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Administration of recombinant Reishi immunomodulatory protein (rLZ-8) diet enhances innate immune responses and elicits protection against nervous necrosis virus in grouper *Epinephelus coioides*

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ABSTRACT

Nervous necrosis virus (NNV) infection during larvae and juvenile stage in grouper (*Epinephelus coioides*) has caused severe economic losses in the aquaculture industry in Asia. The aims of this study were to evaluate the influence of recombinant Reishi protein, rLZ-8, on the innate immune responses and the viral resisting ability in fish. Groupers were fed with rLZ-8 supplemented diet (1.25–37.5 mg $_{rLZ\text{-}8}$/kg $_{diet}$), and the cytokine gene expression, innate immune responses, and survival rate after NNV challenge were examined. The fish fed with rLZ-8 diet showed 6- to 11-fold upregulated TNF-α and IL-1β gene expression, along with significant increased respiratory burst and phagocytic activity. Moreover, feeding the fish with 37.5 mg/kg rLZ-8 diet elicited significant improvement in post viral challenge survival rate (85.7%). These discoveries indicated that rLZ-8 could be utilized as an ant-pathogen immunostimulant, and provided a new candidate to fight against NNV infection in fish.

1. Introduction

Grouper, *Epinephelus coioides*, is one of the most important aquaculture species in Asia. It is noticed that intensive culture of grouper has resulted in rapid deteriorations of rearing environments, which led to increased incidence of diseases. In grouper, nervous necrosis virus (NNV) infection during larvae and juvenile stages has caused severe economic losses in the aquaculture industry. Strategies such as vaccination [1,2] and ozone sterilization of water and eggs [3] have been developed to fight against viral infection, and have been reported to inhibit the severity of NNV infection.

Besides the strategies mentioned above, recent researches have demonstrated that application of immunostimulants might be effective in treating fish diseases. Wang et al. have shown that intraperitoneal injection of antimicrobial peptides was effective in reducing NNV infection in grouper [4]. Dietary administration of sodium alginate [5,6], probiotics [7], and iota-carrageenan [8] have exhibited efficacies in stimulating innate immune responses such as respiratory burst (RB), superoxide dismutase (SOD) activity, complement formations, phagocytic activity, and lysozyme activity in groupers. In addition, feeding grouper with these immune enhancing substances also provided significant resistance against both bacterial and viral challenges in grouper [5–8]. These evidences strongly suggested the relevance between enhanced innate immune response and viral resistance; however, whether application of immunostimulants could elicit resistance against NNV in grouper remained untested.

Reishi, *Ganoderma lucidum*, also known as Ling-zhi, is a medicinal fungus possessing numerous therapeutic effects [9]. The protein purified from Reishi was first discovered by Kino in 1989 and denominated LZ-8 [10]. Since then, the gene of LZ-8 has been cloned and its structure has been characterized [11,12]. Numerous studies have shown that LZ-8 exhibited various immunomodulatory effects such as stimulating cell proliferation and IL-2 secretion in human T lymphocyte [13,14], inducing the activation and maturation of human monocyte-derived dendritic cells [15] and mice dendritic cells [16]. Our previous research also demonstrated that, in mice, recombinant LZ-8 (rLZ-8) could induce TNF-α, IL-1β

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and IL-12p70 cytokine production by macrophage, and that rLZ-8 could stimulate the activation of mice $CD4^+$ and $CD8^+$ T-lymphocytes [17].

In the present study, we evaluated the potential of rLZ-8 expressed by *Saccharomyces cerevisiae* to strengthen grouper innate immunity and its ability to protect groupers against NNV challenge. We first tested the influence of rLZ-8 on fish immune response *in vitro* by measuring the respiratory burst of rLZ-8 stimulated grouper head kidney leukocytes. We then evaluated whether dietary administration of rLZ-8 exhibited immunostimulatory effects *in vivo* by examining cytokine gene expression, respiratory burst, and phagocytic activity of the grouper fed with rLZ-8 diet. Finally, to determine the protective strength elicited by rLZ-8, we conducted NNV viral challenge experiments on the grouper fed with rLZ-8 diet.

2. Materials and methods

2.1. Experimental design

One *in vitro* study and three *in vivo* experiments were conducted. In the *in vitro* study, 5 fish were bled for leukocyte isolation in each experiment. The acquired leukocytes were pooled for LZ-8 induction. The superoxide and reactive oxygen species production were examined.

In the first *in vivo* experiment, the cytokine gene expression study, a total of 160 fish were used. The fish were divided into four groups, each consisted 40 fish, and fed with control or rLZ-8 supplemented diet. Eight fish were sampled at each indicated time point for mRNA extraction.

In the second *in vivo* experiment, the innate immune response study, a total of 140 fish were used. The fish were divided into four groups, each consisted 35 fish, and fed with control or rLZ-8 supplemented diet. Five fish were sampled at each indicated time point for innate immune response analysis.

In the third *in vivo* experiment, the NNV-challenge test, a total of 324 juvenile fish were used in two independent experiments. In each experiment, 162 fish were divided into six groups, each consist 27 fish. In the six groups, five groups were fed with control or rLZ-8 supplemented diet, and nine fish from each group were randomly selected for NNV injection at each indicated time point. The remained group was fed with control diet, and nine fish were injected with PBS rather than NNV at each indicated time point.

2.2. Fish and culture conditions

Groupers (*E. coioides*) were purchased from Aquatic Animal Center in Keelung, Taiwan, and were shipped to the Department of Aquaculture, National Taiwan Ocean University. For *in vitro* experiment, cytokine gene expression analysis, and innate immune response study, the fish were maintained indoors in 96-L tanks (60 × 40 × 40 cm) with recirculating, filtered, ozone-sterilized, aerated seawater (35‰) at 27 ± 1 °C. For NNV-challenge test, the fish were maintained in 91.125-L tanks (45 × 45 × 45 cm) with non-circulating seawater (35‰) at 27 ± 1 °C. In the non-circulating system, water exchange was conducted at five-day intervals, where fresh, ozone-sterilized, aerated seawater replaced the original water in the tanks. All NNV-challenged fish were maintained in one tank to avoid tank-to-tank variation. All fish were fed with the basal diet for one week before conducting each experiment. The average weight of the fish for cytokine gene expression analysis, immune parameter assays, and NNV-challenge tests were 30 ± 5 g, 100 ± 15, and 0.5 ± 0.1 g (mean ± SD), respectively.

2.3. Sample preparation

The rLZ-8 samples used in this study were produced via the heterogeneous expression system described in our previous report [17]. In brief, rLZ-8 was expressed intracellularly by *S. cerevisiae* DBY747 (MAT*a his3-1 leu2-3,-112 trp1-289 ura3-52*). The cultured mycelia were homogenized to release rLZ-8.

To purify the rLZ-8 for the *in vitro* study, the homogenate was further purified by fast protein liquid chromatography system through HiTrap Q column (Amersham Biosciences, Uppsala, Sweden). The purified rLZ-8 was validated via SDS-PAGE and western blot (Fig. 1A) using the methods described in our previous report [17]. The rLZ-8 content in the purified sample was determined by BCA protein concentration analysis using bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) as a standard. The rLZ-8 was dialyzed into Hank's balance salt solution (HBSS) and adjusted to appropriate concentrations (1.25, 6.25, 12.5, 31.25, 62.5 μg/mL) for *in vitro* studies.

To prepare the test diets for *in vivo* experiments, the homogenates from rLZ-8 producing *S. cerevisiae*, were incorporated into the basal diet. The rLZ-8 content in the homogenates was 0.125 mg/mL as determined by quantitative ELISA using standard curve established using the purified rLZ-8 (Fig. 1B). A diet containing homogenates from non-transgenic *S. cerevisiae* was prepared as a control

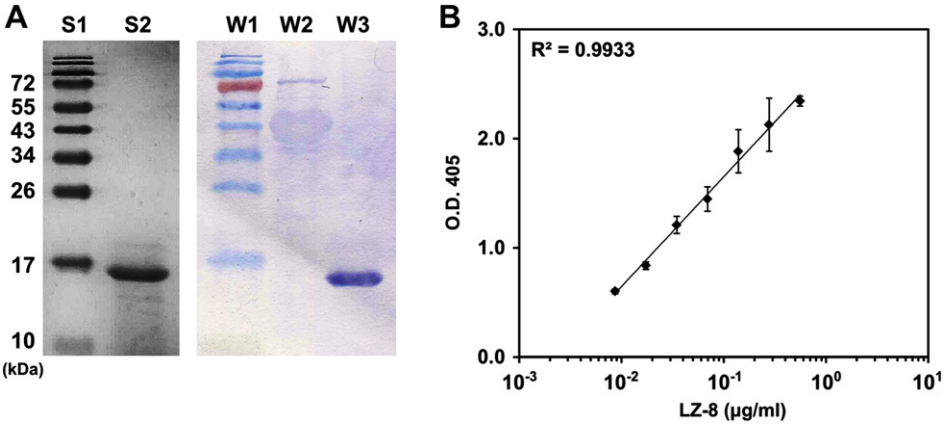

Fig. 1. The SDS-PAGE and western blot analysis of the purified rLZ-8 sample (A) and the rLZ-8 standard curve for quantitative ELISA (B). The content of each lane in the SDS-PAGE (S1-S2) and western blot (W1-W3) were pre-stain protein marker, purified rLZ-8 sample, pre-stain protein marker, ovalbumin, and purified rLZ-8 sample for S1, S2, W1, W2, and W3, respectively.