Changes in the Properties of *Achatina fulica* Mucin at Different Storage Conditions

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Bachelor of Science with Honours
(Resource Biotechonology)
2022

Changes in the Properties of *Achatina fulica* Mucin at Different Storage Conditions

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

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Resource Biotechnology Programme
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2022

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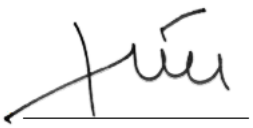
Supervisor's signature  (14 JULY 2022)

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ACKNOWLEDGEMENT

Firstly, I would like to thank God for His blessings, that I was able to face all the challenges during my studies. Secondly, I would like to express my sincere gratitude to my supervisor, Dr. Micky Vincent for his guidance and advice on the final year project. Thanks to the lectures and lab assistants of Faculty Resource Science and Technology for allowing and guiding me throughout my laboratory work. I am also grateful for my postgraduate seniors, Effa and Thracesy who assist and help me in completing my project. I would also like to thank my fellow course mates for their motivation and encouragement, providing a friendly and comfortable atmosphere to carry out the work. Lastly, special thanks to my family members for their inspiration, moral and financial support throughout my study.

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ABSTRACT

Snail mucin is an ingredient of interest in cosmeceutical and pharmaceutical that is discovered to contain important chemical components such as allantoin, antioxidants, glycolic acid, and vitamins. Among all the land snail species, *Achatina fulica* is founded as one of the most suitable snail species for heliciculture in Malaysia. In this study, *A. fulica* mucin was subjected to four storage conditions which were room temperature, 4 °C, with mucin stabilizer and with no added mucin stabilizer for 60 days to study the relationship between different storage conditions and the changes in the chemical/biochemical properties of *A. fulica* mucin. The chemical characterization tests were Bradford assay on protein determination and cetyltrimethylammonium bromide turbidimetric method (CTM) assay for the study of hyaluronic acid concentration. The microbial test was conducted through the spread plate method and antimicrobial properties were tested using the disc diffusion method. Based on the results, storage of *A. fulica* mucin at 4 °C with added mucin stabilizer was the most suitable storage condition to preserve the chemical components in the mucin and to provide antimicrobial properties.

Key words: *Achatina fulica*, snail mucin, storage condition.

ABSTRAK

Lendir siput berjaya mendapat perhatian dalam bidang kosmetik dan farmaseutikal setelah ditemui mengandungi bahan kimia yang penting seperti allantoin, antioksidan, asid glikolik dan vitamin. Antara semua spesies siput tanah, Achatina fulica *didapati sebagai salah satu species siput yang paling sesuai untuk diternakan di Malaysia. Dalam kajian ini, lendir* A. fulica *telah diletakkan bawah empat keadaan penyimpanan iaitu pada suhu bilik, pada 4 °C, dengan bahan penstabil dan tanpa bahan penstabil selama 60 hari untuk mengkaji hubungan antara kaedah penyimpanan dan perubahan atas sifat kimia/biokimia lendir* A. fulica. *Ujian kimia yang telah dilakukan adalah ujian Bradford untuk menetukan kepekatan protein dan ujian turbidimetrik setiltrimetilammonium bromida (CTM) untuk mengkaji kepekatan asid hyaluronik. Ujian mikrob telah dilakukan melalui teknik plat penyebaran manakala ujian antimikrob telah dijalankan menggunakan kaedah penyebaran cakera. Berdasarkan hasil kajian, keadaan penyimpanan lendir* A. fulica *adalah paling sesuai pada suhu 4 °C dengan penambahan bahan penstabil untuk memelihara komponen kimia dan memberikan sifat antimikrob kepada lendir.*

***Kata kunci**:* Achatina fulica, *lendir siput, keadaan penyimpanan..*

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LIST OF ABBREVIATIONS

AST	Antimicrobial susceptibility test
BSA	Bovine serum albumin
CTAB	Cetyltrimethylammonium bromide
CTM	Cetyltrimethylammonium bromide turbidimetric method
EDTA	Ethylenediaminetetraacetic acid
HA	Hyaluronic acid
MH	Mueller-Hinton
NA	Nutrient agar
NB	Nutrient broth
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline

CHAPTER 1

INTRODUCTION

1.1 Introduction

Land snails are gastropod mollusks that live on land. Snails belong to the second-largest phylum in the animal kingdom which is the phylum Mollusca and there are at least 80,000 species worldwide. They possess high adaptability towards a wide range of climates and growth environments, making them available across all continents in the world except Antarctica (Cameron, 2016). Snails are invasive pests to the agricultural fields due to their polyphagous properties and rapid reproduction (Sarma *et al*., 2015). In Australia and the United States, the importation of snails is illegal and only a few snail farms are available (Department of Agriculture, Water and the Environment Australia, 2021; U.S. Department of Agriculture, 2021). However, due to the emerging global snail market demands, heliciculture, or commonly known as snail farming is becoming more popular among other developing African and Asian countries where their meat is made into delicacy due to their high nutritional contents that have the potential to be a source for animal protein (Funmilayo, 2008). According to several research in Europe, the import of snails surged by 49% from 1995 to 2010, with over 100,000 metric tons consumed yearly (Morei, 2012; Oikonomou *et al.*, 2012). Snail farming, however, is not well received by Malaysian farmers and there are limited publications on snail farming in this country (Sahid, 2013). It is reported that there are between 800 to 1000 species of snails in Malaysia. Among these species, *Achatina fulica*, *Macrochlamys indica*, and *Bradybaena similaris* are considered the common garden land

snails (Liew & Foon, 2022). But based on the same report, *Achatina fulica* and *Helix aspersa* are found to be the most suitable species for snail farming in Malaysia.

Another snail product that is highly valued and sought after is its slime or mucin. The market value of snail mucin is estimated to reach $770 million by the year 2025 (McDermott *et al.*, 2021). Snail mucin exhibits antimicrobial and healing properties due to the presence of chemical components such as allantoin, antioxidants, glycolic acid, and vitamins that made it an ingredient of interest in cosmeceutical and pharmaceutical (Mubarak *et al.*, 2013; Perpelek *et al.*, 2021; Song *et al*., 2021). Although several studies have been done to investigate the chemical composition and properties of snail mucin, few resources document the optimal storage conditions and biochemical integrity of stored snail mucin. The extracted snail mucin is required to undergo processing and refining before being made into commercial products, hence it is crucial to know the suitable storage condition to preserve the desired chemical component in the mucin before sending it for processing (Laneri *et al.*, 2019). Therefore, the main aim of this study is to investigate the relationship between different storage conditions and the change in the chemical/biochemical properties of *A. fulica* mucin over time. The results from this research may be able to provide some insights on the suitability and stability of *A. fulica* mucin as a cosmeceutical and pharmaceutical product which may increase the interest of *A. fulica* heliciculture.

1.2 Objectives

The objectives of this study are to:

1. Analyze the chemical and microbiological properties of *A. fulica* mucin.
2. Investigate the relationship between different storage conditions and the changes in the chemical/biochemical properties of *A. fulica* mucin.
3. Determine the suitable storage condition for *A. fulica* mucin.

CHAPTER 2

LITERATURE REVIEW

2.1 *Achatina fulica*

Achatina fulica is well known as the giant African snail. It is a gastropod under the Mollusca phylum. *A. fulica* can be distinguished by its large size (Figure 1), with the length of the shell being approximately 20 to 22 cm and its body length can reach up to 30 cm (Vinci *et al.*, 1988). The shell is conical, thin, and ceramic-like with reddish-brown color in general, depending on the diet and environmental factors. *A. fulica* is native to the coastal area of East Africa, however, it has been introduced to many other countries across the world and was identified as an invasive species to the local agriculture (Sarma *et al.*, 2015; Guo *et al.*, 2019). *A. fulica* favors a warm and humid environment, but it also developed adaptation to thrive in less ideal surroundings, such as hibernation during low temperatures and aestivation during high temperatures. This ability to cope with temperatures between 2 to 30 °C enables the species to be distributed worldwide from temperate to tropical climates (Hoffman & Pirie, 2014). *A. fulica* can survive in different environments such as coastal areas, forests, wetlands, agricultural areas, and urban areas (Pearce & Örstan, 2006). It is a polyphagous pest that feeds on various types of food including decayed matter, crops, ornamental plants, tree barks, lichens, fungi, and algae (Mead, 1961; Raut & Barker, 2002; Jadhav *et al.*, 2016).

Figure 1: *A. fulica* (Liew, 2022).

A. fulica has a lifespan of approximately three to five years, and may live longer until nine years. It is a solitary species that does not often require communication with other species except for mating (Bhattacharyya *et al.*, 2014). *A. fulica* is hermaphroditic and does not self-fertilize. About 1200 eggs can be produced each year (U.S. Department of Agriculture, 2021). *A. fulica* requires moving around looking for mates and food, hence, it produces mucus to enhance its movement across rough surfaces. Aside from being an invasive pest that destroys agricultural products and carries parasites, the organism can also bring ecological and economic importance under controllable conditions (Mead, 1961; Vinci *et al.*, 1988; Grilla *et al.*, 2016).

The giant African snail is considered an important delicacy among communities of West Africa and the elites of many European countries (Nkansah *et al.*, 2021). Since the global food demand is rising due to the increasing human population, conventional animal protein sources are becoming more expensive and declined with the persistent natural disaster and diseases (Funmilayo, 2008). Through multiple research projects, snails are found to have the potential as an alternative source of animal protein due to the high level of protein, iron, magnesium, phosphorus, potassium, and low levels cholesterol, fat, and sodium (Ajayi *et al.*, 1978; Adeyeye, 1996; Fagbuaro *et al.*, 2006).

Snails are also traded in the global and overseas markets. According to Vinci *et al*. (1988), the output of snail farming in Nigeria is exported to France as a delicacy with lucrative prices, yet there is still a shortage of snails that has triggered snail farming in British. Aside from the economic importance, *A. fulica* also plays a role in the ecosystem by assisting in nutrient recycling as it decomposes decaying matter and dead vegetation (Bloch, 2012). *A. fulica* is also part of the food web with a few predators such as *Euglandina rosea*, a predatory snail, and rats while it is also a host to some parasites such as *Angiostrongylus cantonensis*, a rat lungworm (Lv *et al*., 2009; Cowie, 2010).

2.2 Snail Mucin

Mucin is a type of slimy, aqueous substance secreted by glands to cover up the membrane and epithelial surfaces (Smith, 2010). Most gastropods which include snails produce mucus. The main component in mucus is mucin, which is a highly glycosylated protein (Bansil *et al.*, 1995). In a mucin monomer, multiple oligosaccharides are chained onto a protein backbone through O-glycosidic linkages (Figure 2). There are cysteine-rich regions on the protein backbone which are lightly glycosylated but rich in chemical diversity, having cysteine moieties that involve the formation of disulfide linkages among mucin monomers (Authimoolam & Dziubla, 2016). These glycoconjugates are assembled into polymers that form a barrier to molecules and the growth of microbes due to their large size (Cone, 2008; Ensign *et al.*, 2011). This property enables mucin to function as a stable coating. The glycans associated with mucin can actively form hydrogen bonds with water molecules, resulting in high water-holding capacity (Crouzier *et al.*, 2015). The structure, charge, and hydration

property of mucin also contribute to the formation of lubricating elements. Mucin can actively react with biological molecules, microbes, and animal cells (Petrou & Crouzier, 2018).

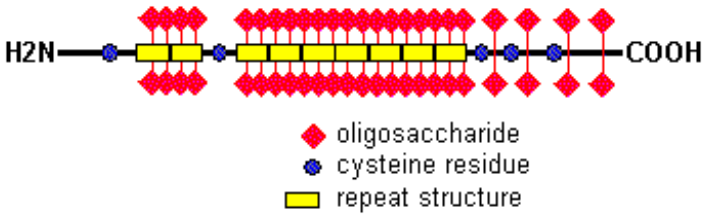

Figure 2: Generic structure of a mucin monomer (Vivo.Colostate.edu, n.d.).

The most easily found mucus from snails can be observed when it is crawling, leaving behind a slimy trail. This mucus produced by its foot exhibit qualities of a lubricant that enables the organisms to crawl through rough and sharp surfaces without injury (Bansil *et al.*, 1995). The mucus also has the qualities of a glue that allows the organism to crawl and attach to walls without falling off (Newar & Ghatak, 2015). Aside from mucus produced by the foot, snails would also produce mucus to cover up their exposed body parts to prevent desiccation. Under unfavorable conditions, snails could produce epiphragm as shown in Figure 3, which consists of dry mucus to cover their aperture and to prevent water loss as 90% of their mucus consists of water (Bhattacharyya *et al.*, 2014; Pearce & Örstan, 2006). Generally, mucin is produced by innate and adaptive immunity which involves inflammatory processes (McGuckin *et al.*, 2015). According to a study by Mafranenda *et al*. (2014), *A. fulica* exhibits antimicrobial properties in its mucin, specifically achasin and mytimacin-AF proteins against *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* which is both oral pathogenic bacteria. The mucus of *A. fulica* also contains allantoin which can promote wound healing, soothe irritation, and stimulate mineralization (Kantawong *et al.*, 2016). Snail mucin is also used in cosmeceuticals, due to the presence of moisturizing agents, growth factors that

trigger collagen production, minerals, and antioxidants such as vitamin A and vitamin E (Mubarak *et al.*, 2013; Perpelek *et al.*, 2021; Song *et al.*, 2021).

Figure 3: Snail with transparent epiphragm made of dried mucus (Geller-Grimm, 2006).

2.2.1 Extraction of Snail Mucin

One of the methods to collect snail mucus is by manually stimulating its pedal glands (Santana *et al.*, 2012; Nantarat *et al.*, 2019). Snails are placed in favorable environments specifically dark, quiet, and humid rooms for optimal mucin production. Phosphate-buffered saline (PBS) can be used to encourage and stimulate mucus production by swabbing its pedal glands (Pitt *et al.*, 2015). Another method of snail mucus isolation is done through the electric shock of about 4 to 12 volts below 60 seconds (Mafranenda *et al.*, 2014; Harti *et al.*, 2018). Ultrasonic baths at 30 °C for five cycles that last for 15 seconds each can lead to irritation of the snail that triggers the production of mucus (Kantawong *et al.*, 2016). In the research conducted by Etim *et al.* (2016), snails are removed from their shell under aseptic conditions and the mucus was squeezed out from the organisms for collection. The direct homogenization method is also used for mucus isolation followed by purification steps (Ulagesan & Kim,

2018). Due to issues on animal ethics when extracting snail mucin, cruelty-free extraction methods have been developed.

Recently, in Italy, snail mucin extracting machines MullerOne were invented to effectively collect snail mucin using a cruelty-free method besides preserving the mucin quality. The machine is equipped with two plexiglass domes, as shown in Figure 4, that can hold 1500 snails at a time and 3.0 to 3.5 kg of mucus can be extracted during a one-hour extraction cycle (Instituto Elicicoltura Cherasco, 2021). The snails are treated with an ozone shower during the one-hour extraction process. The ozone-infused water functions to clean the snails from dirt besides stimulating the organism for a sensation of pleasure that leads to optimal mucus production (Mitzman, 2017). Based on this similar concept, Veroni and Franzoni (2016) invented an ozonization device for snail mucus extraction that comprised of at least a compartment to place the snails, a sanitizing unit, and a compartment for stimulating solution, all connected by a liquid transport system and designed to have a draining system. As stated in the European patent application form, the snails should be sanitized by an ozone shower, and sterilized by either UV lights or reverse osmosis before being exposed to the stimulating solution consisting of citric acid monohydrate, sodium benzoate, potassium sorbate, N,N,N-trimethylglycine and xylitol in purified water. After collecting the snail mucus, mucin should be processed and refined before being made into cosmeceutical (Laneri *et al.*, 2019).

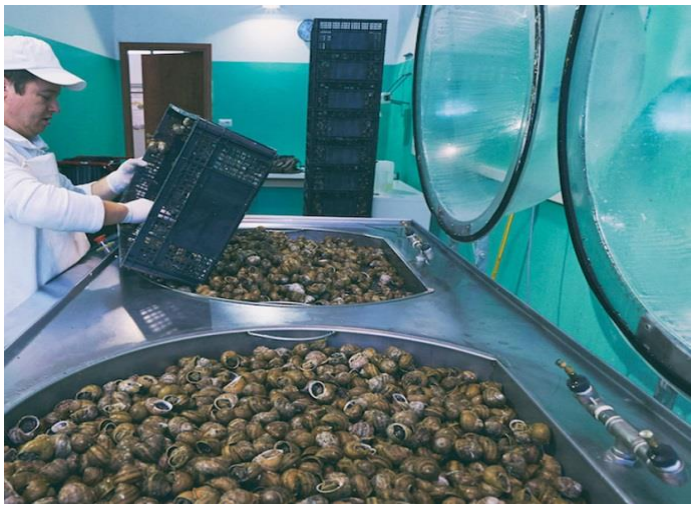

Figure 4: Snails filled into the MullerOne extraction machine (MullerOne, n.d.).

2.2.2 Storage of Snail Mucin

A significant parameter to consider when it comes to storage is the temperature. One of the most common storage temperatures is 4 °C, achieved by refrigeration (Etim *et al.*, 2016; Ulagesan & Kim, 2018; Noothuan *et al.*, 2021). Kantawong *et al.* (2016) suggested that snail mucin can undergo freeze-drying and be kept under -20 °C until use. There are also conditions where snail mucin is kept under an ambient temperature which is around 15 to 25 °C, in a well-ventilated and dry space for a short period (Pitt *et al*., 2015). pH is also an important factor that can affect the quality of mucin upon storage. According to a study by Trapella *et al.* (2018), the pH of snail mucin is between 6.0 to 7.0 which is slightly acidic.

To improve shelf life and to preserve the mineral contents in the mucin, preservatives can be added. The main purpose of adding preservatives to cosmetic products is to inhibit microbial growth and the formation of reactive oxygen species (Nowak *et al.*, 2021). Phenoxyethanol, parabens, isothiazolinones, formaldehyde releasers, triclosan, and chlorhexidine are preservatives that have been widely used in cosmetic products (Halla *et al.*,

2018; Dréno *et al.*, 2019). Aside from the well-known chemical preservatives, plant-derived extracts and essential oils can also exhibit antimicrobial properties when added to cosmetics (Varvaresou *et al.*, 2009).

2.3 Bradford Assay

The Bradford assay is an accurate protein quantification method that is applicable for most proteins, including snail mucins (Harlow & Lane, 2006). The quantification process can be completed within two minutes with little to no interference except a large amount of detergent. Coomassie Brilliant Blue G-250 dye ($C_{47}H_{48}N_3NaO_7S_2$), chemical structure as shown in Figure 5, is used in Bradford assay, which binds to protein and causes a shift in absorbance from 465 to 590 nm that can be detected using a spectrophotometer (Bradford, 1976). The dye binds to the protein via electrostatic interactions with protonated amino acids such as histidine, lysine, and arginine, whereas hydrophobic bonds are formed with aromatic residues (Steinberg, 2009). According to research, the Coomassie Brilliant Blue G-250 dye may exist in four different ionic forms. The cationic form, which is red and green has an absorbance range of 470 to 650 nm, while the anionic blue form, which binds to the protein has a maximum absorbance reading of 590 nm (Kruger, 2009). Bradford reagent consists of an acidified solution of Coomassie Brilliant Blue G-250, hence initially the dye is protonated, and the reagent is red in color. Figure 6 shows the color gradient resulting from bovine serum albumin (BSA) as protein standard solution, added with Bradford reagent.

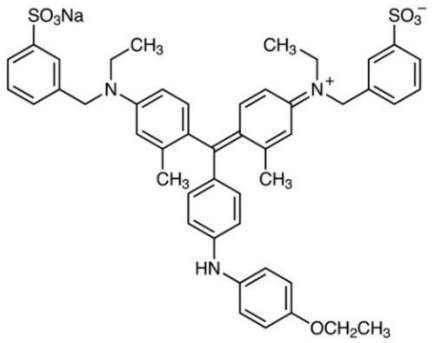

Figure 5: Chemical structure of Coomassie Brilliant Blue G-250 dye (Cymit Química, n.d.).

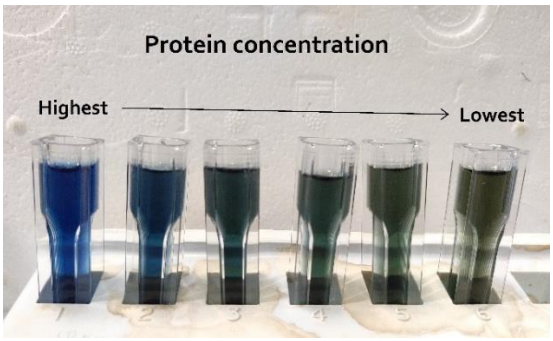

Figure 6: Color gradient resulting from BSA added with Bradford reagent.

2.4 Cetyltrimethylammonium Bromide Turbidimetric Method Assay

Cetyltrimethylammonium bromide (CTAB) turbidimetric method (CTM) is a method developed to quantify the amount of hyaluronic acid (HA). Hyaluronic acid $(C_{14}H_{21}NO_{11})n$, the structural formula shown in Figure 7, is an anionic and non-sulfated glycosaminoglycan that could retain water which made it a key molecule in skin moisturizer and can slow down skin aging (Papakonstantinou *et al*., 2012). HA is naturally found in body tissues and fluid, abundantly in synovial fluid and articular cartilage (Gupta *et al*., 2019).

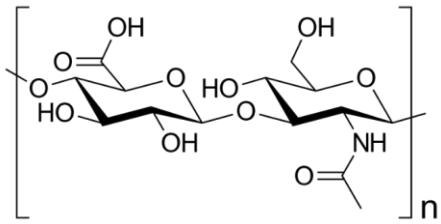

Figure 7: Skeletal formula of hyaluronic acid (Vaccinationist, 2016).

The main reagent used in CTM assay is the CTAB buffer reagent. CTAB buffer reagent consists of CTAB ($C_{19}H_{42}NBr$), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), and tris hydrochloride (Tris-HCl) (Clarke, 2009). CTAB is an organic ammonium cation that can be used to precipitate polyanionic polymers such as HA which the polymer concentration can be calculated through a turbidity test on the precipitate concentration (Oueslati *et al.*, 2014). Figure 8 shows the comparison between phosphate-buffered saline (PBS) blank and sodium hyaluronate solution in the CTAB reagent.

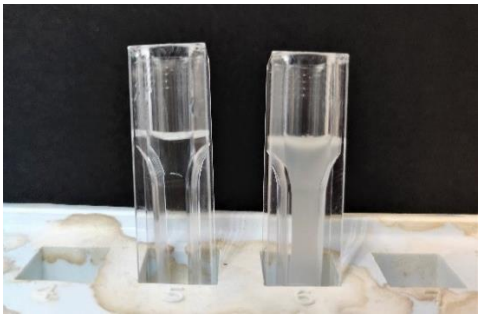

Figure 8: Comparison of blank (left) and HA in CTAB reagent (right).

2.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test (AST) is usually conducted in a clinical laboratory setting to identify the antibiotics suitable against the bacteria or fungus species isolated from patients that enable the appropriate prescribing of treatment (Brown & Kothari, 1975). In diagnostic laboratories, however, known bacterial species are used instead to examine the antimicrobial properties of the biological material. One of the common methods used for AST is the Kirby-Bauer test, or better known as the disk diffusion test. During the disk diffusion test, a known bacteria species is inoculated onto the surface of the Mueller-Hinton (MH) agar plate, and a 6 mm filter paper disk impregnated with an antimicrobial compound is placed onto the agar surface (Hudzicki, 2009). The antimicrobial compound will diffuse into the