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ORIGINAL RESEARCH

Antibacterial screening of epidermal mucus protein extract of freshwater Bornean spotted barb *Barbodes sealei*

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Abstract The epidermal mucus of fish serves as the first line of defence against the microbe-rich aquatic environment, containing various innate immune components, including antimicrobial proteins. However, information regarding the antibacterial properties of skin mucus of Bornean native fish is scarce. This study aims to enhance the understanding of the epidermal mucus of *Barbodes sealei,* a Bornean endemic freshwater fish species. Pooled mucus samples were extracted using saline (aqueous extract) and acetic acid (acidic extract). The extracts were purified and concentrated through ammonium sulfate precipitation. This study presents the antibacterial screening of these mucus extracts against 16 selected bacterial strains. The results revealed that among the bacterial strains tested, only *Salmonella braenderup* ATCC BAA 664 showed sensitivity to the acidic extracts, while none of the aqueous extracts exhibited any antibacterial activity. The findings suggest that higher protein contents in the extracts did not necessarily correlate with better antibacterial activities. To identify the major proteins present in the active extracts and determine the antibacterial proteins, a qualitative bioanalysis was conducted using high-throughput Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). Four antibacterial proteins, namely Histone H2A, Histone H2B, Histone H4, and Heat shock protein 70, were identified based on comparison with existing literature. Further isolation and characterisation of the active components, particularly the antimicrobial proteins, are warranted to gain deeper insight into their role in fish immunity. This study establishes the antibacterial potential of epidermal mucus from *B. sealei* and proposes it as a non-invasive source for the isolation of new biologically active compounds, such as antimicrobial proteins and peptides.

Keywords Antibacterial activities . *Barbodes sealei* . Borneo . Fish epidermal mucus . Histone H2A . Histone H2B . Histone H4

Introduction

Throughout human history, fish has been recognized as a significant resource, serving not only as a nutritious food source rich in protein and lipids but also as valuable trade commodities, including ornaments and medicines (Tilami and Sampels 2017). To date, there have been over 33,000 described and reported fish species (Froese and Pauly 2023), with more than 40% of them thriving in freshwater habitats (Lundberg et al. 2000; Tedesco et al. 2017). This is remarkable considering that freshwater ecosystems cover only a small portion of the Earth's surface, approximately 0.8%, and make up less than 0.02% of global water (Dudgeon et al. 2006). In Borneo, there are 23 families of freshwater fishes that are confined to freshwater systems and exhibit little tolerance to saltwater (Berra 2007). Among these families, Cyprinidae, which

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includes barbs, carps, and minnows, is the most dominant group in freshwater habitats, comprising over two-thirds of the total freshwater fauna (Sulaiman and Mayden 2012). One notable endemic species found in Borneo is *Barbodes sealei*, the Bornean spotted barb locally referred to as "Turungau" (Inger and Chin 1962; Froese and Pauly 2023). This species is typically found in clear or slightly murky, unpolluted forest streams with sandy or gravelly riverbeds (Inger and Chin 1962). It can be identified by a row of equally spaced dark blotches along its flank.

Unlike terrestrial animals, fish spend their entire life in an aquatic environment. Most aquatic habitats are teeming with saprophytic, pathogenic, and non-pathogenic microbes, such as bacteria, viruses, and fungi (Magnadottir 2010). As fish have continuous contact with their aquatic surroundings and rely on gill-breathing, they are more susceptible to a wide range of diseases. The success of an infection largely depends on the ability of pathogens to adhere to the mucosal surfaces of fish (Magariños et al. 1995; Benhamed et al. 2014). Thus, fish rely heavily on their complex and fast-acting innate immune mechanisms to combat the constant threats to their health (Ellis 2001; Arellano et al. 2004). In general, the innate immune system of fish comprises various organs, including scales, gills, gut, and epidermis, along with the mucus secreted by epithelial cells (Esteban 2012). One of the most crucial components of the fish's innate immune response is the mucous layer that covers their body surface. Mucus is a viscous colloid gel that forms an adherent layer cover, serving as the primary interface between the environment and the interior milieu of the fish. It is continuously secreted and sloughed off as fish encounter, monitor, and regulate the vast microflora present in the aquatic environment, thereby preventing the adherence of pathogens to the underlying tissues (Esteban and Cerezuela 2015). Beyond its role as a physical barrier in the innate defence system, fish skin mucus actively prevents microbial infections and is considered a crucial immunological factor. Epidermal mucus in fish primarily consists of approximately 95% water and glycoproteins, along with various other substances (Bansil and Turner 2006). It contains a wide range of innate immune components, including lysozymes, calmodulin, complement, proteolytic enzymes, lectins, C-reactive proteins, immunoglobulins, as well as antimicrobial peptides and proteins (Shephard 1994; Magnadóttir 2006; Alvarez-Pellitero 2008; Esteban 2012).

Presently, a growing body of research on the antimicrobial function of fish skin mucus suggests that it plays a role in preventing the invasion of parasites, bacteria, and fungi (Hellio et al. 2002; Subramanian et al. 2008b; Lee et al. 2020; Tiralongo et al. 2020). Most of the studies have focused on commercially important farmed or marine species such as Atlantic cod (*Gadus morhua*) (Magnadóttir et al. 2018), Atlantic salmon (*Salmo salar*) (Provan et al. 2013), discus fish (*Symphysodon aequifasciata*) (Chong et al. 2006), European seabass (*Dicentrarchus labrax*) (Cordero et al. 2015), and gilthead seabream (*Sparus aurata*) (Cordero et al. 2016). However, the antimicrobial potential of mucus from freshwater fish, particularly the native species in Borneo, remains unexplored. Therefore, knowledge of skin mucus of Bornean fish species and their innate defence mechanisms can be crucial to overcoming the challenge of combating multidrug-resistant pathogens. This study explores the potential antimicrobial properties of the epidermal mucus of a Bornean endemic freshwater fish species, *Barbodes sealei,* by screening antibacterial activities and sequencing peptides of the active mucus extracts using LC-MS/MS.

Materials and methods

Preparation of the fish mucus extracts

In the study, all procedures were conducted with the approval of the UNIMAS Animal Ethics Committee (UNIMAS/AEC/R/F07/020). *Barbodes sealei* were collected from the upstream river near Melaban Village in Kota Samarahan District (1.5025 °N, 110.4080 °E) using homemade minnow traps with commercial fish feed pellets as attractants. After a seven-day acclimatisation period, thirty healthy fish were selected for the collection of epidermal mucus using methods modified from Subramanian and co-workers (2008b). Prior to the mucus collection, the fish were starved for 24 hours and rinsed with sterile distilled water. They were then placed in a sterile zip-locked polyethylene bag containing 30 ml of physiological saline solution (0.85 % NaCl) and massaged gently for 10 to 15 min to slough off the mucus. The fish were returned to a recovery tank afterwards, while the collected mucus samples were immediately pooled and stored at -4 °C.

The aqueous extract was prepared according to the methods modified from Loganathan and colleagues