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Evaluation of Antimicrobial Peptide Isolated from Skin Mucus of Bacterial Infected Fishes

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Abstract

Fish mucus has been recognized as the potential source of antimicrobial proteins and peptides, which is of interest to provide the front line defence against numerous invading pathogens. The antimicrobial peptides (AMPs) have been reported to possess important ecological and biological functions. Fishes are always in intimate contact with the environment containing diverse and higher concentrations of viruses and bacteria. This study reports the isolation and characterization of a potent antimicrobial peptide, from the skin mucus of bacterial infected fishes collected from different fresh water bodies of Kashmir valley. The fish mucus was subjected to column chromatographic techniques for isolation and purification using gel filtration chromatography. The peptide was demonstrated to be single protein band by SDS-PAGE, with the apparent molecular weight of 20 kDa. The peptide exhibited the antimicrobial property as revealed by disc diffusion technique against three pathogenic strains like Klebsiella pneumoniae, Staphylococcus aureus and E.coli. The purified AMP presented the potential anti-microbial activity with all the three strains of bacteria and the maximum inhibition was recorded against Klebsiella pneumonia (19mm). The broad antimicrobial activities of isolated antimicrobial peptide suggest that it contributes to the innate host defence system in bacterial infected fish.

Keywords: Fish mucus; Antimicrobial peptides; Kahmir valley; chromatography.

1. Introduction

Over the past few decades, there are serious challenges in drug-resistant infections in the area of antimicrobial therapies [1]. Biochemical molecules like antimicrobial proteins and peptides (AMPs) are key effectors that function as broad-spectrum anti-infectives [2]. They have an innate- immune defence in mammals [3]. These biochemical molecules (AMPs) directly interact with bacteria and viruses and kill them [4]. The sources of organisms with antimicrobial properties and peptides attributes have gained much interest among the research scientists due to their biochemical diversity, anti-viral, anti-bacterial, anti-fungi, anti-protozoan parasites, and anti-tumoral traits [5]. The presence of these molecules aids in the nonspecific defence

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mechanisms against these microbes [6]. These molecules were initially identified in frogs and insects but later identified diversely in all the animal and plant kingdom [7]. The recent advancement/technologies in the fish cultures of the present day amplified the fish by reducing the pathogenicity to the fish population. Hence, a great number of pharmaceutical antibiotics were fed in the fish farms for preventing the outreach of these infectious diseases, which contributed to the development of bacterial resistance to the antibiotics [8]. Thus, there is a necessity to replace these conventional antibiotics. The antimicrobial peptides are the best possible and useful natural antibiotics for pharmaceutical application [9]. So, Fish mucus containing AMPs being quite defensive against the pathogens affecting this particular population, this study was primarily devised to the study of bioactive molecules of fish mucus (e.g. antimicrobial peptides and immune-related molecules) and associated micro biota because of their potential in aquaculture and human medicine [10-11].

2. Material and methods

2.1 Collection of fish Samples from native habitats:

A network of study citations was selected from various water bodies covering the Kashmir Valley and fishes from the different sites like Manasbal Lake, Khag and Rangil water bodies were collected with the assistance of local fishermen. The collected fishes were identified by means of the numerous taxonomic keys available in the published literature, in addition to the assistance provided by experts in the area.

- Laboratory procedures for bacterial diseases:

Fish samples were brought to lab from different sampling sites. They were kept in freezer to avoid the denaturation of peptides. Following information was proposed for diagnosis of bacterial infection in fish [12].

- Gross clinical signs of disease on individual fishes.
- Internal abnormities during post mortem examination.
- Histo-pathological examination of diseased tissue.

2.2 Collection of fish mucus:

The collected fishes obtained from the different locations of Kashmir valley's kokernag, khag Budgam and Manasbal fish farms were subjected to scrubbing and collecting the surface fish mucus. The fishes were killed with a sharp blow to the head and subjected for further processing. The collection of mucus involves the using of blunt scraper, such as a wooden spatula, to gently take a sample of mucus from fish skin requires taking and preparing a skin scrape. In order to prevent intestinal and spermal contamination, ventral skin mucus was collected. To stop any possible bacterial infection, the skin mucus sample was immediately frozen and aseptic conditions were maintained throughout the study to elude cross contamination.

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2.3 Total Protein content estimation:

The protein concentration of collected fishes were estimated by the method of Lowry with bovine serum albumin as standard [13]. To 5 ml of Lowry reagent, add 1 ml of suitably diluted sample and the mixture is stored for 10 min at room temperature. To this add 0.5ml of Folins reagents and kept at dark condition for 30mins. The absorbance of samples were taken at 640 nm and the total protein was calculated accordingly.

2.4 Purification of peptides:

In order to reveal the presence of different Amps in the fish mucus, various purifying steps were followed to identify and isolate small peptide masses from the collected mucus. The mucus was subjected to centrifugation for removal of unwanted material. The supernatants containing AMPs were filtered through different molecular cut-off filters. All samples were stored at -20 degree Celsius until testing. At the time of analysis, the samples had been thawed by keeping them at 4 degree Celsius for 2 hours and subsequently further subjected to Sephadex G50 gel column chromatography [14]. The eluted fractions further undergo following procedures.

2.5 Protein separation and quantification:

In conjunction with a reduction agent, the powerful anionic detergent SDS is most frequently used and heated to disassociate the proteins until they are loaded onto the gel. The sum of bound SDS is often proportional to the poly peptides' molecular weight and is independent of their sequence individual charge. The protein was separated by SDS-PAGE electrophoresis and by comparing its electrophoretic mobility in SDS-PAGE with mobility marker proteins of known molecular weight were also used [15]. The appropriate weight of isolated microbial peptide was determined and further biological characterization was employed to identify its biological function.

2.6 Anti-microbial properties:

The purified protein fraction was analyzed for the anti-bacterial property in order to ascertain the possible antibacterial role [16], During present study, antimicrobial activity of fish mucus of bacterial infected fishes were determined against strains of E.coli, S.auerus and K. pneumoniae. Three different increasing concentrations of purified peptides like 10, 20 and 30ug/ml were evaluated against Bacterial strains E.coli, S. aureus and K. pneumoniae. In addition to this, antibiotic streptomycin was used as a positive control for bacterial strains. The antibacterial activity was recorded as inhibition zone diameter (IZD), measured in mm. The aqueous (dimethyl sulphoxide) of 10% was used as a negative control. All the strains tested, were found to be resistant against negative control. The inhibition of bacterial strains was presented as the bar diagram and plotted as percent inhibition versus increasing protein fraction concentrations.

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3. Results

3.1 Protein estimation

The estimated protein content in fish mucus using Lowry's method was found to contain 1.33mg/ml of protein.

3.2 Protein separation and Quantification

AMPs are well-known to be proteins in nature and are highly sensitive towards pH and temperature, this hinders their development and utilization and they can be easily denatured during the process. Also, small size of AMPs and their limited presence of amino acids confines the structure elucidation of the AMPs. SDS-PAGE method being the sensitive and effective tool to identify and monitor proteins during the purification process. The pure fraction of fish mucus (Fig 1, lane C) was confirmed as a single protein band with an apparent molecular weight of 20 kDa and crude fish mucus showed many protein bands with molecular size ranging from 9 kDa to 120 kDa as shown in fig 1, lane B. The PAGE reveals that only a specific protein fraction containing the protein of 20 kDa was isolated using column chromatography and further characterized for possible biological activity. Furthermore, the purified AMP exhibited a well defined peak at 276 nm in UV-Visible spectrum (Fig. 2) which is the characteristic of well defined structure and presence of tryptophan residues.

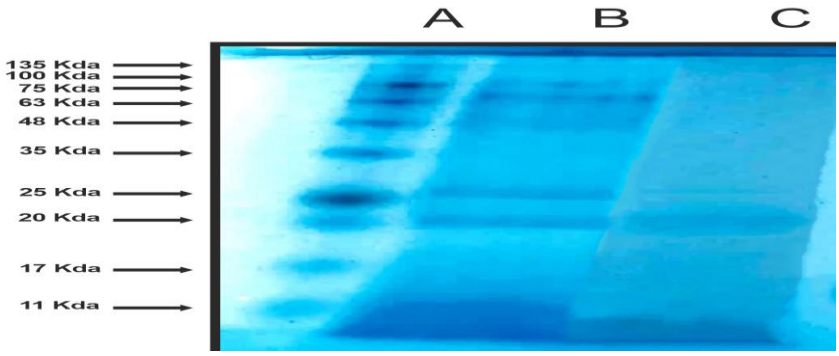

Fig. 1: SDS page profile of purified protein from fish mucus. Lane (A) determines the ladder of different molecular weights, (B) is the crude extract profile of fish mucus and (C) is the purified protein fraction.

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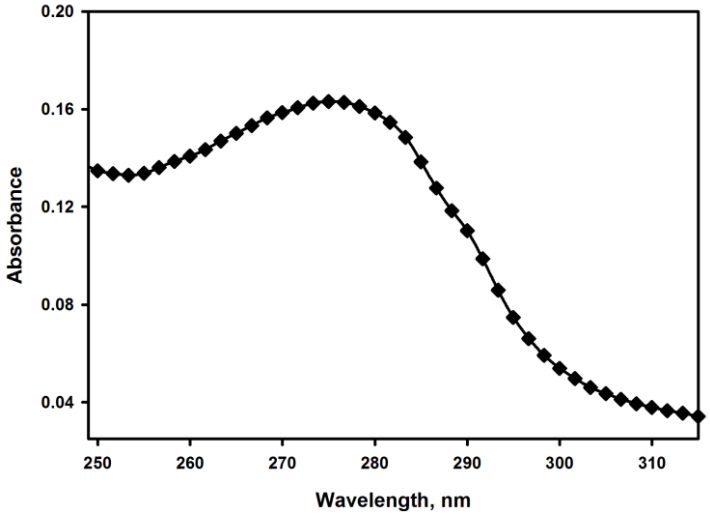

Fig. 2: Absorption spectrum of purified AMP from fish mucus. The peak determines the tryptophan peak at 276 nm.

3.3 Antimicrobial assay

The mucus extract was evaluated against Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus for antimicrobial activity. The results showed that fish mucus protein was active against all tested strains of bacteria (Table 1, fig. 3 and 4). Antibacterial spectrum was attained by disc diffusion process of fish mucus on the agar plates. The samples of purified mucus protein limited the growth of different bacteria on petri plates. Notably, the mucus protein was most sensitive towards K. pneumoniae with the zone of inhibition of 19 mm with activity of 70% at 30 µg concentration to that of streptomycin used as positive control followed by staphylococcus aureus having activity of 69% with zone of inhibition of 18 mm (Table 1, Fig. 3 and 4). Out of tested bacterial strains least activity which is almost equal to that of S. aureus was found in case of E.coli, were highest protein concentration causes inhibition by 68 %, with zone of inhibition of 17 mm which is near to similar to that found with Staphylococcus aureus treated one. So, it seemed that the antibacterial activity of the skin mucus protein was high. The table 1and figure 3 and 4 demonstrate the spectrum of antibacterial activity in fish mucus.

Table 1: Fish mucus protein against various bacterial strains during antibacterial activity assay.

Microorganism	**Mucus sample** **Zone of inhibition**			**Streptomycin**
	10 ul\disc	20 ul\disc	30 ul\disc	100 ug/disc

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K. pneumoniae	14 mm	17 mm	19 mm	27 mm
S. aureus	12 mm	16 mm	18 mm	26 mm
E. coli	12 mm	15 mm	17 mm	25 mm

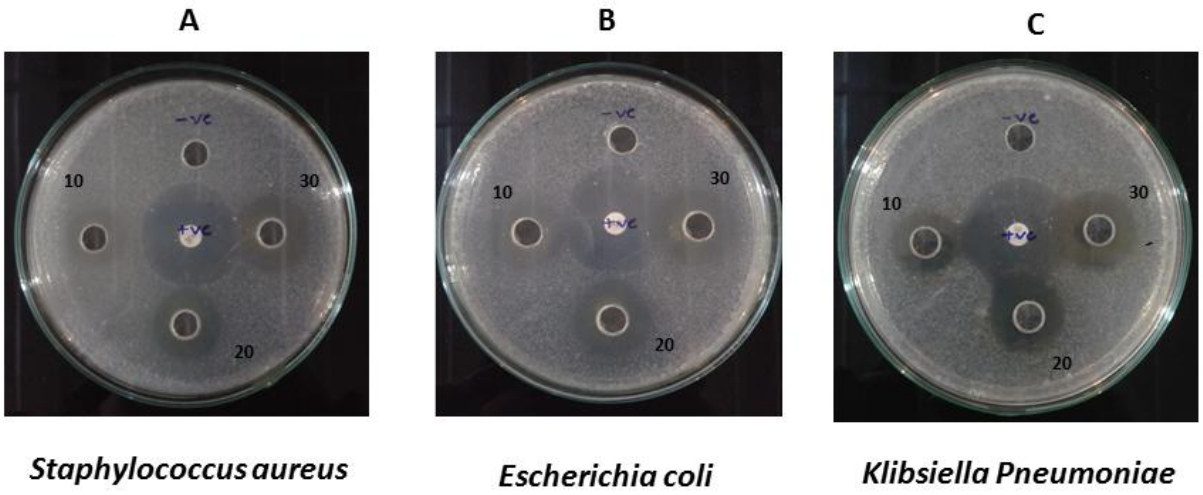
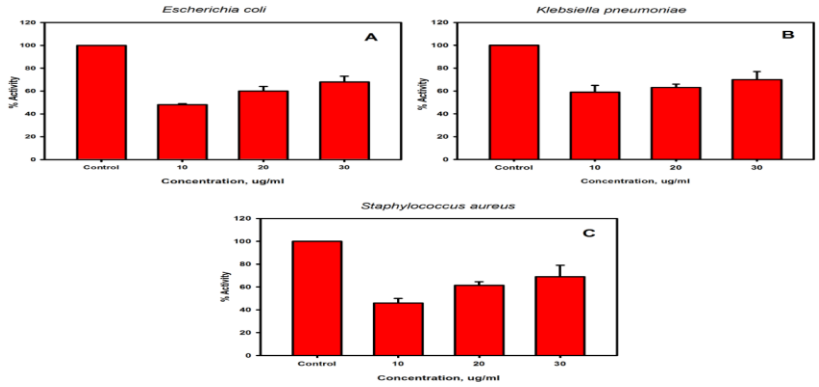


Fig. 3: Antimicrobial activity of fish mucus protein on different strains of bacteria represented by A, B and C. The central disc contains the positive control (streptomycin). Different concentrations of mucus protein from 10-30 µg/ml have been used which is designated as 10, 20 and 30 on inhibition zones.

Fig 4: Bar diagrams representing the antimicrobial activity of purified fish mucus protein against



three bacterial pathogens (A) E. coli (B) K. pneumoniae and (C) S. aureus. The streptomycin has been used as control in all the three strains and consigned as 100 % activity.

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4. Discussion

Organisms live in intimate contact with their environment, which is densely populated with microorganisms. Such organisms have developed the widespread defence mechanisms to counter these microbial infections. Increased bacterial resistance has resulted in the unsystematic widespread use and abuse of antibiotics, causing new threats and challenges to human well-being [17]. As a result, humans face a global recurrence of infectious diseases and a rapid rise in bacteria that are immune to multiple drugs and commercially available antibiotics, making the world return to the pre-antibiotic era [18]. The search of these peculiar molecules with antibacterial activity that might be able to get over the resistance phenomenon is of greatest importance (19). Therefore, these commonly-occurring, antimicrobial peptides (AMPs) are considered the new expectation and mesmerize scientists as suitable markings for the advancement of alternatives to typical antibiotics. We have successfully isolated and purified the antimicrobial protein using column chromatography with a molecular weight of 20 KDa. The isolated protein revealed the antimicrobial property as shown by disc diffusion technique against three pathogenic strains like Klebsiella pneumoniae, Staphylococcus aureus and E.coli. The potential anti-microbial activity was found almost equally with all the strains of bacteria used while, the maximum inhibition was recorded against Klebsiella pneumoniae (19 mm).

Many AMPs are relatively small-scale peptides (< 60 amino acids) with a wide spectrum of action against microorganisms (Gram-positive and Gram-negative bacteria, fungi, viruses, parasites) that are active against pathogenic bacteria and a low probability of commencing resistance [20]. In addition, AMPs play numerous functions that are well beyond the purpose of these peptides as mere antibiotics [21]. In fact, several peptides have anticancer activity, by promoting cytokine release, chemotaxis, antigen presentation, angiogenesis, inflammatory responses, and adaptive immune induction, activating the immune system [22]. Much attention is given to acquiring unique antimicrobial compounds with broad-spread action against a wide variety of pathogenic microorganisms, and existing research is aimed at advancing the acquisition of more effective drugs with modified compounds [23]. They may be involved in the mechanism of breakdown of cell wall or cell membrane dissociation in the bacteria. Important variations from peptide to peptide and importance for particular AMP-bacteria paring are shown by the process used by AMPs to destroy specific bacteria; however, the entire mechanism between AMP structures and killing mechanisms is still not well understood [24].

5. Conclusion

We have successfully isolated and characterized a 20 KDa protein. Our protein is also one of the AMP which presents the potent anti-microbial activity and may be further evaluated for its role in other clinical manifestations. So, our proteomic approach by isolating AMP from fish mucus with a broad spectrum antibacterial activity being antimicrobial agents in fish mucus could be used to formulate new medications for the prevention of infectious diseases caused by

pathogenic and opportunistic microorganisms. Such properties of these isolated proteins may have utility in aquaculture and human health-related applications.

Conflict of interest

The authors state that there is no conflict of interest in the study.

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