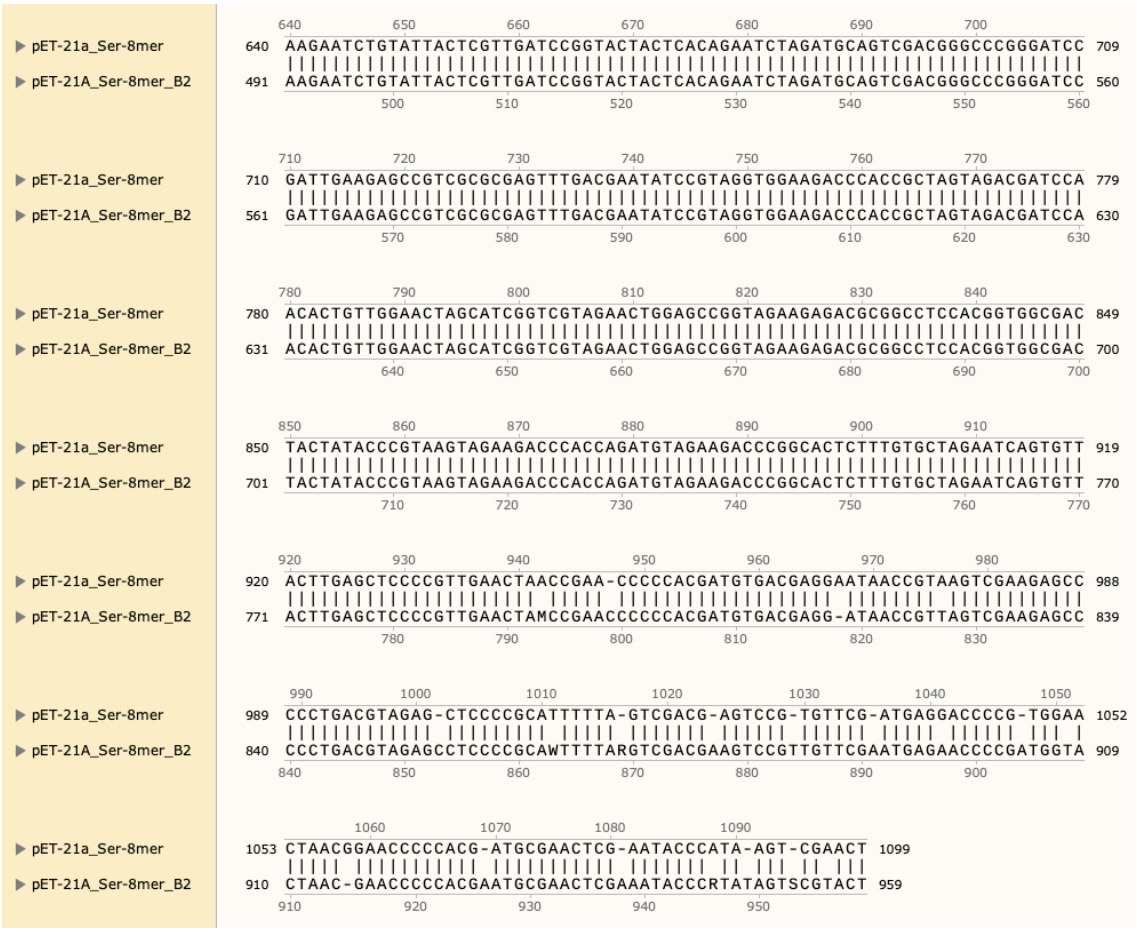
APPENDIX C - Sequence Analysis for pET-21a_Ser-8mer

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491 AAGAATCTGTATTACTCGTTGATCCGGTACTACTCACAGAATCTAGATGCAGTCGACGGGCCCGGGATCC 560
500 510 520 530 540 550 560
710 720 730 740 750 760 770
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570 580 590 600 610 620 630
780 790 800 810 820 830 840
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710 720 730 740 750 760 770
920 930 940 950 960 970 980
920 ACTTGAGCTCCCCGTTGAACTAACCGAA-CCCCCACGATGTGACGAGGAATAACCGTAAGTCGAAGAGCC 988
771 ACTTGAGCTCCCCGTTGAACTAMCCGAACCCCCCACGATGTGACGAGG-ATAACCGTTAGTCGAAGAGCC 839
780 790 800 810 820 830
990 1000 1010 1020 1030 1040 1050
989 CCCTGACGTAGAG-CTCCCCGCATTTTTA-GTCGACG-AGTCCG-TGTTCG-ATGAGGACCCCG-TGGAA 1052
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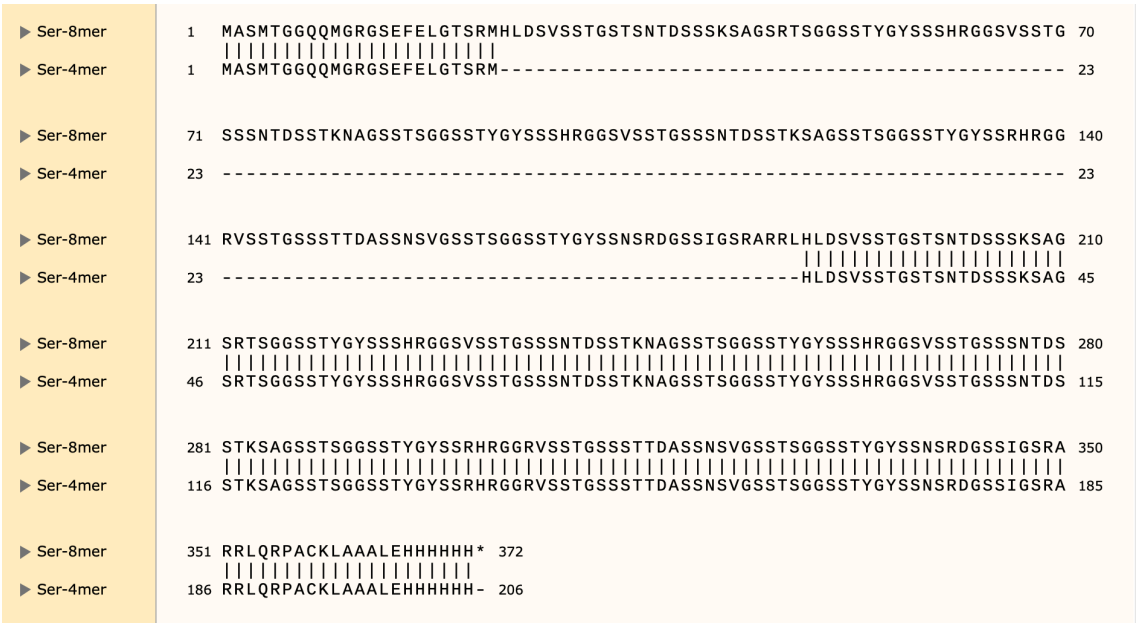
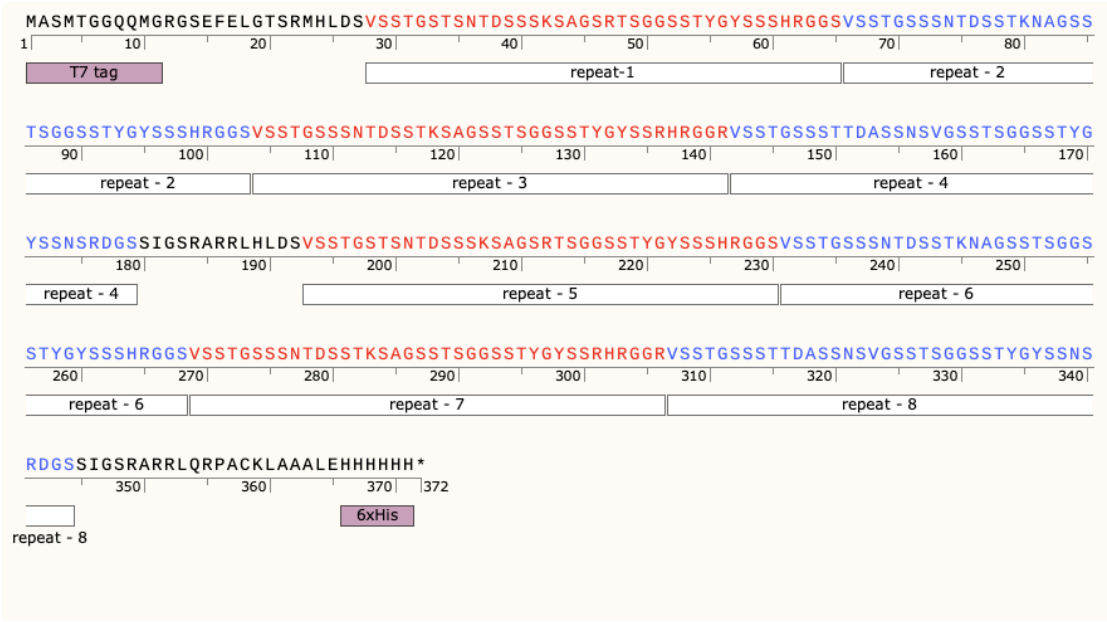
1060 1070 1080 1090
1053 CTAACGGAACCCCCACG-ATGCGAACTCG-AATACCCATA-AGT-CGAACT 1099
910 CTAAC-GAACCCCCACGAATGCGAACTCGAAATACCCRTATAGTSCGTACT 959
910 920 930 940 950

APPENDIX D - Protein Alignment



APPENDIX E - Protein Sequence of Ser-8mer



APPENDIX F - Standard Curve For BCA Assay