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QUANTIFICATION OF MUCOPROTEINS (GLYCOPROTEINS) FROM SLUGS MUCUS, *ARION HORTENSIS* AND *ARION ATER*

P. SWAPNA[1], T. RAVINDER REDDY[2]

1. Department Of Pharmacy Kharvel Subharti College of Pharmacy Swami vivekan and Subharti University Meerut.
2. Laboratory of Eco-physiology and toxicology, Department of Zoology, Kakatiya University, Warangal-506009,Telangana, India

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Abstract: The hydrophilicity of pedal gland secretion (mucus) trails deposited by snails and slugs influences the settlement of other organisms and can potentially influence the trailing and homing mechanisms of terrestrial snails and slugs. A layer of mucus covers the external body surface contributing therefore, among other important biological functions, to the defense system of slugs. The prevention of colonization by parasites, bacteria and fungi is mediated both by immune system compounds (IgM, lysozyme, etc.) and by antibacterial peptides and polypeptides. We have recently shown that only the hydrophobic components of crude epidermal mucus of slugs exhibit strong pore-forming properties, which were well correlated with antibacterial activity. In this paper the authors have isolated by precipitation with ethanol and lyophilization (after precipitation with acetone) mucoproteins from slugs mucus (*Arion hortensis* and *Arion ater*). They were quantified and compared according to the method of isolation (at room temperature and at 4 ^{0}C).

Keywords: Pedal Gland, Mucus, *Arion Hortensis*, *Arion Ater*, Lyophilization

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Corresponding Author: PROF. T. RAVINDER REDDY

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INTRODUCTION

The suprapedal gland or mucous pedal gland is an anatomical feature found in molluscans. It is located inside the front end of the foot of gastropods. The function of this gland is to produce mucus. The gland opens on the front end of the sole, on the ventral side of the foot. The mucus produced by this gland becomes a thin layer covering the sole of the foot, and this helps the gastropod in moving. There are three types of gland cells: 1) cells producing mucoproteins, 2)cells producing lipids and 3) cells producing sulphated mucopolysaccharides. Molluscan slime was traditionally used medicinally from Ancient Greece to the Middle Ages internally against gastrointestinal ulcers, and in the form of syrup, to soothe a cough and it contains rich in proteins of high and low molecular weight hyaluronic acid and antioxidants. The secretion of the molluscans supposedly has a double function when applied to human skin: on one hand it is claimed to stimulate the formation of collagen, elastin and dermal components that repair the signs of photoaging and, second, is claimed to minimize the damage generated by free radicals that are responsible for premature skin aging and slime varies in appearance and quality according to the environmental conditions, season, and food sources used by the snails. These factors supposedly determine the quality of the slime and therefore the properties of a product made with it.

Gastropods, such as slugs and snails, secrete mucus from their pedal gland while travelling across a surface [1]. The unique mechanical properties of snail pedal mucus enable the animal's locomotion while also causing the mucus to function as an adhesive to the substrate [2]. The mucus trail performs a number of other functions, including the provision of mechanisms for re-tracing a path (*i.e*. "homing") and for finding a mate of the same species by following a trail [3]. An understanding of the functionality of trail mucus, including its interactions with water vapor, can therefore lead to a means of controlling the reproduction of snails and thereby limiting their impact on the environment, especially vegetable crops [4]. It has been reported [5] that the presence of a snail's pedal mucus on a surface influences the settlement of other adhering aquatic organisms (such as barnacles) to that surface. The hydrophilicity or hydrophobicity of a substrate, in particular, has been found to influence the settling of other organisms [5-7], which suggests a reason why a mucus trail will have an impact.

When freshly deposited by terrestrial slugs, trails of pedal mucus are reported to be in the range of 10 to 20 mm thick [8]. But since the mucus typically consists of between 90 and 99.7% water by weight, the trails dry to leave a much thinner solid film. The main constituent of gastropod mucus is a complex of proteins and polysaccharides. This complex is usually classified into the broad categories of mucopolysaccharides and glycoprotein’s.

Glycoproteins (GP), physiologically active bio macromolecular structures, widespread in the animal world, are hetero proteins (proteins conjugated) structured from a carbohydrate (polysaccharide with fragments of N-acetyl hexosamine, different monosaccharides, and uronic acids), is called mucopoly-saccharide (immunopolysaccharide) as prostetic group and proteins proper, predominantly quantitatively. Depending on the nature of the linking, in most glycoproteins, there are three glycosidic bridges: (O) – glycosides, (C) – glycosides and (N) glycosides, respectively. **Mucin glycoproteins** are the major macromolecular constituents of epithelial mucus and have long been implicated in health and disease.

An important feature of modern food processing is modelling, respective fractioning natural raw materials and using the products thus obtained as ingredients to obtain "formulated foods". Literature suggests insistently that invertebrate protein fractioning should follow the model supplied by milk processing in protein fractioning by using combined, undenatured methods based particularly on separations with semi-permeable membranes and enzymatic processes, that allow the development of an impressive range of products destined to specific applications in the food industry[9].

MATERIAL AND METHOD:

Mucin isolation from slug mucus: By precipitation with ethanol. Collection of mucus is made with a rod glass, by scraping foot, 200 g of sulgs of each species, *Aron hortensis* (AH), and *Arion ater*(AA), slugs secrete a large amount of mucus. From A.H. slug is obtained 30g mucus, and from A.A.27g. It is collected in Erlenmeyer and over is added 2 to 3 volumes of distilled water. Mucus mixture water is stirred overnight at room temperature (RT^0), for the first experiment, and at 4 0C for the second (figure 1).

After stirring, in both cases, the mixture is centrifuged at 11,000 g (gravitational force), 30 minutes at 4 ^{0}C. After centrifugation remove the precipitate and the supernatant WSF (water soluble fraction) is collected, 110 ml for A.H. and 98ml for A.A at RT^0, and 83.5 ml for A.H and 100 ml for A.A. at 4 ^{0}C.

Supernatant is precipitated with 3 volumes of cooled ethanol, precipitation performed at -20 0C for 2 h. Collection of precipitate is made by centrifugation at 2900 g (gravitational force), 30 minutes and 4 0C.

Before precipitation ethanol and WSF was cool down at -20 0C for 20 minutes. Precipitated with ethanol is kept at -20 0C. Centrifuge used is Avanti J-30i from Merk Company, with the possi- bility of temperature adjustment.

By precipitation with acetone and lyophilization: Isolation of slugs mucins can be done by lyophilization. On the mucus obtained by rubbing is add 2 volumes of dH2O to obtain a mixture

of mucus. The mixture is directly precipitated with 2 volumes of acetone, previously cooled. The precipitate is collect, and lyophilized using Ilshin freeze dryers. Freeze time is 24 h, and temperature -53 0C. Block diagram of operations is shown in (figure 2).

Figure 1. Operations block diagram of Isolation for mucins.

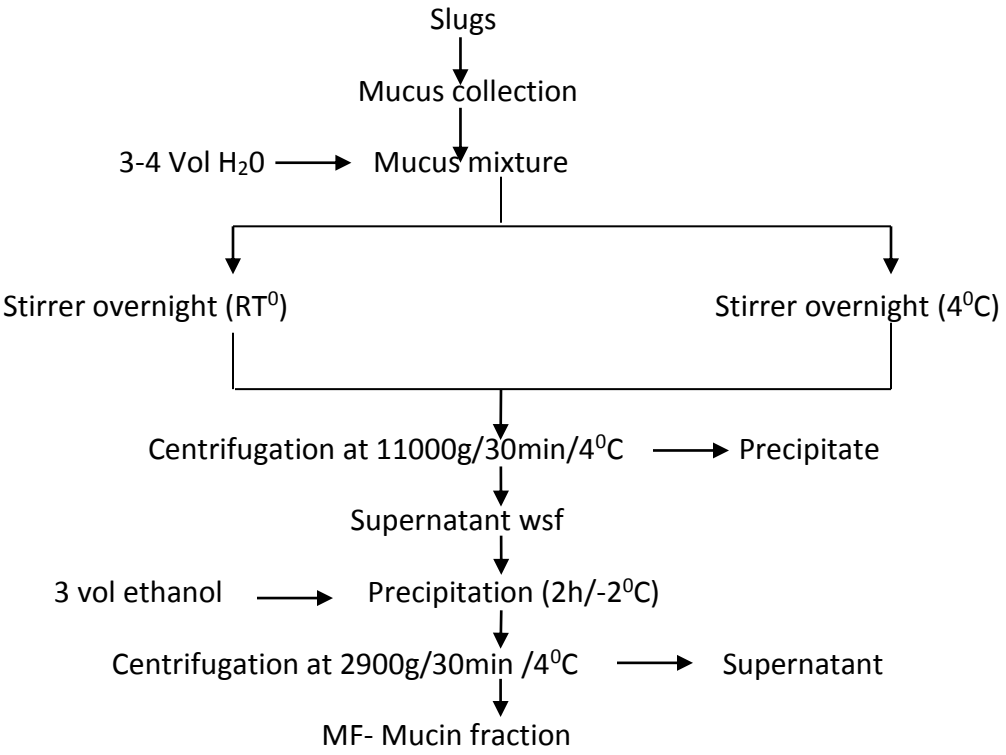
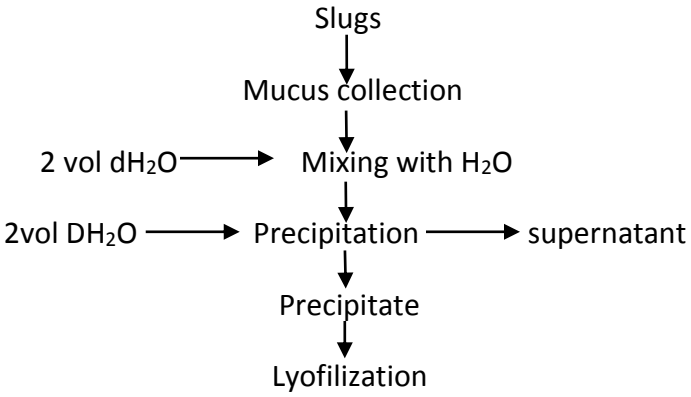


Figure2. Operations block diagram to obtain lyophilized mucin



Precipitated proteins quantification using the method Bradford:

Precipitates, 20 mg of each, are dissolved in 500 ml of urea solution, pH 7.4. For a better dissolve were heated to 90 0C for 5 minutes, shaken in vortex and / or ultrasonic bath maintained for 5 to 10 minutes. The dilution, with water, for sample is made: 1:5, 1:10, 1:20, 1:50. Serum albumin, BSA (2 mg BSA / NaCl 0.9% NaN3), is using as standard. Dilution standard are made as follows:

A – 20 µL urea solution

B – 20 µL BSA + 20 µL urea solution

C – 20 µL din B + 20 µL urea solution

D – 20 µL din C + 20 µL urea solution

E – 20 µL din D + 20 µL urea solution

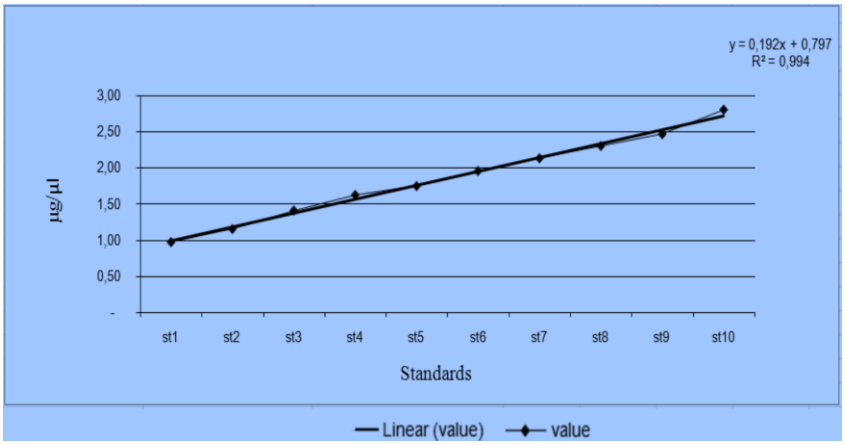
F – 20 µL din E + 20 µL urea solution

On the plate is placed diluted and diluted samples, 3 times each in three wells each. In each well is put 5 µL of sample, over which is added 250 µL of Bradford solution. The plate is read with Magellan program on Tecan Sunrise. Read plate is made at 595 nm. Is made the average absorbances for standard dilutions, linear regression and the chart standard. With linear regression equation is calculate the amount of protein from each sample. The result is multiplied with the dilution and results expressed in mg / mL protein).

RESULTS AND DISCUSSION:

Protein quantification was done according to the manufacturer's protocol [10]. The test was performed in a microtiter plate with BSA calibration curve which ranged between 5 to 50µg/100µl (10 standard points). 100µl of the BSA or unknown sample was mixed with 100µl working reagent. The reaction components in the microtiter plate were incubated at 37°C for 2h and the absorbance was measured spectrophotometrically at 570nm. The protein concentration was quantified by using the BSA calibration curve (Figure 3).

Figure 3: Bovine serum albumin (BSA) standard curve.



Determine the amount of protein from precipitated, was used Bradford method. 20 mg of precipitated sample was dissolved in 500 ml urea buffer. For the precipitate obtained by stirring for 12 h at RT^0 and 4 ^0C, protein concentration was calculated using the standard (serum albumin BSA).

Table 1. The amount of protein, mg/mL, precipitate from A.H and A.A. (20 mg precipitated dissolved in 500 µL urea solution)

Dilution of protein		Absorbance of protein at 570nm	Concentration of protein (mg/Ml)
Room temparature(R^0T)			
A.H1	**1:5**	0.515	2.55
A.H1	**1:10**	0.451	2.78
A.H1	**1:20**	0.33933333	2.78
A.H1	**1:50**	0.29233333	2.47
A.A1	**1:5**	0.579	3.02
A.A1	**1:10**	0.45466667	3.23
A.A1	**1:20**	0.376	3.99
A.A1	**1:50**	0.286	2.77
At 4^0C			
A.H2	**1:5**	0.66033333	3.54
A.H2	**1:10**	0.504	5.24
A.H2	**1:20**	0.44566667	5.70
A.H2	**1:50**	0.32333333	4.44
A.A2	**1:5**	0.69333333	3.68
A.A2	**1:10**	0.51	4.05
A.A2	**1:20**	0.36866667	3.77
A.A2	**1:50**	0.312	3.72

Figure 4. The difference between A.H mucoprotein content of samples at 4^{0}C and R^{0}T

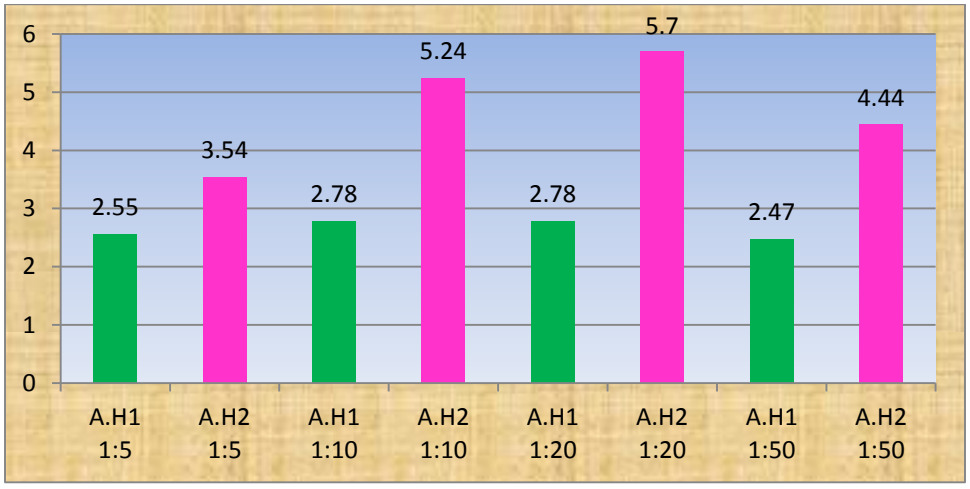
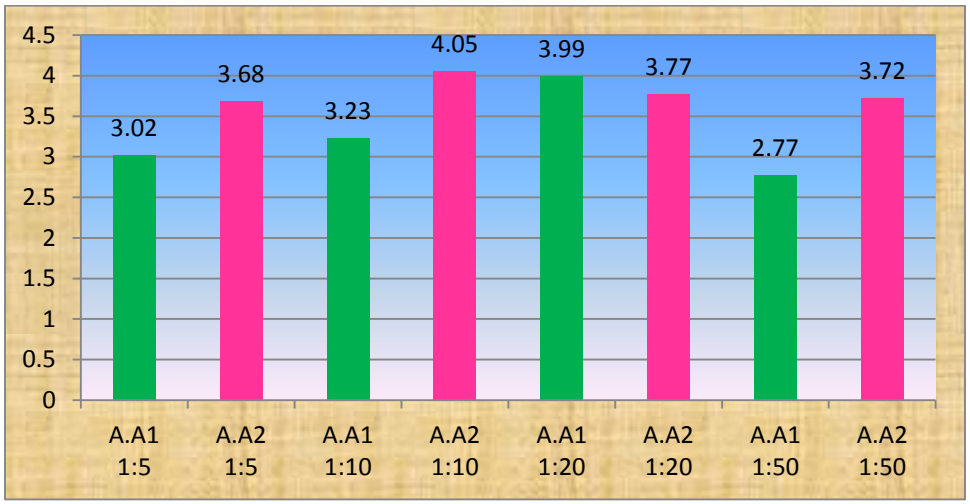


Figure 5. The difference between A.A mucoprotein content of samples at 4 ^{0}C and R^{0}T



From table 2, figure 4, and figure 5, can see that content mucoproteins is higher in precipitate obtained by stirring overnight at 4 ^{0}C, compared to those obtained at RT^0, although by stirring at 4 ^{0}C the amount of precipitation is higher than the amount obtained from RT^0. From the same table can be seen that the amount of mucoproteins obtained by stirring overnight at RT^0 is higher in *Arion* ater (A.A), instead if stirring at 4 ^{0}C, the amount of mucoproteins is higher in Arion hortensis (A.H).

CONCLUSION:

The amount of mucin isolated from *Arion ater* species is higher than mucin isolated from *Arion hortensis*. Also, the amount of mucoproteins isolated from *Arion ater* is higher than the amount isolated from *Arion hortensis*. Given the temperature at which the mixture is stirring (mucus

and water), the quantity of mucoproteins is higher in samples shaken overnight at 4 0C (for the same species), protein is higher for *Arion hortensis* slugs for cold stirring (4 0C). Glycoproteins are also, due to their diversity, biologically active competences, inter- and pluridisciplinary application fields, of real interest for the science of food processing in the near future.

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