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Preliminary investigation on antimicrobial and proteolytic property of the epidermal mucus secretion of marine stingrays

Rathinam Vennila, Kalainesan Rajesh Kumar*, Shankar Kanchana, Muthuvel Arumugam, Shanmugam Vijayalakshmi, Thangavel Balasubramaniam

Center of Advanced Study in Marine Biology, Annamalai University, Portonovo, P.O. Box 608 502, Tamil Nadu, India

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ABSTRACT

Objective: To determine the antibacterial, antifungal, minimum inhibitory concentration (MIC) and the protease activity of the stingray mucus *Dasyatis sephen* (*D. sephen*) and *Himantura gerrardi* (*H. gerrardi*). **Methods:** Antimicrobial activity of crude aqueous, acidic and organic mucus extract was evaluated by disc diffusion method against human pathogens, MIC of the active samples were determined by spectrophotometric method and the protease activity which is responsible for the antimicrobial activity was determined by using zymogram method. **Results:** The crude acidic extracts of both the species showed antibacterial activity against *Salmonella typhi* (*S. typhi*), *Klebsiella pneumoniae*, *Streptococcus aureus*, *Escherichia coli* (*E. coli*), *Vibrio cholerae* (*V. cholerae*) and the acidic extracts of both the species exhibit antifungal activity against all the tested pathogens. Remaining extracts didn't show any inhibitory activity. The acidic extracts of *H. gerrardi* is significantly active against *S. typhi, E. coli, V. cholerae, Trichophyton mentagrophytes* (*T. mentagrophytes*), *Alternaria alternaria* (*A. alternaria*), *Trichophyton rubrum* (*T. rubrum*), *Candida tropicalis* (*C. tropicalis*) at the minimum concentration of 16 μg/mL, but the acidic extract of *D. sephen* required 32 μg/mL of protein to inhibit *S.typhi, E. coli, Aspergillus niger* (*A. niger*), penicillium sp, *T. mentagrophytes*, *A. alternaria*. Both the *D. sephen* and *H. gerrardi* shows the proteolytic activity above the molecular mass of > 66 KDa. The characterization of protease class using inhibitors showed the presence of both serine and metallo protease in the the samples. **Conclusions:** Protease activity present in the sting ray mucus is one of the key factor responsible for the antimicrobial activity and the results proved the role of mucus in the innate immunity.

1. Introduction

The fish skin is covered by epidermal layer and mucus, act as a primary wall between internal and external environment. This heterogeneous group of organisms occupy an apparent crossroads between the innate immune response and the appearance of the adaptive immune response. Importantly, immune organs homologues to those of the mammalian immune system are present in fish. However, their structural complexity is less, potentially limiting the capability to generate fully functional adaptive immune responses against pathogen invasion[1]. Therefore the fishes are depending on their innate immune mechanism for protection against invading pathogens. The innate immune components includes the mucus layer on the skin, gills and Gastrointestinal (GI) tract, constituents of the blood such as natural killer cells and phagocytes[2]. The epidermal mucus contains a key component of innate immunity that protects from the unfavorable conditions and prevents foreign substances from invading. The epidermal mucus is secreted by the epidermal goblet cells composed mainly of water and gel forming macromolecules such as mucin, and other glycoproteins, *etc*[3]. This mucus secretion is thought to perform number of functions including lubricant[4], mechanical protective function, osmoregulation, locomotion, immunological role and intraspecific chemical communication[5]. Mucus protects against attacks by gnathiids, acting like mosquito nets in humans, a function of cocoons and an efficient physiological adaptation for preventing parasite infestation that is not used by any other animal[6]. The mucus layer on the surface of the fish

*Corresponding author: RK Rajesh kumar, CAS in Marine Biology, Faculty of Marine Science, Annamalai University, Portonovo-608 502, India.
Tel: +91-99761 55576
Fax: +91-4144-243555
E-mail: rk.rajeshkumar@yahoo.co.in

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is continuously replaced which possibly prevents stable colonization by parasites, bacteria and fungi. Skin secretions contains a wide variety of polypeptides with antimicrobial properties. Proteases are considered to be a antimicrobial proteins which involved in the regulatory production of antimicrobial peptides. In addition fish mucus also contains a variety of biologically active substances such as lysozyme, lectins, flavoenzymes, immunoglobulins, C-reactive protein, apolipoprotein A-I and antimicrobial peptides which gives protection to fish from potential pathogens[7-11].

Antimicrobial activity of epidermal mucus extracts against a broad range of microbial pathogens was observed by many researchers, but those works were focused towards on marine microbial strains and notably there is no information available on the antimicrobial function of epidermal mucus of stingray. The goal of the present study was to investigate the *in vitro* antimicrobial activity in the epidermal mucus of the stingray family which includes *Dasyatis sephen* (*D. sephen*) and *Himantura gerrardi* (*H. gerrardi*) species against human pathogens (grampositive and gramnegative bacteria, fungi) and assessed the presence of protease activity by using casein as a substrate and we have also determined the protease classes present in the mucus by using various inhibitors.

2. Materials and methods

2.1. Mucus collection and extraction

The stingray *D. sephen* and *H. gerrardi* was freshly collected with the help of fisherman of Portonovo coast, Tamil nadu, India. Mucus secretions were carefully scraped from the dorsal side of the body using spatula. The mucus was immediately transferred to the laboratory and stored at -70 ℃ until use. A portion of mucus was lyophilized and suspended in phosphate buffer (PBS) (pH 7.4) at 1 mg/mL concentration to give the aqueous extract (Extract A).

The mucus samples were mixed with 10% acetic acid in the ratio of 1:1 and placed in a boiling water bath for 5 minutes and it was cooled, centrifuged at 18000 xg for 35 min at 4 ℃. The resulting supernatant was evaporated overnight, mixed in water (Extract B) and then assayed for antimicrobial activity.

The organic extract was prepared by the method of Hellio *et al*[13] with slight modification. The lyophilized powder (1 mg/mL) was suspended in 95% ethanol and centrifuged at 11000 xg for 30 mins. The supernatant was discarded and the pellet was resuspended in 95% ethanol for 3 more times. The ethanol extracts were pooled, evaporated, suspended with distilled water to give 50 mL as final volume and extracted with CH_2Cl_2 (4×50 mL). The aqueous phases was lyophilized (Extract C), while the Dichloromethane phase (Extract D) were pooled and evaporated, the resulting dried samples obtained from both the phases were dissolved in water and 5% dimethylsulfoxide (DMSO) respectively and used for further analysis.

2.2. Microorganisms and media

Bacterial strains used were *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), *Klebsiella pneumonia* (*K. pneumonia*), *Klebsiella oxytoca* (*K. oxytoca*), *Vibrio cholerea* (*V. cholerea*), *Streptococcus aureus*, *Staphylococcus aureus*, *Salmonella paratyphi* (*S. paratyphi*). The fungal pathogens used were *Candida tropicalis* (*C. tropicalis*), *Aspergillus niger* (*A. niger*), *Penicillium* sp., *Trichophyton mentagrophytes* (*T. mentagrophytes*), *Alterneria alteneria* (*A. alteneria*), *Candida albicans* (*C. albicans*), *Rhizopus* sp., *Mucor* sp., *Trichophyton rubrum* (*T. rubrum*). All eight species of bacterial strains were maintained in nutrient agar and the fungal strains were maintained in potato dextrose agar (PDA).

2.3. Agar disc diffusion method

The screening of antimicrobial activity of the mucus extracts were carried out in the agar disc diffusion method using Muller Hinton agar (MHA) medium for antibacterial activity and PDA for antifungal activity.

The bacterial and fungal inocula were prepared from the colonies of 24 h culture on nutrient agar and PDA medium. The inocula was adjusted with McFarland density to obtain final concentration of approximately 10^4 and 10^6 CFU/mL for the fungi and the bacteria respectively. 30 μg of each extracts were imbibed in Whatmann AA filter paper and applied on the test media which were previously inoculated with each test strain. Plates were incubated at 37 ℃ for bacteria or 28 ℃ for fungus. Inhibition zones were measured after 24 h of incubation[13]. Standard disk of erythromycin (15 mcg/disc) and nystatin (100 units/disc) served respectively as the positive antibacterial and antifungal controls.

2.4. Minimum inhibitory concentration (MIC)

MIC was determined by serially diluting the active acidic extracts in the concentration of 8, 16, 24, 32, 40, and 48 μg/mL. Microorganism (2×10^8) were grown in liquid medium consisting of Mueller Hinton for bacteria and RPMI 1640 for fungi. broth at 37 ℃ and media at 28 ℃. MIC represents the lowest concentration required to inhibit the growth of microorganism. All assays were carried out for four times and the control test was carried out with the solvents in the concentration of 5% DMSO[14].

2.5. Protease activity

Effect of protease was determined by using substrate SDS-PAGE analysis (10% acrylamide) gels containing 2 mg/mL casein by the method of Barbaro *et al*[15]. Samples were mixed with non-reducing sample buffer and the gel