参赛队员姓名：	王佳霖
所在中学：	北京大学附属中学
国家/地区：	中国
所在省份：	北京
指导教师姓名：	刘凯
指导教师所在单位：	清华大学
论文题目：	**Preparation and Structural Characterization of High-performance Artificial Spider Silk Fibers**

Abstract

Natural spider silk, with its excellent mechanical properties, holds great promise for applications in various fields. However, to mass-produce spider silk through artificial ways is impractical, which limits its practical application. With the rapid development of synthetic biology technology, this research aims to produce artificial spider silks using recombinant spider silk protein fibers. In previous research, the expression of spider silk proteins containing highly repetitive sequences in E. coli generally forms inclusion bodies, posing challenges for subsequent purification and application processes. Additionally, recombinant spider silk fibers exhibit comparatively inferior mechanical properties relative to natural spider silk.

This study employed a modular protein recombination approach to design and prepare recombinant spider silk proteins with soluble expression characteristics, enhancing the efficiency of expression and purification. Furthermore, the artificial spider silk fiber was successfully prepared by wet spinning technology, and the coagulation bath composition was optimized to increase the mechanical performance of the silk. Moreover, the transmission electron microscope and Fourier transform infrared light were used to characterize the secondary structure and assembly morphology of proteins, confirming that their structure is closely related to performance improvement. Through the introduction of a dynamic covalent bond crosslinking network, the mechanical characteristics of protein fibers were further improved. This study provides a feasible technical path for the large-scale preparation of high-performance artificial spider silk.

Keywords: recombinant spider silk protein, soluble expression, toughness, secondary structure, self-assembly

Table of Contents

Introduction.....6

Literature Review.....7

Structure of Natural Spider Silk.....7

The Spinning Process of Spiders.....9

Recombinant Spider Silk Protein.....11

Preparation Methods of Artificial Spider Silk Fibers.....12

Conclusion.....13

Methodology.....14

Materials and Tools.....14

Procedures.....14

Construction of Recombinant Spider Silk Protein Plasmid.....14

Expression of Recombinant Spider Silk Protein Fibers.....17

Preparation of Recombinant Spider Silk Protein Fibers.....19

Mechanical Properties Testing of Recombinant Spider Silk Protein Fibers.....19

Structural Characterization of Recombinant Spider Silk Protein Fibers.....20

Results and Discussion.....20

Construction of recombinant spider silk protein plasmid.....20

Expression and Purification of Recombinant Spider Silk Protein Plasmid.....21

Mechanical Property Curve Graph.....24

Structural Characterization of Recombinant Spider Silk Protein Fiber.....27

Conclusion.....30

Future Perspectives.....31

Acknowledgement.....32

References.....34

Introduction

Fibers are important in human daily life and have been widely used in the fields of textiles, industry, and medicine. Among these, synthetic fibers such as aramid fiber, carbon fiber, and Kevlar are now the most utilized materials in various applications. However, synthetic fibers generally exhibit poor toughness, and they are derived from petroleum, which is not renewable and will cause negative impacts on the environment and organisms (Suran, 2018).

As a result, researchers are increasingly focused on producing environmentally friendly natural protein fibers with great mechanical properties. Compared to chemical fibers, protein fibers are natural polymers that are abundant in nature, offering outstanding biodegradability and biocompatibility, and are sustainable materials that never run out (Agnieray, Glasson, Chen, Kaur, Domigan, 2021). Specifically, spider silks have been extensively studied due to their excellent mechanical qualities (Zhang et al., 2020). Previous researchers have successfully generated various kinds of artificial spider silks using recombinant spider silk proteins. Granted, this technique still faces challenges: firstly, traditional recombinant proteins usually form insoluble inclusion bodies when expressed in prokaryotic expression systems (such as Escherichia coli), which significantly reduces soluble expression and purification efficiency; secondly, how processing conditions during fiber spinning affect their mechanical performance has not been systematically explored, and the connection between structural control at the microscopic scale and the resulting macroscopic properties is still unclear. Consequently, optimizing the recombinant expression strategy and adjusting the

spinning conditions is crucial for enhancing the soluble expression of proteins and improving the mechanical characteristics of fibers. This study seeks to substantially improve both the robustness and ductility of artificial spider fibers by refining the design and fiber-forming procedures of recombinant spider silk proteins, thereby facilitating broader application in practical uses.

Literature Review

Structure of Natural Spider Silk

The spider's abdominal glands are used for the secretion of spider silks, and new research has shown that a single spider is capable of secreting six unique spider silk fibers and one special silk for egg cases (Eisoldt, Smith, Scheibel, 2011, p. 80). Each type of spider silk fiber and sticky protein has a specific function, and their combined action is important to the spider's survival. The figure below shows the distinct types of silk generated by spiders.

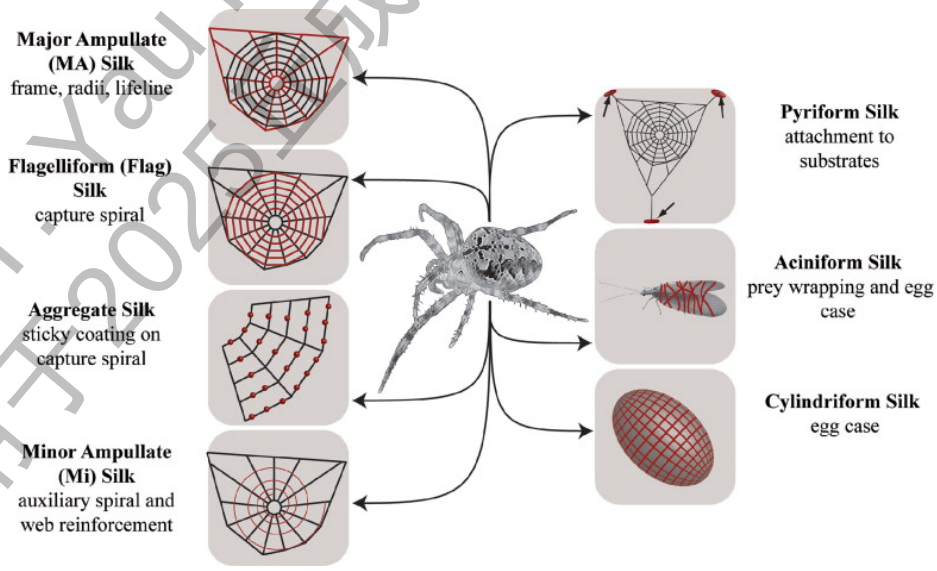

Figure 1. Schematic overview of different silk types and their functions (Eisoldt et al., 2011, p. 81).

The silk generated by the major ampullate gland (dragline), forms the core frame of the spider's net. This silk not only constructs the frame and radius of the spider's web, but it is also an important lifeline for the spider to move through vertical space (Gosline, Guerette, Ortlepp, & Savage, 1999). The dragline silk shows excellent mechanical properties. From its strength, researchers have discovered that the major ampullate silk has a maximum strength of up to 1.7 GPa, which could be seen as a high-tech material (Tokareva, Jacobsen, Buehler, Wong, & Kaplan, 2014, p. 1). While it also shows outstanding toughness. "Indeed, with an energy to breakage (toughness) of 160 MJ m^{-3}, MA silk is 3–10 times tougher than its engineering counterparts" (Gosline et al., 1999).

The molecular mass of the MA silk protein ranges from 200 to 350 kDa, and the MA silk protein is composed of major ampullate spidroins 1 (MaSp1) and major ampullate spidroins 2 (MaSp2) (DeFrancesco, 2017, p. 496). Through the continuous development of Proteomics and sequencing technologies, researchers have identified a third protein, MsSp3, and the variants of these three proteins and several non-protein components. All these components collaborate to form a complex network of proteins.

The secondary structure's feature of all MA silk proteins shows a high degree of consistency, and these features include a central repeating region and the C-terminal non-repeating regions (CTDs), and N-terminal non-repeating regions (NTDs) located at both ends, respectively. NTDs serve as key regulators of spider silk protein self-assembly, whereas the C-terminal domains (CTDs) facilitate their stable storage within the silk glands and promote the formation and organization of secondary structures during self-assembly.

In MaSp1, the repeating region is composed of the hydrophobic poly-alanine domains (poly(A)) and the glycine-rich domains (GGX, where G represents glycine, and X represents a variable amino acid). Poly (A) tends to form the β-sheet structure, which improves strength of the spider silk fibers, whereas GGX forms a 3_{10}-helix structure, which serves as a link between the β-sheet structures and can contribute to the β-sheet structures correctly aligning (Tokareva et al., 2014). In comparison, the repeating region of MaSp2 contains the hydrophobic poly-alanine domains and the hydrophilic GPGXX amino acid domains (P represents proline), and GPGXX can form α-helix structure and β-turn structure, which significantly enhance the toughness and extensibility of spider silks. The figure below illustrates the dragline silk protein structure.

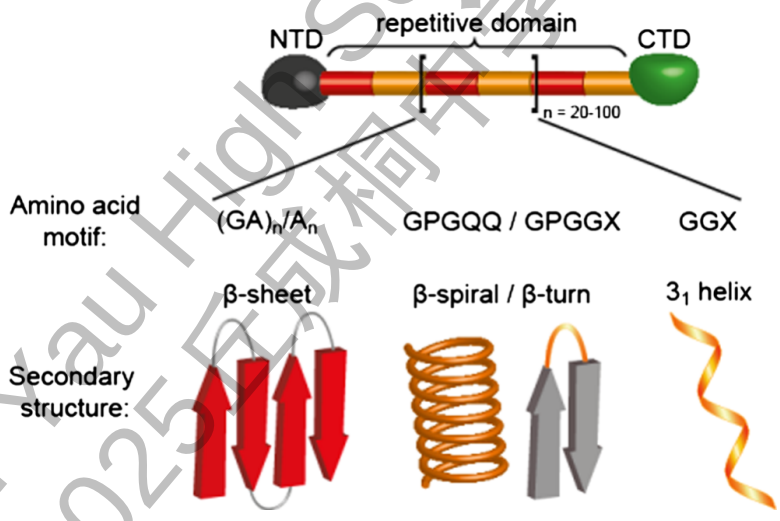

Figure 2. Schematic structure of major ampullate spidroins and the corresponding secondary structure (Doblhofer, Heidebrecht, & Scheibel, 2015, p. 3).

The Spinning Process of Spiders

The diverse secondary structures of spider fiber proteins contribute to their remarkable mechanical characteristics. Concurrently, the delicate mechanism of the spider silk spinning

process in their body also has a significant impact on the construction of secondary structures and the mechanical performances of their fibers. In the spider body, each spinneret is connected to a specific gland, and each gland produces a specific type of silk protein. One of these glands, the major ampullate gland, has been researched a lot because of its large size and the excellent properties of silk it secretes. Figure 3 shows the four main parts of this gland.

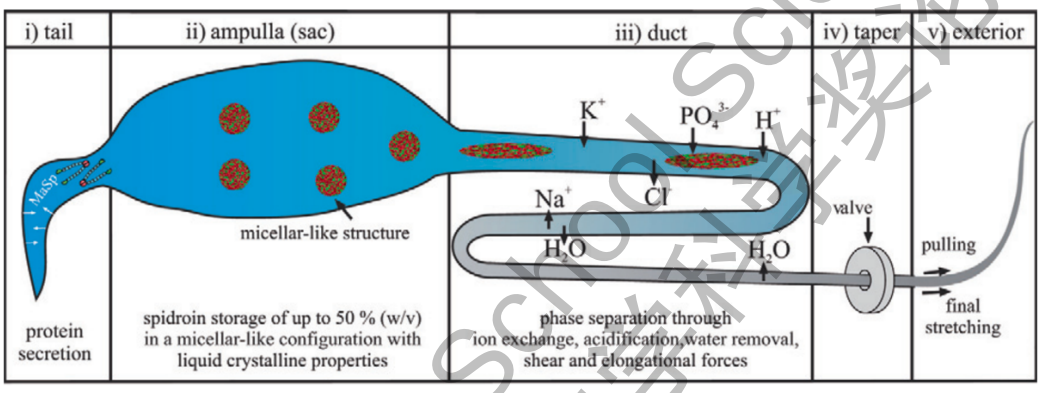

Figure 3. A spider's spinning gland is divided into four parts (Eisoldt et al., 2011, p. 83).

In the first part, the tail, proteins are first secreted and stored in a highly concentrated state in the ampulla region. In subsequent processes, these proteins migrate to the S-shaped duct, where shear forces progressively increase as the duct narrows. At the same time, the concentrations of K^+ and PO_4^{3-} increase while the concentration of Na^+ decreases. In addition, the pH value shows a gradual decrease (Andersson et al., 2014, p. 2). Under the combined influence of shear force, ion concentration, and pH change, the proteins transformed from being dominated by helical and randomly curled structures into fibers being dominated by folded structures (Knight, D. P., Knight, M. M., & Vollrath, 2000). When the proteins leave the spinneret through the valve, the spider will use its hind legs to stretch the fibers that have just left the body. This can prompt the evaporation of water and optimize the arrangement of

molecules inside the fibers, thus enhancing the overall performance of the fibers (Vollrath & Knight, 1999).

Recombinant Spider Silk Protein

Although natural spider silk has excellent properties, it cannot be produced on a large scale. Most spiders are territorial and cannibalistic, so they cannot be raised like silkworms; at the same time, direct acquisition of spider silk from spiders also has problems, such as inefficiency and difficulty in collection. Recent improvements in synthetic biology and genetic engineering have enabled the heterologous expression of recombinant spider silk proteins across diverse host systems, including bacteria, yeast, plants, and mammalian cells.

E. coli is the most common host bacterium that expresses recombinant spider silk proteins, and its advantages include simple operation, short growth cycle, and low cost (Pontrelli et al., 2018; Ma et al., 2021). Nevertheless, E. coli has difficulty isolating recombinant proteins in the extracellular environment. The extracellular secretion of recombinant proteins into the culture medium provides multiple benefits compared to intracellular expression: avoiding the formation of inclusions; promoting the correct folding and stabilization of proteins; simplifying purification steps, reducing costs, and improving protein purity and expression.

Yeast has become a widely used system for expressing recombinant proteins, and it can even express larger recombinant genes than E. coli. Furthermore, yeast can directly secrete recombinant proteins into the growth medium, which might theoretically reduce purification steps and increase yield (Celik & Calık, 2012; Jansson, 2016).

Compared to bacteria and yeast, mammalian cell has the expression capacity closer to that of native biological proteins and can therefore serve as an alternative expression host system for recombinant spider proteins (Lazaris, 2002).

Preparation Methods of Artificial Spider Silk Fibers

Wet spinning technology is a common method for artificial fibers production. When used in recombinant proteins or natural fibers, this technique uses specific solvents to dissolve the proteins. Since spider silk proteins are highly crystalline and strongly adhesive, the dissolution process must be carried out in a highly denaturing environment and usually needs specific organic solvents such as hexafluoroacetone hydrate, trifluoroacetic acid, and hexafluoro isopropanol (Blamires, Wu, Blackledge, & Tso, 2012; Ha, Tonelli, Hudson, 2005).

After dissolving the proteins, introduce the protein solution into a coagulation bath. Subsequently, the fibers are subjected to stretching, a process that induces polypeptide chain precipitation and promotes their orderly alignment. During this process, the structure of the protein was successfully converted into a β-sheet structure, which ultimately led to the formation of solid silk fibers (Lefèvre & Auger, 2016). The silk fibers produced through this process can also be further stretched, which can improve the molecular orientation and thus effectively enhance the mechanical properties of the fibers (Cho, Ki, Oh, Lee, & Um, 2012).

When selecting coagulation bath, a common approach is to use anhydrous ethanol as the primary component. Furthermore, aqueous solutions such as methanol aqueous solution, acetone, isopropanol, and aqueous ammonium sulfate solutions can also be used as

alternative options (Zhou, Shao, Knight, Yan, & Chen, 2009). During the wet spinning process, many factors such as the composition of the coagulation bath, rate of coagulation, temperature, extrusion speed, and rotor stretching speed have a significant impact on the pattern and final performance of the fibers (Anton, 1934).

Conclusion

The spider silk fiber has excellent mechanical properties and a complex structure, and spiders have a complex body structure that supports the form of high-performance fibers. The production of artificial spider fibers critically depends on both the protein expression process and the subsequent fiber-spinning process. With the ongoing advances in biological engineering and spinning methods, many previous researchers have successfully generated artificial spider fibers using recombinant spider silk proteins. However, in traditional recombinant expression systems, spider silk proteins often aggregate into insoluble inclusion bodies, leading to precipitation during processing and a consequent reduction in recovery efficiency. Besides, the produced protein fibers' mechanical properties are lower than natural spider silks, and the influence of varying spinning conditions has been largely overlooked in previous studies. These issues cause the practice of artificial spider silk fibers to be limited.

Based on previous gaps, this study was conducted from the following perspectives: first, by employing genetic engineering techniques, hydrophilic elastin-like polypeptide sequences were fused with native spider silk proteins, which markedly improved the solubility and stability of the recombinant proteins while effectively preventing inclusion body formation. Second, a wet-spinning process was utilized to achieve large-scale fiber production. Then, the

fibers' secondary structures were characterized via Fourier-transform infrared spectroscope, while a polarized optical microscope was employed to observe changes in molecular orientation and ordering within the fibers before and after tensile treatment. Furthermore, taking advantage of the ability of lysine residues in elastin-like domains to undergo rapid crosslinking reactions with aldehyde compounds at room temperature, composite protein fibers with covalently crosslinked networks were constructed, thereby improving their mechanical properties and stability.

Methodology

Materials and Tools

All the materials and tools used in the experiment were provided by Prof. Kai Liu from Tsinghua University and were utilized in standard procedures and satisfied experimental requirements. Materials include: DH5α competent cells, NdeI, EcoRI, T4 DNA ligase, glycerol, Potassium dihydrogen phosphate (KH_2PO_4), DNA gel, SDS-PAGE gel, SDS-PAGE running buffer, SDS-PAGE gel staining solution, Lysogeny Broth Medium, Terrific Broth Medium, Dipotassium hydrogen phosphate (K_2HPO_4), ampicillin antibiotic, IPTG (Isopropyl β-D-thiogalactoside) inducer, formic acid, ethanol, glutaraldehyde, Sodium chloride (NaCl), Imidazole.

Procedures

Construction of Recombinant Spider Silk Protein Plasmid

To construct a recombinant protein, this research selected a fragment of the MaSp2 protein from the natural spider (A. diadematus), which is labeled as C. The detailed DNA sequence and its corresponding protein sequence are listed in Table 1. In this sequence, the poly(A) region provides the necessary strength support for the fiber, while the GPGQQ region confers excellent extensibility and toughness. To further enhance the fiber's toughness and mechanical properties, this research conducted a fusion expression strategy combining 5 elastin-like proteins with spider silk protein C. The DNA sequence of the elastin-like protein named K5 and its corresponding protein sequence are also shown in Table 1. This recombinant protein is named CK5. The hydrophilic elastin-like protein facilitates the soluble expression of spider silk proteins, while its flexible random-coil structures further enhance the extensibility of the resulting fibers; concurrently, it can undergo chemical reactions with the side chains of lysine (K) residues to introduce covalent and non-covalent bonds, thereby further improving the mechanical performances of the silks. Prior studies have shown that increasing the number of sequence repeats in recombinant spider silk proteins results in higher molecular weights and enhanced mechanical properties of the resulting fibers. In this study, a CK5 gene sequence containing one repeat unit will repeat 12 times. Moreover, the carboxy-terminal domain (CTD) plays a huge part fiber’s properties, so this research integrates it to construct the CK5-CTD protein plasmids. To simplify the subsequent purification process, six histidine His tags were added to the end of the CK5-CTD gene sequence. The $(CK5)_{12}$-CTD+His tag gene sequence is synthesized by Suzhou GENEWIZ,

and then the synthesized target gene is ligated into the pET-25b vector containing a T7 strong promoter.

Name	DNA sequence	Protein Sequence
C	GGCTTCTGCGGCTGCGGCGGCAGCGGCGG CATCTGGTCCGTGTGTGTGTGTGTGTGTGT GTCCGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGT	GSSAAAAAAA AASGPGGYGPE NQGPSGGGYG GPPP
K5	GTACCGGGTAAAGGCGTGCCGGGCAAAGG TGTGTGTTCCAAAGGCGTTCCGGGCAAAGG CGTGCCGGGTAAAGGC	$(VPGKG)_5$
CTD	ACTAGTGGTGCGGCATCAGCTGCCGTTTCA GTAGGCGGCTACGGGCCACAAAGTAGCTC GGCACCGGTGGCCTCGGCAGCTGCTTCTCG TTTAAGTAGCCCTGCGGCATCTTCGCGCGT CAGTTCGGCCGTGTCATCGCTGGTAAGTAG CGGTCCTACCAACCAGGCTGCTTTGTCCAA TACTATTAGTTCTGTGGTGAGCCAAGTCTC GGCTTCTAACCCTGGACTGTCTGGCTGCGA CGTCCTGGTTCAAGCCCTGTTGGAGGTAGT TTCTGCGTTAGTTTCCATTTTGGGAAGCTCG AGCATTGGCCAGATTAATTACGGAGCCTCG GCTCAATACACACAGATGGTAGGGCAGAG CGTCGCGCAAGCCTTGGCTGGAACTAGT	GAASAAVSVG GYGPQSSSAPV ASAAASRLSSP AASSRVSSAVS SLVSSGPTNQA ALSNTISSVVSQ VSASNPGLSGC DVLVQALLEV VSALVSILGSSS IGQINYGASAQ YTQMVGQSVA QALAG

Table 1. The DNA sequence and corresponding protein sequence

Detailed steps for gene ligation are as follows. First, using the NheI and SpeI restriction enzymes to perform a 37℃, 1-hour; 65℃, 0.5-hour double restriction enzyme digestion in

the PCR amplifier to obtain the $(CK5)_{12}$-CTD+His target fragment and the pET-25b vector. Then, using agarose gel electrophoresis to separate the target sequence from the digestion products, and using an agarose extraction kit to produce a solution containing the target sequence. Subsequently, using T4 DNA ligase to connect the vector fragment pET-25b with the target gene fragment in the PCR amplifier at 16℃ for 5 hours. To amplify the number of plasmids, the ligation constructs were introduced into *E. coli* DH5α competent cells and subsequently grown on LB agar plates, then a single colony was transferred to a test tube filled with LB medium and incubated at a temperature of 37°C on a 220-rpm shaker for 10 hours. Finally, the target plasmids were extracted using a plasmid extraction kit and sent to Beijing Tsingke Biotechnology Co., Ltd. for in-depth analysis of the DNA sequence. Through the overall procedure, a pET25b-$(CK5)_{12}$-CTD plasmid including 12 repeat units was successfully constructed.

Expression of Recombinant Spider Silk Protein Fibers

The pET25b-$(CK5)_{12}$-CTD plasmid was introduced into E. coli BLR competent cells and cultured on LB solid medium, then a single colony was transferred into a test tube filled with LB medium and incubated at 37 °C on a 220 rpm shaker. When the optical density (OD) achieved approximately 3, 600 μL of the bacterial culture was mixed with 400 μL of 50% (w/w) glycerol stocks in a 1.5 mL EP tube to prepare glycerol bacteria. After thorough mixing, the samples were stored at -80 °C. The 50% (w/w) glycerol solution was sterilized by autoclaving at 121 °C for 30 minutes before use.

Inoculate 1 mL of E. coli stock into 100 mL of LB medium, add 100 μL of ampicillin, and incubate at 37°C on a shaking incubator at 220 rpm. When the optical density (OD) value of the bacterial suspension reaches approximately 3, transfer 20 ml of it to 1 L of TB medium, and add 8 ml of 50% glycerol and 1.5 ml of ampicillin. Continue shaking at the same temperature and speed. When the optical density reaches approximately 3 again, add 100 μL of IPTG and adjust the culture temperature to 28.5°C while maintaining the same rotation speed. After completing a 12-hour incubation cycle, the supernatant was separated from the bacterial culture by centrifuging at 6000 rpm/min for 10 minutes at 4°C. The resulting bacterial cells were then precisely weighed and stored at -80°C in a low-temperature refrigerator to ensure long-term stable storage.

For each gram of bacterial pellet, 10 mL of Ni lysis buffer, 200 μL of DNase, 1 mg/mL of lysozyme, and 0.6 g/100 mL of magnesium chloride hexahydrate were added, followed by stirring at room temperature for 30 minutes. Then, the suspension was disrupted three times using a high-pressure homogenizer, followed by ultrasonic disruption for 30 minutes. The bacterial lysate was centrifuged at 12,000 rpm for 1 hour at 4 °C, and the supernatant was collected, filtered through a 0.45 μm membrane, and subjected to purification. Since the protein carried a histidine tag, it was initially purified through a nickel-affinity chromatography column. As the elastin-like polypeptides are positively charged and can interact with negatively charged resins, further purification was carried out using a cation-exchange (SP) column. Finally, the protein solution was desalted using a size-exclusion chromatography column. After desalting, the protein solution was stored at -80 °C for at least

two days until completely frozen, and then lyophilized using a low-temperature vacuum freeze-dryer.

Preparation of Recombinant Spider Silk Protein Fibers

In wet-spinning, pre-dissolved proteins are injected into a coagulation medium, where the differential diffusion of solvents drives protein solidification and the assembly of fibrous structures. This study designed two coagulation baths with different compositions to generate fibers, one containing 90% ethanol and another containing 90% ethanol and 1.2 ml of 50% glutaraldehyde solution.

First, purified and freeze-dried $(CK5)_{12}$-CTD proteins were reconstituted in formic acid (FA) solution at a concentration of 150 mg/ml. The solution was then centrifuged at 12,000 rpm for 5 minutes, and the supernatant was collected into a 1 ml syringe. The protein solution was then directly extruded into a coagulation bath containing 90% concentration of ethanol and a coagulation bath containing 90% concentration of ethanol plus 1.2 ml of 50% glutaraldehyde solution. The fibers formed during this process were harvested using a specialized collection apparatus.

Mechanical Properties Testing of Recombinant Spider Silk Protein Fibers

A comprehensive test of the mechanical performances of the fibers was conducted using the FAVIMAT+ fiber tensile tester produced by the German company Textechno. During the testing of all raw fibers, the tensile rate was set to 6 mm/min, and the clamping distance was set to 3 mm. The raw fibers were first subjected to 100% stretching, followed by performance

testing under the same conditions as the initial setup: tensile rate of 6 mm/min, and a clamping distance of 3 mm.

Structural Characterization of Recombinant Spider Silk Protein Fibers

(1) Fiber Morphology Characterization

A scanning electron microscope was used to characterize the fiber morphology. The fibers are adhered to the conductive glue at the base of the aluminum sheet and then photographed.

(2) Fiber Internal Orientation Characterization

A polarization light microscope was used to characterize the internal molecular arrangement orientation before and after fiber stretching.

(3) Fourier Transform Infrared Spectroscopy

Fourier infrared instrumentation was used for secondary structural characterization. The amide I band (1600-1700 cm-1) was analyzed using PeakFit, and peak fitted was performed.

(4) Transmission Electron Microscope Characterization

The proteins were characterized by a transmission electron microscope under formic acid conditions. First, the protein is dissolved in formic acid at a concentration of 1 mg/ml. Then, the displacement column was displaced into a 50% ethanol and 50% PBS solution.

Results and Discussion

Construction of Recombinant Spider Silk Protein Plasmid

The sequence of the ligation product showed that the $(CK5)_{12}$-CTD sequence was successfully attached to the pET-25b vector.

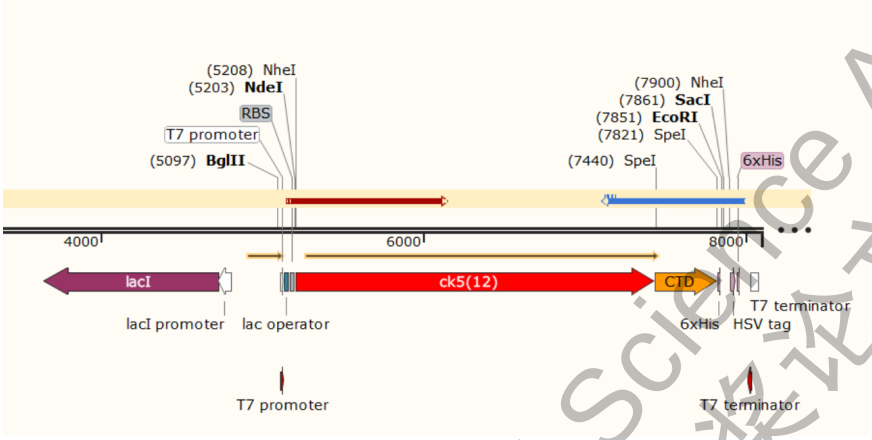

Figure 4. The results of forward and reverse sequencing. The ck5(12) is the target sequence. The red and blue arrows indicate the sequencing results of the ligation product, which corresponds with the sequence of the target gene.

Expression and Purification of Recombinant Spider Silk Protein Plasmid

Through analysis, it was found that the addition of lysine to the recombinant gene sequence improves the solubility of the protein in the expression process. This study employed a fusion expression strategy combining the partial protein sequence (C) of MaSp2 with elastin-like protein (K) to synthesize a recombinant spider silk protein gene sequence containing 12 repeat units. The protein sequence of the elastin-like protein is VPGKG, which contains a hydrophilic lysine. Lysine has an amino group ($-NH_2$) on its side chain, which at physiological pH converts to NH_3+. This positive charge enabled lysine to bind to water molecules through hydrogen bonds, which contribute to protein solubility. By comparing the SDS-PAGE gel electrophoresis bands of pET-25b, bacterial suspension, and supernatant, whether the recombinant protein is dissolved in the supernatant solution can be concluded.

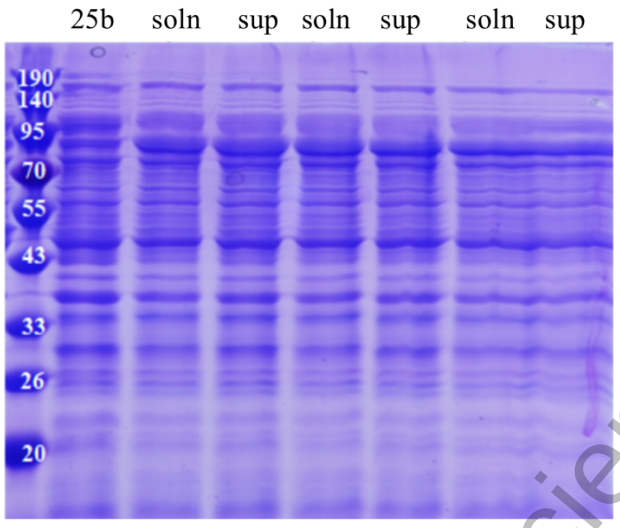

Figure 5. The SDS-PAGE gel electrophoresis of pET-25b, the bacterial suspension solution, and the supernatant after protein expression.

The SDS-PAGE gel electrophoresis diagram of the carrier plasmid pET-25b, bacteria suspension solution, and supernatant is shown in Figure 5. In this research, the designed recombinant spider protein has a molecular weight of approximately 76.27 kDa; therefore, the recombinant proteins were expected to appear between 95 and 70 kDa on the gel electrophoresis. As the graph shows, between 95 and 70 kDa, the bands of the carrier plasmid 25b column and the bacteria solution column show different brightness and width, which means the recombinant protein was successfully expressed and remained in the bacteria solution. Then, comparing the solution column and the supernatant column, it was found that the bands in the two columns show a similar brightness and width. This result indicates that the recombinant protein remained in the supernatant after removing the precipitate, which means the recombinant protein is dissolved in water. This gel electrophoresis points out that the designed recombinant spider silk protein is soluble, and the introduction of an elastin-like protein that contains lysine does contribute to the protein's dissolution.

During the purification process, we utilized the property that the histidine tag carried by the protein can specifically bind to nickel ions. Based on this principle, the first purification step was carried out using a nickel-affinity chromatography column (Ni column). During the experiment, both the flow-through fraction obtained during sample loading and the protein fraction eluted with Ni Elution buffer (containing imidazole) were collected. The underlying mechanism involves imidazole competing with the histidine-tagged protein for coordination with nickel ions; through this competitive interaction, imidazole effectively displaces the protein from the nickel binding sites, therefore achieving protein elution.

To further purify the recombinant spider silk protein, cation-exchange chromatography was employed as the second purification step, taking advantage of the protein's high positive charge. This approach relies on electrostatic forces between the protein and the negatively charged resin, enabling selective adsorption of the target protein. After loading, both the flow-through fraction and the eluted fraction were collected: the flow-through contained unbound proteins, while the target protein was dissociated from the SP column using a high-salt elution buffer (containing a high concentration of Na^+ ions).

However, the protein fractions obtained from ion-exchange chromatography retained relatively high concentrations of Na^+ ions, which could interfere with the structural integrity and fiber-forming behavior of the protein solution during subsequent spinning processes. To eliminate the influence of salt ions, the eluted samples were subjected to desalting, yielding a high-purity, low-ionic-strength protein solution suitable for fiber spinning experiments. Figure 6 shows the results of purification.

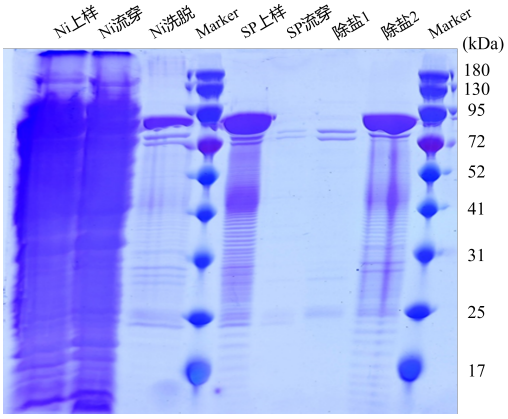

Figure 6. The result of protein purification.

Mechanical Property Curve Graph

After dissolving the protein in formic acid solution, it was subjected to two different spinning treatments, followed by an analysis of the mechanical perforemance of the resulting fibers. First, the dissolved protein was subjected to a coagulation bath composed of a 90% concentration of ethanol solution to produce fibers. After generating the fibers, 5 pieces of fibers from a single silk were collected and tested for their properties using the FAVIMAT+ fiber tensile tester. The graph of the average performance of the fibers was created as shown in Figure 7.

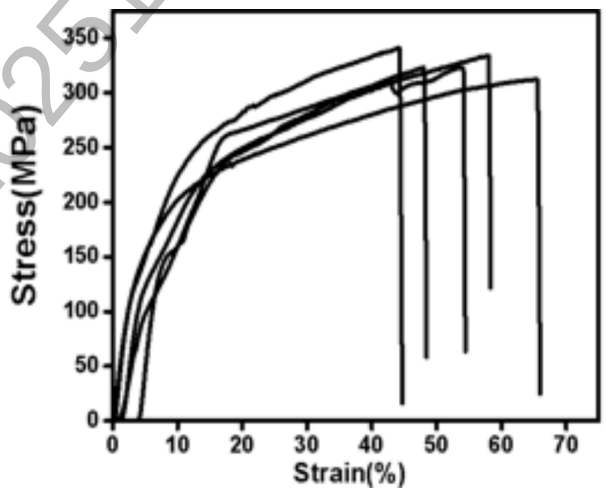

Figure 7. The mechanical property curves of $(CK5)_{12}$-CTD protein fibers that generated using a 90% concentration of ethanol.

	Strength (MPa)	Toughness (MJ/m^3)	Modulus (GPa)	Ductility (%)
Ethanol	326.66±10.99	134.21±22.42	4.63±1.30	55.39±8.36

Table 2. The properties of $(CK5)_{12}$-CTD protein fibers that generated in a 90% concentration of ethanol.

The overall mechanical properties of the fibers, determined through feature analysis, are summarized in Table 2. The maximum stress the fibers can withstand before failure is 326.66 ± 10.99 MPa. The roughness of the fibers is 134.21 ± 22.42 MJ/m^3, indicating that the energy absorbed before fracture is around 134.21 MJ/m^3. The fibers' stiffness is 4.63 ± 1.3 GPa, and the extent of plastic deformation before fracture of the fibers is 55.39 ± 8.36%. The fibers show a relatively great mechanical properties, especially in strength.

Next, utilized the coagulation bath that contains a 90% concentration of ethanol plus 1.2 ml of 50% concentration glutaraldehyde solution to produce silk fibers. Then, selected 5 pieces of fibers from a single silk and measured the properties using the same instrument as before. The graph of the average properties of the fibers is shown in Figure 8.

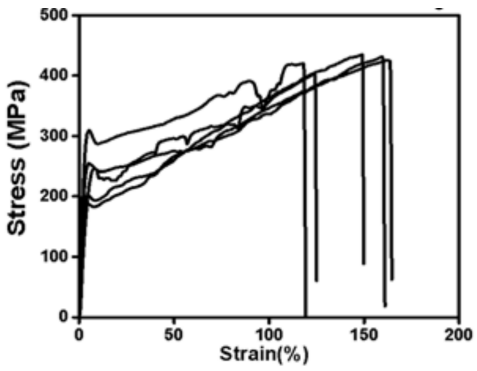

Figure 8. The mechanical property curves of $(CK5)_{12}$-CTD protein fibers that generated using a 90% concentration of ethanol plus 1.2 ml of 50% glutaraldehyde.

	Strength (MPa)	Toughness (MJ/m^3)	Modulus (GPa)	Ductility (%)
Ethanol+ glutaraldehyde	422.99±12.76	452.38±61.06	7.98±1.74	143.98±20.69

Table 3. The properties of $(CK5)_{12}$-CTD protein fibers that generated in a 90% concentration of ethanol plus 1.2 ml of 50% glutaraldehyde.

The overall properties of the fibers generated by a coagulation bath containing glutaraldehyde are shown above. The strength of fibers is 422.99 ± 12.76 MPa, indicating a relatively high resistance to deformation. The roughness of the fibers is 452.38 ± 61.06 MJ/m^3, suggesting excellent energy absorption capacity. The fibers' stiffness is 7.98 ± 1.74 GPa. The ductility of 143.98 ± 20.69% shows the plastic deformation capability prior to failure. In summary, the fibers show excellent mechanical properties both in strength and toughness.

	Strength (MPa)	Toughness (MJ/m^3)	Modulus (GPa)	Ductility (%)
Ethanol	326.66±10.99	134.21±22.42	4.63±1.30	55.39±8.36
Ethanol+ glutaraldehyde	422.99±12.76	452.38±61.06	7.98±1.74	143.98±20.69

Table 4. The comparison of mechanical properties between fibers generated in 90% ethanol and fibers generated in 90% concentration of ethanol plus 1.2 ml of 50% glutaraldehyde.

Comparison of fibers produced under the two spinning conditions revealed that incorporating glutaraldehyde into the coagulation bath enhances the mechanical performance

of the resulting fibers. The aldehyde group in glutaraldehyde can react with the amino group of lysine residues (-NH_2) in proteins through a condensation reaction, causing the formation of a covalent bond, which increases the strength of the fiber. According to Table 4, the fibers produced using ethanol and glutaraldehyde have overall greater mechanical properties than fibers produced using only ethanol. Especially in toughness, ethanol and glutaraldehyde generated fibers have a toughness of about 452.38 MJ/m^3 and a ductility of about 143.98%, which are largely higher than the ethanol generated fibers (toughness of about 134.21 MJ/m^3 and ductility of about 55.39%). In aggregate, the addition of glutaraldehyde in the coagulation bath has contributed to the overall mechanical properties of fibers.

Structural Characterization of Recombinant Spider Silk Protein Fiber

First, the microscopic morphology of the fiber samples prepared under formic acid conditions was observed by scanning electron microscope, and the results are shown in Figure 9. High-resolution imaging reveals that the prepared fibers exhibit a continuous and uniform surface morphology, with no apparent internal defects such as pores, cracks, or inclusions. Particularly, its surface smoothness is high, showing excellent structural regularity and continuity. This morphological feature is highly similar to the typical surface morphology of natural spider silk (Wang, 2022), indicating that formic acid conditions contribute to the formation of fibers with dense structures and controllable faults.

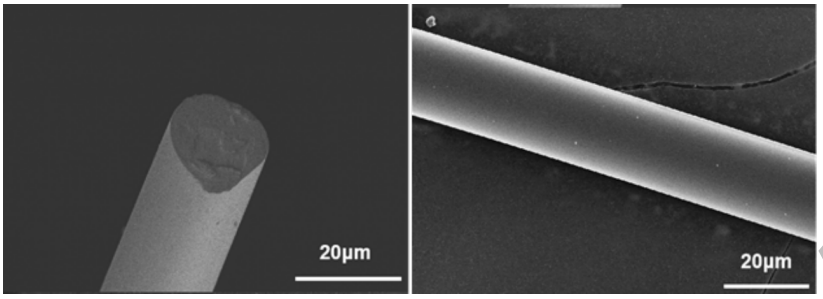

Figure 9. Scanning electron microscope of $(CK5)_{12}$-CTD protein fiber under formic acid conditions.

The orientation of the fiber samples before and after stretching was characterized by a polarized optical microscope (POM), and the results are shown in Figure 10. Comparing the POM images before and after stretching, it can be clearly observed that after tensile orientation, the microcrystalline structure of the β-sheet (β-sheet) inside the fiber is significantly oriented in the direction of tensile, showing obvious birefringence. The increase in birefringent strength directly reflects the increase in the orientation of macromolecular chains within the fiber, indicating that the microstructure orderliness and regularity are improved (He, 2020). This highly oriented structure helps improve the mechanical properties of protein fibers.

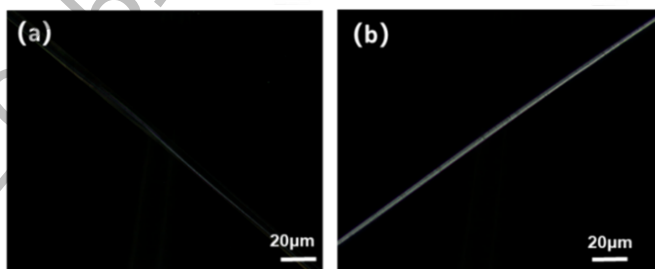

Figure 10. Polarization light microscope of $(CK5)_{12}$-CTD protein fibers before and after stretching under formic acid conditions.

Then, the secondary structural changes of CK5-CTD protein in formic acid solution were analyzed by Fourier transform infrared spectroscopy (FTIR), and the amide I band (1600–1700 cm^{-1}) was subjected to peak fitting, as shown in Figure 11. The results showed that a characteristic absorption peak attributable to the angular structure of the protein occurred at 1691 cm^{-1}, indicating that formic acid can promote the formation of the turn structure in protein molecules (Shao, 2015). The increase in turn structure enhances the flexibility and motion of protein molecular chains, thereby significantly improving the ductility and deformation ability of fiber materials at a macroscopic level. This finding provides key evidence elucidating how formic acid treatment influences the mechanical properties of protein fibers from the perspective of molecular conformation.

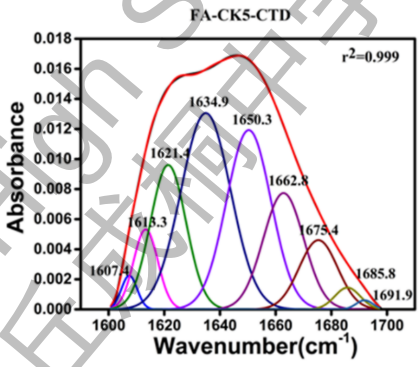

Figure 11. Curve fitting of the amide I region FTIR spectrum of the $(CK5)_{12}$-CTD protein under formic acid conditions shows the red line as the fitted curve and the black line as the original spectrum. The high fitting coefficient ($R^2 > 0.999$) indicates excellent agreement between the fitted and experimental curves.

A transmission electron microscope was used to observe the self-assembly behavior of protein samples dissolved in formic acid during ethanol solvent displacement, as shown in Figure 12. The results showed that under ethanol induction, proteins aggregated significantly

and formed short rod-like or fibrous nanostructures of different lengths. This morphological feature suggests that ethanol may induce targeted aggregation and nucleation of proteins by changing the polarity of solvents. To further explore the internal structure and assembly mechanism of these fibrous aggregates, a low-temperature electron microscope is proposed to analyze their internal secondary structure distribution and molecular arrangement at near-atomic resolution, thereby providing a structural biological basis for understanding the protein self-assembly pathway and fiber formation mechanism.

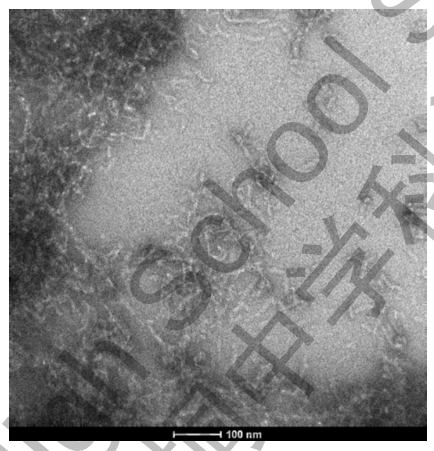

Figure 12. Self-assembly of recombinant proteins during ethanol solvent exchange.

Conclusion

Motivated by the remarkable properties of natural spider silk, this study focuses on the development of recombinant spider silk protein fibers exhibiting enhanced robustness and ductility. Through the design of modular recombinant spider silk proteins, fibers with superior mechanical performance were successfully fabricated. Our results showed that the introduction of elastin-like protein VPGKG, which contains lysine, to the recombinant spider silk protein helps produce a soluble recombinant protein, and this improvement greatly

increases the efficiency in the protein expression process. In addition, formic acid acts as a solvent to effectively promote the formation of β-corner structures, thereby imparting excellent toughness to the fibers. Moreover, it was found that the addition of glutaraldehyde to the spinning coagulation base has enhanced the aggregate mechanical performance of the spider silk fibers. This study offers a viable approach for the fabrication of biomimetic, enhanced-performance protein fibers and demonstrates potential applicability in the field of materials science.

Future Perspectives

While the E. coli expression system enables the soluble production of recombinant spider silk proteins, its expression level is still limited, and the host lacks an efficient secretion mechanism, resulting in complex purification processes and low yields. Therefore, there is an urgent need to develop more advantageous recombinant expression systems to significantly increase protein yield and promote their secretive expression, thereby simplifying purification steps, reducing costs, and improving yield efficiency. In addition, advanced structural biology techniques such as cryo-electron microscopy and solid-state nuclear magnetic resonance can be employed to systematically analyze the assembly process of recombinant spider silk proteins during the transition from solution to fiber state at near-atomic resolution. This high-precision structural information will deeply reveal the molecular basis of its macroscopic mechanical properties and provide a theoretical basis and technical path for rationally designing biomimetic fiber materials with customizable mechanical functions.

Acknowledgement

When I first came to Professor Liu Kai's lab in 2023, I was obsessed with the amazing process of the formation of artificial fibers: from various chemical agents to finally physical fibers. Since I had anticipated the IRP course at my high school, I had a chance to be accepted learning in Professor Liu's lab for artificial spider silk fibers. From August 2024 to August 2025, I spent a lot of time and effort on the experiment: constructing the plasmids, culturing E. coli, expressing and purifying the protein, and spinning the fibers. I did every step again and again until I got the results that I wanted, and then I wrote this paper. During the experiment, I faced some challenges, such as many theories involved in the experiment that were beyond the scope of high school knowledge, and some steps in the experiment often failed to get the desired result. To overcome these difficulties, I kept learning new knowledge and theories through reading books and literature, and I continually explored the reasons when I failed an experiment and then tried to fix it.

During my research, the person who helped me the most was my supervisor, Professor Liu Kai. I would like to thank him for his careful guidance and full support in topic selection, theoretical learning, data analysis, and paper writing, so that I was able to successfully complete the whole research. Especially, he explained the theoretical knowledge to me and taught me how to organize and connect this knowledge. In addition, I would like to thank Professor Liu Kai's research team for providing me with free experimental conditions, instruments, and selfless guidance and help.

Doctor Tang Lingfang is a biology teacher at my school, and after my thesis and essay were completed, she put forward many pertinent revisions to help me continue to polish and improve.

Moreover, I would like to express my heartfelt gratitude to Senior Qin Dawen for providing great help to my research and experimental progress. He helped me understand the details of the experiment more deeply, patiently guided me on how to use the instruments in the laboratory, carefully helped me improve many experimental operations, so that the research was more rigorous and accurate, and the research process could proceed smoothly, and finally achieved the ideal experimental results.

Finally, I would like to thank my parents for their support and concern for me. Their love and warmth have given me great strength, so that I can seriously and persistently complete the research project and constantly revise and improve the thesis.

References

Andersson, M., Chen, G., Otikovs, M., Landreh, M., Nordling, K., Kronqvist, N.,Westermark, P., Jörnvall, H., Knight, S., Ridderstråle, Y., Holm, L., Meng, Q., Jaudzems, K., Chesler, M., Johansson, J., & Rising, A. (2014). Carbonic anhydrase generates CO2 and H+ that drive spider silk formation via opposite effects on the terminal domains. *PLoS biology*, *12*(8), e1001921.

Agnieray, H., Glasson, J. L., Chen, Q., Kaur, M., & Domigan, L. J. (2021). Recent developments in sustainably sourced protein-based biomaterials. *Biochemical Society transactions*, *49*(2), 953–964.

Blamires, S. J., Wu, C. L., Blackledge, T. A., & Tso, I. M. (2012). Post-secretion processing influences spider silk performance. *Journal of the Royal Society, Interface*, *9*(75), 2479–2487.

Celik, E., & Calık, P. (2012). Production of recombinant proteins by yeast cells. *Biotechnology advances*, *30*(5), 1108–1118.

Cho, H. J., Ki, C. S., Oh, H., Lee, K. H., & Um, I. C. (2012). Molecular weight distribution and solution properties of silk fibroins with different dissolution conditions. *International journal of biological macromolecules*, *51*(3), 336–341.

Doblhofer, E., Heidebrecht, A., & Scheibel, T. (2015). To spin or not to spin: spider silk fibers and more. *Applied microbiology and biotechnology*, *99*(22), 9361–9380.

DeFrancesco, L. (2017). Hanging on a thread. *Nature biotechnology*, *35*(6), 496–499.

Eisoldt, L., Smith, A., & Scheibel, T. (2011). Decoding the secrets of spider silk. *Materials Today*, *14*(3), 80-86.

Formhals, A. (1934). Process and Apparatus for Preparing Artificial Threads. *US Patent* No. 1975504.

Gosline, J. M., Guerette, P. A., Ortlepp, C. S., & Savage, K. N. (1999). The mechanical design of spider silks: from fibroin sequence to mechanical function. *The Journal of experimental biology*, *202*(Pt 23), 3295–3303.

Ha, S. W., Tonelli, A. E., & Hudson, S. M. (2005). Structural studies of Bombyx mori silk fibroin during regeneration from solutions and wet fiber spinning. *Biomacromolecules*, *6*(3), 1722–1731.

He, H., Yang, C., Wang, F., Wei, Z., Shen, J., Chen, D., Fan, C., Zhang, H., & Liu, K. (2020). Mechanically Strong Globular-Protein-Based Fibers Obtained Using a Microfluidic Spinning Technique. *Angewandte Chemie (International ed. In English)*, *59*(11), 4344–4348.

Jansson, R., Lau, C. H., Ishida, T., Ramström, M., Sandgren, M., & Hedhammar, M. (2016). Functionalized silk assembled from a recombinant spider silk fusion protein (Z-4RepCT) produced in the methylotrophic yeast Pichia pastoris. *Biotechnology journal*, *11*(5), 687 -699.

Knight, D. P., Knight, M. M., & Vollrath, F. (2000). Beta transition and stress-induced phase separation in the spinning of spider dragline silk. *International journal of biological macromolecules*, *27*(3), 205–210.

Lazaris, A., Arcidiacono, S., Huang, Y., Zhou, J. F., Duguay, F., Chretien, N., Welsh, E. A., Soares, J. W., & Karatzas, C. N. (2002). Spider silk fibers spun from soluble recombinant silk produced in mammalian cells. *Science (New York, N.Y.)*, *295*(5554), 472–476.

Lefèvre, T., & Auger, M. (2016). Spider silk as a blueprint for greener materials: A review. *International Materials Reviews*.

Ma, C., Sun, J., Li, B., Feng, Y., Sun, Y., Xiang, L., Wu, B., Xiao, L., Liu, B., Petrovskii, V. S., Liu, B., Zhang, J., Wang, Z., Li, H., Zhang, L., Li, J., Wang, F., Göstl, R., Potemkin, I. I., . . . Herrmann, A. (2021). Ultra-strong bio-glue from genetically engineered polypeptides. *Nature Communications*, *12*(1), 1-14.

Pontrelli, S., Chiu, T. Y., Lan, E. I., Chen, F. Y., Chang, P., & Liao, J. C. (2018). Escherichia coli as a host for metabolic engineering. *Metabolic engineering*, *50*, 16–46.

Suran, M. (2018). A planet too rich in fibre: Microfibre pollution may have major consequences on the environment and human health. *EMBO reports*, *19*(9), e46701.

Tokareva, O., Jacobsen, M., Buehler, M., Wong, J., & Kaplan, D. L. (2014). Structure function-property-design interplay in biopolymers: spider silk. *Acta biomaterialia*, *10*(4), 1612–1626.

Vollrath, F., & Knight, D. P. (1999). Structure and function of the silk production pathway in the spider Nephila edulis. *International journal of biological macromolecules*, *24*(2-3), 243–249.

Wang, Q., McArdle, P., Wang, S. L., Wilmington, R. L., Xing, Z., Greenwood, A., Cotten, M. L., Qazilbash, M. M., & Schniepp, H. C. (2022). Protein secondary structure in spider silk nanofibrils. *Nature Communications*, *13*(1), 1-12.

Zhou, G., Shao, Z., Knight, D. P., Yan, J., & Chen, X. (2009). Silk Fibers Extruded Artificially from Aqueous Solutions of Regenerated Bombyx mori Silk Fibroin are Tougher than their Natural Counterparts. *Adv. Mater.*, *21*(3), 366–370.

Shao, Z.-Z. (2015). *Silk, silkworm and spider silks and their silk proteins* [M]. Chemical Industry Press.

Zhang, J., Sun, J., Li, B., Yang, C., Shen, J., Wang, N., Gu, R., Wang, D., Chen, D., Hu, H., Fan, C., Zhang, H., & Liu, K. (2020). Robust Biological Fibers Based on Widely Available Proteins: Facile Fabrication and Suturing Application. *Small*, *16*(8), 1907598.