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The serum of rabbitfish (*Siganus oramin*) has antimicrobial activity to some pathogenic organisms and a novel serum L-amino acid oxidase is isolated

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ABSTRACT

The serum of rabbitfish (*Siganus oramin*) has been confirmed previously to have killing effect to *Cryptocaryon irritans*, an important marine ciliate protozoan that causes a disease referred to as "marine white spot disease". Herein, we find the serum of the rabbitfish also shows antibacterial activity against both gram-positive and gram-negative bacteria and has killing effect on two other parasites: *Trypanosoma brucei brucei*, *Ichthyophthirius multifiliis*. Results of scanning electron microscopy indicated that after treating with rabbitfish serum, the surface of the *Staphylococcus aureus* was wrinkled and pores were formed on the surface of *Escherichia coli*. Serum of the rabbitfish possesses a strong killing effect to *Ichthyophthirius multifiliis* in vitro, causing a similar effect as to *C. irritans*. The serum of rabbitfish also showed strong killing effect to *T. b. brucei* in vitro, with the minimus trypanocidal titre (MTT) only to be 1.5% in 1 h. Results of laser confocal fluorescence microscopy indicated that rabbitfish serum could also induce cell rupture of *T. b. brucei*. A novel antimicrobial protein (SR-LAAO) was isolated from the serum of rabbitfish by using ultrafiltration, reversed phase high performance liquid chromatography (RP-HPLC) and Native polyacrylamide gel electrophoresis (Native-PAGE). Results of gel overlay assay showed that the protein could act alone to inhibit the growth of *S. aureus* and *E. coli*. Results of western blot and automated Edman degradation showed that it was the same as the antiparasitic protein (APP) reported before to have killing effect on *C. irritans*. Full length cDNA sequence of the SR-LAAO was cloned. BLAST research suggested that the cDNA of SR-LAAO has a close similarity with a number of L-amino acid oxidases (LAAOs) and possesses two conserved motifs that exist in LAAOs. Combined, these results demonstrate that this protein which has antimicrobial activity to some pathogenic organisms was a novel LAAO found in the serum of rabbitfish. Immunohistochemical analysis demonstrated tissue specific expression and localization of SR-LAAO in the spleen, kidney, gill and blood of the rabbitfish, but was not found in other tissues. These results suggest that this protein may contribute considerably to the host non-specific immune defense mechanism to combat microbes of the rabbitfish and has the potency for using in future drug development.

1. Introduction

The rabbitfish *Siganus oramin* (also known as *Siganus canaliculatus*) is a commercially important and widespread species of fish along the coast of southeast China [1]. They are economically important as a human food resource as well as a popular aquarium fish. Interest in cultivation is growing rapidly in several areas [2]. In previous studies, we have found the serum of rabbitfish to have a strong killing effect on *Cryptocaryon irritans*, an important marine ciliate protozoan that causes a disease commonly referred to as "marine white spot disease" [3]. The present study will focus on what effect the serum of rabbitfish has on other parasites or pathogenic microorganisms and whether the active factor in the serum has the same protein as the one of killing *C. irritans* as reported before. Therefore, several gram positive and negative bacteria were selected to test the antibacterial activity of the serum. Meanwhile, two species of parasites: *Trypanosoma brucei brucei*, a causative agent of domestic and game animal African trypanosomiasis (also called Nagana) and *I. multifiliis*, a serious pathogenic parasitic cilia of freshwater fish were selected for testing as well.

L-amino acid oxidase (LAAO) is a flavoenzyme which catalyses the stereospecific oxidative deamination of an L-amino acid substrate to yield ammonia, hydrogen peroxide, and ketoacids with

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oxygen consumed [4,5]. LAAO family proteins share a common dinucleotide-binding motif and a GG motif (R-x-G-G-R-x-x-T/S) [6]. They are usually homo dimeric, flavin adenine dinucleotide (FAD) binding glycoproteins with molecular mass around 50–70 kDa when assayed by SDS-PAGE [7]. FAD catalyzes electron transfer during the enzymatic reaction [8]. It has been reported that LAAOs have bioactivities such as apoptosis, cytotoxicity, hemolysis, platelet aggregation and antiviral, antibacterial, anti-protozoal activities in a variety of animal fluids [7]. Moreover, they are widely distributed across diverse phyla from bacteria to mammals, which appear to be important in both vertebrate and invertebrate host defenses [9]. Most of the antibacterial LAAOs reported to date derived from secretory glands, such as venom in snakes [10–13], body surface mucous secretion in terrestrial snails [9], the albumen gland and ink in sea hares [14–16], epidermal mucus in fish [17–19], and milk in mice [20,21]. It was first reported in teleost fish by Jung et al. (2000) that the presence of LAAO as an apoptosis inducing protein (AIP) in *Mackerel viscera* infected with the nematode Anisakis simplex [22]. After this, LAAOs have now been found in various fish species, such as the skin mucus of rockfish *Sebastes schlegeli* [4], skin mucus of the great sculpin *Myoxocephalus polyacanthocephalus* [18], epidermal mucus of flounder *Platichthys stellatus* [19] and serum of rockfish *S. schlegeli* [23]. Previous studies have suggested that the bioactivity of LAAOs were elicited by hydrogen peroxide generated from L-amino acid oxidation [15,24] and the binding of LAAO to bacterial cells and viruses [9,25]. However, the exact mechanism of the antimicrobial activity and their biological role are still unclear.

This paper reports a unique antibacterial spectrum of the serum of rabbitfish that was active against a wide range of gram-positive and negative bacteria from both fish pathogens and non-aquatic species. Meanwhile, the serum has killing effects on two parasites *Trypanosoma brucei brucei* and *Ichthyophthirius multifiliis.* Moreover, a novel L-amino acid oxidase was purified and demonstrated that it's the same protein as reported previously that has a killing effect to *C. irritans*. The identification and characterization of proteins involved in immune responses are now considered to be essential for the elucidation of immune defense mechanisms and for disease control because of their potential use as therapeutic agents and genetic improvement biomarkers on disease resistant strain selection. A better understanding of resistance mechanisms could lead to better control of fish diseases and thus increased efficiency and production of fish in aquaculture.

2. Materials and methods

2.1. Sera sample collection

12 species of cultured fish (Table 1) were obtained from the marine culture region in Daya Bay, Guangdong Province, China and blood samples were collected from randomly selected fish. Fish were anaesthetized using 200 mg/l MS-222 and bled from the caudal vein with a 23-gauge needle. Blood was placed in separate 1.5 ml eppendorf tubes and allowed to clot at room temperature. Serum was collected following centrifugation at 3000×*g* for 10 min. The serum was heat inactivated at 56 °C for 30 min and stored at −80 °C for further testing.

2.2. Determination the antibacterial spectrum of the rabbitfish serum by inhibition zone assay

Analysis of the antibacterial spectrum of the serum samples was done by inhibition zone assay on agar plates as previously reported [26]. The pathogens, growth medium and culture conditions are shown in the following text.

Table 1
The antibacterial and antiparasitic activity of serum from different fish species used in this study.

Families	Species	Antibacterial activity	Antiparasitic activity
Haemulidae	Yellow Spotted Grunt (*Plectorhynchus cinctus*)	No	No
Sparidae	Sparidae Black Seabream (*Sparus macrocephalus*)	No	No
Carangidae	Snubnose Pompano (*Trachinotus blochii*)	No	No
Serranidae	Grouper (*Epinephelus coioides*)	Yes	No
Lutjanidae	Red Snapper (*Lutianus argentimaculatus*)	No	No
Lutjanidae	Crimson Snapper (*Lutjanus erythopterus*)	No	No
Lutjanidae	White-Spotted Snapper (*Lutjanus stellatus*)	No	No
Sciaenidae	Red Drum (*Sciaenops ocellatus*)	No	No
Cichlidae	Tilapia (*Oreochromis mossambicus*)	No	No
Rachycentridae	Cobia (*Rachycentron canadum*)	No	No
Tetraodontidae	Fugu Obscurus (*Takifugu obscurus*)	No	No
Siganidae	Rabbitfish (*Siganus oramin*)	Yes	Yes

Different bacterial groups were selected: *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus iniae* for gram-positive bacteria and *Escherichia coli*, *Vibrio cholerae*, *Aeromonas sobria*, *Edwardsiella tarda*, *Vibrio alginolyticus* and *Photobacterium damselae subsp. piscicida* for gram-negative bacteria. All the bacteria strains were from stored strains in our lab. These bacteria were grown overnight to mid-logarithmic phase. Agarose (1.5%) in Luria-Bertani (LB) medium was mixed with bacterial cultures to achieve a final density of 1×10^5 CFU/ml. This mixture was poured into petri dishes to make a 4 mm layer of agarose. Wells of 0.538 cm in diameter were punched in the agarose layer and 20 μl serum from each sampled fish species were loaded into each well separately. 0.01 M phosphate-buffered saline (PBS) alone was used as negative control. After incubation at 37 °C for 24 h, the diameter of growth inhibition zone was measured. The assay for some strains (*Streptococcus iniae*, *Vibrio cholerae*, *Aeromonas sobria*, *Edwardsiella tarda*, *Vibrio alginolyticus*, *Photobacterium damselae subsp. piscicida*) was performed in the same manner but with Brain Heart Infusion Agar (BHI) (BHIA; HuanKai, China) broth. After incubation overnight at 28 °C, the diameters of inhibition zones were recorded. 30 random selected serum samples per fish species were chosen for this experiment. The differences in antibacterial activity were deemed significant when the probability was less than 0.05.

Assays for serum antifungal activity were examined by disc diffusion on potato dextrose agar (PDA) plates as above reported with little changes. Two fungi strain *Aspergillus niger* and *Fusarium gramineaum* were used. The fungi was cultured overnight in PDA broth medium, then a small piece of lawn was seeded on the center of fresh new PDA plate. Sterile paper discs (0.538 cm diameter), impregnated with 20 μl of the serum sample were placed on the PDA plate. After incubation at 35 °C or 28 °C for 3 days respectively, the diameter of the growth inhibition zone was measured. 30 randomly selected serum samples per fish species were chosen for this experiment as previously mentioned.

2.3. Determination of the minimum inhibition titre of the serum to bacteria

The minimum inhibition titre (MIT) of the serum was examined in sterile 96-well plates (Nunc F96 microtiter plates, Jet). Serial twofold dilutions of sera samples in 0.01 M PBS were prepared and 150 μl was added into each well of 96-well tissue culture plates. Addition of PBS alone was used as a negative control. The bacteria