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Biochemival Characterization of an Antibactrial Glycoprotein from *Achatina fulica ferussac* Snail Mucus Local Isolate and Their Implication on Bacterial Dental Infection

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Abstract.

Snails crawl over a variety of potentially contaminated surfaces and their foot is the primary site of entry for pathogens, parasites and a range of opportunistic organisms, so it is a little wonder that they must have a defensive system to protect them. The mucus secreted on the body surfaces of mollusks is known to play crucial role in locomotion, feeding, osmoregulation, reproduction and protection of epithelial surfaces. The snail mucus also contains Glycoaminoglycans (GAGs) which are complex polysaccharides that participate in the regulation of physiological processes through the interactions with a wide variety of proteins. GAGs, such as heparin, serve as key to biological response modifiers, in example for acting asa a target for pathogen and parasitic factors for attachment, invasion, and immune system.

For years, it has been known that the mucus secretions from snails *Achatina fulica ferussac* local isolate can be used as a medication, and even empirically it is used to treat infected teeth tahat is suffered by people in rural area. The antibacterial factor was surveyed in the aqueous extract and the mucin fraction of snail *Achatina fulica ferussac*, and they exhibited positive antibacterial for Gram-positive, *Escherichia coli* and Gram negative, *Streptococcus mutans*. In the following study, it has been proved that an antibacterial content in the mucus was a Glycoprotein. It was composed of two subunits of Molecular Weight (MW) 71-73 kDa. The GelCode Glycoprotein Staining Kit detected glycoprotein sugar moieties in polyacrylamide gel and on nitrocellulose membrane, while the glycoprotein carbohydrate estimation kit detected glycoprotein and estimated carbohydrate content. The glycoprotein content was 4.537 ± 0.876 for carbohydrate and 6.420 ± 1.242 for protein.

Keywords : characterization, glycoprotein, *Achatina fullica* Ferussac snail mucus, galur Jawa, antibacterial factor

Introduction

The recent appearance of growing number of bacteria resistant to conventional antibiotics has become a serious medical problem. The continual use of antibiotics has resulted in multi resistant bacterial strains all over the world and as expected, hospitals have become breeding grounds for human-associated microorganisms (Mainous and Pomeroy, 2001). Similarly, the same time-bomb effect is slowly developing with animal associated pathogens in commercially driven activities such as aquaculture and confined poultry breeding where the indiscriminate use of antibiotics is perceived as essential for industrial survival (Marshall and Arenas, 2003). As pathogenic microorganism continues to evolve resistance to conventional antibiotics, the development of novel antimicrobial agent

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will become a pressing issue. Consequently, there is an urgent need to search for alternatives synthetic antibiotics.

Peptide antimicrobial agents are existing candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to the peptides (Mainous and Pomeroy, 2001; Tincu, 2004). A wide variety of organisms produce antimicrobial peptides as part of their first line of defense (Hancock, 1998). This day, hundreds of such peptides have been identified, indicating their importance in the innate immune system. The discovery of two classes antimicrobial peptides, non-ribosomally synthesized (Hancock and Chapple, 1999) present in bacteria-lower eucaryotes and plants and ribosomally synthesized peptides of wider distribution (Boman, 1995; Broekaert, *et al* 1997; Hancock and Lehrer, 1998; Hoffmann, *et al*, 1999; Thevissen, *et al*. 1999; Zasloff, 2002; and Ezekovitz and Hoffmann, 2003), provided a new therapeutic strategy to fight micro organisms. Recent studies show that several cationic and non cationic peptides expressed in many vertebrata, invertebrate and bacterial species (Luders, *et al*. 2003) act synergistically to improve immune responses.

The snail species *Achatina fulica ferussac* local isolate is an invertebrate belonging to the phylum Mollusca. They have been known as a parasite because they take nutrient from plants grown by human. However, today they have been cultivated for consumption in Indonesia and other countries as well. The snail mucus also contains Glycoaminoglycans (GAGs), complex polysaccharides that participate in the regulation of physiological processes through the interactions with a wide variety of protein. GAGs are primarily located inside granules in the snail's body tissues and in the shell, and are secreted onto the surface as a mucous material. GAGs, through their binding and regulation of a large number of ligands and receptors, are important mediators of normal and tumor cell behavior, such as proliferation, differentiation, migration, and adhesion. The specific structure of GAG chains and their binding proteins influence tumor cell proliferation, metastasis and cancer progression. GAGs, such as heparin, heparin sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid, serve as key biological response modifiers by acting as stabilizer cofactors, and/or coreceptors for growth factors, cytokines, and chemokines; regulator of enzyme activity; signaling molecules in response to cellular damages, such as wound healing, infection, and tumorigenesis; and target for bacterial, viral, and parasitic virulence factors for attachment, invasion, and immune system.

Their bodies are characterized by rich mucus which covers their surface; apparently the mucus may serve in preventing the moisture evaporation, in helping smooth movement and in protecting the body from mechanical injuries. Snail crawl over a variety of potentially contaminated surfaces and the snail foot is the primary site of entry for pathogens, parasites and a range of opportunistic organism, so it is a little wonder that they have a defensive system to protect themselves. In addition, some unknown biochemical function may be involved in the mucus. Empirically this mucus has been known by people in rural area for years to treat their teeth infected by dropping the mucus secretion in the cavity.

Tincu *et al.* (2004), said that phylum mollusca from marine invertebrate have an antimicrobial peptides that are a major component of innate immune defense system.

Chisholm (1992) in their investigation also said that antimicrobial peptides are

widespread in invertebrates, especially in tissues such as gut and respiratory organs in invertebrates, where exposure to pathogenic microorganism is likely. These peptides generally act by forming pores in microbial membranes or otherwise disrupting membrane integrity (Tam *et al.* 2000), which is facilitated by their amphiphilic structure. This mode of action is unlikely to lead to the development of resistance (Bax *et al.*, 2000), although it must be noted that this presumption is debatable.

In the folowing paper, an antibacterial activity was surveyed in the aqueous extract of snail *Achatina fullica Ferussac* local isolate, and the molecular weight of the antibacterial factor found in the body surface of the snail was determined to be about 71.3 kDa (Berniyanti *et al*, 2005). In present study, the antibacterial factor was separated and its biochemical properties were revealed. The biochemical characterization tried to identify the properties of glycoprotein in snail mucus *Achatina fullica ferussac* local isolate that has been isolated: that is protein, and carbohydrate contained in glycoprotein. These methods would prove that the mucus from snail Achatina fullica Ferussac local isolate was glycoprotein.

Material and Methods

Isolation of snail mucus Achatina fullica ferussac local isolate

Land snail, *Achatina fulica ferussac* local isolate around 10-20 heads were captured in East Java, transported via ground to laboratory and fed. Mucus secretion was stimulated by exposing the snails to a direct current electric shock (0.6-0.8 mA) on the voltage of 5-10, at interval 30-60 s. The water soluble fraction (WSF) of the mucus was obtained by the following procedure. Two volumes of water were added to the above mucus, stirred overnight in refrigerator, and centrifuged at 11.000 g (Beckman Model J-21) for 30 min. The supernatant was referred to as WSF. Three volumes of ethanol were added to the supernatant and the mixture was centrifuged again at 2900 g (Yuan Kr 422) for 30 min. The precipitation obtained was referred to as ethanol percipitated fraction (EtP).

Purification of glycoprotein with ion exchange chromatography

The glycoprotein factor of snail mucus was purified in the following way. EtP was redissolved in 50 nM Tris-HCL buffer (pH 8.0). Purification of glycoprotein was conducted by ion exchange chromatography. Matrix used in this purification was anion exchange (sepharose in 50 mM Tris-HCL buffer (pH 8.0)), and column used was 17 cm length and 4 cm in diameter. The column was packed by matrix as much as needed. About ± 3.5 cm^3 of the sample was put in the column, and we waited until all were dissolved in the matrix, and then it was calibrated with 120 cm^3 of the same buffer to remove all insoluble materials. The column was eluted with 200 cm^3 buffer over a linear gradient from 0-0.8 M NaCl. The gradient was run in 40 km/h speed in each fraction as much as 50 drops. The affluent will fill the fraction collector according to their ion charged. The glycoprotein that has been separated according to their charged then measured in UV spectrophotometer l 280 . Antibacterial activity was examined and protein concentration was determined.

Glycoprotein Characterization with SDS-PAGE

The characterization of glycoprotein purification with positive antibacterial activity from snail mucus has been carried out with sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) technique. The composition of separating gel was 10% (1.2 g acrylamide; 0.032 g bis-acrylamide ; 3 ml 1.5 M Tris ph 8.8

; 0.12 ml SDS 10% ; 8.88 aquades, 7 µl TEMED and 80 µl APS 10%) and stacking gel 3% (0.9 g acrylamide ; 0.024 g bis-acrylamide; 2.52 ml 1.5 M Tris ph 6.8 ; 0.3 ml SDS 10% ; 17.18 distilled water, 3.5 µl TEMED and 50 µl APS 10%). Separating gel 10 % solution was put in gel plate in vertical position and butanol was added on the top, and then allowed to be polymerized. Stacking gel 3% was added in glass and the comb was put, and being left until all became polymerized.

The gel plate was put on the Minigel Twin G-42 slab and poured with electrophoresis buffer (0.0248 M Tris; 0.19 M glycin ; 0.1% SDS 10%) in the electrode field. Fifty microliter of glycoprotein sample plus 12.5 µl 5 x SDS sampel-buffer ((2.5 ml 1.5 M Tris pH 6.8 ; 2 g SDS ; 0.5 g DTT (Dithiothretol) / 5 ml merchaptoethanol); 10 mg bromphenol blue ; 10 ml glycerine and 2.5 ml aquades))were prepared in ependorff, boiled around 2 min, removed and stored in the abundance of ice, and poured in the gel little by little. Protein marker was used with range around 6.5-205 kDa (Sigma). The power supply was turned on with electrical current 99.9 volt, 50 mA and 12 W.

Biochemical characterization of glycoprotein (GelCode Glycoprotein Staining)

Glycoprotein Sugar moieties in polyacrylamide gel were detected with *GelCodeStaining Kit* (Pierce no.Cat.24562). When treated with oxidizing reagent (periodic acid), glicols present in glycoproteins were oxidized to aldehydes. After completing the electrophoresis procedure, the gel was fixed by completely immersing it in 100 ml of 50 % methanol for 30 min. The gel was washed by gently agitating with 100 ml of 3% acetic acid for 10 min, and this step was repeated once (2x10min). The gel was left in water overnight at 4°C. The gel was transferred to 25 ml oxidizing solution and agigated gently for 15 min. Wash gel by gently agitating with 100 ml of 3% acetic acid for 5 min and repeat this step twice (3x5 min). The gel was transferred to 25 ml GelCode glycoprotein staining reagent and agitated gently for 15 min. If crystal is formed in the glycoprotein stain, then the solution was centrifuged and the supernatant was removed for use. The gel was transferred to 25 ml of reducing solution and agigated gently for 5 min. The gel was washed by gently agitation with 100 ml of 3% acetic acid and then with ultrapure water. Glycoprotein appears as magenta bands. The gel was stored in 3% acetic acid.

Glycoprotein carbohydrate concentration

Protein and carbohydrate in each sample were determined with *Glycoprotein Carbohydrate Estimation Kit* (Pierce no. Cat. 23260) which is a simple, rapid method for glycoprotein detection and carbohydrate content estimation and Biuret method.

Three eppendorfs were prepared. Two eppendorfs were filled with 0.4 ml glycoprotein assay buffer as a blank and 1 eppendorf with 16.7 l achasin (glycoprotein) sample was added to glycoprotein assay buffer solution in a volume of 0.4 ml. Each of eppendorf was added with 0.2 ml kalium periodate 10 mM, homogenized with vortex, and incubated at room temperature for 10 minutes, added with 0.6 ml glycoprotein detection reagent 0.5%, then homogenized with vortex and incubated at room temperature for 1 hour. Then the absorbance was measured with spectrophotometer UV-Vis atmaximum value of 550 nm. This step was repeated three times.

The glycoprotein and carbohydrate content in sample could be detected by comparing with their protein solution standard by using the following formula:

Glycoprotein (mg/ml) =
absorbance of sample C protein(mg/ml) Solution
Absorbance of standard

Carbohydrate (mg/ml) =
glycoprotein content % carbohydrate total standard

The determination of protein using Biuret Method was begun with standard curve of BSA. Protein preparation from sample was measured with the following procedure:

Achasin 0.5 ml was added to 1 ml solution standard of 5000 ppm BSA and 4 ml biuret reagent, mixed and left for 30 min in room temperature. The absorbance with spectrophotometer UV – VIS was measured at maximum value of 550 nm. As a blank, 1.5 ml water was used as well as solution standard, repeated three times. Protein content was determined by estimating linear regression (conversing the absorbance data to concentration) standard curve of BSA, from Hamilton, (1992) as follows:

$$Y = aX \quad , \quad X = \quad ;$$

X= Total Protein Concentration

Result

Achasin fractionation with ion exchange chromathography/ DEAE Sepharose column chromatography (Anion Exchanger)

The precipitate obtained was referred to as an Ethanol precipitation, a solid and viscous precipitation, but become a liquid when redissolved in 50 mm Tris-HCl buffer (pH 8.0). Protein obtained was clearly white, filled in *fraction collector* about ± 120 tubes, that was seperated according to their ion charge. Protein was determined by spectrophotometer OD 280 UV and illustrated as U.V spectra.

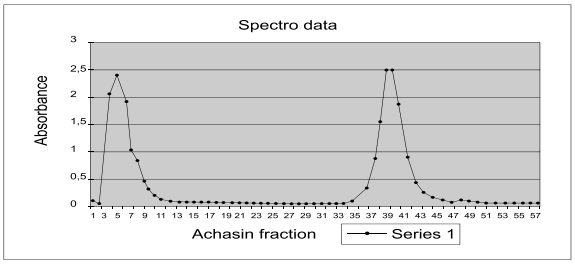

Figure 1. The elution pattern of the column purified fraction on chromatography ion exchange. The column purified fraction was applied on a sepharose matrix (4 q x 17 cm) previously equilibrated with 50 mM Tris-HCL buffer (pH 8.0) and eluted at flow rate of 1.5 cm^3 / min at 20°C

Analytical ion Exchange Chromatography of the purified sample was performed using a sepharose matrix column eluted with 50 mM Tris-HCL buffer (pH 8.0). As the figure shows, metabolic inhibition activity was detected at a certain peak of protein. Fraction on the first and second peak shows higher absorbance compared to the others.

Biochemical characterisation

Glycoprotein characterization with staining

To determine the molecular weight of achasin from glycoprotein band on snail mucus from glycoprotein bands contained in mucus obtained from purification, we used staining using GelCode Glycoprotein Staining Kit. Figure 2 shows the result of glylocprotein staining before and after purification

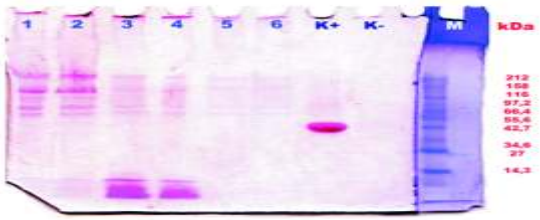

Figure 2. Glycoprotein bands from Achasin using SDS-PAGE 12%
Notes: S: Supernatan from mucus sample ; Ept: Precipitation from ethanol
Ach: Achasin sample from purification with ion exchange chromatography and SDS-PAGEStaining

was done to mucus supernatant, to mucus from purification with ethanol that produced precipitated ethanol, as well as to mucus after purification with ethanol, ion exchange purification and SDS-PAGE.

Table 1. Gel Measurement Values as the Result of SDS

Distance	Rf	MW (Y KDa)	MW (Y Da)	Log Y (Da)
2.6	0.321	212	212000	2.326
2.9	0.358	158	158000	2.198
3.4	0.420	116	116000	2.064
3.7	0.457	97.6	97600	1.989
4.1	0.506	66.4	66400	1.822
4.4	0.543	55.6	55600	1.745
4.8	0.593	42.7	42700	1.63
5.4	0.667	34.6	34600	1.539
5.8	0.716	27.2	27200	1.434
6.8	0.840	14.3	14300	1.155
		log Y Da (y=-2,2027x + 2,984)	Antilog y BM Da	BM kDa
4.1	0.506	1.8691	73970	73.970

The result of staining allows the measurement of the length of band and gel in order to make assessment using linear regression to obtain the glycoprotein with certain molecular weight. Obtained measurement results are presented in Table 1. The curve in Figure 3 represents data linearity before being converted into regression formula.

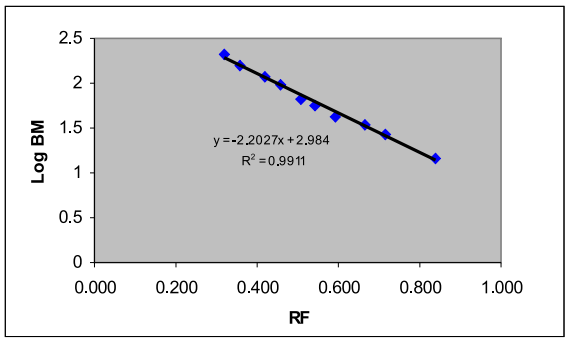

Figure 3. Fit Curve, SDS-PAGE measurement result.

The results of measurement revealed several bands. Based on the measurement of the gel picture, the molecular weight could be determined using regression equation Y = a + bx. By means of distance data and Rf, the result of assessment using SPSS revealed the following result: The regression equation was Y = -2.984 x + 2.2027 (in which Y= molecular weight, X = concentration / band appearance). The result of prediction showed that molecular weight of the existing band was 73.970 kDa.

Characterization of glycoprotein level

Biochemical characterization was not performed only with staining, but also to the level (Table 2).

Mean content in the mucus	Total mucus	Achasin	Methods
Glycoprotein	23.820 ± 7.249	10.960 ± 2.112	PAS method
Carbohydrate	9.862 ± 3.001	4.537 ± 0.876	PAS method
Protein	13.958 ± 4.248	6.420 ± 1.242	Biuret method
Relative molecular weight (kDa)	5 bands	106.557 ± 44.519	SDS-PAGE with PAS staining

Characterization was carried out to find the biochemical properties of isolated mucosal glycoprotein, i.e., the protein, carbohydrate, and glycoprotein content (Walker, 1994 ; Sumitro *et al.* 1998), by means of Glycoprotein Carbohydrate Estimation Kit from Pierce (No. cat. 23260) and Biuret method spectrophotometrically. The results of the characterization level can be seen in Table 2. Glycoprotein and carbohydrate measurement was based on the principle that glycoprotein is oxidized by Calium Perlodate (KIO4) to become aldehyde. Furthermore, aldehyde was detected by using PAS reagent, while protein content was measured using Biuret method.

Discussion

This research tried to prove the antibacterial factor contained in glycoprotein isolated from snail mucus *Achatina fulica Ferussac* local isolate. To

illustrate in detail the expected target, this research has been done in following steps. The first step was the characterization of protein molecular weight. The next step was the biochemical characterization of glycoprotein molecule by measuring the contents of glycoprotein, protein, and carbohydrate as well as determining the molecular weight by using one-dimentional electrophoresis method. Previous characterization using SDS-Polyacrylamide Gel Electrophoresis revealed that the molecular weight was 71 kDa with protein staining.

The determination of glycoprotein molecular weight was conducted to purified achasin using ion-exchange chromatography, and by means of SDS-Page electrophoresis using glycoprotein staining from Pierce co. (No. Cat. 24562). Glycoprotein staining principle is the occurrence of carbohydrate oxidation bound through proteoglycan binding within the glycoprotein to become aldehyde and the staining appears as light red band. There were 5 bands in total protein and 4 bands in purified Achasin. The first band had molecular weight of 177.732 kDa, the second 114.659 kDa, the third 101.163 kDa, the fourth 73.970 kDa and the fifth 65.263 kDa. The emergence of these five bands is likely related to the presence of other protein which was not visible in previous protein staining. However, from several bands, those with 73 kDa molecular weight were glycoprotein bands that could be expected as antibacterial protein.

This finding proved that the mucus of local isolate (Java strain) *Achatina fulica Ferussac* snail is a glycoprotein with molecular weight of 71-73 kDa, using protein and carbohydrate staining. This was slightly different from snail mucus *Achatina fulica Ferussac* Giant African found by Iguchi *et al* (1982), which was a glycoprotein with molecular weight of 7-80 kDa with protein and carbohydrate staining. Glycoprotein and carbohydrate measurement based on principle that glycoprotein is oxidized by kalium perodate to become aldehyde. The aldehyde was detected using glycoprotein-detecting antigen, i.e. the reactant from Pierce (cat. no. 23260). With aldehyde, glycoprotein detection reagent shows purple color and it has maximum absorbance in 550 nm. Absorbance in 550 nm is equal to the percentage of carbohydrate component within glycoprotein. This was also confirmed by the fact that protein not containing carbohydrate, such as lysozyme and BSA (in standard) provides lower absorbance in 550 nm.

Glycoprotein in the samples could be detected using PAS (Periodic Acid Schiff) reaction, a reaction based on the effect of periodic acid oxidative (HIO4) in 1.2-glycol cluster in carbohydrate residual producing aldehyde cluster, whose reaction can be described as follows:

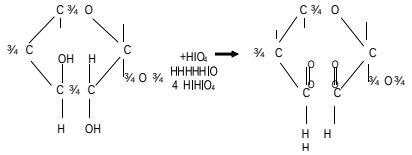

Figure 4. 1,2-glycol cluster in glucose aldehyde cluster PAS reaction (Periodic Acid Schiff)

Achasin glycoprotein isolate contains carbohydrate as much as 4.537 ± 0.876. The content of carbohydrate compound measured in the sample shows that the sample contains glycoprotein. Carbohydrate content in glycoprotein is highly important in intercellular recognition, in which the carbohydrate is bound to polypeptide chain through N-linked and O-linked oligosaccharide. N-linked olygosaccharide is a formation of bi-; tri- and tetra-antennary, so that the molecular weight of the carbohydrate may reach 40% of the total weight of glycoprotein molecule. Olygosaccharide attachment to the peptide may also increase solvability,

include the antigenic domain and protect backbone against protease (Bhavanadan and Furukawa, 1995). Similarly, the polysialic acid that attaches to Neural Cell Adhesion Molecule (N-CAM), carbohydrate also often modulates protein function. The composition of 40% carbohydrate and 60% protein was evidently found in mucus of the snail *Achatina fulica ferussac*, local isolate (Java strain), in which the carbohydrate was 4.537 ± 0.876 and protein was 6.420 ± 1.242.

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