

Biomimetic Manufacturing of Fibers: Materials Development

Michael Ellison, leader; Albert Abbott, Jackie Palmer, Gary Lickfield, William Marcotte, Robert Latour, Cécile Aubé, Jamie Wood (Clemson)

The Golden Orb Weaver spider *Nephila clavipes* (see right) manufactures seven types of silk of different compositions that exhibit different physical properties adapted for a specific use (e.g., web, cocoon, and dragline). These silks are natural protein-based fiber-forming polymers resulting from the self-assembly of many individual fiber protein molecules into a complex and stable network. Physical and chemical studies performed on spider dragline silk, produced by the major ampullate glands of the spider, suggest that this silk exhibits unique properties that are superior to those displayed by the silkworm silk and even to most man made fibers.

Nephila clavipes



The most highly studied is the dragline silk, a fiber whose significant toughness results from the combination of high extensibility and high strength. Extensive information is available regarding *Nephila clavipes*' dragline silk composition, as well as physical property data on the silk fiber. Spider silk is an excellent model to investigate the relationship between the structure of the individual repetitive motifs present in the fiber proteins and their mechanical function in the resulting fiber. Spidroins, the spider dragline silk protein components, are characterized by an amino acid repeat containing a glycine rich motif, resulting in an amorphous fraction, followed by an alanine rich motif (crystalline fraction); protein conformation plays a critical role. Understanding the rules governing this composition/ structure/function relationship is also the first step towards fiber protein engineering for the production of designed novel protein-based biomaterials.

We generated Spidroin 1-like genes encoding for proteins containing a variable amount of alanine motifs (normal = ALA, less = LA, and none = NA) using synthetic oligonucleotides that were designed to span the region encod-

Using spider silk and collagen as a model, we are investigating the role that protein primary structural components play in protein expression and in fiber production and properties.

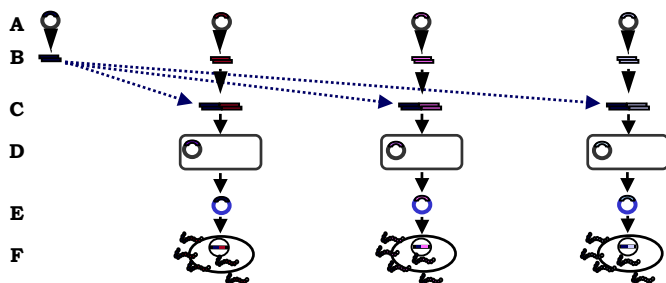
ing for the amino acid consensus repeat of the major ampullate Spidroin 1 protein. We produced three types of Spidroin 1-like proteins homogenous in size and only differing by the number of alanine motifs present in their sequence. In addition, we produced copolymeric proteins from collagen/spidroin1-like genes with selected alanine content that were generated using the sequences which encode the helical part of a cuticle collagen from a parasitic nematode in combination with each of our synthetic Spidroin 1-like genes (see Figure at bottom left).

Our goal was to determine the secondary structure adopted by the alanine motifs present in Spidroins and the copolymer variant produced *in vivo*. Thus, our engineered genes were cloned in yeast and plants (tobacco). This allowed study of the feasibility of both a simple eukaryote such as yeast and a higher plant organism to manufacture, handle and secrete such fiber proteins. The three types of genes ranging between 500 and 600bp were first cloned in *E. coli* for sequence verification and then cloned in *Pichia pastoris* for production of the three types of homopolymer proteins (Spidroin 1-ALA, -LA, or -NA).

We want to determine the secondary structure that is influenced by the alanine motifs present in modified amounts in the transgenically produced silk-like proteins and their possible role in the formation of the natural silk fiber structure. Using *in-situ* polarized Raman spectroscopy, we found for the silk protein structure that step-strain deformation tension, resulted in a peak at 1656 cm^{-1} in the amide I region suggesting the formation of α -helix. Similarly, new peaks appeared at 1290 cm^{-1} (amide III region) and 1056 cm^{-1} (backbone stretch region). However, all these peaks disappeared after relaxation indicating that the elasticity of spider silk might originate from the formation of a helix structure.

We developed a large-scale bioreactor¹ as a "protein factory" that will allow for continuous growth of yeast, with the production of the collagen-spidroin 1 copolymer protein. We used *Pichia Pastoris* to express a recombinant copolymer of *Nephila clavipes* spider dragline silk and collagen under a methanol-regulated promoter (AOX1). The gene for this copolymer protein, inserted into the AOX1 gene, is under the control of the methanol inducible AOX1 promoter. Therefore, when methanol is present cell growth should be proportional to copolymer protein production.

From the bioreactor we obtained a supernatant sample and raw cells, which need to be extracted using a French Press. Heating at 95°C for 20 minutes separated the copolymer spider silk recombinant proteins from most other yeast proteins. Then, we concentrated the recombinant spi-



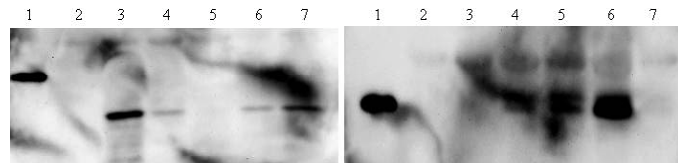
Copolymer Gene Engineering in Yeast

- Collagen, spidroin-1 ALA, spidroin 1-LA, and spidroin 1-NA cloned genes.
- Polymerase chain reaction with specific primers to add restrictions sites and cloning in *E. coli* for sequencing.
- Ligation of the collagen gene to each of the three spidroin 1-like genes to form the copolymer genes.
- Cloning of the copolymer genes in an expression vector in *E. coli* and sequencing the chosen plasmid vector allows the production of a recombinant protein with a *myc* epitope and a polyhistidine tag in its carboxyl terminal for immunological detection and purification by affinity chromatography and allows the secretion of fusion proteins in the culture media, facilitating the purification process).
- Recombinant vector carrying the copolymer genes was used to transform yeast.
- Selection of three types of yeast transformants that each produces and secretes one of the three types of collagen-spidroin 1 like proteins.

¹ aid of Dr. Sarah Harcum (Dept of Chemical Engineering) acknowledged.

der proteins, visualized them by Coomassie Blue staining and subjected them to Western blot analysis for detection.

The major progress of the transgenic plant work was to successfully detect recombinant proteins in extracts from leaves for both the Sp1LA and modCOP proteins (see Immunoblots below showing protein expression). These plants are being propagated further for larger scale protein extraction and development of a purification procedure.



Immunoblot Analysis: T₀ Sp1LA (left) and modCOP Proteins

[Detection with an anti-myc antibody, then a secondary antibody to mouse.]
 Lane 1: Recombinant GST-Em-myc protein as a positive control, ~35-40 kD.
 Lane 2: Wild-type protein sample.
 Lanes 3-7 (T₀ Sp1LA, specifically plants 1.1, 1.2, 1.4, 4.3 and 4.4):
 According to sequence data, the Sp1LA protein is ~23 kilodaltons (kD).
 Lanes 3-7 (T₀ modCOP, specifically plants 1.1, 1.2, 1.5, 2.1 and 3.1):
 Protein is ~21 kD based on sequence data, but signal much closer in size to positive control indicating that modCOP protein is forming dimer in the plant.
 For both immunoblots: Some cross reactivity is seen in the WT lane.
 Protein observed correlates with RNA detected in Northern blot.

We continue to test the function of alanine sequences and their conformational motifs in fiber mechanical properties.

Contributing Graduate Students: *Cécile Aubé*, *Gaurang Narasimhan*, *Xiaomeng Shi*, *Jamie Wood* (Clemson), *Florance Teulé* (U.Wyoming); **Postdoc:** *Xiaofeng Cui*; **Adjunct Faculty:** *Jacqueline Palmer* (Clemson, Bio Sciences).

Industry Interactions: 4 [Agricultural Consultants Inc. (formerly Agritech); **Other:** Academic; Government

Project Web Address:

<http://hubcap.clemson.edu/~ellisom/biomimeticmaterials>

Michael S. Ellison, a Professor of Textile and Polymer Science at Clemson, joined the faculty in 1984. Mike received a Ph.D. in polymer fiber physics at the Univ. of California (Davis) in 1982. His research interests include structure/property relationships in melt extrusion of fibers, tensile and non-tensile loading during mechanical property testing of fibers, electrical properties of polymers and application of chaos theory to polymer physics.



M92-A01, M93-C08*, M94-C04*, M94-S02, M96-C01*, F98-C04*, M98-CL05*, M01-CL01, M02-CL04*, M02-CL05, M04-CL11
ellisom@clemson.edu
 (864)-656-5966

<http://mse.clemson.edu/htm/faculty/Ellison.htm>

Gary C. Lickfield, an Associate Professor in Textiles, Fiber & Polymer Science at Clemson, joined the faculty in 1986. He earned a Ph.D. there in physical chemistry in 1983 and a B.S. in chemistry from Ursinus College in 1978. Gary's research interests include molecular modeling, polymer surfaces and interfaces modification and characterization, wetting and adhesion.



F92-S12, M95-S22, C97-C03*, M98-CL05, C99-CL03, C00-CL01*, C01-CL01, M02-CL04
lgary@clemson.edu
 (864)-656-5964

<http://mse.clemson.edu/htm/faculty/Lickfield.htm>

Albert G. Abbott, a Professor of Biological Sciences at Clemson, joined the faculty in 1984. He earned a Ph.D. in cell and molecular biology from Brown University in 1980 and a B.S. in biological sciences from Univ. of Connecticut in 1976. He was a Fellow at the Rockefeller Foundation's Plant Breeding Institute in Cambridge (England). Bert's research interests include basic gene structure and function, improving plant products through genetic manipulation and genetic engineering to produce novel proteins.



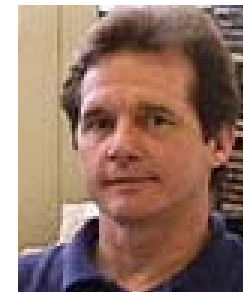
M98-CL05, M02-CD05, M02-CL04

aalbert@clemson.edu

(864)-656-3060

<http://www.clemson.edu/biosci/abbott.html>

Robert A. Latour Jr, a Professor of Bio-engineering at Clemson, joined the faculty upon earning a Ph.D. in bioengineering in 1989 at Univ. of Pennsylvania. Bob also earned a B.S. in chemical engineering at Univ. of Virginia in 1979, then spent 5 years as an oil refinery operations engineer at Amoco. His research interests include thermodynamics of biomolecular interactions, specifically protein-surface adsorption and ligand-receptor binding behavior.



M98-CL05, M02-C04

latour@clemson.edu

(864)-656-5552

<http://www.ces.clemson.edu/bio/people/latour.htm>

William R. Marcotte, Jr, an Associate Professor of Biological Sciences and Genetics at Clemson, joined the staff in 1992. He received his B.S. in biochemistry in 1980 from Virginia Polytech and his Ph.D. in microbiology from Virginia in 1986. He was a Visiting Scientist at DuPont from 1986-9 and a Research Associate at North Carolina from 1989-92. Bill's research interests include molecular genetics and molecular physiology of gene and protein expression in plants.



M98-CL05, M02-C04

marcotw@clemson.edu

(864)-656-0119

<http://www.clemson.edu/genbiochem/People-Faculty-Marcotte.html>

Cécile Aubé, a masters student in Genetics and Biochemistry Life Science Studies at Clemson, earned a B.S. in biomedical research and biotechnology in 1999 from "École supérieure des Techniques de Biologie Appliquées" (Paris). Cécile's research interest is the production and purification of spider silk proteins for novel synthetic fibers.



M02-CL04
mailto:caube@clemson.edu,

(864)-656-3060

Jacqueline Palmer

Need bio, photo, phone,

M98-CL05, M02-CL04

palmerj@gte.net

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