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Short communication

L-amino acid oxidase expression profile and biochemical responses of rabbitfish (*Siganus oramin*) after exposure to a high dose of *Cryptocaryon irritans*

Biao Jiang [a], Jing Wang [a], Heng-Li Luo [a], Ge-Ling Lu [a], Yan-Wei Li [b], An-Xing Li [a, *]

[a] State Key Laboratory of Biocontrol/Guangdong Provincial key Lab for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, Guangdong Province, PR China
[b] College of Marine Sciences, South China Agricultural University, Guangzhou 510642, Guangdong Province, PR China

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ABSTRACT

Cryptocaryon irritans is an important protozoan parasite which infects almost all kinds of marine teleosts, causing heavy economic losses. In our previous studies, we found that rabbitfish (*Siganus oramin*) displayed high resistance to *C. irritans* infection, and a novel protein, L-amino acid oxidase (LAAO), was identified from the serum that was lethal to *C. irritans*. In this study, the rabbitfish were firstly infected with a high dose of *C. irritans*, then the LAAO mRNA expression pattern and the activity of three enzymes [superoxide dismutase (SOD), Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase] were measured in various tissues. The results indicated that, after infection, the feeding and swimming of rabbitfish was normal, and the infection intensity in the host was low. Tissue distribution analysis showed that LAAO mRNA was most pronounced in the head kidney and gill, with lower expression observed in the muscle. After infection with *C. irritans*, the LAAO mRNA was up-regulated early post infection (from 6 to 24 h) in both gill and spleen, but then returned to normal levels, implying that LAAO may play an important role in the host's early immune response. The SOD activity in the liver was significantly higher in the infection group than in the control group by 48 h post infection, while Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities in the gill were decreased by 12 and 24 h after infection; no significant difference was detected at the other time points throughout the experiment. Together, these results suggest that biochemical responses of rabbitfish are relatively mild after infection with a high dose of parasite, and the LAAO may play an important role in the host's defense against *C. irritans*.

1. Introduction

Cryptocaryon irritans is an obligate ciliated parasite which can infect almost all kinds of marine teleosts in tropical and subtropical regions [1,2]. This protozoa parasitizes the skin, gill, fin and cornea of its host, and forms a large number of small white spots that causes a disease commonly known as “white spot disease”. This can then severely impair the physiological functions of skin and gill [3,4]. In recent decades, the marine aquaculture industry has developed rapidly in China; unfortunately, with the increase in fish density, disease caused by *C. irritans* has become a primary parasitic disease, causing huge economic losses [5].

To find effective prevention methods, many physical and chemical methods have been investigated to help prevent and control this disease; heat treatment, freshwater immersion, drying treatment, removing tomonts, ultraviolet radiation and oral chemotherapeutic agents have proven to be somewhat effective [6-10]. However, all of these treatments have some shortcomings. For example, these methods are usually not applicable to large water bodies, cause environmental pollution, are toxic to fish or create chemical residues that build up in the fish flesh. In recent years, researchers have focused on exploring the immune response to *C. irritans*. Some have demonstrated that prior infection or immunization can bestow acquired protection to marine fish species against *C. irritans* [11-13]. However, with primary infections, protection of the fish against *C. irritans* depends heavily on the innate

* Corresponding author. State Key Laboratory of Biocontrol/Guangdong Provincial key Lab for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, 135 Xingang West Street, Haizhu District, Guangzhou 510275, Guangdong Province, PR China.
E-mail address: lianxing@mail.sysu.edu.cn (A.-X. Li).

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immune system [14]. For teleosts, antimicrobial factors are among the earliest molecular facilitators of innate immunity, which play a critical role in defence against pathogens.

In a previous study, we found that rabbitfish (*Siganus oramin*) displayed strong resistance to *C. irritans* infection and their serum presented a strong killing effect on *C. irritans in vitro* [15]. Then a novel antiparasitic protein (L-amino acid oxidase, LAAO) was isolated and purified from the serum [16]. Later studies showed that recombinant LAAO of rabbitfish expressed in *Escherichia coli* is also lethal to *C. irritans* [17]. However, few investigations have reported the expression pattern of LAAO mRNA post *C. irritans* infection; also, the biochemical responses of rabbitfish to parasite infection are largely unknown. In this study, rabbitfish were infected with *C. irritans* at a high dose of 30,000 theronts/fish and various parameters were measured: LAAO mRNA expression level in the gill and spleen, and superoxide dismutase (SOD), Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities in the gill and liver. The hypothesis was that LAAO may play a more important role than other biochemical factors in the host's defence when facing a *C. irritans* infection challenge.

2. Materials and methods

2.1. Cryptocaryon irritans and experimental fish

The *C. irritans* strain was isolated from gills of naturally-infected *Larimichthys crocea* (large yellow croaker), and parasite propagation and collection of the tomonts were conducted by infecting *L. crocea* (average body mass of 100 g) according to Jiang et al. [8].

Healthy juvenile rabbitfish were obtained from a bay in Qiuzhu Village, Ningde City, Fujian Province, PR China. The fish were housed in a 1000-L round plastic tank to acclimatize for 1 month; then fish of a uniform size (21.53 ± 2.12 g) were selected for the experiment. They were fed twice daily with commercial pellet feed and their faeces were siphoned off before feeding. During the trial, sand-filtered seawater was used that had the following characteristics: salinity of 29–31‰, temperature of 27 ± 2 °C, 5.0–7.0 mg/L dissolved oxygen, < 0.2 mg/L inorganic nitrogen and 100 L/h water flow rate.

2.2. Experimental methods and sampling

The rabbitfish were randomly divided into two groups: infected and control, each group containing three similar subgroups, with 25 fish per subgroup. The fish in the infected group were challenged with *C. irritans* at 30,000 theronts/fish as previously described by Dan et al. [18]; non-infected fish served as controls. The whole experiment lasted for 7 days, with food intake and motility observed daily. At 48 h post challenge, the second right gill of three fish from each subgroup were excised and trophonts were counted under a microscope. The infection intensity was described as the number of trophonts on the second right gill divided by the body weight. In addition, the gill, liver and spleen of three fish per subgroup (randomly selected) were sampled at 0 h, 6 h, 12 h, 1 d, 2 d, 3 d, 5 d and 7 d after challenge. The gill and liver were stored at −80 °C; in addition, parts of the gill and spleen samples were stored in RNAsafer Stabilizer Reagent (Takara, Dalian, China).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue samples using Trizol Reagent (Taraka) following the manufacturer's protocol. The total RNA concentration and quality were determined by agarose gel electrophoresis and by using an OD260/280 test, respectively. An aliquot (1 μg) of the total RNA was used to synthesize the first-strand cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. The cDNA was then stored at −20 °C until needed.

2.4. LAAO expression analysis

Real-time PCR was used to detect various tissue expression profiles of LAAO in healthy fish, and the temporal expressions of gill and spleen samples post *C. irritans* infection. The LAAO and β-actin cDNA sequence (GenBank no. HQ540313.1 and EU107278.1) of rabbitfish was downloaded from the NCBI database. The primers Q LAAO F: 5′- GCTGCTGCTTGCTCTGTT-3′, R: 5′-CGTGGTTGGATGTGTTGATG T-3′ with 140 bp and β-actin F: 5′-AATCGTGCGTGACATCAAG-3′, R: 5′-GGAAGGAAGGCTGGAAGA-3′ with 179 bp were designed using Beacon Designer 7.80, and β-actin was used as the internal control. Real-time PCR was performed using a Roche LightCycler 480 Real-time PCR Detection System with SYBR Green Real-time PCR MasterMix (Takara). The PCR cycles were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and at 60 °C for 30 s. The specificities of the PCR products were assessed by melting-curve analysis and sequencing. Each sample was amplified in triplicate. The mRNA expression level of the target gene was calculated relative to the reference gene using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen [19].

2.5. Detection of enzyme activities

The superoxide dismutase (SOD), Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities of gill and liver were measured with assay kits (developed by the Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol supplied with the kits. The samples were homogenized with normal saline (1:9) using an oscillator. Next, the suspension was centrifuged for 10 min at 3000 rpm at 4 °C and the supernatant was used for determination of enzyme activity. To measure SOD activity, the optical density was measured at 550 nm. One unit (U) of SOD activity was defined as the amount required for inhibiting the rate of xanthine reduction by 50% in a 1-mL reaction system, and specific activity was expressed as SOD units per mg protein. The Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities were determined using a colorimetric method. The activity unit was defined as 1 enzyme activity unit being equal to the amount of ATPase required to produce 1 μmol of inorganic phosphorus from the decomposition of ATP in tissues containing 1 mg protein within 1 h (μmol phosphorus/mg of protein/hour), according to Yin *et al.* [20]. The total protein contents of the measured supernatant were determined using the Bradford method [21] with bovine serum albumin as the standard.

2.6. Statistical analysis

All data were analysed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by Duncan's test. The results were presented as mean ± standard error (SE) and graphed using GraphPad Prism5.

3. Results and discussion

3.1. Symptoms

Infection with *C. irritans* at 30,000 theronts/fish did not affect feed intake or mobility of the rabbitfish; the fish were infected at a low intensity of 0.84 ± 0.12. In contrast, in other studies, the same dose of *C. irritans* given to the same size or larger fish sizes can cause serious death and damage, such as with *Epinephelus coioides*, *Sebastiscus marmoratus*, *Trachinotus ovatus* and *L. crocea* [15,20,22].