Biomimetic Production Techniques for Mechanical and Chemical Characterization of Sucker Ring Teeth Isoform-12 From the Dosidicus Gigas Squid

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BIOMIMETIC PRODUCTION TECHNIQUES FOR MECHANICAL AND CHEMICAL CHARACTERIZATION OF SUCKER RING TEETH PROTEIN ISOFORM-12 FROM THE DOSIDICUS GIGAS SQUID

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY MARCUS T. GRANT ENTITLED Biomimetic Production Techniques for Mechanical and Chemical Characterization of Sucker Ring Teeth Suckerin Protein Isoform-12 from the Dosidicus Gigas BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Grant, Marcus T. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2016. Biomimetic Production Techniques for Mechanical and Chemical Characterization of Sucker Ring Teeth Suckerin Protein Isoform-12 from the Dosidicus Gigas Squid.

The unique protein-based structure of Sucker Ring Teeth (SRT) of cephalopods have spurred research into the molecular design, physical characteristics, functionality and mechanical properties to explore biomimetic engineering and biochemical potential for eventual industrial production. Previous research has elucidated the potential for scientific and industrial exploitation. However, much of the previous research focused on the most abundant protein isoform of the sucker ring teeth, suckerin-19 (also known as suckerin-39) from the Jumbo or Humboldt Squid (Dosidicus Gigas). There is little known about the characteristics of the other 37 protein isoforms of Sucker Ring Teeth. Although the other isoforms have similar modular repeats in the primary and secondary structures, the other isoforms are smaller and may provide some additional clues into the biochemical characteristics of the suckerin genes.

Of the 37 protein isoforms, the suckerin-12 isoform displayed some sequence and modular similarities to suckerin-19 that warranted further evaluation. The procedures and techniques used to study suckerin-12 focused on the expression and purification techniques, mechanical and structural analysis, and fine-tuning strategies for future
functionalization. Experiments were performed to evaluate protein isoform suckerin-12 as a candidate to provide a suitable biopolymer for development of highly durable and strong biomaterials that rival other suckerin isoforms and may provide some insight into protein functionality in both dry and wet environments.

By mimicking post-transcriptional cellular processes in an aqueous and dry environment, suckerin-12 displayed special physical and chemical characteristics to those seen in suckerin-19. Specifically, the procedures used to form testable suckerin-12 based materials via di-Tyrosine cross-linking required alternate methods than the ruthenium-based cross-linking observed in suckerin-19 studies. This study presents a method that increases the stability of the suckerin-12 protein structure through enzymatically cross-linking di-tyrosine to create sclerotized hydrogel structures. Hydrogen-bonding and induced hydrophobic and non-polar interactions are important in suckerin protein aggregation and protein solubility in various solvents. Utilizing salting-in and salting out techniques with Hofmeister series anions allowed fine-tuning and protein structural and conformational manipulation through fine-tuning of concentrations, pH, and ionic strengths.
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1.0 INTRODUCTION

1.1 Introduction to Proteins as Biomaterials

Over the past 30 years [1] protein-based composite biomaterials have been vigorously sought after in order to leverage their physical and biological properties to suit an extensive range of industrial and mechanical needs. For example, protein-based biomaterials offer versatility to bolster engineering of assorted tissues in regenerative medicine as well as electrochemical gradients that may be useful for optical fibers. Biomaterials in general have been used for medication conveyance, medical scaffolding, plastics, biosensors, and issue recovery [2]. Biomaterials are specifically useful due to their diverse range of structural, bioactive and mechanical properties that make them ideal for developing environmentally friendly materials that can be easily functionalized and tunable outside of their natural settings. It is desirable to have inexpensive and abundant materials that can be easily blended with specific enzymes or structural matrices to easily form fibers, films, and powders for incorporation into textiles, paints, and other products [3].

Extracellular proteins show bioactive properties that have systematically evolved in nature for many years thus providing a strong framework for biotechnological applications. Over millions of years, various organisms have developed systems that evolved to produce composite proteins [2] that supports both mechanical and structural intra- and extracellular multifunctional needs. For instance, collagen and elastin are regularly co-expressed
together in the body allowing for the proteins to coalesce for increased mechanical strength required for particular tissue functions.

In order support efficient procedures for developing biomaterials from proteins, investigators require further understanding of specialized natural processes unique for specific organism functional and mechanical needs. Studies of the biomaterials are needed to create robust, strong, and flexible biomaterials that can rival and exceed the properties of synthetic products [4], but still have the ability to overcome the current economical limitations of industrially scaling up protein production by refining biotechnical protocols.

1.2 Protein Structure and Function

The unique properties of proteins have stimulated research into the molecular design and functional purposes of proteins encoded from a diverse range of organisms. Protein-based materials provide material engineers and researchers vast options for development of both highly functional and structurally stable materials. Proteins structures are comprised of amino acids that help build higher order secondary, tertiary and quaternary structures. Secondary structures and tertiary structures that form proteins can self-adhere and interact with other proteins. The higher order structures help support both the structural needs of the organism, but they also support mechanical and other biochemical needs within an organism. Charged groups help proteins cross-link within their structures and bind to metallic elements like heme groups for various functional
needs. Proteins are also lightweight and strong that make them ideal as load bearing materials.

The information gained from the natural state of proteins can support efforts to fine-tune protein conformations to meet design specifications for mechanical and functional biomaterials [5]. Protein engineering for the purpose of biotechnology and material science also provides dialogue for developing renewable resources and physiologically based strategies consistent with green chemistry of biological systems [6]. By studying post-translational modifications of protein sequences, improved understanding into the mechanisms that manipulate chemical properties, folding, structure, solubility and functionality will help mimic natural processes in nature that can be exploited for specific technological or synthetic material development advancements. For example, chemical modification of secondary structure residues by ligation of charged groups located on protein backbones can have electrochemical implications [7]. Staudinger ligations, which involves conjugation of azido-amino groups on protein side chains, has several applications for uses as diverse as florigenic labeling, epitope tagging of G-protein-coupled receptors and the installation of photo-switches [8]. Through continued research, additional information can be gained that will support facile methods to produce novel functional materials.
1.2.1 Silk

Silk has become the bio-inspiration for advance biomaterial research due to its outstanding structural and mechanical properties. The extraordinary mechanical elements and biocompatibility are reasons why silk has been utilized for wound dressing, materials and sutures [9]. Silk forms an ordered complex assemblage of $\beta$-structures that form fiber networks [10] and have proven to be suitable for an extreme range of both textile and high-technology applications. Silk is characterized by its extraordinary mechanical properties rivaling synthetic materials, such as, Kevlar, nylon and high-tensile steel. With new innovations in the fields of recombinant protein expression and biomaterial synthesis, silk is consistently revisited for exploration for biomaterial and biomedical examination.

Silk protein-based fibers are created by arthropods [11] for various purposes such as building nests and traps for prey. Numerous silk-based strands are composed of continuous amino-acid repeats of glycine, alanine, serine, and tyrosine that can organize into hardened crystalline structures. Silk from the silkworm species Bombyx mori show that silk fibers are formed by two microfilaments implanted in a sticky glycoprotein named sericin which functions as a covering. Every microfilament results from the gathering of a hydrophobic 370 kDa heavy-chain fibroin protein, a moderately hydrophilic 25 kDa light-chain fibroin and a 30 kDa P25 protein [12]. Spider dragline silk has a diverse structure with a center fiber formed by two spidroin molecules, major ampullate spidroin protein 1 (MaSp1) and 2 (MaSp2), covered by glycoproteins and lipids [13].
The outstanding mechanical properties of the diverse types of silk are to some extent a consequence of the location of the $\alpha$-helix and $\beta$-turns that provide the protein its viscoelastic properties. These elastic areas exchange with $\beta$-sheet motifs, which help to develop parallel/anti-parallel formed $\beta$-sheets and amide-amide associations. These amide-amide associations in the $\beta$-sheet crystalline areas are thought to contribute to the astounding firmness of silk filaments [14]. In B. mori silk, the hexapeptide repeats (GAGAGS) helps to contribute to the formation of the $\beta$-sheets. Other poly-Ala and GA sequences also contribute these $\beta$-structure arrangements within the protein. There are additional poly-Ala segments, GGX repeats (X can be Tyr, Leu or Gln) and GPGXX repeats that contribute for the arrangement of anti-parallel $\beta$-sheets [14]. These poly-Ala and GA themes are implanted in indistinct motifs shaped by either GGX (X can be Tyr, Leu or Gln) or GPGXX repeats [15].

Although met with challenges, the B. mori silk is extensively available in limitless quantities of reconstituted supply from sericulture and thus general proportion of material can be utilized for biotechnological studies. However, one limitation is that the silk protein is large and requires extensive genetic engineering to express recombinantly. Recombinant expression in a compatible vector has become challenging, prompting research into alternative means for material development. Gene instability and some translational pausing are problems seen when attempting to recombinantly express large silk proteins. Finally, harsh chemical environments are required to solubilize into aqueous solutions [16].
However, with the development of biotechnology apparatuses, it is presently conceivable to engineer insect silk qualities to deliver silk-like proteins [17] for tissue building [18], cell culturing [19], nerve recovery [20] [21] and wound dressing [22] applications.

### 1.2.2 Marine Organisms

Recently, some marine organisms have gained traction as a link between biological science and materials science. For example, marine sessile organisms like ocean barnacles show adhesive insolubility, and hence durability, resulting from the aggregation and cross-linking of cement proteins in aqueous saline environments [23][24][25]. Moreover, barnacles are composed of approximately 90% protein [23][25][26] with the remainder as carbohydrate (1%), lipid (1%) and inorganic ash (4%; 30% of the inorganic ash is calcium) [26]. Barnacle cement is an aggregate of at least 10 major proteins, a portion of which have been isolated and sequenced [27][28]. They have the unique ability to permanently adhere to hard substrates derived typically derived from a secreted adhesive with specific chemical properties. Their strong structure can be attributed to the secreted cement-like adhesives that can displace water via bulk and surface hydrophobic interactions, spread and form adhesive bonds with substrates [29]. The proteins then coagulate and cross-link that provides unique stability on to inorganic and organic materials as an adhesive [29]. Adhesive cross-linking via metal binding of adhesive proteins has been shown in multiple marine glues including that of marine mussels and gastropod mollusks [30].
1.3 Discovery of Sucker Ring Teeth (SRT) Proteins and Identification of Isoforms

Initial research into marine organisms centrally focused on mineralized structures such as bone structures, mollusk shells, sponge spicules, and echinoderm ossicles. There have been several other species of marine animals found to have hardened structures devoid of mineralized components. Squids have four extracellular hard tissues of research interest: the pen, the beak, the cartilaginous cranium, and the sucker ring teeth (SRT). The pen, composed of mainly β-chitin, is a rod-like structure that strengthens the structure of the long body mantle. The beak, composed of mainly α-chitin is that is encompassed by the buccal mass, which is utilized for prey dissection. The buccal mass is made of a composite structure matrix of chitin and cross-linked proteins with gradient-like hydration effects [31]. The SRT are ring-like teeth structures inside the suckers of the tentacles of cephalopod [32]. They originate from the inside the suckers of the tentacles and arms of all Decapodiformes species used for predation, laceration, grappling and object manipulation [33].

The squid has eight arms and two tentacles that are lined by suckers with accompanying crown-like rigid SRT. Squid teeth are comprised exclusively of proteins (named “suckerin”) organized into a supramolecular network, reinforced into nano-confined β-sheets that are embedded in an amorphous matrix [33]. The SRT provides functionality to the suckers allowing increased shear forces that support separating the seal created by the infundibulum of the sucker [33]. On the tentacle, several suckers are
supported by circular muscle tissue that helps provide sucker contraction. The very sharp, moderately sized teeth are bent inward and will subsequently penetrate the skin or scales of its prey, such as fish [33].

1.3.1 Biomolecular Design and Genetic Origins of SRT

It normally takes years for cDNA cloning and sequencing (i.e., templating enzymes that direct bio mineralization or silk spinning mechanisms) [33][34] of biomaterials resulting in delays in identifying the capacity for scientific and industrial exploitation. Nevertheless, through RNA-sequencing technology, these limitations have been bypassed with current proteomic, biophysical and mechanical characterization tools. To explore biomimetic engineering and biochemical potential for industrial production of suckerin proteins, it is important to understand the biomolecular design and genetic origins of SRT [33]. To limit the requirements to sacrifice squids, efficient recombinant processes serve as a humane alternative to study genes from the tissues that form SRT. Specifically, recombinant expression requires understanding of primary protein sequences, pathways, and structure-function information related to SRT protein secretion and conformation. From a molecular standpoint, it is important to understand the molecular design and origins of SRT to expound the function and purpose of these proteins. From an engineering standpoint, it is important to explore and compare the range of molecular designs to exploit any mechanical and structural properties and compare any commonalities to previously studied biological materials; furthermore, providing opportunity to explore options to fine-
tune the molecular structure to provide an informed analysis of aforementioned molecular design.

To determine composition and molecular design of SRT, several suckerin coding genes were isolated from tissue samples taken from the suckers of three distantly related decapodiform cephalopods, the Jumbo Squid, also known as the Humboldt Squid, Dosidicus gigas (Order Oegopsida), the Bigfin Reef Squid, Sepioteuthis lessoniana (S. lessoniana, Order Myopsida), and the Golden Cuttlefish Sepia escuelenta (S.esculenta, Order Sepiida) [34]. Phylogenetic analysis indicated that suckerin proteins were encoded by an ancient gene family that gave rise to five distinct architectures divergences conserved across the three cephalopod species [34]. The proteins were arranged via de novo transcript assembly to generate a transcriptome library of the sucker tissue surrounding the SRT [34]. Through Rapid Amplification of cDNA Ends Polymerase Chain Reaction (RACE-PCR) and multiple techniques involving analysis of amino acid composition against predicted SRT protein transcripts, N-terminal Sanger sequencing, LC-MS/MS, and identification and verification techniques [34]. Utilizing RNA-sequencing technologies, 37 unique isoforms were identified that encoded higher modular molecular proteins ranging from 5 to 57 kDa and pI’s between the 7-10 range with varying expression levels [16][33]. The 39.4 kDa isoform from D.gigas labeled suckerin-19 was found to contain the preponderance of the repertoire of proteins isoforms identified from the three species of squid [33] making it the focus of the majority of the research performed on SRT [16][33][34].
1.3.2 Structural and Mechanical Properties of SRT Proteins

Structural analysis of the SRT showed unique structural components. The structural integrity of the SRT were shown to be held together by proteins that are bonded by hydrophobic, hydrogen bonds, and nano-confined modular β-structure assemblies [33]. Through Synchrotron Wide Angel X-ray Scattering (WAXS), SRT was found to be isotopically oriented into sheet nanocrystals of precise dimensions that helps to reinforce its amorphous network [34]. It was also highlighted in previous research that SRT lacks mineralization and metallic ions; yet, it has a unique ability to maintain stiffness within aqueous environments. Typically, organisms utilize chitin cross-linking as the common microstructural strategy to make hard tissues and maintain its structural integrity in saline or aqueous environments. Conversely, suckerin protein stiffness was found to be based primarily on secondary structure stacking and the conformational changes maintained by H-bonds in aqueous environments. The molecular structure of suckerin proteins are similar to that of silk, where amorphous domains in turn surround hydrophobic β-sheet nanocrystals that are likely to repel water [35]. However, unlike silk, processing of SRT does not require harsh processing conditions to solubilize [33] and offer a great alternative to industries looking water soluble, biodegradable, and bio compatible structures that can be recombinantly expressed.

There is a direct correlation between β-sheet disruption, hydrogen and hydrophobic bonding. The stability of hydrogen bonds and the effects on stability was tested in urea. When placed in urea, hydrogen bonds are disrupted and the stability, strength, and structure
of the SRT and suckerin proteins will weaken [34]. However, when chemically cross-linked with ruthenium-based photo cross-linking, (rec) suckerin-19 proteins had a higher elastic modulus of 9 GPa, in wet or dry conditions, with a greater elastic modulus than the native SRT [34]. Photo cross-linking recombinant suckerin-19 with APS, permanently sclerotizes (rec) suckerin thin films making it resistant to external condition changes. Although cross-linking chemistry is not necessary to develop structural materials and films, most recently, it was shown that suckerin-based films maintained its structure in water spanning 7-orders of magnitude via di-tyrosine cross-linking chemistry [34][36]. By varying the amounts of APS, researchers also demonstrated the ability to fine-tune the stiffness gradients of on the nano-confined β-structures instead of utilizing organic solvents, water or acid [36].

Micro-Raman spectroscopy spectra of ultramicrotomed SRT cross-sections showed SRT is comprised of random isotopically distributed β-sheet formations with Amide-I bands matching Bombyx mori silk. FTIR analysis of thin films further confirmed that SRT is comprised of proteins that organize into primarily β-sheet secondary structures. Structural characterization performed with polarized micro-Raman spectroscopy revealed randomly oriented β-sheets that stabilize a silk-like protein polymer network [34]. These random nanoconfined β-sheet domain orientations, isotropic distribution and modular repeats allows SRT to have resistance to compression, shear, torsional, and load-bearing forces beneficial for cephalopod predation functions.

Suckerin ring teeth protein structures display impressive mechanical properties. The
arrangement and interactions of suckerin isoforms is thought to support the nanotubular structure and its tensile strength [33]. Independently, suckerin proteins were found to have a variation of modular amino acid repeats and proline disruptors that allows for stable nano-confined structures. The variation of amino acids repeats and proline organization supports non-covalent isoform-to-isoform binding and stacking to form inter-structural hydrogen bonding giving the SRT additional strength and stability [33][34][42]. Yet, despite the lack of covalent bonds and weak molecular interactions the elastic modulus recombinant dry suckerin is roughly 8 GPa, confirmed by evaluating modulus strength versus urea concentrations in both dry and hydrated states [34].

Finally, SRT was shown to display thermoplastic behavior [36] by heating the material to over 42 °C and using templating techniques in order to create nano tubule structures, spectroscopic techniques confirmed thermoplastic behavior [33]. Verified by FTIR, the thermoplastic behavior is due to the weak interactions within the structure allowing for the formation of specific shapes. Although thermoplasticity is not uncommon in biopolymers, the latest research demonstrated for the first time the potential to use suckerin proteins as a thermoplastic biopolymer for 3D printing applications [36].

1.4 Humboldt Squid’s Suckerin-19 Isoforms and Orthologs

1.4.1 D.gigas Isoforms

As previously discussed, due to the higher representation of suckerin-19 and the full-
length expression identified during RACE-PCR analysis suckerin-19 became the central focus for structural, mechanical, and biochemical analysis. The D.gigas protein isoforms were found to be comprised of 17 to 23 amino acid signal peptides with conserved full-length protein sequences and amino acid composition similarities [34]. All D.gigas isoforms exhibit di-block copolymer-like molecular structures with β-sheet forming modular blocks about 12 residues long flanked by longer amorphous-forming alternate modules rich in Gly, Tyr, and His and lesser represented blocks of Ala, Leu, Thr, and Val [33][34]. The majority of [M1] sequences contribute to the β-sheet conformations with a length 3.1-3.5 nm long obtained from WAXS data [34].

The D.gigas SRT has 21 suckerin isoforms, the highest represented catalogue found out of the three species of cephalopods. There is a 3-fold expansion in its suckerin repertoire and a concomitant increase in modularity and the number of tandem repeats within individual genes and proteins [34]. D.gigas teeth are found to be larger than the other species as well which may contribute to the display of suckerin isoforms represented. D.gigas suckerin are found in Clades 1 through 6, but mostly dominated in Clade 1 and Clade 2 which may have some evolutionary implications. The D.gigas isoforms were numbered 1 thru 21 based on their size. Each isoform has unique secondary structures that vary based on length and modularity. However, there are similarities based on the represented amino acid repeat structures and modular structures of GGY and GGLY, with a greater representation of glycine-rich modules than Ala, Ser, Thr, Val, and His modules. Observations show that the D.gigas suckerin-1 and D.gigas suckerin-2 are small in size
and have a uniform distribution of glycine across the secondary structure, with little or no [M1] motif. The D.gigas suckerin-3 through 21 was observed to have a mix of [M1] and [M2] motifs, with D.gigas suckerin-10, 12, 14, 18 through 21 having a more uniform distribution of motifs. D.gigas suckerin-10, suckerin-12, suckerin-19, and suckerin-20 have similar modular structures ranging in size.

Although D.gigas suckerin-19 and suckerin-12 lack Asp and Glu, studies have explored Tyr redox-reactions as a means of functionalization. Tyrosine is known to be weakly acidic and is partially deprotonated at pH 11 [34]. The high Tyr content of suckerin-12 and suckerin-19 have been shown to produce of gold nanoparticles in suckerin-based films and fibers [34]. Suckerin-12 and suckerin-19 β-sheet folds exhibit a strong affinity for Au$^{3+}$ surfaces. The resulting tyrosinates reduce Au$^{3+}$, but have been shown to absorb into facets of the β-sheet structures allowing for opportunities to trap composite materials when increasing concentrations and forcing oligomeric changes in the protein structure to assist with functionalization [34]. Suckerin was created into electrical scaffolds to allow for efficient electron transfer from the ionized Tyr phenolic groups at high pH to a metal ion from a solution state. The results of this research allows for physicochemical pathways for biopolymer and electrophysiological suckerin proteins in order to synthesize inorganic nanomaterials applications in bioengineering [37].

The D.gigas isoforms of suckerin-19 and suckerin-12 can be easily precipitated via pH and salt concentration adjustments and yet be solubilized in various mild organic solvents such as 5% acetic acid, water, and HFIP. For example, previous studies showed
some suckerin-19 primary structure changes in colloidal suspensions indicating that as the concentration increases from 0.5 to above 8 mg/mL, there is a transition from a monomeric dispersed state (≈6 nm hydrodynamic radius) into colloidal oligomers stabilized by inter-protein sheets [34].

1.4.2 S.Esculuenta and S.lessoniana Isoforms

The S. esculuenta SRT and S.lessoniana were found to have less total isoforms, smaller in size, and contained different modular sequence compositions than D.gigas species SRT proteins. S.esculuenta was found to have 9 isoforms. The S. lessoniana SRT was found to have 8 isoforms, the least out of the three species of cephalopods. The S.esculuenta suckerin isoforms were found in all clades, except for Clade 4, and represented the smallest percentage of other species isoforms. The S. lessoniana suckerin were found in clades 1-6, but only represented a small percentage of other species isoforms. The S.esculuenta isoforms were numbered 1 through 9 based on their size and S.lessoniana isoforms were numbered 1 thru 8 [33].

When observing each isoform, each isoform has unique secondary structures that vary based on length and modularity [33]. However, observation indicates that both species are dissimilar to D.gigas isoforms due to the lesser representation of amino acid repeat structures and modular structures of GGY and GGLY. Through observation, there is a higher abundance of [M1] module Ala, Ser, Thr, Val, and His modules counter of the [M2] modules seen in D. gigas. The S.esculuenta suckerin-2, 4, and 8 has a uniformed
appearance of GGY and GGLY similar to the structures found in D. Gigas suckerin, with slightly more abundance of [M2] modules [33]. However, the other isoforms are not uniform in appearance, but all S.esculenta suckerin have a mix of [M1] and [M2] motifs [34]. Observations also indicated that S. lessoniana suckerin-1 through 7 are not uniform in appearance; however, S.lessoniana suckerin-8 has more of a uniformed appearance of GGY and GGLY similar to the structures found in D. gigas suckerin. All S.lessoniana have a mix of [M1] and [M2] motifs [34].

1.5 Isoform Similarities to Suckerin-19

There is limited research on other isoforms similar to suckerin-19. Several isoforms mentioned represented in the D.gigas clade have similar motif structures to suckerin-19 and may provide additional options for biomolecular, structural and mechanical exploitation. For example, suckerin-12 is similar to suckerin-19 due to the order and consistency of modular GGLY and GGY modules and alanine repeats (Figure 1). Suckerin-10 is another example of a smaller isoform structurally similar to suckerin-19. Suckerin-12 is specifically unique as it is 60% the size of suckerin-19 with a molecular weight of 22.9 kDa compared to the 39.4 kDa seen in suckerin-19 [37]. Suckerin-12 has roughly the same ratio of Ala, Thr, His, and Tyr and has a di-block modular architecture that is identical to suckerin-19 [34][37]. Both suckerin-12 and suckerin-19 have similar maximum solubility at pH 5 [33]. Suckerin-19 is soluble at high concentrations (70.9 mg/ml), [16] however, the smaller molecular weight of suckerin-12 leads to a greater
solubility at lower concentrations (~60 mg/ml) in aqueous solutions making it more ideal for film processing and better film quality [37]. Considering the limited information on other isoforms, more research is needed to support identification of structural, mechanical, and biochemical similarities to suckerin-19, other SRT isoforms, and silk fibroin.

Figure 1. Suckerin-19 versus Suckerin-12 Sequence Comparison. Representation of the amino acid sequence of suckerin-19 and suckerin-12 protein structures encoded from the suckerin-19 and suckerin-12 coding regions. The table on the right shows suckerin-19 and suckerin-12 amino acid percent content.

1.6 Specific Aims

1.6.1 Aim 1

The goal is to determine an optimized process for bacterial expression and purification of recombinant proteins from the Sucker Ring teeth of the Humboldt Squid (Dosidicus gigas). Utilizing suckerin-19 as a baseline, understand molecular methods and
biochemical techniques for isolation, cloning, and expression of suckerin genes.

1.6.2 Aim 2

The goal is to develop methods to create practical materials for mechanical testing and structural analysis. Through structural, mechanical, and bio processing analysis strategies, the information will seek to provide valuable insight into developing environmentally benign routes for eventual synthesizing of novel materials.

1.6.3 Aim 3

The goal is to investigate conditions whereby suckerin-12 materials are condensed and sclerotized. Through analysis of conformational changes and fine-tuning of physical form within various Hofmeister series salts, the information from this research will hopefully provide insight into various methods for creating and manipulating hardened materials within biological by understanding hydrophobic effects of protein-protein interactions, the chemical properties of anionic salt exchange within β-sheet secondary structures.
2.0 MATERIALS AND METHODS

2.1 Plasmid Construction

2.1.1 Subcloning

All methods described for each study employed the use of full-length suckerin-19 and suckerin-12 recombinantly expressed proteins. The full-length open reading frame (ORF) of suckerin-19 and suckerin-12 was originally obtained from the transcriptome assembly of the Humboldt squid sucker tissue and confirmed by RACE-PCR [16]. Expression plasmids were previously purchased from Genescript, and were constructed using the methods described below.

The PCR products were isolated using a QIAquick Gel Extraction Kit (Qiagen) [16] and cloned into the LIC sites of the pET-15b plasmid vector that included sequences encoding a 5’ N-terminal polyhistidine tag (six His residues) and a linker peptide containing a thrombin protease cleavage to the open reading frame (Genescript) via overlap polymerase chain reaction (PCR). A stop codon was added at the 3’ end of the gene to prevent expression of the C-terminal His tag encoded in the expression vector. The PCR fragment of suckerin-12 (750 bp) and suckerin-19 (1250 bp) was then cloned into the Nde I/BamH I site of the pET-15b (5708bp) vector (GenScript) (shown in figure 2) to create a suckerin-coding plasmid that encodes replicas (clones) of the native suckerin-19 and suckerin-12 protein sequences.
2.1.2 DNA purification

Maxi prep protocol

In order to create sufficient quantities of suckerin protein to conduct studies, a maxi prep protocol was accomplished. 100 grams of Luria Bertani (LB) media (Sigma Aldrich) media and 3500 mL of water was transferred to a 4 L flask and stirred for 20 minutes. The 4 L flask of LB broth mix was separated into four 2 L flasks containing 1 L of the LB broth media mix. Each of the four 2 L flasks were autoclaved for 1.5 hours and allowed to cool to room temperature.

2.2 Recombinant Protein Expression

2.2.1 Transformation of Bacteria

Suckerin plasmids were transformed into competent (BL21) DE3 E. coli cells for recombinant expression of the cloned vector. The BL21 (DE3) cells and suckerin plasmid prep (suckerin-19 or 12, pET15b-His tagged) was placed in 2 ml Eppendorf tubes and placed on ice to prepare for heat shock transfer methods. Empty Eppendorf tubes were cooled by placing into ice for a minimum of 30 mins. 30 µls of competent BL21 (DE3) cells (transfer efficiency of 1–5 x 10⁷ cfu/µg) and 2 µls of suckerin-plasmid prep (0.05 ng/µl) were transferred to cold Eppendorf tubes, vortexed, and maintained on ice. The tubes were heat-shocked at 42°C for 60 seconds, and immediately placed back into ice for 1-2 minutes. 500 µls of LB media was pipetted to the cell mixture, then vortexed, and
incubated at 37 °C for an hour. The cells were streaked onto LB agar plates containing ampicillin (100 μg/mL) and 0.5 mM IPTG and incubated overnight at 37°C.

2.2.2 Culturing and Induction

Random colonies were selected and inoculated into separate selective LB media (starter cultures) while performing autoclaving. Each colony was screened via SDS-PAGE for identification of highest expression colony or strain (blue/white screening was not performed). An overnight bacterial starter seed culture of 10 ml LB and 20 μl of 100 μg/ml ampicillin was grown from selected colonies at 37°C and shaken at 220 rpm (Figure 2). The following day, the 10 ml starter culture was transferred (sub-cultured) into a 4L beaker with 8 mls of 0.5 g/10 ml ampicillin, starter culture, and 250 μl of antifoam to aerate cells. The aerated cultures then were placed in an incubator overnight @ 37°C and shaken at 220 rpm to achieve higher optical density growth compared to classical shaking growth without the decrease in pH value and bacterial viability. Once the optical density (OD) reached between OD_{600} to OD_{800}, 0.5 mM of isopropyl β−δ-thiogalactopyranoside (IPTG) was used to induce protein expression, allowed to incubate and aerate at 37 °C for more than 4 hours. Subsequently, bacterial cells were centrifuged at 4 °C with a speed of 14000 rpm for 10 minutes. Following centrifugation, cells were stored at -80 °C overnight for additional processing.
Figure 2. Protein Expression in E. Coli. Cells obtained from transformant screening were placed into high throughput protein production with aeration to increase protein expression yields.

2.2.3 Extraction and Inclusion Body Purification

Suckerin inclusion bodies were extracted by re-suspending cells into lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 100 µg/ml lysozyme and Triton-X). Cells were then sonicated (45 % amplitude, 90 secs on/off, 15 sec pulses), centrifuged at 5000 rpm, for 20 minutes at 5 °C for three intervals before resuspending into plain buffer (50 mM Tris, 150 mM NaCl, pH 8.0). All resuspended inclusion bodies were combined into a 400 mL beaker.

For the columnless inclusion body purification techniques for suckerin-19, crude inclusion bodies were further purified by a simple acid washing techniques. 3.5mM acetic acid was used to wash crude material. Crude material was transferred to 1.5 ml centrifuge tubes, and centrifuged (8000 rpm/5 min). The majority of the inclusion bodies were located
in the pellet, devoid of the suckerin protein. After centrifugation (8000 rpm/5 min), the pellet was discarded. The suckerin-19 was retained in the supernatant. The supernatant was washed and centrifuged for three cycles again until the purest form was accomplished. The solubilized material was dialyzed overnight and then lyophilized for a final powdered form.

For the columnless inclusion body purification techniques for suckerin-12, cells were centrifuged again (5000 rpm, 5 °C) and inclusion bodies pellets were resuspended in 20 mls of deionized water. The pH in the suckerin-19 crude system was adjusted to 3.0 with HCl for suckerin-12 precipitation. The crude solution was transferred to 2.5 mL Eppendorf tubes and centrifuged (8000 rpm/10 min). The supernatant was transferred to a beaker and pellets discarded. A buffer solution (100 mM NaCl, 1 M Tris, pH 8.0) was added to the beaker until solution reached a pH of 8.0. The solution was then centrifuged (8000 rpm/10 min). The pellet was transferred to another 400 mL beaker where glacial acetic acid was added to pH to 3.0. The solubilized material was dialyzed overnight and then lyophilized for final powdered form.

2.3 Protein-Based Material Processing

2.3.1 Immobilized Metal Affinity Chromatography (IMAC) Purification

Suckerin protein purification was conducted by washing and column binding of the histidine tag with TALON beads. Inclusion bodies were transferred into a 400 ml beaker
where they were denatured with a binding buffer (8 M urea, 20 mM NaH$_2$PO$_4$, 0.5 M NaCl, pH 7.8). Ni-NTA (TALON beads) were added to the clarified non-detergent solubilized proteins. The inclusion bodies were solubilized in urea and lysate were collected and loaded onto a metal chelating column (with a 10-micron filter), and purified by affinity chromatography. A flow rate of 0.1 ml per minute was used for loading the column and for follow-on washes. The column was equilibrated with binding buffer (8 M urea, 20 mM NaH$_2$PO$_4$, 0.5 M NaCl, pH 7.8), thus removing any unbound protein from the column. In order to elute the bound protein of interest from the TALON column and to take advantage of the His-tag non-binding at low pH, the suckerin buffer was eluted with a low pH buffer and ran though the IMAC column at each step to remove unwanted proteins. The eluate was collected in 10 ml fractions to ensure complete removal of pure protein from the TALON column, multiple 1 ml elutes were collected. Each purification step in the IMAC elution column was screened for purity. The final eluate was dialyzed for 24 hours and then lyophilized to obtain pure suckerin in the powdered form.

2.3.2 SDS-PAGE Analysis

In order to determine the purity of the suckerin protein, the effectiveness of the purification process, and colony expression and fraction screening levels, a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (settings constant amperage 200V, 60 min, 40 mA) with Coomassie blue staining analysis was performed. Protein samples (100 µl) were taken at various stages of the purification process and the
specific colony was tracked throughout the process to identify the highest expressing batch product that will support greater protein yields. Additionally, the purity of suckerin-12 proteins was carefully monitored via SDS-PAGE for fractionation during columnless purification protocol.

SDS-PAGE gels of the acid-based purification process was accomplished by testing samples at various stages. 100 µl of 6% samples (60 mg/ml) of suckerin-19 was adjusted to pH of 3.0, 5.0 and 8.0 and centrifuged to determine solution retention based on pH. At each pH, 1 µl samples were taken of the crude inclusion bodies (prior to centrifugation), supernatant, and pellet. Additional 5 µl and 10 µl samples were taken at a pH of 5.0 to determine protein concentration changes based on volume. Protein samples were treated with a sample buffer (0.4g of SDS, 2 ml of 100% glycerol, 10% β-mercaptoethanol, and 8 ml of cathode buffer) and boiled for 2 minutes. Samples were loaded onto the 12% SDS gel and compared to a molecular standard [5 µL full range rainbow, ECL MW-RPN-800E (Amersham)]. Approximately 100 ml of electrode buffer was added to the electrophoresis chamber containing 100 mM Tricine powder, 100 mM Tris base, 0.1% SDS and 250 ml of anode buffer containing 200 mM Tris pH of 8.9. The gel was imaged using a Fujifilm LAS-4000 Imaging System. The highest expressing colony seen on the gel was chosen for additional processing.
2.3.3 Thin Film Casting

Suckerin-12 and suckerin-19 were drop-casted (air dried) into films in mild acidic utilizing both hexafluoro-2-propanol (HFIP) and 5% acetic acid. The 5% acetic acid films were used in experiments. The dry casted suckerin films were generated by the addition of 10 µl of 5% acetic acid and 10 µl of HFIP at 20 mg/ml protein solution 5 mm² cylindrical centrifuge tube caps. The samples were allowed to dry for 12 hours resulting in approximately 5 µm thick films. Films placed back into water to test stability in aqueous environments.

Suckerin-19 thin films were also created via ruthenium-based photo cross-linking. 20 mg/ml recombinant suckerin-19 in 5% acetic acid with 2.5 mM APS and 0.2 mM Ru(bpy)₃Cl₂ in 20 µl reaction volume was used to create photo cross-linked bars. HFIP films and photo cross-linked bars retained stability in water. Suckerin-12 did not photo cross-link. The HFIP and suckerin-19 photo cross-linked films were both created as proof of concepts and controls.

2.3.4 HRP Cross-linking

Figure 6 shows the process for creating HRP cross-linked hydrogels. Glass plates were made using scoring tool and polydimethylsiloxane (PDMS) strips as molds to create wax molds. Ethanol and 6N HCl was used to clean the glass. PDMS strips were placed on glass slides and consequently dipped in wax to form a wax/PDMS mold. The PDMS strip was removed from the glass for form 3D well-like imprints (2 mm diameter x 2 mm
Suckerin-12 hydrogels were generated by the combining of 100 μl of 6% suckerin-12 (60 mg/ml) protein solution, 2 μl hydrogen peroxide, and 4 μl of horseradish peroxidase (HRP) (2000 U/μl) into a 1.5 ml Eppendorf tube. After each chemical addition, a swift vortex was accomplished and the total solution was transferred into custom wax-based 3D printed mini-wells to make hydrogel bar. Hydrogels were then placed inside a humidity chamber overnight to allow for cross-linking. The samples were allowed to cross-link for 4-24 hours resulting in hydrogels. Hydrogels were manually tested with a clean pipet tip to determine stability and extent of cross-linking.

2.4 Protein-based Material Characterization

2.4.1 Circular Dichroism Analysis

Each compound was prepared at a concentration of 10 μg/ml with a volume of 300 ml in a cuvette. Suckerin protein concentration in water was kept low (<1 mg/ml) to minimize ionic strength signal noise of the system. This reduced the presence and absorbance of ionized salts (below 195 nm) thus resolving the resolution of the secondary structure signals above 180 nm. The JASCO J-815 CD Spectrometer with Spectra Manager 2 software was used to analyze triplicate wavelengths for specific secondary structure signals. The far ultraviolet region (240-180 nm) that include secondary structures like α-helices, β-sheets, turns and unordered structures (peptide bond groups), and the near ultraviolet (320-260 nm) that include aromatic side chains (tertiary
structures) were studied.

2.4.2 FTIR Analysis

FTIR analysis of suckerin-12 drop cast films was accomplished by Dr. Joseph Slocik, (AFRL/RXAS) to compare secondary structure composition to suckerin-19 and identify the structural components of Hofmeister series contracted hydrogels. The structure of drop cast films and contracted hydrogels was analyzed by a Bruker Alpha-P Fourier-transform infrared (FTIR) spectrometer equipped with a MIRacle™ attenuated total reflection (ATR) Germanium crystal cell in reflection mode. For each measurement, 32 scans were coded with resolution 4 cm\(^{-1}\), with the wave number ranging from 400–4000 cm\(^{-1}\). Fourier self-deconvolution (FSD) of the infrared spectra covering the amide I region (1595–1705 cm\(^{-1}\)) was performed by Opus 5.0 software to identify secondary structures. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm\(^{-1}\) and a noise reduction factor of 0.3. The FSD spectra were curve-fitted to measure the relative areas of the Amide-I region components.

2.4.3 SEM Analysis

In order to investigate the micro features of contracted material in an aqueous environment, scanning electron microscopic micrographs was obtained by Dr. Joseph Slocik, AFRL/RXAS. HRP cross-linked suckerin-12 hydrogels were contracted in 4 mM sodium acetate, soaked in water and freeze-dried (fractured pieces of 0.4 – 1.0 mm in
length, corresponding to half-diameter of each hydrogel). The bars were fractured to show micro ribbing (crazing), separation, viscoelasticity properties, layering and deamination around the fractured surface. The hydrogel was mounted onto an aluminum stud, and gold coated by plasma vapor deposition. The contracted hydrogel surface and interior were recorded by a field emission scanning electron microscope FEI Quanta eSEM at 15.0 kV and an aspect ratio (AR) was given to measure representative pore size distributions in a wet versus dry environment. The AR is the diameter of a pores obtained by averaging its major and minor axes.

2.4.4 Mechanical Analysis

In order to acquire viscoelastic properties of dry-casted films and contracted hydrogels, at least 5 samples were manually tested with a manual tensile tester (TA Instruments, Model Rheometrics Series RSA III) with a 0.2 kN load cell by Dr. Christina Harsch, AFRL/RXAS. Digital self-calibrating load cells were used with calibrating analog load cells using external weights. The samples were placed into the grips of the tensile tester and aligned to the long axis of the specimen with an imaginary line joining the points of attachment of the grips to the machine. The specimen was aligned in the direction of pull to reduce the slippage in the grips. The testing machine recorded the tensile load and the amount of separation of the attachments (grips). Measurements had an accuracy of ± 2% with a 0 to 50 mm/min adjustment capability [38].
Calculations. An arithmetic mean and standard deviation of each property for the samples with the five highest tensile strengths were calculated to the proper number of significant figures. This is done on the basis that the expected errors (nicks or flaws in the specimen, breaks within the grips, specimen slippage, etc.) would all tend to produce lower results. The standard deviation is calculated as follows:

\[
S_x = \sqrt{\frac{N \sum x_i^2 - \left( \sum x_i \right)^2}{N(N-1)}}
\]

Xi is the value of a single observation (i = 1 through N), N is the number of observations, and S is the estimated standard deviation [38].

Tensile Strength. Tensile strength was calculated by dividing the load at break by the original minimum cross-sectional area. The results are expressed in gigapascals (GPa).

\[
Tensile\ Strength = \frac{\text{(load at break)}}{(original\ width)(original\ thickness)}
\]

Percent Elongation. Percent elongation was calculated by dividing the elongation at the moment of rupture by the initial gauge length and multiplying by 100. The gauge marks or extensometers were used to define a specific test section and the recorded length is used in the calculation. The result was expressed in percent and reported to two significant figures [38].
percent elongation = \frac{(elongation \ at \ rupture) \times 100}{(initial \ gauge \ length)}

Young’s (Elastic) Modulus. Young’s modulus was calculated by drawing a tangent to the initial linear portion of the stress-strain curve, selecting any point on this tangent, and dividing the tensile stress by the corresponding strain. For purposes of this calculation, the tensile stress was calculated by dividing the load by the average original cross section of the test sample. The result is expressed in gigapascals (GPa) and reported to three significant figures [38].

![Typical stress/strain curve](image)

**Figure 3. Typical stress/strain curve.** Region AC indicates the “toe region” caused by a take-up of slack, and alignment or seating of the film and contracted hydrogel samples corrected by an added zero point on the strain or extension axis. The linear line represents Hookean (linear) behavior with a continuation of the linear (CD) region of the curve constructed through the zero-stress axis. The intersection (B) is the corrected zero-strain point from which all extensions or strains must be measured, including the yield point. The elastic modulus can be determined by dividing the stress at any point along line CD (or its extension) by the strain at the same point (measured from point B, defined as zero-strain) [38].
3.0 RESULTS

3.1 Suckerin Expression and Purification

3.1.1 Screening of Bacterial Transformants

Previous studies demonstrated that suckerin isoforms can be expressed recombinantly in E. coli expression systems [33][34]. In order to obtain scalable amounts of individual suckerin isoforms for future study, suckerin-19 and suckerin-12 coding regions were sub-cloned into an E. coli expression vector. Both suckerins 19 and 12 cDNA
were subcloned using the BamH/NdeI sites of the pET 15b vector (Invitrogen). Suckerin-19 and 12 plasmids were transformed into competent E. coli (BL21) DE3 cells for recombinant expression of the cloned vectors (Figure 4). The transformed cells were streaked onto LB agar plates containing ampicillin (100 ug/mL) and grown overnight at 37 °C. At least eight single colonies were selected for screening and individually inoculated into 10 mls LB media. After an initial period of growth where the cultures reached an optical density of 600 at 600nm, the cultures were extracted and the inclusion bodies prepared for a TALON pull-down assay (see Materials and Methods). TALON purified inclusion body preparations of the individual clones were analyzed with SDS-PAGE followed by Coomassie Brilliant Blue staining. Figure 5 shows the process for screening of suckerin protein transformants.

**Figure 5. Colony Screening Process.** Schematic showing the preparation and screening of recombinant suckerin protein transformants with SDS-PAGE and Coomassie Brilliant Blue staining.
SDS-PAGE analysis of the purified inclusion bodies from the Suckerin-19 and 12 cultured clones showed expression in all of the selected clones in that each clone demonstrated a stained band at the predicted molecular weight (Figure 6). However, most of the suckerin clones gave low expression of the respective isoform. Of the clones selected, two suckerin-19 clones demonstrated relatively high expression (Figure 7a) while only one of the suckerin-12 clones indicated high expression (Figure 7b). These results indicate that the levels of suckerin expression within bacterial clones is very unstable and screening must be carried out to assure that high expressing clones are selected for future studies. The high expressing clones from each suckerin isoform screens were saved as glycerol stocks for future inoculation of starter cultures (see Materials and Methods).

Figure 6. Suckerin Protein Purification Gels. SDS-PAGE of recombinantly expressed His-tagged suckerin-19 (41.6kDa) and suckerin-12 (25.2 kDa) clones stained with Coomassie Brilliant Blue.
3.1.2 IMAC Purification of Suckerin-19

SDS-PAGE analysis of lysed cell pellets performed in previous research, showed that His-tagged suckerin-19 in urea can be selectively purified through binding to Ni-NTA or TALON resin in a column and eluted under denaturing conditions [16]. Elution fractions can later be dialyzed, lyophilized and reconstituted under 5% acetic acid for subsequent use. Although it was demonstrated that the IMAC purification process of suckerin-19 was straightforward, further analysis into the lost protein yields obtained from IMAC purification (4.3 mg/L) compared to what was obtained from acid-based purification techniques (20.2 mg/L) [16]. SDS-PAGE analysis of the protein load, flow through (FT), eluate, and TALON beads helped to further clarification the limitations of IMAC purification process compared to the greater yields seen in an acid-based purification process [16][34]. The inclusion bodies were solubilized in urea and lysate were collected.
and loaded onto a metal chelating column (with a 10-micron filter), and purified by IMAC (see Materials and Methods). Figure 8 shows the process for performing IMAC.

**Figure 8. IMAC Purification Process.** Schematic shows steps used to purify (rec) suckerin-12-His-tagged and (rec) suckerin-19-His-tagged proteins from inclusion bodies utilizing Immobilized Metal Affinity Chromatography.

SDS-PAGE analysis of lysed cell pellets of each stage of the purification showed protein loss in the IMAC purification process. Utilizing a molecular weight marker (Figure 9, Lane 1) and a purified (rec) His-suckerin-19 control (Figure 9, Lane 2), the SDS-PAGE gel confirms purification of the His-suckerin-19. In order to understand the quality and the protein available for final elution, the load (Figure 9, Lane 3) showed the His-suckerin-19 and other less desired inclusion bodies prior to loading onto the column. Samples of
contaminating proteins from the load washed from the column showed that the protein remained on the column and the binding buffer and adjustment of the pH was effective in removing undesirable proteins (Figure 9, Lane 4). The quantity of protein seen in the purified His-suckerin-19 eluate (Figure 9, Lane 5) was markedly lower than the protein that remained on the column (Figure 9, Lane 6). Roughly 60% of the available protein in the column remained trapped in the Ni-NTA (TALON) resin indicating a high binding affinity, crowding and loss of the protein. The results of the SDS-PAGE analysis indicate that IMAC is not the most ideal method to purify suckerin protein for large-scale production due to a large percentage of the material adhering to the column resin and other methods should be considered to increase protein yields.

![Figure 9. SDS-PAGE of IMAC Purification of Suckerin-19.](image)

SDS-PAGE analysis of (rec) His-suckerin-19 stained with Coomassie Brilliant Blue fractionation of the inclusion bodies processed through a IMAC column.
3.1.3 Columnless Purification of Suckerin-19

As mentioned in the previous section, the results from previously published research revealed that cell lysis though acid-based purification techniques (6 cycles) is high throughput and is the preferred method for scaling up production of (rec) suckerin-19 [34]. As observed in previous research, acid-based purification significantly improves the yield and efficiency for purification of (rec) suckerin-19 from inclusion bodies, requires minimal levels of urea (40 mL of a 2M urea/L culture) [16] or other solvents and removes the time and physical bottlenecks of the affinity columns. Although previous researcher explored the basic effect of resolubilization of (rec) suckerin-19 in 5% acetic acid, this technique did not specify the employment of taking advantage of the pKa of His (5.8) by adjusting the pH as part of the acid-based technique in absence of urea.

In efforts to reclaim the purified His-tagged protein trapped on the column, SDS-PAGE analysis was performed on TALON bead samples (Figure 10, Lane 1) from previous IMAC purification of (rec) His-suckerin-19 as control for visual comparison of purified (rec) His-suckerin-19 yielded from an acid-based purification process (see Materials and Methods). The SDS-PAGE gel shows the results of two acids used to purify inclusion bodies obtained from lysed cell pellets utilizing a simple acid washing technique. The (rec) His-suckerin-19 was solubilized into 10 mM HCl (Figure 10, Lane 2) and (Figure 10, Lane 3) 3.6 mM 5% acetic acid prior to performing additional washes indicating solubility at different pH levels (Figure 10, Lane 4). After performing a purification cycle (solubilization, sonication and centrifugation) (rec) His-suckerin-19 protein remained in
the supernatant (Figure 10, Lane 5 and Lane 8). The supernatant was later clarified through another purification cycle, showing that the (rec) His-suckerin-19 was retained in the supernatant (Figure 10, Lane 6 and Lane 9). There is minimum protein in the pellet and most of contaminating material is located in the pellet (Figure 10, Lane 7 and Lane 10). The 5% acetic acid shows greater yields of retained suckerin than the IMAC TALON beads tested in the IMAC purification process. In absence of urea, the results demonstrate that the effects of adjusting the pH to maximize the (rec) His-suckerin-19 purification yields during acid-based purification techniques exceeds IMAC yields and resulted in protein yields equivalent to what was previously reported (20.2 mg/L). However, the results did not show the effectiveness of these procedures on other identified isoforms.

Figure 10. SDS-PAGE of Columnless Purification of Suckerin-19. Utilizing TALON beads as a control, SDS-PAGE of (rec) His-suckerin-19 in 10 mM HCl and 3.6 mM 5% acetic acid stained with Coomassie Brilliant Blue to show purification with a simple acid washing technique.
3.1.4 Columnless Purification of Suckerin-12

Previously, it was shown that (rec) His-suckerin-19 shows remarkable stability and solubility in 5% acetic acid solutions. As an alternative utilizing the His-tag in a Ni-NTA (TALON) purification column, purification and clarification through pH-based purification techniques showed that increased protein expression yields of (rec) His-suckerin-19 proteins in 5% acetic acid. However, further investigation is needed to analyze the effectiveness of a columnless acid-based purification protocol on other suckerin isoforms. Inclusion bodies from lysed cells containing (rec) His-suckerin-12 isoform proteins were placed into a 5% solution of acetic acid and was carefully monitored via SDS-PAGE for fractionation by analyzing SDS-PAGE samples of both the supernatant and pellets after adjusting pH to 3.0, 5.0 and 8.0. The level of stability was also analyzed by increasing the volume of the (rec) His-suckerin-12 and 5% acetic acid at a pH of 5.0 (see Materials and Methods).

Utilizing a marker (Figure 11, Lane 1), (rec) His-suckerin-12 protein was present in the supernatant of crude unprocessed (rec) His-suckerin-12 when solubilized in 5% acetic acid (Figure 11, Lane 2). After adjusting the pH of the solubilized (rec) His-suckerin-12 in 5% acetic acid to 3.0 with HCl, (rec) His-suckerin-12 remained in the supernatant (Figure 11, Lane 3) and was not present in the pellet (Figure 11, Lane 4) after centrifugation. When raising the crude inclusion bodies from the lysed cells to a pH of 8.0, (rec) His-suckerin-12 was not present in the supernatant (Figure 11, Lane 5), yet, it was seen as a precipitant and remained in the pellet after centrifugation (Figure 11, Lane 6).
When solubilizing the crude inclusion bodies from lysed cells into 5% acetic acid in a pH of 5.0 and incrementally increasing the volume of the supernatant at 1 µl, 5 µl, and 10 µl, (Figure 11, Lanes 7-9) (rec) His-suckerin was shown to be highly retained in the supernatant.

The results indicate that (rec) His-suckerin-12 is retained in the supernatant when precipitated at a pH of 3.0 and is retained in the pellet at a pH of 8.0 and the amount of protein can be scaled up based on the volume of solvent used to purify this isoform. Based on the results, a pH-based columnless purification protocol was created to take advantage of the stability of the retention of (rec) His-suckerin-12 protein in the supernatant at a pH of 3.0 and 5.0. Additionally, the protocol requires adjusting the pH to 8.0 in order to remove additional contaminants (see Figure 12). The result demonstrates there is a 12-fold increase in (rec) His-suckerin-12 (250 mg/L vs 20.2 mg/L) protein yields in comparison to (rec) His-suckerin-19 protein when purifying with a pH-based purification protocol. Sufficient concentrations of purified (rec) suckerin proteins are required for development of basic forms of materials supporting further studies involving mechanical and structural analysis.
Figure 11. SDS-PAGE of (cec) Suckerin-12 Acid Wash Fractions. Inclusion bodies from (rec) His-suckerin-12 cells were carefully monitored via SDS-PAGE stained with Coomassie Brilliant to determine (rec) His-suckerin protein presence during acid based purification.
3.2 Suckerin Thin Film Analysis

3.2.1 CD Analysis of Solvated Suckerins

Circular Dichroism (CD) analysis performed on solvated (rec) His-suckerin-19 previously demonstrated the influence of aqueous-based environments on assembly and secondary structure [16] and secondary structures were analogous to native SRT. In acidic conditions (5% acetic acid), water, and 0.05 MES, pH of 5.5, and at low protein
concentrations (1 mg/mL a conformational switch was observed with increased concentrations [16] [33]. When concentrations were increased from 1 mg/mL to 6 mg/mL, the CD signature peaks presented random-coil structure with a minimum at around 195-200 nm changing to a minimum at 215 nm and a maximum at 202 nm at higher concentration (6 mg/mL) indicating a conformational shift and refolded into β-sheet enriched structures in aqueous-based solutions. It was demonstrated in the previous section that (rec) His-suckerin-19 is soluble in 5% acetic acid and pH of 5.0 further characterizing secondary structure behavior in various aqueous environments. However, SDS-PAGE analysis of (rec) His-suckerin-19 and (rec) His-suckerin-12 shows that there are slight differences in the quantities of (rec) His-suckerin material retained in the supernatant when varying the pH in 5% acetic acid (Figure 11). Further analysis of β-sheet assembly changes in (rec) His-suckerin-12 due to pH changes in a soluble acid (5% acetic acid) provides insight into smaller isoform self-assemblage, and interactions with larger (and more abundant) isoforms such as (rec) His-suckerin-19. The (rec) His-suckerin-12 samples were prepared for CD spectroscopy at a concentration of 10 μg/ml in a pH of 3.0 and 5.0 with 300 μl in a cuvette and was analyzed with a JASCO J-815 CD Spectrometer with Spectra Manager 2 software (see Materials and Methods).

Utilizing a CD standard and (rec) His-suckerin-19 as a control, the far ultraviolet region (240-180 nm), near ultraviolet region (320-260 nm) were analyzed for characteristic minimums and maximum peaks that correspond to the CD spectrum to support identification of secondary structures, peptide bond groups, and aromatic side chains [16].
Secondary structures were identified in the CD spectra at 10 μg/ml and compared to the CD standard. There were no α-helical profile peaks identified with peaks around 190 nm (high) and two additional lower peaks around 210 nm and 220 nm (low). Random coil peaks at a longer wavelength around 215 nm (high) and has a low peak around 200 nm (low) are also not represented in the (rec) His-suckerin-12 CD spectrum. Similar to (rec) His-suckerin-19, the spectra presented classical β-structure wavelength peaks mdeg around 190 nm (high) and 220 nm (low) at a pH of 5.0. However, when changing the pH to 3.0, minimal β-sheet structure is retained. Conformational changes are seen in (rec) His-suckerin-12 when adjusting the pH between 3.0 and 5.0 in aqueous conditions (Figure 13).

Figure 13. Recombinant Suckerin-19 and Suckerin-12 CD Analysis. (a) Circular Dichroism (CD) analysis was performed (rec) His-suckerin-19 as a control to identify characteristic secondary structures. (b) CD was later performed on (rec) His-suckerin-12 isoform in 5% acetic acid using in pH 3.0 and pH 5.0 to view conformational changes.
3.2.2 FTIR Analysis of Suckerin Thin Films

Previous research demonstrated that representative secondary structure abundances in thin films fabricated with solubilized (rec) His-suckerin-19 in 5% acetic acid (20 mg/ml) showed a high content of β-sheet structures (>50% abundance) where other structures were generally less abundant (helix, random coil, and β-turns). Analysis of (rec) His-suckerin-19 in solution also showed the presence of relatively high content of β-sheet conformations in 5% acetic acid (26%), water (34%), and 0.05 M MES at pH 5.5 (42.7%). However, to perform ATR-FTIR, higher concentrations (>10 mg/ml) were needed to create films. At a lower concentration (6 mg/ml), (rec) His-suckerin remained in liquid form. The ATR-FTIR results in combination with CD analysis results showed high β-structure signal in acetic acid, but greater signal in the 0.05 M MES in pH 5.5. When comparing the (rec) His-suckerin-19 thin-films generated at higher concentrations, it becomes evident that there are concentration dependent structural changes and oligomerization that is enabled by the formation of inter-chain β-sheets through solvent, concentration and pH adjustments. However, previous ATR-FTIR research did not demonstrate any effects on smaller isoform secondary structures in high concentrations (20 mg/ml) in 6% acetic acid or their stability in aqueous solutions (water).

Analysis of thin films of (rec) His-suckerin-12 was performed by Dr. Joe Slocik (AFRL/RXAS) to determine if β-sheet formation is consistent with (rec) His-suckerin-19 structures and to identify other secondary structure peaks in (rec) His-suckerin-12 thin
films (see Materials and Methods). Recombinant His-suckerin-12 β-sheet formation was found to be consistent with (rec) His-suckerin-19 structures. The suckerin-12 isoform was shown to be roughly 40% smaller than suckerin-19, yet, it has a larger number of random coils, and less α-helical structures (Figure 14). There is a greater preponderance of random coil, β-turn, and β-sheet formation in the film structure compared to (rec) His-suckerin-19 films; however, α-helix and undefined regions are more represented in (rec) His-suckerin-19 film structures (Figure 14).

![FTIR Analysis of Suckerin-12 and Suckerin-19 Thin Films](image)

**Figure 14. FTIR Analysis of Suckerin-12 and Suckerin-19 Thin Films.** FTIR analysis and comparison of drop casted films in 5% acetic acid show formation of (rec) His-suckerin-12 and (rec) His-suckerin-19 secondary structures.

### 3.2.3 Mechanical Testing of Suckerin Thin Films

It was demonstrated in earlier work that (rec) His-suckerin-19 displays remarkable mechanical properties similar to silk where there is a large window of opportunity for
stiffness tuning [33] mimicking stiffness gradients [32] of the native material. The elastic modulus of (rec) His-suckerin-19 thin films in dry and wet state was similar to silk’s considerable range in dry versus hydrated modulus [33]. In a dry state, (rec) His-suckerin-19 films displayed an elastic modulus comparable to the native SRT (~7.5 GPa), and under hydrated conditions the elastic modulus decreased to 5-8 MPa (similar to silk ~10 MPa) and remained less than native hydrated SRT (4 GPa) [33]. Stiffness gradients were demonstrated in the native SRT from the periphery (2.75 GPa) of the teeth to the core (1.75 GPa) and the hardness between 0.25 and 0.15 GPa [32], providing additional information regarding the direct effects of water of the β-sheet structures. However, it was also shown that it is possible to use ruthenium-based photo cross-linking to take advantage of the high tyrosine amino acid content (15%) in suckerin-19 proteins to create permanently cross-linked films thus increasing the elastic modulus of suckerin proteins in hydrated states. B.mori silk films were shown to have less tyrosine residues (5%) and di-tyrosine photo-cross-linked films remained stable in water with 2-3 orders of magnitude greater elastic modulus than silk and (rec) His-suckerin-19 films in aqueous solutions. Recombinant suckerin-12 films were created to investigate the elastic modulus in both a dry and hydrated state to compare to the mechanical properties demonstrated in (rec) His-suckerin-19 thin films.

Five μm thick dry casted (rec) His-suckerin-19 and (rec) His-suckerin-12 films were created and subjected to tensile testing methods (see Materials and Methods section) to determine the elastic modulus in a dry state. The elastic modulus for (rec) His-suckerin-
19 films was found to be 2.4 +/- 0.7 GPa. The elastic modulus for (rec) His-suckerin-12 films was found to be 5.1 +/- 1.0 GPa (Figure 15). The (rec) His-suckerin-19 and (rec) His-suckerin-12 films were placed into water to determine stability and mechanical testing in a hydrated state. The (rec) His-suckerin-12 films show a 2-fold greater elastic modulus than (rec) His-suckerin-19 films. The films dissolved in water and indicating instability in a hydrated state and not a suitable condition to perform mechanical tests. Although it was previously shown that the ruthenium-based photo cross-linking was successful for (rec) His-suckerin-19 thin film fabrication, (rec) His-suckerin-12 based thin film photo cross-linking attempts were unsuccessful requiring other methods to determine stability and mechanical behavior in a hydrated state.

<table>
<thead>
<tr>
<th>Film</th>
<th>Elastic Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc-12 (OHAc)</td>
<td>5 +/- 1</td>
</tr>
<tr>
<td>Suc-19 (OHAc)</td>
<td>2.4 +/- 0.7</td>
</tr>
</tbody>
</table>

Figure 15. Tensile Tests of Suckerin-19 and Suckerin-12 Thin Films. This figure shows the results from performing tensile tests on thin films dry casted in 5% acetic acid at concentrations of 10 mg/ml.

3.3 Suckerin-12 Hydrogel Analysis

3.3.1 HRP Cross-linking of Suckerin-12

As previously shown, (rec) His-suckerin-19 and (rec) His-suckerin-12-based films are unstable in aqueous solutions, other routes to test mechanical and structural properties
should be developed to create testable materials in aqueous environments. Hydrogels are widely used as reservoirs in drug delivery and scaffolds for tissue engineering [39] [40]. Previous research has shown the ability to fine-tune cross-linking density in protein-based hydrogels through the enzymatic activity of horseradish peroxidase (HRP) with \( \text{H}_2\text{O}_2 \) an oxidizing agent [39] [40]. Some additional advantages of HRP is that it is active in a pH of 5.0, it amplifies detectability of a weak signal from a target molecule giving it a brownish-yellow indicator of cross-linking density, and it has a small size (44.4 kDa) making it a suitable to support interaction with other small and monomeric proteins like suckerin-12 [39] [40]. A solution of HRP, \( \text{H}_2\text{O}_2 \) and (rec) His-suckerin-19 and (rec) His-suckerin-12 was tested as a means to generate hydrogels by testing the ability to catalyze cross-linking reactions of phenol conjugates (tyrosine residues) in the presence of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)).

A solution of 6% (rec) His-suckerin-12 in 5% acetic acid (100 µl), 2000 U/µl of HRP (2 µls) and (2 µls) of 2% \( \text{H}_2\text{O}_2 \) concentrations was placed onto a wax-indented slide and placed into a humidity chamber overnight for stabilization (described in the Materials and methods section). The ability to cross-link and therefore create stable (gel-like) (rec) His-suckerin hydrogel bars for contraction studies depended on the concentration of hydrogen-peroxide used during the mixing process. Hydrogel creation success rate fell by 50% when using volumes of 4 µl of \( \text{H}_2\text{O}_2 \). Although there were some hydrogen peroxide concentrations limitations were identified during the hydrogel casting process and (rec) His-suckerin-19 was not able to be HRP cross-linked, the results represented facile method
to create highly concentrated stable suckerin-based materials in aqueous environments for additional mechanical testing and encapsulation studies.

3.3.2 Contraction and Expansion of Suckerin-12 Hydrogels

It was shown in previous research that SRT maintains the nanoconfined and amorphous beta structures due to hydrogen bonds that increase protein-protein interactions and hydrophobic effects from the high content of Gly, Tyr and Ala. The Gly and Ala residues is thought to help drive bulk water from the internal conformations of suckerin-19 and other isoforms of SRT causing overlapping and packing of $\beta$-structures [9][14]. Urea was also shown to weaken the structure of films further supporting the ability for Cl$^-$ and other anionic interactions to interact with H-bonds within the protein structures [34]. The previous section shows that (rec) His-suckerin-12 can be cross-linked into a stable form in a hydrated form. The hydrogels represent a protein and water rich environment that is devoid of salt molecules that is cross-linked to form a di-Tyr linked structure. The hydrogel that contains the (rec) His-suckerin-12 protein has an abundance of hydrophilic amino acids and a single charged amino acid that can now interact with the water in the buffer solution. To test the ability to fine-tune gelation and cross-linking density through manipulation of water and H-bonds in a hydrated state, HRP cross-linked hydrogels were soaked in an anionic solution for a minimum of 30 min.

Measurements of the cross-linked hydrogels were taken after 30 minutes to observe anionic effect on cross-linking. In NaCl, the casted hydrogel contracted by a maximum of
33%. The contracted hydrogel was later placed in 10 mM HCl to observe expansion (Figure 16). In HCl, the casted HRP cross-linked hydrogel expanded to a maximum area at 4 hours. The expanded HCl hydrogel was placed back into 500 mM NaCl and contracted down to the maximum contraction area, 7% of the expanded volume (Figure 16) (See Materials and Methods). Initially, hydrogels showed minimal swelling, however, immediately prior to 30 minutes, a yellow-brownish color change was observed representing increased HRP and (rec) His-suckerin-12 protein contraction density and a suitable indication for removal from the glass slide. Removing the hydrogel from the slide maximized contraction and expansion in anionic solutions. When the hydrogel is added to a salt solution containing anions, the charged and hydrophilic amino acids interact with water causing less solvent water molecules to interact with the protein. As a result, suckerin molecules begin to aggregate and collapse as the hydrophobic and nonpolar amino acids extrude water out of the core of the hydrogel center. However, alternatively, HCl reverses the effects by creating more H-bond interactions with Cl⁻ that within the hydrogel allowing more water to solvate the water to protein-backbone interactions. The results indicate that salts of various charges enter the protein matrix to interact with the H-bonds within the structure. This causes the nonpolar and hydrophobic amino acid components of the protein start to aggregate together, becoming insoluble in the buffer solution but can be reversed in sufficient concentrations of HCl.
Figure 16. HRP Cross-linking in Anionic Solutions. Tests were conducted to determine the extent of (rec) His-suckerin-12 based hydrogel contraction and expansion in anionic solutions.

3.3.3 PBS Expansion and Contraction Hydrogel Study.

The contraction and expansion study of (rec) His-suckerin-12 hydrogels in anions showed the ability to condense hydrogels from a casted HRP-cross-linked material. However, more information can be gathered by testing in physiological conditions. Phosphate buffered saline solutions (PBS) are solutions that support evaluation of materials in physiological conditions that is representative of the original organism. Hydrogels were placed in 1 x PBS and individual components to determine the extent of contraction in a non-toxic/physiological environment for over a 24-hour period.

Hydrogels were placed into a 1x PBS solution (Figure 17) and one casted hydrogel was placed into individual PBS component solutions (1 mM solutions of NaCl, KCl, NaPO₄, and KPO₄) (Figure 18) to determine the extent of contraction in a non-
toxic/physiological environment in a 24-hour period. Measurements of the hydrogels in each PBS component were taken in one hour increments up to four hours, and at 24 hours (See Materials and Methods section).

**Figure 17. PBS Expansion and Contraction Hydrogel Study.** Recombinant His-suckerin-12 based hydrogels were placed into 1 x phosphate buffered saline (PBS) to observe the extent of contraction and expansion in physiological and non-toxic conditions. Three measurements of the extent of contraction was performed on each hydrogel bar inside 1 x PBS solvents by utilizing Pixelstick application.
Figure 18. PBS Component Expansion and Contraction Hydrogel Study. Recombinant His-suckerin-12 based hydrogels were placed into components of the PBS solution to observe the extent of contraction and expansion in physiological and non-toxic conditions. Measurements of the extent of contraction was performed on each hydrogel bar inside each PBS component solvents in 4 hour increments and overnight by utilizing Pixelstick application.

The results indicate that the rate of contraction in 1 x PBS is greater within the first 2 hours. There was a ~50% decrease in hydrogel size (contraction) observed at 24 hours. Phosphate buffer saline contracts hydrogels by 60% in a 24-hour period. There is a greater rate of contraction in the first 2 hours indicating immediate conformational changes of β-structures when exposed to physiological environments with greater stabilization of β-structure interactions after 2 hours. Once contracted, the results indicate that the contracted gels show stability in physiological and non-toxic conditions, but specifically, balanced natural anionic molecules can increase stability of the (rec) His-suckerin-12 proteins and potentially allow for contraction gradients within HRP-cross-linked hydrogels. The individual components contraction results show that the phosphate groups cause this immediate contraction and it is the PO$_4^{3-}$ ion that is responsible for most of the contraction.
of the (rec) His-suckerin-12-based hydrogel initially followed by the Cl\(^-\) groups. The Cl\(^-\) groups causes swelling of the hydrogel within the first hour of placing in the solution. Each component of the PBS condenses to roughly the same area < 10 mm\(^2\) in a 24-hour period. Based on the ability to increase protein-protein interaction and break H-bonds in NaCl solutions, further investigation into Hofmeister series may provide different levels of contraction.

3.3.4 Suckerin-12 Contraction with Hofmeister Series Salts

Hofmeister series salt effects on the behavior of protein-protein interactions has been studied since 1888 [41], where previous research has demonstrated the influence of anions on ordering and compaction of protein structures. Anions were ranked based on the ability to influence breaking of H-bond interactions and bonding with water and its ability to stabilize protein structures [41]. The species to the left of Cl\(^-\) were named kosmotropes (water structure makers) and those species to the right of Cl\(^-\) were named chaotropes (water structure breakers) [41]. Kosmotropic anions bond to protein side-chains and jealously guard their water shells resulting in salting-out effects while to chaotropes attract water causing salting-in effects [41]. To determine if there are direct ion to protein interactions that can explain (rec) His-suckerin-12 contraction in anion solutions, hydrogels were placed in Hofmeister series solutions to observe the rate and level of contraction.

Hydrogels were placed into ranked Hofmeister series ions solutions (1.33 mM citrate, 4 mM acetate, 2 mM NaPO\(_4\), and 2 mM NaSO\(_4\), and 4 mM NaCl) to determine the
extent of contraction inorganic and inorganic solvents (Figure 19). Measurements of the hydrogels in each anionic solution were taken in one hour increments up to four hours. Later, all contracted hydrogels were later placed into water to test the stability (see Materials and Methods section).

![Figure 19. Organic and Inorganic Hofmeister Suckerin-12 Hydrogel Series Study.](image)

The (rec) His-suckerin-12 HRP cross-linked hydrogels were sclerotized with low salt concentrations of Hofmeister series ions to determine the contraction kinetics in a 4-hour time frame (Figure 20). The citrate, phosphate, and sulfate contracted hydrogels condensed at a faster rate and was easily removed from the wax-based slide within 30 minutes of placing in solution. The acetate condensed hydrogel swelled within the first 30
minutes, and was found to be difficult to remove from the wax-based slide within the first 30 minutes but contracted shortly after with a moderate delay in contraction rate. The NaCl condensed hydrogels swelled for the first 4 hours and eventually fell off the slide into the solution before contracting after 24 hours. All sclerotized (rec) His-suckerin-12 bars observed condensed to roughly the same size in 4 hours and remained sclerotized in water, except for NaCl. The NaCl contracted bars swelled when placed in water indicating that there is minimal structural stability from NaCl condensed hydrogels in aqueous solutions. Results show adherence to Hofmeiser series salting out utilizing kosmotrophic anions to increase the stability of hydrogen bonds and increase in hydrophobic interactions within the contracted HRP cross-linked (rec) His-suckerin-12 hydrogels. Contracted hydrogels remained stable in water (except for NaCl contracted hydrogels) and provided an opportunity to perform mechanical and structural analysis in aqueous environments.
Figure 20. Contraction Kinetics of Suckerin-12 Hydrogels in Hofmeister Series Buffered Solutions. This figure shows contraction of (rec) His-suckerin-12 HRP cross-linked hydrogels after being placed into low concentrations of Hofmeister series inorganic and organic salts after 4 hours. Measurements were taken in one hour increments up to 4 hours with the Pixelstick application.

3.3.5 Analysis of Dried Suckerin-12 Hydrogels

FTIR analysis on thin films showed a ponderous of β-sheet and random-coil formation in 5% acetic acid in lower concentration. However, as previously mentioned, (rec) His-suckerin-12 thin films were found to be instable in water negating the ability to test thin films in water and test (rec) His-suckerin-12 in a hydrated or cross-linked state. Additionally, the information presented in earlier sections only demonstrates mechanical properties in the dry state at low concentrations (10 mg/ml). It was also shown that parallel
channel-like ultrastructural organization of the sucker rings has a direct effect on their mechanical properties [42], showing the important of nanoindentation and micro feature analysis with SEM. Stable materials with increased concentrations (>60 mg/ml) in both dry and hydrated states presents opportunities to perform both structural and mechanical studies on materials recombinately created to compare with the native SRT properties. Structural analysis of secondary structures represented in Hofmiester series contracted (rec) His-suckerin-12 hydrogels was accomplished by FTIR analysis, followed by physical microstructure analysis utilizing SEM. Mechanical tensile tests and qualitative assessment of Hofmeister series contracted (rec) His-suckerin-12 hydrogels was also accomplished.

FTIR analysis of (rec) His-suckerin-12 Hofmeister series hydrogels contracted in 1.33 mM citrate, 4 mM acetate, 2 mM sulfate, and 2 mM phosphate was performed by Dr. Matt Dickerson (AFRL/RXAS) to determine if β-sheet formation is consistent with suckerin-19 and suckerin-12 structures found on thin films, and to identify other secondary structure peaks in (rec) His-suckerin-12 contracted hydrogels. FTIR scanning methods used for hydrogels were equivalent to those utilized in thin film analysis (see Materials and Methods section). The results from the FTIR analysis shows high abundance of β-sheets and β-turns and random coils. FTIR also shows some structural shifting of secondary structure Hofmeister Series contracted bars in phosphate buffer. The sulfate ion buffer has increased the α-helical structure compared to other Hofmeister series anions (Figure 21, insert). There is less β-turn representation in sulfate ion solution and potential α-helical coil-to-coil to β-sheet switch in the sulfate. The secondary structures were conserved and
shown to have an overall increase in percentage of secondary structures in the hydrogel in comparison to both (rec) His-suckerin-12 and (rec) His-suckerin-19 thin films.

![FTIR Analysis](image)

**Figure 21. FTIR Analysis of Suckerin-12 Hydrogels Contracted with Hofmeister Series Ions.** FTIR analysis was performed on Hofmeister series contracted (rec) His-suckerin-12 hydrogel to determine specific anionic effects on secondary structures. The image on the left represents the Amide I band. The image on the top right corner displays secondary structure representation based on the anionic solution utilized to contract (rec) His-suckerin-12 hydrogels.

A representative (rec) His-suckerin-12 hydrogel contracted with Na-acetate was freeze fractured to analyze microstructures that may influence on the mechanical structures in an aqueous environment (Figure 22). SEM images were provided aspect ratios (AR) (the ratio of the width to the height of a micro feature) and evaluated for small micro features such as micro ribbing (crazing), delamination, small trenches and holes. The results show an AR of 6, allowing trenches, hole and sectional analysis. When analyzing
the microfilm, microstructures become elongated in an aqueous environment supporting
the hypothesis that β-structures have elastic features in aqueous environments that allows
for changes in structure. Freeze fractured dried bars show micro ribbing (crazing)
indicating that separation occurred prior to fracturing when material was warmer. Crazing
is often associated with viscoelastic properties. In a fracture plane, we see evidence of
layering, and delamination, and orientation with microstructure. Based on the differences
in AR in wet or anionic solution (5.8 +/- 0.4) compared to contracted hydrogel that were
freeze dried (5.0 +/- 0.3), the hydrogel dries longitudinally then laterally. The results
shown support additional hypothesis into mechanically properties we are seeing in the wet
state. The micron structure may be trapping small amounts of water into micro domains.
Further investigation will be needed to investigate if water could be interacting with peptide
backbone thus increasing the elastic modulus and stability in water.
Figure 22. SEM Analysis of Fractured Contracted Suckerin-12 Hydrogels Bars. The SEM microfilm image shows a freeze fractured (rec) His-suckerin-12 hydrogel bar contracted with 2 mM of Na-acetate. Samples were imaged in the dry and hydrated states to investigate micro indentations and features.

Dry tensile tests on Hofmeister series contracted (rec) His-suckerin-12 hydrogels were accomplished by Dr. Christina Harsch (AFRL/RXAS) with methods similar to films (Figure 23). Mechanical tests show an elastic modulus of (0.4 +/- 0.1 GPa) in citrate indicating that mechanical and structural strength is inconsistent with Hofmiester series. Hydrogels contracted with sulfate, showed some softening and lower rigidity once removed from solution compared to the other contracted bars tested; however, hardened within 2 hours and retained its elastic modulus. The elastic modulus from acetate contracted (rec) His-suckerin-12 bars does not exceed the elastic modulus seen in thin (rec) His-suckerin-12 and (rec) His-suckerin-19 films casted with 5% acetic acid, nevertheless,
as indicated in (rec) His-suckerin-19 studies [33], acetate has a profound effect on solubility and structural stability. The elastic modulus (0.48 +/- 0.7 GPa) of (rec) His-suckerin-12 acetate contracted hydrogen bars and strength may highlight the importance of amide interactions to support stability in aqueous solutions.

**Dried Suckerin-12 Bars**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young’s Modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S12 NaCitrate</td>
<td>(4 ± 1) E+08</td>
</tr>
<tr>
<td>S12 NaSulfate</td>
<td>(3.5 ± 0.5) E+08*</td>
</tr>
<tr>
<td>S12 NaPhosphate</td>
<td>(2.7 ± 0.5) E+08</td>
</tr>
<tr>
<td>S12 NaAcetate</td>
<td>(4.8 ± 0.7) E+08</td>
</tr>
</tbody>
</table>

*5 Days in salt

Figure 23. Dry Tensile Testing of Contracted Suckerin-12 Hydrogel Bars. Mechanical testing was accomplished on Hofmeister series contracted His-suckerin-12 hydrogels to determine consistency with Hofmeister series and to measure tensile strength and structural stability in aqueous solutions.

Qualitatively assessments on contracted (rec) His-suckerin-12 hydrogel bars was accomplished to test for rigidity. Rigidity was tested through physical bending, pulling, and manipulation to determine sturdiness when taken out of Hofmeister series anionic solutions. Rigidity values were given ranging from *-weak rigidity to ****-strong rigidity (Figure 24). The results support stability observed in Hofmeister series contracted bar data showing increased rigidity in citrate and phosphate; however, there is large rigidity variability in sulfate contracted bars. Salting out via Hofmeiser series buffers show increased rigidity for anions located to the far left of the Hofmeister series. The kosmotropic anions changes surface tension at the water polymer interface thus causes
hydrophobic collapse and dries water out of material. Different anions are causing stiffening around peptide bonds.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rigidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S12 NaCitrate</td>
<td>****</td>
</tr>
<tr>
<td>S12 NaSulfate</td>
<td>*</td>
</tr>
<tr>
<td>S12 NaPhosphate</td>
<td>****</td>
</tr>
<tr>
<td>S12 NaAcetate</td>
<td>***</td>
</tr>
<tr>
<td>S12 ZnAcetate</td>
<td>***</td>
</tr>
</tbody>
</table>

Figure 24. Qualitative Assessment of Contracted Suckerin-12 Hydrogel Bars. Qualitative tests were performed on contracted hydrogels to determine rigidity and consistency with Hofmeister series ions.

3.3.6 Suckerin-12 Hydrogel Concentration Effects on Contraction

Recombinant His-suckerin-12 hydrogels were casted at 3% and 6% concentrations and placed in 10 mM and 100 mM Na-acetate, measured with the Pixelstick application at casting and 24 hours. The results demonstrate (rec) His-suckerin-12 hydrogel concentration dependency based on a specific anionic condition or concentration (Figure 25). Hydrogels in concentrations (3% and 6%) contracted by ~50% in both 10 mM and 100 mM Na-acetate. The extent of (rec) His-suckerin-12 hydrogel contraction is dependent on the salt concentration and the concentration of the hydrogel within the solution. This is further demonstrated in Figure 26 where the sizes of Na-acetate condensed (rec) His-suckerin-12 molds casted with 3% and 6% concentrations can be manipulated based on placing in a specific concentration of Na-acetate or another anionic solution. Specific concentrations may provide various contraction percentages therefore providing ability to
create gradients. Based on the results, concentrations of (rec) His-suckerin-12 proteins and anionic solutions that may be used to create spatially-designed micro features in structural materials but has yet to be tested.

![Figure 25. Hydrogel Concentration Contraction in Adjusted Salt Concentrations.](image)

**Figure 25. Hydrogel Concentration Contraction in Adjusted Salt Concentrations.** Hydrogels were casted with 3% and 5% concentrations of 5% acetic acid and placed into 10 mM and 100 mM of Na-acetate to determine the extent of contraction based on protein concentrations and changes in anionic environments. Measurements were taken after 24 hours.

![Figure 26. Suckerin-12 Condensed with 100 mM Na-acetate, pH 7.](image)

**Figure 26. Suckerin-12 Condensed with 100 mM Na-acetate, pH 7.** Recombinant His-suckerin-12 based molds were casted with different concentrations to determine specific contraction effects in an anionic solution.
4.0 DISCUSSION

4.1 Suckerin-12 Expression and Purification

As the results indicated, BL21 (DE3) cells with a pET-15b vector was shown to be a suitable option for suckerin-12 cloning and expression. Suckerin-12 has favorable expression and purification of cloned suckerin-12 sequences when utilizing acid-based purification techniques compared to suckerin-19 possibly due to the suckerin-12’s smaller size. The smaller sequence size may minimize the frequency of mis-folding, tRNA pool depletions, and transcription errors [16][34]. Additionally, utilizing large silk proteins as an example, protein aggregation of the larger proteins may limit the efficiency of production within E. coli [16][34]; additionally, GC sequences can cause some unstable expression in E. coli during recombinant protein expressions [33]. However, this research shows that colony screening techniques can increase the fidelity for material production by identifying a high yielding and stable colonies prior to cloned expression (Figure 5 and 7). Colony screening allowed for the identification of higher expressing colonies amongst hundreds of colonies.

Initial studies involved traditional methods for expression and purification of (rec) His-suckerin-12 by utilizing the poly-histidine tag while utilizing previously successful (rec) His-suckerin-19 protocols as a control. Performing traditional purification protocols on (rec) His-suckerin-19 helped to highlight potential challenges and limitations of purifying suckerin proteins through the use of the His-tag. As studies with (rec) His-
suckerin-19 indicated, the His-tag may be problematic when desiring self-assembly applications. The His-tag may also contribute to some instability in expression. The His-tagged proteins were shown to have increased loading and aggregation within the column that restricted elution yields. The SDS-PAGE gels (Figure 9) showed that IMAC is not an ideal method to purify (rec) His-suckerin protein for large-scale production due to a large percentage of the material adhering to the column resin (Ni-NTA or TALON beads). The data presented demonstrates that there is approximately a 60% loss in protein yields when purifying in a column indicating that this process is inefficient.

Suckerin-19 research gave insights into conformational behavior as a result of pH adjustments. Through adjustments of pH, it was concluded that a columnless purification protocol is ideal for purifying (rec) His-suckerin-19 and (rec) His-suckerin-12 proteins [34]. Microfluidation techniques used in the purification process, took advantage of (rec) His-suckerin-12 and (rec) His-suckerin-19 solubility and stability in 5% acetic acid. The acid-based fraction studies showed that His-suckerin-12 was primarily found in the inclusion bodies in very low and high pH and was confirmed by SDS-PAGE (Figure 11). Recombinant His-suckerin-12 was found be more stable in mild acidic conditions, and exhibited higher solubility in 5% acetic acid, but specifically at concentrations greater than 10 mg/ml (similar to (rec) His-suckerin-19, at a pH of 5.0) [16]. Performing purification in mild acidic conditions provides higher protein yields in a purified form when comparing to purifying with a IMAC column. The requirement for a IMAC is negated due to the successful demonstration of the columnless protocol. Removal of the polyhistidine tag on
the (rec) His-suckerin-12 protein sequence should be considered for large scale production. Performing acid-based purification required less steps and time, and eliminated the need for bottlenecking of columns purification processes. This process also provided a greater protein yield of ~250 mg/L versus ~ 10 mg/L cited in previous studies [16][33][34].

4.2 Beta Structure

The primary sequence modularity of suckerin illustrates extreme modularity, with alternating Ala/His and Gly/Tyr rich domains. Through observation of amino acid sequences and modular structure, it is evident that suckerin-19 is homologous to suckerin-12 (Figure 27); however, there are some differences. The sequences in the [M1] domain of both suckerin-12 and suckerin-19 are similar to β-sheet forming poly-Ala sequences of spider dragline silks and the Val-Thr motifs that form β-sheets in spider viscid silk, but has less similar repeat sequences than spidroin or silkworm [34]. However, both suckerin proteins are devoid of serine repeats and silk lacks leucine repeats. Suckerin-12 is roughly 40% smaller than suckerin-19 (25.2 kDa His-suckerin-12 compared to 41.6 kDa His-suckerin-19) and there are minor differences in the composition of the amino acid repeats (Figure 1).

The majority of suckerin-19 protein sequences include GGY and GGLY modules and β-sheets found to have an amorphous semi crystalline structures strengthened by randomly oriented nanoconfined β-sheet. Glycine residues maintain conformational flexibility and often reduce the propensity for β-sheet formation, it cannot be fully ruled
out that the Gly-rich suckerin domains may also participate in \( \beta \)-sheet formation [34]. \( \beta \)-sheets are 2.4-2.6 nm for H-bond directions and 3.5 nm for the peptide backbone direction. Each wedge is having a five strands width and 8-10 amino acids in length [34]. Most of the \( \beta \)-sheets will be comprised of the GGY and GGLY modules, but will be supported by other modules like TTHHA and AVSHTTHHA, which are alanine, tyrosine and histidine-rich sequences which are 20-30 residues long mostly separated and flanked by proline [33]. Proline disruptors and other modules limit the \( \beta \)-sheet size are intertwined into larger modules every 12-13 residues that range 15 - 68 amino acids. Prolines directly adjacent to modular repeats are said to contribute to the disordered structure or \( \beta \)-turns and limit the size of the protein. These amino acid sequences can be seen in 90% of all isoforms [33], however, there are variations in the tyrosine availability for cross-linking considering that suckerin-19 has a greater prevalence of tyrosine residues compared to suckerin-12 due to its size.

The modularity profile of the suckerin proteins may account for the differences in conformational changes, solubility, and cross-linking to form nano-confined structures. Figure 27 depicts suckerin-19 and suckerin-12 modular repeat comparisons. Suckerin-19 modular repeats often seen in other highly structured biomaterials such as silk. There is an abundance of GGY (22), GGLY (15), AVSHT (8) and (10) TTHHA repeats separated by proline intermediates. The suckerin-12 modular repeats are shown to be very similar to suckerin-19, with differences in the number of GGY (11), GGLY (8) and TTHHA (6) repeats. Suckerin-12 also lacks the larger AVSH repeats and has repeats GLGAYGFGY
(3). Suckerin-12 has less TTHHA repeats. The larger GLGAYGFGY repeat may require further investigation into its role in enzymatic cross-linking with HRP.

**Figure 27. Suckerin-19 and Suckerin-12 Beta Structure Modular Repeat Comparison.** This schematic shows suckerin-19 comparisons with suckerin-12 modular repeats that contribute to organize into highly structured biomaterials such as silk.

### 4.3 What Causes Contraction

Hofmeister series is more pronounced for anions than for cations. Ordering of anion series is based on its influence on hydrogen bonds within the protein structure as indicated in Figure 28. Suckerin-12 interacts with hydrogen of water that help to collapse the structure of these molecules. Proteins will have specific monomers around the polymer surface that can hydrogen bond.
Figure 28. Hofmeister Series Effects. This figure shows the Hofmeister series ionic salt solution influences on hydrogen bonds within the protein structure.

Provided sufficient (rec) His-suckerin-12 concentrations (~60 mg/ml), it can be shown that kosmotropic anionic salts causes a salting out effect on beta structures in hydrogels making them structure makers. Citrates and sulfate ions are well hydrated, two ions can hydrogen bind with water around them, so they jealously guard their hydration shells [41]. These are waters that are directly bound to these anions in aqueous solutions. The anions located to the right in the Hofmeister series, ions that have charges of $-1$ do not guard their water molecules well, and they are less hydrated [41]. For example, if add $SO_4^{2-}$ to solution, it will not come close to the polymer surface. There will be a region around this polymer in which the $SO_4$ ion does not enter. Consequently, the $SO_4^{2-}$ molecules can remain on the exterior of the polymer, but the ion will never come close to
the polymer chain mainly because it wants to conserve its hydration waters. It is costly to the system to expel water from the surface therefore minimizing the surface area of the polymer causing a collapsed or aggregated state [41]. In phase transitions in the presence of ions like NaSO₄, it becomes easier enter a collapsing process. As a consequence, the actual phase transition from the expanded to the contracted phase will be influenced by external stimuli such as pH and temperature specific for the binding sites of the biopolymer [41]. In (rec) His-suckerin-12, this pH was found to be 5.0.

Chlorides (NaCl) is ranked in the middle of the Hofmeister series where the macromolecules behave differently than anions ranked on the far left of the series, like citrate (Figure 28). The Cl⁻ will bind directly to specific binding sites of a polymer chain, making it more difficult to collapse. As observed in hydrogel bars (Figure 20), the Cl⁻ maintain keeps polymers in the swollen states for longer periods making NaCl a "structure breaker". An adjustment in the external stimuli (i.e. raising temp, pH, or concentrations) can reverse these effects (Figure 19).

In the case of suckerin-12, a method is presented that increases the stability of the protein structure through enzymatic cross-linking di-tyrosines to support comparative studies in aqueous and dry environments. Once in a hydrogel state, the hydrogels are in a state in which they can be sclerotized by applying salting-in and salting out techniques utilizing Hofmeister series anions to fine-tune and change the structure of the protein based on the concentrations, pH, and ionic strengths of the H-bonding and induced hydrophobic and non-polar interactions.
The extent to which proteins dissolve in water depends on the amount of hydrophilic amino acids present in a specific protein structure. When salts are added to a solution of protein dissolved in water, the solubility of protein decreases. When salt concentrations increase, the protein will eventually become insoluble in solution and will precipitate out of the solution. This precipitating out is called salting out. This is a standard method for purifying proteins, but also have other applications for manipulating the stability of structures from the aggregation (precipitation) caused by salting out.

As demonstrated in the schematic for creating cross-linked hydrogels (Figure 29), Hofmeister series anions enter the protein matrix to interact with the H-bonds within the structure. This causes the non-polar and hydrophobic amino acid components of the protein start to aggregate together, becoming insoluble to the buffer solution it is in. The hydrogels represent a protein and water rich environment that is devoid of salt molecules that is cross-linked to form a di-Tyr linked structure. The hydrogel that contains the (rec) His-suckerin-12 protein has sufficient of hydrophilic amino acids and a single charged amino acid that can now interact with the water in the buffer solution.

By placing the hydrogel to a salt solution containing Hofmeister anions, the charged and hydrophilic amino acids interact with water. This results in less solvent water molecules available to interact with the protein and suckerin molecules begin to aggregate/collapse as the hydrophobic and nonpolar amino acids extrude water out of the core of the hydrogel center.
Figure 29. Hydrogel Contractions with Hofmeister Series Anions. The schematic shows the process and hydrophobic interactions observations seen when placing (rec) His-suckerin-12-based hydrogels into Hofmeister series anions.

4.4 Fine Tuning through Acid Solubility and pH

It was demonstrated that suckerin protein-protein interactions and H-bonds within β-structures can be manipulated by acetate and various ionic salt levels. Through chaotropic and kosmotropic phase control, suckerin proteins can be fine-tuned to sclerotized structures that rival other biomaterials like silk. The solubility of these acetates depend on pH levels indicating strong influence of hydrogen bonds and structures within the internal secondary and tertiary structures of nano-confined structures of suckerin ring teeth. It is suggested that the hydrogel studies mimicked cephalopods sucker tissue external salt ion environment causing contraction of the hydrogel that may be equivalent
to the natural salt-based environment. The proteins expressed from the sucker tissue experience aggregation and thus contract to form highly compact structures that may be degraded through breaking of H-bond structures. This follows Hofmeister ion interaction series where surface tension and H-bonding of water effects the stability of the protein. The Na-phosphate concentration contraction of hydrogel studies also showed characteristic contraction based off of concentration (Figure 25 and 26). Utilizing this information, researchers can manipulate the external environment to perform fine-tuning in biological systems as shown with PBS hydrogel contraction studies by varying concentrations of sodium phosphate for gradient studies of suckerin models.

It is suggested that smaller isoforms may help to solubilize or harden other isoforms synergistically in SRT, thus providing an argument to explain why (rec) His-suckerin-12 is shown to provide higher yields during acid-based purification. Considering that suckerin-12 was found to be molecularly smaller than suckerin-19, there may be different self-adherence, aggregation and suckerin isoform-to-isoform interactions as observed in the failed attempts to enzymatically cross-link (rec) His-suckerin-19.

There may be some protein structural implications from the natural secretions of suckerin-12 as compared to suckerin-19. Suckerin-19 is secreted naturally in larger abundance and concentrations than suckerin-12, [34], thus requiring minimal amounts of suckerin-12 to augment hierarchical bulking of these proteins to create nano-confined β-structures. Higher concentrations of suckerin-12 synthetically or recombinantly expressed as a pure protein may interact differently in various external conditions and thus more
favorably when self-aggregating as compared to intracellular expression. Suckerin-12 may be secreted to further stabilize and the larger suckerin-19 isoform intracellularly, but may not form structures independent of the other isoforms. In ideal physiological conditions, with saline with pH levels ranging between 5.0-7.0, (rec) His-suckerin-12 precipitates and thus allows for aggregation and formation of structures in-vitro. Changing pH can help break H-bonds within suckerin proteins thus most likely allowing aggregation. However, within the tissue, cells are kept at physiological conditions allowing solubility and may not form material with the elastic modulus levels shown for (rec) His-suckerin-12 in Figures 15 and Figure 23.

It is suggested that vesicles within the marine organisms provide microenvironments that prevents aggregation of protein by keeping water out and keeping the pH at levels similar to the 5% acetic acid levels used in this research. Within the cell, the protein is kept in a passivated form (percolated hydrogel), swollen and water rich. In this state, molecules are stable, connected, and at a primed state. Transporters outside vesicles can change ion, counter-ions concentrations, ionic water and therefore pH to effect the solubility of the protein and assemblage. As observed with the HRP contraction studies in this research, when suckerin proteins are secreted, extracellular enzymes and ions can potentially change conformation and pattern of structure of the protein. The proteins changes and water condensation allows for post-translational modification where ions and an enzyme like lysozyme, which has a pH of 5.0, can help cells can achieve optimal conformation within the cell just prior to being translated. By changing the pH and the
concentration of ion within these cells, the squid can easily control the formation and thus hardness of their teeth.

Condensed protein processes in terrestrial environments differ from what is witnessed from water-borne/marine-based organisms. Evaporation of water is crucial to the crystallization of proteins in both aquatic and non-aquatic animals. In aquatic animals like the squid, dehydration of the protein interactions may cause stronger hydrophobic interactions and strength that can be reversible in the correct set of conditions. There may be opportunities to change the rate of hydrogel sclerotizing, but this effect might require greater concentrations of substrate and anionic solutions than what was employed in the experiments previously presented. By placing a (rec) His-suckerin-based material environment of choice, water can be instantaneously extruded out to create instantaneously hardened materials, similar to how arachnids are able to create aquamelt-like (instantaneous drying) material instantaneously as silk is exposed to the environment.

4.5 Model

Similar to (rec) His-suckerin-19 and other biomimetic materials, (rec) His-suckerin-12 proteins show promise for creating functional materials that exhibit thermoplastic properties and physical and mechanical structures that are “fine-tunable” through manipulation of the external environment. Figure 30 shows a model and concept utilizing a wax mold with hydrogel to demonstrate the ability to manipulate the elastic properties of
suckerin by varying pH, anionic solutions, or (rec) His-suckerin-12 protein concentrations to demonstrate a desired effect.

Figure 30. Model for Suckerin-12 Material 3D Print and Fine-Tuned Effects. This figure shows a model for creating 3D printed (rec) His-suckerin-12 based materials in a (rec) His-suckerin-12 based mold by inserting different concentrations of anionic solutions to form sclerotized materials.

4.6 Future

Experimental techniques showed that (rec) His-suckerin-12 proteins may be utilized as a suitable biopolymer for development of highly durable and strong biomaterials that rival other suckerin isoforms and may provide some insight into protein-mechanical properties in both dry and wet environments. Pre and post-translational cellular process used to secrete SRT proteins allow for stabilization in their nanoconfined condensed form in aqueous environments. It was determined that condensed protein structures found in suckerin proteins are similar to that of silk. Silk proteins, although terrestrial, is initially
spun aqueously eventually crystallizing once exposed to extra-cellular environments such as air. This aquamelt behavior provides some interest for showing the hydrate effects on biopolymers versus non-hydrated states and determining any relation to cellular hydrophobic effects on sucker ring teeth exhibited by the Humboldt squid.

This research has demonstrated novel properties of β-sheet secondary structures, protein-protein interactions, anionic solution hydrophobic effects, material characterization, and efficient methods for recombinantly expressing and purifying protein for industrial production of nano-confined highly elastic material. More studies are recommended to continue to elucidate the extent of suckerin-12 and combined interactions (dual expression) with isoforms of SRT of other cephalopod species with and without the His-tag. Future studies should explore Matrix-Aided Laser Desorption/Ionization Time of Flight (MALDI-TOF) analysis to determine dimerization and how other isoforms may oligomerize with suckerin-19 and suckerin-12. Other studies should also explore potential 3D printer technologies and functionalization of histidine amino acids on the primary structures.
5.0 REFERENCES


38. The Institute for Interconnecting and Packaging Circuits, www.ipc.org, IPC-TM-650 Test Methods Manual, 2.5.5.6


