Isothermal Assembly of a Multidomain Spider Silk Gene

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Reviewed on 29 April 2017; Accepted on 19 June 2017; Published on 10 November 2017

Researchers have been exploring the use of spider silk to make synthetic textiles; however, the spider silk is composed of a highly repetitive multi-domain protein and this makes the gene hard to synthesize using traditional commercial techniques. We explored a procedure for a technique using restriction enzymes and DNA ligase in an isothermal reaction which ran overnight. We accomplished this by using restriction enzymes a and b, which when ligated together are no longer subjected to further digest. These reactions were viewed on a flash gel to verify bandwidths. When the products of this reaction were viewed on a gel, multiple bands appeared. The size of the fragments depended on the relative concentration of the gene segments. This appears to be a fairly efficient technique for assembling a gene for encoding a complex spider silk protein. Future experiments could involve creating more complex assemblies and products.

Keywords: Gene assembly, gene fragment lengths, spider silk gene, multi domain protein, isothermal assembly, synthetic textiles

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Watch a video introduction by the authors at
http://bit.ly/2uWuQ3e

When one thinks of a spider, either the horrific thought of a hairy tarantula, or the cute spider from Charlotte's Web arises; however, by studying spiders, specifically their webs, scientists have come to understand not only the miracles that are these spider silk proteins, but also how they can provide a myriad of options and answers to problems that face society.

To understand the genetic nature of these proteins, one must first understand genetics itself. Genetics, by definition, is the study of heredity, or the way that traits are passed from parent to offspring. As time moves forward, science has learned that DNA is the fundamental component of heredity that determines which traits are expressed in an individual. Alterations to these sequences of nucleotides can lead to mutations. Consequently, these positive, negative, or neutral changes in proteins are responsible for different traits. It is through these mutations, however, that scientists can understand both positive and negative outcomes; scientists can discover how mutations strengthen the gene pool and how these mutations can create unique products that could be used in further research.

Throughout history, spider silk has been quite useful in many different areas; the ancient Greeks used this silk to stop bleeding wounds, Aborigines used the silk for fishing, and more

recently, many types of guns and telescopes used in battle (during World War II for example) have used spider silk for the crosshairs in their optical devices (Spider Silk, n.d.). More current research has shown that spider webs can be extremely useful in textile manufacturing as they are comprised of fibers that are extremely strong and versatile. Spider silk would be ideal for textiles that need to be of the highest strength, such as bullet proof vests and catch lines on aircraft carriers. Therefore, it can be proposed that through the genetic engineering of such webs and the chemical and mechanical manufacturing techniques available today, a sufficient and ideal material could be synthesized to help society, while would still represent an environmentally- and cost-friendly option that can be constructed without the use of strong chemical reagents.

The purpose of Frederick High School's research this year was to begin engineering a spider silk gene that is capable of producing a fluorescent web. Building on prior research conducted at Frederick High School, we planned to use our sequences that encode the fluorescent LOV protein to help create our fluorescent spider silk (Bollinger et al, 2016). Unfortunately, we first had to overcome issues with the commercial synthesis of the spider silk gene, as the spider silk protein sequence is extremely repetitive, which makes it difficult to synthesize the gene commercially. The repetitive region was a problem because the company that we initially planned on having synthesize the gene for us, said that they would not be able to synthesize the gene because the process they used wouldn't work on a gene that was as repetitive as ours. As a result, we came up with our own solution involving the use of competing restriction enzymes and ligases to assemble our gene. We were able to ligate together our fragments, obtained from a company that commercially synthesized them, through the addition of ligase to our solution.

In this project, we decided to create the constructs ourselves through the use of overlapping repeated sequences and restriction enzymes that, while cutting the DNA fragments, would allow the overlay to be ligated together, thus creating our spider silk protein sequence. Beyond this year's project, the team plans to engineer a spinning device to begin the spinning of actual spider silk, and to begin using the silk in tangible products. Several products currently underway that use this technology in commercial application include bullet-proof clothing, wear-resistant clothing, ropes, nets, seat belts, parachutes, and biodegradable bottles among many other items (Spider Silk, n.d.).

Materials and Methods

Isothermal assembly of our spider silk gene

Materials needed for this experiment include a thermocycler, a Smart Cut Buffer, microtubes, micropipettes and tips, and 10 µM of each of the six oligonucleotides that comprise Segments A, B, and C. Four tubes were created before the thermocycling process began. In the first tube, 40 µL of nuclease-free water, 2.5 µL each of 10 µM FWD-A, REV-A, FWD-B, and REV-B were added. Tube 2 consisted of 28 µL of nuclease-free water, 2.5 µL each of 10 µM FWD-A, REV-A, FWD-B, and REV-B, 5 µL Smart Cut Buffer (New England Biolabs [NEB]), 5 µL of 10 mM RiboATP (NEB), 1 µL of XmaI (NEB) and 1 µL of BspI (NEB). In tube 3, 22 µL of nuclease-free water, 2.5 µL each of 10 µM FWD-A, REV-A, FWD-B, REV-B, FWD-C, and REV-C, 5 µL Smart Cut buffer, 5 µL of 10 mM RiboATP, 1 µL XmaI, 1 µL BspI and 1 µL T4 DNA Ligase. Tube 4 consisted of 2 µL nuclease-free water, 12.5 µL each of 10 µM FWD-C and REV-C, 2.5 µL each of 10 µM FWD-A, REV-A, FWD-B, and REV-B, 5 µL Smart Cut Buffer, 5 µL of 10 mM RiboATP, 1 µL XmaI, 1 µL BspI, and 1 µL T4 DNA ligase. Each of these tubes was placed inside a thermocycler set to hold at 25°C for 12 hours to allow restriction enzymes to cut and ligases to bring the DNA sequence back together; 37°C for 1 hour to promote full cleavage of any remaining restriction sites; 65°C for 20 minutes in order to heat inactivate all the enzymes; and finally, 4°C to keep all samples chilled until recovery.

Visualization confirmation of our spider silk assembly and extraction of DNA bands

After samples were retrieved from the thermocycler, it needed to be determined whether or not the DNA had successfully combined to form the sequence that would produce our preferred spider silk gene. In this part of the experiment, we visualized the polymerase chain reaction (PCR) assembled fragments on a Flashgel (Lonza). Materials needed for this part of the experiment are the four products from the thermocycler, micropipettes and tips, a FlashGel System, FlashGel cassettes along with a specific marker and loading dye. In Well 1 of the gel, 5 µL of DNA marker was pipetted to create our standard for measurements. In four separate microcentrifuge tubes, 1 µL of 5 X FlashGel loading dye was added to each tube containing 5 µL of samples from the PCR procedure. Afterwards, 4 µL of the total volume was pipetted into the corresponding wells of the FlashGel cassette. When all samples were added, the cassette was loaded into the FlashGel apparatus. The voltage cables were then plugged into a power source. The UV light housed within the apparatus was turned on along with the power source (dialed up to 275 V) to begin the movement of DNA bands through the gel. According to the product's protocol: Never plug in the low voltage power supply and turn on the power supply to the UV light when a cassette is not in the dock. Approximately five minutes after initiation, the bands reached their optimal distance from the wells and we turned off the power source. Because we wanted to extract only the DNA that was viable for us, we used a Flashgel with collection ports. When the bands reached the open site on the gel, the power was turned off and a pipette was inserted into the port to remove the DNA from that band. This DNA was then deposited in a microcentrifuge tube to await further testing.

Safety

Throughout our experimentation and in various labs, proper safety methods were practiced: safety gear was worn and safety procedures were followed. Every student was trained in the use and care of all tools, preventing any damage to the materials or the students themselves. Routine checks were completed on eyewash stations and chemical showers to ensure proper functionality. Disposable gloves and goggles were worn when handling any of the lab materials. To eliminate any

contamination issues, all lab surfaces were cleaned with Q18 disinfectant, and Clorox wipes were used for any surfaces in the lab that would've come into direct contact with lab equipment. All labs were overseen by our mentor, Mark Trice, and occasionally by our associate, Dr. David Rozak, and all lab designs were finalized by both of them to prevent any faulty procedures. Thermocycler and vortex training were completed so that neither would be damaged or cause any injury.

Results

Designing our gene segments

When designing our segments, we had to carefully consider the effects of our restriction enzymes on the strength of the final product. First and foremost these restriction enzymes will actually cut our DNA, hence serving their purpose to splice everything together. Following the lead of Teulé et al. (2009), we chose two enzymes (XmaI and BspEI) for their abilities to create a permanent bond upon annealing that would not be recognized again by the enzymes throughout the experiment. The thought process behind this procedure was due to the fact that the two enzymes produce DNA fragments with compatible cohesive ends that can be ligated together. However, the resulting ligation site is no longer recognized by either of the restriction enzymes and won't be re-cut.

Throughout the project we inserted multiple C segments into the gene as this is the final segment and most critical to be picked up, since segment C is the segment that actually produces the spider silk, while the other segments are the operons that allow the spider silk to be made. Additionally, it is seen in our results that the more C segments that were added to the solution, the larger the band on the gel, which results in a higher concentration of DNA fragments. It could therefore be theorized that the higher the concentration of DNA, the more spider web could ultimately be produced.

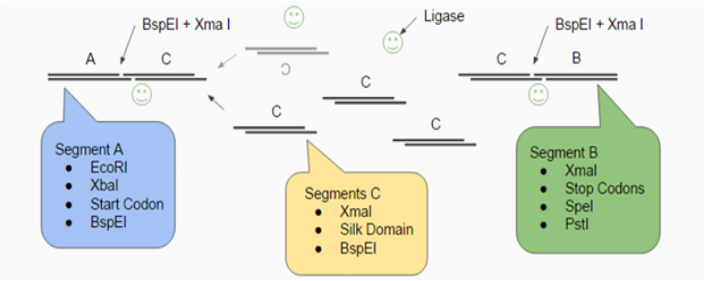

Figure 1. Components of each segment used in our research. With the restriction digest and subsequent ligation, we were able to form a repetitive chain that could later be separated through electrophoresis to verify our results.

Designing the isothermal reaction conditions

Tuele, et al. (2009) proposed propagating the growing gene fragment in *Escherichia coli* after each ligation step. However, we decided to explore a different approach in order to avoid culturing *E. coli*. Rather than continually transforming bacteria to produce small segments, we had multiple digests and ligations within one reaction that could ultimately produce one larger recombinant fragment. Figure 1 shows how each fragment was restricted and then ligated to form the recombinant fragment that includes all segments. Table 1 shows the specific primers that were used that correspond to segments A, B, and C within the experimental protocol.

Table 1.
Primers used to create Segments A, B, and C.

FWD-A	GGTCGGAGAATTCTCTAGAATGT
REV-A	CCGGACATTCTAGAGAATTCTCCGACC
FWB-B	CCGGGTAATGAACTAGTCTGCAGTCGAG
REV-B	CTCGACTGCAGACTAGTTCATTAC
FWD-C	CCGGGCAGGGAGCAGGACAGGGTGGATATG GTGGTTTAGGTGGGCAAGGTGCAGGACAAG-GTGCAGGTGCCGCGGCAGCAGCCGCAT
REV-C	CCGGATGCGGCTGCCGCGGACCTGCACCTTG TCCTGCACCTTGCCCACCTAAACCACCATATC-CACCCTGTCCTGCTCCCTGC

Observing reaction products on a gel

In Figure 2, the Flash Gel showing segments A, B, and C can be seen. Well 1 has the DNA ladder. Well 2 contains a mixture of segments A and B without the enzyme. Well 3 contain A and B with the polymerase. Wells 4 and 5 add increasing amounts of segment C. The number and sizes of the bands increase as we increase the concentration of segment C, suggesting that multiple copies are being successfully inserted into the assembly.

When looking at Wells 4 and 5, the gel is a brighter color, indicating higher DNA concentration, and the bands also travel farther than the rest, indicating that there are more, smaller fragments due to the C segments being added. The appearance of distinct bands allowed us to count the number of segments integrated in the assembly and ultimately indicate the presence of each segment and their respective concentrations.

Discussions

After completing the digest and ligations through the thermocycling process, we found that our DNA had successfully been cut and re-annealed to produce larger fragments. We determined this on the basis that the size and fragment pattern of our bands increased as we move from left to right on the gel. As we observe from left to right, as more DNA was ligated together, more strands appear at the top of the gel due to the increased weight and size of the new DNA fragments as opposed to individual components. This appears to be in direct correlation with segment C, as the amount of this fragment increased as we move right across the gel. We concluded that we would be able to continue to the next portion of the experiment, which would be to isolate the DNA we are specifically interested in.

Because we are interested in ultimately creating the spider

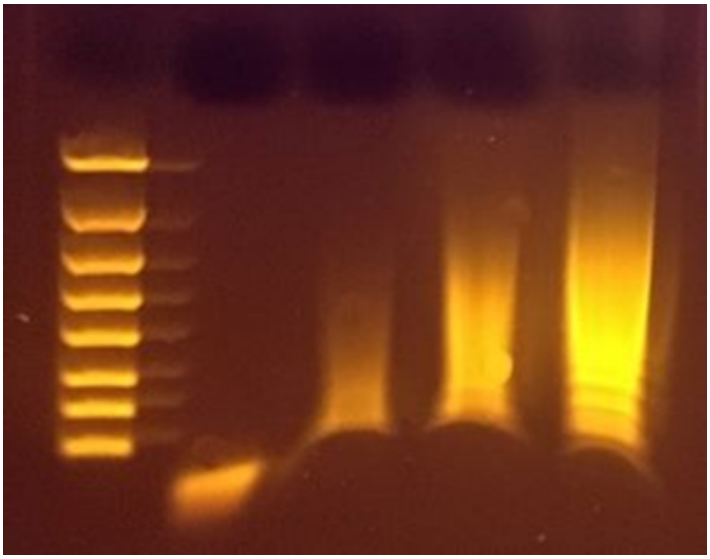

Figure 2. The gel shows segments A, B, and C assembled correctly. Well 1 has the FlashGel standard DNA ladder (100-4000 bp). Well 2 contains a mixture of segments A and B without the enzyme. Well 3 contains A and B with the polymerase. Wells 4 and 5 add increasing amounts of segment C, with well 4 having 5 µL of C and well 5 having 25 µL of C.

silk sequence, we wanted to isolate the DNA product that produces the silk apart from the other segments in the tubes, which could, in theory, result in less efficient and lower textile strengths. Further into our research, we completed a second trial of gel electrophoresis to duplicate the results to ensure that not only did we have our bands, but that we could also extract the specific bands needed to further reach the goal of the specific silk sequence. We successfully extracted the DNA and have placed each of the samples in storage to wait for further analysis.

While commercial synthesis of the highly repetitive spider silk gene was the greatest hurdle for us to overcome, our team, with the help of Dr. Dave Rozak, was able to develop an alternative procedure to enable us to move forward in our research. When the commercial application didn't work, our team worked together to find a different path. This ultimately helped us to learn to think "outside the box" and facilitated the very core ideals of our after-school program with engineering aspects and ideas leading to the creation of a novel solution.

In the future, we hope to place the DNA into E. coli to grow colonies with this specific sequence in the resulting cells. If we can successfully transform them into E. coli and then effectively isolate the protein, we will know we have a functioning spider silk protein. After synthesizing the product, we hope to continue to increase the number of proteins we are able to produce and ultimately create a machine that would help to spin the spider silk, resulting in a high tensile strength textile.

Acknowledgements

First and foremost, we would like to thank our advisors, Mr. Mark Trice and Dr. Dave Rozak. We would also like to thank New England BioLabs for providing reagents and Frederick High School for accommodating our lab needs.

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