CLONING OF NOVEL SERICIN LIKE PROTEINS AND OPTIMIZATION OF THEIR EXPRESSION

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ABSTRACT

CLONING OF NOVEL SERICIN LIKE PROTEINS AND
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Sericin is a protein that helps fibroin fibers link together to create the cocoon, and it forms silk with fibroin. Fibroin is used in textile production as well as in biomaterial applications. Sericin has essential biocompatibility, biodegradability, cryoprotection, antioxidant properties, and a multi-component structure. Thanks to these properties, its use in biomaterial and biomedical fields is expanding and also used in cosmetic research and wound healing, and drug delivery thanks to its high fiber structure. Sericin can be obtained from silkworm cocoons by degumming method but changes the structure of the obtained protein since the protein is exposed to high temperatures. Sericin produced in different batches is obtained in different quantities and these create inconsistencies in the quality of the biomaterials obtained from the sericin, limiting the use of the sericin as a biomaterial. Besides, obtaining protein by recombinant production provides the advantage that the repetitive chain length can adjust as desired and the protein can be standardized. In this thesis, recombinantly generated and optimized a novel sericin-like protein (Ser-12mer) with the native sericin sequence encoding twelve repeats of recombinantly conserved 38 amino acid motifs in *Escherichia coli* and characterized its structural properties. In addition, the effects of induction cell density and cell culture media on the expression of the previously produced sericin-like protein (Ser-4mer) was investigated and its expression and concentration were increased by optimization. Recombinant production of a sericin-like protein will provide an understanding of the sequence-structure relationships and significantly expand their applications as biomaterials.

ÖZET

SERİSİN BENZERİ YENİ PROTEİNLERİN KLONLANMASI VE ÜRETİMLERİNİN OPTİMİZASYONU

Serisin, fibroin liflerinin koza oluşturmak için birbirine bağlanmasına yardımcı olan bir proteindir ve fibroin ile birlikte ipeği oluşturur. Fibroin, tekstil üretiminde ve biyomateryal uygulamalarında kullanılmaktadır. Serisin, temel biyouyumluluk, biyolojik olarak parçalanabilirlik ve antioksidan özelliklere ve çok bileşenli bir yapıya sahiptir. Bu özellikleri sayesinde biyomateryal ve biyomedikal alanlarda kullanımı yaygınlaşmakta ve ayrıca yüksek lif yapısı sayesinde kozmetik araştırmalarda ve yara iyileşmesinde, ilaç dağıtımında da kullanılmaktadır. Serisin ipekböceği kozalarından zamk giderme yöntemiyle elde edilebilir ancak bu metodlar proteinin yüksek sıcaklıklara maruz kalması sebebiyle elde edilen proteinin yapısında değişiklikler oluşmasına yol açar. Farklı partilerde üretilen serisinin farklı miktarlarda ve öngörülemeyen özelliklerde elde edilmesi, serisinden elde edilen biyomateryallerin kalitesinde tutarsızlıklar yaratarak serisinin biyomateryal olarak kullanımını sınırlandırmaktadır. Ayrıca rekombinant üretim ile protein elde edilmesi, tekrarlayan zincir uzunluğunun istenildiği gibi ayarlanabilmesi ve proteinin standardize edilebilmesi avantajını sağlar. Bu tez çalışmasında, doğal serisinin tekrar eden 38 amino asit dizisinin on iki tekrarını içeren yeni bir serisin benzeri protein (Ser-12mer) klonlanmış ve Escherichia coli bakterisinde ilk olarak üretilerek optimize edilmiş ve yapısal özellikleri karakterize edilmiştir. Ayrıca indüksiyondaki farklı hücre yoğunluğu ve farklı hücre kültürü ortamının daha önce üretilmiş serisin benzeri proteininin (Ser-4mer) ekspresyonu üzerindeki etkileri araştırılmış ve optimize edilerek ekspresyonunun arttırılması başarılmıştır. Serisin benzeri bir proteinin rekombinant üretimi, dizi-yapı ilişkilerinin anlaşılmasını sağlayarak literatüre katkı sağlayacak ve biyomalzemeler olarak uygulamalarını önemli ölçüde genişletecektir.

TABLE OF CONTENTS

ABSTRACT iii
ÖZET iv
TABLE OF CONTENTS viii
LIST OF FIGURES xi
LIST OF TABLES xiii
CHAPTER 1. INTRODUCTION 1
1.1 Silkworm 1
1.2 Silk Proteins 1
1.2.1 Fibroin 2
1.2.2 Sericin 3
1.2.2.1 Biochemical Properties of Sericin 3
1.2.2.2 Structural Analysis of Sericin Genes 4
1.3 Potential Applications of Sericin 4
1.3.1 Biodegradability 5
1.3.2 Biocompatibility 5
1.3.3 Antioxidant 6
1.3.4 Effect of Sericin on Culture Media and Culture Media 6
1.3.5 Cryoprotective Effects of Sericin 7
1.3.6 Biomaterial Applications of Sericin 7
1.4 Degumming Methods for Extraction of Sericin 7
1.4.1 Chemical Treatment of Sericin 8
1.4.2 Sericin Boiling in Water 8
1.4.3 Sericin Enzymatic Extraction 9
1.5 Problems of Extraction and Processing of Sericin 9
1.6 Recombinant Production of Sericin 10
1.7 Aim of the Study 11
CHAPTER 2. MATERIALS AND METHODS 12
2.1 Materials 12
2.1.1 Ser-4mer, Ser-8mer, Vectors, Primers, and Restriction Enzymes 12
2.1.2 Cells 13
2.1.3 Instruments 13
2.2 Method 13

2.2.1 Optimization of Ser-4mer Production in E.coli BL21 (DE3)..... 14
2.2.1.1 Small Scale Expression of Ser-4mer 14
2.2.1.2 Optimization of Ser-4mer Expression 15
2.2.1.3 Purification of Large-Scale Ser-4mer Expression 16
2.2.2 Cloning of Ser-12mer Gene..... 17
2.2.2.1 Double Digestion of the pRT_SRC4 Plasmid with PstI and NsiI Restriction Enzymes..... 18
2.2.2.2 Single Digestion of Plasmid pRT_SRC8 with NsiI Restriction Enzyme 19
2.2.2.3 Ligation of Isolated Ser-4mer Insert into pRT_SRC8 Plasmid..... 20
2.2.2.4 Colony Polymerase Chain Reaction (cPCR) for Prt_SRC12 . 22
2.2.3 Cloning of Ser-12mer Gene into Expression Vector..... 24
2.2.3.1 Double Digestion of the pRT_SRC12 Plasmid with EcoRI and HindIII Restriction Enzymes..... 25
2.2.3.2 Sequential Digestion of Pet-21a(+) Plasmid with EcoRI and HindIII Restriction Enzymes..... 26
2.2.3.3 Ligation of Isolated Prt_SRC12 insert into Sequentially Digested pET-21a(+) Expression Vector 28
2.2.3.4 Colony Polymerase Chain Reaction (cPCR) for Pet-21a_Ser12 29
2.2.4 Expression of Ser-12mer Protein in E. coli BL21 (DE3) 30
2.2.4.1 Ser-12mer Small Scale Expression 31
2.2.4.2 Ser-12mer Large Scale Expression 31
2.2.5 Optimization of Ser-12mer Protein Expression..... 31
2.2.5.1 Optimization with different antibiotics 32
2.2.6 Purification of Ser-12mer Protein..... 32
2.2.6.1 Small Scale Isolation of Ser-12mer protein..... 32
2.2.6.2 Large Scale Purification of Ser-12mer Protein 33
2.2.6.3 The Determination of the Concentration Ser-12mer Protein .. 34
2.2.7 Characterization of Ser-12mer Protein 34
2.2.7.1 The Dialysis of Ser-12mer Protein against Deionized Water and Phosphate Buffer 34
2.2.8 Particle Size Analysis 35
2.2.8.1 Agarose Gel Electrophores..... 35

2.2.8.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis 36
CHAPTER 3. RESULTS AND DISCUSSION 37
3.1 Ser-4mer Expression and Optimization 37
3.1.1 Expression Test of Ser-4mer 37
3.1.2 Effects of Different Growth Media on Ser-4mer protein expression 39
3.1.3 Different Cell Densities at the Start Induction of Ser-4mer Expression 40
3.1.4 Small and Middle Scale Production for Optimization of Ser-4mer . 41
3.1.5 Large Scale Production of Ser-4mer 47
3.2 Construction of pRT_SRC12 48
3.2.1 Double Digestion of PRT_SRC4 plasmid 48
3.2.2 Ligation with pRT_SRC4 and pRT_SRC8 48
3.3 Construction of Ser-12mer Protein 49
3.3.1 Double digestion of PRT_SRC12 plasmid 50
3.3.2 Ligation with PRT_SRC12 and Pet21A 51
3.4 Expression Test of Ser-12mer Protein 52
3.5 Optimization of Ser-12mer Expression 54
3.6 Purification of Ser-12mer protein 55
3.6.1 The Small Scale Isolation of Ser-12mer Protein 56
3.6.2 The Large Scale Isolation of Ser-12mer Protein 56
3.6.3 Determination of Concentration 58
3.7 Amino Acid Composition and Extinction Coefficient of Ser-12mer 59
3.8 Dialysis 60
CHAPTER 4. CONCLUSION 62
REFERENCES 63
APPENDICES 68
APPENDIX A 68
APPENDIX B 69
APPENDIX C 70
APPENDIX D 71
APPENDIX E 72
APPENDIX F 73
APPENDIX G 74
APPENDIX H 75
APPENDIX I 76

LIST OF FIGURES

Figure **Page**

Figure 1.1 Component of Silk..... 2
Figure 2.1 *PstI* and *NsiI* restriction Enzymes Recognition Sites..... 17
Figure 2.2 Cloning of pRT_SRC12 with RDL method (Created with BioRender) 18
Figure 2.3 Restriction enzymes recognition sites..... 24
Figure 2.4Construction of pET21a_SRC12..... 25
Figure 3.1 15% SDS-PAGE analysis for expression test of ser-4mer protein 38
Figure 3.2 15 % SDS-PAGE analysis for expression test of ser-4mer protein. 39
Figure 3.3 15% SDS-PAGE analysis for expression test of ser-4mer protein with different growth media..... 40
Figure 3.4 15% SDS-PAGE analysis for expression test of ser-4mer protein with different growth media..... 42
Figure 3.5 15% SDS-PAGE analysis for expression test of ser-4mer protein with TB and LB growth media with different OD_{600} values of induction. 43
Figure 3.6 15% SDS-PAGE analysis for expression test of ser-4mer protein with 2xYT growth media with different OD_{600} values of induction..... 44
Figure 3.7 Different Cell Densities at the Different Induction..... 45
Figure 3.8 Cell Density graphic for Induction when OD600 value at 0.5..... 46
Figure 3.9 Cell Density graphic for Induction when OD600 value at 0.6..... 46
Figure 3.10 Cell Density graphic for Induction when OD600 value at 0.8..... 47
Figure 3.11 1% Agarose gel image of bands including Ser-4mer gene obtained from double digestion..... 48
Figure 3.12 Colonies include pRT_SRC12 plasmid after ligation in 1% agarose gel.... 49
Figure 3.13 1% Agarose gel image of bands including pRT_Ser12 gene obtained from double digestion..... 50
Figure 3.14 Colonies include pET21a_SRC12 plasmid after ligation in 1% agarose gel..... 51
Figure 3.15 1% Agarose gel image of bands including Ser-12mer gene obtained from double digestion..... 52
Figure 3.16 SDS-PAGE gel analysis of the expression of Ser-12mer protein (15% SDS-PAGE) 53

Figure **Page**

Figure 3.17 15% SDS-PAGE gel analysis for expression with chloramphenicol 55
Figure 3.18 15% SDS-PAGE gel analysis for small scale isolation..... 56
Figure 3.19 SDS-PAGE gel analysis for large scale protein expression 57
Figure 3.20 %15 SDS - PAGE gel analysis after isolation with ampicillin 58
Figure 3.21 Graph of absorbance values obtained against the concentration of Albumin (BSA) standards at 562 nm. 59
Figure 3.22 15% SDS-PAGE gel analysis after dialysis 61

LIST OF TABLES

Table **Page**

Table 1.1 Composition of Silk 2
Table 2.1 Phosphate Buffer Saline (PBS) components 16
Table 2.2 Buffer components of a purification step 17
Table 2.3 The components of PRT_SRC4 double digestion mixture 19
Table 2.4 The components of PRT_SRC8 single digestion mixture. 20
Table 2.5 The components of dephosphorylation of digested PRT_SRC8 DNA. 20
Table 2.6 The component of ligation of digested PRT_SRC4 and digested PRT_SRC8 ... 21
Table 2.7 cPcr Method for Prt_SRC12 plasmid 22
Table 2.8 The components of the pRT_SRC12 cPCR mix. 23
Table 2.9 The Designed Primers To Amplify pET21a-SRC12 23
Table 2.10 The components of the Ser-12mer double digestion mixture. 26
Table 2.11 The components of pET-21a(+) digestion mixtures. 27
Table 2.12 The components of dephosphorylation of digested pET-21a(+) 27
Table 2.13 The component of ligation of digested ser-12mer and digested pET21a. 28
Table 2.14 cPcr Method for Pet-21a_Ser12 plasmid 29
Table 2.15 The components of the pET-21a-SRC12 cPCR mix. 29
Table 2.16 The designed primers to amplify pET-21a-SRC12. 30
Table 2.17 1% Agarose Gel Components 35
Table 2.18 Gels Mixtures for SDS PAGE analysis 36
Table 3.1 Amino acid sequence of Ser-12mer protein 54
Table 3.2 Amino acid composition of Ser-12mer protein 60

CHAPTER 1

INTRODUCTION

1.1 Silkworm

Bombyx mori is an insect from the *Bombycidae* moth family, known as the silk moth. The silkworm is the caterpillar or larva of a silk moth and it is economically significant because of the secretory of silk.

After the butterfly lays its eggs, the caterpillars that emerge as larvae from the eggs, when they develop, secrete a filament-like liquid from the hole in their upper lip and begin to form a cocoon. The caterpillars surround themselves with their cocoon. after a few weeks, the butterfly is formed, and pierces the cocoon, and comes out. When obtaining silk, it is undesirable to the pie of the cocoon. Before the butterfly emerges, the cocoons are placed in hot water and the butterflies are killed. Silk fibers are obtained from these cocoons. Silkworm cocoons consist of two main proteins that Silk fibroin, and silk sericin. (Eslah et al. 2015). While 25-30% of silk protein is form from sericin, 70-75% of fibroin, and around 5% other materials (Rui, 1998).

1.2 Silk Proteins

Silk is a soft, shiny polymer synthesized in nature. The cocoon of the silkworm Bombyx mori is made up of a continuous strand of two filaments fused together. Silk filament is a double strand of fibroin bound together by silk sericin or silk gum, a sticky material (Figure 1.1). Because of its unique chemical and mechanical qualities, including self-assembly, biocompatibility, biodegradability, and controlled structure, natural-silkderived proteins are increasingly being exploited as biomaterials for tissue engineering and drug delivery applications (Lamboni, Gauthier, Yang, & Wang, 2015a).

B. mori's silk gland synthesizes and secretes two kinds of silk proteins; sericin and fibroin (Huang, Bini, Valluzzi, Vernaglia, & Kaplan, 2003).

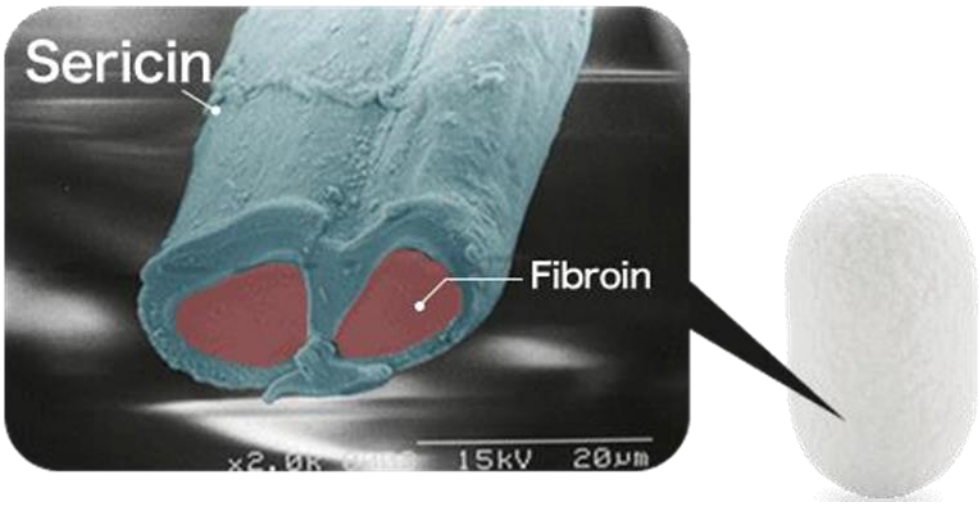

Figure 1.1 Component of Silk

Table 1.1 Composition of Silk

(Source: Rui, 1998)

Component	%
Fibroin	70-80
Sericin	20-30
Carbohydrates	1.2-1.6
Inorganic matter	0.7
Wax matter	0.4-0.8
Pigment	0.2
Total	100

1.2.1 Fibroin

Silk fibroin, a natural fibrous protein with a semi-crystalline structure that gives hardness and strength makes up more than 70% of the cocoon (Table 1.1) (Koh et al. 2015; Sinohara, Asano, & Fukui, 1971). Fibroin is produced in the posterior silk gland (PSG). Fibrin is secreted from the posterior to the central region of the gland for storage. Fibroin is covered with sericin, which functions as an adhesive between two silk strands (Neves & Reis, 2016). A light chain (L) (25 kDa) and heavy chain (H) (350 kDa) are joined by a single disulfide bond at the H-C-terminus chain's to form a H–L complex.

Silk I (water-soluble state) and silk II (crystalline state) are the two primary crystal structures found in silkworm SF (Vepari & Kaplan, 2007; Mori et al. 1995). Gly (43%), Ala (30%), and Ser (12%) are the most common amino acids found in SF from *Bombyx mori* (Koh et al. 2015).

1.2.2 Sericin

Sericin, one of the two proteins that make up silk together with fibroin, aids in the binding of the fibroins and the development of the cocoon. Sericin is produced in the middle silk gland (MSG). Sericin is a protein that has a high concentration of hydrophilic amino acids, particularly serine.

Sericin contains several beneficial qualities, including antibacterial, UV resistance, oxidative resistance, and moisturizing capabilities. It has a wide range of applications including textile, cosmetics, polymer materials, and biomaterials (Shen, Johnson, & Martin, 1998; Pérez Rigueiro, Elices, Llorca, & Viney, 2001).

A degumming method separates fibroin from sericin, the other principal component of the cocoon, to produce shiny silk from dried silkworm cocoons, while sericin is primarily discarded in the wastewater. From the waste solution of one million tons of cocoon (fresh weight) or 400,000 tones of dried cocoon, roughly 50,000 tons of sericin can be recovered (Y. Q. Zhang, 2002; Y. Q. Zhang, 2002).

1.2.2.1 Biochemical Properties of Sericin

Sericin is a sticky, hydrophilic protein that holds two fibroin fibers together. This ensures the stability of the cocoon (Huang et al. 2003; Y. Q. Zhang, 2002). This glue-like property of sericin is thought to come from the hydrogen bonding of sericin in the structure of fibroin with each other. Sericin is a hydrophilic glue protein that links two fibroin fibers together to produce a stable cocoon structure composite. It accounts for around 20-30% of the cocoon's weight (Y. Q. Zhang, 2002; Huang et al. 2003).

Sericin be able to extracted by many different methods. As a result, the amino acid compositions also differ (Aramwit, Damrongsakkul, Kanokpanont, & Srichana, 2010a). Thus sericin is obtained with different molecular properties (Takasu, Yamada, &

Tsubouchi,2002). Depending on factors such as temperature, pH, processing time in extraction methods, its molecular weight has yielded results in the range of 10 to 400 kDa (Vaithanomsat & Kitpreechavanich, 2008; Kato et al. 1998; Freddi, Mossotti, & Innocenti, 2003; Y. Q. Zhang, 2002).

1.2.2.2 Structural Analysis of Sericin Genes

In the structural analysis of the sericin gene, it was observed that there is a repeating module containing 114 nucleotides in the gene. These repeating bases encode a polypeptide chain of 38 amino acids with a high amount of serine in them.

Sericin is produced by three genes known as Ser1, Ser2, and Ser3. Sericin accounts for 25-30% of the cocoon's weight.

The Ser1 gene is a single copy of 9 exons, approximately 23 kb in length, found on chromosome 11, and through the RNA splicing (splicing) process, four main mRNAs (10.5, 9.0, 4.0, and 2.8) kb) are encoded by the Ser1 gene (Garel et al. 1997). The gene was found by Michael et al. (1990), Ser2 extending from 28 to 2574 bp, having 13 exons, and encoding two mRNAs (3.1 and 5.0-6.4 kb). Ser2 gene is more complicated and changeable. Takasu et al. (2007) discovered that the Ser3 gene, the final gene implicated in sericin production, is likewise situated on chromosome 11 and is roughly 3.5 bp in size and encodes a simple 4.5 kb transcript.

1.3 Potential Applications of Sericin

Research has confirmed that sericin is promising for biomedical applications, thanks to its low inflammatory petticoat. Studies, it has been shown that sericin does not have a cytotoxic effect in dissolved form, and it has also been found that dissolved sericin protein increases cell growth (Aramwit et al. 2009). Thanks to its unique properties such as pharmacological functions and inhibition of tyrosinase activity (e.g. anticoagulant, digestion support, anticancer activities and cryoprotection) compatibility, antioxidant effect, antibacterial property, ability to absorb and release moisture, UV protective property, sericin protein is used in tissue engineering applications, wound dressing. It is a natural polymer that continues to be used in many biotechnological fields such as drug

release systems, remediation processes, textile engineering (Aramwit et al. 2011; Kuns et al. 2016).

Sericin is high effective for enhancing polymer materials such as polyamides, polyesters, polyolefins, and polyacrylonitrile due to its characteristics. It may also be used to make bio-membrane materials, fibers, biodegradable materials, biomedical materials, and hydrogels etc (T. T. Cao & Zhang, 2016).

1.3.1 Biodegradability

Biodegradability is substantial for a material to be utilized as a biomaterial for tissue engineering studies. The degradation rate is the rate at which the desired new tissue is formed. Toxic compounds should not be formed when biomaterial is degraded. This material should be easily absorbed by the body (Lloyd, 2002).

Powder of sericin is first dissolved in an organic solvent for example dioxane or tetrahydrofuran in one method. Sericin reacts with a polyisocyanate in the solution. The resulting polyurethane is biodegradable and contains biodegradable sericin segments.

The resultant polyurethane be able to used to make films and fibers (Hatakeyama, 1996).

1.3.2 Biocompatibility

Any biomaterial's propensity to activate the immune system, such as its biocompatibility, is an important factor in its application (Panilaitis et al. 2003). The capacity of a material to function with adequate host response in tissue engineering is known as biocompatibility (Barrer, Mahmood, De Groot, & Van Blitterswijk, 2008). This is, in fact, one of the most crucial aspects determining a biomaterial's success or failure in regenerative medicine and tissue engineering applications (Brown & Badylak, 2012). In the work, electrospinning was used to create novel biodegradable poly (L-lactic-cocaprolactone)-sericin (PLCL-SC) co-polymer membrane (Inthanon et al. 2016).

1.3.3 Antioxidant

Serine inhibits lipid peroxidation and tyrosinase activity, which has been shown in prior research (Kato et al. 1998).

Furthermore, several research shows that sericin inhibits both UV radiation-induced mouse skin and colon carcinogenesis and chemical. According to the mechanism, sericin be able to guard to oxidative stress (Zhaorigetu, Yanaka, Sasaki, Watanabe, & Kato, 2003; Zhaorigetu, Sasaki, Watanabe, & KATo, 2001;). By sulfonating sericin and fibroin, silk protein may be converted into biomaterials having anticoagulant characteristics (Tamada, 1997).

Another research looked at the free radical activity of silk sericin and antioxidative activity and concluded that it possesses excellent antioxidant properties. As a result, it shows B. mori is a natural antioxidant that obtain silk sericin from the silkworm B. mori is a natural antioxidant (T. T. Cao & Zhang, 2016).

1.3.4 Effect of Sericin on Culture Media and Culture Media

Cell culture studies are widely used today and fetal bovine serum (FBS) or Bovine serum albumin (BSA) is suplemented to the culture medium. These materials can be replaced by cheaper ones (Terada et al. 2007). Experiment has shown that sericin be able to used as an alternative (Terada, Nishimura, Sasaki, Yamada, & Miki, 2002).

The following study found that sericin effectively promoted T lymphocyte cell line growth. As a result, Terada and colleagues explained that sericin is an important component in making serum-free cell culture (Terada et al. 2007). A medium containing 0.5 percent sericin protects cells from oxidative stress, promotes cell survival and collagen production, and improves the quality of individual bovine embryos produced. (Mahesh N Padamwar, Pawar, Daithankar, & Mahadik, 2005; Isobe et al. 2012; Terada et al. 2002). Sericin sped up cell proliferation by reducing the lag phase of the cell cycle and leading the cultures to reach the logarithmic phase sooner.

1.3.5 Cryoprotective Effects of Sericin

As is known, freezing and storage of cells are important for transplantation. For this purpose, Cryoprotectant solutions containing 10% DMSO and 10% FBS are widely utilized. Research determined that silk sericin peptide, which is a repeat of 38 amino acid residues produced in E. coli, had a cryoprotective effect utilizing freeze-thaw in vitro experiments (Tsujimoto, Takagi, Takahashi, Yamada, & Nakamori, 2001) Tsujimoto et al. 2001).

Sericin enhanced the growth of mammalian insect cells in previous studies (Terada et al. 2002). In addition, sericin was found to protect cells against the toxicity of dimethyl sulfoxide (DMSO) in a study. These studies show that sericin can replace substances used for cell freezing (Takahashi, Tsujimoto, Yamada, Takagi, & Nakamori, 2003). In this way, cell death caused by DMSO can be prevented.

1.3.6 Biomaterial Applications of Sericin

In the textile business, sericin is regarded as a waste product. According to recent research, this waste product be able to be employed as a biomaterial in a lot of variety of disciplines ranging from cosmetics to medicines, drug delivery, and the development of regenerative medicine (Lamboni et al. 2015a). Natural sericin formulations in aqueous or solvent form could be used to make a variety of biomaterials for other biomedical purposes and tissue engineering. Sericin must be extracted and then reprocessed into the appropriate material forms, such as electron spin fibers, films, surface modifications, nanoparticles, hydrogels and porous 3D sponges (Lamboni et al. 2015a).

1.4 Degumming Methods for Extraction of Sericin

Sericin can be extracted using many different methods and these methods are entitled degumming method. Degumming is the process of removing gum from a material, particularly sericin from silk. Degumming could be accomplished in a variety of methods (Takasu et al. 2002). Choosing and applying one of these techniques is related

to the area in which sericin will be used. Because sericin, which is obtained as a result of all these methods, differs in properties such as amino acid sequence and molecular weights. All methods have some advantages and disadvantages. For instance, methods used under heat or pressure have the advantage that they do not cause impurities. However, this approach is extremely time-consuming and causes severe fibroin damage. therefore, these methods are not preferred especially in industrial production. (Freddi et al. 2003). For another example, ethanol has been shown to successfully precipitate sericin from silk effluent, and higher ethanol concentrations may increase product yields. When applied on a large scale, however, it is harmful to the environment (Wu et al. 2007).

1.4.1 Chemical Treatment of Sericin

Acid or alkali degradation is employed to separate sericin from fibroin in this treatment procedure.

Degumming agents include acids like tartaric, citric acids, sulphuric and hydrochloric (Jiang et al. 2006). For the acid degradation process, Cocoons are cut and cooked for 30 minutes in a 1.25 % citric acid solution. After paper filtration removes insoluble fibers, the clean filtrate is dialyzed against distilled water using cellulose tubing to remove all citric acid (Aramwit et al. 2009).Sericin is extracted from cocoon fragments for a sodium carbonate solution (Na2CO3) and high heat in the alkali degradation process. To remove alkali solution, the supernatant is collected, filtered, and dialyzed numerous times with a 3 kDa dialysis membrane (Nayak, Talukdar, & Kundu, 2012).

1.4.2 Sericin Boiling in Water

The simplest extraction procedure is to boil it in water. Autoclaving chopped cocoons in water at a high (100°C) temperature and also pressure to produce a sericin solution. After that, silk cocoons are collected. Then filtered, frozen, and lyophilized (M N Padamwar & Pawar, 2004). However, the butterflies perish before they emerge from the cocoon.

1.4.3 Sericin Enzymatic Extraction

Sericin is extracted using a variety of enzymes (papain, alkylate, alkaline protease, trypsin) at varying temperatures and doses (M N Padamwar & Pawar, 2004; Freddi et al. 2003). In addition to this procedure, additional methods for degumming sericin have been tried, such as freezing and membrane filtering (Vaithanomsat and Kitpreechavanich (2008) Capar et al. (2008); Fabiani et al. (1996))

1.5 Problems of Extraction and Processing of Sericin

The degumming process of silk cocoons, which is carried out in different ways, differs in terms of quality and structure of the product obtained. The technique chosen affects the properties of the final product in the degumming process, such as isoelectric point, amino acid sequence and molecular weight (Aramwit et al. 2010a).

Studies show that unlike acid and heat extraction, which yields sericin compounds with molecular weights ranging from 35 to 150 kDa, alkali solution extraction yields products with molecular weights ranging from 15 to 75 kDa.

Chemical treatments make recovering high-quality sericin for future research challenging since extra purification techniques are required to eliminate chemical contaminants. The degumming solution have a high concentration of Na2CO3, making separation from the sericin protein problematic (T. Cao, Wang, and Zhang 2013).

To extract sericin from the silk cocoon, enzymatic degumming can be helpful. This procedure, therefore, is expensive and regulates specific proteolytic hydrolysis of the core sericin chain structure, resulting in a molecular weight reduction (Aramwit et al. 2012; Freddi et al. 2003).

The study's findings revealed that degumming procedures did not produce highquality sericin. The final product yields give different outcomes than the previous one, which is a significant negative.

1.6 Recombinant Production of Sericin

Since a standard product could not be obtained as a result of extraction with the degumming method, alternative methods were tried. In addition, production with degumming takes a very long time and is environmentally polluting.

Silk protein is encoded by very large genes (up to 15 Mbp). It contains a lot of repetitive DNA sequences. Moreover, bacteria have difficulty expressing very large genes. Therefore, it can be said as the biggest obstacle in front of silk protein production in these bacteria (*E. coli*) (Altman et al. 2003). In addition, insects' codon usage differs from bacteria.

As a result, two procedures for producing silk proteins have been developed (Vendrely & Scheibel, 2007). The first is the creation of a cDNA library from mRNA isolated from silk-producing insect glands, and the second is the use of synthetic oligonucleotides designed out from sequence of silk protein optimized for codon usage in bacteria.

Sericin is a gene that has repetitive sequences that code for 38 amino acids and contains a lot of serine (Altman et al. 2003). Previous research looked at a repetitive motif and came up with a 38-amino-acid motif to represent repetitive areas (SSTGSSSNTDSNSNSVGSSTSGGSSTYGYSSNSRDGSV). When synthesized, the designed sericin-like protein did not have the requisite stability and also structural features. The utilization of repetitive sequences in the creation of sericin protein creates a barrier to cloning and protein production of the necessary function and length (Huang et al. 2003).

Despite all the difficulties, because of the inexpensive cost and large amount of sericin available for commercial usage, bacteria are more suitable to use. As a result, bacterial synthesis of silk proteins is the favourite approach (S R Fahnestock & Bedzyk, 1997; Lewis, Hinman, Kothakota, & Fournier, 1996; Lazaris et al. 2002).

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-negative and negative bacteria.

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 then 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.

1.7 Aim of the Study

This study aims to produce a novel dodecamer repeat sericin-like protein in *E.coli* by recombinant production method and optimization, isolation, and characterization of structural properties of Ser-12mer and previously made sericin-like proteins. Optimization of the previously produced sericin-like protein will be attempted to higher efficiency.

In cloning studies, previously produced sericin-like proteins and recursive directional ligation (RDL) method planned to be used. The dodecamer sericin protein to be formed is expected to contain 12 repeats of the 38 amino acid repeat region in the native sericin. With the production of sericin by recombinant methods, it was desired to obtain the sericin with cheaper, more consistent, and predictable properties than other extraction methods. It is aimed that the new sericin protein, which protein will experience first in literature, will be used in studies in various fields such as biomaterial and biomedical in the future.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The Geneaid PrestoTM Mini Plasmid kit was utilized to purify the plasmid from cultured cells. The Geneaid Gel / PCR DNA Extraction Kit is a Gel Extraction Kit, and a PCR clean-up kit in one and kit was used to purify bound oligonucleotides and digested plasmids as well as recover DNA fragments from agarose gels. The PierceTM Bicinchoninic Acid Protein Assay kit was utilized to calculate the protein concentration from Thermo Scientific. SoluLyseTM Reagent was acquired from Genlantis. Lysogeny Broth (LB) Low Salt used for the growth of bacteria was purchased from Duchefa Biochemie. A disposable 5 mL polypropylene Ni-NTA column and Ninitrilotriacetic acid (Ni-NTA) agarose resin were acquired from Thermo Scientific to perform protein isolation. Ampicillin sodium salt for selection for ampicillin resistance in mutated and transformed cells was purchased from Fisher Scientific. Isopropyl β- d-1-thiogalactopyranoside (IPTG) utilized to induce protein expression were purchased from VWR Chemicals. Dialysis membrane, Syringe Filter 0.2 μm Cellulose

Acetate Membrane was obtained from Spectra / Por 1 VWR. Standard RC Tube MWCO: 68 kDa was utilized for dialysis of protein. Urea -99.5 and acetone -99.5 were purchased from Isolab. Imidazole 99% was purchased from Alfa Aesar. Agarose, sodium chloride (NaCl) - 99.5% and ethanol - 99.9 were bought from Sigma Aldrich.

2.1.1 Ser-4mer, Ser-8mer, Vectors, Primers, and Restriction Enzymes

SENTEGEN manufactured the pET-21a(+) reverse and forward primers. NEB provided all restriction enzymes (*HindIII*, *NsiI*, *PstI*, and *EcoRI*), T4 polynucleotide kinase buffer, T4 buffer, 2X Taq master (mix), T4 DNA ligase, T4 polynucleotide kinase enzyme and NEB 3.1 buffer and Calf intestine alkaline phosphatase (*CIP*), CutSmartTM

buffers. 1 kB DNA ladder and 6X purple gel loading dye was obtained from New England Biolabs.

2.1.2 Cells

The Ser-4mer and Ser-12mer gene sequence was cloned using *E. coli* DH5α competent cells. The Ser-4mer and Ser-12mer gene was expressed in *E. coli* BL21 (DE3) plysis competent cells.

2.1.3 Instruments

Incubator (N-Biotek Shaking Incubator Cooling (NB-205LF)), Spectrophotometer (VWR $UV1_{600}$ PC), μDrop Spectrophotometer (Thermo Scientific Multiskan GO), Centrifuge (Gyrozen Multipurpose High-Speed Centrifugate 1580 R), thermal cycler (Thermo Scientific) vertical and horizontal electrophoresis systems (Cleaver Scientific and Bio-Rad Power Pac Basic), water bath (Nuve),were used for this study.

2.2 Method

In the method of this study, first the expression of previously obtained Ser-4mer protein was optimized and its production increased. Next, the gene encoding the Ser-12mer protein was cloned using general molecular biology tools, after which the generated plasmid was tested to confirm that sufficient amount of the protein was expressed. The recombinantly obtained protein was produced and analyzed on a large scale.

2.2.1 Optimization of Ser-4mer Production in *E.coli* BL21 (DE3)

In the previous study, an optimized synthetic vector containing a native sericin repetitive sequence was synthesized by SENTEGEN, and the plasmid called pRT_SRC4 containing the Ser-4mer sequence was transformed into *E. coli* DH5α. The vector pET-21a(+) was digested with the restriction enzymes *EcoRI* and *HindIII,* and the Ser-4mer gene was ligated with the enzyme T4 ligase. The plasmid pET21a_SRC4 constructed by ligation was transformed into the *E. coli* DH5α. (Bostan, F. & Surmeli, N. B. (2020)).

Due to the decrease in expression efficiency of the previously synthesized and generated Ser-4mer protein, several optimization experiments were carried out. To optimize Ser-4mer expression, production with various growth media was tried, induction was started with different optical density (OD_{600}) values, and their expression times were tested many times, pET-21a(+) stability test was performed, and competent cells were renewed. As a result of a series of experiments with all these variables, the conditions with the highest production were determined.

2.2.1.1 Small Scale Expression of Ser-4mer

pET-21a_Ser4 plasmids were isolated from *E. coli* DH5*α* cells using Geneaid PrestoTM plasmid isolation kit according to manufacturer's instruc- tion. Then, plasmids were transformed using the standard method into the 50 µL chemically competent BL21(DE)3 pLysS *E. coli* cells. After transformation, four colonies were selected from Lysogeny broth (LB) agar plates with ampicillin and colonies were individually cultured in 6 mL of LB (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing ampicillin (100 µg/mL) as a starter culture and grown overnight at 37 °C in a 220 rpm shaking incubator. Then cells were transferred (ratio 1:100) into 8 mL of LB medium as a growing culture under the same conditions, and protein expression was induced by adding 0.5 mM IPTG at the early log phase when the optical density 600 nm (OD_{600}) reached 0.5. IPTG is a non-metabolizable analog of galactose and induces the synthesis of β-galactosidase by inactivating the lac repressor in *E. coli Cells* were harvested by centrifugation at 3900 rpm for 30 minutes after 2 hours of protein expression at 37 °C.

Cell pellets were frozen at −80 °C. Samples taken before and after IPTG induction were centrifuged at 15000g for 30 minutes and stored at -20 °C for SDS-PAGE analysis.

2.2.1.2 Optimization of Ser-4mer Expression

A series of experiments were performed to increase and optimize expression levels of Ser-4mer protein. LB, terrific broth (TB), and 2xYT were used as growth media under identical conditions; 0.5 mM IPTG was added to all these growth media when the OD_{600} value was reached 0.5, 0.6, and 0.8 separately. All experimental groups were performed in both 10 mL and 100 mL cell culture volumes containing ampicillin (100 μg/mL). As a new variable, to remove tryptones and peptones in the media, three washes were made with 1X phosphate-buffered saline (PBS) at pH 7.4 (table 2.1). The mentioned 18 variable experimental groups were repeated 3 sets each. Samples were taken before and after IPTG induction with calculating OD_{600} normalization and visualized by 15% SDS-PAGE analysis to compare the expression levels of each. Then cell pellets were dissolved with lysis buffer with 5 mL per g and sonicated. After centrifugation, protein concentrations were determined via Pierce™ BCA Protein Assay Kit for compared with each experimental group according to manufacturer's instructions.

Table 2.1Phosphate Buffer Saline (PBS) components

Phosphate Buffer Saline (PBS)
0.137 M NaCl
0.0027 M KCl
0.01 M Na_2HPO_4
0.0018 M KH_2PO_4

2.2.1.3 Purification of Large-Scale Ser-4mer Expression

Expressing colony was determined as a result of the expression test, and Ser-4mer was expressed in 2 L E. coli BL21 (DE3) culture, as mentioned in the previous part.

In the purification step, the frozen cell pellet was resuspended with 5 times its weight in lysis buffer (pH 8.0). After sonication and centrifugation, the protein was transferred to the supernatant as soluble in the urea medium. The Ni-NTA resin was rotated with the supernatant at room temperature for 1 hour. Using the Ni-NTA column, Ni-NTA was bound to the resin column and washed with 20 mL of wash buffer (pH 8.0). At a wavelength of 280 nm, the UV-Visible spectra of protein attached to the Ni-NTA column were monitored. After the washing step, the Ni-NTA column was filled with 10 mL elution buffer (pH 8.0). Table 2.2 include all buffer components. In the elution buffer, Ser-4mer protein was isolated. Ser4mer protein, which was isolated, was visualized with 15% SDS-PAGE analysis, and BCA analysis was performed to calculate the concentration of purified protein.

Table 2.2 Buffer components of a purification step

Lysis Buffer	**Elution Buffer**	**Wash Buffer**
8 M urea	8 M urea	8 M urea
300 mM NaCl	300 mM NaCl	300 mM NaCl
100 mM $Na_2H_2PO_4$	100 mM $Na_2H_2PO_4$	100 mM $Na_2H_2PO_4$
-	250 mM imidazole	20 mM imidazole

2.2.2 Cloning of Ser-12mer Gene

The repetitive directional ligation (RDL) method was used for the Ser-12mer sequence to be obtained with recombinant DNA techniques by using the previously synthesized Ser-4mer and Ser-8mer sequences. This method; Although the recognition sites of the two enzymes used are different, it was chosen because of the advantage that the sticky ends obtained after cutting are the same.

The RDL method was performed using the enzymes given in figure 2.1 to generate a Ser-12mer sequence by ligation of the Ser-4mer and Ser-8mer sequences. The pRT_SRC4 plasmid has *PstI* and *NsiI* recognition sites. The schematic representation of the cloning methodology of pRT_SRC12 can be seen in the Figure 2.2.

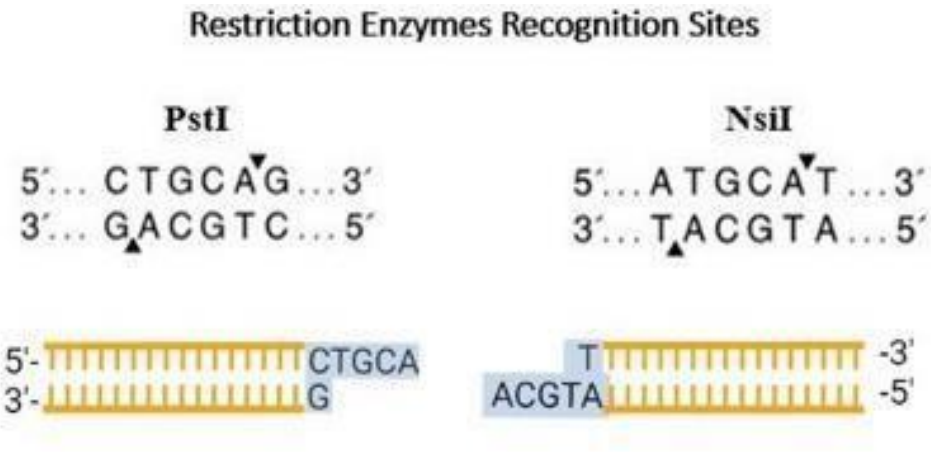

Figure 2.1 *PstI* and *NsiI* restriction Enzymes Recognition Sites

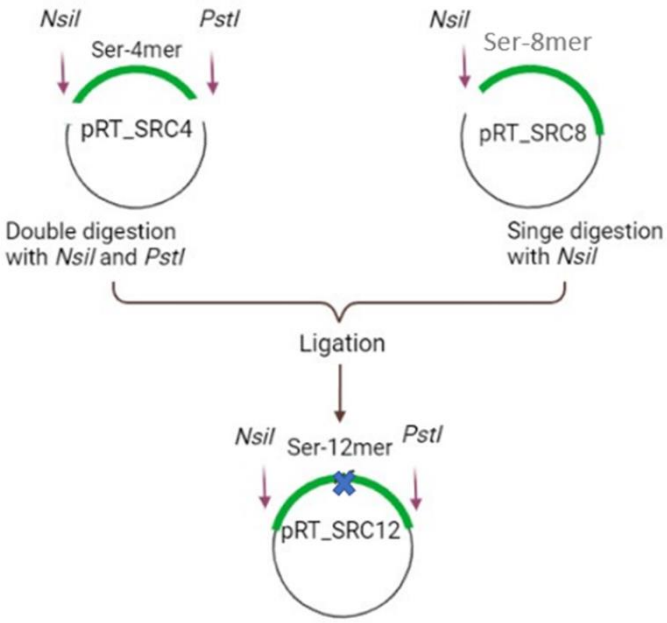

Figure 2.2 Cloning of pRT_SRC12 with RDL method (Created with BioRender)

2.2.2.1 Double Digestion of the pRT_SRC4 Plasmid with *PstI* and *NsiI* Restriction Enzymes

In the first stage of cloning the Ser-12mer gene with the RDL method, it was isolated from the pRT_SRC4 plasmid of the Ser-4mer gene by applying the double cut method using *PstI* and *NsiI* restriction enzymes.

As the first step, pRT_SRC4, which is previously produced recombinantly, is grown into a 6 mL LB medium (20 g/L) with 100 µg/mL ampicillin at 37 °C 220 rpm by shaking incubator overnight. Then, cells were pelleted by centrifuge at 3200 rpm for 30 minutes, and plasmid isolation was performed by applying the plasmid isolation procedure according to the Presto TM Mini Plasmid kit. The concentration of purified plasmid DNA was measured with a µDrop spectrophotometer. In the next step for double digestion with *PstI* and *NsiI* restriction enzymes, 17,239 µg pRT_SRC4 DNA sample was incubated for 3 hours at 37 °C in the dry bath and enzyme mixture shown in Table 2.3. pRT_SRC4 DNA samples were loaded into 1% agarose gel. After the agarose analysis, the agarose gel extraction step was performed to isolate digested Ser-4mer insert from the mixture using the Gel/PCR DNA Extraction Kit protocol. The concentration of DNA

samples was calculated with a μDrop spectrophotometer. After all these steps, the insert was obtained.

Table 2.3 The components of PRT_SRC4 double digestion mixture.

Components	Volume
PRT_SRC4 DNA	45 μL (17.234 μg)
NEB Buffer 3.1 (10X)	6 μL
PstI	2 μL
NsiI	3 μL
ddH_2O	4 μL
Total volume	60 μL

2.2.2.2 Single Digestion of Plasmid pRT_SRC8 with NsiI Restriction Enzyme

In continuing the cloning stages of the Ser-12mer gene by the RDL method, a single digestion method was applied to plasmid pRT_SRC8. The single digestion process was done using the NsiI restriction enzyme.

pRT_SRC8, which was produced recombinantly, is grown into 6 mL LB medium (20 g/L) with 100 μg/mL ampicillin and then incubate at 37 °C 220 rpm by shaking incubator overnight. Growing cells were pelleted by centrifuge at 3200 rpm for 30 minutes, and plasmid isolation was performed by applying the plasmid isolation procedure according to the Presto TM Mini Plasmid kit. The concentration of purified plasmid DNA was measured in a μDrop spectrophotometer.

For single digestion with NsiI restriction enzymes of pRT_SRC8, NEBuffer 3.1 buffer solution was selected over the NEBcloner application, and 12.51 μg pRT_SRC8 DNA sample was incubated for 3 hours at 37 °C. The reaction mixture is indicated in table 2.4. Then, the clean-up procedure was applied to digested pRT_SRC8 to make the reaction mixture enzyme-free using the kit. After the digestion process, the phosphate groups at the 5' ends were cut with the reaction mixture given in table 2.5 so that the

digested plasmid would not become circular again. The dephosphorylation process was carried out at 37 °C for 3 hours. Then the clean-up process was repeated using the kit. The concentration of isolated plasmid DNA was measured in a µDrop spectrophotometer.

The resulting plasmid was used as a vector for cloning the Ser-12Mer protein.

Table 2.4 The components of PRT_SRC8 single digestion mixture.

Components	**Volume**
PRT_SRC4 DNA	45 µL (17.234 µg)
NEB Buffer 3.1 (10X)	6 µL
PstI	2 µL
NsiI	3 µL
ddH_2O	4 µL
Total volume	60 µL

Table 2.5 The components of dephosphorylation of digested PRT_SRC8 DNA.

Components	**Volume**
PRT_SRC8 DNA	18 µL
Cut Smart Buffer (10X)	2.5 µL
CIP enzyme	2,5 µL
ddH_2O	2 µL
Total volume	25 µL

2.2.2.3 Ligation of Isolated Ser-4mer Insert into pRT_SRC8 Plasmid

The Ser-4mer gene was double digested with PstI and NsiI restriction enzymes and then isolated by gel extraction method, used as an insert for Ser-12mer cloning. The

pRT_SRC8 plasmid, which was digested with NsiI restriction enzyme and applied the cleanup procedure described above, followed by the second clean-up procedure with dephosphorylation, was used as a vector for the cloning of the Ser-12mer. The Ser-4mer gene and the plasmid pRT_SRC8 were ligated with the NEB T4 DNA ligase enzyme. The NEB ligation calculation tool was used to determine the optimal ligation, and a 5:1 insert/vector ratio was used. In addition to the insert and vector in the ligation mixture, NEB T4 DNA ligase enzyme and NEB T4 buffer were used, and the reaction mixture is shown in table 2.6. Ligation was conducted at 16°C with a dry bath for 16 hours. In addition, a second ligation mixture containing water instead of the insert was prepared as the negative control. Except for this difference, the amount of the ligation mix is the same and is indicated in table 2.5 and incubated left to incubate for 16 hours at 16°C under the same conditions.

The plasmid was named as pRT_SRC12 containing the Ser-12mer gene obtained after ligation was transformed into chemically competent *E. coli* DH5α cells. For this transformation step, 5 µL of the ligation reaction mix was transformed into 50 µL *E. coli* DH5α competent cells and inoculated into LB agar Petri dishes, and incubated for 16 hours at 37 °C.

Table 2.6 The component of ligation of digested PRT_SRC4 and digested PRT_SRC8

Ligation Components	**Volume**	**Control Components**	**Volume**
İnsert (Prt_SRC4)	3,3 µL	Prt_SRC8	3,3 µL
Vector (Prt_SRC8)	4,7 µL	ddH_2O	4,7 µL
T4 Ligase Buffer (10X)	1 µL	T4 Ligase Buffer(10X)	1 µL
T4 Ligase enzyme	1 µL	T4 Ligase enzyme	1 µL
Total volume	10 µL	Total volume	10 µL

2.2.2.4 Colony Polymerase Chain Reaction (cPCR) for Prt_SRC12

Colony polymerase chain reaction (cPCR) was applied to control the presence of DNA belonging to the Ser-12mer gene in the colonies obtained from the plates after the transformation. The cPCR method quickly and efficiently replicates the multiple cloning site (multiple cloning site) of the pRT_SRC8 vector and is used to check for insert Ser-4mer. For cPCR application, 12 ligations and two control groups were picked from to grown colonies overnight in Petri dishes. These established colonies were dissolved in 100 µL of ultra-pure water. The resuspend colonies were utilized in the cPCR application. cPCR method steps shown in table 2.7 and PCR mixture is given in table 2.8. cPCR was performed with primers designed as forward and reverse M13 primers (Table 2.9). In general, the cPCR procedure created for the Ser-4mer gene was applied precisely. Still, the elongation step was determined as 90 seconds instead of 30 seconds since the designed insert is theoretically expected to be three times longer than the Ser-4mer insert. In addition, the temperature calculation according to the primer used was made with a Thermo fisher calculator, and the annealing step was performed at 53 °C.

Table 2.7 cPcr Method for Prt_SRC12 plasmid

Step	**Temperature, Duration, Number of cycles**
Initial denaturation	95 °C for 10 min (1 cycle)
Denaturation	95 °C for 30 sec (30 cycles)
Annealing	53 °C for 40 sec (30 cycles),
Extension	72 °C for 90 sec (30 cycles)
Final extension	72 °C for 5 min (1 cycle)

Table 2.8 The components of the pRT_SRC12 cPCR mix.

Components	Volume
Cell mixture (colony)	5 µL
2X Taq master (Mix)	7 µL
M13 Forward (10 µM)	1 µL
M13 Reverse (10 µM)	1 µL
Total	14 µL

Table 2.9 The Designed Primers To Amplify pET21a-SRC12s

Name (F/R)	Target-specific primer	Length	GC%	T_m
M13 Forward primer	TGTAAAACGACGGCCAGT	18 bp	50%	63.59°C
M13 Reverse primer	CAGGAAACAGCTATGACC	18 bp	50 %	63.59°C

1% agarose gel electrophoresis was applied to control the genes amplified after cPCR. The size of the Ser-12mer gene, which is expected to be formed after the RDL method, is expected to be three times larger than the Ser-4mer gene. Its weight was determined by agarose gel electrophoresis after cPCR. The colonies, whose ligation was completed successfully after imaging, was grown in 2 mL LB media medium containing 100 µg/ mL ampicillin at 37 °C overnight shaking incubation at 220 rpm, then colonies were stored in -80 °C in the presence of 50% glycerol.

The plasmid DNAs were isolated from overnight cultures using the Presto TM Mini Plasmid kit to validate the proper plasmid DNA sequence. A µDrop spectrophotometer was used to assess the concentration of plasmid DNA extracted.

The base sequence was made by TRIOGEN with the Sanger sequencing method. The Sanger technique is based on the employment of four different forms of 2,-3-dideoxynucleotides (ddATP, ddGTP, ddCTP, ddTTP). If these substances enter into a sequence during synthesis, the absence of oxygen in the OH molecules in the 2nd and 3rd positions of deoxyribose, and the presence of only H in the structures of these substances, inhibits the polymerization. They are unable to generate phosphodiester linkages with nearby molecules. DNA sequencing using a primer designed as forwarding M13 primers was used to determine the correct clones of the recombinant expression plasmid pRT_SRC12. The GENEIOUS R11 program was used to analyze the sequence analysis results.

2.2.3 Cloning of Ser-12mer Gene into Expression Vector

Recombinantly obtained pRT_SRC12 with RDL method and the pET-21a(+) vector was double cut with EcoRI and HindIII enzymes, and ligation was performed using T4 DNA ligase enzyme. The resulting plasmid was named pET21a_SRC12 and transformed into DH5α. Colonies with expected weight on agarose gel after cPCR were sent to sequencing to validate the succession of sequence insertion. Finally, plasmid pET21a_SRC12 was transformed into *E. coli* BL21 (DE3) cells for expression, and all these stages are explained in detail in this section. Figure 2.3 shows the enzymes to be used in the RDL method and formation of pET21a_SRC12 was schematized in Figure2.4

Figure 2.3 Restriction enzymes recognition sites

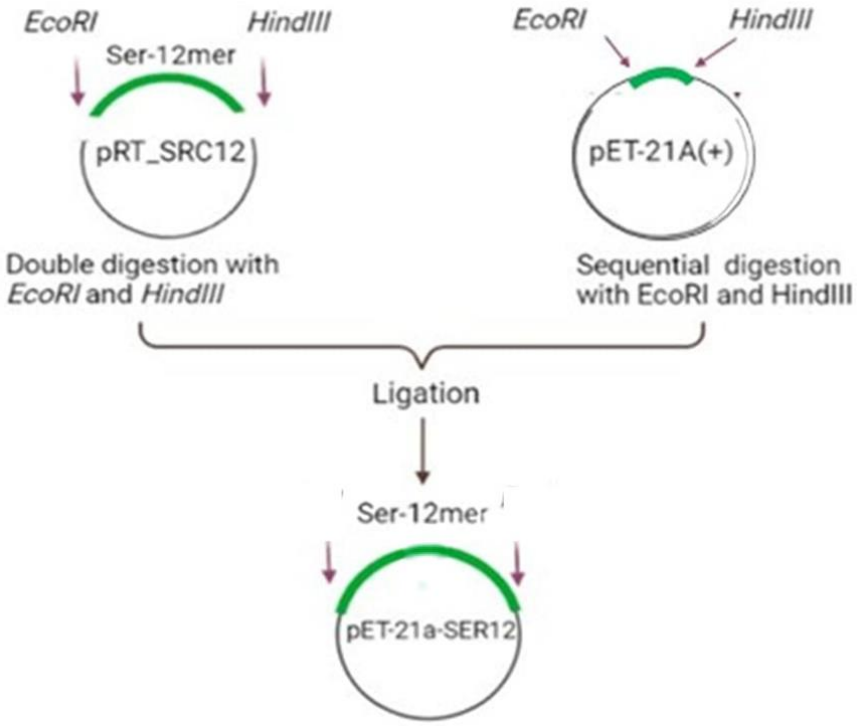

Figure 2.4 Construction of pET21a_SRC12

2.2.3.1 Double Digestion of the pRT_SRC12 Plasmid with *EcoRI* and *HindIII* Restriction Enzymes

Recombinantly obtained pRT_SRC12 was inoculated into 6 mL of LB media include 100 µg/ mL ampicillin. Then, incubated overnight in a shaking incubator at 220 rpm at 37°C. After an overnight incubation period, growing cells were pelleted by centrifugation at 3200 rpm for 30 minutes, and plasmid isolation was carried out using the Presto TM Mini Plasmid kit, followed by concentration measurements in a µDrop spectrophotometer for recombinantly obtained pRT_SRC12.

For the double digestion step of recombinantly generated pRT_SRC12, 14.78 µg of pRT_SRC12 DNA was incubated at 37 °C for 3 hours with *EcoRI* and *HindIII* restrictions enzymes, and the double digestion mixture was given in table 2.10. After digestion, pRT_SRC12 DNA samples were loaded on 1% agarose gel. Following the agarose analysis, the agarose gel extraction was performed using the Genesis gel/PCR DNA Fragments Extraction Kit procedure. The concentration was measured in a µDrop

spectrophotometer after the agarose gel extraction. The insert was obtained once all of these phases had been completed.

Table 2.10 The components of the Ser-12mer double digestion mixture.

Components	Volume
Ser-12mer DNA	48 µL (14.78 µg)
NEB Buffer 3.1 (10X)	6 µL
EcoRI	1 µL
HindIII	1 µL
ddH_2O	4 µL
Total volume	60 µL

2.2.3.2 Sequential Digestion of Pet-21a(+) Plasmid with *EcoRI* and *HindIII* Restriction Enzymes

The pET-21a(+) plysis vector was inoculated into 6 ml of LB media involving 100 µg/ mL ampicillin concentration and left overnight in a shaking incubator at 220 rpm at 37°C. Then, growing cells were pelleted by centrifugation at 3200 rpm for 30 minutes, and plasmid isolation was carried out using the Presto TM Mini Plasmid kit, followed by concentration measurements in a µDrop spectrophotometer for pET-21a(+).

Double digestion of pET-21a(+) was not accomplished with the simultaneous addition of two restriction enzymes like the digestion mentioned in the previous chapter. This is because the restriction enzyme recognition sites on the pET-21a(+) vector are very close to each other. As a result, a sequential method was used in which the plasmid pET-21a(+) was digested with the *EcoRI* restriction enzyme for 2 hours in the first step at 37°C. *HindIII* restriction enzyme was then added to the mixture and digested for an additional hour at 37°C for total digestion of 3 hours. 14.78 µg of pET-21a(+) DNA was added to the sequential digestion and digestion mixture have given in table 2.11

A clean-up kit was used to remove enzymes from digested pET-21a(+). The phosphate groups at the 5' ends of the digested plasmid were cut with the reaction mixture after digestion to prevent the plasmid from becoming circular again. The dephosphorylation was carried out for 3 hours at 37 °C table 2.12. The cleaning process was then repeated with the kit. The concentration of extracted plasmid DNA was determined using a μDrop spectrophotometer. The plasmid that resulted was employed as a vector for cloning the sericin DNAs into the expression plasmid.

Table 2.11 The components of pET-21a(+) digestion mixtures.

Sequential Digestion	
Components	**Volume**
pET21a DNA	48 μL (11.76 μg)
NEB Buffer 3.1 (10X)	6 μL
EcoRI	1 μL
HindIII	1 μL
ddH_2O	4 μL
Total volume	60 μL

Table 2.12 The components of dephosphorylation of digested pET-21a(+)

Components	**Volume**
pET-21a(+)	18 μL
Cut Smart Buffer (10X)	2.5 μL
CIP enzyme	1 μL
ddH_2O	3.5 μL
Total volume	25 μL

2.2.3.3 Ligation of Isolated Prt_SRC12 insert into Sequentially Digested pET-21a(+) Expression Vector

The NEB ligation calculation algorithm was used to compute optimal DNA concentrations for the ligation stage. SRC-12mer DNA and pET-21a(+) were ligated overnight at 16 °C with T4 DNA ligase enzyme. After the ligation, 5 µl of the ligation reaction mix was transformed into 50 µL E. coli DH5α competent cells and inoculated into LB agar Petri dishes, and incubated for 16 hours at 37 °C. The transformation mixture was given in table 2.13.

DNA from the pET21a-SRC12 gene in the colonies after transformation was checked using colony polymerase chain reaction (cPCR).

Table 2.13 The component of ligation of digested Ser-12mer and digested pET21a.

Ligation	**Volume**	**Control**	**Volume**
Insert (Ser-12mer)	3.7 µL (70,57 ng)	Ultra pure water	3.7 µL
Vector (pET-21a(+))	4.3 µL (44.90 ng)	Vector (pET-21a(+))	4.3 µL (44.90 ng)
T4 DNA Ligase Buffer (10X)	1 µL	T4 DNA Ligase Buffer (10X)	1 µL
T4 DNA Ligase enzyme	1 µL	T4 DNA Ligase enzyme	1 µL
Total volume	10 µL	Total volume	10 µL

2.2.3.4 Colony Polymerase Chain Reaction (cPCR) for Pet-21a_Ser12

Selected LB agar Petri colonies were dissolved in 0.1 mL deionized distilled water, and a cPCR was performed. cPCR procedure was applied as given table 2.14. and PCR mixture (table 2.15) prepared with a primer designed as forward and reverse pET-21a(+) primers (Table 2.16).

After cPCR, samples were analyzed using 1% agarose gel electrophoresis, 50 µL of the colonies that show the presence of successfully cloned pET-21a_Ser12 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.

The converted cPCR products were placed onto a 1% agarose gel. Growing cells were grown overnight in a 2 mL LB medium containing 100 µg/mL ampicillin in a shaking incubator at 37 °C 220 rpm. To validate the proper plasmid DNA sequence, the plasmid DNAs were recovered from overnight cultures after centrifuge and using the Presto TM Mini Plasmid kit. µDrop spectrophotometer using for concentration of plasmid DNA was measured .

Table 2.14 cPcr Method for Pet-21a_Ser12 plasmid

Step	**Temperature, Duration, Number of cycles**
initial denaturation	95 °C for 10 min (1 cycle)
Denaturation	95 °C for 30 sec (30 cycles)
Annealing	48 °C for 40 sec (30 cycles),
Extension	72 °C for 90 sec (30 cycles)
final extension	72 °C for 5 min (1 cycle)

Table 2.15 The components of the pET-21a-SRC12 cPCR mix.

Components	**Volume**
Cell mixture (colony)	5 µL
2X Taq master (Mix)	7 µL
pET21A Forward (10 µM)	1 µL
PET21A Reverse (10 µM)	1 µL
Total	14 µL

Table 2.16 The designed primers to amplify pET-21a-SRC12.

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

2.2.4 Expression of Ser-12mer Protein in E. coli BL21 (DE3)

Expression experiments of his-tagged Ser-12mer protein produced recombinantly were performed for the first time. Cloning and expression of the recombinantly obtained Ser-12mer protein have never been attempted in previous studies. Small-scale expression testing was conducted first, subsequently by large-scale expression.

2.2.4.1 Ser-12mer Small Scale Expression

The obtained Ser-12mer was isolated and transformed into an expression vector *E. coli* BL21 (DE3) pLysis vector, and colony growth was performed on LB agar plates with ampicillin.

Ser-12mer was transformed with *E. coli* BL21 (DE3) cells. Colonies that select the pET-21a(+) SRC12 plasmid for the expression assay were chosen from an LB-agar plate. Four colonies were cultivated independently in 8 mL LB medium with 100 µg/mL ampicillin overnight in a shaking incubator at 37 °C 220 rpm. 100 µL of growing colonies were transferred in 10 mL LB media with 100 µg/mL ampicillin at 37°C 220 rpm in a shaking incubator. Protein expression is initiated by adding IPTG to the cells after the OD_{600} reaches 0.5 and protein expression is stimulated by adding 0.5 mM IPTG. Then

cells were incubated at 220 rpm at 37°C for 2 hours by shaking incubator for expression. Expressed cells were harvested after centrifugation at 3800 rpm for 30 minutes. The cell lysate was weighed and frozen at -80°C. Before and after adding IPTG, 1 mL of sample was taken and centrifuged at 15000 rpm for 30 minutes, and the cells pellets were stored at -20°C for 15% SDS-PAGE analysis.

2.2.4.2 Ser-12mer Large Scale Expression

An expressed colony was determined as a result of the expression test, and Ser-12mer was expressed in 2 L volume *E. coli* BL21 (DE3) culture, as mentioned in the previous part.

2.2.5 Optimization of Ser-12mer Protein Expression

The optimization phase includes the addition of 0.5-1% glucose to the medium to maintain plasmid stability, carbenicillin, chloramphenicol with ampicillin for plysis strain, and the plasmid stability test experiments.

2.2.5.1 Optimization with different antibiotics

Colonies were selected from LB agar plate with ampicillin and chloramphenicol and cultured in 8 mL of LB containing ampicillin and chloramphenicol (100 μg/mL) as a starter culture and grown overnight at 37 °C in a 220 rpm shaking incubator. Cell culture transfer to 40 mL LB media (cell: media, 1:100) containing ampicillin and chloramphenicol (100 μg/mL) when OD_{600} reached 0.5, IPTG induction was added to regulate expression. After 2 hours, the cell pellet was harvested with centrifugation. 1 mL samples were taken before and after IPTG induction for 15% SDS-PAGE analysis. A similar experiment was carried out using carbenicillin and glucose.

2.2.6 Purification of Ser-12mer Protein

The SoluLyse™ technique was used to assess the solubility of expressed Ser12mer protein. Small and large-scale protein isolation of histidine-tagged Ser-12mer protein at the C terminus was examined with different volumes and techniques and using Ni-NTA Column. The concentration of isolated Ser-12mer was measured using the Bicinchoninic Acid (BCA) Assay.

2.2.6.1 Small Scale Isolation of Ser-12mer protein

20 mL scale of expression was performed and, according to SoluLyze™ reagent protocol, resuspended the pellet in 150 µl of SoluLyse™ Reagent and rotated the tube 10 minutes at room temperature. Centrifuged at 14,000 rpm for 5 minutes, so the soluble protein was obtained in the supernatant and transferred the supernatant to a clean tube, and resuspended the insoluble fraction in 300 µl SoluLyse™ Reagent.

For HisPurTM Ni-NTA (2:1), SoluLyse™ Reagent was used, and it was washed by centrifuge at 800 xg for 2 minutes. Following centrifugation, the pellet was dissolved in 800 µl SoluLyse™ reagent and centrifugation at 800 xg for 2 minutes was performed twice.

Ser-12mer protein was incubated in the rotator for 1 hour at +4 °C with 540 µL Ni-NTA resin. Ser-4mer-Resin was centrifuged at 800 xg for 2 minutes after passing through the rotator. The pellets were dissolved in 800 µl of SoluLyseTM reagent and centrifuged at 800 g for 2 minutes. The washing procedure was performed three times.

100 mM imidazole was added to the SoluLyse™ reagent during the elution step. The pellets were dissolved in 800 µL SoluLyse™ reagent containing 100 mM imidazole and centrifuged for 3 minutes at 800 xg. The elution procedure was carried out three times. All samples were collected and kept at -80 °C during the washing and elution processes with resin added to the supernatant and pellet. To concentrate the wash and elution samples, the acetone precipitation (4:1) method was used. SDS-PAGE analysis of all collected samples was performed at a concentration of 15%.

In addition, the small scale isolation experiment was applied by dissolving the cell pellet with lysis buffer including 8 M urea, breaking the cell wall by sonication, and

combining the protein that passed into the supernatant by centrifugation with the Ni-NTA, was performed both washing and elution steps were applied with the same protocol.

2.2.6.2 Large Scale Purification of Ser-12mer Protein

Ser-12mer protein was expressed in 1.5 L culture of BL21(DE)3 pLysS *E. coli* cells. Cells were harvested by centrifugation for purification and frozen at -80 °C for more effectiveness. The harvesting cells were resuspended with lysis buffer (pH 8.0) calculated to be 5 ml per gram wet weight, sonicated several times for 30 seconds and at one-minute intervals, and centrifuged at 3900 rpm for 60 minutes. After centrifugation, Ser-12mer protein was obtained in the supernatant in the presence of urea. Ni-NTA column was used to purify the protein. It was calculated as 1 mL of HisPur^{TM} Ni-NTA resin in 8 mL of supernatant and incubated with a rotator for 1 hour at room temperature.

By binding to the HisPur^{TM} Ni-NTA resin column, 20 mL wash buffer (pH 8.0) was loaded into the column and samples were collected until UV visible spectra results at 280 nm were stabilized and remained close to zero. 10 mL of elution buffer (pH 8.0) was put onto the Ni-NTA column following the washing phase. In the elution buffer, Ser-12mer protein was obtained. The UV-Visible spectra of protein attached to the Ni-NTA column were monitored at 280 nm. The isolated Ser-12mer protein was observed using a 15% SDS-PAGE analysis with collected samples between all these steps.

2.2.6.3 The Determination of the Concentration Ser-12mer Protein

Ser-12mer protein concentration was determined via Pierce™ BCA Protein Assay Kit according to manufacturer's instructions. This assay was performed against the known bovine serum albumin (BSA) standard.

According to the BCA procedure, dilution is made in certain proportions to obtain a certain standard. in this way, a control group in the range of 25-2000 µg/mL is obtained. The BCA working reagent consists of reagent a and b (50:1, Reagent A: B). Add 10 µl

each of the control groups and protein samples to the wells. 200 µl of working reagent is then added and mixed. The well plate is covered with aluminum foil and incubated at 37 °C for 30 minutes. It is then incubated at room temperature and absorbance is measured at 562 nm in a plate reader.

2.2.7 Characterization of Ser-12mer Protein

The isolated Ser-12mer protein was dialyzed against water and KPI buffer pH 8 containing 8 M of urea to examine its solubility and structure in water and KPI buffer containing urea with 3,5 kDa and 50 kDa dialyzer membranes.

2.2.7.1 The Dialysis of Ser-12mer Protein against Deionized Water and Phosphate Buffer

Membranes (MW:6-8 kDa) were used for dialysis. Ser-12mer protein was dialyzed against in deionized water at + 4 °C. Besides, Ser-12mer protein was dialyzed against 1 mM phosphate buffer with 8 M urea at pH 8 at + 4 °C. Furthermore, the BCA assay determined the concentration of dialyzed Ser-12mer protein.

2.2.8 Particle Size Analysis

Gel electrophoresis is a technique for sorting charged molecules like DNA and proteins into different sizes. Smaller molecules migrate more quickly across the gel and go further than bigger fragments that move more slowly. Consequently, the molecules are divided according to their sizes (Koontz, 2013).

2.2.8.1 Agarose Gel Electrophores

The success of plasmid creation was demonstrated using agarose gel electrophoresis. TAE buffer is a buffer solution made composed of Tris base, acetic acid, and EDTA that is used to maintain a neutral pH in the DNA solution. As stated in table 2.17, a 1% agarose gel was constructed. SYBR Green attaches to double-stranded DNA's minor groove. It becomes free when the DNA strands are separated. Purple gel loading dye (6X) was used to stain colony PCR samples and a 1 kb DNA ladder (6:1). Following the colony PCR samples, the samples were separated at 100 V for about 30 minutes.

Table 2.17 1% Agarose Gel Components

Agarose Gel
0.3 g Agarose
333 µL 50X TAE
30 mL ddH2O
3 µL SYBR Safe DNA gel strain dye

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

SDS-PAGE was utilized to observe the presence of the Ser-12mer protein band, to compare the expression of the protein, and control the efficiency of isolation and purification steps. For SDS-PAGE analysis, separating gel was prepared (table 2.18) and quickly 400 µL isopropanol was added to avoid oxidation of the gel. The monomer of the gel polymer is acrylamide. Free radical polymerization is used to polymerize it. It has neurotoxic properties. A cross-linking agent is N,N'-methylene-bis (acrylamide). It helps with gelling. SDS denatures proteins and distorts their three-dimensional structure, causing them to become linear and negatively charged. Tetramethylenediamine (TEMED) is a free radical stabilizer. Ammonium persulfate (APS) is a free radical source

that causes gel formation. Glycerol gives the density for examples. Mercaptoethanol aids denaturation and disulfide bond breaking. Then the gel was incubated to polymerize for 30 minutes. The stacking gel was prepared (table 2.18) and incubated during polymerization. Protein samples were prepared by heating 90 °C for 3 minutes with 450 µL 6X loading dye solution (375 mM tris.HCl, , 0.03 percent bromophenol blue, 9% SDS, 50% glycerol) and 50 µL 1 M DTT solution. The gel is filled with 10L protein samples and operated at 30 Volt for 1 hour, then 100 Volt for 2 hours.

Table 2.18 Gels Mixtures for SDS PAGE analysis

Separating Gel	**Stacking Gel**
450 µL ddH_2O	2.100 mL ddH_2O
2.5 mL 30% acrylamide,	500 µL 30% acrylamide
1.950 mL 1M tris (pH 8.8)	380µL 1M tris (pH 6.8)
50 µL 10% SDS	30 µL 10% SDS
50 µL 10% ammonium persulfate (APSs)	30 µL 10% APS
4 µL tetramethylethylenediamine (TEMED)	3 µL TEMED

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Ser-4mer Expression and Optimization

The Ser-4mer gene must be inserted into an expression vector to be expressed. The T7 gene 10/lac promoter controls the expression of the pET-21a(+) expression vector, which has a histidine tag at the C-terminus. Therefore, the pET-21a(+) expression vector provides a tight expression control and a high amount of protein expression. Since there is no Rho termination, the T7 polymerase ensures transcription and translation of the whole gene (Stephen R Fahnestock, Yao, & Bedzyk, 2000).

In bacterial cells, the Rho factor is a protein that mediates transcription termination at certain locations. Furthermore, affinity chromatography with the histidine tag makes it easier to isolate the obtained protein. The expression conditions were optimized by changing different parameters such as growth media, and the cell density at the start of induction of protein expression.

3.1.1 Expression Test of Ser-4mer

Four of the transformed colonies were chosen from the LB agar plate and 8 mL cultures were prepared per colony; protein expression was induced with 0.5 mM IPTG. Two samples were collected from each culture; one before IPTG induction and one after two hours of expression. All collected samples were analyzed using SDS-PAGE to verify protein expression.

In comparison to prior research, SDS-PAGE gel analysis revealed that the protein expression after induction by IPTG was insufficient (figure 3.1) The molecular weight of Ser-4mer is approximately 25 kDa.

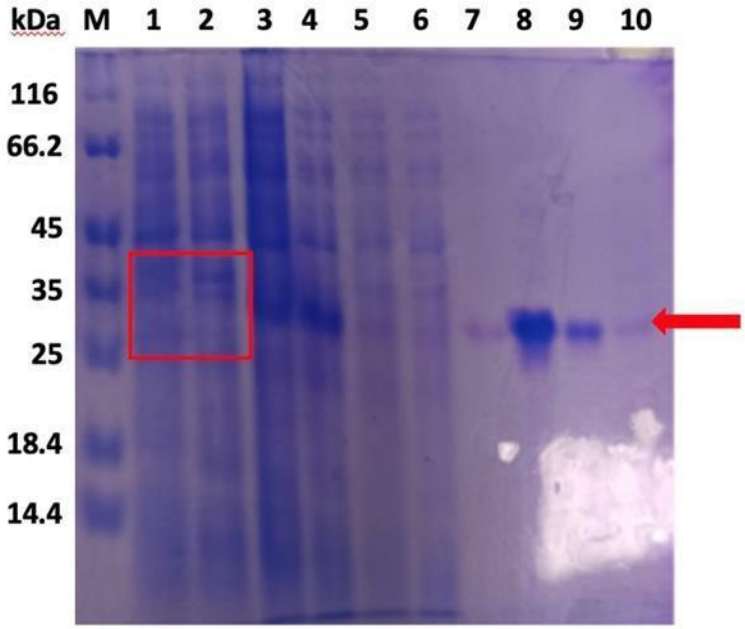

Figure 3.1 15% SDS-PAGE analysis for expression test of ser-4mer protein (M: Unstained Protein MW Marker (Pierce) Line 1- 3- 5: before IPTG induction, 2-4: after IPTG induction. 6: wash step 7: flow-through 8-9-10:elution step. Arrow indicates the expected location of the Ser-4mer protein.

As seen in the SDS-PAGE analysis, the expected increase in the expression band was not observed after IPTG induction. A plasmid stability test was performed to understand the reason for lack of expression. Against the loss of plasmid, the transformation was renewed. The expression vector pET-21a(+) plasmid was isolated from the *E. coli* DH5α cell and subjected to sequence analysis to solve this problem. No mutation was found in the gene as a result of sequence analysis. After sequence analysis pET-21a(+) plasmid was transformed again into *E. coli* BL21(DE3) pLysS bacteria, four selected colonies were tested for expression, and 15% SDS-PAGE gel analysis of expression results are shown in figure 3.2. As a result of this analysis, in these new colonies expression bands expected to be induced by IPTG addition were observed, and the expression problem of the Ser-4mer gene was solved.

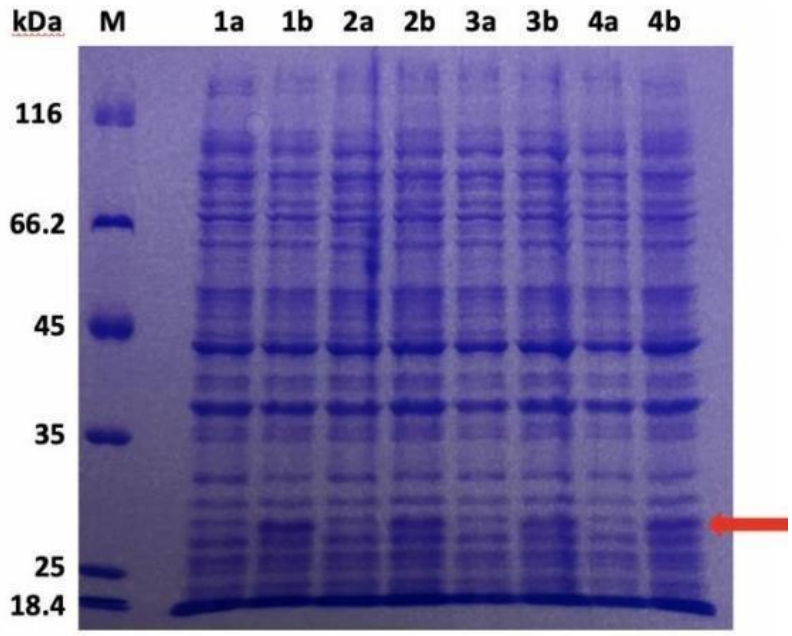

Figure 3.2 15 % SDS-PAGE analysis for expression test of ser-4mer protein. (M: Unstained Protein MW Marker (Pierce) Colonies were numbered 1-4. Label “a” represents before IPTG, Label “b” represents after IPTG induction of the colonies. Arrow indicates the expected location of the Ser-4mer protein.

3.1.2 Effects of Different Growth Media on Ser-4mer protein expression

A series of experiments were carried out to investigate the effect of LB, TB, and 2xYT growth media on Ser-4mer expression. Growth media LB, TB, and 2xYT were produced keeping all other production conditions the same and SDS-PAGE analysis of expression tests are shown at figure 3.3. As a result of this analysis, a slightly more intense expression band was observed in the production with 2xYT and TB medium than in the production with LB medium. However, the changes are not significant enough to draw any conclusions.

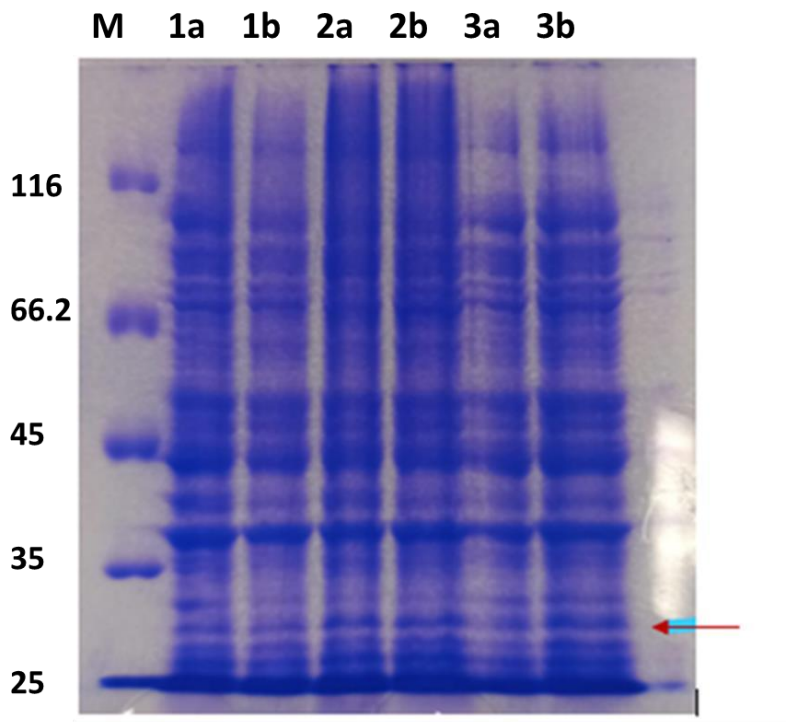

Figure 3.3 15% SDS-PAGE analysis for expression test of ser-4mer protein with different growth media. M: Unstained Protein MW Marker (Pierce) 1: LB medium. 2: 2xYT Medium. 3:TB medium Label "a"represents before IPTG induction. Label "b" represents after IPTG ınduction. Arrow indicates the expected location of the Ser-4mer protein.

3.1.3 Different Cell Densities at the Start Induction of Ser-4mer Expression

The effect of cell density at the induction of expression was tested. The cell density was determined by spectrometer at 600 nm. In this experiment, the difference in expression that will occur with the induction with 0.5 mM IPTG after OD_{600} reaches 0.5, 0.6, and 0.8 values were tested.

An expression test was performed to compare the expression results with different growth media (LB, TB, 2xYT) and different induction initial values of OD_{600} (OD_{600} 0.5, 0.6, 0.8). Then 800 µl the SoluLyse™ Protein Extraction Reagent was added, and BCA analysis was performed to compare the protein concentration obtained for all variables.

These values showed that the highest protein production was when OD_{600} was 0.5, adding IPTG and using 2xYT as a growth medium. Cell growth with the previously used LB medium had the lowest concentration observed. According to these results, the amount of protein increased. The experiments mentioned are diversified and their results are added to the next section.

3.1.4 Small and Middle Scale Production for Optimization of Ser- 4mer

After it was understood that different growth cultures could be important in increasing the production of Ser-4mer protein, the experiments were repeated 3 times for all three media separately (LB, TB, and 2xYT) under the same conditions in 10 ml medium. Protein expression was induced with 0.5 mM IPTG. Two samples were collected from each culture; One was before IPTG induction and the other was IPTG-induced colonies after 2 hours of expression. All collected samples were analyzed using 15% SDS-PAGE (figure 3.4) to compare protein expression levels.

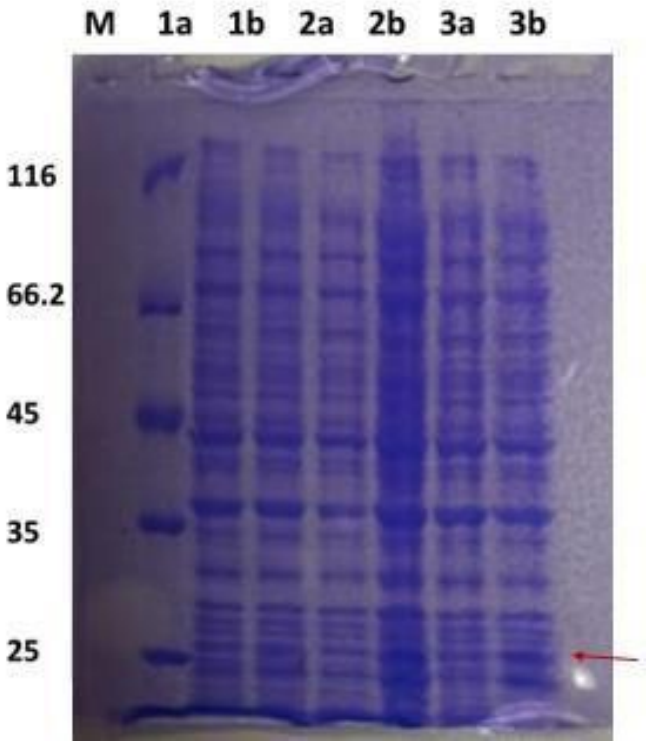

Figure 3.4 15% SDS-PAGE analysis for expression test of ser-4mer protein with different growth media. M: Unstained Protein MW Marker (Pierce) 1: with LB medium. 2: with TB Medium. 3:with 2xYT medium. Label “a” represent before IPTG induction. Label “b” represent after IPTG ınduction Arrow indicates the expected location of the Ser-4mer protein

With this repeated analysis, it was observed again that the expression band of Ser-4mer was slightly more intense in TB and 2xYT mediums than in the LB medium. To further confirm this significant result, medium-scale protein production was carried out again. Protein production in 100 mL volume was made separately for LB, TB, and 2xYT media. The cultures were induced with 0.5 mM IPTG when the OD_{600} value reached 0.5. Samples before and after IPTG were collected for SDS-PAGE analysis.

By the previous experiments on the OD_{600} value, production was started with 3 different media in 100 ml volume. In these experiments, when the OD_{600} measurements reached 0.5, 0.6, and 0.8 values separately, 0.5 mM IPTG induction was added and the expression time was 2 hours. During this whole process, cell growth was monitored by following at OD_{600} nm. The SDS-PAGE analysis of the experiment with different value

inductions of growth cultures is shown in figures 3.5 and 3.6. These results are consistent with the previous observations and it was observed that the expression level was increased when TB and 2xYT were used as a medium. In addition, induction of IPTG adding when OD_{600} value of 0.8 for TB and 2xYT also showed a slightly higher expression level.

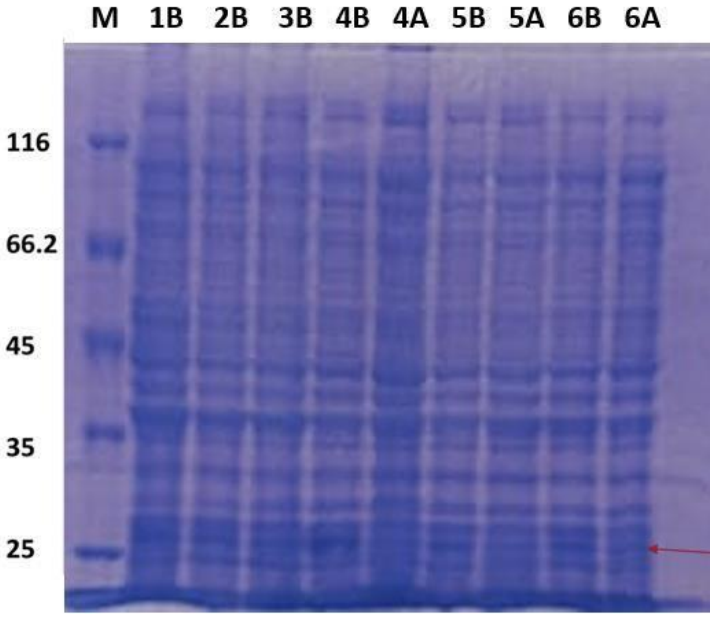

Figure 3.5 15% SDS-PAGE analysis for expression test of ser-4mer protein with TB and LB growth media with different OD600 values of induction. M: Unstained Protein MW Marker (Pierce) 1, 2 and 3 represent: Samples grown in LB Media 1: induction at OD600 0.8, 2: induction at OD600 0.6, 3: induction at OD600 0.5. 4, 5 and 6 represents: Samples grown in TB media. 4: induction at OD600 0.8 medium 5: OD600 0,6 with TB medium 6: OD600 0,5 with TB medium. Label "A" represent: Before IPTG induction. Label "B" represent After IPTG ınduction Arrow indicates the expected location of the Ser-4mer protein

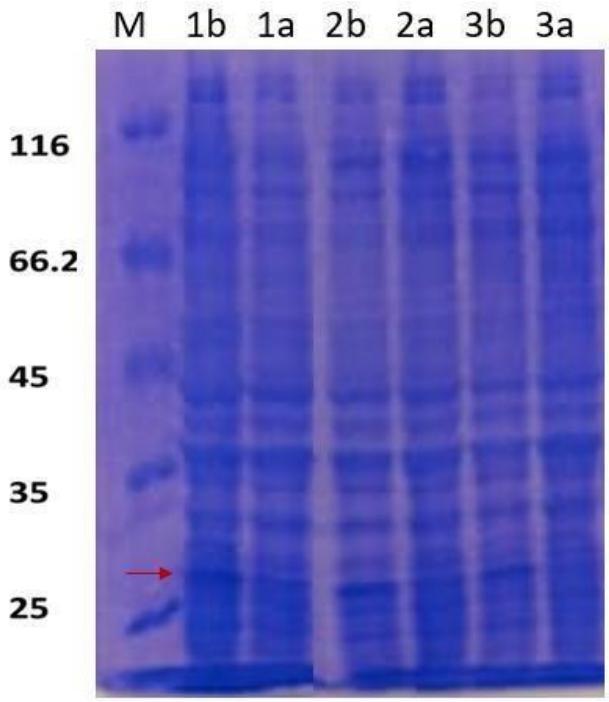

Figure 3.6 15% SDS-PAGE analysis for expression test of ser-4mer protein with 2xYT growth media with different OD_{600} values of induction. M: Unstained Protein MW Marker (Pierce) Label “a” represent: Before IPTG induction. Label “b” represent: After IPTG ınduction 1: OD600 0,8. 2: OD600 0,6. 3: OD600 0,5. Arrow indicates the expected location of the Ser-4mer protein

In addition to all these experiments, three washes with PBS were carried out to eliminate the peptone and trypsin content in different media. After the PBS wash whole cell pellet obtained was dissolved with Lysis buffer and sonicated, protein concentration was determined via Pierce™ BCA Protein Assay Kit according to manufacturer’s instructions.

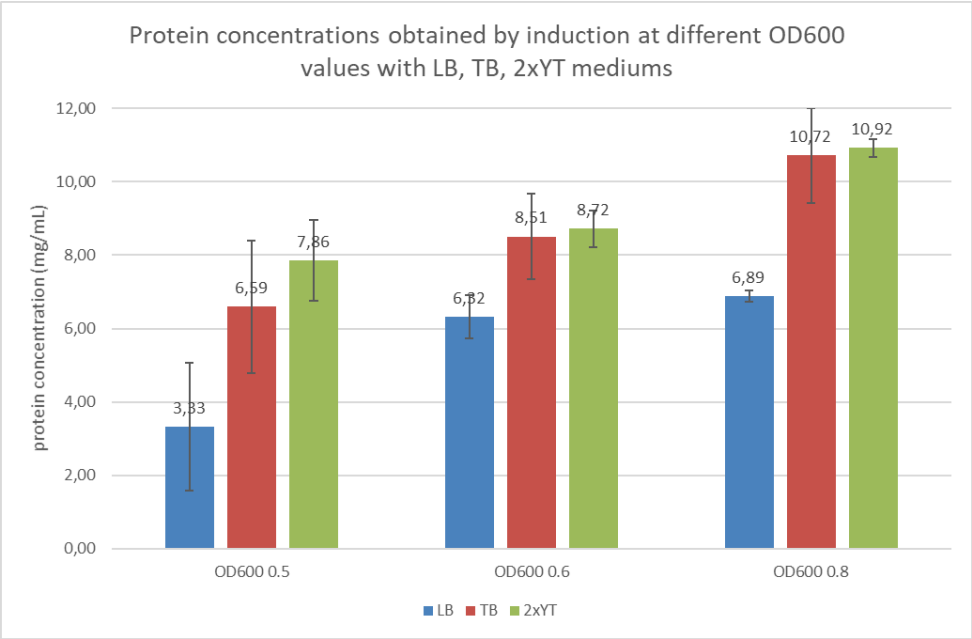

Figure 3.7 Different Cell Densities at the Different Induction. Different growth media and different induction of IPTG when OD600 value reach 0.5, 0.6 and 0.8 with three experimental group for each factor with standart deviation.

Nine different sets of experiments were performed by initiating IPTG induction at three different cell growth values of OD_{600} with three different media. These 9 experimental sets were applied. In addition, each mentioned experiment was repeated 3 times for control purposes.

As seen in the graph (figüre 3.7), when the OD_{600} value was 0.8, IPTG induction significantly increased the total protein concentration in TB and 2xYT media. This is very important for future studies on sericin.

To control cell growth, cell density was measured every half hour during protein expression and the relationship between medium and IPTG addition time was shown with a graph. Representative growth curves are shown in figure 3.8, 3.9, 3.10. The growth experiments were repeated three times all growth curves are included in Appendix E,F,G. As seen in Figure 3.10 highest cell density is observed in 2xYT medium when expression was induced at 0.8 OD_{600} nm which is consistent with all experiment results.

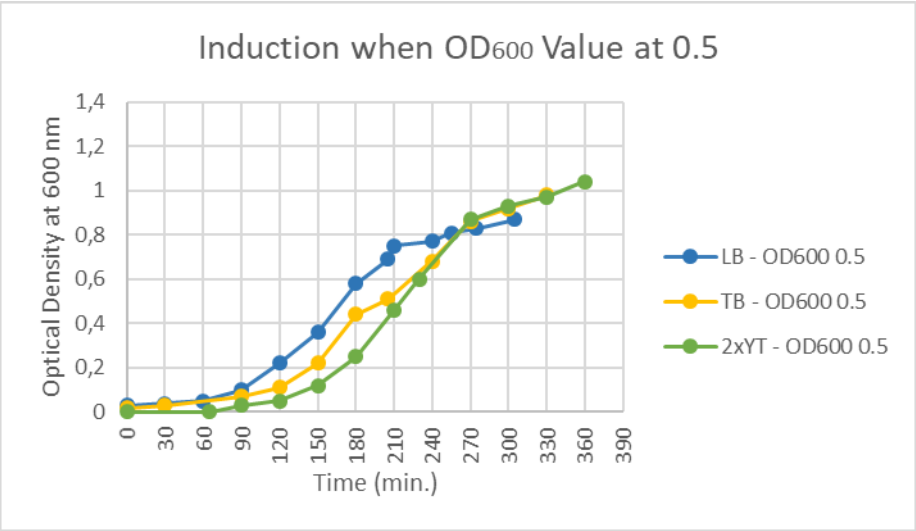

Figure 3.8 Cell Density graphic for induction when OD_{600} value at 0.5

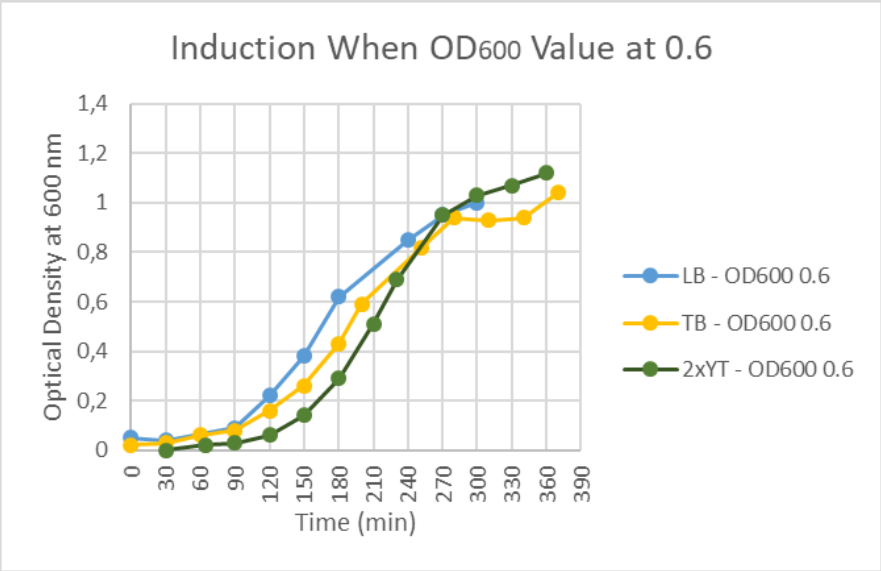

Figure 3.9 Cell Density graphic for induction when OD_{600} value at 0.6

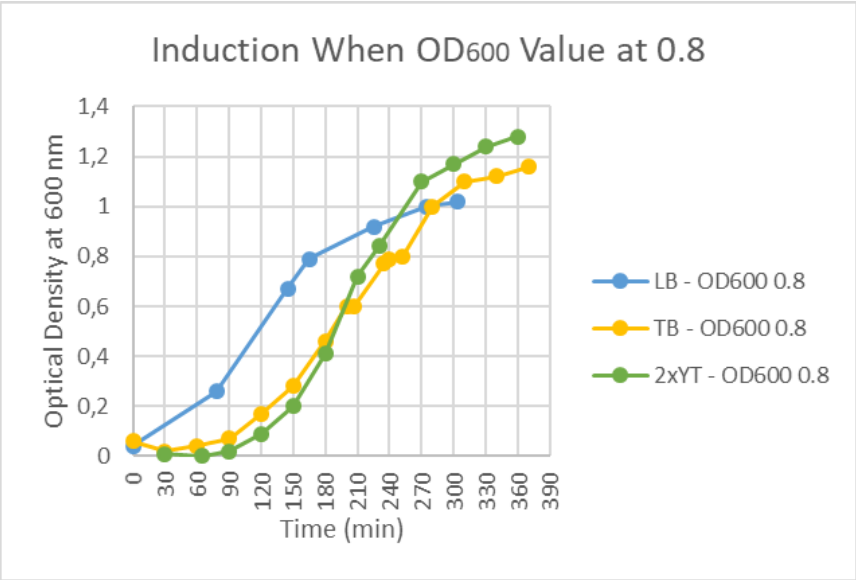

Figure 3.10 Cell Density graphic for induction when OD_{600} value at 0.8

3.1.5 Large Scale Production of Ser-4mer

Optimizations such as stock replenishment and transformation to new BL21 (DE3) *E. coli* cells also increased the amount of Ser-4mer obtained in large-scale production of the protein. In large-scale productions before these changes only 3 mg/mL was obtained, while a protein concentration of 8.72 mg/mL was calculated in the new production. As a result, the amount of protein increased 3 fold.

3.2 Construction of pRT_SRC12

The repetitive directional ligation (RDL) method was used for the Ser-12mer sequence to be obtained with recombinant DNA techniques by using the previously synthesized Ser-4mer and Ser-8mer sequences. RDL technology was specifically designed to build plasmid libraries for repeating polypeptides like sericin and was employed in this investigation. RDL allows for controlled and progressive oligomerization of DNA, as well as the adjustment of desired chain length by copying a repeated number of oligomers from the beginning DNA (Meyer and Chilkoti 2002)

To construct pRT_SRC12 with the RDL method, pRT_SRC4 was double digested with *PstI* and *NsiI*, and pRT_SRC8 was single digested with *NsiI* restriction enzymes and used as vector and insert.

3.2.1 Double Digestion of PRT_SRC4 plasmid

The previously obtained pRT_SCR4 plasmid containing the Ser-4mer gene was double digested with *NsiI* and *PstI* enzymes and observed on 1% Agarose gel electrophoresis in Figure 3.11. Ser-4mer gene is seen at about 0.5 kb, and molecular weight was found to be the same as in previous studies.

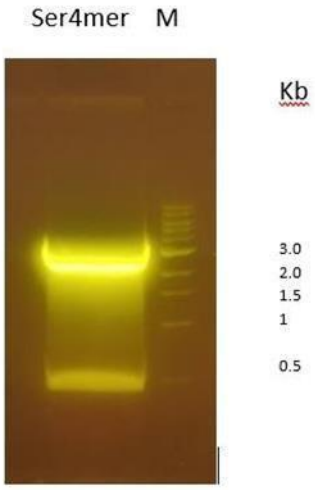

Figure 3.11 1% Agarose gel image of bands including Ser-4mer gene obtained from double digestion. (M: Marker, 1 kb DNA Ladder)

3.2.2 Ligation with pRT_SRC4 and pRT_SRC8

Previously obtained pRT_SCR8 plasmid containing the Ser-8mer gene were single digested with *NsiI* enzyme. Then by applying the clean-up protocol, it was dephosphorylated to ensure that the vector does not re-circularize during ligation. pRT_SCR8 plasmid, which was cut with *NsiI* restriction enzyme and Ser-4mer gene, was ligated with T4 ligase enzyme. pRT_SRC12 plasmid obtained by ligation was transformed into *E. coli* DH5α. After this step, colony PCR was employed to test the

presence of the Ser-12mer gene from transformed pRT_SRC12 colonies and visualized on 1% agarose gel electrophoresis in Figure 3.12.

Since the previously obtained Ser-4mer gene weighed 0.5 kb, after the cPCR, a band of at about 1.5 kb was expected in pRT_SRC12. Figure 3.12 shows in LP3, LP7, LP10 samples of pRT_SRC12 were observed about 1.5 kb. These samples, which were the same size as the predicted size, were sent for sequence analysis after plasmid isolation (appendix A,B).

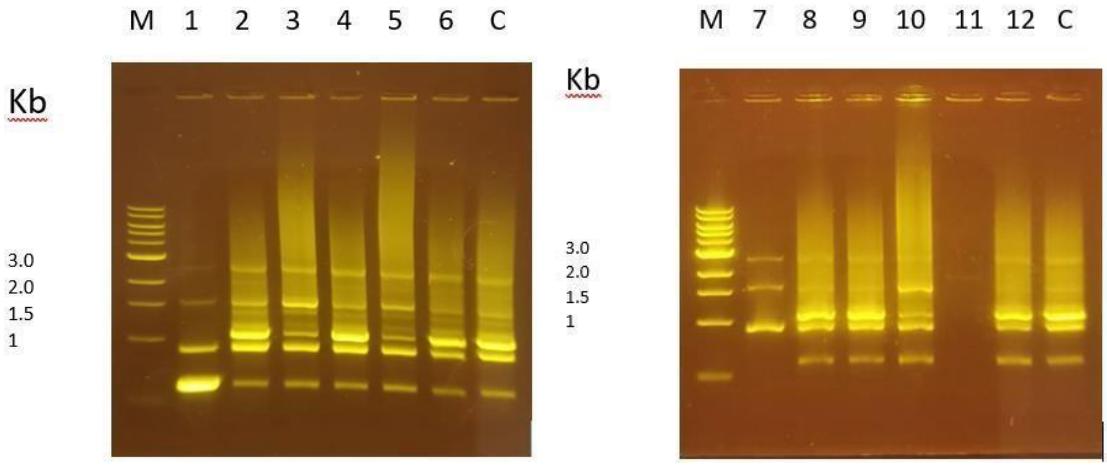

Figure 3.12 Colonies include pRT_SRC12 plasmid after ligation in 1% agarose gel (M: Marker,1 kb DNA Ladder).

3.3 Construction of Ser-12mer Protein

The pET-21a(+) vector, is widely used in cloning and expressing recombinant proteins, since it contains histidine tag for easy purification. The synthesized gene should be transferred into an expression vector in order to achieve large-scale protein expression. The ability of pET-21a(+) to encode for polyhistidine tag (6xHis-tag) at the C terminus of the protein, which will be useful for protein purification by immobilized metal affinity chromatography (IMAC) at a later stage, was one of the reasons for its selection. The capacity to encode T7 expression system and lacI repressor, which will be eased by IPTG induction and change the plasmid into a specific expression system for Ser-12mer protein, is the vector's second advantage. Ser-12mer will also be cloned into pET-21a(+) for expression.

The pET21-a(+) and pRT_SRC12 were double cut with *EcoRI* and *HindIII* enzymes, and ligation was conducted with the T4 DNA ligase enzyme. The plasmid that resulted was given the name pET21a_SRC12 and was transformed into DH5α. After cPCR, colonies that showed expected bands on an agarose gel were submitted to sequencing.

3.3.1 Double digestion of PRT_SRC12 plasmid

The sequencing result of the previously obtained sample LP 7 (Figure 3.12) was noisy. Therefore, to confirm insertion the vectors obtained were double digested with *HindIII* and *EcoRI* enzymes after the plasmid isolation and displayed on Agarose in figure 3.13. As seen in Figure 3.13. LP3 and LP 10 gave a band of 1.5 kDa as expected, but not LP7. This image is consistent with the sequencing results. Sequencing analysis alsı confirmed insertion in LP 10 and LP 3 samples. As a result, plasmid pRT_SRC12 was produced, and the insert was obtained by applying the gel extraction protocol.

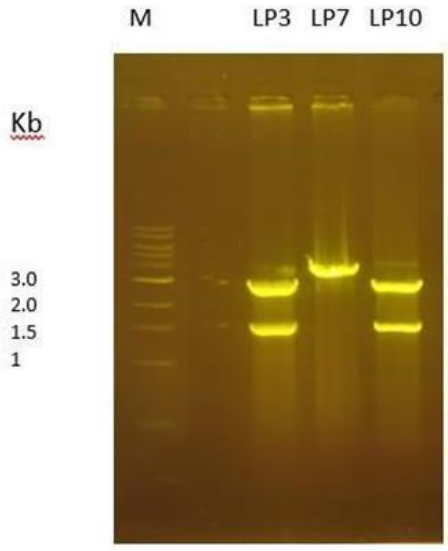

Figure 3.13 1% Agarose gel image of bands including pRT_Ser12 gene obtained from double digestion. (M: Marker, 1 kb DNA Ladder)

3.3.2 Ligation with PRT_SRC12 and Pet21A

The resulting pRT_SRC12 was double cut with *EcoRI* and *HindIII* after plasmid isolation, andloaded on 1% Agarose and showing 1.5 kDa bands were obtained as inserts by gel extraction.

The pET-21a(+) expression vector must be double digested with the *EcoRI* and *HindIII* restriction enzymes. Still, the recognition sites of these two enzymes are very near to each other in the pET-21a(+) expression vector. Fort his reason, each digestion was made separately instead of double digestion as described in the method section. The pET21-a(+) vector was dephosphorylated to prevent it from recirculating during ligation (Bergkessel & Guthrie, 2013). pET21-a(+) vector, which was digested with *EcoRI* and *HindIII* enzymes and pRT_SER12 plasmid, was ligated with T4 ligase enzyme. pET21a_SRC12 plasmid obtained by ligation was transformed into *E. coli* DH5α. After that, colony PCR was utilized to test the presence of the Ser-12mer gene from transformed pET21a_SRC12 colonies and visualized on 1% agarose gel electrophoresis in Figure 3.15.

As shown in Figure 3.14, colonies 1, 2, 3, 4, 5, 9, and 11 of pET21a_SRC12 were observed at about 1.5 kb. Plasmid sample colony 11 was sent for sequence analysis, and the sequence analysis shows that it contains Ser-12mer gene sequence in pET21a_SRC12

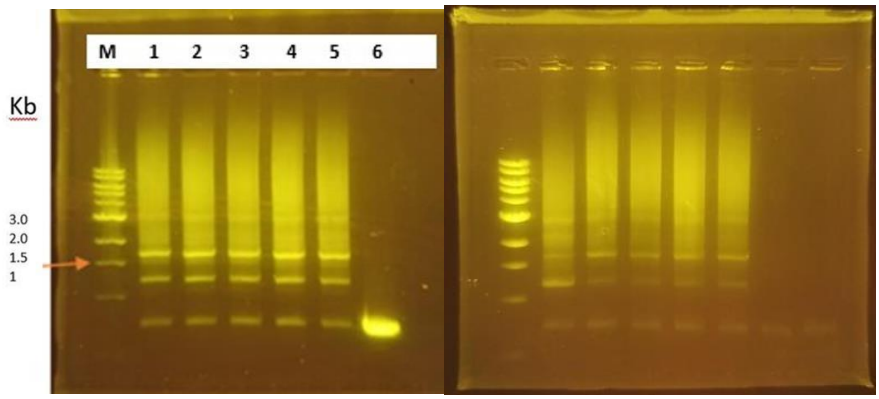

Figure 3.14 Colonies include pET21a_SRC12 plasmid after ligation in 1% agarose gel (M: Marker,1 kb DNA Ladder).

The obtained Ser-12mer samples were double cut with *EcoRI* and *HindIII* enzymes for justification after plasmid isolation, and agarose was also visualized as in figure 3.15 and again weighed 1.5 kDa.

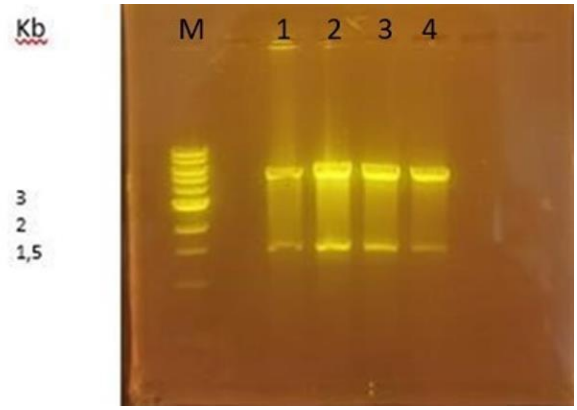

Figure 3.15 1% Agarose gel image of bands including Ser-12mer gene obtained from double digestion. (M: Marker, 1 kb DNA Ladder)

Ser-12mer was transformed into *E. coli* BL21 (DE3), and then plasmid isolation was performed and sent for sequence analysis.

3.4 Expression Test of Ser-12mer protein

The pET21a Ser12 plasmid must be converted into an expression host. Because BL21(DE3) pLysS cells possess the pLysS plasmid, which encodes for T7 lysozyme, these cells' basal metabolism and background production of the target protein are reduced, making them ideal for the development of hazardous proteins. BL21(DE3) pLysS cells were chosen as the protein expression host for this investigation since the sericin protein has antibacterial effects on E. coli cells and may be harmful.

The expression test was performed for the first time for the novel Ser-12mer protein, and samples taken before and after IPTG induction are shown by 15% SDS-PAGE analysis (figure 3.16). The weight of Ser-12mer was estimated to be around 66 kDa. According to the results of this analysis, a new band was observed between 45 kDa and 66 kDa, but the increase after IPTG induction was not sufficient. To increase the

expression level, many experiments were carried out by changing the variables in the production. Amino acid sequence of Ser-12mer given in table 3.1

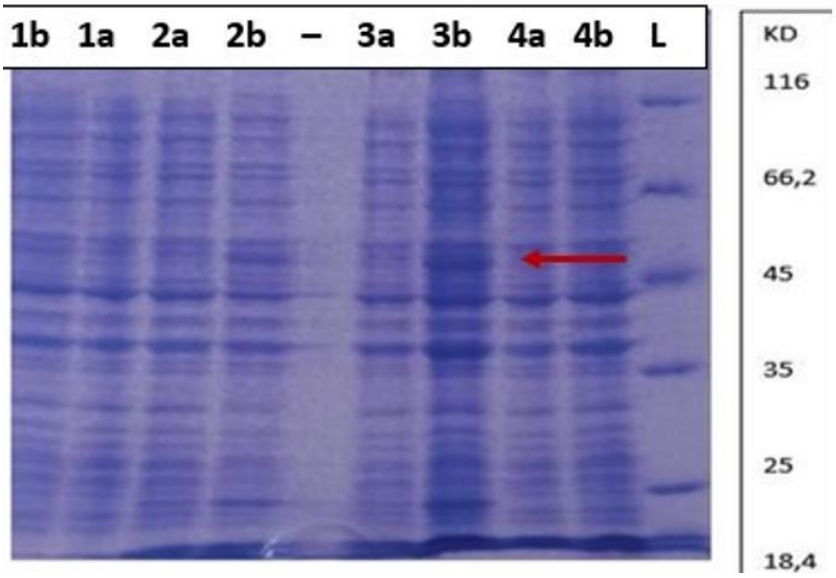

Figure 3.16 SDS-PAGE gel analysis of the expression of Ser-12mer protein (15% SDS-PAGE) L: Unstained Protein MW Marker (Pierce) Label “a” represent: Before IPTG induction. Label “b” represent: After IPTG ınduction. Arrow indicates the expected location of the Ser-12mer protein

Table 3.1 Amino acid sequence of Ser-12mer protein (bold sequence shows the 38 aa repeat sequence, other sequences show 8 repeats of repeat sequence of native protein)

MASMTGGQQMGRGSEFELGTSRMHLDS
VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGR
VSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS
SIGSRARRLHLDS
VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGR
VSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS
SIGSRARRLHLDS
VSSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGS
VSSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGR
VSSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS
SIGSRARRLQRPACKLAAALEHHHHHH

3.5 Optimization of Ser-12mer expression

Any recombinant protein expressed in *E. coli* may interfere with the cell's normal functioning and, therefore, may be toxic to the bacteria. The BL21 (DE3) pLysS competent cells are resistant to chloramphenicol and have tighter control of protein expression for the production of hazardous proteins. For this reason, protein expression previously performed by adding only ampicillin was tested in the presence of both ampicillin and chloramphenicol. Chloramphenicol was added to both solid and liquid mediums for this purpose. This experiment was performed with the best expressing colony in the first expression test. Although the expression level was not at the desired level, a band was seen at approximately 66 kDa in the SDS-PAGE analysis (figüre 3.17)

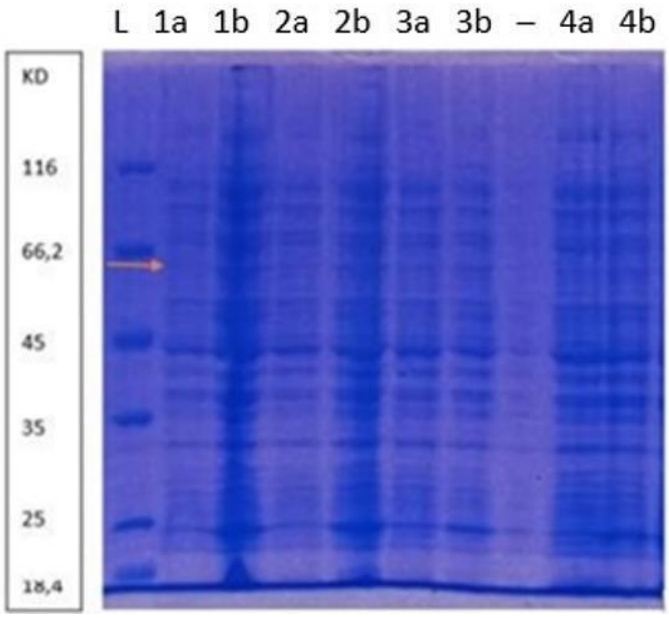

Figure 3.17 15% SDS-PAGE gel analysis for expression test with chloramphenicom. L: Unstained Protein MW Marker (Pierce) Label “a” represent: Before IPTG induction. Label “b” represents: After IPTG induction. Arrow indicates the expected location of the Ser-12mer protein

3.6 Purification of Ser-12mer protein

The histidine tag at the C-terminal of Ser-12mer protein was used in protein purification. The Ser-12mer protein may be insoluble during isolation from bacteria, but the proteins can be made soluble by the denaturation process. Therefore, isolation is performed using urea or guanidine hydrochloride as denaturants. In small-scale protein isolation of Ser-12mer protein containing the histidine tag at its C-terminus was tested with The SoluLyse™ Protein Extraction Reagent and with lysis buffer that contains urea. The SoluLyse™ Protein Extraction Reagent (50 mM phosphate buffer, pH 7.4) is used to extract soluble proteins from bacterial cells and ensures the perforation of the cell wall without denaturing the proteins. Moreover, there is no need for another process such as sonication.

Ser-12mer was isolated by binding to the Ni-NTA resin and this step was followed by SDS-PAGE analysis.

3.6.1 The Small Scale Isolation of Ser-12mer Protein

The protein obtained from the production wherechloramphenicol and ampicillin were used as antibiotics, was used in solid and liquid media. By doubling the volume, expression was performed with a 40 mL cell culture. Also, differently, it was dissolved in elution buffer and subjected to sonication because since the production volume increased, the amount of soluLyze reagent needed to be used increased, and this increased the production cost. Figure 3.18 shows that in elution steps, lanes Ser-12mer protein was observed but not purified more because of the long repetitive sequence. Ser-12mer protein was bound to the Ni-NTA column with his-tag.

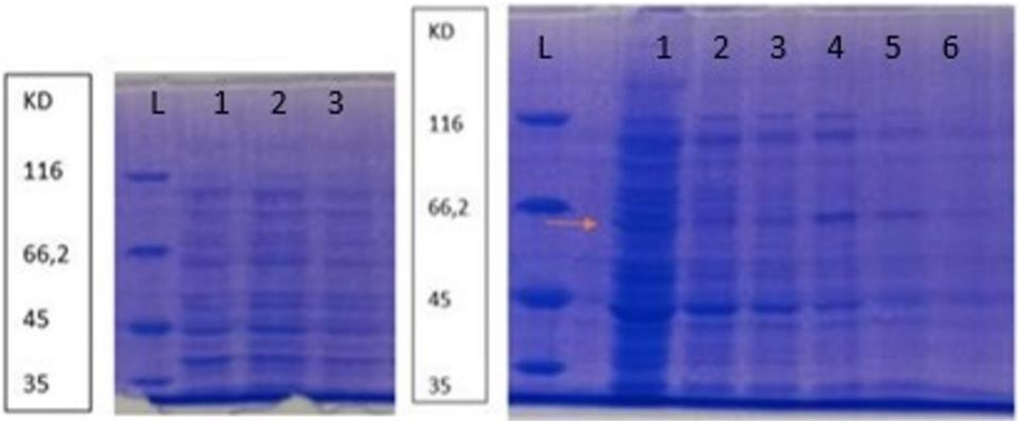

Figure 3.18 15% SDS-PAGE gel analysis for analyze small scale isolation L: Unstained Protein MW Marker (Pierce) Left Gel 1: pellet after sonication 2: supernatant after sonication 3: Flowthrought. Right Gel: 1,2,3: Sample from Wash. 4,5,6: Sample from Elution

3.6.2 The Large Scale Isolation of Ser-12mer Protein

Ser-12mer was expressed in 1.5 L in *E. coli* BL21 (DE3) culture which was grown by adding carbenicillin and gave a band at 66 kDa in small-scale isolation, After protein expression, approximately 7.71 g cells were harvested. Isolation of Ser-12mer protein

was followed by SDS-PAGE analysis in Figure 3.19. Ser-12mer protein was obtained at about 66 kDa as seen in SDS-PAGE. Unfortunately, the sericin produced was not pure. Another band was observed at around 25 kDa which is the weight of Ser-4mer protein.

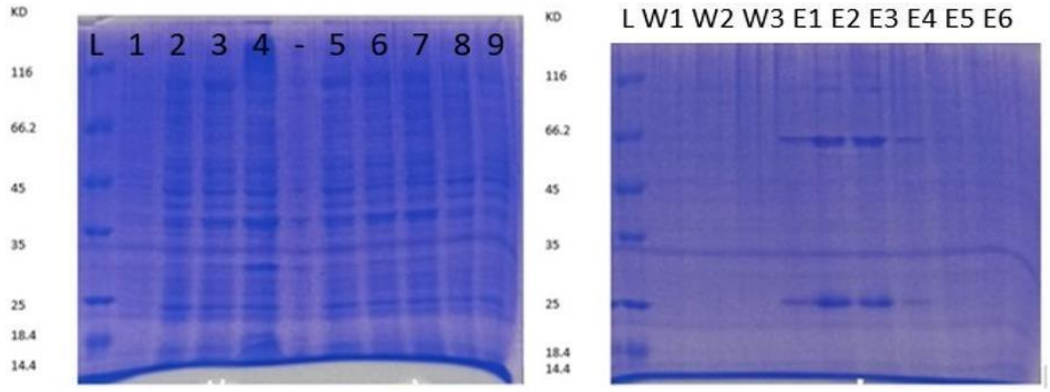

Figure 3.19 SDS-PAGE gel analysis L: Ladder, Line 1-2: before IPTG line3- 4:after IPTG W: wash samples E: elution samples

A new production scheme was carried out to compare the amount of produced protein in the presence of only ampicillin or chloramphenicol and ampicillin-used conditions. This was made with the example that showed the expression band in the expression made for the first expression test in this study. Ser-12mer was expressed in 2 L *E. coli* BL21 (DE3) pLysS cell culture. After expression, a 6.90 g pellet was harvested. Due to the SDS analysis (figüre 3.20), the production of impure Ser-12mer was observed at approximately 66 kDa. The band weights seen are the same as the previous production, which is also positive.

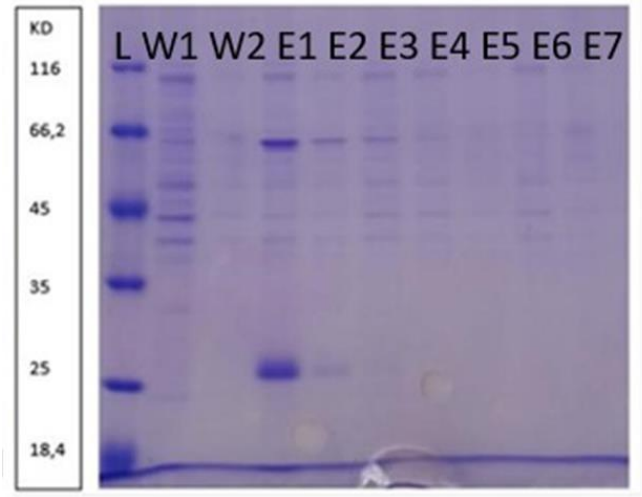

Figure 3.20 %15 SDS - PAGE gel analysis after isolation with ampicillin

3.6.3 Determination of Concentration

The BCA protein assay was used to assess the protein content of isolated Ser-12mer, as stated in the materials and methods section.

The standard curve obtained with the BCA method is shown in Figure 3.21. As a result, 2.83 mg/mL of Ser-12mer protein concentration was obtained from the expression with carbenicillin and ampicillin including 1.5 L cell culture. After the second production, 1.59 mg/mL protein was obtained from 2 L production. Comparing these two results, it was determined that carbenicillin production in solid and liquid mediums yielded an approximately 2 fold increase in protein production. In total, 15 mg of Ser-12mer protein were obtained from a total of 3.5 L cell culture.

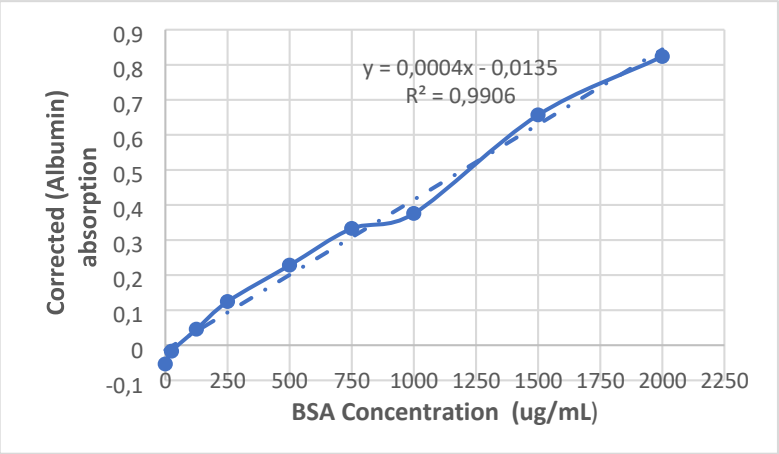

Figure 3.21 Graph of absorbance values obtained against the concentration of Albumin (BSA) standards at 562 nm.

3.7 Amino Acid Composition and Extinction Coefficient of Ser-12mer

In order to have information about the structure of the obtained protein, the protein sequence was analyzed using the ExPASy ProtParam tool and aligned with the Ser-4mer sequence. Additionally, the extinction coefficient of the protein was estimated as 51984.68 M^{-1} cm^{-1}.

When the Table 3.2 is examined, hydrophilic amino acid composition of the Ser-12mer protein is found to be 38.1% serine, 11.6% threonine and 4.5% 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-12mer protein

Amino acid		**Number**	**%**	**Amino acid**		**Number**	**%**
Ala	(A)	20	3.7%	Gln	(Q)	3	0.6%
Arg	(R)	33	6.2%	Glu	(E)	3	0.6%
Asn	(N)	18	3.4%	Gly	(G)	83	16.6%
Asp	(D)	18	3.4%	His	(H)	18	3.4%
Cys	(C)	1	0.2%	Ile	(I)	3	0.6%
Leu	(L)	9	1.7%	Ser	(S)	204	38.1%
Lys	(K)	10	1.9%	Thr	(T)	62	11.6%
Met	(M)	4	0.7%	Trp	(W)	0	0.0%
Phe	(F)	1	0.2%	Tyr	(Y)	24	4.5%
Pro	(P)	1	0.2%	Val	(V)	15	2.8%

3.8 Dialysis

100 µl Ser-12mer protein was dialyzed against in deionized water at room temperature. Besides, Ser-12mer protein was dialyzed against 1 mM phosphate buffer with 8 M urea at pH 8 at room temperature. As shown in figure 3.22, It was observed that the 66 kDa band of the Ser-12mer protein was dialyzed with water. The Ser-12mer protein was insoluble in water, and no protein was observed in the soluble fraction after dialyzsis to buffer as well. Hence the protein was thought to be in the precipitated state after dialysis. Then, the concentrations were checked with BCA analysis. After the dialysis concentrations were 0.409 mg/ml, 0.465 mg/ml, and 3.112 mg/ml, respectively, and high protein concentration in the experiment with urea is related to

protein precipitation. This result is an expected result due to the long DNA sequence of the protein and the protein was insoluble.

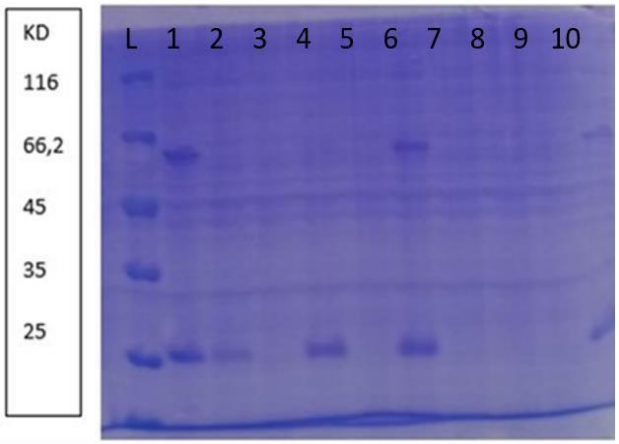

Figure 3.22 15% SDS-PAGE gel analysis L: Ladder. 1: Before dialysis (chl) 2: After dialysis with water 3: buffer sample 4: after dialysis with water 5: buffer sample 6: After dialysis with water 7:buffer 8 after dialysis with urea

CHAPTER 4

CONCLUSION

Sericin is one of the major proteins which make up natural silk, it is useful for wound healing studies due to its fibrillar structure. In this study, firstly, the expression of the sericin-like protein, Ser-4mer, which was produced previously in our laboratory was optimized. As a result, the production of Ser-4mer protein in a TB and 2xYT growth medium and IPTG induction when OD_{600} value at 0.8 increased the protein concentration 3,5 fold. The use of a soluble sericin-like protein as a biomaterial will be greatly expanded thanks to recombinant synthesis Moreover, we cloned recombinantly the native sericin sequence for the first time, encoding twelve repeats of the 38 amino acid repeat motif in *Escherichia coli*. The previously constructed gene for Ser-4mer protein was taken as the basis and the new gene was constructed successfully using recursive directional ligation methodology. The cloned Ser-12mer protein was expressed in *E. coli* BL21(DE3) cells and isolated using immobilized metal affinity chromatography. We also characterized their structural properties by expression and optimization. The base sequence of the Ser-12mer protein is very long and therefore difficult to express in E. coli. The low expression levels achieved are explained by this. It shows that it is insoluble in characterization experiments. Dialysis should be tried at different temperatures and different pH values to increase solubility. The protein was purified to its purest form in the purification step. Difficulties were encountered in the purification step because it contains too many repeat sequences. All obtained results are in agreement with the literature. Cloning studies and experiments will provide ideas for the development of sericin-like proteins with desired composition and molecular weight. The novel sericin protein (Ser-12mer), which will be included in the literature for the first time, is expected to be used in studies in various fields such as biomaterials and biomedicine in the future.

REFERENCES

Altman, G. H, Diaz, F., Jakuba, C., Calabro, T., Horan, R. L., Chen, J., … Kaplan, D. L. (2003). Silk-based biomaterials. Biomaterials, 24(3), 401–416.

Aramwit, P, Kanokpanont, S., Punyarit, P., & Srichana, T. (2009). Effectiveness of inflammatory cytokines induced by sericin compared to sericin in combination with silver sulfadiazine cream on wound healing. Wounds, 21(8), 198.

Aramwit, P, Siritientong, T., & Srichana, T. (2012). Potential applications of silk sericin, a natural protein from textile industry by-products. Waste Management and Research, 30(3), 217–224. https://doi.org/10.1177/0734242X11404733.

Barrere, F, Mahmood, T. A., De Groot, K., & Van Blitterswijk, C. A. (2008). Advanced biomaterials for skeletal tissue regeneration: Instructive and smart functions. Materials Science and Engineering: R: Reports, 59(1–6), 38–71.

Bostan, F, & Surmeli, N. B. (2020). Cloning, expression, and characterization of a novel sericin like protein. Biotechnology and Applied Biochemistry.

Brown, B. N, & Badylak, S. F. (2012). Acta Biomaterialia Expanded applications, shifting paradigms, and an improved understanding of host – biomaterial interactions. Acta Biomaterialia, 1–8. https://doi.org/10.1016/j.actbio.2012.10.025.

Cao, T. T, & Zhang, Y. Q. (2016). Processing and characterization of silk sericin from Bombyx mori and its application in biomaterials and biomedicines. Materials Science and Engineering C, 61, 940–952. https://doi.org/10.1016/j.msec. 2015.12.082.

Cao, T.-T, Wang, Y.-J, & Zhang, Y.-Q. (2013). Effect of strongly alkaline electrolyzed water on silk degumming and the physical properties of the fibroin fiber. PLoS One, 8(6), e65654.

Eslah, S, Tavanai, H, Morshed, M. 2016. Electrospinning and characterization of poly(vinyl alcohol)-sericin nanofibers as a potential for tissue engineering applications, The Journal of The Textile Institute 107, 949-957. http://dx.doi.org/10.1080/00405000.2015.1072328

Fahnestock, Stephen R, Yao, Z, & Bedzyk, L. A. (2000). Microbial production of spider silk proteins. Reviews in Molecular Biotechnology, 74(2), 105– 119.

Freddi, G., Mossotti, R., & Innocenti, R. (2003). Degumming of silk fabric with several proteases, 106, 101–112. https://doi.org/10.1016/j.jbiotec.2003.09.006.

Freddi, G., Mossotti, R., & Innocenti, R. (2003). Degumming of silk fabric with several proteases, 106, 101–112. https://doi.org/10.1016/j.jbiotec.2003.09.006.

Hatakeyama, H. (1996). Biodegradable sericin-containing polyurethane and its production. Japan Patent.

Huang, J., Valluzzi, R., Bini, E., Vernaglia, B., & Kaplan, D. L. (2003). Cloning, Expression, and Assembly of Sericin-like Protein. Journal of Biological Chemistry, 278(46), 46117–46123. https://doi.org/10.1074/jbc.M307792200.

Inthanon, K., Daranarong, D., Techaikool, P., Punyodom, W., Khaniyao, V., Bernstein, A. M., & Wongkham, W. (2016). Biocompatibility Assessment of PLCL-Sericin Copolymer Membranes Using Wharton's Jelly Mesenchymal Stem Cells. Stem Cells International, 2016, 5309484. https://doi.org/10.1155/2016/5309484.

Isobe, T., Ikebata, Y., Onitsuka, T., Wittayarat, M., Sato, Y., Taniguchi, M., & Otoi, T. (2012). Effect of sericin on preimplantation development of bovine embryos cultured individually. Theriogenology, 78(4), 747–752.

Kato, N., Sato, S., Yamanaka, A., Yamada, H., Fuwa, N., & Nomura, M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. Bioscience, Biotechnology and Biochemistry. https://doi.org/10.1271/bbb.62.145.

Kato, N., Sato, S., Yamanaka, A., Yamada, H., Fuwa, N., & Nomura, M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. Bioscience, Biotechnology and Biochemistry. https://doi.org/10.1271/bbb.62.14

Koh, L. D., Cheng, Y., Teng, C. P., Khin, Y. W., Loh, X. J., Tee, S. Y. Han, M. Y. (2015). Structures, mechanical properties and applications of silk fibroin materials. Progress in Polymer Science, 46, 86–110. https://doi.org/10.1016/j.progpolymsci. 2015.02.001.

Koontz, L. (2013). Agarose gel electrophoresis. Methods Enzymol, 529, 35–45.

Kunz, R. I., Brancalhão, R. M. C., Ribeiro, L. D. F. C., & Natali, M. R. M. (2016). Silkworm Sericin: Properties and Biomedical Applications. BioMed Research International, 2016. https://doi.org/10.1155/2016/8175701

Lamboni, L., Gauthier, M., Yang, G., & Wang, Q. (2015a). Silk sericin : A versatile material for tissue engineering and drug delivery. Biotechnology Advances, 33(8), 1855–1867. https://doi.org/10.1016/j.biotechadv.2015.10.014

Lamboni, L., Gauthier, M., Yang, G., & Wang, Q. (2015a). Silk sericin : A versatile material for tissue engineering and drug delivery. Biotechnology Advances, 33(8), 1855–1867. https://doi.org/10.1016/j.biotechadv.2015.10.014

Lamboni, L., Gauthier, M., Yang, G., & Wang, Q. (2015b). Silk sericin: a versatile material for tissue engineering and drug delivery. Biotechnology Advances, 33(8), 1855–1867.

Lloyd, A. W. (2002). Interfacial bioengineering to enhance surface biocompatibility. Medical Device Technology, 13(1), 18–21.

Meyer, Dan E., and Ashutosh Chilkoti. 2002. “Genetically Encoded Synthesis of ProteinBased Polymers with Precisely Specified Molecular Weight and Sequence by Recursive Directional Ligation: Examples from the Elastin-like Polypeptide System.” Biomacromolecules 3 (2): 357–67.

Mori, K., Tanaka, K., Kikuchi, Y., Waga, M., Waga, S., & Mizuno, S. (1995). Production of a chimeric fibroin light-chain polypeptide in a fibroin secretion-deficient naked pupa mutant of the silkworm Bombyx mori. Journal of Molecular Biology, 251(2), 217–228. https://doi.org/10.1006/jmbi.1995.0429

Nayak, S., Talukdar, S., & Kundu, S. C. (2012). Potential of 2D crosslinked sericin membranes with improved biostability for skin tissue engineering. Cell and Tissue Research, 347(3), 783–794.

Neves, N. M., & Reis, R. L. (2016). Biomaterials from Nature for Advanced Devices and Therapies. Biomaterials from Nature for Advanced Devices and Therapies. https://doi.org/10.1002/9781119126218

Padamwar, M N, & Pawar, A. P. (2004). Silk sericin and its applications: A review.

Panilaitis, B., Altman, G. H., Chen, J., Jin, H.-J., Karageorgiou, V., & Kaplan, D. L. (2003). Macrophage responses to silk. Biomaterials, 24(18), 3079– 3085.

Pérez-Rigueiro, J., Elices, M., Llorca, J., & Viney, C. (2001). Tensile properties of silkworm silk obtained by forced silking. Journal of Applied Polymer Science, 82(8), 1928–1935.

Rui, H. G. (1998). Quality of Cocoon Filament. Oxford & IBH Publication Co. Pvt. Ltd., New Delhi, 58-69.

Shen, Y., Johnson, M. A., & Martin, D. C. (1998). Microstructural characterization of Bombyx mori silk fibers. Macromolecules, 31(25), 8857–8864.

Sinohara, H., Asano, Y., & Fukui, A. (1971). Glycopeptides from silk fibroin of Bombyx mori. BBA - General Subjects, 237(2), 273–279. https://doi.org/10.1016/0304- 4165(71)90317-5

Takasu, Y., Yamada, H., & Tsubouchi, K. (2002). Extraction and chromatographic analysis of cocoon sericin of the silkworm, Bombyx mori. Journal of Insect Biotechnology and Sericology, 71(3), 151–156.

Tamada, Y. (1997). Anticoagulant and its production. Japan Patent, 9227402.

Terada, S., Nishimura, T., Sasaki, M., Yamada, H., & Miki, M. (2002). Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. Cytotechnology, 40(1–3), 3–12.

Terada, S., Takada, N., Itoh, K., Saitoh, T., Sasaki, M., & Yamada, H. (2007). Silk protein sericin improves mammalian cell culture. In Cell technology for cell products (pp. 397–401). Springer.

Tsujimoto, K., Takagi, H., Takahashi, M., Yamada, H., & Nakamori, S. (2001). Cryoprotective effect of the serine-rich repetitive sequence in silk protein sericin. The Journal of Biochemistry, 129(6), 979–986.

Vaithanomsat, P., & Kitpreechavanich, V. (2008). Sericin separation from silk degumming wastewater, 59, 129–133. https://doi.org/10.1016/j.seppur.2007.05.039

Vaithanomsat, P., & Kitpreechavanich, V. (2008). Sericin separation from silk degumming wastewater, 59, 129–133. https://doi.org/10.1016/j.seppur.2007.05.039

Vepari, C., & Kaplan, D. L. (2007). Silk as a biomaterial. Progress in Polymer Science (Oxford), 32(8–9), 991–1007. https://doi.org/10.1016/j.progpolymsci.2007.05.013

Zhaorigetu, S., Sasaki, M., Watanabe, H., & KATo, N. (2001). Supplemental silk protein, sericin, suppresses colon tumorigenesis in 1, 2dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. Bioscience, Biotechnology, and Biochemistry, 65(10), 2181–2186.

Zhaorigetu, S., Yanaka, N., Sasaki, M., Watanabe, H., & Kato, N. (2003). Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. Journal of Photochemistry and Photobiology B: Biology, 71(1–3), 11–17.

APPENDICES

APPENDIX A

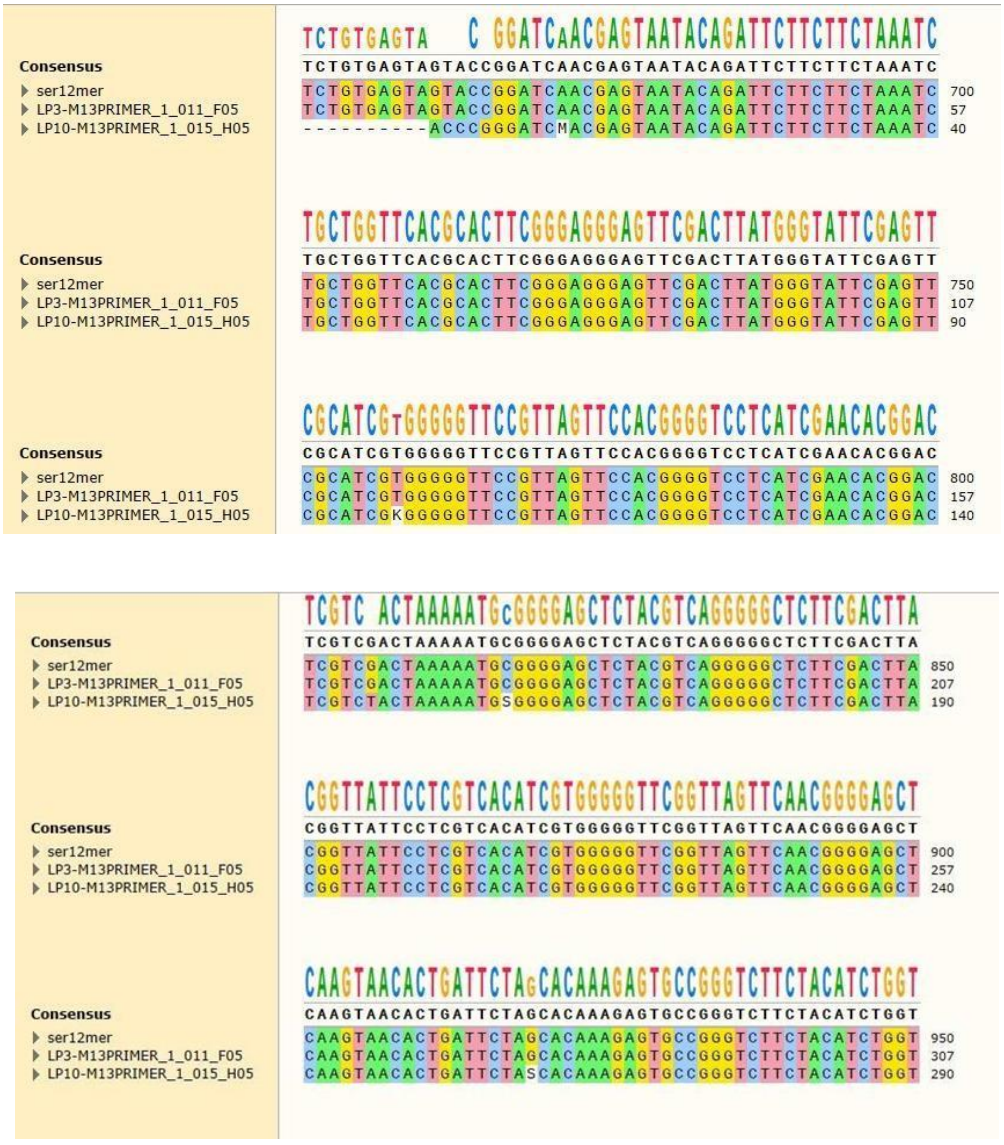

Figure: Comparison of the obtained pRT_SRC12 plasmid with Ser-12mer

APPENDIX B

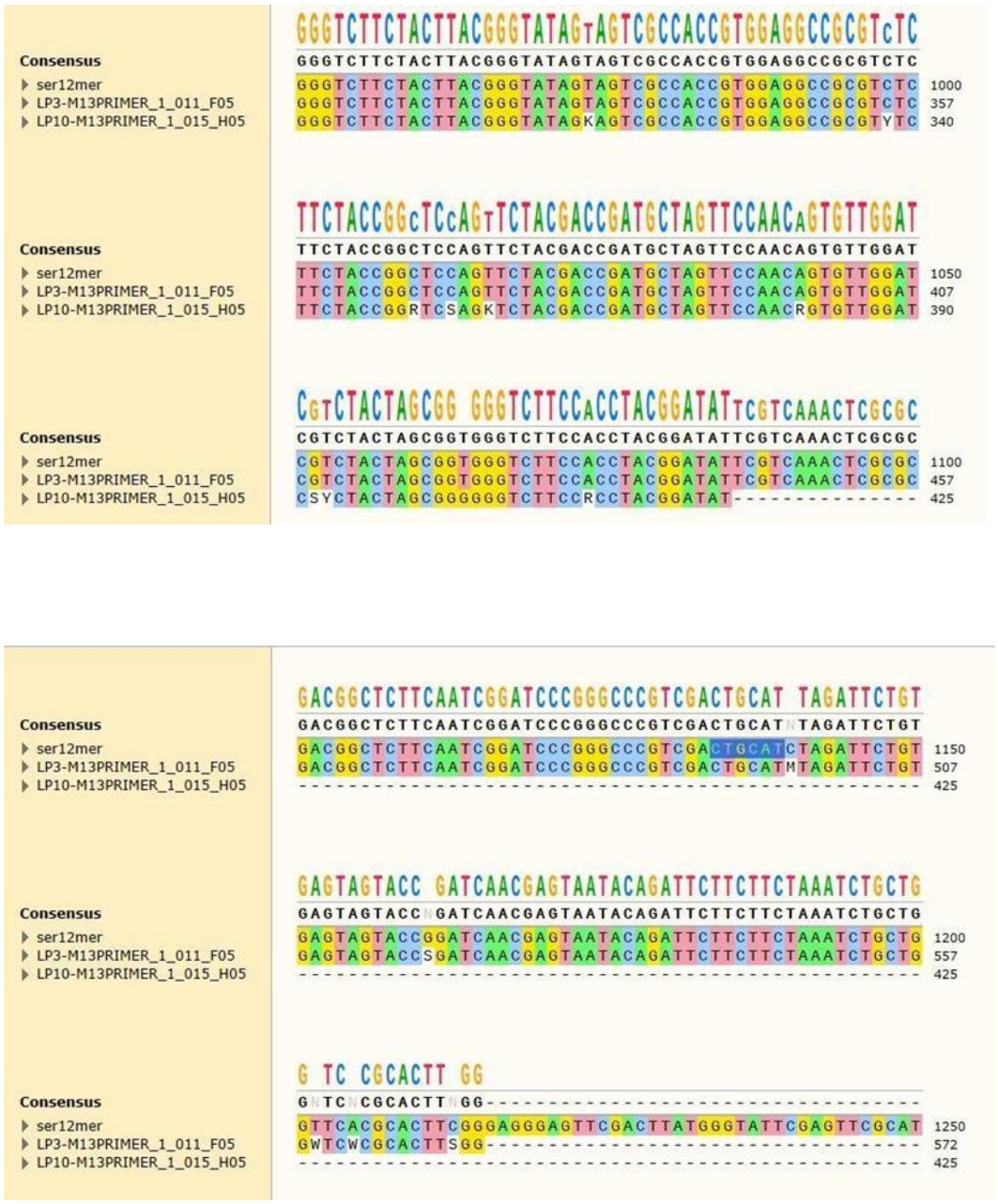

Figure: Comparison of the obtained pRT_SRC12 plasmid with Ser-12mer. Showed the ligation site with blue.

APPENDIX C

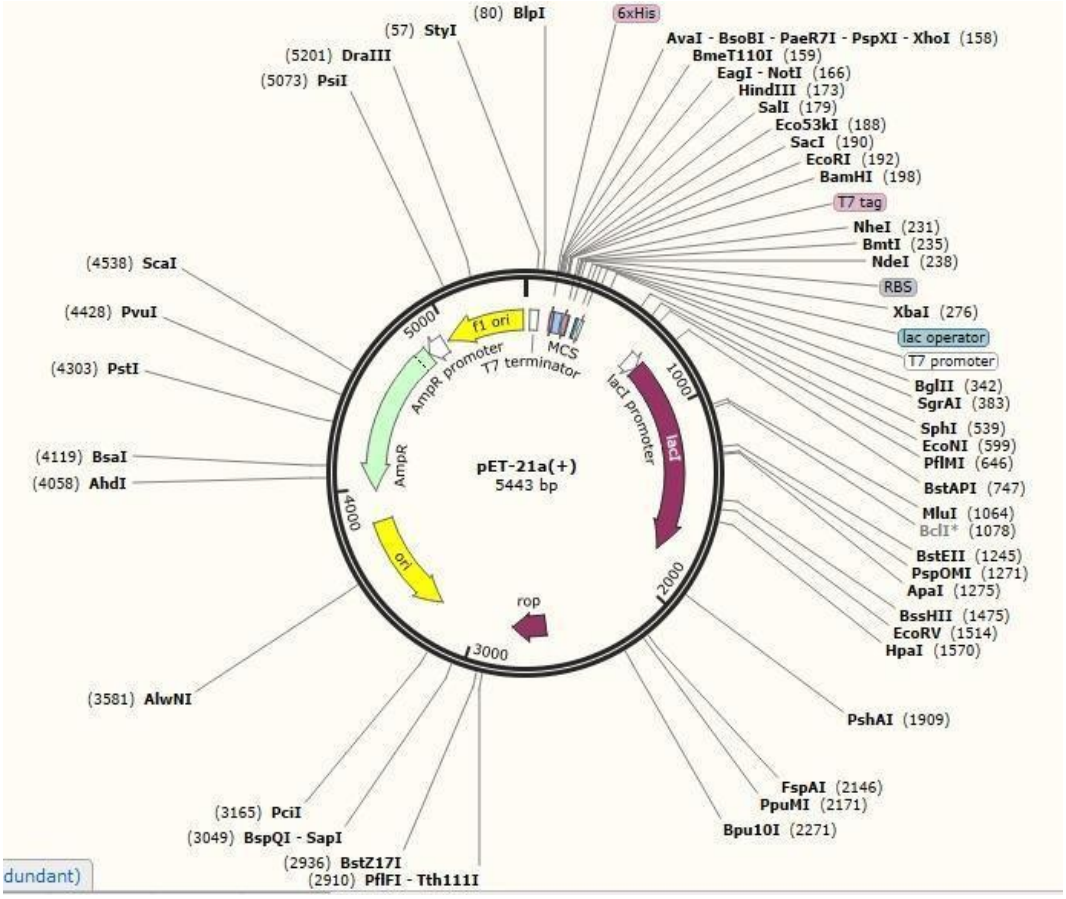

Figure: Pet-21A(+) vector map

APPENDIX D

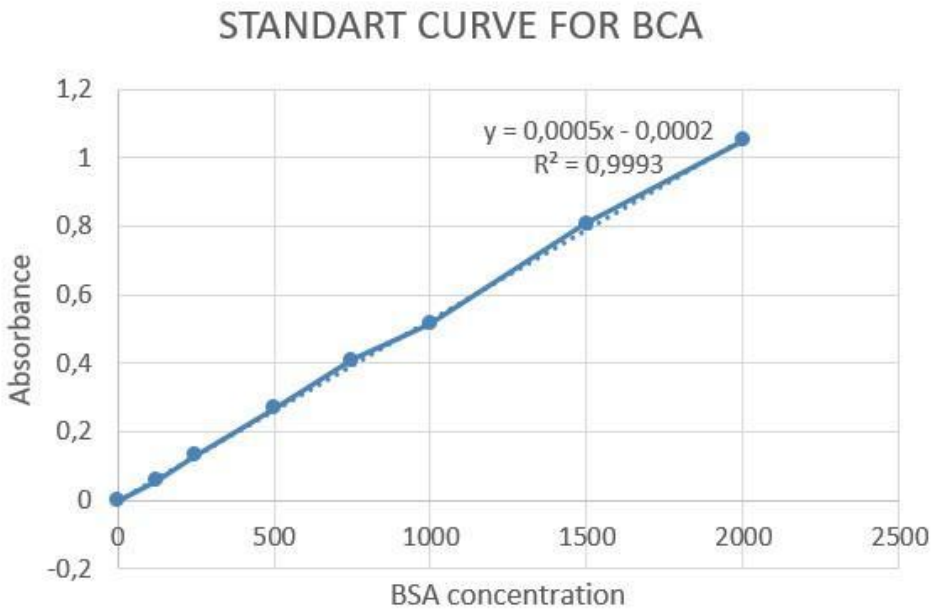

Figure: Standard Curve For BCA Assay

APPENDIX E

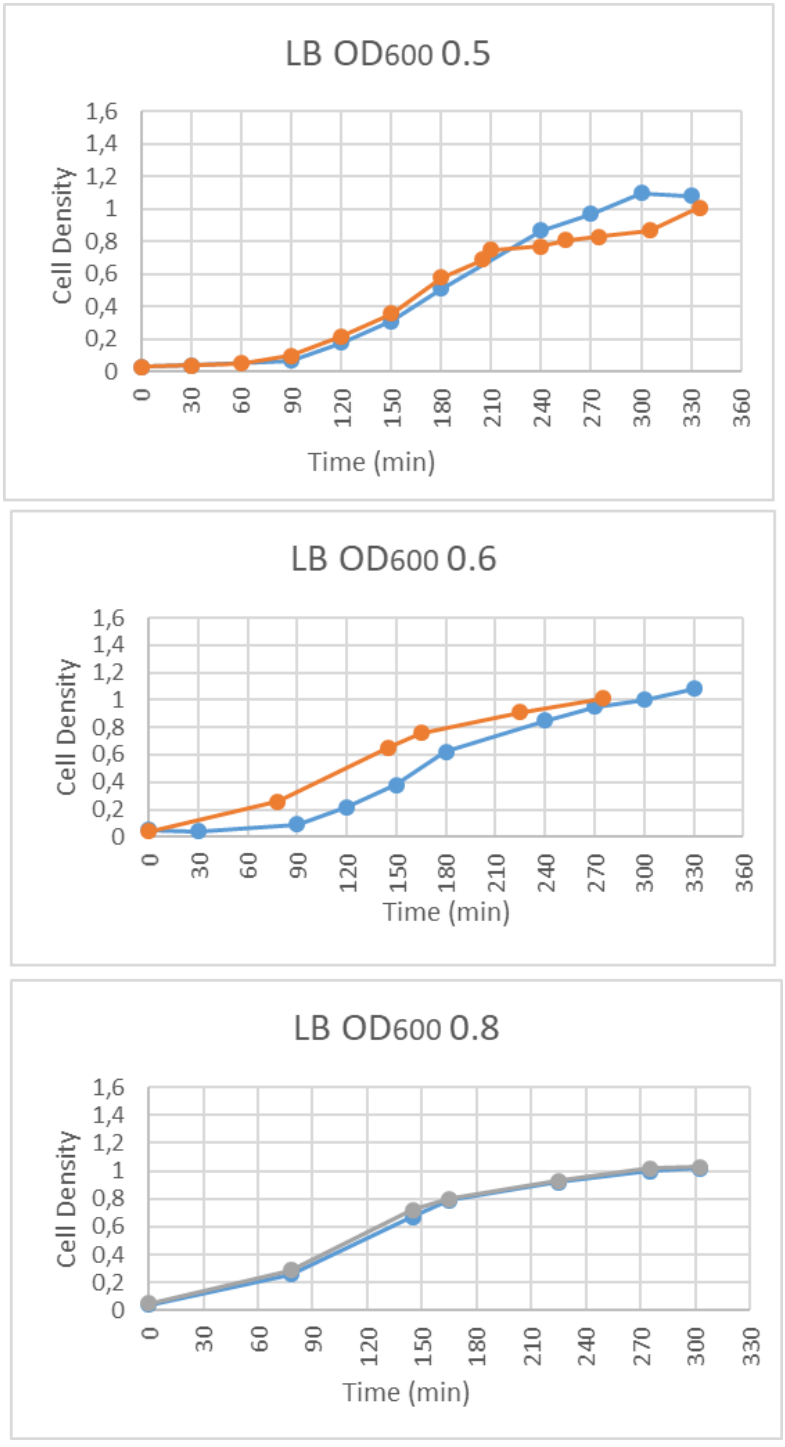

Figure: Cell Density graphics IPTG induction at OD_{600} value reaches 0.5, 0.6 and 0.8 with LB medium with two different production

APPENDIX F

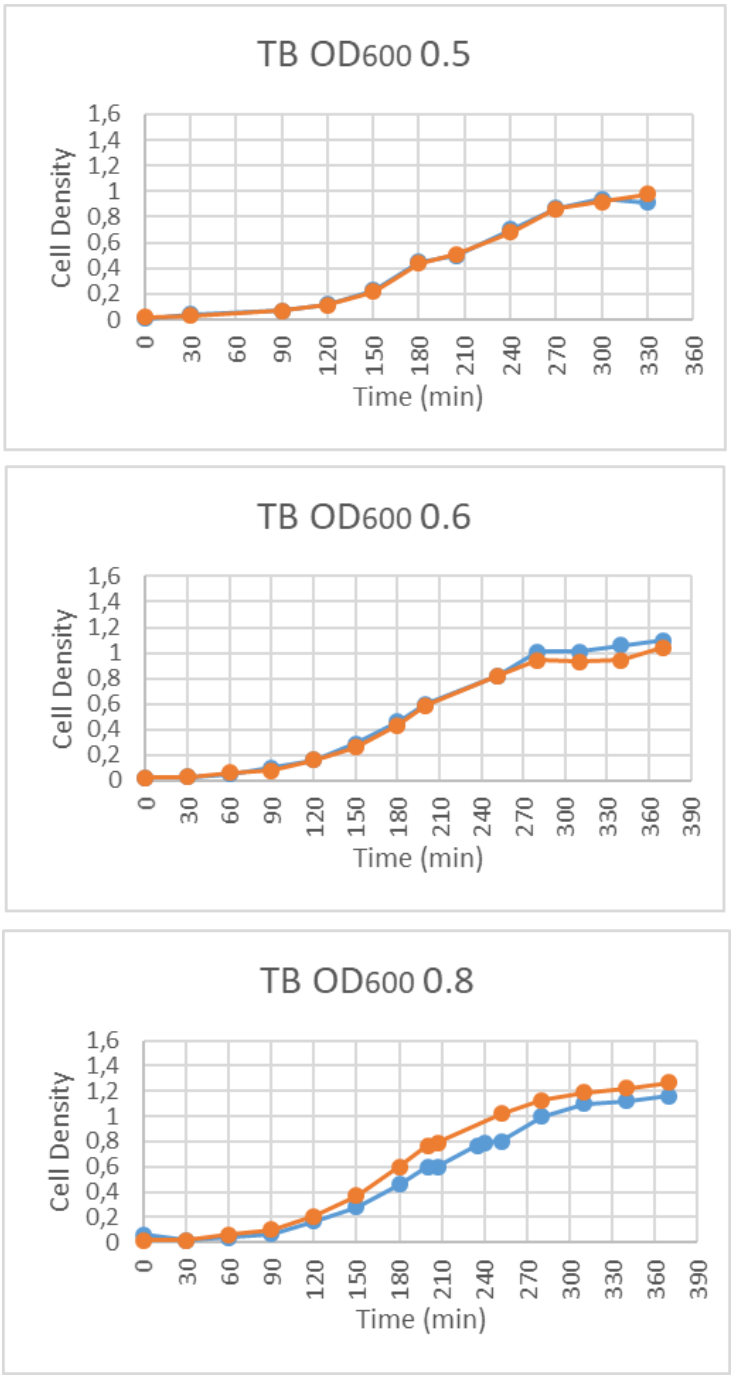

Figure: Cell Density graphics IPTG induction at OD_{600} value reaches 0.5, 0.6 and 0.8 with TB medium with two different production

APPENDIX G

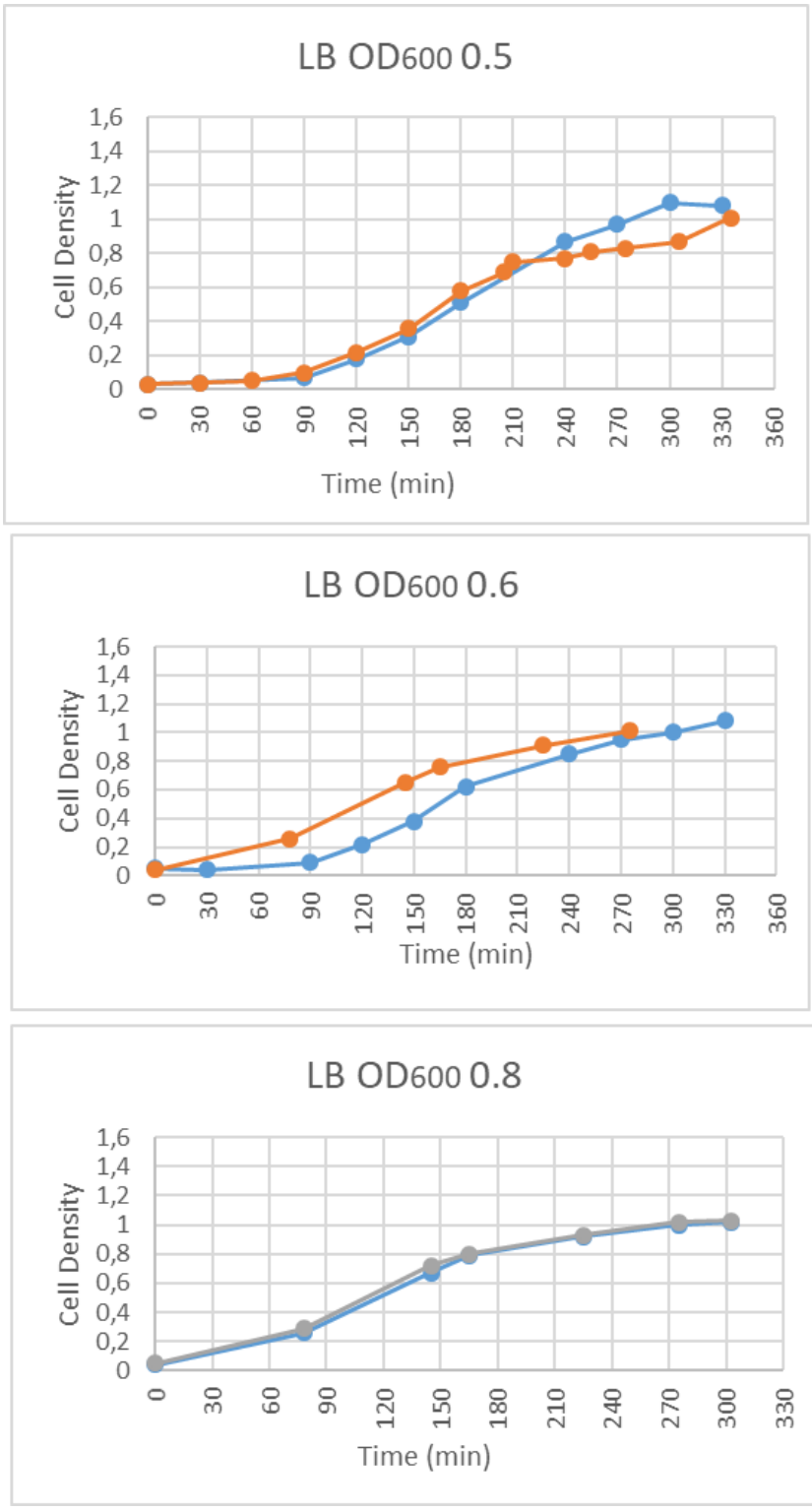

Figure: Cell Density graphics IPTG induction at OD_{600} value reaches 0.5, 0.6 and 0.8 with TB medium with two different production

APPENDIX H

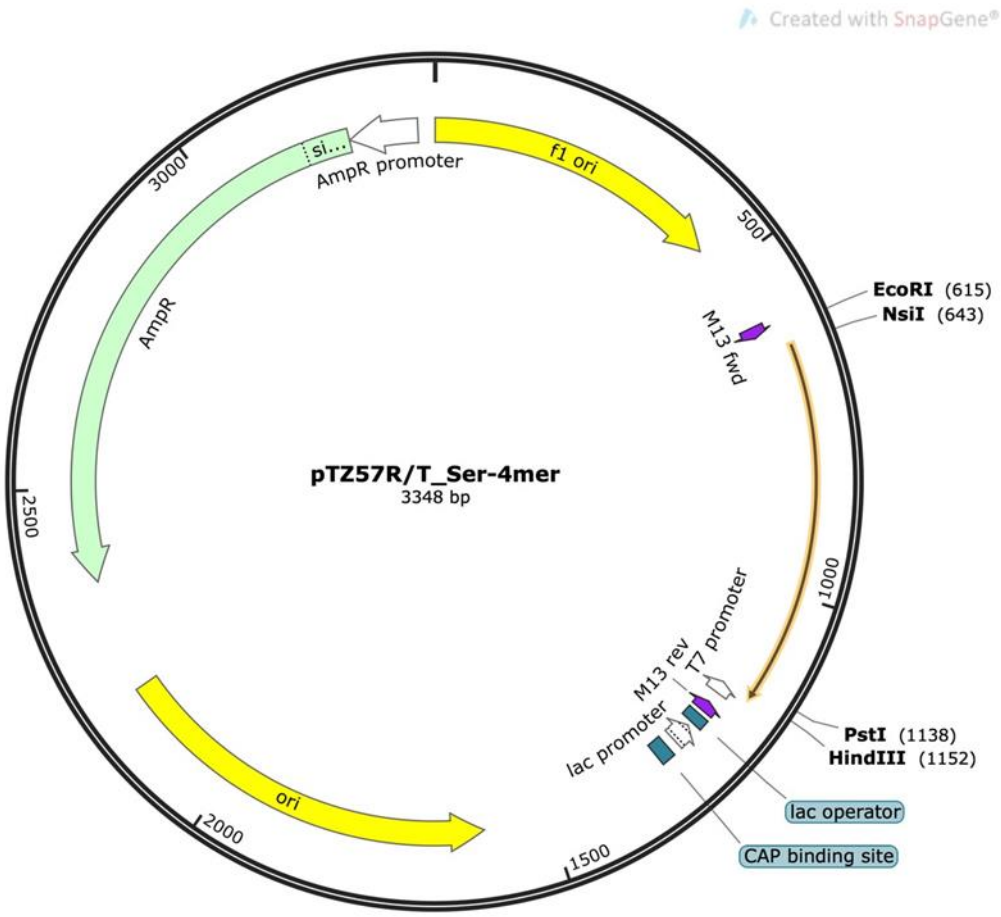

Figure: Plasmid map for Prt_SRC4 which encodes for Ser-4mer (Created with SnapGene)

APPENDIX I

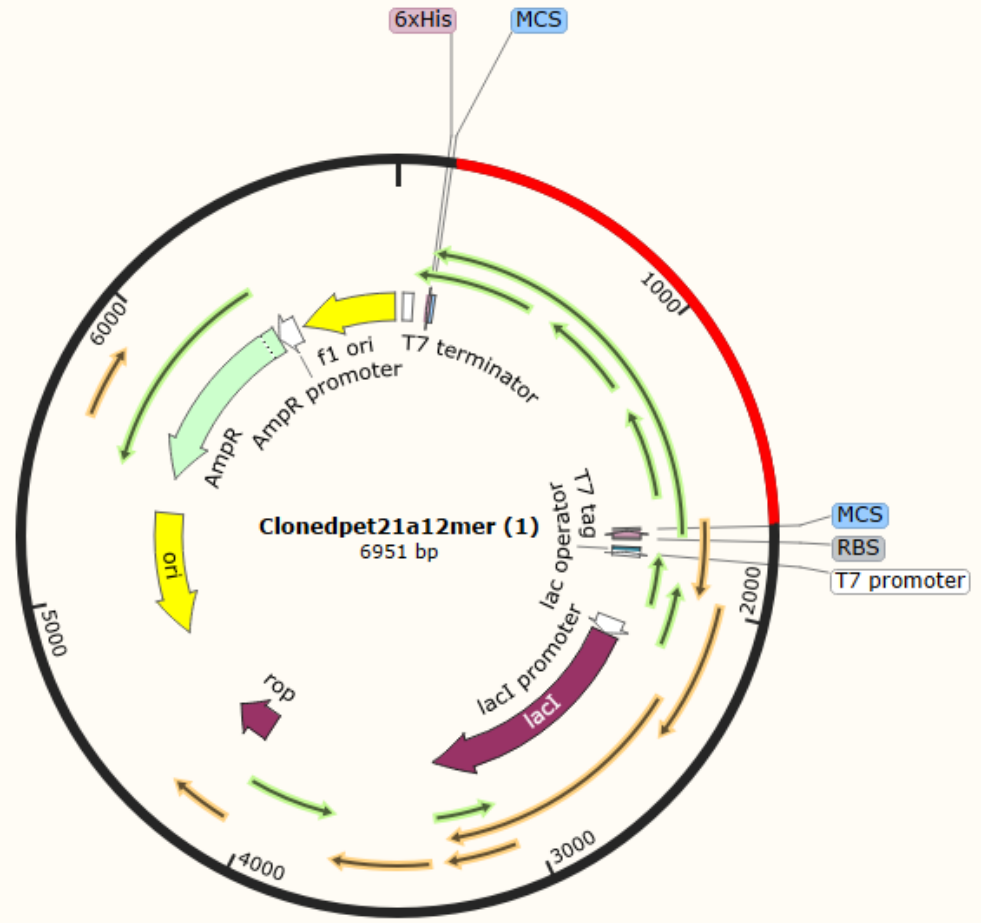

Figure: Plasmid map for Pet-21a-Ser12mer which encodes for Ser-12mer (Created with SnapGene 6.0)