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EXPRESSION SYSTEMS FOR SYNTHETIC SPIDER SILK PROTEIN

PRODUCTION

by

Michaela R. Hugie

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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2019

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ABSTRACT

Expression Systems for Synthetic Spider Silk Protein Production

by

Michaela R. Hugie, Doctor of Philosophy

Utah State University, 2019

Major Professor: Dr. Randolph V. Lewis
Department: Biological Engineering

Spider silk is a biodegradable and biocompatible material that is stronger than steel and more elastic than nylon. These properties make spider silk a desirable material for many commercial avenues, ranging from textiles to biomedical materials. Due to spiders' cannibalistic and territorial nature it is not feasible to farm them to procure spider silk in large quantities. Therefore, a bioengineered synthetic process is necessary to produce spider silk. To date synthetic spider silk has been produced in *E. coli*, goats, yeast, plants, mammalian cells and silkworms, but none of these processes has provided a commercially available product. More specifically, limiting factors include: 1) low purification yields and 2) recombinant spider silk proteins (rSSps) that are much smaller than the natural proteins and create synthetic fibers with mechanical properties inferior to those of natural fibers. The size of the protein is directly correlated to the mechanical characteristics of the resulting fiber.

This research aimed to address the optimization of spider silk production in varying hosts to attain a commercially viable process, while improving the synthetic spider silk's mechanical characteristics through both genetic engineering and expression

techniques. More specifically, efforts were made to increase the yield and improve the mechanical characteristics of the rSSps produced in *Escherichia coli*, *Medicago sativa* (alfalfa) and *Spodoptera frugiperdes* (insect cells). Each host was used to address different needs pertaining to spider silk research: *E. coli* provides a quick production method to produce a large amount of synthetic protein in a relatively short time frame, *M. sativa* may provide a high yield production system that can be used in long-term commercialization, and *S. frugiperdes* enables post-transcriptional modifications, specifically glycosylation, that are necessary to create a synthetic aggregate spider silk with properties that properly mimics the native silk.

In summary, the overall objective of this research was to utilize and improve several synthetic production systems. Improvements were aimed towards a commercialization process for rSSps with mechanical features comparable to native silks. While both yields and properties were improved, further research is necessary to create a commercially viable process and product.

(123 Pages)

PUBLIC ABSTRACT

Expression Systems for Synthetic Spider Silk Protein Production

Michaela R. Hugie

Spider silk is a biodegradable and biocompatible natural material that is stronger than steel and more elastic than nylon. These properties make spider silk a desirable material for many commercial products, ranging from textiles to biomedical materials. Due to spiders' cannibalistic and territorial nature it is impossible to farm them to produce spider silk at a high enough yield to meet product demands. Therefore, a bioengineered synthetic process is necessary to produce spider silk. Synthetic spider silk has been produced in bacteria, goats, yeast, plants, mammalian cells and silkworms, but none of these processes provided a commercially viable yield or were able to express recombinant spider silk proteins (rSSPs) that can mechanically imitate the natural spider silks. The overall goal of this research was to increase the yield and mechanical characteristics, e.g. strength and elasticity, to create a commercially viable spider silk. Three different hosts were used: *E. coli*, alfalfa and an insect cell line. Each host addresses issues with synthetic protein production in both the short-term and long-term scheme. Through this research yields were increased, while the mechanical properties of the synthetic silks were improved and groundwork for future research into the improvement of synthetic spider silk production were identified.

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Overall, I am thankful to all the laboratory members that have cycled through during the course of my doctorate. More specifically undergraduate researchers Cameron Brock, Josh Worley, Bronson Kunzler and Josie Metcalf. My fellow graduate students have also been instrumental in any success that has come of my research, of note being Dr. Thomas Harris, Danielle Gaztambide and Nate Herbert.

Last but not least I am thankful for the continued emotional support of my family and friends. My Grandpa and Grandma Hugie, Fred and LuDean, have always been the source of my motivation and number one fans. Both my parents, Paul Hugie and Carrie Wallace, have stood by and for me throughout this experience, as well as step parents, step grandparents and friends.

Michaela R. Hugie

CONTENTS

	Page
ABSTRACT	iii
PUBLIC ABSTRACT	v
ACKNOWLEDGMENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER	
1. INTRODUCTION	1
References	19
2. IMPROVING SYNTHETIC SPIDER SILK PRODUCTION IN ESCHERICHIA COLI UTILIZING VECTOR ADAPTATIONS	27
Chapter Preface	27
Introduction	29
Materials and Methods	32
Results and Discussion	36
Other Work	42
Conclusion	42
References	43
3. SPIDER SILK PRODUCTION IN AGROBACTERIUM TRANSFORMED MEDICAGO SATIVA	46
Chapter Preface	46
Introduction	49
Materials and Methods	51
Results and Discussion	63
Future Research	66
Conclusion	67
References	69
4. EUKARYOTIC CELL CULTURE PRODUCTION OF GLYCOSYLATED AGGREGATE SPIDER SILK PROTEINS IN SPODOPTERA FRUGIPERDES	71
Chapter Preface	71
Introduction	74
Materials and Methods	76
Results and Discussion	84

Future Research	90
Conclusion	91
References	93
5. ENGINEERING SIGNIFICANCE	95
6. SUMMARY AND CONCLUSION	99
APPENDICES	102

LIST OF TABLES

Table	Page
1-1 Mechanical Properties Comparison of Silks and Various Synthetic and Natural Materials	3
1-2 Synthetic Spider Silk Production Yields and Size in Comparison to Native Spider silk.....	9
2-1 Vector Composition Breakdown	33
3-1 List of rSSp Production Systems.....	49
3-2 List of Tags and Sequences Necessary for <i>Agrobacterium</i> Tumor Inducing Expression Vectors.....	52
4-1 Synthetic Aggregate Spider Silk Proteins Sizes When Glycosylated Versus Non-glycosylated.....	83
5-1 Engineering Significance	98

LIST OF FIGURES

Figure	Page
1-1 Spider Silks and Their Purposes	3
1-2 Schematic of Silk Types and the Nucleotide Motifs Driving Their Secondary Structures.....	6
1-3 History of Synthetic Spider Silk Protein in Transgenic Hosts.....	8
1-4 Potential Synthetic Spider Silk Materials	10
1-5 Diagram Showing the Effect of Spider Silk Protein Size on Mechanical Properties.....	12
2-1 Data Accrued Showing Spider Silk Protein Yields Effect on Cost	30
2-2 Schematic of <i>Escherichia coli</i> Expression Vectors	33
2-3 Nucleotide Sequence for a Synthetic Major Ampullate Spidroin.....	34
2-4 Gel Analysis Confirming Successful Integration of MaSp2 into the pET19SX Vector.....	37
2-5 Western Blot Analyses of MaSp2 Production Via pET19SX in <i>Escherichia coli</i> Expression Assays.....	38
2-6 Western Blot Comparison of MaSp2 Production in pET19k and pET19SX vectors in <i>Escherichia coli</i> Expression Assays	39
2-7 Western Blot Comparison of MaSp2 Production in Varying Expression Vectors in <i>Escherichia coli</i> Expression Assays.....	40
2-8 AKTA Advant Purification Graph of Absorbance Over Time for MaSp2 Production in pET19K and pET19SX in <i>Escherichia coli</i> 5 L Bench Fermentations	41
3-1 N- and C-term Adaptors Nucleotide Sequences	52
3-2 Nucleotide Sequence for a Synthetic Major Ampullate Spidroin with a Conserved C-term Sequence.....	54
3-3 Images of Alfalfa Callus and Boxed Cultivars	56

3-4	Flow Diagram Showing Chlorophyllase Experiments on Transgenic Alfalfa Leaf Purifications.....	62
3-5	Western Blot Analyses of MaSp2 Produced in a Transgenic Alfalfa Plant and Crudely Extracted	63
3-6	Pictures of Chlorophyllase and Precipitation Experiments Conducted on Alfalfa Plants Containing MaSp2 + C-term	65
4-1	Cloning Scheme to Produce a 6X Aggregate Via a Doubling Strategy with 3X Aggregate.....	77
4-2	Cloning Schemes to Produce a 9X and 12X Aggregates Using Compatible Non-Regenerable Sites	78
4-3	Microscopic Images of SF9 Cells Over a Three-Day Expression of 3X Aggregate.....	86
4-4	Coomassie's Conducted on Two Expression Assays of 3X Aggregate in SF9 Cells at Different MOIs Over a Four Day Period.....	87
4-5	Western Blot Analyses of 3X Aggregate Produced in SF9 Cells	88
4-6	Western Blot Analyses of Deglycosylated 3X Aggregate	90

CHAPTER 1

INTRODUCTION

Spider Silk

Since spider silk's remarkable mechanical and biological characteristics were first observed, humans have been attempting to utilize it in varying applications. Spider silk has been shown to be a material that contains not only considerable strength, but is also highly extensible¹⁻³. Spider silk has been proven to be stronger than steel⁴⁻⁶ and as elastic as rubber^{7,8}, but spider silk's mechanical characteristics are not the only novel features it possesses. Even though the term biocompatible was not coined until the 1970s⁹, the characteristic was observed in spider silk thousands of years ago, leading to people harvesting entire orb webs and using them to dress wounds. Spider silk's biocompatibility¹⁰⁻¹³ and biodegradability² characteristics make it an ideal material for many medical materials including: sutures^{14,15}, tendon/ligament replacements^{16,17}, drug delivery devices¹⁸⁻²⁰, and coatings for catheters²¹ to name a few and much like silkworm silk, spider silk has been utilized as a textile on occasions since the 1700's²².

Over the last 450 million years, spiders have evolved specialized silks for a variety of purposes^{23,24}. Research has focused on orb weaving spiders, specifically *Nephila clavipes* of the Araneidae family, rather than non-orb weaving spiders, who lack fiber versatility⁴. *N. clavipes* has six different types of silk and one glue: aciniform, major ampullate, minor ampullate, piriform, tubiliform, flagelliform and aggregate⁵ (Figure 1-1). Each of these silk proteins mechanical properties varies according to their function within the orb web and may have more than one protein forming them^{5,25-27}. Major

ampullate silk, more commonly known as dragline silk, has a tensile strength that is comparable to Kevlar ©^{4,5,28}, while flagelliform silk has a strain higher than a human ligament combined with a toughness greater than rubber and the ability to elongate to more than 200% its original size while being able to recover back to its original length^{7,8} (Table 1.1). While we know that native spider silk's mechanical properties are due to their genetic sequences, past research has shown that synthetic silks' mechanical features can be tuned by both genetic sequence alteration and post-spin manipulation of the synthetic protein materials. While a synthetic spider silk fiber has been created that has comparable mechanical properties to that of native spider silk, to date a process that can reliably produce the fibers in large, commercially viable quantities has not been established²⁹.

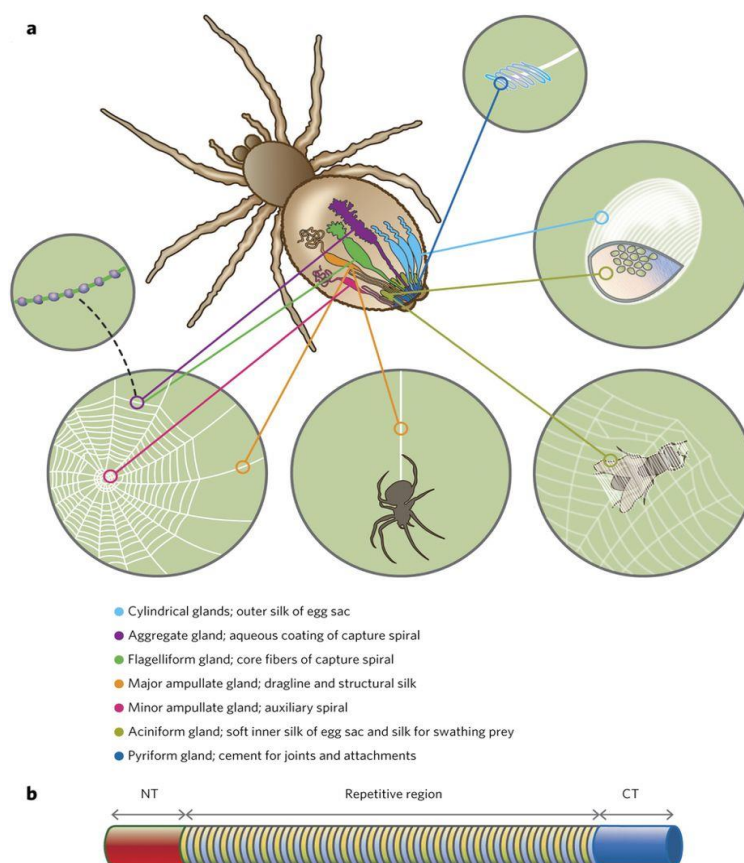


Fig. Error! No text of specified style in document.1-1 Diagram representing Araneidae spider silk types, glands and function³⁰

Table 1-1 | Mechanical property table comparing spider silk and other materials

Material	Strength (MPa)	Strain (%)	Toughness (KJ/kg)
Dragline silk	4000	35	400
Minor ampullate silk	1000	5	30
Flagelliform	1000	>200	400
Tubuliform silk	1000	20	100
<i>Bombyx mori</i> silk	600	20	60
Kevlar 49	3600	5	30
Rubber	50	850	80
Tendon	150	5	5
Ligament	20	150	1.6
Bone	160	3	3
^a Data from ref. (Gosline), (Lewis), (Kaplan)			

In order to understand spider silks' extraordinary mechanical properties, researchers' focus turned to the chemistry and secondary structure of the proteins. Through amino acid analyses (AAA), protein sequencing and gene cloning the primary structure was determined for various orb weaving spiders' silk proteins. Dragline silk was among the first silks examined and was shown to have a high glycine and alanine content, accounting for greater than 50% of the amino acid composition³¹. Glycine and alanine were not the only amino acids that were found in great abundance within dragline silk; over 90% of the silks' amino acid composition is accounted for by only six amino acids: glycine, alanine, serine, glutamine, proline and arginine. Upon further investigation into the other spider silk proteins it was discovered that a high glycine and alanine content was common among all the proteins, although with some variation. Some differences among the silk proteins include: 1) the absence of proline in minor ampullate silk³², 2) a low alanine concentration in aciniform³³, 3) proline in abundance in piriform³⁴ and 4) a high serine content in tubuliform^{35,36}. These variations in amino acid composition found in the different silk types were believed to contribute to the differences in mechanical properties present. Through X-ray diffraction, amino acid analyses, nuclear magnetic resonance (NMR), circular dichroism and Raman spectroscopy the primary structure for spider silk was assigned to specific secondary structures that attributed elasticity and strength to the fibers³⁷⁻⁴⁵.

Within the spider silk protein's sequence there are three notable structural motifs (Figure 1.2). These motifs govern the elasticity (GPGXX/GPGGX) and strength $[(GA)_n/(A)_n$ and GGX] characteristics exhibited by the fibers⁴⁶. The fibers' motif concentration quantitatively determines its mechanical properties, making synthetic

spider silks tunable at the genetic level. If the poly-alanine motif is reiterated at a higher concentration in a particular silk, than the correlating mechanical property, in this case strength, will be higher, and so forth. Due to high repetitions of these motifs in silks it follows that the larger, in kDa, the protein is, the better or higher the mechanical properties. Specifically, the more the strength motifs are repeated the greater the strength, and the same for the elastic motifs^{8,33}.

Once the primary structure was elucidated, researchers could determine the secondary structure that helped drive the protein's function. The GPGXX/GPPGX motif forms a β -spiral secondary structure, due to the prolines. The β -spiral acts like a slinky, allowing the fiber to stretch and retract without permanent deformation, leading to a high elasticity in proteins containing these motifs^{40,41,47,48}. GGX repeats create Gly II helices, creating a rigid structure within the fiber. As the strength characteristics observed in spider silk have been assigned to the $(GA)_n/A_n$ motifs, it is not surprising that X-ray diffraction^{43,45,49,49}, NMR^{40,41,48}, Raman spectroscopy^{42,50}, and Fourier transform infrared spectroscopy (FTIR)⁵¹ studies show that these motifs lead to a secondary structures that forms stiff crystalline β -sheets. These crystalline β -sheet regions act as the zipper, backbone in the proteins and when they are aligned upon spinning, cause the fiber to have an increased tensile strength (Figure 1.2).

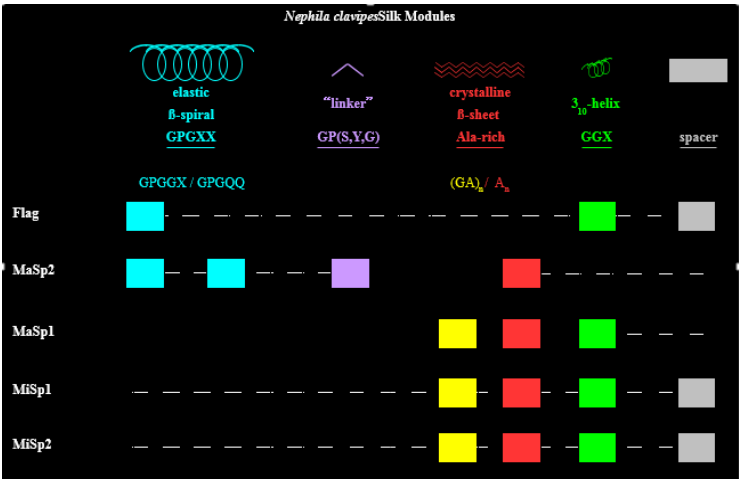


Fig. 2-2 A representation of the mechanical properties for each spider silk type and the motifs that drive secondary structure within the silk's sequences ¹⁴

As would be expected there are some additional differences found in aggregate spider silk, which is the adhesive protein that the spider secretes and uses to capture prey^{52,53}. The aggregate protein is excreted as droplets onto the web. One main difference in aggregate silk versus other silks is that it is a glycoprotein and while it is known that aggregate is glycosylated post-translationally, researchers has proven that the glycosylation is both O- and N-linked^{54–57,25,53,58–61}. Other than the glycosylation found in the aggregate, the gene follows many of the same genetic patterns as the other silks, such as a conserved C-terminal and glycine/proline rich repetitive motifs^{25,58}.

Synthetic Production and Current Limitations

Due to orb weaving spiders' cannibalistic and territorial nature, it is impossible to successfully farm them to procure spider silk commercially. Therefore, a bioengineered synthetic process is necessary to efficiently produce spider silk. To date synthetic spider silk has been produced in *E. coli*^{5,62–66}, goats (Nexia Biotechnology, unpublished),

yeast⁶⁷, plants^{68,69}, mammalian cells⁷⁰ and silkworms^{64,71,72}, but none of these processes provided commercially viable yields or are able to express recombinant spider silk proteins (rSSps) that can mimic the sizable natural silks^{63,67,71,73–75} (Figure 1-3).

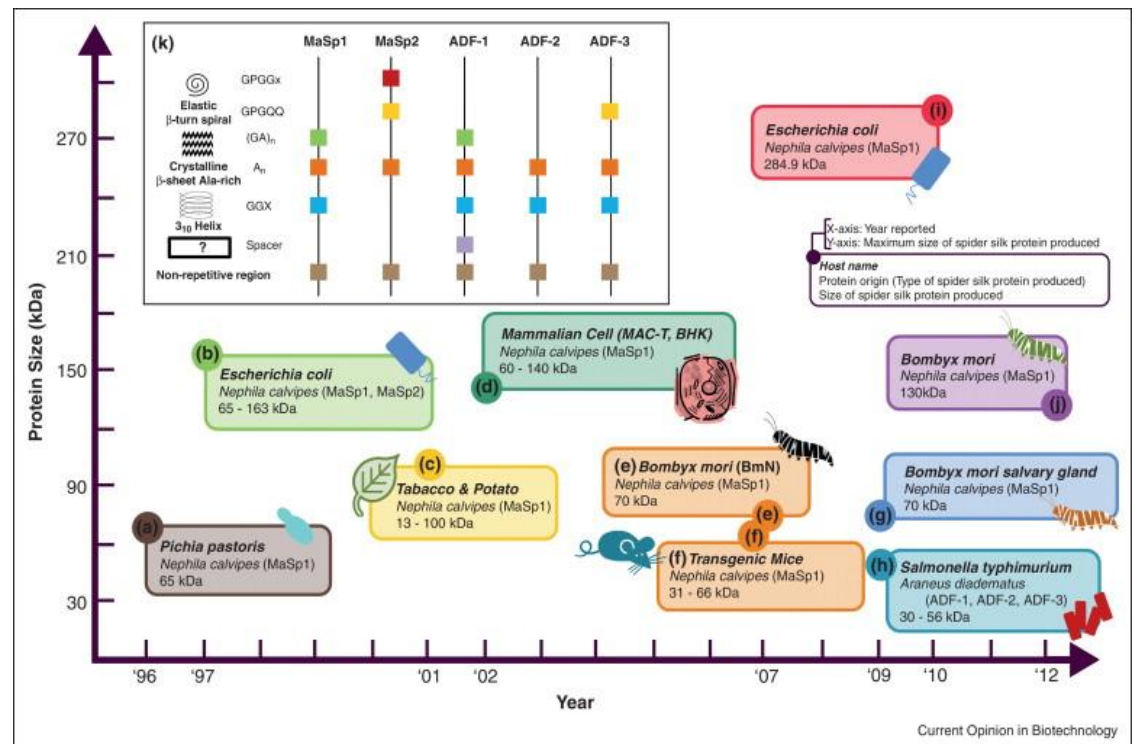


Fig. 1-3 | Brief history of recombinant spider silk protein production (a) Production of synthetic spider dragline silk protein in *Pichia pastoris* in 1996¹³, (b) synthetic spider dragline silk proteins and their production in *Escherichia coli* in 1997¹⁷, (c) production of spider silk protein in tobacco and potato in 2001¹², (d) spider silk fibers spun from soluble recombinant silk produced in mammalian cells in 2002¹⁸, (e) expression of EGFP-spider dragline silk fusion protein in BmN cells and larvae of silkworm, which showed that solubility is the primary limitation for the yield of spider silk protein, in 2007¹⁹, (f) construct synthetic gene encoding artificial spider dragline silk protein and its expression in the milk of transgenic mice in 2007²⁰, (g) transgenic silkworms (*B. mori*) producing recombinant spider dragline silk in cocoons in 2009²¹, (h) engineering the *Salmonella* type III secretion system to export spider silk monomers in 2009²², (i) native-sized recombinant spider silk protein produced for the first time in metabolically engineered *E. coli* resulting in a Kevlar-strength fiber in 2010²³, and (j) transgenic silkworms transformed with chimeric silkworm-spider silk genes producing composite silk fibers with improved mechanical properties in 2012²⁴. The organization of the important motifs in spider silk proteins, which is modified from Hayashi *et al.*²⁵ and Teule *et al.*¹⁴. The colored-squares indicate the modules contained in each silk protein. The proteins are: MaSp1 and MaSp2, major ampullate spidroin 1 and 2 from *Nephila clavipes*; ADF-1, ADF-2, and ADF-3, minor ampullate, putative cylindrical, and major ampullate, respectively, from *Araneus diadematus*²⁵ (Reprinted from Chung *et al.*⁷ with permission)⁷⁶

The redundant motifs in spider silk lead to a protein composition of up to 80% glycine, proline, and alanine⁷⁷. Spider silk protein's large molecular weight and repetitive nature cause some primary difficulties in synthetic production. Literature indicates that the limitations in synthetic production can be explained by analyzing the silks' genetic sequences and the innate biochemistry of the hosts^{62,78}. The native spider silk proteins are large, ranging from 200-350 kDa. Due to the spider silk genes highly repetitive nature and large size, there has been limited success in expressing a native-sized recombinant silk protein in any host. Glycine, proline and alanine in particular are vital to producing a full-length spider silk protein. The two limiting factors in spider silk production are: 1) there is no commercially viable process that produces native-sized proteins and 2) synthetic fibers have been unable to match the mechanical properties of natural fibers (Table 1-2).

Table 1-2 | Synthetic spider silk yields, sizes and goals in different production hosts versus the orb-weaving spider *Nephila clavipes*

Source	Spider Silk Size (kDa)	Yield	Goals
<i>N. clavipes</i>	250-500	NA	NA
Goats	127	0.5-2 g/L	2-5 g/L
<i>E. coli</i>	127	0.7 g/L	1 g/L
Silkworms	240	10-20%	50% spider silk
Alfalfa	80-120	unknown	1% yield

Not only are the characteristics found in different spider silks widely variable and promising, but so are the diverse materials that can be made from their protein components. This is mostly due to the plethora of ways that spider silk can be processed, turning spider silk into more than just a fiber. Once the synthetic proteins have been

produced into a dried protein after purification and lyophilization there are many ways of processing. It can be spun into a fiber^{5,79–81} or resolubilized with heat and pressure to make a dope that under different conditions can create hydrogels^{19,82–88}, sponges, films, and aerogels⁸⁹ (Figure 1-4). In order to make all of these novel materials, a large amount of spider silk protein will need to be made to make product development viable.

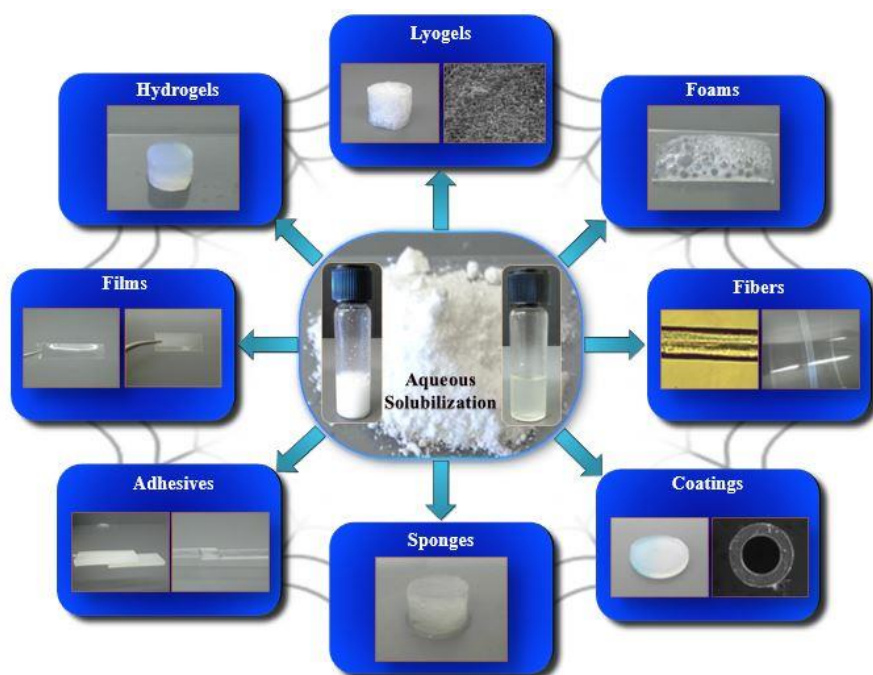


Fig. 1-4 | Schematic showing some potential materials that can be made using synthetic spider silk⁹⁰

E. coli (Bacteria)

Escherichia coli is of particular interest as a host for spider silk production due to its relatively fast production time, cost and it is commonly used as a host in industry and therefore has been well studied as a host for synthetic production. One issue in producing spider silk proteins in *E. coli* is that the bacteria does not naturally produce proteins that

are in the same molecular weight range or proteins that require such high amounts glycine, and innately have a smaller concentration tRNA pool available to them than that which is found in spiders^{5,63,66,91,92}. During translation, a deficiency in either amino acid availability or the correct tRNAs can cause the ribosome to “fall off”. If the ribosome falls off prior to reaching the stop codon, premature translational termination occurs and a truncated protein is the result.

Many different methods have been employed to improve spider silk protein production in *E. coli*, including: the addition of supplementary amino acids, specifically glycine, alanine and proline to the media, to make them more available to the bacteria for translation, this ultimately failed to improve expression and protein size⁶⁷. To alleviate these problems one study showed that the addition of glycine tRNAs in *E. coli* leads to successful production of larger proteins with consequently, better mechanical characteristics (Figure1-5). These results were not reproduced until recently, and originally were only conducted for major spidroin 1 (MaSp1) protein with no additional proline tRNAs, which are necessary for major spidroin 2 (MaSp2) protein production⁶².

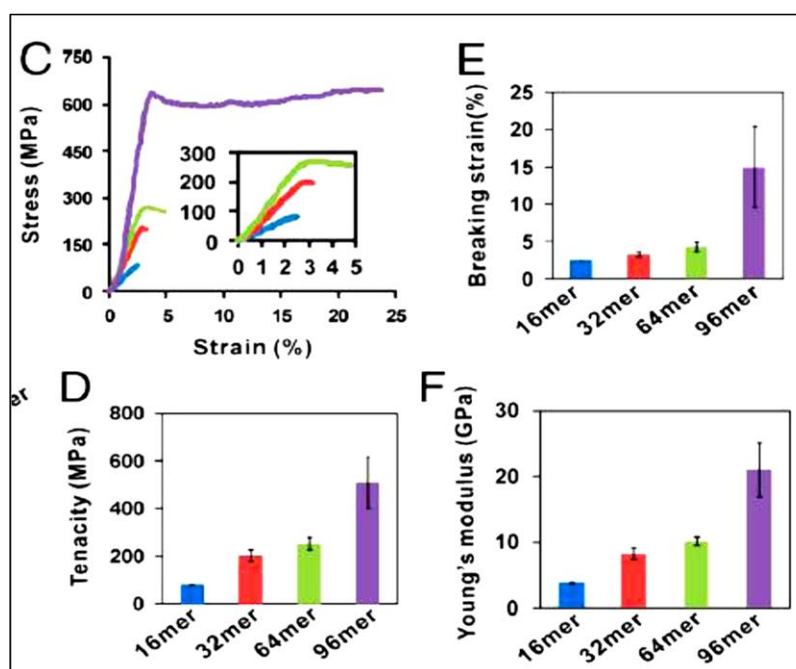


Fig. 1-5 | Diagram showing the research results from a study conducted in 2010 by Xia et. al. Where synthetic silk constructs ranging in size from 16 motif repeats (16mer) to 96 motif repeats (96mer). C) shows the Young's modulus plot for each construct, D) shows tenacity (MPa), E) shows the percent breaking strain, or elasticity, in each construct, and F) shows a calculation of the Young's modulus plot for each construct^{62,93,94}

Medicago sativa (Alfalfa)

Medicago sativa, alfalfa, is another host that has the capability to produce spider silk at a yield that is commercially viable and more economic than some other production hosts⁶⁸. Alfalfa is a perennial crop that can be harvested up to four or more times a year and has been utilized as a genetic engineering host since the 1980s with great success in both protein production and yield. The USDA has approved alfalfa as an appropriate genetic host for protein and peptide production for years and companies, such as Medicago ® and Forage Genetics ®, currently grow transgenic fields with commercial

product⁹⁵⁻⁹⁹. Total protein content in alfalfa is 24% dry weight, and past research has shown yields of up to 5% dry weight synthetic protein. A 2% dry weight spider silk yield in alfalfa would produce 218 kg spider silk per acre per year, a yield that supports commercialization.

The main setback encountered in spider silk production in alfalfa comes when the spider silk protein is extracted from the harvested material. In all the hosts a poly-histidine tag is employed at the C- or N- termini, more commonly the N-terminal, as a means to extract the desired rSSps via HPLC with a column charged with nickel (Ni^{2+}), also known as Nickel immobilized metal affinity chromatography (IMAC). When the cell lysate is sent over the column the charged poly-his6 tag should bind to the oppositely charged nickel, which acts as a ligand, and the rest of the lysate should flow through into the waste, then using imidazole the protein can be eluted from the column and collected to be processed into a final pure product. In the alfalfa's case there is the complication of chlorophyll, which can also bind to the metal ion and is difficult to remove due to the molecule's phytol tail. In order to successfully produce spider silk in alfalfa, creating a commercially viable process, this problem must be resolved.

Bombyx mori (Silkworms)

Bombyx mori, silkworms, have also been employed as a host for synthetic spider silk production. While synthetic production in silkworms is advantageous in that the rSSps are spun into fibers, there are several negative aspects to this production system. Research using CRISPR/Cas9 initiated non-homologous end joining, native-size spider silk genes have been integrated at defined locations in the fibroin genes of the silkworm.

The mechanical properties of these transgenic spider/silkworm fibers are similar to those found in the native silks¹⁰⁰. This 15% integration greatly increases the mechanical properties, so that they approach that of native spider silks. (manuscript submitted and under review as of the time of this writing)

Some issues that are innate to production via silkworms is that while it is convenient that the silkworm spins the spider silk proteins as a chimera with silkworm silk^{71,72,101}, it is nearly impossible to deconstruct the fibers to make any other material, such as sponges, hydrogels etc. Another issue with production in silkworms is that their silk is coated with a protein called sericin that is not biocompatible, rendering any material made with these synthetic fibers useless in medical applications unless a degumming process is employed. The degumming process itself does not always have a 100% efficiency and upon degumming research has shown a decrease in mechanical characteristics of the fibers¹⁰².

Goats

Utilizing goats as a synthetic spider silk host is an attractive option due to the fact that the targeted protein can be expressed in tandem with the goat's milk proteins and produced in the milk. Goats are relatively small, in comparison to other milk producing farm animals, they produce high protein content milk at a comparatively high volume, and it is only a two-year waiting time from birth to reproduction, and therefore milk-producing, age. In 2002 Nexia Biotechnologies, along with Randy Lewis' laboratory, created transgenic goats containing the spider silk gene, creating transgenic goats capable

of producing spider silk in their milk (Nexia Biotechnologies, unpublished). The spider silk genes are heritable and can be passed on to progeny.

Currently this is the highest producing system for synthetic spider silk, although the spider silk protein being expressed is only a 65 kDa protein and they are only producing the major ampullate silk proteins. The high production rate in these transgenic goats has allowed to for improvements to the fiber spinning process⁷⁹, creating fibers that more closely mimic the native spider silk, and other methods for material development to be discovered: including hydrogels, aerogels, sponges and films^{89,90}. Despite the current success using goats as a host for spider silk production, upscaling the goat herds is a concern due to high costs for non-therapeutic protein, especially for markets that require a large amount of material.

Eukaryotic Cell Culture (*SF9* and *BmN*)

One potential method to synthetically produce spider silk is through cell culture in various eukaryotic cells, namely *Spodoptera frugiperdes* (SF9/SF21) and *Bombyx mori* (BmN) cells, which have already produced spider silk-like proteins. Although only a 70 kDa synthetic silk has been produced with a very low yield of 5% total cell protein in BmN cells, resulting synthetic spider silk proteins produced in SF9 cells have proved very weak or fragile, making it difficult to form tangible products with them or mechanically⁷⁰. There are concerns that, like with *E. coli*, the eukaryotic cell lines will have difficulties producing spider silk proteins that are as large as native proteins, not just due to the size of the proteins, but also the repetitive nature contained in the genetic sequence for the proteins which can cause a depletion in the aminoacyl-tRNA pool

creating truncated proteins. Cell cultures costs is also a concern, with the cost being expensive in comparison to other production processes. While research in our laboratory has shown that spider silk gland cells may produce spider silk proteins in culture, a glandular cell line has remained unattainable due to difficulties with proliferation (unpublished).

Modern research into spider silk has only heightened the materials status as a super biomaterial and given insight into the applications able to be engineered using these novel proteins. Now that the genetic code has been unraveled, it has revealed that the mechanical properties can be manipulated to fit a wide variety of applications and it has become necessary to continue research into developing synthetic spider silk at a rate that can meet the pressing demands to create spider silk biomaterials. With newly developed materials making headway and prototypes in demand, this necessity is only intensified. Once processes to create synthetic spider silk that are capable of meeting these demands are produced, centuries of human perseverance and curiosity will have paid off. It is a far step from those in the past that would collect full spider webs with sticks to use as wound dressings or those in the more recent past that would sit for hours forcibly silking spiders to obtain enough to make small textiles. There will now be a facile process to create multiple materials to fit varying needs, all from synthetic spider silk.

Research Aims

The overall goal this research aims to achieve is **optimizing spider silk production in a variety of hosts to attain a commercially viable process, while improving the synthetic spider silk's mechanical characteristics through both**

genetic engineering and expression techniques. There are three different projects that are discussed below for expressing and purifying spider silk in varying hosts. The resulting expressed protein will be used for other material-oriented projects throughout the laboratory and the mechanical data acquired will be integrated to further research into improving both production and product characteristics.

Objective #1: *E. coli*

The first objective was to increase the expression capability in *E. coli*, both by increasing expression yields and the molecular weight of the synthetic spider silk protein produced. By applying molecular cloning methods glycine, alanine and proline tRNAs were inserted into an expression vector, therefore being expressed in tandem with selected spider silk genes. The poly-histidine tag placement was also experimented with, to see if a C-terminal or a N-terminal tag would improve expression and/or full-length protein purification.

Objective #2: Alfalfa

A second objective was to produce recombinant spider silk proteins (rSSps) in *Medicago sativa*, alfalfa. Using molecular cloning and bioengineering methods an agro-transformation vector was created ensuring selected proteins would be retained in the endoplasmic reticulum, resulting in synthetic protein expression being contained within the plant's leaves and increasing the proteins expression. A new purification method was necessary due to the interaction from the chlorophyll molecule and the resin used for HPLC.

Objective #3: *Spodoptera frugiperda*

The third, and final, objective of this research was producing a synthetic aggregate spider silk protein in *Spodoptera frugiperda*, the SF9 cells. Since aggregate is an adhesive protein and, like most biological protein-based glues, is glycosylated it must be expressed in a host that has the ability to post-translationally modify the synthetic protein with glycans. While *E. coli* does not post-translationally modify proteins to have N- or O- glycosylation both *Medicago sativa* and *Spodoptera frugiperda* do. Due to the rapid genetic integration and expression of selected synthetic proteins into SF9 cells versus the agro-transformation method used to insert and express synthetic proteins in alfalfa it was decided to express aggregate in SF9 cells for initial research. Two other reasons that SF9 cells are attractive is due to their reduced generation times and potential easy rSSp purification.

In summary, the projects listed are an effort to address production issues, namely creating a yield capable of supporting commercialization and increasing the rSSps molecular weight, and in a proportionate way the mechanical characteristics, to more closely mimic the native spider silk genes. By addressing these issues in multiple hosts both a short-term and a long-term approach to production can be established. The resulting rSSps will support product research and eventually commercialization.

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CHAPTER 2

IMPROVING SYNTHETIC SPIDER SILK PROTEIN PRODUCTION IN *ESCHERICHIA COLI* UTILIZING VECTOR ADAPTATIONS

Chapter Preface

This chapter describes the research conducted into improving recombinant synthetic spider silk protein (rSSp) production in *Escherichia coli* through molecular cloning with the expression vector pET19b. In order to address production issues, molecular engineering was utilized creating four different vectors: 1) pET19-SX, a pET19b-derived vector containing additional glycine and proline tRNAs and a serine hydroxyl methyl transferase (SHMT) enzyme that turns serine into glycine, 2) pET19-SXC, which is identical to the pET19-SX vector with the poly-histidine tag being moved from the N-terminal to the C-terminal, 3) pET19K, a kanamycin resistant version of the commercially available ampicillin resistant pET19b and 4) pET19KC, this vector is the same as the commercially available pET19b vector from Invitrogen ® except having kanamycin resistance, rather than ampicillin, and a C-terminal poly-histidine tag. These vectors were hypothesized to increase rSSp yields and mechanical characteristics and also allow for larger proteins to be produced. Strength and elasticity would increase based on the increased capability of *E. coli* to produce larger rSSps, that are more similar in size to native spider silk proteins.

The Invitrogen ® commercially available vector pET19b was used as a base vector for comparison in expression, as well as the vector genetically modified with inserts creating the pET19SX, pET19SXC, pET19k and pET19KC vectors. The

modifications applied to the above vectors resulted in very large vectors, which has often led to difficulty in expression and fermentation of *E. coli*. Shortly after these vectors were constructed a co-expression system was also created, with one vector containing the extra tRNAs and SHMT sequences, and the second vector containing the target spider silk gene. This research was not conducted for this specific dissertation, but bears mention. The research presented in this chapter was conducted in 2012-2014, so it is not current and further research has been conducted leading to a more successful bacterial production system for rSSps. Regardless, the research presented in this chapter is presented as it was a precursor to the current production process and success in *E. coli*.

Introduction

One of the most popular systems used for synthetic protein production is the gram-negative bacterium *Escherichia coli*, this is due to fermentation being relatively low cost, in regard to other synthetic protein production processes. *E. coli* has a fast generation time that ensures rapid biomass accumulation, and the process is straight forward to scale up¹⁰³. These particular characteristics make *E. coli* one of the first and most common production systems for spider silk proteins. Since spiders are cannibalistic and territorial a synthetic production system is needed and *E. coli* provides a quick and relatively cheap expression system. To date, synthetic spider silk has been produced in *E. coli*^{5,62-66}, goats (Nexia Biotechnology, unpublished), yeast⁶⁷, plants^{68,69}, mammalian cells⁷⁰ and silkworms^{64,71,72}, but due to the comparatively low cost and fast processing time production in bacteria is attractive in the long-term.

While recombinant spider silk proteins (rSSp) have been produced in *E. coli* through fermentation, the resulting yields are not high enough to meet commercial or research demands, and the synthetic proteins produced did not create fibers of the same strength as the native spider silks. The inferior mechanical properties innate to the rSSps were due to the smaller protein size versus the large native spider silks, ~25-65 kDa in comparison to 250-500 kDa. Additionally, the highly repetitive nature of spider silk proteins leads to ribosomal fall-off and, therefore, truncated-versions of the protein are produced^{66,104,105}.

Many researchers attempted to address the low yield and truncation issues by optimizing the codons in the gene sequences to more closely match that of the heterologous host¹⁰⁶ and by altering the expression vectors to contain kanamycin

resistance, rather than the less stable ampicillin resistance^{63,66,106,107}. While this led to a 10X increase in production, it did not resolve the issues with truncated protein nor did these attempts improve yields.

In order to make a commercially viable synthetic spider silk production process the fermentation costs need to be lowered. While fermentation is generally a low-cost production process, this was not proving to be the case with spider silk. Factors driving the high cost were believed to be one or a combination of the following factors: 1) materials, 2) equipment, 3) labor and 4) yields. By increasing the rSSp yields an inverse in production costs is achieved, due to there being no effect on the materials, equipment or labor costs, therefore the most feasible way to lower production costs is by improving spider silk yields (Figure 2-1). Through computer simulation it was determined that a rSSp yield of even 2 g/L would meet the economic and material demands for high end commercialization¹⁰⁸.

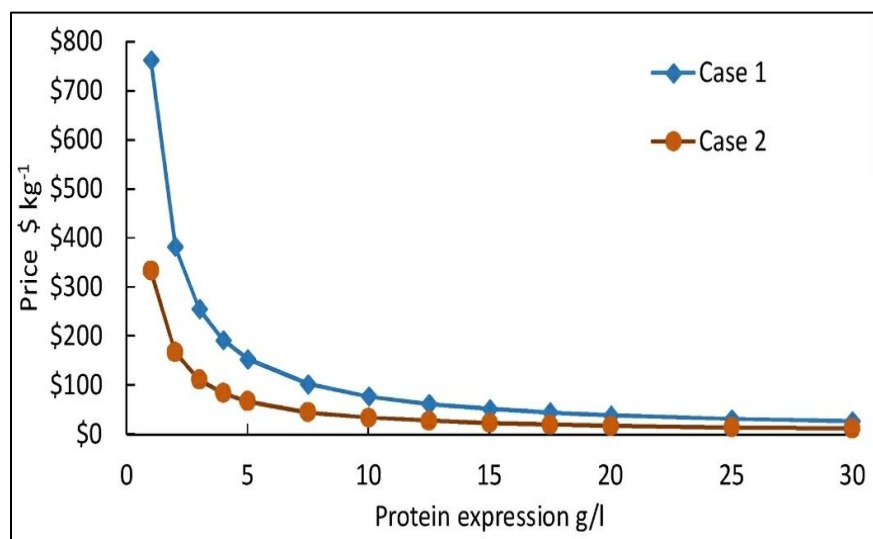


Fig. 2-1 | Data accrued showing spider silk protein yields effect on cost¹⁰⁸

Research was conducted based on the theory that the main limiting factor in spider silk production in *E. coli* was the availability, or in this case unavailability, of the most highly used amino acids found in the gene's repetitive motifs. Doping the media with glycine, proline and alanine, the three most common amino acids in most spider silks⁹⁻¹³, may lead to complete translation of the proteins. However, the resulting rSSps from these experiments were only marginally larger than those produced without doping the media, and the yields were not significantly increased. These results led to a second theory: the limiting factor in spider silk protein production in *E.coli* was not the amino acid concentration, but a deficit in the t-RNAs that coded for these amino acids⁷³. If the t-RNA pool in *E.coli* has a lower glycine, proline and alanine concentration, the result would be translational pauses that would lead to ribosomal fall off and truncated proteins^{66,73,106,110-112}.

The main focus of this research was to improve synthetic spider silk yields in *E. coli* through genetic manipulation of the expression vector. More specifically, the effect of adding glycine and proline t-RNAs within the expression vector and the translocation of the histidine tag used to purify the targeted proteins from the N-term to the C-term on both protein yields and size. By relocating the purification tag to the end of the sequence it was theorized that only full-length proteins would be purified from the fermentations. The additional t-RNAs and a serine hydroxyl methyl transferase (SHMT) had been shown in past experiments to increase both the size of synthetic protein produced and the resulting yield⁶². SHMT is a ubiquitous pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes the reversible interconversion of L-serine and glycine, therefore providing an enzymatic pathway for the conversion of serine into glycine.

Four vectors were created based on Invitrogen's pET19b ®: 1) pET19-SX, containing additional t-RNAs and an SHMT, 2) pET19-KC, has a C-terminal histidine tag rather than the generic N-terminal tag, and pET19-SXC, has a C-terminal histidine tag and the additional t-RNAs and SHMT. A MaSp2 rSSp was inserted into each novel vector, as well as the original pET19k, and expression was compared for both flask and bench culture. Comparison was conducted using Western blot analyses and by the resulting yields from the fermentations of the different constructs. Results from this research were implemental in the concurrent and present research and production of rSSps via bacterial fermentation.

Materials and Methods

pET19 Vector Evolution:

For this research four different vectors were created via molecular cloning methods and utilized to examine the effects of additional t-RNAs, a serine hydroxyl methyl transferase (SHMT) and relocating the histidine tag used for purification from the N-term to the C-term would have on rSSp production in *E. coli* (Figure 2-2). These additions and alterations were used in tandem, as well as individually to examine what exactly was improving protein yields and/or limiting protein truncation. The four vectors are: 1) pET19k, a modified Invitrogen pET19b vector with kanamycin resistance, rather than ampicillin, 2) pET19-KC, this vector has translocated his-tag from the N-terminal side of the multiple cloning sites (MCS) to the C-terminal, 3) pET19-SX, which has additional glycine and alanine t-RNAs, as well as, a SHMT cloned in after the MCS but prior to the stop codon, and 4) pET19-SXC which is the same as pET19-SX but has a relocated his-tag on the C-terminal (Table 2-1).

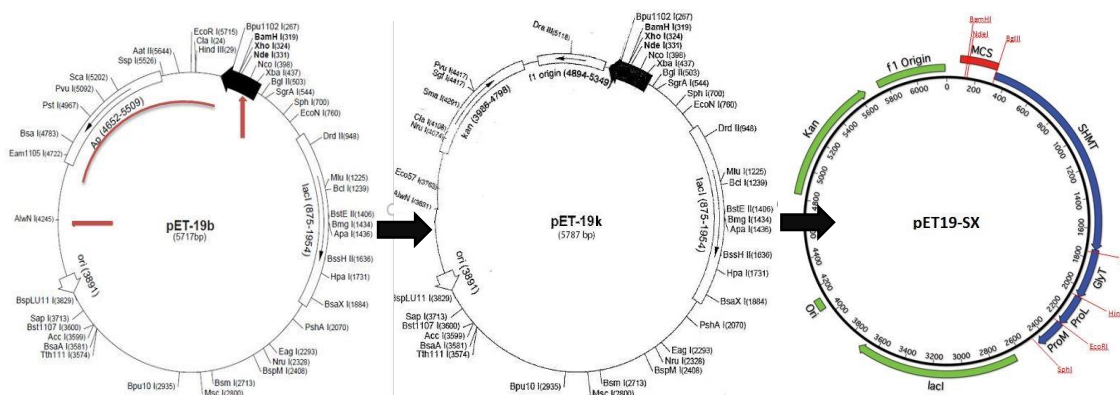


Fig. 2-2 | Vector evolution from Invitrogen's pET19b® to pET19k to pET19SX

Table 2-1 | Vector composition breakdown including: the location of the histidine-tag and presence of glycine and proline t-RNAs and a serine hydroxyl methyl transferase (SHMT)

Vector	Tag loci	Gly t-RNA	Pro t-RNA	SHMT
pET19k	N-term	No	No	No
pET19-KC	C-term	No	No	No
pET19-SX	N-term	Yes	Yes	Yes
pET19-SXC	C-term	Yes	Yes	Yes

Spider Silk Protein Gene Cloning

This research used a 16-Mer, 16 repeats of a major spidroin monomer, MaSp2 (National Biosciences), 1825 bp major ampullate silk spidroin, sequence created by Dr. Michael Hinman (Figure 2-3). The MaSp2 sequence and vectors were cut with the restriction enzymes BamHI and NdeI, at the C and N-terminus respectively) and then ligated using a T4 DNA ligase (New England BioLabs M0202S); the resulting ligations were electroporated into XL1-Blue Supercompetent Cells (Agilent/Stratagene C2992) at

1800V. Once clones were screened and the sequences verified, they were transformed into BL21 competent *E. coli* (New England BioLabs C2530H) for expression studies.

5' GATCCC(CCGGGTGGCTATGGTCCTGGACAGCAAGGTCCTGGCCG
GTCCTGGCCAACAGGGTCCCTCTGGTCCAGGCAGTGCAGCTGCCG
CGCAGCGGGT)₁₆CCGGATCAAGCTTATCGATACCGTCGGGATC^{3'}

Fig. 2-3 | Nucleotide sequence for a synthetic major ampullate spidroin (MaSp2). Nucleotides in red represent the repeated monomer, this monomer is repeated 16X making this sequence a 16-mer.

Expression Assays and Western Blot Analyses

Expression assays were conducted using 5 mL kanamycin (100 mg/m) LB media (Miller 71753-6) in 15 mL culture tubes and were inoculated with 1 colony forming unit from a kanamycin (100 mg/mL) agar plate containing the MaSp2 construct: including pET19k-MaSp2, pET19KC-MaSp2, pET19SX-MaSp2 and pET19SXC-MaSp2. The culture tubes were grown in a shaking incubator at 37°C until an OD₆₀₀ of 0.8 was reached and then expression was stimulated with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown an additional 4 hours. A 1 mL sample was harvested every hour starting at 0 hours, after inoculation to test for protein expression. The harvested cells were centrifuged and the pellets were resuspended in 100 μL of 2X SABU, sonicated for 15 seconds at 0.5-1W, centrifuged and the lysate was collected for examination.

Western Blots were conducted on all processed cell pellets from the expression assays to determine MaSp2 production over the course of the culture. A 35 μL aliquot was taken from each sample and loaded onto a 4-20% tris-glycine SDS-PAGE gel

(Invitrogen XP04200BOX) and run at 135 V for 90 minutes. Gels were transferred to nitrocellulose membranes overnight at 25 mA in a wet transfer apparatus. The blots were conducted on the nitrocellulose membranes with a primary antibody produced in mice that targets the histidine tag (Rockland 200-303-382, 1:1000 dilution). A rabbit anti-mouse antibody conjugated with Alkaline Phosphatase (AP) was used at a 1:5000 dilution (Abcam ab6729). The membranes were developed using 1-StepTM NBT/BCIP Substrate Solution (ThermoFisher Scientific 34042) for imaging.

Bench Fermentations and HPLC

A New Brunswick BioFlo/CelliGen 115 benchtop bioreactor (BF115) was used for all culture experiments for expression of the MaSp2 in the various vectors. Fermentations were conducted using fed-batch aerobic fermentation conditions. A cell culture seed of 100 mL was used to inoculate media containing LB, glucose (25 g/L), trace metals (1X), magnesium sulfate (.002 M), yeast extract (5 g/L), phosphate (10 g/L), thiamine (2.5 g/L), kanamycin (100 mg/mL) and 0.3% v/v antifoam A (Sigma-Aldrich A6582). The fermentation was grown until OD₆₀₀ of 80 was reached and then inoculated with 1 mM IPTG and grown for an additional 4 hours. Bacteria was harvested via centrifugation at 6000 rpms for 30 minutes and the cell pellet was processed in an 8M Urea binding buffer (8M Urea, 20 mM Tris-HCL, 0.5 M NaCl, 2 mM EDTA) at 1:3. Sonication was conducted at 1 W for 4 minutes, a minute rest, and repeated 3 times (QSonica 3/4" diameter probe). The cell lysate was harvested through centrifugation at 6000 rpms for 30 minutes, retaining the supernatant as the final cell lysate.

Protein purification was conducted using immobilized metal affinity high performance liquid chromatography (IMAC-HPLC) utilizing the polyhistidine (6X) tag

on the MaSp2 constructs, either at the N- or C-terminus depending on the vector. An AKTA Avant was used with Ni-NTA resin (GE XK-16, bed height 14 cm). Sample was applied to column at 1 mL/minute and clarified lysate was applied across the column from both the top and bottom, separately to ensure complete application. The column was washed with running buffer (0.5 M NaCl, 20 mM Tris-HCL, 0.5 mM EDTA) and wash fractions were collected. 5 mL elution fractions were collected for 3 column volumes at 5%, 10% and 100% imidazole. Samples of these fractions were mixed 1:1 with 2X SABU with 6 M Urea and heated for 15 minutes at 100°C. These samples were analyzed by SDS-PAGE and western blot as described above.

Results and Discussion

Cloning and Expression Assays

The MaSp2 16-mer used as the rSSp to examine the effects additional t-RNAs, SHMT and relocation of the his-tag to the C-term had on spider silk expression was cut with the restriction endonucleases BamHI and NdeI then ligated into pET19k, pET19SX, pET19KC, or pET19SXC cut with the same enzymes. After transformation into XLBlues *E.coli* cells, transformants were examined to determine whether the constructs had been successfully ligated and transformed. Clones were screened by digestion with BamHI and NdeI, which would yield two bands: the vector used and the other running at 1825 bp for the MaSp2 16-mer (Figure 2-4). Cloning the MaSp2 into the pET19k, pET19SX, pET19SXC and pET19KC were successful. The resulting clones were then transformed into BL21 *E. coli* enabling protein expression experiments.

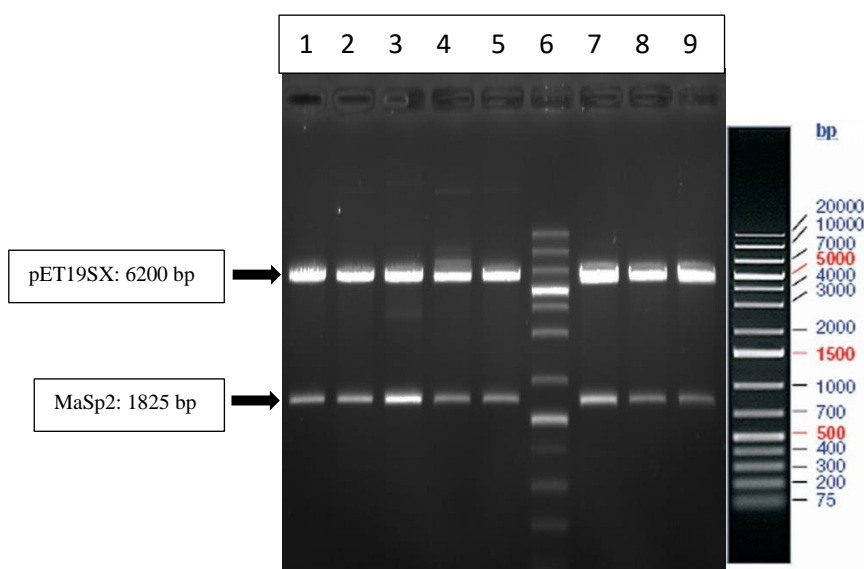


Fig. 2-4 | Confirmation of plasmid construction of MaSp2 + pET19SX via gel electrophoresis. The MaSp2 + pET19SX plasmid was cut with the restriction enzymes BamHI and NdeI. Two bands are present: 1) the top band is the linearized 6200 bp pET19SX vector and 2) the lower band is the MaSp2 spider silk insert at 1825 bp.

Expression assays were conducted using MaSp2+pET19k, MaSp2+pET19SX, MaSp2+pET19SXC and MaSp2+pET19SXC clones with samples taken every hour from induction, starting at hour 0 to hour 6, to characterize protein expression in a qualitative fashion over time and to ultimately compare the different vectors expression of the rSSp. There was some protein expression in the assays before induction, but the 65 kDa MaSp2 was expressed with minimal truncation for 6 hours (Figure 2-5). Western blots showed protein production over all 6 hours after induction, so it was decided to run the rest of the experiments under these conditions.

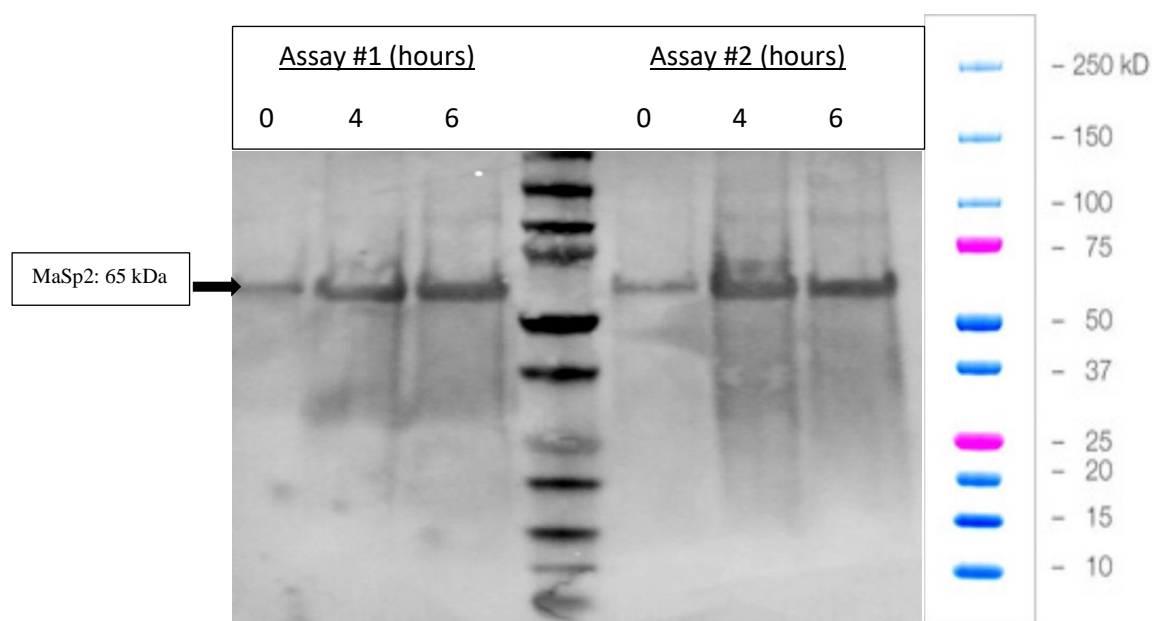


Fig. 2-5 | Western blot of a 65 kDa MaSp2 in pET19SX expression assay conducted in BL21 cells. Samples were taken at 0, 4, and 6 hours post-induction from two different assay cultures.

Western blot analyses of the expression assays conducted on MaSp2+pET19k and MaSp2+pET19SX showed a significant increase in protein production both over time and when using the pET19-SX vector (Figure 2-6). Although these were only qualitative results, they were promising enough to move to benchtop fermentation and full-scale purification to determine quantitatively how much the yield increased when using the pET19SX vector. These preliminary results showed that the addition of glycine and proline tRNAs, as well as the SHMT, increased the yield spider silk protein produced in *E. coli*.

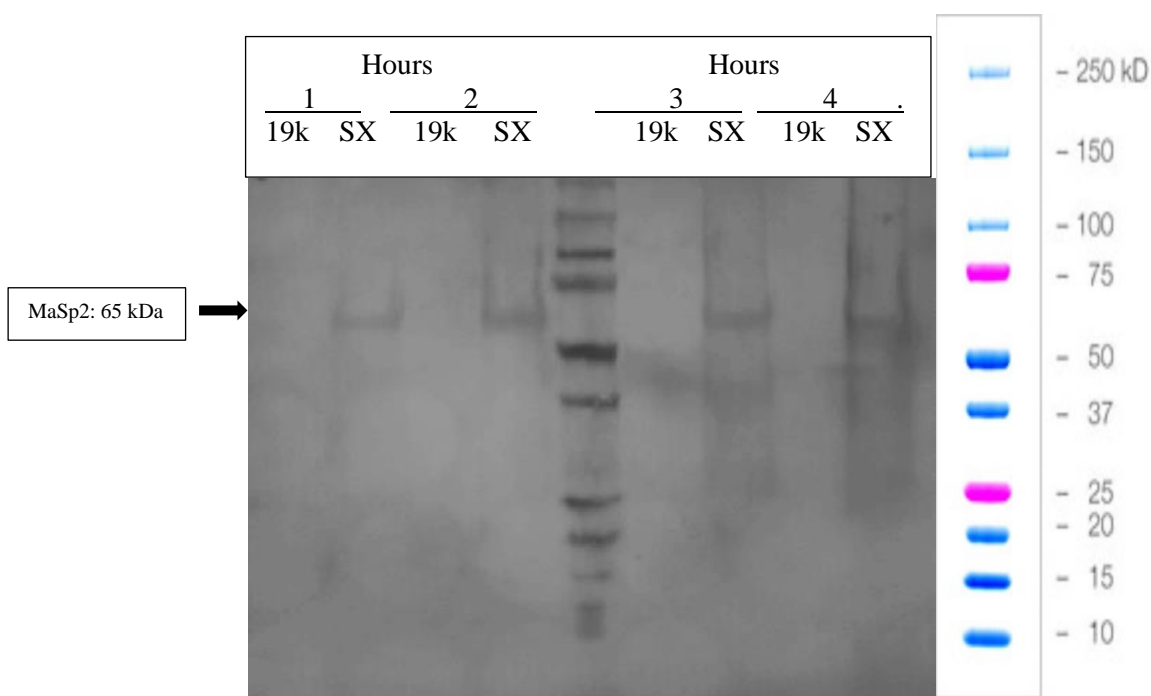


Fig. 2-6 | Western blot showing a protein growth assay comparison between expression of MaSp2 (65 kDa) in the expression vectors pET19k and pET19SX. Samples were taken 1-, 2-, 3- and 4-hours post-induction to show protein accumulation over time.

Western blot analyses on the expression assays of all the constructs (MaSp2+pET19k, MaSp2+pET19SX, MaSp2+pET19KC, and MaSp2+pET19SXC) were used to assess if moving the his-tag to the C-termini had any affect on protein production or to limit protein truncation. The western blots showed that when MaSp2 was produced in the pET19k and pET19KC vectors there was much lower protein accumulated than when using the vectors containing the extra tRNAs and SHMT, pET19SX and pET19SXC (Figure 2-7). Despite there being minimal truncated protein produced in any of the expression assays using the different vectors, both pET19KC and pET19SXC, the vectors with the relocated his-tag, did not produce as much MaSp2. After several more trials and experiments it was determined that moving the his-tag to the C-terminal had no

beneficial effects on protein production, and, in fact, was detrimental to spider silk production. Due to this discovery MaSp2+pET19SXC and MaSp2+pET19KC were not scaled up to the benchtop fermentation, as it was determined this would be a waste of resources and not beneficial to spider silk protein production.

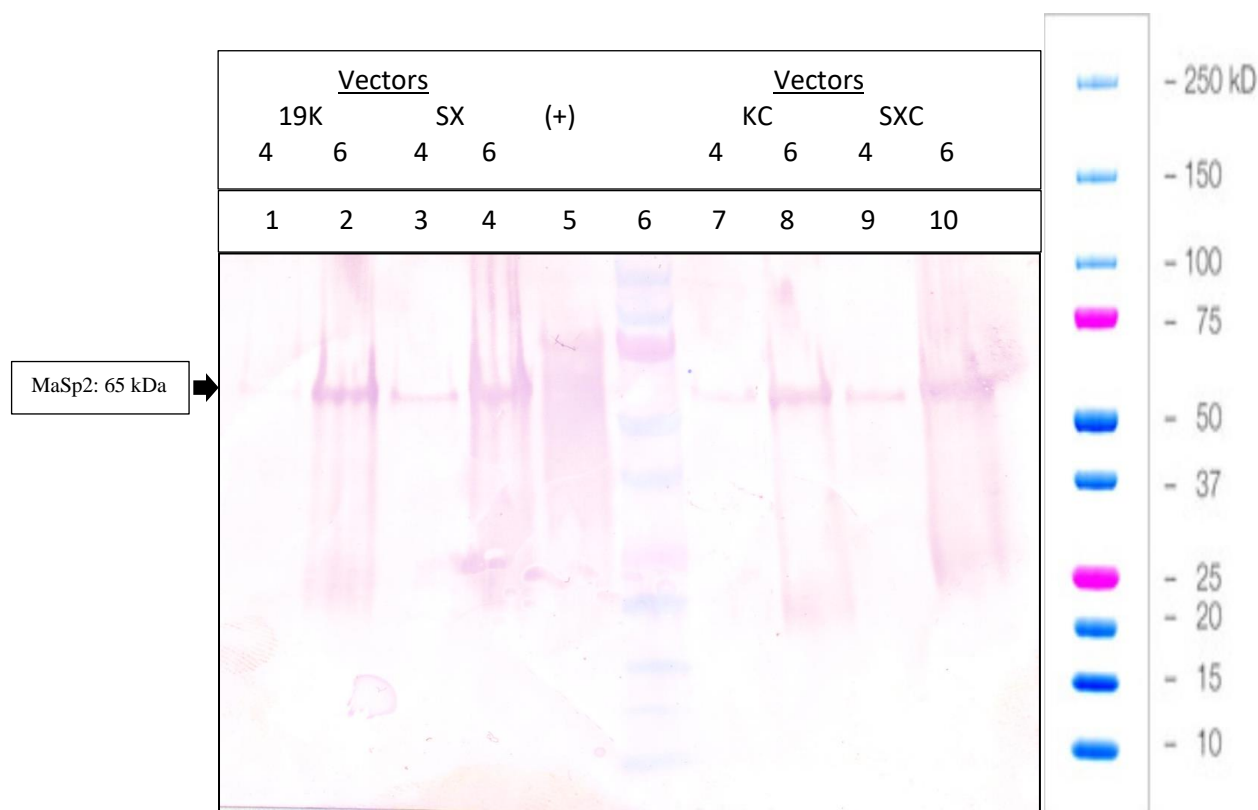


Fig. 2-7 | Western blot showing a MaSp2 spider silk protein (65 kDa) production. Growth assay comparisons between pET19k (wells 1 and 2), pET19SX (wells 3 and 4), pET19KC (wells 7 and 8) and pET19SXC (wells 9 and 10) with samples taken 4- and 6-hours post-induction.

Bench Fermentations and HPLC

To quantify yields and determine the difference in protein production using pET19k or pET19SX, fermentations were conducted at a benchtop scale with a volume of 1-5 L, depending on the fermentation vessel used. *E. coli* was harvested and processed on an AKTA Advant, MaSp2 production was verified with Western blot analyses. The

AKTA purifications showed MaSp2 was eluted with a significantly higher absorbance when the pET19SX vector was used, rather than the pET19k (Figure 2-8). Once the elution fractions were freeze-dried and the protein was verified on a Western blot analyses it was determined that by adding the gly-tRNAs, pro-tRNA and SHMT present in the pET19SX vector yields could be increased from 100 mg/L to 500 mg/L, a 5X increase in production. These results were only based on the fermentations run under the same conditions listed in the Materials and Methods above, there was no further exploration into altering fermentation conditions to improve yield conducted for this particular research project.

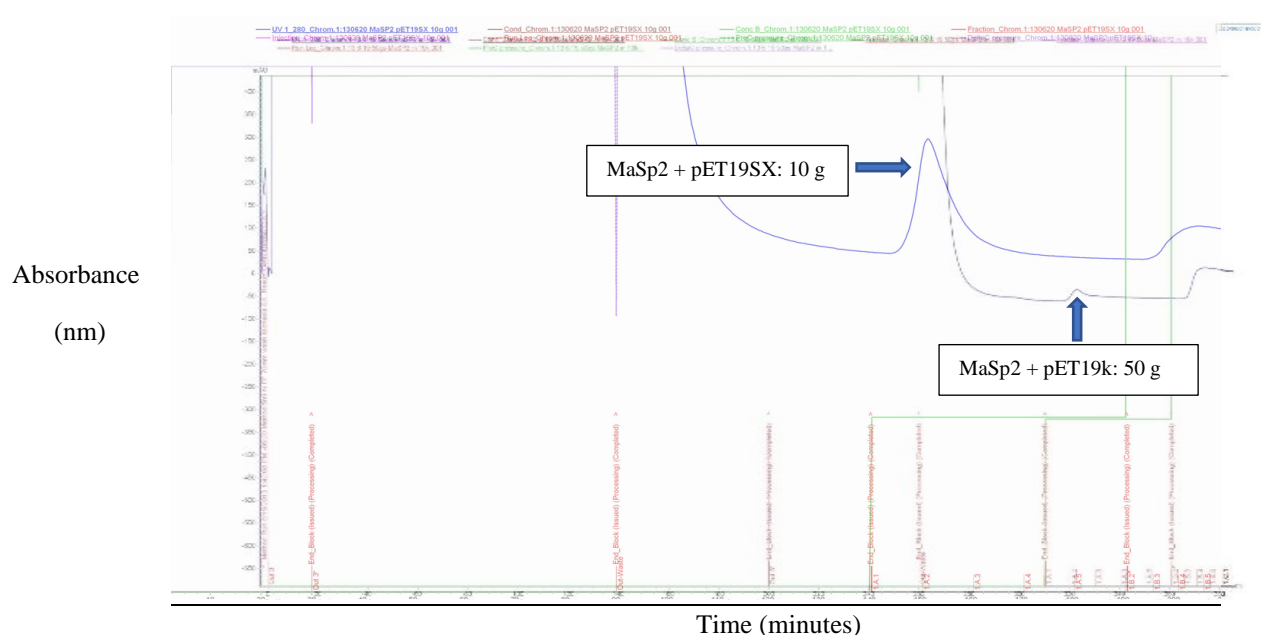


Fig. 2-8 | HPLC purifications using the AKTA Advant on MaSp2 (65 kDa) in either the pET19k (50 g cell pellet) or pET19SX (10 g cell pellet) expression vectors. The overlapped purifications are shown on a graph of absorbance (nm) over the purification time (s), the peaks represent the protein being eluted from the nickel column and fractionated into collection tubes.

Other Work

This research was conducted in 2012-2014 and there has been a significant amount of research conducted since then on spider silk production in *E. coli*, some based on this research's results. Some further research worth mentioning includes: changes in the media to increase glucose availability, lowering the temperature at which the fermentations were performed and using a co-expression system with two different vectors, one containing the additional tRNAs and the other containing the targeted silk gene. The field has changed by substantially, researchers can now attain spider silk that more closely mimics the native silk in properties and size, as well as, attain consistent yields of greater than or equal to 1 g/L^{94,113,114}. A manuscript is currently in progress from members of our spider silk laboratory, with Sreevidhya T K, PhD as the primary author.

Conclusion

While there was a lot more research required, some of which has since been conducted, a commercially viable rSSp process has proven to be attainable in *E. coli*. By altering fermentation conditions and utilizing varying vectors to increase tRNA availability, rSSps can be produced that more closely mimic the mechanical properties innate to native spider silk. The research conducted for this dissertation alone improved rSSp yields by five-fold. These extraordinary rSSps can now be produced at 1 g/L in *E. coli* fermentations, therefore are now commercially viable products.

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CHAPTER 3

SPIDER SILK PRODUCTION IN AGROBACTERIUM TRANSFORMED *MEDICAGO SATIVA*

Chapter Preface

This chapter presents research conducted to develop transgenic *Medicago sativa*, more commonly known as alfalfa, that produces recombinant spider silk proteins (rSSps). Due to alfalfa's high protein content (24% dry weight), it is a great option for long-term commercial production of synthetic spider silk. Past research has seen yields as high as 2% dry weight rSSps produced. While most synthetic materials produced in alfalfa are only peptides (50-100 bp), in this research much larger spider silk genes (2000-6000 bp) were integrated into the plant's genome. These genes translate into 80-110 kD rSSps. The goal of this research project was to create an economically feasible system to produce rSSps, utilizing alfalfa as the transgenic host.

Using molecular cloning and bioengineering methods an *Agrobacterium tumefaciens* mediated transformation was conducted using spider silk genes inserted into a newly constructed plant tumor inducing vector, MpRI201AN, which was part of this research and based on the commercially available pRI201AN from Takara ®. MpRI201AN contains all the necessary DNA elements and tags to increase targeted gene expression and retain the proteins in the endoplasmic reticulum (ER), which will ensure that the synthetic proteins will only be expressed in the leaves of the genetically modified plants. This modified vector not only simplifies the synthetic protein purification process, but it also removes waste management costs and creates a secondary source of revenue,

as the stems and other waste from the plants can be used as either feed for livestock or ethanol production. Unlike the stems and roots, the leaves are much easier to harvest and process.

During the course of this research issues arose during purification via a N-terminal poly-histidine tag due to the plant's chlorophylls interacting with the resin containing the nickel ions used for immobilized metal ion high performance liquid chromatography (IMAC/HPLC). In order to successfully extract and purify rSSps from alfalfa, a new purification process was created. In collaboration with Dr. Charles Miller from Utah State University's Biological Engineering department, a bacterially derived synthetic chlorophyllase was used to cleave the phytol tail, the portion of the chlorophyll molecule that was adversely interacting with the resin of the nickel packed column, from the chlorin magnesium ring. By conducting a sequential precipitation, the rSSps were successfully separated from the phytol tail. Whether this enzymatic reaction combined with an alcohol/salt precipitation is sufficient to produce a pure enough synthetic protein is yet to be seen. The synthetic protein may still need to be further processed to ensure purity, either through ammonium sulfate precipitations or HPLC. We have hypothesized that HPLC will prove unnecessary and the purified rSSps can be attained through ammonium sulfate precipitations. It should be noted that each additional step added to the purification process causes a decrease in yield, due to rSSp loss in each step. These issues will need to be analyzed and possibly addressed if proven significant, via downstream processing methods to create a viable production process in alfalfa.

As has been observed in bacterially derived spider silk, alfalfa derived spider silk does not store well when frozen, rendering the rSSps completely insoluble that make extraction and purification, as of yet, impossible. Due to this and some other unforeseen issues with machinery malfunctioning and plant health issues, a pure rSSp and quantifiable yield has not been obtained. Currently new plants are in development including: 1) a MaSp2 + C-term plant, which is the protein that is used throughout this research and 2) 3X and a 12X aggregate silk protein plants.

This research lays the groundwork toward creating transgenic alfalfa plants capable of producing native-sized spider silk proteins at a commercially viable yield. This current and future research is being conducted to produce a successful rSSp production process using alfalfa as the genetic host and answer questions into the production processes' feasibility for long-term production system.

Introduction

Medicago sativa, more commonly known as alfalfa, is a perennial crop that can be harvested in most locations four to ten times a year and has been utilized as a genetic engineering host since the 1980s with great success, producing many different synthetic proteins and peptides at a high yield¹¹⁵. Alfalfa was approved by the USDA more than a decade ago as an appropriate transgenic host for synthetic protein and peptide production. Companies, such as Medicago® and Forage Genetics®, currently grow transgenic fields for commercial product^{95–97,99,116–119}. Total protein content in alfalfa is up to 24% dry weight, and past research has shown yields of up to 5% dry weight synthetic protein. Other attributes that make alfalfa desirable as a host for synthetic protein production, is that it has no common relatives and, therefore, cannot cross-breed with any other plant species¹²⁰. A buffer area of 50 feet has proven to adequately ensure that the synthetic crop is isolated from other non-genetically modified alfalfa fields; this is due to the area of cross-pollination that is inherent to alfalfa. Our laboratory has determined that a 2% dry weight spider silk yield in alfalfa would produce 218 kg protein per acre per year, a yield that supports commercialization (Table 3-1).

Table 3-1 | A comparison of synthetic spider silk protein production hosts, current predicted yields and measured rSSp sizes

Comparison of Protein Yields		
System	Protein Yield	Protein Size (kDa)
Bacteria	3 kg per 30,000 L	50-70
Goats	18 kg per goat per year	65
Silkworms	2-5% spider silk	80-240
Alfalfa	218 kg per acre per year	80
Data from Randolph Lewis Laboratory		

An added benefit is that all spider silk protein will be produced in the leaves, so the “waste” can be used as either livestock feed or for ethanol production, which are already developed processes in alfalfa^{98,121}. These two processes essentially eliminate all waste removal costs associated with synthetic protein production and provide a secondary source of revenue. Alfalfa is also hypothesized to be capable of producing the large (200-350 kDa) and highly repetitive spider silk proteins without premature translational termination, since some native alfalfa proteins are of a similar size.

A few attempts have been made to produce spider silk-like proteins in plants^{68,69,122}. Recombinant silk proteins were produced in potatoes and tobacco, but they were not purified or mechanically tested, and they were small monomers of only ~35 kDa. Production in alfalfa provides a much more promising system for recombinant spider silk proteins (rSSps) than other plants that have been utilized due to its’ high protein content and previous synthetic protein production approvals through the USDA.

The main focus of this research project was to improve rSSp yields and create a long-term commercially sustainable process for synthetic production by implementing spider silk genes into alfalfa. Spider silk genes were inserted into alfalfa’s genome via an *Agrobacterium tumefaciens* mediated transformation using an in-house Ti vector, MpRI201AN, of alfalfa cultivars. A poly-histidine tag, other various protein expression tags and regulators were cloned into a new expression vector to amplify protein expression, ease purification techniques, and cause targeted rSSp expression. The rSSps will be retained in the endoplasmic reticulum of the cells, ensuring that vast majority of expressed protein will be present in the leaves and not in the stems or roots of the plants. The theory for this research was that a new production method for rSSps could be created

that would improve yields and expand rSSp production capabilities, by expressing native sized synthetic proteins that would have improved mechanical properties.

Materials and Methods

Cloning

For this research a new expression vector was created with the ability to transform into both *E. coli* and *A. tumefaciens*, and ensuring that targeted genes would be retained in the endoplasmic reticulum (ER), causing the protein to only be expressed in the leaves of the transformed cultivars. Some modifications that needed to be added to the commercially available pRI201AN from Takara ® were: 1) the two multiple cloning sites needed to be combined into one multiple cloning site that would contain both a NdeI site on the N-term and a BamHI site on the C-term, as these are the sites used for cloning of all in-laboratory rSSps, 2) the 5' end of the vector, upstream from the NdeI site, needed to contain a Leb 4, His₆-tag, Xpress Epitope, while retaining a viable NdeI site, so the enzyme used in the adaptor needed to be compatible/nonregenerable to NdeI in this case the MseI restriction endonuclease was utilized, 3) a Gly-Lys-Lys-Gly sequence was added between the Leb 4 and his₆-tag, to address any purification issues and 4) the 3' end of the vector, downstream from the BamHI site, needed to contain ELP repeats, KDEL, a SacI site, and a KpnI site so that a HSP (heat shock protein) terminator could be integrated there (Table 3-2 and Figure 3-1). These N-term and C-term adaptor sequences were codon optimized for *Medicago sativa* and synthesized (Life Technologies).

Table 3-2 | List of tags and sequences necessary for *Agrobacterium* transformation into *Medicago sativa* that will result in targeted protein production in the leaves of the transgenic plants. The name of the sequence and their function within the vector and plant are described.

TERM	FUNCTION
Cam35S promoter	A promoter from the cauliflower mosaic virus. It is a very powerful promoter and is not greatly influenced by environmental conditions or tissue types
HSP terminator	Heat shock protein derived terminator that allows for higher target gene expression. Has a higher expression rate than the traditional NOS terminator
Leb 4	Permits the translocation of the protein to the endoplasmic reticulum
Xpress Isotope	Helps increase protein expression
ELP repeats	This is used as a tag and also increases protein expression
KDEL	This causes the protein to be retained in the endoplasmic reticulum. Research has shown that ER retention allows the most protein to be accumulated
Gly-Lys-Lys-Gly	Removes the N-term with trypsin. Backup plan for if the N-term causes problems with protein purification and/or alters the spider silk protein's properties

N-term Insert:	
5'TTAAATGGCTTCCAAACCTTTCTATCTTTGCTTTCACCTTCCTTGCTTCTCTTTACAAGCACATGTTTAGCA GGAAAAAAGGGACACCATCACCATCACCACGATCTTTATGATGATGACGATAAGCATATG ^{3'}	
Leb 4	Gly-Lys-Lys-Gly
	His ₆ -tag
	Xpress Epitope Tag
C-term Insert:	
5' GGATCC(GTTGGTGTTCTGGA) ₅ AAAGACGAACCTAGGAGCTCTAGGGTACCGAATTC ^{3'}	
ELP repeats	KDEL stop

Fig. 3-1 | N- and C- term adaptors synthesized by Life Technologies. These amplify targeted protein production and cause the synthetic proteins to be retained in the endoplasmic reticulum, therefore the leaves, of the alfalfa plant.

The N- term adaptor was cloned into pRI201AN by first digesting both the pRI201AN vector and the N-term adaptor with the restriction enzymes MseI and NdeI, and then ligating the vector and adaptor together using a T4 DNA ligase (New England

Biolabs M0202S); the resulting ligations were electrotransformed into XLBlues at 1800V. Ultimately, these transformations proved unsuccessful. It was determined that because the MpRI201AN DNA was so large, 10,719 bp total, that any cloning to occur to build and design the vector or to insert genes into the expression vector once it was constructed needed to be treated differently. Due to folding issues within the DNA pieces used to build the vector 0.2% DMSO was added to the ligation mixture and, to slow the ligation down, it was conducted on ice for 15 minutes, rather than at room temperature for 10 minutes. These changes in the ligations still did not yield any successful clones, despite showing the correct ligation upon enzymatic digestion checks on 0.8% agarose gels.

Ultimately, another variation of competent cell was needed that was specifically for building and transforming large ($\geq 10,000$ bp) constructs: chemically competent β -10s. These proved far more successful when transformed with the ligated pRI201AN and N- and C-term adaptors, as well as any further cloning within the newly built MpRI201AN. Once clones with the N-term adaptor were screened and verified, the C-term was inserted into the BamHI and EcoRI restriction enzyme sites through the same process. The new vector was named MpRI201AN and verified via DNA sequencing.

This research was a continuance of research conducted at the University of Wyoming by Holly Steinkraus. During the prior research a MaSp2 16-mer was inserted into the BamHI and NdeI sites of an expression vector, R111, obtained from Forage Genetics®, this construct was electrotransformed into XL1-Blue Supercompetent Cells (Agilent/Stratagene C2992) at 1800V (Figure 3-2). Once the plasmid was verified via

DNA sequencing it was electrotransformed into LBA4404 *Agrobacterium* competent cells (Thermo Fisher Scientific 18313015). Upon successful transformation into *Agrobacterium*, the cells could then be used to agrotransform alfalfa.

5' AAGCTTGGATCCACGCC(GGCCCGGGTAGCGCTGCCGCAGCGGC
AGCTGCAGCTGGACCTGGGGGCTATGGACCAGGTCAACAGGGAC
CAGGAGGTTACGGTCCTGGCCAGCAAGGTCCTTCC)₁₆GGAGATGAT
GACGATAAGTCTAGAGGATCCACTAGT^{3'}

Fig. 3-2 | Nucleotide sequence for a synthetic major ampullate spidroin (MaSp2) with a conserved C-term sequence. Nucleotides in red represent the repeated monomer, this monomer is repeated 16X making this sequence a 16-mer.

Other rSSp genes that were ligated into the BamHI and NdeI restriction enzyme sites of MpRI201AN included: 3x aggregate + linker, 9X aggregate + linker, 12X aggregate + linker, and (MaSp2)₁₆ + C-term. All spider silk sequences were extracted from their base vectors (pMK or pBluescriptII) by digesting with BamHI and NdeI restriction enzymes from NEB and then running gel electrophoresis on a 1.2% agarose DNA gels at 50V for 1 hour to separate the cut plasmid and the targeted inserts were cut out with a razor blade. A QIAquick gel extraction kit (QIAGEN 28704) was used to extract the DNA from the gel slices according to company protocol.

Agrobacterium transformation and *Medicago sativa* Cell Culture

Alfalfa agrotransformation protocols were obtained from Forage Genetics International. The appropriate strain of *Agrobacterium* (LBA4404, ElectroMAX™ A. *tumefaciens* Thermo Fisher Scientific 1810315) was streaked out on a plate of YEP (10g/L Peptone, 10 g/L yeast extract, 5 g/L NaCl with 50 µg/mL kanamycin) and incubated at 28°C for 2-3 days. At 12 pm the day before transformation a flamed loop

was used to scoop up 2-3 colony forming units and grown overnight in 125 mL YEP flask, shaking at 27-28°C and ~200 rpms. The OD₆₆₀ was checked before transformation, typically a healthy OD₆₆₀ should be approximately 1.4-1.5. *Agrobacterium* was pelleted for 10 minutes at 3700 rpm in a 50 mL tube. 40 mL room temperature 1/10th SHDN (Macronutrients, micronutrient's, iron-EDTA, vitamin, sucrose, agar 2,4 D and kinetic) bacterial resuspension media was used to resuspend the cells.

1 mL of liquid SHO (Iron-EDTA, alfalfa vitamins and nutrients, sucrose and agar) was pipetted directly onto the SHDN (SHO, 2,4-D, and Kinetin) plate and a sterile Whatman filter paper was placed on plate. Using forceps and a sharp scalpel, leaves were removed from R2336 box plant (Forage Genetics) and put into the *Agrobacterium*. The leaves were sliced into 2-3 mm pieces, blotted on the Whatman paper and transferred to paper-lined SHDN plate (40-50 pieces/plate) with no antibiotic selected. Plates were sealed with masking tape and incubated at 25-26°C, without light, for 3 days.

After 3 days, the plants were transferred to SHDN + Timentin (150 µg/mL). If overgrown with *Agrobacterium* they were washed with Timentin. After another 3 days, the explants were thinned to 20-25 per plate. Plates were thinned as calli grow to 10-15 per plate, depending on their size. This is the point when kanamycin is added to the plates at 50 µg/mL. After 6 weeks growth if plants are clearly dead, they are discarded. Callus starts to develop green buds or embryos and at this point they are transferred to regeneration or induction media containing hormones to promote leaf and root development. Embryos are separated from the campus after 2-3 weeks (Figure 3-3 A). After three additional transfers on kanamycin selective media well-developed embryos

were transferred onto SHO Timentin (50 $\mu\text{g/mL}$). Now embryos are transferred to new media every two weeks. Once roots were sufficiently developed the plantlets were transferred to magenta boxes containing SHO Timentin (100 $\mu\text{g/mL}$) (Figure 3-3 B). Rooted plants were transferred to soil and maintained in a Conviron standing incubator. Optimal conditions include: 25°C, 16 hours light and humidity.

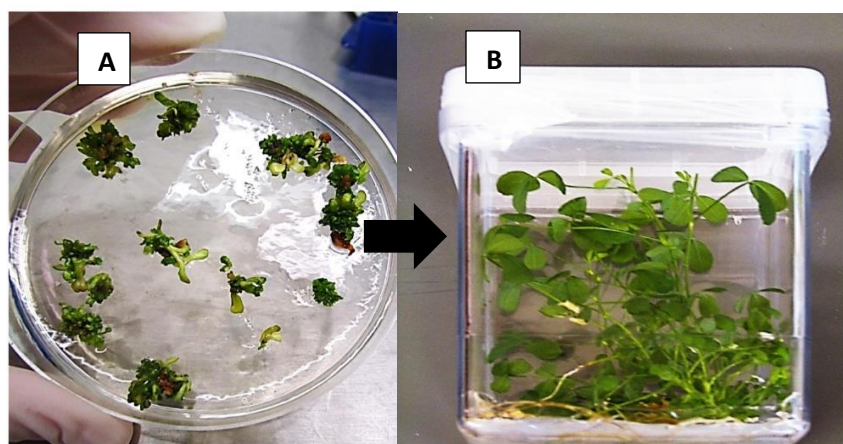


Fig. 3-3 | Photographic images of the stages in alfalfa plant cell culture. A) Is a picture of a developed *Agrobacterium* transformed callus that has started to develop leaves and roots due to growth hormone stimulation. B) Shows a fully developed alfalfa cultivar in a magenta box of a transgenic plant containing the MaSp2+C-term protein/gene in the leaves.

Western Blot Analyses

All Western Blots conducted in this research to determine protein production, for both rSSps and chlorophyllase followed the following protocol: 35 μL loading samples were prepared with a 1:1 of protein sample to be analyzed to 2XSABU, samples were boiled for 10 minutes in a water bath and then loaded onto a 4-20% tris-glycine SDS-

PAGE gel (Invitrogen XP04200BOX) and run at 135 V for 90 minutes. Gels were transferred to nitrocellulose membranes overnight in a 4°C freezer room on ice at 25 mA in a wet transfer apparatus. The blots were conducted on the nitrocellulose membranes with a primary antibody produced in mice that targets the histidine tag (Rockland 200-303-382, 1:1000 dilution). A rabbit anti-mouse antibody conjugated with Alkaline Phosphatase (AP) was used at a 1:5000 dilution (Abcam ab6729). The membranes were developed using 1-StepTM NBT/BCIP Substrate Solution (Thermo Fisher Scientific 34042) for imaging.

Purification

Leaves were harvested from transgenic alfalfa plants manually and stored in Ziploc ® bags at -80°C. In order to extract the proteins, the leaves were ground up using a mortar and pestle in a 1:3 (wt/v) binding buffer (8M Urea, 20 mM Tris-HCL, 0.5 M NaCl, 2 mM EDTA). Three freeze (-80°C for 10 minutes), thaw and grind cycles were conducted to homogenize the plant cell tissue adequately. The ground plant material was centrifuged at 16,000 rpms for 30 minutes and the cell lysate (supernatant) was taken off the plant cell tissue pellet as the cell lysate. This process was repeated until the cell lysate was confluent or clear of all solid plant material, on average four times. When done at room temperature there was inadequate tissue emulsification, leading to a lot of solid plant tissue that wasn't processed. After some experimentation a freeze/thaw cycle was concluded to lead to the best plant tissue processing method, with no solid pieces of plant tissue present after the final cycle. Three freeze (-80°C for 10 minutes), thaw and grind cycles were conducted to fully homogenize the plant cell tissue adequately.

Originally, it was believed that this alfalfa cell lysate could be purified using the poly histidine tag, so purification was attempted using immobilized metal affinity high performance liquid chromatography (IMAC-HPLC). An AKTA Avant was used with Ni-NTA resin (GE XK-16, bed height 14 cm). The sample was applied to column at 1 mL/minute and clarified lysate was applied across the column from both the top and bottom. Halfway through all attempts to purify using this strategy the pressure would get so high in the column that the run would automatically abort. The Ni-NTA resin would turn completely green, the exact color of the chlorophyll in the plant extracts, and all efforts to remove the attached chlorophyll molecules were unsuccessful and it was determined that in order to purify rSSps from alfalfa the chlorophyll molecules would have to be removed from the cell lysate prior to any processing and purifying using HPLC.

Chlorophyllase Production and Purification

After much literary research and thought it was determined that the reason the chlorophyll was interacting with the Ni-NTA resin was because of the phytol tail portion of the chlorophyll molecule. Several chlorophyll removal processes were considered as options, including: chemically removing chlorophyll, precipitating the alfalfa cell lysate to separate the chlorophyll from the protein, size exclusion chromatography and an enzymatic reaction that would cleave the phytol tail of the chlorophyll molecule from the chlorin ring. The chemical removal of chlorophyll from the protein lysate was determined to be too expensive to upscale, due to chemical costs and waste removal costs, since some of the chemicals used in this reaction are non-reusable and very caustic. The

precipitation option turned out to be a fruitless option, as the rSSp would end up in the same phase as the chlorophyll due to their similar charges and other characteristics. Size exclusion chromatography was also not a valid option, since there are some rSSps that are similar in size to that of the chlorophyll, ~250-300 kDa. It was decided that using an enzyme to break up the chlorophyll molecule was the best route and fortunately, Dr. Charles Miller from the Biological Engineering Department at Utah State University had just concluded an IGEM project that cloned a synthetic chlorophyllase (TaChl) into an expression vector for production in *E. coli*. The TaCHL is a chlorophyllase enzyme from a common wheat species and is a 37 kDa enzyme that has a histidine tag and can, therefore be purified using IMAC¹²³.

This chlorophyllase (TaCHL) acts by cleaving the chlorophyll molecule into two subunits: the chlorin magnesium ring and the phytol tail. In order to remove the chlorophyll molecules from the alfalfa cell lysate, deterring any interactions with the charged rSSps and HPLC purification, an enzymatic reaction was conducted followed by an alcohol precipitation.

This synthetic chlorophyllase was produced in a 1 L *E.coli* flask culture. The culture was inoculated with 1 colony forming unit from a chloramphenicol (50mg/mL) agar plate containing the synthetic chlorophyllase construct. Cultures were grown in 1L LB Broth media (Miller 71753-6) and selection was conducted with chloramphenicol (50 mg/mL). Flask cultures were grown until an OD₆₀₀ of 0.8 was reached and then expression was initiated with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) and cultured an additional 4 hours. Cells were harvested from the flask culture by centrifuging at 6000 rpms for 30 minutes and the cell pellet was processed in an 8M Urea

binding buffer (8M Urea, 20 mM Tris-HCL, 0.5 M NaCl, 2 mM EDTA) at 1:3 weight to volume dilution. Sonication was conducted on ice at 1 W for 4 minutes, followed by a minute rest, and this process was repeated 3 times (QSonica $\frac{3}{4}$ " diameter probe). The cell lysate was harvested through centrifugation at 6000 rpms for 30 minutes, retaining the supernatant as the final cell lysate.

Protein purification was conducted using immobilized metal affinity high performance liquid chromatography (IMAC-HPLC) utilizing the polyhistidine (6X) tag on the TaChl (35 kDa) constructs, either at the N- or C-terminus depending on the vector. An AKTA Avant was used with Ni-NTA resin (GE XK-16, bed height 14 cm). Sample was applied to column at 1 mL/minute and clarified lysate was applied across the column from both the top and bottom, separately to ensure complete application. The column was washed with running buffer (0.5 M NaCl, 20 mM Tris-HCL, 0.5 mM EDTA) and wash fractions were collected. 5 mL elution fractions were collected for 3 column volumes at 5%, 10% and 100% imidazole. Samples of these fractions were mixed 1:1 with 2X SABU with 6 M Urea and heated for 15 minutes at 100°C. These samples were analyzed by SDS-PAGE and western blot as described above to verify protein purification.

Chlorophyllase Reaction

Attempts were made to separate the chlorophyll, or at least the phytol tail portion that was causing issues with rSSp purification, from the alfalfa derived rSSp. Synthetic chlorophyllase (TaChl) was combined with the protein lysate, and in order to induce enzymatic activity of the TaChl 1:1 acetone:water was added to the solution and shaken at 2500 rpms, to ensure distribution, at 37°C, which is the optimal temperature for the chlorophyllase enzyme. After 30 minutes of enzymatic activity a precipitation was

conducted at room temperature using two volumes 3:4 (heptane:acetone). Separation was aided by centrifuging the reactions for 30 minutes at 3500 rpms. This precipitation was conducted in glass vials or glass capped tubes due to the erosive nature of heptane. At this point the chlorophyllase had enzymatically cleaved the phytol tail from the chlorin magnesium ring and the two resulting molecules had been separated by precipitation into different phases. Two controls were used to verify that rSSp separation was due to the chlorophyllase reaction, and not just the precipitation: 1) a precipitation control with no alfalfa lysate and 2) an alfalfa lysate precipitation with no TaChl (Figure 3-4). This particular precipitation protocol was used because, despite heptane being a harsh chemical it is reusable and is contained in the upper phase of the precipitation. The heptane can be decanted off of the other two phases and placed in a glass vial until needed for the next precipitation.

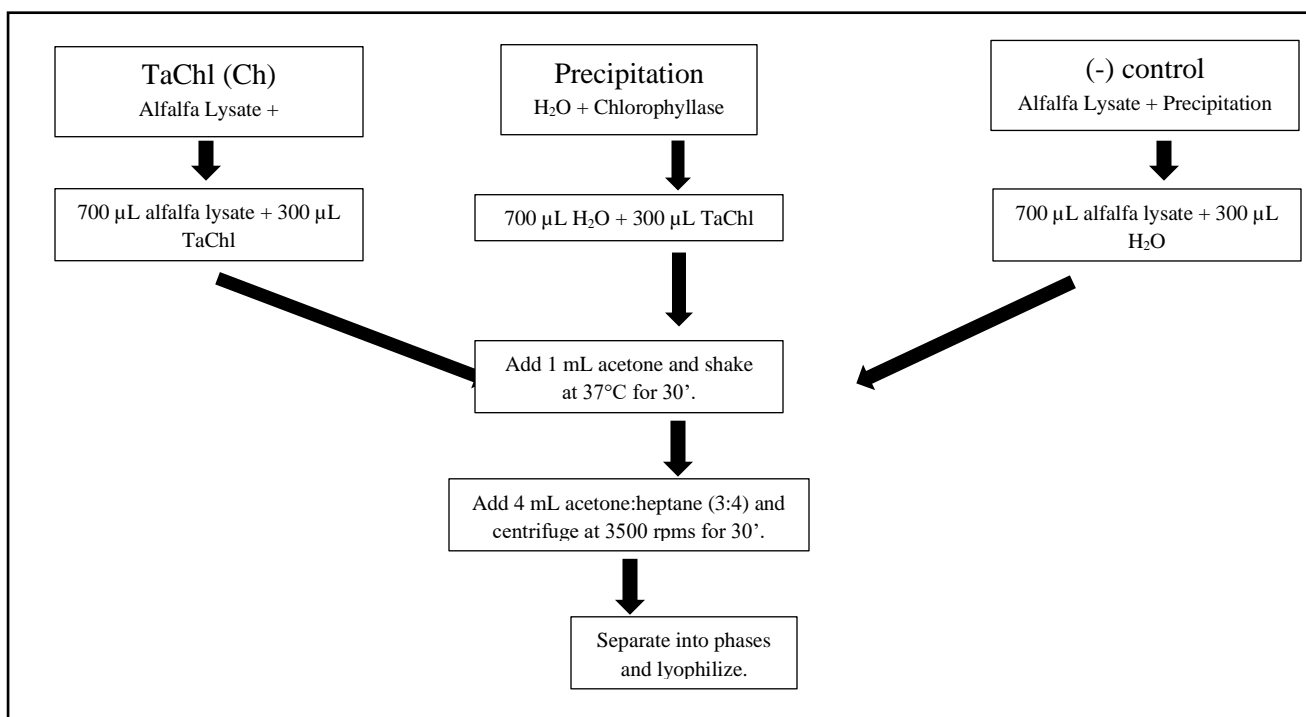


Fig. 3-4 | Flow diagram showing the chlorophyllase experiment progression conducted on alfalfa protein lysate containing the synthetic MaSp2.

The phases were separated into an upper phase, which includes the heptane and phytol tail portion of the chlorophyll molecule, an intermediate phase, where the chlorin magnesium ring would separate into, and a lower phase, where it was theorized that the rSSps would precipitate to. These separated phases were dried overnight, until all liquid was removed, and resuspended in 200 µL 2X SABU. All samples were analyzed using the western blot analyses protocol listed above, to determine which phase the rSSp precipitated into.

Results and Discussion

The MaSp2+C-term plants originally from University of Wyoming were tested by extracting protein from the alfalfa and running the lysate across an SDS-PAGE gel and analyses on a nitrocellulose membrane via western blot analyses. The MaSp2+C-term is an ~80kDa protein and was shown to be present in the *Agrobacterium* transformed plants that Holly Steinkraus had made (Figure 3-5). Now that we had proof that a spider silk protein was being produced in the alfalfa plants, a purification process and yield determination were needed.

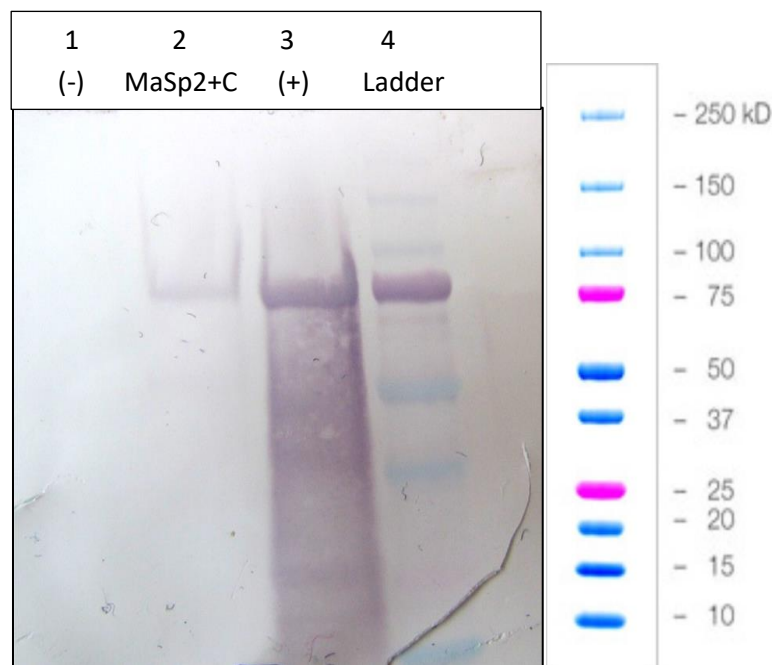


Fig. 3-5 | Western blot showing a MaSp2 spider silk protein with a C-term (80 kDa).

Proteins were produced and crudely extracted from a transgenic alfalfa plant in an 8M Urea buffer (well 2), in comparison to a plant cultivar that has not been transformed (well 1) and a positive his-tag spider silk produced in *E. coli* (well 3).

All purification attempts on the AKTA Avant (HPLC) with the cell lysate caused the nickel columns to be bound and clogged up by the chlorophyll molecules, causing the columns to turn green, and ultimately the program would auto abort due to the increase in pressure. Once the columns were bound up with the chlorophyll, we were unable to recover the spider silk or clean the columns with any amount of treatment with imidazole or other solvents. We were not only unable to obtain any spider silk protein from this purification method, but we were also ruining hundreds of dollars of materials upon each attempt. At this point in the research an alternative purification method was needed and it was decided to utilize a chlorophyllase enzyme to break apart the chlorophyll molecule, followed by a precipitation to separate the two chlorophyll components from the spider silk proteins.

Chlorophyllase experiments yielded three different phases for the chlorophyllase reactions and two phases for the precipitation controls. In the chlorophyllase reactions the upper phase or layer consists of the heptane and, if successfully removed in the chlorophyllase reaction, the phytol tail, the intermediate phase contains the alcohol and chlorin ring of the cleaved chlorophyll, and the lower phase is all the precipitates. Visually the precipitations of the different experiments show that when the chlorophyllase is used there is a definite alteration in the color of the upper and intermediate phases, versus the experiment with no enzyme; this is a good visualization that the enzyme was working (Figure 3-6). In order to determine if this color difference is actually a response to TaChl activity and separation of the phytol tail, chlorin magnesium ring and the rSSp a western blot analyses was conducted on the lyophilized phases resuspended in 2XSABU. Western blot analyses showed the presence of the TaChl (35

kDa) as a positive control and in the intermediate phases of the enzyme and no enzyme reactions and the lower phase of the precipitation control. The MaSp2 + C-term is precipitated into the lower phase of both the chlorophyllase and no chlorophyllase precipitations (Figure 4-7). Since we know that the phytol tail remains in the heptane upper phase and the chlorin magnesium ring precipitates to the intermediate phase, and the rSSp shows up in the lower phase, this means that the rSSp has been effectively separated from the chlorophyll, specifically the phytol tail that interacts with the nickel column during HPLC.

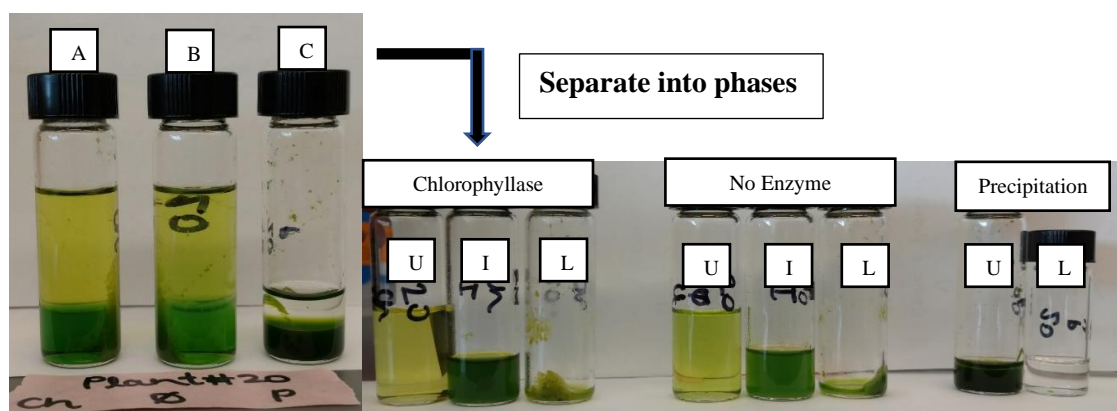


Fig. 3-6 | Chlorophyllase experiments conducted on alfalfa plants containing MaSp2+C spider silk protein. A) A chlorophyllase reaction conducted on a plant protein extract with a precipitation, B) a negative control that was just a precipitation on a plant protein extract containing heptane, but no enzyme, and C) a precipitation control with no heptane added and no chlorophyllase reaction. The precipitations were separated into phases designated U (upper phase), I (intermediate phase) and L (lower phase).

Some issues arose with mold contamination of the spider silk cultivars and a power surge in the incubator that caused the lights to stay on 24-hours a day rather than following the program caused all original MaSp2 + C-terminal plants to wilt and eventually all died before the issues could be addressed. Efforts are being made creating

new MaSp2 and MaSp1 spider silk plants that will take 6 months to go to soil once the genes are successfully agrotransformed into R2336 trifoliate. Also, now that 3X aggregate has been integrated into the MpRI201AN vector and 6X, 9X and 12X aggregate are currently being cloned into the MpRI201AN transformation vector, new spider silk plants with rSSps that are similar in size to native spider silk proteins will be agrotransformed into alfalfa cultivars soon by future members of the laboratory.

Another setback that has occurred, is that much like is the case in *E. coli* produced rSSps, the rSSps become insoluble over time when the leaves are stored at -80°C. Efforts have been made to solubilize the rSSps, including: altering the binding buffer used to contain a higher concentration of Urea, EDTA, etc., after the leaves were grinded in the binding buffers they were subjected to sonication and microwaving before being further processed by centrifugation and 2% Sarkosyl was added to the 2XSABU used for western blot analyses. None of these attempts were successful in solubilizing the rSSp. rSSp presence is known to be in these specific plants, because past research and western blot analyses have shown this, but they are as of now undetectable and unobtainable. These findings have stalled efforts in defining a rSSp yield and obtaining a pure protein product from the alfalfa. While this is unfortunate, the efforts listed above at creating new plants agrotransformed with rSSps are currently being performed in an effort to develop a yield and define a purification process.

Future Research

Future research should include the *Agrobacterium* transformation of other rSSps into alfalfa, namely spider silk genes of varying size and type. One spider silk protein that

would be of particular interest, is aggregate. Aggregate is a glycosylated glue-like spider silk protein that has previously been produced in *Spodoptera frugiperda* (SF9 cells), and due to alfalfa's innate ability to post-translationally modify proteins to be glycosylated, could be successfully produced in alfalfa at a much higher yield and lifetime production than in the SF9 cells.

Other interesting rSSps would be any that are on the same size scale as those that are native silks, 250-500 kDa. While other rSSp hosts have produced novel proteins, with mechanical properties in-line with those found in native silks, none have produced them on the same size scale. Since spider silk's mechanical properties are directly related to the size⁴, and therefore quantitative repetitiveness of the mechanical characteristic's motifs, the rSSps with the same size should inherently have superior mechanical characteristics. The mechanical characteristics currently exhibited by rSSp products are due to post-production manipulations, so in theory, if proteins more closely mimicking the native proteins are created then the products will be far superior to those that are smaller rSSps.

Conclusion

This research has shown, for the first time, that rSSps can be successfully produced in alfalfa. By genetically engineering a new expression vector and creating a downstream process for purification, spider silk is now being produced in alfalfa and a long-term commercially viable production system is on its way to being characterized. A protocol was developed and tested to separate the rSSp from the chlorophyll, and for the first time a synthetic protein was separated from the chlorophyll in the alfalfa, utilizing a synthetic chlorophyllase enzyme and precipitation. Efforts are being made to determine a

rSSp yield. A publication is in the works and the only data needed to finalize is a rSSp yield from the alfalfa. Overall this research has shown that large rSSp can be successfully produced and extracted from alfalfa.

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CHAPTER 4

EUKARYOTIC CELL CULTURE PRODUCTION OF GLYCOSYLATED AGGREGATE SPIDER SILK PROTEINS IN *SPODOPTERA FRUGIPERDES*

Chapter Preface

The following chapter describes the production of synthetic aggregate spider silk proteins in the fall army worm, *Spodoptera frugiperdes* (SF9 cells), using eukaryotic adherent cell culture techniques and in *Escherichia coli*. The aggregate spider silk protein is a glue-like protein that forms into droplets dispersed across the spider's web, and functions as an adhesive assisting in prey capture. Like many naturally occurring proteinaceous glues, aggregate is a post-translationally glycosylated protein.

Aggregate silk proteins have never been synthetically produced or purified in their natural glycosylated state. Researchers have shown a direct correlation to “stickiness” or the glue-like adhesion properties in proteins via glycosylation. Due to this knowledge, we deemed it necessary to create a glycosylated synthetic aggregate silk protein that would more closely mimic the native aggregate glue-like protein. As there are no prior studies creating glycosylated spider silk proteins, a new production process was necessary.

In order to create a synthetically derived aggregate protein that has N- and O-glycosylation, a production system was needed that had the capability to engineer these post-translational modifications. *Spodoptera frugiperdes* was chosen as a host to create these glycosylated spider silks, due to its' ease of culture and inherent capability to post-translationally modify proteins. In order to compare and contrast the mechanical

influence glycosylation lends to aggregate silk, the synthetic aggregate proteins were also expressed and purified from *Escherichia coli*, which is a common production method for recombinant spider silk proteins (rSSPs), but does not post-translationally modify proteins to be glycosylated.

Spodoptera frugiperdes SF9 cells were used as the cell culture line to produce the glycosylated aggregates, and the bac-to-bac baculovirus transfection system was used to create a recombinant cell line containing the genes for the aggregate silks. Using an aggregate spider silk DNA sequence, plus a linker and C-terminal, which is highly conserved within all spider silks and among different species of spiders, a synthetic sequence was synthesized by LifeTechnologies ®. Four different synthetic sequences were then cloned, ranging from a 3X repeat of the monomer sequence to a 12X repeat of the monomer. These synthetic aggregate DNA sequences were cloned into the baculovector pOET2 and the resulting plasmids were used to transfect SF9 cells using the bac-to-bac transfection system. The infected SF9 cells were grown to create viral titers, which were then used to infect other SF9 cells that would in response produce the synthetic aggregate proteins under the correct culture parameters. Glycosylation of the aggregate proteins were determined upon treatment with a deglycosylation kit and subsequent analysis via Western blot and also by comparison to bacterially derived synthetic aggregate proteins.

The research presented in this chapter shows that synthetic aggregate spider silk proteins can be produced utilizing eukaryotic cell culture in SF9 cells. Evidence is also shown indicating that the synthetic aggregate proteins are glycosylated. A purification process utilizing a histidine tag and IMAC/HPLC is presented, as well as, some

difficulties that arose in purifications due to the aggregate protein being retained within the SF9 cells. The overall aim of this research was to create a novel synthetic aggregate protein that was glycosylated, purify the protein and create materials that could be mechanically tested, so that the strength of the synthetic aggregates could be determined and compared to those of other glues.

Research towards this project is ongoing, with aims towards defining a yield and creating testable glues using the synthetic aggregate proteins. Both the SF9 cell produced glycosylated aggregate and the bacterially derived non-glycosylated aggregate will then be mechanically tested and compared to the mechanical characteristics of both common glues and other synthetic spider silk glues. It is important to note that while aggregate production in SF9 cells is currently advantageous for bench top research and material characterization, there may be insurmountable yield issues when scaling up to a commercially viable level that will require an alternative production system also capable of post-translationally glycosylating proteins. While the research presented in this chapter is mostly a preliminary expression system as a proof of concept for synthetic aggregate production, it is essential to future research to mass produce aggregate spider silk proteins for material development and subsequent material testing.

Introduction

Among orb-weaving spiders there are two different methods that add stickiness to the web, allowing for prey capture and lashing different silks together¹²⁴. Cribellate silk is extruded onto the web, specifically the capture spiral, and aggregate silk proteins are secreted onto the web as droplets. Cribellate silk forms into bundles after being extruded from the cribellum and acts as a core fiber in pseudo-flagelliform silk. Cribellate silk is very elastic and, despite the core fiber rupturing at moderate extension, the rest of the bundle can extend to 500% of its original strength while maintaining fiber integrity¹²⁵. Over time spiders have evolved from using cribellate capture threads to adhesive capture threads containing aggregate protein^{126,127}. This is due to aggregates superior adhesion properties allowing greater prey capture potential^{128,129}.

Aggregate proteins are secreted as droplets forming a coating on flagelliform silk. The size and density of the droplets is dependent on the species of spider. At the center of each aggregate protein droplet there is a granule, which was originally thought to contain the glycoprotein glue responsible for the adhesive characteristics seen in aggregate⁵⁵. This was later disproved and it was theorized that the aggregate silk proteins form three layers: 1) the granule, which serves as an anchor for the protein, 2) an aqueous coating, and 3) a glycoprotein layer^{54,57,126}.

Aggregate proteins are particularly interesting due to their ability to vary the Young's Modulus depending on both the relative humidity and the speed with which the fiber is stretched. Aggregate proteins have a higher stress at a 40% relative humidity (RH) and lower stress at 15% and 90% RH^{130–132}. This makes aggregate spider silk

proteins highly desirable with their ability to still have a high adhesion in aqueous conditions. Of particular interest are underwater adhesives, since currently there is not a commercially available aquatic glue available, and surgical glues, as spider silk has been shown to be biocompatible and biodegradable^{2,10,12,133,134}.

Synthetic aggregate silk has never been produced and only recently has the nucleotide sequence been elucidated⁵⁸. Based on what is known of naturally occurring glues and adhesives, it can be surmised that glycosylation of the aggregate proteins is a driving factor in stickiness characteristics. A host system was needed to create synthetic aggregate proteins that were post-translationally modified to be glycosylated. As *Escherichia coli*, a common production system for synthetic spider silks, is not capable of these modifications, another fast production system was needed for research into aggregate production¹³⁵.

Spodoptera frugiperda (SF9 or SF21 cells) is the larval life stage of the fall army worm and has been used in eukaryotic cell culture for years to produce synthetic proteins, even to produce some recombinant spider silk proteins (rSSps)¹³⁶. The novelty in production via SF9/21 cells comes in the relative ease of culturing, fast generation time and, of particular interest for this research, the ability to post-translationally modify proteins, such as with glycosylation⁹³.

In this research SF9 cells were transfected using the bac-to-bac system to contain various synthetic aggregate genes. The synthetic proteins were expressed, purified and verified using western blot analyses and Coomassie staining of SDS PAGE gels. The findings in this study establish a production method for synthetic aggregate proteins that

are post-translationally modified to be glycosylated on the N- and O- sides of the amino acids. Studies are ongoing to define a yield and create products containing both glycosylated aggregate and non-glycosylated aggregate to compare and contrast their mechanical characteristics.

Synthetic aggregate genes were also inserted into an *E.coli* expression vector, pET19KT, and the rSSps were expressed in flask culture. These synthetic proteins would not have any post-translational modifications, namely glycosylation, and could, therefore, be used as a comparison to the SF9 cell derived rSSps. Both the size (kD) and the mechanical characteristics should be altered when glycosylation is present.

Materials and Methods

Cloning

For this research aggregate DNA sequences based on the research conducted by Hayashi et.al were used to create synthetic monomers with a linker and highly conserved C-terminal sequence⁵⁸. The synthetic aggregate monomers were synthesized by Life Technologies®. Four iterations of the aggregate monomer were desired: a 3X aggregate (3 repeats of the monomer sequence and a linker), 6X aggregate, 9X aggregate and 12X aggregate. In order to clone these sequences compatible non-regenerable sites were used²². Specifically, NdeI/AgeI and BspEI/XhoI restriction endonucleases were used. Aliquots of isolated plasmid were digested with NcoI and AgeI or NcoI and BspEI. The digests were run on an agarose electrophoresis gel at 50 V for 90 minutes to ensure band separation and the desired bands were extracted and purified using a gel extraction kit

(QIAGEN 28704). Using T4 DNA ligase the two extracted bands were ligated together (Thermo Fisher Scientific EL0011). Upon ligation the resulting plasmid should have 6X repeat of the aggregate repetitive region (Figure 4-1).

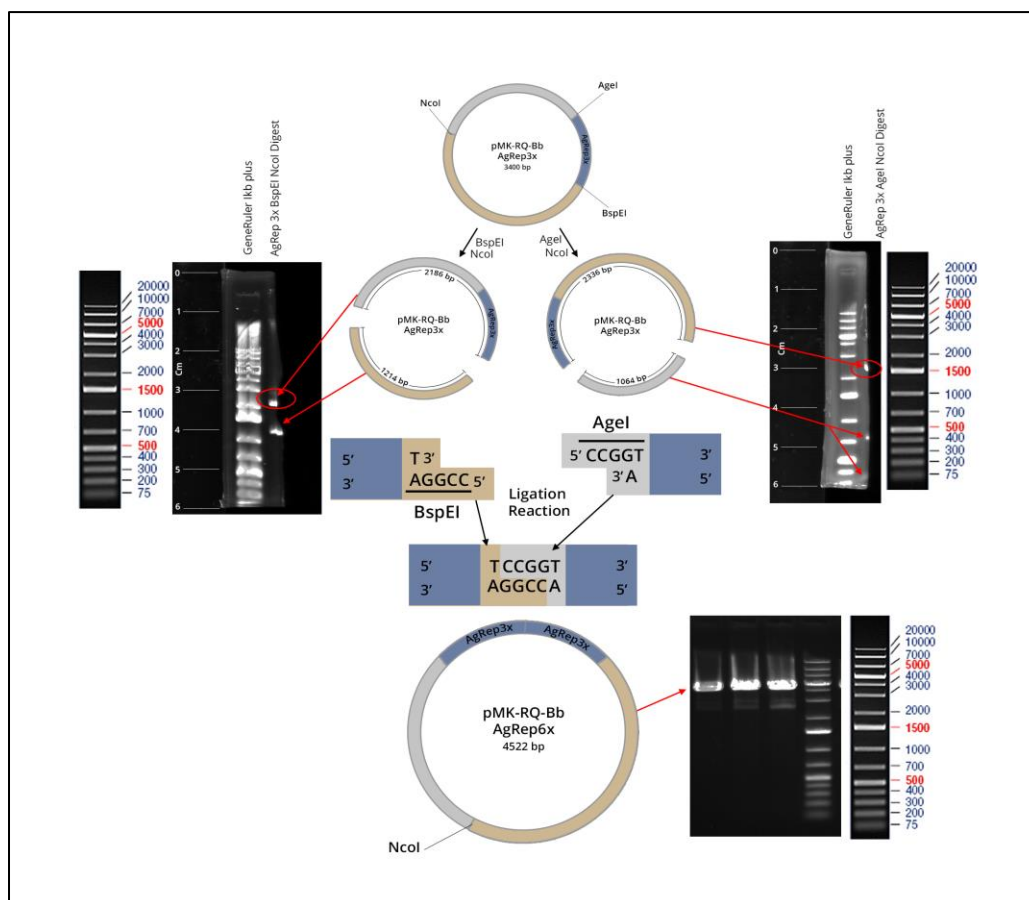


Fig. 4-1 | Formation of the 6X aggregate repetitive region. The respective aggregate monomer is in red. The figure shows destruction of the AgeI and BspEI sites during ligation. Red arrows indicate the gel electrophoresis bands that represent the specified sequences. Segments excised and purified are circled in red. Reprinted with permission from Kyle Berg.

Using the same cloning scheme, a 9X aggregate sequence was made from a 3X aggregate monomer + 6X aggregate polymer, and a 12X aggregate sequence was made from two 6X aggregate polymers cut with the same enzyme combinations (Figure 4-2).

All plasmid constructs were verified via digestion with BamHI and NdeI, as well as DNA sequencing. Once clones were verified, the aggregate sequences were cloned utilizing the KpnI and BamHI sites in pOET2 (Oxford Expression Systems 2001031), a baculovirus transfer vector capable of transfecting the SF9 cells. The genes were also cloned into the BamHI and NdeI sites of pET19KT, an *E.coli* expression vector.

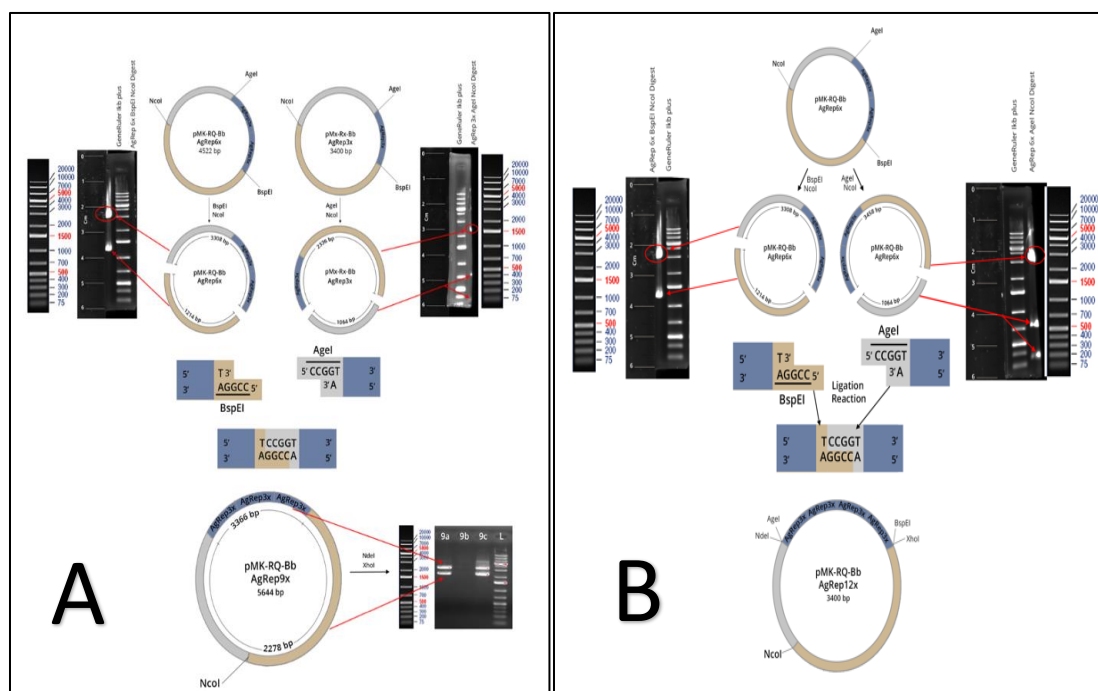


Fig. 4-2 | Formation of the 9X (A) and 12X (B) aggregate repetitive regions. The respective aggregate monomer is in red. The figure shows destruction of the AgeI and BspEI sites during ligation. Red arrows indicate the gel electrophoresis bands that represent the specified sequences. Segments excised and purified are circled in red. Reprinted with permission from Kyle Berg.

Bac-to-bac transfection

For successful transfection SF9 cells need to be in log phase ($1.5-2.5 \times 10^6$ cells/mL) with greater than 95% viability, this was verified on a Vi-cell counter (Beckman Coulter Vi-CELL Cell Counter). 2 mL Grace's Insect Medium,

Unsupplemented was added to each well (6-well plate). Cells were seeded at 8×10^5 per well and allowed to adhere for 15 minutes at room temperature. For each transfection 8 μL Cellfectin II (Thermo Fisher Scientific 10362100) was added to 100 μL Grace's Medium, 1 μL baculovirus DNA was added to 100 μL Grace's Medium and the two mixtures were combined. The DNA-lipid mixture was added dropwise onto the cells and they were incubated for 3-5 hours at 27°C . The transfection mixture was removed and replaced with 2 mL SF-900 II media (SF-900 IITM SFM Thermo Fisher Scientific 10902096). The transfected cells were allowed to grow for 3 days at 27°C . After 3 days the media was collected from each well and centrifuged at $500 \times g$ for 5 minutes to remove cells and large debris. The clarified supernatant was transferred to a 15 mL conical, this is the P1 viral stock.

To amplify the viral stock to a higher titer, cells were plated at 2×10^6 cells/well (6 well plate) and incubated at room temperature for 1 hour. After an hour 0.5-1 MOI (multiplicity of infection) P1 stock was added to the wells. The cells were incubated at 27°C for 2 days, then the media was removed and centrifuged at $500 \times g$ for 5 minutes to remove cells and large debris, this is the P2 viral stock.

Culturing and Expression: SF9 cells

In order to express the aggregate proteins, 6×10^5 cells/well were plated in a 24 well-plate and allowed to attach for 30 minutes. The media was removed and the cells were washed with fresh media. 300 μL fresh media was placed on the cells and baculoviral stock was added at the desired MOI. The cells were incubated at 27°C for up to four days. When conducting assays MOIs of 1, 2, 5, 10 and 20 were tested, as well as

culturing times of 1 day, 2 days, 3 days and 4 days. When the expression cultures were ready to be harvested the cells were resuspended in the media and transferred to 15 mL tubes. Cultures were centrifuged at 1000 rpms for 5 minutes and the supernatant and cells were separated. The SF9 cells were resuspended in a 1:3 (g:v) binding buffer (8M Urea, 20 mM Tris-HCL, 0.5 M NaCl, 2 mM EDTA). Sonication was conducted for 30 seconds at 1 W (QSonica ¾” diameter probe). The cell waste was separated from the cell lysate by centrifugation at 14,000 rpms for 10 minutes.

Once expression assays confirmed the optimal MOI and time (days) to perform culturing for aggregate expression, the process was scaled up to T25 and T75 flask culture. These cultures were conducted using 7 mL and 12 mL SF900 II media, respectively. Within the cells was determined to be where the protein was contained and were sonicated accordingly.

Culturing and Expression: *E.coli*

Two-liter flask cultures were used to express aggregate proteins in *E.coli*. A 5 mL LB media (Miller 71753-6) with kanamycin (100 mg/L) tube culture was grown for 8 hours at 37°C and 2500 rpms, and used to inoculate the two-liter flask culture containing 750 mLs LB media with kanamycin. Flask cultures were grown for 16 hours under the optimal conditions listed above. The flask culture was grown until OD₆₀₀ of 80 was reached and then inoculated with 1 mM IPTG and grown for an additional 4 hours. Bacteria was harvested via centrifugation at 6000 rpms for 30 minutes and the cell pellet was processed in an 8M Urea binding buffer (8M Urea, 20 mM Tris-HCL, 0.5 M NaCl, 2 mM EDTA) at 1:3. Sonication was conducted at 1 W for 4 minutes, a minute rest, and

repeated 3 times (QSonica 3/4" diameter probe). The cell lysate was harvested through centrifugation at 6000 rpms for 30 minutes, retaining the supernatant as the final cell lysate.

Purification

Some issues arose with purification when the cultures were scaled up to the T25 and T75 flasks. All the small scale, Eppendorf sized, purifications yielded recombinant aggregate proteins that were visible when analyzed via western blot or dot blot, but any larger expressions that were processed in plastic conicals showed no protein on the western blots. It was determined that the aggregate proteins were sticking to the conical tubes used for purification, this is most likely due to either the highly hydrophobic nature of spider silk proteins or the post-translational glycosylation of the aggregates, or a combination of both characteristics. In order to remove the proteins a wash with 200 μ L 5 mM NaOH and then the solution was placed in Eppendorf tubes that the protein did not stick to, and neutralized with 200 μ L 5 mM Tris. These samples were lyophilized and the resulting protein powder was suspended in 500 μ L water. Another option to interrupt any interactions between the protein and the container is to use a siliconizing agent readily available from companies such as Thermo Fisher Scientific to pre-treat the conicals or other containers used when processing the SF9s containing aggregate silk protein.

Western Blot Analyses and Coomassie

Western Blots were conducted on all processed cell pellets from the expression assays and cell cultures to determine aggregate production in the culture. A 15 μ L aliquot was taken from each sample and a 1:1 volume 2XSABU (125 mM Tris-HCL, 8M Urea, 6

mM EDTA, 10% SDS, 10% glycerin, 0.4% Bromophenol) was added to the sample. Samples were boiled for 10 minutes and then loaded onto a 4-20% tris-glycine SDS-PAGE gel (Invitrogen XP04200BOX) and run at 135 V for 90 minutes. Gels were transferred to nitrocellulose membranes overnight at 25 mA in a wet transfer apparatus. The blots were conducted on the nitrocellulose membranes with a primary antibody produced in mice that targets the histidine tag (Rockland 200-303-382, 1:1000 dilution). Some blots were conducted using a rabbit anti-mouse antibody conjugated with Alkaline Phosphatase (AP) was used at a 1:5000 dilution (Abcam ab6729) and other blots were conducted using a custom-built C-term antibody made in rabbits conjugated with Alkaline Phosphatase (1:5000). The membranes were developed using 1-StepTM NBT/BCIP Substrate Solution (Thermo Fisher Scientific 34042) for imaging.

Coomassie staining was also conducted on the samples with the same loading onto a 4-20% gradient tris-glycine gel. The SDS-PAGE gels were run at 135 V for 90 minutes. Overnight stains were conducted with Coomassie Blue stain (Bio-rad 161-0786) and were destained (40% methanol, 10% acetic acid, 50% water) for two hours with two washes and then the gels were imaged with Azure Biosystems c200 gel documentation platform.

Deglycosylation

In order to determine if the synthetic aggregate proteins produced in the SF9 cells contained glycosylation the molecular weights of the synthetic proteins were calculated with no glycosylation, just N-glycosylation, just O-glycosylation and glycosylation at both the N- and O- locations. To calculate the different molecular weight of the protein

with and without glycosylation an online calculator was used that added the sugars to the amino group of asparagines (N-linked), or added a monosaccharaide to the hydroxyl group of serine and threonine (O-linked) and a combination of the two types of glycosylation (Table 4-1).

Table 4-1 | Table showing the expected relative size of aggregate spider silk proteins when glycosylated versus when no glycosylation is present.

Protein	N-glycosylation	O-glycosylation	MW (kDa)
3X aggregate + linker	-	-	79.5
	√	-	79.9
	-	√	105.7
	√	√	106.1
6X aggregate + linker	-	-	123.4
	√	-	123.8
	-	√	168.3
	√	√	168.8
9X aggregate + linker	-	-	167.3
	√	-	167.8
	-	√	230.9
	√	√	231.4
12X aggregate + linker	-	-	211.3
	√	-	211.7
	-	√	293.6
	√	√	294

Deglycosylation of aggregate proteins produced in SF9 cells was conducted using Protein Deglycosylation Mix II (New England BioLabs P6044S). This deglycosylation kit deglycosylates proteins at both N- and O- amino acids. 100µg of the glycoprotein,

aggregate, was dissolved into 40 μ L water and 5 μ L 10X Deglycosylation Mix Buffer 1 was added, as well as, 5 μ L Protein Deglycosylation Mix II, solution was gently mixed. Samples were incubated at room temperature for 30 minutes, then transferred to 37°C for 16 hours before analysis via Western blot. The protocol listed here does not denature the protein. To denature the protein an incubation at 75°C for 10 minutes, followed by a cooling step are conducted after the solution is made and before the deglycosylation mix is added. Both of these methods were used for this research.

Results and Discussion

Four synthetic aggregate genes were created for this research, 3X, 6X, 9X and 12X, the 3X aggregate is a three repeat of the monomer repetitive sequence and was synthesized and used to create the other larger proteins as described above. Both the 3X and 12X aggregate genes were successfully cloned into the baculoviral transfer vector pOET2. Work is being continued to insert the 6X and 9X aggregate into the vector, which to date for reasons that have yet to be determined have not been successfully cloned under any attempted ligation and transformation protocol parameters. Using the bac-to-bac transfection techniques SF9 cells were transfected with the 3X and 12X aggregate sequences. Expression studies were conducted using multiple MOIs (multiplicity of infection), over the course of several days and by harvesting and processing both the supernatant and the cells from the expressions to determine what the best parameters for aggregate protein production were.

Expression assays conducted on 3X aggregate showed increasing crystallization within the SF9 cells over a 4-day period, seen as an increase in cell size and change in

morphology, (Figure 4-3), demonstrating successful production of the viral capsid protein. After the expression assays were finished the supernatants and cell lysates were analyzed on Coomassie's to determine when protein was being expressed and if the aggregate protein was being secreted into the media or being retained in the cell. It was determined that the optimal MOIs for protein production were 5, 10 and 20. After 3 days of expression all of the protein was still being accumulated in the cells and none was secreted into the media until day 4, but even on day 4 there was still protein retained in the cells (Figure 4-4). Based on these results it was concluded that the best parameters for 3X aggregate protein expression in SF9 cells was at a MOI of 5 for 3 days, using only the cells from the cultures to purify the protein.

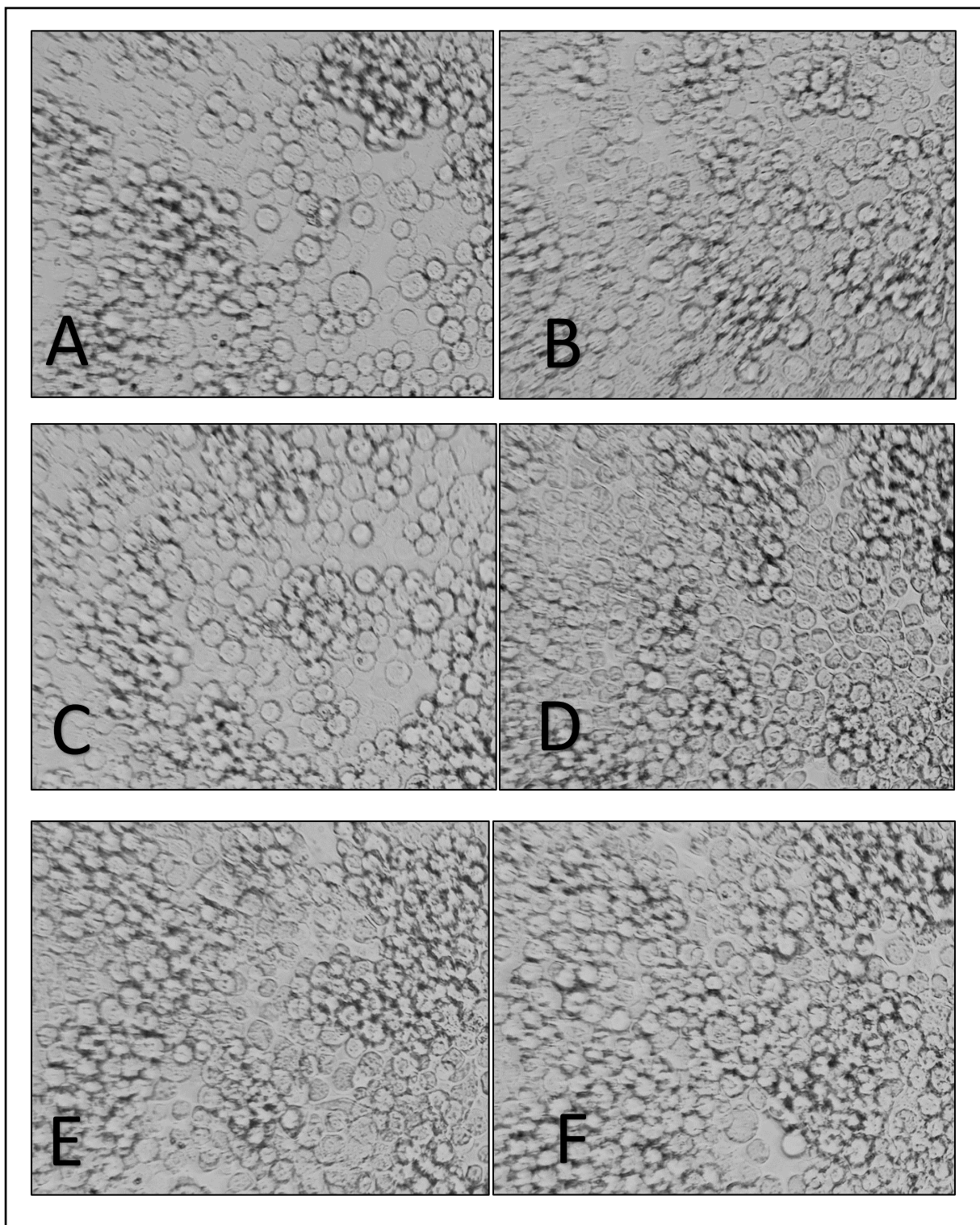


Fig. 4-3 | Microscopic images of SF9 cells transfected with 3X aggregate. A and B are images taken 1 day after expression, C and D are images taken 2 days after expression and E and F are 3 days after induced expression, when cells are harvested.

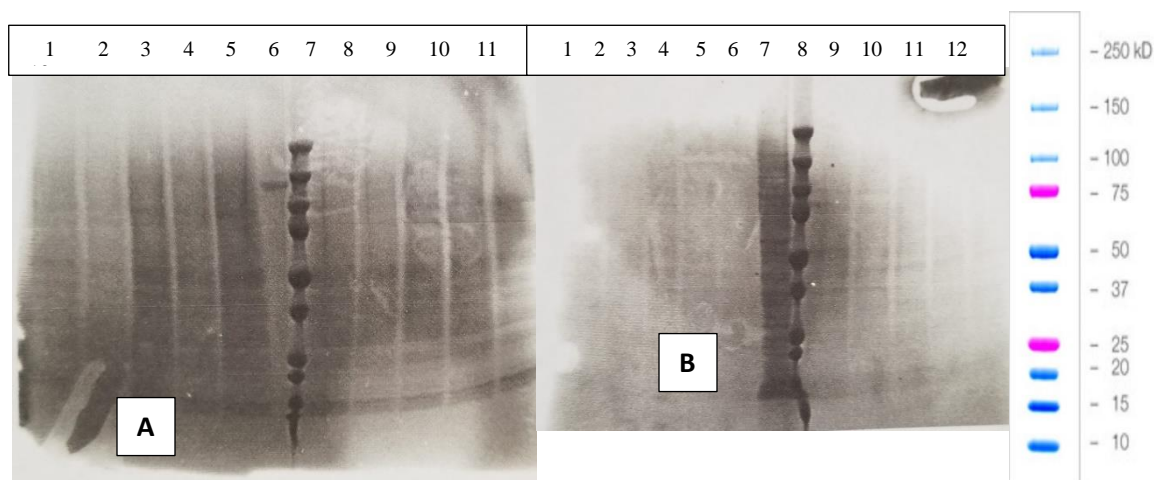


Fig. 4-4 | Coomassie blue analyses of 3X aggregate protein expression in SF9 cells.

A) processed cell pellets from MOIs 1 (wells 1 and 8), 2 (wells 2 and 9), 5 (wells 3 and 10), 10 (wells 4 and 11) and 20 (wells 5 and 12) taken 3 days after induced protein expression. B) the media, or supernatant, taken after 3 days induced protein expression in two SF9 cell cultures at MOIs 1 (wells 1 and 8), 2 (wells 2 and 9), 5 (wells 3 and 10), 10 (wells 4 and 11) and 20 (wells 5 and 12). A positive control from a bacterially expressed his-tagged protein is in well 6 in each sample.

Expression assays were also conducted using varying MOIs of P2 viral titers of 12X aggregate. Much like is seen in *E.coli*, there was no production of this much larger rSSp seen in either Coomassie, western blot analyses or dot blot analyses. 12X aggregate protein is anywhere from 211-294 kDa and it is hypothesized that this specific recombinant protein is too large for the small eukaryotic SF9 cells to produce, which is why we see production of the 109 kDa 3X aggregate protein but not of this protein. As mentioned above, this is a common problem in *E.coli* production due to the host organisms incapability to make a protein not only that large, but also as repetitive as rSSps tend to be. This theory will be further examined once the 6X and 9X aggregate

genes are successfully cloned into the pOET2 baculovirus vector and expression of the genes can be examined.

Western blot analyses

Once it was determined what the best parameters for aggregate spider silk production, western blot analyses were conducted on the cell lysates to verify aggregate production (Figure 4-5). If the aggregate protein was unglycosylated it would be a 79.5 kDa protein, but if glycosylation were present then the protein would express at 106 kDa. All of the 3X aggregate produced was 106 kDa, demonstrating that the protein is glycosylated.

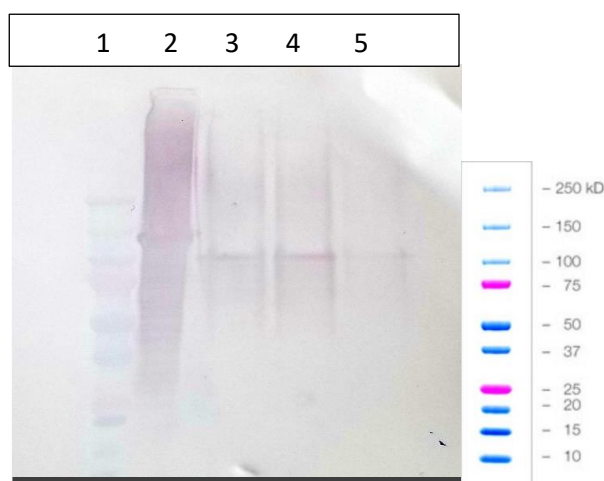


Fig. 4-5 | Western blot analyses of a 106 kDa aggregate protein expressed in SF9 cells. A positive control is shown in well 2 of a bacterially derived synthetic his-tagged protein. The wells show the following: 3) 3X aggregate expressed in SF9 cells with a viral MOI of 5, 4) 3X aggregate expressed in SF9 cells with a viral MOI of 10, and 5) 3X aggregate expressed in SF9 cells with a viral MOI of 20.

In order to further develop that the aggregate proteins being produced by the SF9 cells were glycosylated a deglycosylation kit was used on the cell lysates. The deglycosylation was conducted on two different 3X aggregate protein expressions that were previously verified as 106 kDa on a western blot analyses, two samples from each was used to compare if deglycosylation was altered when the protein was denatured first or under conditions that did not denature the protein. These deglycosylated samples were examined via western blot analyses and showed that some deglycosylation took place in all the samples and that denaturing the protein had little, if any, effect on the amount deglycosylation achieved (Figure 4-6). All the samples showed the glycosylated protein at 106 kDa and laddering down to what would be an unglycosylated 3X aggregate protein at ~80 kDa. This incomplete deglycosylation could be due to several factors, including: 1) the concentration of the protein was too high to have 100% deglycosylation, 2) the number of attached sugars to the N- and O-linked regions proved too many for the enzyme to detach them all and 3) secondary structures inhibited the deglycosylation reaction by hiding the glycosylated sites within the structure of the protein.

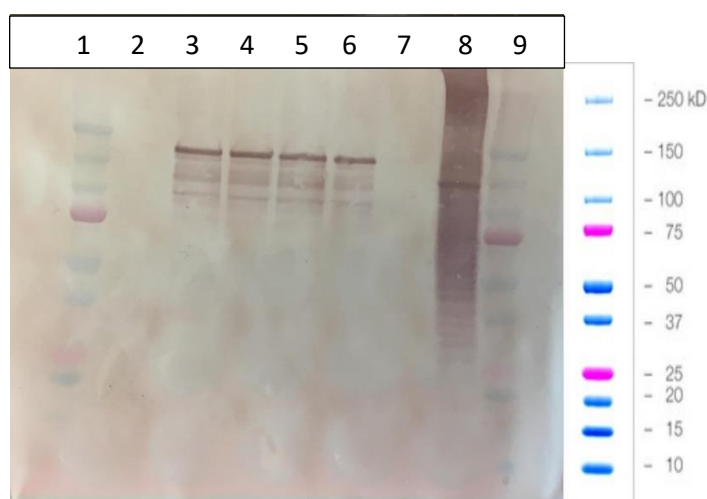


Fig. 4-6 | Western blot analyses of a 106 kDa aggregate protein expressed in SF9 cells and then treated with a deglycosylation kit. A positive control is shown in well 8 of a bacterially derived synthetic his-tagged protein. A negative control of water is in well 7. The wells show the following: 3) 3X aggregate T75 #1 expressed in SF9 cells treated with deglycosylation kit, 4) 3X aggregate T75 #1 expressed in SF9 cells denatured and treated with the deglycosylation kit, 5) 3X aggregate T75 #3 expressed in SF9 cells and treated with the deglycosylation kit and 6) 3X aggregate T75 #3 expressed in SF9 cells denatured and treated with the deglycosylation kit.

Future Research

Synthetic aggregate protein production research has only begun, and there are still many interesting and scientifically significant experiments to be performed. While a synthetic aggregate has been produced there are other larger constructs that have been created, but as of yet unsuccessfully expressed in *Spodoptera frugiperda* (SF9 cells) or any other host system. It will be interesting to obtain mechanical data on synthetically derived aggregate, this would either prove or disprove the theory that a synthetic aggregate would still be of use as a glue and whether the rSSps would behave similarly to native aggregate. Of particular interest, is whether or not synthetic aggregate can be used

in an aqueous environment like the native aggregate. If aggregate rSSps can be used as a marine/aqueous glue, there will not only be a whole new avenue of research and product development available for discovery, but a much higher demand commercially.

Another avenue for future research is the insertion of the synthetic aggregate proteins into alternative hosts to see if the protein inherently has the same properties and can be produced at a higher yield. As of right now 3X, 12X and 9X aggregate genes have been inserted into pET19KT, an in-laboratory expression vector for *E.coli*. *E.coli* does not post-translationally modify proteins to contain glycosylation. Aggregate proteins created in *E.coli* should be an excellent comparison to aggregate proteins produced in SF9 cells. Another interesting host is *Medicago sativa*, alfalfa, which naturally produces glycosylated proteins and contains a much higher concentration protein and is capable of producing higher yields synthetic protein. As the aggregate rSSp sequences have already been cloned into an agrotransformation mediating vector, it would be an ideal host for long term production.

Conclusion

This research has shown, for the first time, that a synthetic aggregate protein can be produced in SF9 cells. The synthetic aggregate proteins were shown to be glycosylated and were partially purified, and while a synthetic aggregate has been produced before there is no evidence that anyone else has been able to produce a glycosylated synthetic aggregate protein. While production via eukaryotic cell culture, specifically SF9 cells, has been useful for preliminary research into synthetic aggregate production, other production methods, such as alfalfa, need to be examined to create a commercially viable

product. Also, research into material development and characterization has a long way to go in order to delve into the full potential of aggregate as an industrial aqueous glue or any other products. Now that glycosylated and non-glycosylated synthetic aggregates have been produced and extracted, many new projects can be created and aggregate can fully be examined.

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CHAPTER 5

ENGINEERING SIGNIFICANCE

The field of biological engineering has always aimed at creating commercially viable engineered systems, designed using biology, applied sciences and technology in innovative ways to produce tangible materials and processes. This interdisciplinary field combines engineering principles, for designing, building and testing products and processes, with research and development by utilizing biology, medical and technology practices and knowledge. The research I conducted uses all of these principles and methodologies to develop processes for production and purification of recombinant spider silk proteins (rSSps) at a commercially viable level. Due to spider silk's remarkable strength and elasticity, as well as biocompatibility and biodegradability characteristics, it is an ideal material for producing a plethora of diverse materials, more specifically biomaterials for medical uses. One of the major limiting factors in rSSp production is a bioengineered process capable of producing commercially viable quantities of spider silk with mechanical characteristics similar to those found in the native silks. The research projects presented in this dissertation all are aimed at designing, building and testing both the processes to create rSSps and the resulting rSSps themselves, ultimately aiming to create a superior and marketable rSSp production system.

Spider silk production in *E. coli* was improved through molecular engineering and bioprocessing. Molecular cloning was conducted by designing an expression vector that included additional glycine and proline t-RNAs and a SHMT enzyme, increasing the

glycine concentration within the cells. This redesigned vector was tested and demonstrated an expression method capable of producing increased quantities rSSps. Other research conducted in the spider silk laboratory since this particular research project was completed in 2014, has improved rSSp bacterial production further through modifying downstream processing and fermentation protocol redesign. Yields of greater than or equal to 1 g/L rSSp are now common when using *E.coli* as the expression host. This is a yield that was theorized to meet commercial demands using bacteria, although efforts are currently being made to successfully upscale the process further, while retaining high yields and maintaining costs.

Another system that was designed and engineered to create rSSps was *Medicago sativa*, more commonly known as alfalfa. Research had been conducted in the past to insert rSSps into plant hosts, but none of these processes ever resulted in native-sized rSSps, in fact they were very small at ~25-40 kDa, or a purified product that could be used to make marketable materials. The research presented in Chapter 3 shows the expression of rSSps via a newly developed molecular engineered vector that was used to transform alfalfa via *Agrobacterium tumefaciens*. This vector and agrotransformation resulted in spider silk plants with rSSps ranging from 85-110 kDa. Not only was a new expression system designed and built, but we were able to extract a crude product from the alfalfa and create a new downstream process system using chlorophyllase and precipitation to purify the rSSps. While work is ongoing to quantify the yield in the transgenic plants, we now know that we can successfully create spider silk in plants and that it is a viable production option for future work. This is the first demonstration of plant-based spider silk production.

Aggregate spider silk had never been synthetically produced prior to the research presented in this dissertation. By designing a production process that yields synthetic aggregate proteins that are post-translationally glycosylated, now materials can be produced using the glue-like protein. Mechanical testing on aggregate materials are theorized to show “stickiness” and remarkable adhesion properties, similar to those seen in the native aggregate proteins. A novel characteristic of aggregate is the ability to work as an adhesive even in the presence of water, allowing any products made with this protein to be used in aquatic or marine conditions. There are no commercially available marine glues that don’t rapidly breakdown, and a glue made with aggregate would produce a tangible material for application in aquatic environments. Native aggregate also is a naturally biodegradable material, which increases its’ appeal as a biological marine glue. Utilizing *Spodoptera frugiperdes* as a host for aggregate spider silk production has created a bioengineered process for creating a material with applications in many technological fields. While there is still much work to be done to characterize the synthetic aggregate proteins and some downstream processing necessary to increase yields, this innovative research created synthetic aggregate that was glycosylated for the first time through a bioengineered process.

In Biological Engineering, a driving theme is to use biology to design, test and modify materials and processes. In my research I was able to design and modify three different spider silk synthesis systems: 1) bacteria, 2) alfalfa, and 3) insect cells. Through testing and further modification of the processes, I was able to determine that: 1) spider silk production in *E.coli* could be improved upon the addition of glycine and alanine t-RNAs, 2) spider silk could be successfully synthesized and purified utilizing alfalfa as a

host, and 3) that the glue-like aggregate spider silk could be produced and post-translationally modified with glycosylation in SF9 cells (Table 5-1). Using design, testing and modification principles of biological engineering, synthetic spider silk production was improved and new production systems were created through the research described in this dissertation.

Table 5-1 | Table showing the engineering significance achieved in this dissertation.

Engineering Significance		
<u>E. coli</u>	<u>Alfalfa</u>	<u>SF9 cells</u>
Engineered and built new vectors that increased rSSp production	Built a new expression vector for alfalfa	Built 3X, 6X, 9X and 12X aggregate sequences
Tested rSSp production in the different vectors	Tested transformed cultivars for rSSp	Designed a purification process for rSSp produced in SF9 cells
	Designed a purification protocol to extract and purify rSSp from alfalfa	Tested synthetic aggregate for posttranslational glycosylation
	Tested precipitation phases to verify that the phytol tail of the chlorophyll separated from the rSSp	

CHAPTER 6

SUMMARY AND CONCLUSIONS

Historically, synthetic spider silk protein production is a low yielding process in goats, *E. coli*, silkworms, plants, and all other biological organisms. There are two main limiting factors in spider silk production: 1) low purification yields and 2) rSSps are not capable of being produced at the same size as the native spider silks. Therefore, the mechanical properties that synthetic proteins have are not as high as those seen in natural spider silks. This research was able to address both of these issues through production in three different hosts.

Spider silk yields in *E.coli* presented in Chapter 2 were increased by the integration of glycine and proline t-RNAs and a SHMT enzyme in the expression vectors. It was shown that by transferring the his-tag from the N-term to the C-term, no additional protein was produced, although little to no protein truncation was seen. The work conducted specifically for this dissertation created rSSp yields five times higher than other vectors used. This research was combined with other projects conducted by several post-doctorates in the spider silk laboratory, ultimately leading to a fermentation system that could produce spider silk at a yield of up to 2 g/L, with a common yield of 1 g/L. This is a yield that has been determined to meet commercialization demands if it can be maintained when scaled up to larger fermentation volumes.

Spider silk genes were also inserted into alfalfa cultivars and a purification process was developed utilizing a bacterial derived chlorophyllase molecule. This research is the first evidence that rSSp can be produced in alfalfa leaves only and can be

extracted. Yields have not been attainable in alfalfa due to the insolubility of the rSSp under storage conditions, but alfalfa has been shown to be able to produce up to a 5% yield synthetic protein and even a 2% yield spider silk would be enough to meet commercial demands. The separation protocol designed for this research successfully separates the rSSp from chlorophyll, which is a crucial step towards obtaining the first pure rSSp from alfalfa.

For the first time a glycosylated synthetic spider aggregate silk was produced. Due to aggregates glue-like characteristics and ability to be used in water, there are now many different avenues for product development open to discovery. Now that aggregate silk proteins have been synthetically produced in *Spodoptera frugiperdes* cells and a non-glycosylated version was produced in *E.coli*, research can be conducted to increase yields and characterize the proteins.

Overall this research has increased rSSp yields in two different systems and created a new synthetic spider silk in insect cells. Although further work is needed for all of these projects, the work completed in this dissertation has been instrumental in establishing and creating several synthetic production systems for rSSp production.

APPENDICES

APPENDIX A

FIGURES AND PERMISSIONS

Fig. 1-1:



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Licensed Content Date	Apr 17, 2015
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Format	print and electronic

Fig. 1-2



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Title: Adverse Reactions to Virgin Silk Sutures in Cataract Surgery

Author: H. Kaz Soong, Kenneth R. Kenyon

Publication: Ophthalmology: Journal of the American Academy of Ophthalmology

Publisher: Elsevier

Date: May 1984

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Fig. 1-3



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Title: Recent advances in production of recombinant spider silk proteins

Author: Hannah Chung,Tae Yong Kim,Sang Yup Lee

Publication: Current Opinion in Biotechnology

Publisher: Elsevier

Date: December 2012

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Licensed Content Author	Hannah Chung,Tae Yong Kim,Sang Yup Lee
Licensed Content Date	Dec 1, 2012
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Fig. 1-4



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Title: More Than Just Fibers: An Aqueous Method for the Production of Innovative Recombinant Spider Silk Protein Materials

Author: Justin A. Jones, Thomas I. Harris, Chauncey L. Tucker, et al

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Apr 1, 2015

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Fig. 1-5**Requesting Permission**

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Fig. 2-1



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- Title:** Economic feasibility and environmental impact of synthetic spider silk production from escherichia coli
- Author:** Alan M. Edlund, Justin Jones, Randolph Lewis, Jason C. Quinn
- Publication:** New Biotechnology
- Publisher:** Elsevier
- Date:** 25 May 2018

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Licensed Content Author	Alan M. Edlund, Justin Jones, Randolph Lewis, Jason C. Quinn
Licensed Content Date	May 25, 2018
Licensed Content Volume	42

VITAE

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Education

Utah State University (USU), Logan, UT

May 2019

Ph.D, Biological Engineering

GPA: 3.64

Thesis: Expression Systems for Synthetic Spider Silk Proteins

B.S., Biological Engineering

May 2011

GPA: 3.31

Engineering Projects

- Investigated the effect of laser-induced microstructure of metal and polymer surfaces on the adhesion and proliferation of eukaryotic progenitor cells and bacterial cells
- Designed and optimized 2 synthetic spider silk protein production and purification systems for spider silk using alfalfa and insect cell culture
- Utilized molecular biology and downstream processing methods to improve recombinant spider silk protein yields in bacterial fermentation

Relevant Skills

Molecular Biology

- Plant and Insect Cell Culture
- Bacterial Cell Culture
- Molecular Cloning
- PCR

Protein Production

- SDS-Page Analysis
- Western Blotting
- Agrotransformation
- Bac-to-bac Transfection

Characterization Techniques

- Mechanical Property Testing
- AFM
- HPLC
- Biocompatibility/Biodegradation

Research and Development Experience

Graduate Research Assistant, Spider Silk Lab, USU, Logan, UT

May 2006 – Present

- Researched and optimized alternative production methods to increase recombinant spider silk protein yields in engineered *Escherichia coli*, *Medicago sativa*, and *Spodoptera frugiperdes*
- Successfully produced the first glycosylated synthetic aggregate spider silk protein glue
- Engineered a method to produce large spider silk proteins in alfalfa leaves only and designed a purification process to obtain the recombinant proteins
- Improved yields in *Escherichia coli* by 5 times
- Supervised and mentored 10 undergraduate researchers

Willing to Relocate

Undergraduate Research Assistant, David Britt Lab, USU, Logan, UT

August 2010 – May 2011

- Analyzed cell growth and characteristics on biomaterials laser engraved to induce microstructuring
- Completed testing and analyses of the microstructured prototypes and determined the efficacy for applications as a biomaterial
- Conducted biocompatibility testing of biomaterials with surface alterations, successfully demonstrating biocompatibility and biointegration

Teaching and Mentoring Experience

Supervisor Experience

Home Depot, Logan UT

- Head Cashier: supervision duties for all cashiers 2009-2012

Undergraduate Student Mentor

Spider Silk Lab, Biological Engineering, Utah State University, Logan UT

- Direct training, supervision and instruction of over 10 students 2011-2019
- Guideline and assistance of 2 UCUR projects 2014-2019

Professional Affiliations

American Chemical Society (ACS)

Institute of Biological Engineering (IBE)

Honors and Awards

Graduate Assistantship Award	2011-2019
Dean's Scholarship	2011-2015
New Century Scholarship	2011-2013

Conferences and Presentations

Materials Research Society (MRS) Meeting and Exhibit	2017
Rocky Mountain Bioengineering Symposium	2015
Utah Science and Technology Research Initiative (USTAR) Annual Conference	2014-2016
USU Synthetic Biomanufacturing Center (SBC) Annual Meeting	2014-2016
Annual USU Student Research Symposium (SRS)	2011-2017
NanoUtah Conference	2012
Senior Design Thesis Capstone Presentation	2011

Research on Capitol Hill	2010
Utah Conference on Undergraduate Research (UCUR)	2011

Service and Volunteer

- | | |
|---------------------------------------------------------------------------------------------------------------------|-----------|
| • Performed tours, community outreach and education sessions
Regarding research performed at the spider silk lab | 2013-2019 |
| • Volunteer at Antelope Island Spider Festival | 2016-2019 |