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Identification of Proteins in Tissue Fluids of the sea mussel *Isognomon alatus*

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Abstract

The aim of this preliminary study was to investigate the presence of proteins in the tissue fluids of the sea mussel *Isognomon alatus*. Protein extraction was done by the chloroform-cold ethanol technique. Immunization for production of antibody to be used as reagents in Western blotting, assessment of the protein concentration by the Bradford method, protein characterization by native polyacrylamide gel electrophoresis (PAGE) were also performed as a part of the methodology of this study. The results showed a protein content of 65 mg/ml in tissue fluids and a protein band approximately of 220 kDa in PAGE that was further confirmed by the Western blotting. Future work should investigate the structure and function of the proteins separated from the tissue fluids and we considered it as a limitation of this investigation. The sea bivalve literature is scanty. However the limitation of this work we still can conclude that there are high molecular weight proteins in large concentrations in tissue fluids of the sea mussel *Isognomomalatus*.

Keywords: Sea mussel; *Isognomon alatus*; Polyacrylamide gel electrophoresis (PAGE); Protein

Introduction

The mussel *Isognomon alatus* a bivalve mollusc. This species inhabits warm marine littoral habitats [1]. Despite the economic importance of this mussel, which is used for human consumption in many countries, there are no reports on the protein content of the tissue fluids of this organism. High protein content has been described in other species of mussels, e.g. the horse mussel *Modiolus modiolus* [2]. One approach to understanding the nutritional value of the *Isognomon alatus* was to determine the protein presence and concentrations in extracellular fluids of the mussel.

In addition we studied the presence of *Salmonella* spp. In the mussel, which is eaten row by the Jamaican and other countries populations, constitute a risk to human health. Other microorganisms have been isolated from the mussel *I. alatus*. This research paper resumes the work done in a moderate amount of *I. alatus* (40 mussels), which is known for the Jamaican population as a "flat oyster" and considered a sexual stimulant. *I. alatus* protein concentration in extracellular fluids and Salmonella contamination were assessed in this study. The scientific literature of *I. alatus* is limited to a few studies and that was a hindrance to our study that has to present a little information about the mussel but with this work we are contributing to expand the knowledge of the sea mussel *I. alatus* and stimulating to other scientists to research on this topic.

Material and Methods

Collection of tissue fluids from the *Isognomon alatus*

The extracellular fluid secreted from the *I. alatus* was collected in a 50 ml sterile plastic tube and it was placed in sterile plastic bags and transported to the laboratory in ice packs, for analysis.

Protein extractions from the tissue fluids of the mussel *Isognomonalatus*

One volume of chloroform (15 ml) was added to 15 ml of tissue fluids from 10 mussels and the mixture centrifuged at 1500×g (low gravity) for 5 min at 4°C. The clear supernatant (top layer) was separated from lipids (bottom layer) and treated with cold ethanol (100%) and centrifuged for another 5 min at 1500×g and 4°C. A large white pellet (protein extract) (5 ml) was resuspended in one volume (5 ml) of phosphate buffered saline (PBS), and dialyzed against BPS, pH=7.4 for 24 hours at 4°C, with 3 buffer changes and stored at -20°C until further analysis.

The protein concentration was assessed by Bradford method

Duplicate aliquots 0.5 mg/ml of bovine serum albumin (BSA) (5, 10, 15, 20 µl) was added to micro centrifuge tubes and the volume in each tube brought to 100 µl with 0.15 M NaCl. Two blank tubes were also prepared. One ml of Coomassie brilliant blue solution was added to each tube and each vortexed and left to stand for 2 min at room temperature (RT). The absorbance readings were taken at 595 nm against a 0.15 M NaCl blank using a 1 cm path-length microcuvette. A standard curve was then constructed by plotting a graph of absorbance against protein concentration and the protein concentration was determined from the standard curve.

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Preparation of a reagent for immunoblotting (production of anti-*I. alutus* protein extract antibody in rats and titre measurement)

Two male rats of approximately 1800 g were intramuscularly immunized with 0.5 ml (1 mg/ml) of the protein extract containing 0.3 ml of incomplete Freund's adjuvant, (that was used to immune response stimulant). They were immunized in day zero, day 7 and day 15. The antibody titter was measured by doubled immuno diffusion (Ouchterlony technique) as follows 1% agarose gel was prepared and wells cut into the gel using a template. A 25 µl (1 mg/ml) of the protein extract were added to the centre well. The peripheral wells were filled with serial dilutions (from 1:4 to 1:512) of 25 µl of immunized rat serum. The gels were incubated at -RT for 48-72 hrs and then examined for precipitin lines. The positive results were taken as the presence of precipitin lines and negative results the absence of precipitin lines. The rat sera were treated with ammonium sulphate to precipitate the antibody-containing protein fraction until its further use [3].

Native polyacrilamide gel electrophoresis (PAGE) of the protein extract

The protein extract was separated by PAGE as described by Neville [4] using an acrylamide concentration of 10%. Aliquots of 10 µl of protein (0.5 µg/µl) in sample buffer (0.125 M TrisHCl, 20% glycerol, pH 6.8) were done. The sample was then electrophoresed at 150 V for 1 h. A molecular weight (MW) marker (10-220 kDa, Sigma-Aldrich Co, San Louis Missouri) was included with each run. The gels were stained with Coomassie blue.

Western blotting analysis of the protein extract

Following a new electrophoresis the *I. alatus* protein extract was blotted onto nitrocellulose membranes (approximately 75 minutes at 40 mAmps using running buffer: 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, 0.5% SDS). The membranes were blocked by incubating 2 h in 10% non-fat dry milk in PBS (with 0.05% Tween 20, pH 7.4). The membranes were washed 4×10 min with PBS-Tween 20 and incubated overnight at 4°C with an anti-*I. alatus* protein extract antibody developed in rats. The following day membranes were again washed 4X 10 min with PBS-Tween 20. SpLA-HRP (Sigma-Aldrich Co, San Louis Missouri) diluted 1:5000 was added to the membranes, which were incubated for 3 h at RT and the washing procedure was repeated. Tetramethylbenzidine (TMB) solution (Sigma) was added to the membranes, which were then incubated in the dark at RT for 5 min after which the membranes were shaken gently, rinsed thoroughly in de-ionised water, dried and photographed.

Isolation of Salmonella from *I. alatus*

The isolation of Salmonella was carried out using previously described procedures [5-8]. The swabs from the body of mussel and tissue fluids were collected from fisherman at the coast line in the north coast of Jamaica. The swabs were immediately placed in sterile screw cap test tube containing 9 ml of universal pre-enrichment broth. The swab was completely submerged in the broth to ensure optimal recovery of the organism. At least 25 g of each type of specimen was dissolved in 250 ml of universal pre-enrichment broth (buffered peptone water 1%, Difco).

The inoculated universal pre-enrichment broth was incubated at 37°C for 24 hours following this incubation the pre-enrichment broth was thoroughly mixed using a vortex mixer. A 1 ml aliquot of the pre-enrichment broth was added to 9 ml of enrichment broth (Selenite broth, Selenite cystein broth, and Tetrathionate broth) and further incubated at 37°C for 24 hours. After vortexing 0.5 ml and a 3 mm loopful of inoculums were used to inoculate differential plating media such as MacConkey agar, Salmonella-Shigella agar selective media Bismuth Sulphite and Brilliant green agar and incubated at 37°C for 24-48 hours.

Following incubation the cultures were examined. Non-lactose fermenting colonies were selected and used to inoculate Kligler iron agar and urea agar slants. After a further 24 hours incubation period at 37°C, colonies that gave the typical Salmonella/Shigella agar reaction were further investigated for confirmation, using slide agglutination with somatic "O" and flagella "H" antigens of Salmonella. Serological typing was performed to determine the Salmonella serovar [9].

Identification by slide agglutination

Presumption Salmonella isolates were stored on tryptose agar a room temperature until confirmation as previously described (Kauffman-White Schema, Difco, Laboratory, Detroit, and Michigan U.S.A). For each isolate 2 loopfuls of the growth on tryptose agar was emulsified in one drop of normal saline solution (0.9%) on a clean microscope slide. The preparation was examined for autoagglutination.

If the organism was not self-agglutinating one drop of either "H" antiserum or "O" antiserum was added to each spot. After mixing the slide was agitated by gently rocking back and forth for 2 to 3 minutes. The slide was examined for agglutination (Kauffman-White Schema, Difco, Laboratory, Detroit, and Michigan U.S.A). Identification of Salmonella serovar was performed in the Salmonella reference laboratory, Department of Microbiology, Faculty of Medical Sciences, The University of the West Indies.

Antibiotic susceptibility test

All Salmonella isolates tested were investigated for their antibiotic susceptibility with the disc diffusion test using the following discs (Difco): gentamicin (10 µg), kanamycin (30 µg), ampicillin (10 µg), amikacin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), cefazolin (30 µg), cephalothin (30 µg), cefepime (30 µg), cefotaxime (30 µg), streptomycin (10 µg), ceftazidime (30 µg), cefoxitin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), tetracycline (30 µg) and imipenem (10 µg).

Results and Discussion

The chloroform-cold ethanol method was effective to separate proteins from lipids produced by the mussel *I. alatus* and secreted as tissue fluids. The Bradford method showed a protein concentration of 65 mg/ml and the protein concentration was adjusted to 1 mg/ml and a protein extract with a suitable adjuvant was used to immunized 2 male rats to produce an anti-*I. alatus* protein antibody to be further used in the protein characterization by Western blotting. The radial immunodiffusion test demonstrated the successful production of the said anti-serum in titres up to 1:256, which can be considered as high titres.

The native PAGE showed in Figure 1 the presence of a protein band with a high molecular weight, approximately 220 kDa and the Western blotting confirmed the result. The presence of protein extracts of tissue fluids of the mussel *I. alatus* has not been previously reported. The presence of proteins has been reported in other species of bivalves including the pacific oyster *Crassostre agigas*, the horse mussel *Modiolus modiolus* [2], Mytilusedulis [5,6] and the sea mussel *Crenomytilus grayanus* [7] and some of these proteins are lectins that bind to

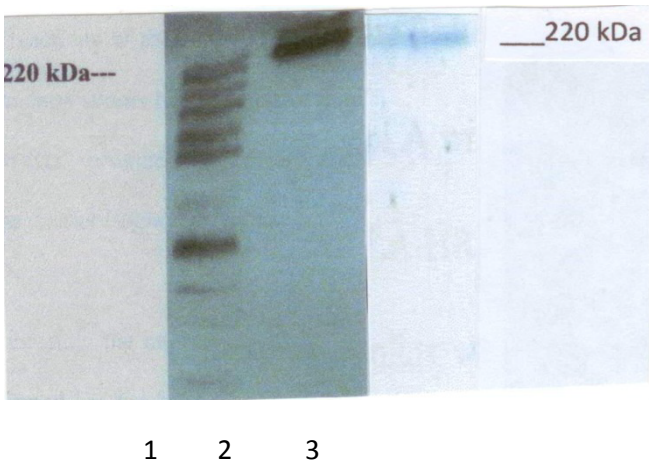

Figure 1: 10% Native PAGE of a protein extract from tissue fluids of the mussel *Isognomom alatus*: lane 1 molecular weight (MW) marker (Sigma-Aldrich Co); lane 2 protein extract with MW of 220 kDa and lane 3 western blotting confirmation of the protein extract presence and MW of the proteins in the tissue fluids of the mussel *I. alatus*. The western blotting used an anti-*Isognomon alatus* tissue fluid protein extract antibody developed in rats and a peroxidase-labeled protein LA conjugate (Sigma-Aldrich Co) as tools.

carbohydrates and it is not conclusive if they have protective capacity against several antigens or microorganisms that they encounter in their environment [2,5]. Future work should investigate the structure and function of proteins in the tissue fluids of the sea mussel *I. alatus* and we considered it as a limitation of this investigation. Vareltzis and Undeland, 2012 reported that the pH shift method can be successfully used to extract functional proteins from mussels and add value to blue mussels unsuitable for human consumption [8].

The role of *I. alatus* potential sources of infection is clearly documented [9]. It was reported salmonellosis in Venezuelan where *I. alatus* was originally linked to contaminated raw shellfish. However in our study Salmonella Spp was not isolated from the bivalve that was suitable for human consumption, because the mussels were collected in non-fecally contaminated waters. Other studies have demonstrated the presence of other microorganisms in *I. alatus* for example *Escherichia coli*, the fecal contamination of raw seafood by indicators and opportunistic pathogenic microorganisms represents a public health concern in country as Venezuela, where the *I. alatus* is eaten as a raw food [9].

In the literature review pure cultures of Gram-negative straight rods with fimbriae from *Isognomon alatus* and *Crassostrea rhizophorae* was reported [9]. In the same studyresults show that *P. mirabilis* was predominant under our culture conditions. Other enterobacteria such as *Morganella morganii* and *Klebsiella pneumoniae* were also isolated from seawater mussels and oysters. The presence of pathogenic bacteria in bivalve could have serious epidemiological implications and a potential human health risk associated with consumption of raw seafood [9]. Although the mussel *I. alatus* was found to be free of *Salmonella* spp contamination we did not rule out other infections by other microorganisms but it can be done in future.

Conclusion

The sea mussel *I. alatus* is of economic importance because is part of the consumption of the Jamaica population. It has high protein value but potentially can be infected with enterobacteria representing a hazard to human consumers. In this work we search only for Salmonellosis and it was free of this enterobacteria.

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Competing Interests

Authors have declared that no competing interests exist.

Authors' Contributions

Authors AJV designed the study and wrote the protocol, carried out the laboratory studies. All authors wrote and revised the manuscript, all authors managed the literature searches and read and approved the final manuscript.

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