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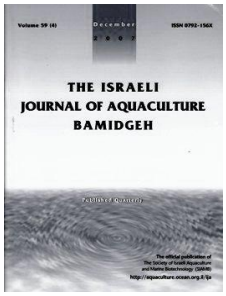

Effect of Marine Red Yeast *Rhodosporidium paludigenum* on Antioxidant-Related Gene Expression in *Litopenaeus vannamei*

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Abstract

The effects of orally-administered dry and live marine red yeast *Rhodosporidium paludigenum* on antioxidant-related gene expression in the hepatopancreas and hemocytes of *Litopenaeus vannamei* were investigated by RT-PCR. In the hepatopancreas of *L. vannamei* fed dry yeast, manganese superoxidate dismutase (SODMn) and catalase (CAT) were enhanced while glutathione peroxidase (GPx) and ferritin remained at the same levels as in the control group. In the hepatopancreas of *L. vannamei* fed live yeast, SODMn and ferritin were higher than in the control. In hemocytes of *L. vannamei* fed dry yeast, SODMn was lower, ferritin was similar, and CAT and GPx fluctuated, in comparison to the control. In hemocytes of *L. vannamei* fed live yeast, SODMn was lower, ferritin was higher, and CAT and GPx tended to be lower than in the control group. Results suggest that consumption of the marine red yeast, *R. paludigenum*, can stimulate antioxidant gene expression in the hepatopancreas of shrimp.

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Introduction

Reared shrimps are exposed to pathogens of many kinds (Mohankumar and Ramasamy, 2006; Flegel, 2009; Hameed, 2009), hypoxia (Zenteno-Savín et al., 2006), and other environmental perturbations (Le Moullac and Haffner, 2000; Lesser, 2006) that can lead to physiological and/or antioxidant system malfunctions. Pathogenic micro-organisms such as *Vibrio alginolyticus* and environmental stress can directly affect the expression of antioxidant genes in aquatic animals (Liu et al., 2007a; Zhou et al., 2008; Wang et al., 2009). Thus, enhancing defensive abilities is important in shrimp culture. Vitamin E (Liu et al., 2007b), carotenoids (Pan et al., 2003), mannan oligosaccharide (Özlüer-Hunt et al., 2011a), selenium (Ozluer-Hunt et al., 2011b), and probiotics (Chiu et al., 2007; Castex et al., 2010, Yang et al., 2010) can affect the antioxidant competence of cultured shrimp.

Red yeasts are microbial species normally found in the natural environments of shrimp culture. They include carotenoids and mannan oligosaccharide that can promote antioxidant competence in aquatic animals. For example, the red yeast, *Phaffa rhodozyma*, has a reducing effect on oxidized oil-induced oxidative stress in fish (Nakano et al., 1999). In an earlier study, we found that a diet supplemented with marine red yeast (*Rhodosporidium paludigenum*) enhances the growth performance and antioxidant competence of *Litopenaeus vannamei*, and has the potential to be a promising probiotic (Yang et al., 2010). In the present study, we investigated changes in antioxidant-related gene expression in *L. vannamei* fed dry or live *R. paludigenum*. These genes include manganese superoxidate dismutase (SODMn), catalase (CAT), glutathione peroxidase (GPx), and ferritin, which play important roles in the antioxidant defense system of shrimp (Zhou et al., 2008; Wang et al., 2009).

Materials and Methods

Rhodosporidium paludigenum was selected as a test candidate due to its high carotenoid content and innocuous presence in the intestinal mucus of *L. vannamei*. The yeast was isolated from coastal waters at Zhanjiang, China, cultured in a yeast/peptone/dextrose medium at 28°C, and harvested by centrifugation at 4000 *g* for 10 min at 4°C (Yang et al., 2010). Two experimental diets were prepared from a commercial pelleted diet (Guangdong Yuehai Feed Group Co., Ltd.): (a) dry yeast at 1 g/100 g diet and (b) live yeast at 10^8 yeast cells (hemocytometer counts)/1 g diet. The commercial diet without yeast was used as the control diet. The diets were air-dried for 2 h and stored at -20°C until use.

A batch of apparently healthy *L. vannamei* (6.0±0.51 g) was brought to the laboratory from the Yuehai Shrimp Farm located at Donghai Island, Zhanjiang, China. After 7 d quarantine, the shrimps were transferred to nine plastic 200-l tanks (three per treatment) at a density of 40 shrimp per tank. The diets were given four times a day at a daily rate of 8% body weight. The water was aerated continuously and always saturated with oxygen. Feces and food remains were removed daily by siphoning. The water temperature was maintained at 28.0-29.0°C, salinity at 0.5%, and pH at 7.4-8.0.

Samples from each group were collected on days 0, 1, 4, 7, 10, and 14. Shrimps were anesthetized in cold water (Luedemana and Lightnera, 1992), then tissues from the hepatopancreas were excised by stainless scissors under cold conditions. Hemolymph was withdrawn aseptically from the ventral blood sinus using a sterile syringe containing 0.5 ml sterile anticoagulant and transferred to a sterile microcentrifuge tube. The hemolymph of three shrimp from each tank was pooled and immediately centrifuged at 800 *g*, 4°C, for 10 min to harvest the hemocytes. The hemocytes were washed twice with sterile anticoagulant and centrifuged again. The hemocyte pellets and hepatopancreas tissues were collected and immediately suspended in TRIzol (Invitrogen, USA) for total RNA extraction according to the manufacturer's instructions.

First-strand cDNA synthesis was based on reverse transcriptase (Takara, Japan) using total RNA treated with DNase I (NEB, USA) as the template. Reaction conditions recommended by the manufacturer were followed.

Table. 1. Oligonucleotide primer sequences.

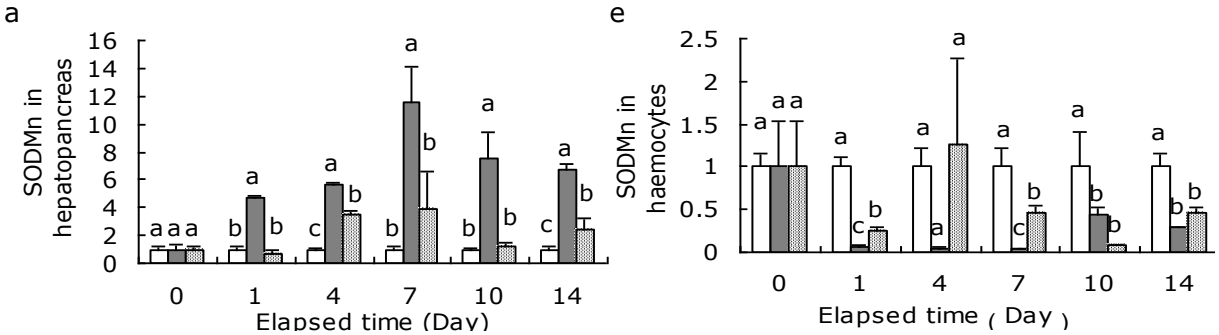
Target gene	*Accession no.*	*Primer sequence (5′ → 3′)*	*Position*	*Product (bp)*
SOD_{Mn}Fr	DQ005531	AAT TGG AGT GAA AGG CTC TGG CT	583-735	153
SOD_{Mn}Rr		ACG GAG GTT CTT GTA CTG AAG GT		
CATFr	AY518322	TCA GCG TTT GGT GGA GAA	1428-1574	147
CATRr		GCC TGG CTC ATC TTT ATC		
GPxFr	AY973252	AGG GAC TTC CAC CAG ATG	237-353	117
GPxRr		CAA CAA CTC CCC TTC GGT A		
FerritinFr	AY955373	CAA GTC CGC CAG AAC TAC	142–280	139
FerritinRr		TGG CAA ATC CAG GTA GAG		
actinFr	AF300705	GCC CAT CTA CGA GGG ATA	522-642	121
actinRr		GGT GGT CGT GAA GGT GTA A		

Specific primer pairs of SOD_{Mn}, CAT, GPx, ferritin, and β-actin were used to quantify antioxidant-related genes (Table 1). Specific primer pairs for SOD_{Mn} were based on the NCBI sequence DQ005531. Specific primer pairs for CAT, GPx, and β-actin were described by Wang et al. (2009). The specific primer pair for ferritin was described by Zhou et al. (2008). All primers were produced by Sangon (Shanghai, China). RT-PCR assays were carried out in an ABI7300 real-time PCR system (Applied Biosystems, USA). Amplifications were performed in a 96-well plate in 25-μl reaction volume containing 12.5 ml of 2 × SYBR Premix Ex Taq (Takara, Japan), 0.5 μl forward primer (10 mM), 0.5 μl reverse primer (10 mM), and 11.5 μl diluted cDNA. The thermal profile for RT-PCR was 94°C for 3 min followed by 40 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s. Dissociation curve analysis of the amplification products was performed at the end of each PCR reaction. The related gene expressions were determined according to the ΔΔCt method using IQ5 software (Bio-Rad; Tovar-Ramírez et al., 2010). Individual samples were analyzed in triplicate and results are expressed as fold-changes in the experimental groups as compared to the control group.

Gene expression data are presented as relative values using β-actin as a housekeeping gene. Data are expressed as means±SD. The effects of the marine red yeast on the antioxidant-related gene expression were assessed by one-way ANOVA using SPSS v13.0 (SPSS, Chicago, IL, USA). The level of statistical significance was set at $p \leq 0.05$.

Results

On day 14, SOD_{Mn} expression was higher in the hepatopancreas and lower in hemocytes of shrimps fed the dry or live yeast than in those fed the control diet, while GPx expression did not differ from the control (Fig. 1). CAT expression fluctuated in the hepatopancreas and hemocytes of shrimp fed the dry or live yeast diets. Ferritin was significantly higher in the hepatopancreas and hemocytes of shrimps fed the live yeast than in those fed the dry yeast or control diets.



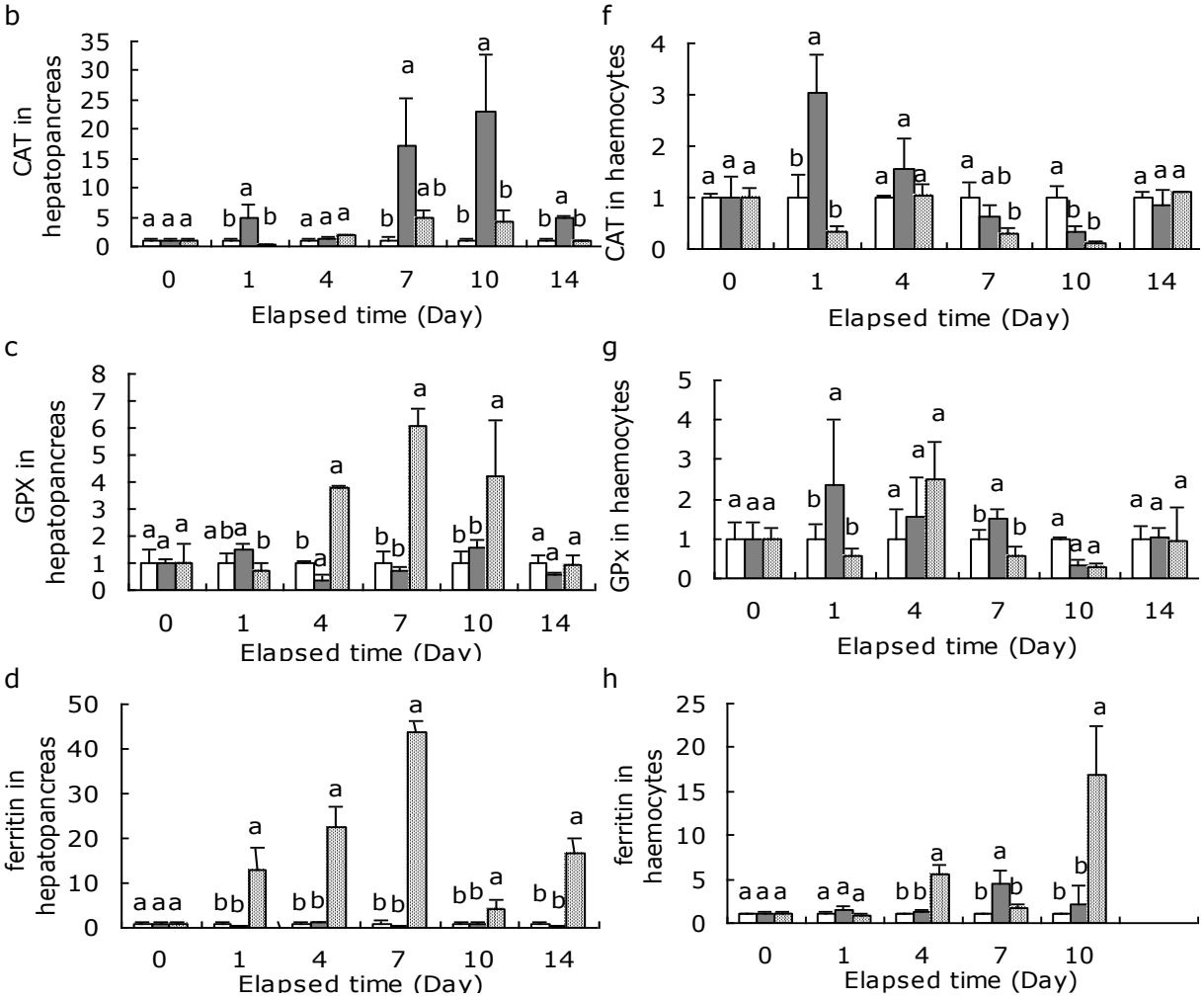


Fig. 1. Expression of (a) SOD_{Mn}, (b) CAT, (c) GPx, and (d) ferritin in the hepatopancreas, and (e) SOD_{Mn}, (f) CAT, (g) GPx, and (h) ferritin in hemocytes of *Litopenaeus vannamei* fed dry or live red yeast *Rhodosporidium paludigenum*. Different lower case letters above bars indicate significant differences on that day (p<0.05), □ = control, ■ = dry yeast, ▒= live yeast.

Discussion

There are reports concerning the effects and mechanisms of probiotics on the activity of antioxidant enzymes in aquacultured animals (Chiu et al., 2007; Castex et al., 2010; Ganguly et al., 2010; Yang et al., 2010). However, few reports mention the effects of probiotics on expression of antioxidant-related genes in aquacultured animals. The expression level of prophenoloxidase (proPO) mRNA in *L. vannamei* fed diets containing *Lactobacillus plantarum* was significantly higher than that of shrimps fed a control diet after 168 h (Chiu et al., 2007). But *Dicentrarchus labrax* larvae fed *Debaryomyces hansenii* showed lower activity and gene expression levels of glutathione peroxidase (GPx) and superoxidate dismutase (SOD) than fish fed a control diet (Tovar-Ramírez et al., 2010).

SOD, CAT, and GPx play an important role in the antioxidant defense of organisms. Dietary inclusion of β-1,3-glucan significantly enhanced SODMn in the hepatopancreas of *L. vannamei*; it rose during the first 12 h, peaked at 12 h (p<0.05), and returned to the control level at 24 h (Wang et al., 2008). SOD and GPx activity in the hepatopancreas was higher in *L. vannamei* fed marine red yeast (Yang et al., 2010). *Litopenaeus stylirostris* fed probiotic *Pediococcus acidilactici* for one month, then challenged with *Vibrio nigripulchritudo*, had higher GPx activity in the digestive gland at 24 and 48 h than the control (Castex et al., 2010).

In the present experiment, expression of CAT and SOD was higher in the hepatopancreas and lower in the hemocytes of shrimps fed dry or live yeast than in shrimps fed the control diet. The hepatopancreas is the major metabolic center for production of reactive oxygen species and important tissues for immune defense in crustaceans (Arun and Subramanian, 1998). In our study, supplementation of *R. paludigenum* enhanced expression of some antioxidant genes in the hepatopancreas and, subsequently, oxidative stress in the shrimps decreased. Meanwhile, SODMn and CAT expression in the hemocytes decreased, indicating that marine red yeast can enhance the antioxidant competence of *L. vannamei* by increasing the gene expression of antioxidant enzymes.

Ferritin is a cytosolic iron storage protein involved in iron metabolism. Ferritin mRNA is expressed in the hemocytes, midgut gland, brain ganglion, gills, hepatopancreas, abdominal ganglion, eyestalk, muscle, thoracic ganglion, and heart of shrimp (Hsieh et al., 2006). Ferritin, the protein that orchestrates cellular defense against stress and inflammation, can repress the Fenton reaction by restoring excess iron and controlling ROS generation (Torti and Torti, 2002). In our study, dietary dry yeast did not influence ferritin expression in the hepatopancreas but ferritin expression in the hepatopancreas and hemocytes of *L. vannamei* fed live yeast was higher than in the control group. Some red yeasts can produce siderophores and rhodotorulic acid, and siderophores are related to iron metabolism (Atkin et al., 1970). Further research is needed to determine if they affect the metabolism of iron and ferritin gene expression in *L. vannamei*.

In conclusion, oral administration of the marine red yeast, *R. paludigenum*, enhanced the expression of some antioxidant-related genes in the hepatopancreas of *L. vannamei*. When compared to the control group, ferritin expression in the hepatopancreas and hemocytes of *L. vannamei* fed live yeast increased. Results indicate that *R. paludigenum* can enhance some antioxidant genes and the antioxidant competence of shrimp.

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