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Mucinomics: A Bioinformatic Analysis of Snail Mucins, and Their Function

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Mucinomics: A Bioinformatic Analysis of Snail Mucins, and Their Function

by

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Abstract: Mucins are a diverse family of proteins that have a wide variety of functions and properties. Snails in particular highlight the diversity of mucins found in nature, having multiple functions that are critical to the snail's survival, all of which have industrial significance. While mucins are a unique protein family found ubiquitously in nature, they are well not understood. This thesis outlines the current research on secreted snail mucins, highlighting their potential as a biopolymer, and demonstrating a multi-Omic strategy to examine the hierarchical structures that lead to the enormous biological and chemical diversity of snail mucus genes.

Chapter 1- Advancing Discovery of Snail Mucins Function and Application

Author Contribution: The following chapter is a review paper pending publication. Author contributed to the conception and design of the review, wrote the first draft of the manuscript, and contributed to manuscript revision.

Abstract: Mucins are a highly glycosylated protein family that are secreted by animals for adhesion, hydration, lubrication, and other functions. Despite their ubiquity, animal mucins are largely uncharacterized. Snails produce mucin proteins in their mucous for a wide array of biological functions, including microbial protection, adhesion and lubrication. Recently, snail mucins have also become a lucrative source of innovation with wide ranging applications across chemistry, biology, biotechnology, and biomedicine. Specifically, snail mucuses have been applied as skin care products, wound healing agents, surgical glues, and to combat gastric ulcers. Recent advances in integrated omics (genomic, transcriptomic, proteomic, glycomic) technologies have improved the characterization of gastropod mucins, increasing the generation of novel biomaterials. This perspective describes the current research on secreted snail mucus, highlighting the potential of this biopolymer, and also outlines a research strategy to fulfill the unmet need of examining the hierarchical structures that lead to the enormous biological and chemical diversity of snail mucus genes.

Introduction:

Intrigue in the mucus slime trails left by snails and slugs date back to ancient Greece, where they utilized the mucus for its ability to reduce inflammation and the signs of aging[1]. Today snail

mucus is still used in skin care products by various companies and is a growing market whose value is expected to approach $770 million by 2025[2]. Despite its commercial applications, the field of mucus research remains surprisingly underdeveloped. The primary constituent that is responsible for the properties of mucus are secreted mucins, a family of heavily glycosylated proteins produced in epithelial cells in most animals. Mucins are either bound to the plasma membrane or secreted out of the cell, and each type has major differences in their functions and capabilities[3]. Membrane-bound mucins are glycolipids that act as markers for cell signaling and also protect the cell from extracellular affronts that might lead to damage, such as infections and physical strain[4]. Secreted mucins can be either gel forming or non-gel forming biopolymers. Secreted biopolymers create mucous membranes that account for 99% of the human body's surface area[5–7]. Each snail species secretes multiple distinct functional mucuses. The mucus produced by a snail's foot is used for adhesion and for lubrication, allowing the snail to stick onto or walk across any surface, even while inverted. Additionally, the mucus produced on the back of the snail is used for microbial defense and tissue hydration. Certain snail species have specialized uses for mucus. For example, *Falsilunatia eltanini* (Moon Snail) uses mucus to protect their eggs, and *Tikoconus costarricanus* (Costa Rican Land Snail), uses mucus for load-bearing activities, such as to hide from the sun on the bottom of leaves during droughts [8,9]. Recent advances in omics (genomic, transcriptomic, proteomic, glycomics) technologies have expanded the exploration of gastropod mucins as a scientific resource with wide ranging applications across chemistry, biology, biotechnology, and medicine. For example, the antimicrobial properties of snail mucus are being used to combat human disorders from gastric ulcers, to surgery-related infections [10,11]. Mucins are also being coupled with approved therapeutics in order to potentiate the drug's abilities to cure diseases, such as diabetes and ulcerative colitis[12]. Additionally, snail mucins are

being investigated in a vast array of other biotechnical applications that exploit their surfactant-like properties[13]. Despite their potential, little is known about how the hierarchical mucin structures account for their diverse functional properties. There is an unmet need to examine the biological and chemical diversity of snail mucin genes to elucidate the guiding principles that determine the diverse properties associated with each gene. This perspective article will highlight current applications of secreted snail mucus that demonstrate the potential of this biopolymer as a resource for biotechnological and biomedical advancements. We will also describe an integrated

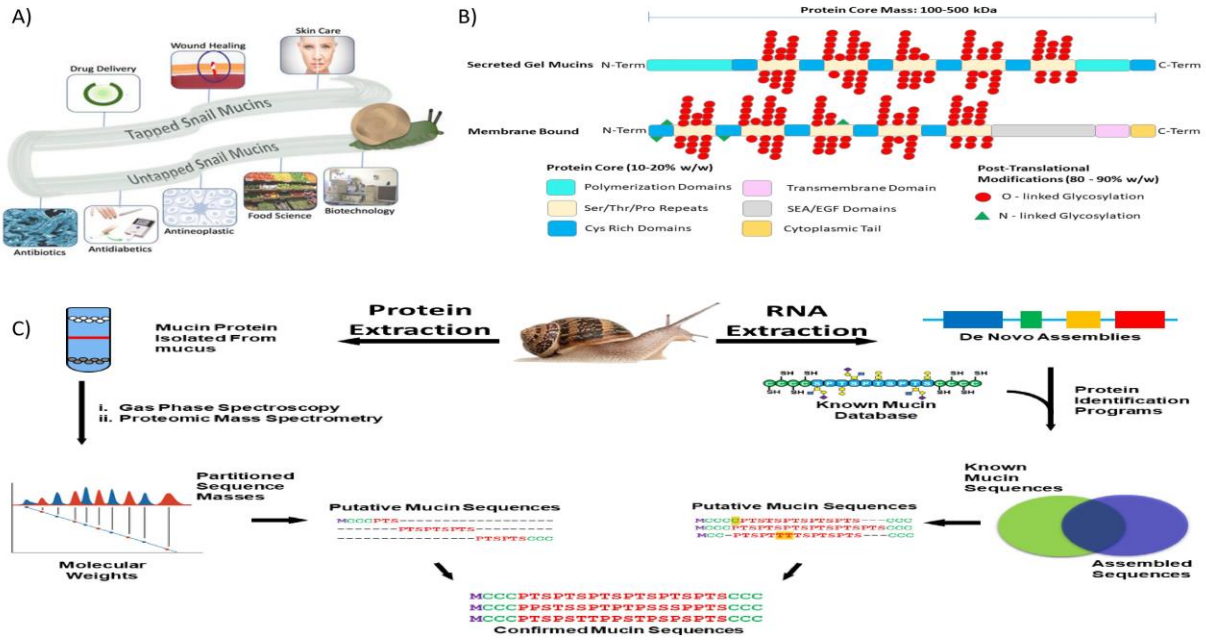

Figure 1.1 A) Applications of snail mucus. Snail mucus has been used for skin care, wound healing and rejuvenation, and drug delivery. Snail mucus is being explored in food science, implant coatings, and other biotechnical sectors are currently researching mucins to be explored for potential use. B) A 2-dimensional representation of the mucin structures. Mucins are characterized by two parts of their structure, their protein core, and their glycan branching. The protein core is a protein sequence of variable length depending on the mucin gene, which has been further modified with glycosylation branches. The Protein structure, however has multiple domains, and these domains vary depending on the function and the cellular location of the mucin. The glycan branches are sugar branches ranging from 3-18 sugars, and make up the majority of the mucin mass. Shown are 2 dimensional representations of the different types of mucins, and their stereotypical features. C) Applying an integrated omics approach to identify snail mucin sequence, structure, and function. Path 1(left) extract crude mucin proteins and separate from the cellular debris to obtain sequence masses from spectroscopic and mass spectrometric analyses. Path 2(right) RNA extraction from mucus glands or whole animal followed by de novo assembly of mucin gene sequences to generate a database to BLAST against by a comparison of assembled sequences to a known mucin database, we obtain putative mucin sequences. Combining the proteomic and RNA pipelines we confirm the native type mucin sequence for further analysis.

omics strategy for investigating the biological and chemical diversity of snail mucus genes (Figure 1.1).

Structural variations of mucins

Mucins contain several domains that contribute to their overall function (Figure 1). The structural variation allows for their extensive biological diversity and unique physical characteristics. A tandem repeat domain located in the center of the protein backbone, rich in serine, threonine, and proline, serves an as anchor for glycosylation. Mucin glycans are predominantly O-linked, but minor amounts of N-linked glycans can be present[14]. The length of the glycosylation domain and number of repeats differs between mucins and imparts different chemical characteristics. Secreted mucins have cysteine-rich regions on both ends of the tandem repeat domain that are used for stabilization, providing disulfide bridge points for both inter- and intramolecular bonding. Additionally, these regions serve both to provide additional structural diversification, and allow for multimerization of mucins and other sulfur-rich biomolecules[15].

Typically, N-acetylgalactosamine (GalNAc) is attached to the protein core via O-glycosidic bonds between the monosaccharide and either Ser or Thr residues (GalNAc[α1]-Ser/Thr). This forms the T_N Antigen, which is commonly upregulated in certain cancers[16]. From there, galactose is appended to the structure (Gal[β1-3]GalNAc[α1]-Ser/Thr), forming the mucin core 1 O-glycan. O-glycans vary in size, from 2-20 sugar residues, and in composition, as other sugars such as N-acetylglucosamine (GlcNAc) and fucose (Fuc) are appended sequentially[17]. Sialic acids and mannose are also found in trace amounts. Sialic acids in particular, have been to known to play a major role in the immune properties of mucins. Sialic acid mediates cell-to-cell interactions, along with being able to mask antigens from macrophages[18]. Further, sialic acids are the major binding

points for lectins, a common protein family found in the innate immune system[19]. Additionally, secreted mucins also exhibit C-mannosylation, where C1 of mannose bonds with the indole ring in tryptophan, allowing for greater variation of tertiary structure[20].

Subtle changes within the mucin structure, in particular the amino acid sequence and glycosylation, can correspond to vastly different biological function[21]. While these proteins are predominantly carbohydrates by weight, up to 90%, both protein and glycan structures provide overall functional characteristics to the mucin[20]. Additionally, individual mucins can have multiple glycoforms in normal and diseased states, and different populations of a single species can exhibit distinct glycoforms[22]. This diversity allows for organisms to individualize each mucin for specific physiological and environmental conditions. While not much is known about the genotype-to-phenotype connection of mucin genes that leads to the various functional properties, several mucin genes have been identified. Humans for example, have at least 21 unique mucin coding genes, each with different biological activities[23], while *Xenopus laevis* (Western Clawed Frog) has 26 gel-forming mucin genes alone[24]. While less is known about the genetics and structural differences between snail mucuses, this has not precluded their application to address pressing medical and biotechnological materials needs. Table 1.1 illustrates several industrial sector applications of mucus from various Molluscan species.

Table 1.1 Snails species and their industry applications.

Mollusca Species	Common Name	Applicable Sectors	Uses	Development Stage
Cornu aspersum 	Garden Snail	Cosmetics Medication Antimicrobial	Skin Care Cancer Treatment Topical Antibiotic	Commercially available(Benton, Mizon, Cos Rx, Biopelle, Missha)
Archachatina marginata 	Banana Rasp Snail	Antimicrobial Pharmacology Wound Care	Antibiotic Drug Delivery & Medication Wound Dressing	Patented for Use (US patent #: WO2000068258A2)
Achatina fulica 	Kalutara snail	Antimicrobial Pharmacology Wound Care	Antibiotic Drug Delivery Medication Wound Dressing	Patented for Use (US patent #: WO2000068258A2)
Arion subfuscus 	Dusky Arion	Medical Equipment	Surgical Glue	Active Research (University of Pennsylvania Lehigh University)
Helix pomatia 	Burgundy snail	Personal care	Shampoo	Commercially available (Royer)
Tikoconus Costarricanus 	*T. Costarricanus*	Biotech	Adhesion and Lubrication	Reported in literature

Snail mucins as antimicrobial agents.

Antibiotic-resistant bacteria are becoming an increasingly prevalent issue without many viable solutions. Because mollusks lack adaptive immunity, they depend on physical barriers and innate immunity for protection against pathogenic agents[25]. For most snails, the foot has the most contact with surfaces that are contaminated with pathogens and parasites, and secretion of mucus along the feet protects against such microbes. One of the earliest mucuses evaluated for antimicrobial activity was that of *Achatina fulica* (Giant African Land Snail) (Table 1)[26]. Mucus from *A. fulica* [27] demonstrated promising antibacterial activity against the Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The mucus secretions of *A. fulica* inhibited the bacterial growth of both *S. aureus* and *S. epidermidis* when applied via wound dressing films on a mouse model[28]. The wound dressings improved the maturation of granulation tissue and the rate of collagen deposition, which are known to expedite the healing process[29]. In a similar study, the mucus of *Helix aspersa* demonstrated antimicrobial activity against several strains of *Pseudomonas aeruginosa*[30]. Further, the mucus of both *A. marginata* and *A. fulica*, were utilized as wound dressinsg on 28 clinical wound samples collected with known common infections[31]. The mucus showed anti-bacterial potency against *Staphylococcus*, *Streptococcus*, and *Pseudomonas* isolated from wounds. In the same study, when compared to seven common antibiotics, including amoxicillin, streptomycin, and chloramphenicol, some of the mucus secretions were more inhibitory to infections than commercial antibiotics. Understanding the antimicrobial properties of snail mucus is an active and growing area of research.

Snail mucin as drug delivery vehicles

The adaptability of snail mucin biopolymers makes them uniquely promising candidates for novel drug delivery systems [32,33]. During mating, male snails shoot a dart to deliver mucus containing accessory proteins into the female, which in turn increases the fertility of the female snail[34]. This process relies on a multifunctional system, with each component playing a defined role. The dart acts as a needle, piercing tissue and injecting the mucus that carries the accessory proteins into the female snail. In a similar manner, mucus could be adapted to act as a delivery vector for bioactive molecules. Snail mucus are known to pair exceptionally well with any medication that is absorbed via mucosal membranes because of their ability to facilitate diffusion across membranes[35]. For example, metformin hydrochloride, a diabetes medication, was attached to Giant African Land Snail mucin using polyethylene glycol (PEG) to increase bioavailability of the drug[36]. Metformin-loaded PEGylated-mucin improved pharmacokinetics and pharmacodynamics of the normally poorly absorbed drug, increasing release to 92% compared to the 81% currently used in market. In another application, whole *Costus afer* (ginger lily) flowers combined with snail mucus showed reduction of blood glucose levels in diabetic *Mus musculus* (Swiss albino mice) in a dose-dependent fashion, which showed the possible anti-diabetic potential of snail mucin[37].

Drug-binding polymer matrix and mucin-containing vaterite crystals have been used as drug delivery carriers for effective loading and controlled release of small anti-cancer drugs and protein-based therapeutics[35]. Vaterite microcrystals, when crystallized in mucin concentrations ranging between 1-6 mg/ml, have better retention of cationic bioactivities and stability in physiological conditions Additionally, mucins have been coupled with photosensitizers in order to enhance targeting and optimize control of delivery into cancerous cells [38]. Self-assembled mucin multilayer capsules and mucin-containing microparticles are of particular interest for future studies

of controlled release drug delivery mechanisms, particularly to overcome challenges of biocompatibility, biodegradability, and mucoadhesion[39].

Snail mucins as anti-tumor agents

Snail mucin has shown therapeutic potential against melanoma, one of the most dangerous skin cancers[40]. While new developments in cancer therapy have resulted in greater remission rates and longer life expectancies for those afflicted, these developments have not shown similar yields for melanoma[41]. As treatment resistance is common for this cancer, there is an urgent need to find effective novel approaches for treating melanoma. To address this need, the effect of *H. apersa* mucus on melanoma cell lines were investigated[42]. This study reported inhibition in melanin overexpression and decreased tyrosinase activity, two phenomena associated with cell-level oxidative stress and the proliferation of melanoma cells[43]. In another study, *H. aspersa* mucin directly inhibited the growth of two human melanoma cell lines, directly demonstrating its anti-melanogenic properties [44]. While still in the early stages of development, the application of snail mucins as anti-tumor agents is of growing interest in the biomedical community.

Snail mucins facilitate wound healing

Snail mucus can facilitate healing and has become an important resource in wound research [31,45]. Mucins from the *Helix aspersa/Cornu aspersum* (Garden Snail) have been shown to help with skin regeneration after acute radiodermatitis, a common side effect from radiotherapy[46]. Garden snail mucus reportedly increased healing rates through antioxidant and free radical regulation[47]. Mucus from garden snails also significantly improved erythema in rat models, and, when applied daily for 3 months, showed a reduction of photoaging signs in rats[48]. As well as

being able to treat superficial injuries, mucins have shown the ability to be used on internal wounds. Mucins have been incorporated alongside oral non-steroidal anti-inflammatory drugs (NSAIDs), the most commonly prescribed classes of medication, to reduce or eliminate gastric mucosal injury[49]. NSAIDs reduce inflammation, but have adverse side effects related to gastrointestinal injury and liver damage, which prevented medicinal use and in turn, caused pharmaceutical companies to look for ways to mitigate the side effects. Many companies have turned to natural products to counteract these side effects. Mucin have been shown to treat peptic ulcers, a side effects caused by NSAIDs[50]. Traditional treatment of peptic ulcers is done via a combination of antibiotics with anti-ulcer medications. These anti-ulcer medications are classified into two groups, those that decrease pepsin secretion and those that help mucosal integrity[51]. A combination of the antibiotic, clarithromycin, and *A. fulica* mucin has shown positive results in treatment of peptic ulcer disease[52,53]. In addition to anti-ulcer properties, the healing rate of ulcers increased with concentration of mucin and was faster than clarithromycin alone.

Snail mucus used for bioinspired materials

Studying naturally occurring substances as a platform to build new materials has resulted in multiple revolutionary products, such as Lipitor, Penicillin, and Morphine. Similarly, mucins have been used as a biomaterial coating in order to reduce rejection of inorganic implants. Rejection of surgical implants due to infection results in over 1 million medical cases per year with the cost of the original surgery only being a fraction of the cost of treating the corresponding infection[54]. Applying mucin-based films to polyethylene terephthalate, a common material used in medical implants, greatly reduced the immune response triggered by IgG and IgM absorption into the plastic[55,56]. The same study also showed that it reduced the activation of fibrinogen, a known

inflammatory agent, when contacting the mucin coating as compared to the uncoated plastic. Mucins have been shown in other studies to reduce microbe reproduction on implanted devices.[57]. Mucin-based technologies show immense promise for advancements in the field of biomaterials.

An example of mucins being used as biomaterials is the application of mucins in the synthesis of water-soluble hydrocarbons. By ligating mucin and/or mucin-mimicking compounds with a hydrophobic lipid chain, the hydrocarbon complex remained suspended in aqueous conditions, even after several months, while the non-complexed hydrocarbon would precipitate rapidly from solution[58]. In another related study conducted by Combaa's group, this property was applied to enhance glucose detection by creating a stabilized suspension of carbon nanotube-mucin complex for a sandwich-type glucose biosensor. The resulting bioanalytical device is 20% more sensitive and 40% faster than conventional devices that do not include this sensor design matrix[59].

Mucins, which come into contact with medications absorbed through mucosal membranes, can also be used in chromatography to assist in determining bioavailability and absorption through the membrane[60]. Porcine gastric mucin, bound to the silica column via amino propyl linkers, allowed for separation of drug molecules by the drug's mucus membrane binding affinity. In another study mucin was anchored to a column using an ion-exchange with calcium-alginate, the mucin is immobilized, mimicking biological mucus membranes. Longer retention time of the molecule within the mucin column indicated high drug-mucin interaction, which is correlated to delayed bioavailability *in vivo*[61]. This adds another dimension to evaluate medications used in specific diseases that affect mucin production, such as cystic fibrosis[62].

The same porcine gastric mucin column has been used to evaluate flavor retention by the food industry. The mucin column was shown to mimic a bovine tongue for flavor retention, which reduces the need for and could potentially eliminate animal testing[63]. Mucins have been extensively studied in their role with flavor perception[64]. The presence of mucins within the oral cavity has been directly correlated to increased sedimentation of flavor-producing compounds, which in turn increases flavor perception[65]. This phenomenon is also being examinned as the cause for the loss of taste in old age[66]. Decreased levels of MUC7 in saliva has been noted in older individuals decreased taste[67]. This downregulation is believed to reduce mucoadhesion of the flavor molecules, leading to attenuated taste perception.

A snail's pace to characterization of mucin molecules

Despite growing interest in the field, there are still many obstacles that prevent advancements in snail mucin research. Many snail species that have the potential for novel mucin discovery are often inaccessible because of their habitat. The lack of readily accessible biological material samples and difficulty in identifying mucin structures prevents the reliable synthesis of mucins in quantities sufficient for repeated experimentation. Several groups are investigating sustainable, scalable approaches to producing synthetic mucins, however the field is in its infancy[13] While mucins that have been isolated from the *A. fulica* have been extensively studied, other species remain neglected [68].

The most viable method for commercial mucin production remains extraction and isolation from animals, which does not allow for substantial yields for application without abundant animal capital and generally involves invasive methods. The complexity, abundance, and localization of glycosylation patterns on each mucin, in addition to various mucin glycoforms cause difficulty in

employing common separation methods to purify, synthesize, and analyze mucin samples [69]. Mucins often undergo posttranslational modifications, such as O-sulfation, N-sulfation, and N-deacetylation that differentiate function between proteins[70]. These posttranslational glycan modifications are a hurdle to mucin sample purification, characterization, and synthesis. A promising synthetic approach involves using recombinant bacteria, glycosyltransferase(GT)-mediated polymerization, and trans-glycosylation[71]. However, these methods are insufficient to achieve industrially practical yields and will fail to generate the exhaustive set of glycoforms that comprise natural mucus gels. There is still difficulty in creating the O-glycosylation in yeast, and there are challenges involved in transferring glycosylation branches to chosen protein residues. These issues present a need for developing viable and high yield methods for synthesizing mucins using scalable chemistries, which would be the first step in using mucins as targeted therapies or treatments[72].

Recent years have seen the emergence of -omic technologies (genomics, transcriptomics, proteomics, glycomics) that require minimal amounts of samples, allowing for the characterization of rare or poorly accessible snail samples[73]. A similar strategy to what has been done with snail venoms using venomics[74–76], which pairs transcriptomic and proteomic methods with de novo sequence bioinformatic assembly programs to identify the genetic structure of snail venom putative peptide toxins, can be applied to characterize mucin genes and mucus proteins (Figure 1). Specifically, by taking the nucleotide sequences of assembled exomes, and then pairing that with proteomic mass values, we can confirm linear mucin protein structures. In this approach we extract mRNA from mucus glands or whole animal and through a bioinformatic pipeline, identify mucin genes and primary mucin protein sequences. A new initiative, the Comparative Animal Mucomics

Project (CAMP) will apply a systematic comparative analyses of mucin genes and mucus hydrogels to determine the hierarchical structures and properties of distinct mucuses[7].

Despite the promise of omic methods for producing robust databases of mucins, major hurdles still remain for their study. One such hurdle are the algorithms used to assemble sequenced genes. De Bruijn graphs, which is the algorithm sequence most assemblers use, have difficulty mapping the repeated domains due to the multiplicity of similar overlapped sequences[77]. Multiple tools are currently being developed to overcome this problem[78]. Each program changes the weighting of the k-mers that are used to construct the De Bruijn graphs in order to accommodate for the tandem repeats. For mucin proteomic study, the intermolecular interactions of mucins with other mucins causes an additional degree of difficulty. Mucins naturally will create multimers of themselves, connecting multiple proteins together in order to form a larger structure, which is regularly observed in nature[79]. In order to then obtain a single protein, the cojoined bonds must be broken, without also breaking the bonds of the single protein. However, mucin multimer bonds are difficult to reduce without also having an effect on the rest of a single mucin chain's secondary structure. A trial and error procedure is currently used in mucin proteomic studies to generate single protein masses. New characterization and synthesis techniques will need to be established to accurately identify and fabricate snail mucins, and with an omics approach we may be able to determine the genotype to phenotype mapping necessary to understand and decipher the functionality differences found in each mucin sample.

Concluding Remarks and Future Perspectives

Snails are found in nearly every biome, and environmental conditions appear to drive the diversity of mucin genes and versatility of mucus functions (Figure 1)[80,81]. Snail mucins have

demonstrated biomedical and biotechnology potential, and are a bioinspired resource of significant promise. Characterization of snail mucins are limited not by their inherent value, but by access and the complexity of the molecule's identification, purification and investigation. There are still several questions left to be answered about the properties of mucins and mucuses in relation to the applicable uses. This prospective demonstrates the high yield potential of snail mucins, and by utilizing an adaptable comparative omics pipeline, we can better understand these unique proteins, and their advantageous biological and chemical properties.

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Chapter 2- Characterization of Snail Mucin Genes from the Common Garden Snail

Introduction

All animals secrete mucus hydrogels that are complex mixtures of water, proteins, and electrolytes[82]. The primary protein components of these gels are high molecular weight, densely glycosylated proteins called mucins[83]. Animals use mucus hydrogels to fill diverse roles, including as adhesives, lubricants, protective barriers, and as mineralizing agents[7]. As such, mucus plays a central role in key aspects of the biology of animals, including for defense, mediating predator-prey dynamics, and in homeostasis. An indication of how useful and important mucus hydrogels are to animals is demonstrated by the fact that all animals secrete at least 3, and as many as 23, distinct mucus hydrogels[81]. The garden snail, *Cornu aspersum*, for example, secretes three distinct mucuses from its exterior surface to manipulate interactions with its environment, a lubricating mucus from its foot that facilitates the animal's movement across flat surfaces; an adhesive mucus also secreted from the foot, that lets the snail adhere to vertical or inverted surfaces; and the third, a mucus secreted from the back, that hydrates the organism and protects it from external hazards. To characterize the diversity of functional properties of secreted *C. aspersum* mucus, it is necessary to address the paucity of data on the hierarchical structures (genotype) and physical properties (phenotype) of snail mucuses that could be used to understand the origins of their beneficial properties and recreate them in biomimetics.

Mucus of *C. aspersum* is harvested for pharmaceutical and cosmetic purposes, including as treatments for burn management, formulations in moisturizing products, and is known to induce in vitro cell proliferation[84–86]. These applications leverage the secretion's abilities to inhibit

bacterial growth, enhance elasticity in composite materials, and incorporate water into its structure. Because of its beneficial properties, the global market for snail mucus is expected to grow to $750 million US dollars by the year 2025. Despite the prevalence and commercial importance of mucins, these biopolymers remains largely unresolved. Data, primarily derived from mammalian mucins, suggests that mucin proteins have common structural elements, including discrete cysteine-rich N- and C-terminal domains that are involved in disulfide-based multimerization and a heavily glycosylated, repetitive serine/threonine-rich interior region. The data on *C. aspersum* mucus is not nearly as abundant or detailed as that on the mammalian mucuses, with details of the protein structures and hierarchical organization remaining largely unresolved. Investigations on the mucus of *C. aspersum* have focused on extraction and quantification of bulk mucoprotein, bioactivity, or influence on snail behavior[87–89]. These studies found that *C. aspersum* snails utilize mucus during mating to induce physiological changes that promote reproduction, and that snails are more likely to follow mucus trails of other snails, leading to mating and evolution between and geographically distant populations despite high energy costs. Regarding the structural properties of snail mucus, research focuses mainly on antimicrobial peptides within the secretions, with several studies reporting multiple peptides of 30-100 kDa, each selectively inhibiting common bacterial strains[30,83,90]. Analysis of snail O-glycosylation has revealed four common monosacchardies: GalNAc, Gal, Man, and Fuc, and additionally identified a core (4OMe-Gal)2GalNAc trisaccharide, but these studies were on glycans from animal tissue and not mucus[91–93]. A remarkable idiosyncrasy of invertebrate O-glycans is the abundance of methylated residues[94,95]. Previous studies on snail mucus proteins have been unable to identify any particular peptides as mucins[96]. According to the NCBI protein database, only four gastropod mucin sequences have been reported, from species freshwater and marine snails as well

as a sea slug[97]. Molecular characterization of snail mucins is limited to basic compositional analysis of amino acid and glycan components, as well as general insights into the size of these biopolymer networks. Particularly, this study found mucin samples to be composed primarily of Ser/Thr residues; GalNAc, Gal, and Fuc glycans; and had masses of approximately 30 kDa. One study correlated an increase in CaCO3 secretion with an increase in mucin particle aggregation[98]. Another reported that alkaline conditions increase mucus adhesive strength by increasing water uptake water ability[99]. Notably, the investigated species vary widely; the lack of a consistent model organism greatly limits comparability between studies and slows progress in the field.

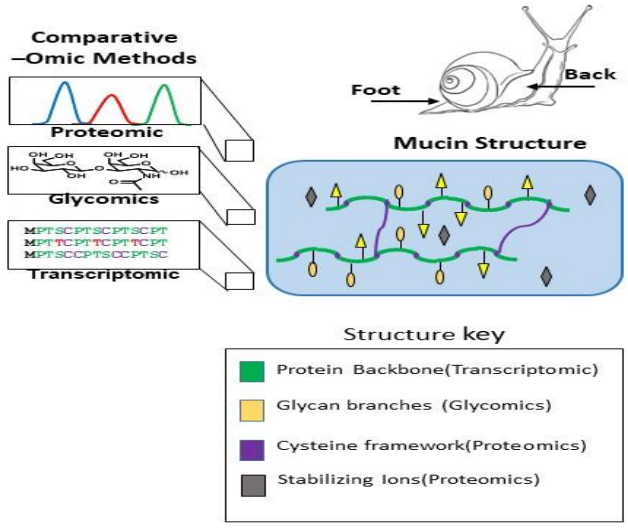


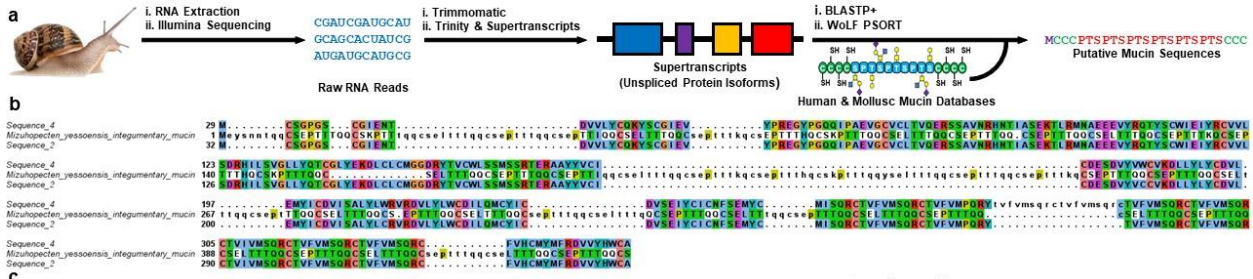
Figure 2.1 Mucomics methods to characterize snail mucuses. A given species of snail will produce at least three functional mucuses that provide lubrication, adhesion, or protection for the animal's soft exterior tissue. In this work, we applied several independent analytical techniques to characterize the mechanical properties of snail mucuses at a genetic and molecular level using omic techniques (transcriptomics, glycomics, and proteomics).

Investigating *C. aspersum* mucuses is a promising avenue for devising nature inspired biomaterials for biomedical applications[31,100]. Snail mucus has been the topic of several studies over the past few decades, and despite the diversity in their properties and functions, the secreted mucins are believed to be constituted primarily from a small set of conventional and glycosylated amino acids, and have many similarities in terms of sequence and size. However the biomolecular mechanisms underlying the secretions' diverse properties have yet to be revealed.

Comprehensive characterization of mucus function requires understanding its complex formulation and determining the hierarchical genotype to phenotype relationship of mucin genes that derives this material. Ideally, thorough examination of a given mucus involves the determination of the peptide sequence and glycan composition of individual mucin proteins, characterization of the non-mucin components of the heterogeneous mucus mixtures, and measurement of the material properties of the macroscopic gel. To conduct truly rigorous comparative analysis between mucuses of differing functions and origins, all samples should be subjected to a standardized experimental approach. As stated previously, standardization of a "comparative mucomics" research strategy to generate comparable datasets between samples and species will lead to establishment of robust mucus structure-property relationships, so that scientists can understand the origins and breadth of these fascinating substances[7].

Here we report a comparative analysis of the hierarchical structures of three different mucuses collected from *C. aspersum* snail mucuses in an effort to begin to unravel the genotype to phenotype connection and to dissect what determines the functional properties found in each mucin gene product. Specifically, we characterized the biomolecular structure of the underlying mucin glycoproteins comprising these hydrogels, determined the composition of the bulk materials, and quantified the mechanical properties of each mucus. By using our novel mucomic approach, we have found solutions to persistent challenges in mucus research, such as sequencing repetitive mucin proteins. Using this omics-style strategy, *C. aspersum* snail mucins were systemically characterized and compared by integrating transcriptomic, glycomic, and spectroscopic analyses on three functionally distinct mucuses. Using tissue-specific transcriptomic analyses, we were able to sequester sequences based on localization of collection from the snail's anatomy (foot, mantle, back), and with bioinformatic methods, reconstructed a phylogenetic tree of multiple snail mucins,

suggesting possible trajectories of mucus evolution. Additionally, the O-glycans from *C. aspersum* mucins have been identified. Using analytical chromatography, we bridged protein sequence and glycosylation to understand the heterogeneity of snail mucins. Finally, by using electron and atomic force microscopies, we are able to connect the molecular structures of these secretions to the macroscopic behavior. By characterizing the structures of *C. aspersum* mucins we can address a knowledge gap that can be used to discover bioinspired materials that meet real world needs and



Prospective Mucin	Mw (kDa)	Length	Ser Total	Thr Total	Pro Total	Ser %	Thr %	Pro %	Ser Fold	Thr Fold	Pro Fold
Sequence 1	64.65	549	32	18	37	5.8	3.3	6.7	0.72	0.63	1.81
Sequence 2	55.88	506	40	27	6	7.9	5.3	1.2	0.98	1.02	0.32
Sequence 3	41.20	352	28	18	5	8.0	5.1	1.4	0.99	0.98	0.38
Sequence 4	42.64	339	10	14	4	2.9	4.1	1.2	0.37	0.79	0.32
Sequence 5	31.78	298	17	37	15	5.7	12.4	5.0	0.71	2.37	1.35
Sequence 6	30.13	257	27	36	0	10.5	14.0	0.0	1.31	2.67	0.00
Sequence 7	28.02	257	24	20	14	9.3	7.8	5.4	1.16	1.49	1.46
Sequence 8	27.87	249	13	28	28	5.2	11.2	11.2	0.65	2.15	3.02
Sequence 9	21.36	188	21	23	19	11.2	12.2	10.1	1.39	2.33	2.72
Sequence 10	16.67	133	2	9	2	1.5	6.8	1.5	0.19	1.29	0.40
Sequence 11	15.80	132	19	1	7	14.4	0.8	5.3	1.79	0.14	1.43
Sequence 12	14.94	132	19	2	2	14.4	1.5	1.5	1.79	0.29	0.41
Sequence 13	14.06	125	16	19	2	12.8	15.2	1.6	1.59	2.90	0.43
Sequence 14	12.75	116	16	7	4	13.8	6.0	0.0	1.71	1.15	0.00
Sequence 15	14.33	113	2	0	0	1.8	0.0	0.0	0.22	0.00	0.00
Sequence 16	11.56	102	6	9	0	5.9	8.8	0.0	0.73	1.68	0.00
Sequence 17	11.57	97	8	8	0	8.2	8.2	0.0	1.02	1.57	0.00
Sequence 18	10.70	97	6	10	13	6.2	10.3	13.4	0.77	1.97	3.60
Sequence 19	9.35	86	13	11	7	15.1	12.8	8.1	1.88	2.44	2.19
Sequence 20	9.75	84	14	2	10	16.7	2.4	11.9	2.07	0.45	3.20

Figure 2.2 Transcriptomic assembly and analysis of de novo assembled putative mucin genes from C. aspersum snails. A) bioinformatic pipeline for processing mucin sequence data. RNAseq results are subjected to Trimmomatic, Trinity, and Supertranscripts to assemble a transcriptome de novo, from which putative mucin sequences are extracted via database searches and criteria-based filtration, using BLAST and WoLF PSORT. B) Multiple sequence alignment between putative mucins 2 and 4 as well as their highest similarity homolog from known molluscan mucins. C) Sequence composition data from all 20 potential mucin candidates. Backbone length and theoretical masses are shown in addition to the amounts of Ser, Thr, Pro, and Cys residues and their fold increase over the natural abundance for invertebrate non-membrane proteins. Green highlighted cells indicate >25% increase over natural abundance, while red highlighted cells indicate <75% of the natural frequency for the given amino acid. D) Phylogenetic tree of all 20 putative mucin candidates and their highest similarity molluscan mucin matches. Human mucins are used as the outgroup**.**

synthetic chemists will be able to replicate more effectively the structures and properties of these functional hydrogels. This Thesis chapter is primarily focused on my contribution of transcriptome sequencing analysis for mucin genes, showing the value of incorporating a multiomic approach to characterize mucin hierarchal structure.

Results

To sequence and identify mucin genes expressed in *C. aspersum* mucus, we extracted RNA from 6 samples for RNASeq transcriptome analyses. As there is no solved genome for *C. aspersum*, we de novo assembled the transcriptome using TRINITY and applied a streamlined bioinformatics pipeline to identify 20 putative mucin sequences (Figure 2A)[101,102]. Our putative sequences were confirmed using homology BLASTto known mucins, and WoLF PSORT to focus on secretory mucins using cell localization probability[103,104]. The 20 putative mucin genes were aligned against known mucin sequences (Figure 2.2B) via MUSCLE[105]. We completed a sequence analysis of these 20 genes to determine peptide backbone abundance, focusing specifically on Ser, Thr, Pro, and Cys, which are well-known to be upregulated residues in mucin proteins (Figure 2C). Comparing against natural amino acid frequencies for invertebrate non-membrane proteins, we find that out of our 20 sequences, 8 are rich in Ser, 11 in Thr, 9 in Pro, and 17 in Cys, exhibiting more than 25%, and even over 400% of the natural frequencies for these amino acids. Using the HMMER package, we identified domains and regions of interest in the 20 putative sequences, including potential transmembrane regions and repeated regions[106]. Two of the putative mucin sequences (sequence 12 , sequence 13) contain VWA domains, and 17 of the 20 sequences had regions with between 2 and 15 repetitions. These repetitions showed upregulations of the residues which we would expect to find for mucin repeat domains, namely the glycosylation branching points residues.

Because of variability in sizes of putative mucin sequences we clustered the sequences into smaller groups with similar structures. The new clusters were then aligned against one another to determine sequence homology, after which all of the sequences, along with their BLAST-matched sequences, were used to create a phylogenetic tree to determine conserved evolutionary traits (Figure 2.2D).

Discussion

Transcriptomic Analysis of C. aspersum mucins

Characterizing the peptide backbones of mucins is paramount to understanding their function. Prior efforts using conventional proteomic approaches were limited due to the numerous repeat domains common to this protein family. We used a transcriptomic approach that circumvents these challenges, allowing sequencing of mucin genes. Our pipeline extracts and selects sequences that show statistically significant similarity to known mucins, with many of these genes exhibit high abundance in mucin-critical residues. Additionally, using domain identification via HMMER to identify domains not found in membrane bound mucins, we confirmed that the majority of the putative genes identified encode gel-forming secreted mucins. Interestingly, the putative mucin sequences identified all have lower molecular masses than previously reported mucins found in higher evolutionary species[107]. This difference highlights the fact that mucins can be highly adaptive biopolymers that can be a new avenue for biomimic polymers. Two putative mucin sequences (sequence 5, sequence 6) exhibited VWA domains, which are commonly found in secreted mucins and participate in multimerization, an activity not found in membrane-bound mucins. Finally, the vast majority of the sequences had discernable repeated regions, showing anywhere between 2 and 15 instances of the particular region, with all repeats containing Ser, Thr, or Pro, indicating potential invertebrate homologs of STP domains. It is important to note the snail

mucin repeat domains were not as clearly defined as in vertebrate mucin sequences, and this may be due to the fact that *de novo* assemblers, including Trinity, are ill equipped to handle tandem repeats, as there is no reference genome informing the process for how many repeats are to be expected. Additionally, while these mucins sequences are not strong homologs to human mucins, we do see a similar lack of organize repeats in other invertebrate mucins, suggesting that the repeat order could be part of an evolutionary process[108].

Notably, two of the putative mucins (sequence 2 and sequence 4) showed high similarity to one another, and showed many of the known sequence characteristics of mucins. These two sequences were assembled within two different samples transcriptomes and differ in length by 200 residues. Their sequences are nearly identical with a 95% alignment match, however one of the genes has an extended tail potentially making it a longer isoform of the small potential mucin gene. Mucins isoforms are a common occurrence, making the mucin gene multifunctional, allowing for the genome to be more compact. These two genes in fact also matched to the same known mucin sequence, found in the *Mizuhopecten yessoensis* (Yesso scallop), in the Mollusca database. CD-Hit clustering showed that certain sequences (sequence 2 and 4) had high sequence homology, showing a promising indicator as the clustered sequences would regularly group with proteins of different assemblies. For tree generation these two sequences clustered together independently to the CD-hit program, and with a high bootstrap value(figure 2.2)[109]. The overall bootstrap values of the phylogenetic tree however, while promising did not show as ideal consistencies. The most likely explanation for this occurrence is that the sequences in question vary greatly in size. When compared to mucins from humans, and even from certain invertebrates, these mucins are considerably smaller, and even within the punitive mucins generated there is a relative size variation. This size generation could potentially lead to poor bootstrapping for these sequences,

even when using a maximum likelihood phylogenetic analysis. When an outlier group containing humans mucins was included in the analysis, the group consistently formed its own clade, suggesting that these mucins are evolutionary diverse compared to the heavily studied mammalian mucins. This further illustrates that snail mucins can serve as an avenue of a biomimic polymer that would be unique from other methods currently being used.

Methods

RNA Extraction and Sequencing: Snails provided by Peconic Escargot in February 2020 were sacrificed on-site via freezing in a dry ice-ethanol mixture. Whole snails were stored in Invitrogen RNAlater™ (Thermo Fisher, AM7021) and frozen at –80 °C until used. Total RNA was extracted using a Qiagen RNeasy Micro kit (Qiagen, 74004), from 6 individual tissue slices from different snails. The integrity of total RNA was confirmed using nanodrop and Agilent 2100 BioAnalyzer analysis. Total RNA was used as a template to perform polyA enriched first strand cDNA synthesis using the HiSeq RNA sample preparation kit for Illumina Sequencing (Illumina Inc., CA) following manufacturer's instructions. The cDNA libraries were sequenced using Illumina HiSeq 1000 technology using a paired end flow cell and 80 x 2 cycle sequencing.

Read Processing, De Novo Assembly, and Putative Mucin Identification: Raw reads were quality checked with FastQC v0.11[110]. Adapter sequences and low-quality reads (Phred score <33) were removed using Trimmomatic v0.36 and trimmed reads were re-evaluated with FastQC to ensure the high quality of the data after the trimming process[111]. Due to the lack of a reference genome, the processed reads were de novo assembled using Trinity v2.4.0[102].

De novo assembled transcriptomes were analyzed to identify putative mucins, and Transcriptomes were translated with Trinity Super Transcripts[112]. Transcripts were searched using the BLASTP

tool against an in-house database that includes all known mollusk mucins, and the 3 most common human mucins available in NCBI databases[103]. Hits with an e-value of 1x10-20 or smaller were maintained. Those maintained punitive mucins were then checked for secretion likelihood using WoLF PSORT v1.4[104]. All transcripts that had their most likely cellular localization being in the extracellular matrix were kept. Additionally they were checked for transmembrane domains using TMHMMR v1.7[113]. Transcript abundance rates were also analyzed using the Kallisto v1.8[114][114].

Phylogenetic Reconstruction of Putative Mucins: Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 10. Phylogenetic analyses of individual mucin families including homologs were performed to validate the homology searches predicted using BLAST to investigate evolutionary relationships across taxa. Selected putative mucins (i.e. non-redundant contigs that were identified as a toxin by BLAST search methods) and homologous sequences from other mollusk species NCBI and clustered via CD-HIT. The created clusters were aligned with MUSCLE v7.31[105].

Conclusion

Mucins, while being an incredibly understudied protein family, have already shown to be highly important to biological function. Mucins themselves, have been linked to a number of diseases including cancers, and cystic fibrosis. While we know that these proteins are important, we have had the ability to study them until recently. With next generation sequencing, we have begun to open up the door onto understanding these diverse biopolymers.

While we are still researching the function of these molecules in humans, other organisms are falling on the way side. Snail mucins have demonstrated biomedical and biotechnology potential, and are a bioinspired resource of significant promise. Characterization of snail mucins are limited not by their inherent value, but by access and the complexity of the molecule's identification, purification and investigation. Mucins could be added to a long list of protein families that have been used to create bio mimics that revolutionize industries. With open access to genomic database, tied to the now having a functional pipeline to identify mucins, we can begin to focus on our explorations onto species that could present with unique molecularly diverse proteins that could help to better our understanding of mucins, and their complex biological functions.

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