What can we do with fish mucus?: Proteomic profiles of *Lepomis macrochirus*, *Micropterus salmoides* and *Pomoxis nigromaculatus*

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INTRODUCTION

Living in an aquatic environment, fish are subject to constant exposure to microorganisms like bacteria which comprise a large majority of the biomass (Tort). To combat infection, fish have developed mechanisms in their innate and adaptive immune system (e.g. mucus and bactericidal peptides) to deter disease. However, teleosteans exhibit poor antibody affinity, antibody response and memory response, so the non-specific immunity is heavily relied on as a mode of protection, such as mucus and the integument. By acting as a physical barrier, the skin of the fish prevents microorganism from entering the body. Also, the skin consists of goblet cells that secrete the mucus which plays a vital role in the fish’s immune system. The glycoproteins not only serve as the medium in which the bacteria killing mechanisms can occur but also the mucus entraps the bacteria in the gelatinous mass to prevent further ingress (Tort). Portions of the fish mucus have antibacterial properties. For example, lysozymes can lyse bacteria by degrading the peptidoglycan layer, and lectins aid in glycosylation by adding carbohydrates for cell wall interface. The goal of this project was to map the proteomic profiles of *Lepomis macrochirus, Micropterus salmoides* and *Pomoxis nigromaculatus* and discover any novels peptides vital in antibacterial or scent activity.

MATERIALS AND METHODS

Mucus Sample Collection

Fish were captured by the conventional hook and line method from Swift Creek Reservoir, Briery Creek Lake, Sandy Creek Reservoir and a private pond. To prevent cross contamination of the mucus with the outside environment, the following was performed: crushed hooks barbs to prevent minimal bleeding, wore gloves to prevent hand secretions to mix with the mucus, cleaned the spatula after every fish and fish sides were rinsed with deionized water. Mucus sample collections were performed by gently scraping the dorsal sides of the fish with a micro spatula, starting from the opercular flap and trailing down to the tail. Mucus was then transferred to a centrifuge tube and immediately put on ice. Each tube contained samples from 2 fish (approximately 0.75 mL). Centrifuge tubes were frozen at -4ºC.

SDS-PAGE Gel

Mucus samples were thawed and centrifuged for 5 minutes at 12000 rpm. Clear supernatant was extracted and transferred to new Eppendorf tubes, and the pelleted cell debris at the bottom was discarded. 10x SDS buffer was diluted to 1x by mixing 100 mL of 10x SDS buffer with 900 mL of deionized water. Loading dye was prepared by mixing 950 µL of 5x western loading dye with 50 µL of β-mercaptoethanol. Afterwards, a 4-15% Mini-PROTEAN TGX Precast Protein Gel was mounted into the electrophoresis chamber bracket and rinsed with deionized water to flush unpolymerized SDS. 25 µL of the dye mixture was pipetted into each of the mucus sample tubes. The newly mixed Eppendorf tubes with dye and an Eppendorf tube of 10 µL of precision plus western standard were heated for five minutes at 90ºC and subsequently snap cooled. The protein ladder and the mucus sample were then pipetted into the gel wells. In this instance, the gel was loaded in the following order: protein ladder, *L. macrochirus*, *L. macrochirus*, *M. salmoides*, *M. salmoides*, *P. nigromaculatus*. The gel was then set to run at 100 volts for one hour. The gel casing was opened, and a corner of the gel was cut to help identify orientation of the wells for later reference.

Staining (Coomassie and Silver Stain)

For Coomassie staining, the gel was washed in approximately 200 mL of ddH_2O. This step was repeated three times. All the ddH_2O was discarded, and the staining container was filled with 50 mL of Bio-Safe Coomassie. The container was gently agitated with a shaker then the gel was rinsed with 200 mL of ddH_2O. Stained gels were placed on a viewing light for pictures, and later stored in 0ºC.

For silver staining, the agarose gel was air dried. Reagent preparation required mixing 950 mL of ddH_2O and 50 g of the development accelerator. After mixture was dissolved, ddH_2O was added to make one liter. Steps for the silver stain were as follows: fixative step required mixing methanol (50% V/V), glacial acetic (10% V/V), fixative enhancer (10% V/V) and ddH_2O (30% V/V), rinse step required removing the fixative enhancer and rinsing the gel twice with 400 mL of ddH_2O, developing step required mixing 35 mL of deionized water with 5 mL of silver complex solution, 5 mL of reduction moderator solution and 5 mL of image development reagent and the stop solution required a 5% acetic acid solution.

MS-MS

Visible protein bands were excised from the gel and shipped to Keith Ray at Virginia Tech for analysis.

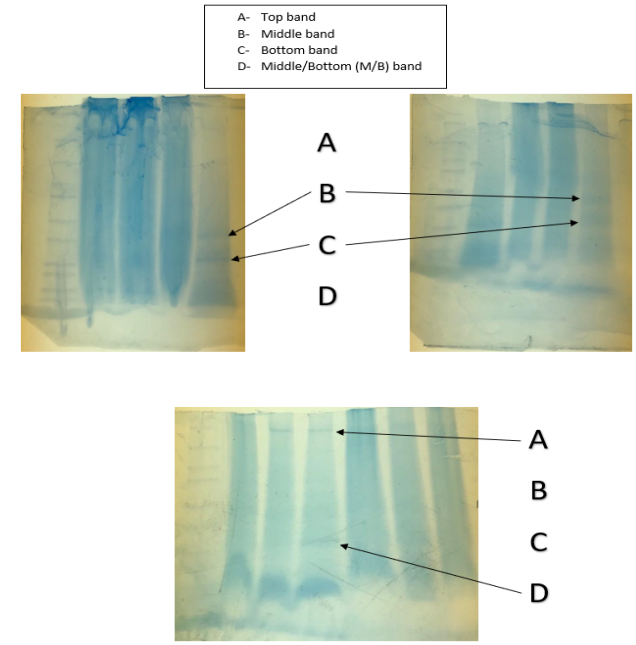
The MASCOT search engine was searched against the NCBIprot and SwissProt databases to confirm identity of peptides.

RESULTS

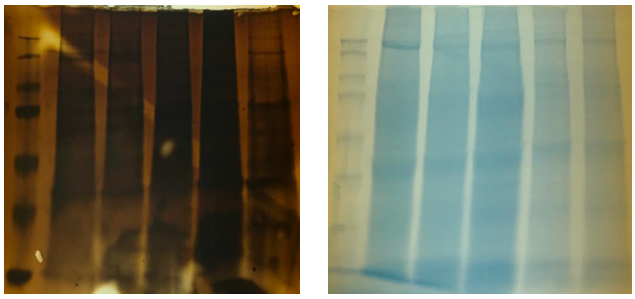
High levels of streaking were evident in initial gels. Subsequent gels yielded crisper band detection and increased band detection. For the early gels, bands were labeled top band, middle band, bottom band and middle/bottom band. The top band (A) had a weight of 150 to 250 kD; the middle bands (B) measures at 50 kD; the bottom band (C) weighed 35 kD; the middle/bottom band (D) weighed around 37-50 kD. There were six protein hits for band A: keratin, type II cytoskeletal 8-like [Haplochromis burtoni], intermediate filament protein ON3 [Astyanax mexicanus], keratin, type II cytoskeletal 8-like [Lates calcarifer], keratin type IIE [Acipenser baerii], keratin, type II cytoskeletal 75 [Podiceps cristatus] and keratin, type II cytoskeletal cochleal-like [Clupea harengus]. Three proteins had 0% false discovery rate. There were five hits for band B: keratin type IIE [Acipenser baerii], keratin type II E3 [Epinephelus coioides], type II keratin [Solea senegalensis], keratin, type II cytoskeletal 75 [Podiceps cristatus] and keratin, type II cytoskeletal cochleal-like [Clupea harengus]. Two proteins matched above identity threshold. Band C and D received no significant protein hits, but band C was likely to be actin filaments. Subsequent staining of gels with Coomassie and silver stain yielded greater enhanced detection of bands, but samples were not sent for analysis.

DISCUSSION AND CONCLUSION

Initial Coomassie stained gels exhibited heavy amounts of streaking which could be attributed to debris that could not be distinguished from each other or degradation. Increased band detection could have been due to better aseptic technique and being more familiarized with protein isolation techniques. Though most proteins identified in gels did not match with typical proteins associated with the immune system detected in similar studies, it is proof of concept that a crude model of protein isolation and identification could be established. Most of the proteins identified were structural proteins as opposed to immune-related ones, such as glutathione-S-transferase omega 1, proteasome 26S subunit and beta 2-tubulin, found in a study with infecting *Gadus morhua* with *Vibrio Anguillarum* (Rajan). However, Tr65 keratins in rainbow trout have exhibited ionophore-type activity (Molle). The keratins identified in band A and B did not belong to a *Micropterus salmoides*, but it was matched to a closely related species. This was probably due to



Three above Coomassie gel stains represent the earlier stain trials



Above gels represent the subsequent silver stain and Coomassie gel stains (note increased band detection)

lack of data collected for glycoproteins for *M. salmoides*. Many proteins were identified for one single band because a 1D gel was performed which only isolated proteins based off molecular weight. If a 2D gel had been attempted the added parameter would have been able to separate more proteins by their isoelectric point, the pH at which the protein has no net charge. Therefore, a 2D-GE coupled with MALDI would increase protein acquisition. For future reference, enzymatic deglycosylation could also be attempted to removed post translational attached glycans while still preserving the homogeny of the protein core. This can be achieved via use of a protein deglycosylation mix, consisting of PNGase F, TFMS, sialidase or more (Edge).

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