Superoxide dismutase as modulator of immune function in American white shrimp (*Litopenaeus vannamei*)

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Abstract

The immunomodulatory action of superoxide dismutase (SOD) and its possible use as an indicator of immune responses in American white shrimp (*Litopenaeus vannamei*) were studied. Juvenile shrimp were immersed in aerated β-glucan and sulfated polysaccharide solutions for 6 h. SOD activity in haemocytes and muscle was quantified to evaluate whether β-glucan and sulfated polysaccharide induce immunostimulatory activity. Haemocytes and muscle showed similar increased levels of SOD activity (1.5- and 1.4-fold that of control, respectively). Total haemocyte count decreased within the first 24 h after challenge with immunostimulants, but total haemocyte count and total soluble haemocyte protein increased over normal values after 48–120 h. Single immunostimulation with β-glucan and sulfated polysaccharide is sufficient to generate an increase in the antioxidant activity of *L. vannamei* SOD.

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1. Introduction

In shrimp, microbial surface antigens, such as lipopolysaccharides (LPS) and β-glucans, activate cellular functions, acting directly on the non-specific immune system, and are used as an alternative approach to antibiotic treatment and vaccination in the prevention of diseases in aquaculture species. The efficacy of oral administration of sulfated polysaccharides against viral infections has been proven (Baba et al., 1988). The mechanism of action of these compounds has been attributed to the inhibition of virus adsorption to host cells (Takahashi et al., 1998). In contrast, some authors have demonstrated that excessive doses of LPS or glucan may be toxic to organisms (Sung et al., 1994; Lorenzon et al., 1999).

To initiate an immune response, some compounds have to be present in the body of the shrimp, which recognises foreign material that has gained entrance into the body. The blood cells of invertebrates are the primary effectors of host defence and are involved in numerous immune processes, as demonstrated in phagocytosis, melanisation, encapsulation and coagulation studies (Söderhäll and Cerenius, 1992). The blood cells of decapod crustaceans, called haemocytes, have generally been classified into three categories, hyaline cells, and small and large granular cells (Söderhäll and Smith, 1983; Hose et al., 1990; Vargas-Albores, 1995; Le Moullac et al., 1997).
In vertebrates, upon suitable stimulation of macrophages, phagocytosis is associated with the production of reactive oxygen species (ROS), such as superoxide anion (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), singlet oxygen (\(O_3\)) and hydroxyl radical (\(OH^-\)), that are highly microbiocidal (Bachère et al., 1995; Muñoz et al., 2000). The process starts when stimulation leads to increased consumption of oxygen, the reduction of which, catalysed by a membrane-bound NADPH oxidase, gives rise to superoxide anion. The antioxidant superoxide dismutase (SOD) converts this microbiocidal metabolite into hydrogen peroxide that passes freely through membranes. The antioxidants catalase and glutathione peroxidase remove the hydrogen peroxide from cells. Although these microbiocidal agents are generated in the phagocytic vacuoles, an important quantity crosses into the extravascular and extracellular environment and may cause damage to cells (Warner, 1994). To prevent this damage, cells and organisms use three defence strategies. One involves low-molecular-weight antioxidants, such as ascorbate, \(\alpha\)-tocopherol and glutathione, that can directly interact with ROS to neutralise them. The other two involve a variety of enzymes that either metabolise ROS (SOD, catalase, glutathione peroxidase) or repair macromolecular damage to nucleic acids, proteins and lipids (DNA repair enzymes, proteases, lipases, etc.) caused by ROS (Warner, 1994).

The complex antioxidant system of aerobic organisms has been developed not only to prevent the effects of ROS, but also to play a vital role in protecting cells from oxidative stress (De la Fuente and Victor, 2000; Downs et al., 2001) draining the cellular pool of reducing agents (NAD, NADPH, GSH) to maintain a reduced environment in cells (Muñoz et al., 2000; Downs et al., 2001).

While production of \(O_2^-\) (respiratory burst) has been reported as an accurate method to measure the effectiveness of potential immunostimulants (Song and Hsieh, 1994; Muñoz et al., 2000), antioxidants are potential indicators of oxidative stress on marine organisms (Agius et al., 1998; Neves, et al., 2000; Downs et al., 2001).

This paper focuses on the effect of \(\beta\)-1,6 glucan and sulfated polysaccharide on Mn–SOD activity from haemocytes and muscle of \(L.\ vannamei\), and discusses the role of this enzyme in modulating oxidative cellular products and oxidative stress in response to immunostimulants. This leads to increased or decreased circulating haemocytes (THC) and cellular proteins that may have a protective effect against infectious agents.

2. Materials and methods

2.1. Experimental animals

Healthy juvenile American white shrimp (\(Lito\- penaeus\) \(\text{vannamei}\)) were obtained from a commercial shrimp laboratory (Acuacultores de la Peninsula, La Paz, B.C.S., Mexico). Shrimp were transported in seawater (27 \(^\circ\)C, pH 7.8–8.2, and 32\% salinity) in a polystyrene box from the commercial shrimp facility to the CIBNOR wet laboratory. Shrimp were acclimated in a 1500-l fibreglass tank with filtered (0.2 \(\mu\)m) seawater containing 10 mg l\(^{-1}\) of EDTA disodium salt under constant aeration. Filtered seawater was maintained at pH 7.8–8.2, 27 \(^\circ\)C, and 35\% salinity. Shrimp were acclimated for 15 days prior to the experiments and were fed daily with a commercial pellet containing proteins, carbohydrates, vitamins and minerals. Only shrimp weighing 10–12 g were used.

2.2. Immunostimulants

\(\beta\)-1,6-Glucan from \(Saccharomyces\) \(\text{cerevisiae}\) (Biotec Mackzymal, Tromsø) and sulfated polysaccharide isolated from \(\text{Cyanothece}\) sp. PE 14 cyanobacteria strain, as previously reported (De Philippis et al., 1998), were used as immunostimulants. \(\beta\)-1,6-Glucan and sulfated polysaccharide suspensions were mixed with 50 l of seawater to obtain final concentrations of 0.5 mg ml\(^{-1}\) and 1 \(\mu\)g ml\(^{-1}\), respectively.

Cyanobacteria strains isolated from hypersaline environments were photoautotrophically grown in enriched seawater or in Zarrouk medium, as previously described (De Philippis et al., 1998). Exopolysaccharides were separated following the methods described by De Philippis et al. (1993). Briefly, cells were removed from the culture medium by centrifugation (14 000 \(\times\) g, 10 min at 10 \(^\circ\)C). Soluble polysaccharides were obtained from the supernatants by the addition of two volumes of isopropanol, and the precipitate was harvested and dried for 3 h at 50 \(^\circ\)C.
2.3. Experimental protocol (β-1,6-glucan, and sulfated polysaccharide)

Treatment groups of 25 shrimp were housed in 200-l fibreglass containers. One group (BG-1) was immersed for 6 h in 0.5 mg ml⁻¹ of β-glucan, based on a previous report by Sung et al. (1994), and another group (SP-1) in 1 μg ml⁻¹ of sulfated polysaccharide, based on previous bioassays performed to select the optimal concentration. A second dose of β-glucan was applied at 24 h to group BG-1. A control group (C-1) was immersed in seawater free of β-glucan and sulfated polysaccharide under the same conditions as the experimental groups. Following β-glucan or sulfated polysaccharide exposure, shrimp were kept in aerated seawater and fed three times daily. Three shrimp per treatment were randomly sampled after the exposure to immunostimulants at 6, 24, 48, 72, and 120 h (and at 18 h in some experimental series). For each sample, haemolymph was immediately analysed and 2.0 g of muscle (extracted from tail) was stored at −80 °C.

2.4. Haemocyte collection

Approximately 1.0 ml of haemolymph was collected from the pleopod base of the first abdominal segment near the genital pore. Haemolymph was obtained using a 27-gauge hypodermic needle on a 3.00-ml syringe, containing 600 μl of cooled (4 °C) anticoagulant solution with ionic and osmotic strength values of shrimp haemolymph (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na₂, 10 mM HEPES, pH 7.3, 850 mOsm kg⁻¹) (Vargas-Albores and Ochoa, 1992; Vargas-Albores et al., 1993, 1997). The haemolymph was placed in sterile glass tubes and kept in an ice bath. Total haemocyte count (THC) was performed immediately in a haemocytometer using a phase-contrast microscope (Nikon, Optiphot-2). The ratio of the THC from the stimulated shrimp to the THC of control shrimp was used as an index for comparing the effects of different treatments on the haemocyte content. The results were expressed as relative haemocyte count (RHC).

2.5. SOD extraction

For cell disruption, 100 mg of frozen shrimp muscle or 100 μl of haemocyte suspension was added to a mechanical homogenizer containing 0.5 ml of phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged (5724 × g for 5 min at 4 °C), and the supernatant was recovered and heated for 5 min at 65 °C. A new supernatant was obtained after a second centrifugation (crude extract) and stored at −20 °C. Samples were maintained on ice at all times.

2.6. SOD activity assay

SOD activity was determined according to Beauchamp and Fridovich (1971) using nitroblue tetrazolium (NBT) in the presence of riboflavin. For this, 2 ml of reaction mixture (0.1 mM EDTA, 13 μM methionine, 0.75 mM NBT and 20 μM riboflavin in 50 mM phosphate buffer at pH 7.8) and 0–100 μl of crude extract were placed under fluorescent light for 2 min or until A₅₆₀ in control tubes reached an optical density (OD) value of 0.2–0.25. The specific activity (units per mg of protein) was calculated using a computer program (Vazquez-Juarez et al., 1993). The ratio of specific activity from the stimulated shrimp treatments to specific activity of the controls was used as an index for comparing the effects of different treatments in Mn–SOD activity. The results were expressed as relative enzyme activity (SODA).

2.7. Protein

Total soluble protein concentration in crude extracts was measured according to Bradford (1976) with serum albumin (BSA) as a standard. The ratio of OD₅₉₅ from the stimulated samples to the OD₅₉₅ of control sample haemocytes or muscle was used as an index for comparing the effects of different treatments on protein content. The results were expressed as relative protein content (RPC).

2.8. Statistical analysis

All the measurements were made in triplicate. The results were studied by analysis of variance (ANOVA) using the Tukey test to analyse the differences (STATISTICA software). Values of P < 0.05 were considered significantly different.

3. Results

3.1. SOD activity

Exposure of shrimp to sulfated polysaccharide or β-glucan for 6 h induced a non-significant (P >
0.05) SOD increase in muscle and haemocytes (Fig. 1a,b, respectively).

SODA in muscle increased 1.4-fold higher than the control group at 24 h after exposure to immunostimulants (Fig. 1a). In addition, SOD decreased in the control group 48 h after challenge and dropped below control values at 72 h (Fig. 1a).

Fig. 1b shows SOD activity in shrimp haemocytes in response to single or double exposure to β-glucan for 6 h. The SOD response was 1.5-fold higher than the control group at time 6. In haemocytes, there were increased levels of SOD at 6 h, but similar values to those in the control group were reached at 24 h (Fig. 1b), while SOD in muscle had maximum values at 24 h (Fig. 1a). SOD decreased significantly \( (P<0.05) \) in shrimp haemocytes at 72 h after challenge (Fig. 1b).

3.2. Soluble protein

RPC in shrimp muscle remained constant over time after the challenge with β-glucan and sulfated polysaccharide.
polysaccharide. No significant differences ($P > 0.05$) in protein content of shrimp muscle were observed (Fig. 2a). However, there was a small increase, approximately 1.2-fold higher than the control group, at 48 h following 6-h immersion of shrimp in $\beta$-glucan (Fig. 2a). In contrast, RPC of shrimp haemocytes increased significantly ($P < 0.05$) 72 h after double exposure and 120 h after single exposure to $\beta$-glucan (Fig. 2b). The increase in RPC in shrimp haemocytes was faster with double than with single exposure to $\beta$-glucan (Fig. 2b).

### 3.3. Haemocyte count

The increase in RHC observed 72 h after exposure with $\beta$-glucan was not statistically significant ($P > 0.05$) (Fig. 3).
RHC of shrimp haemolymph (Fig. 3) showed the same pattern as RPC (Fig. 2b). That is, the highest circulating haemocyte count was obtained at 120 h post-treatment of shrimp using single or double glucan (2E) exposure (Fig. 3). Single exposure with β-glucan decreased THC values at time 6 (Fig. 3). Following the same tendency, the lowest values of RHC remained for the control group using double activation with β-glucan at 6 and 24 h (Fig. 3).

4. Discussion

Immunostimulants are applicable to larval, juvenile and adult stages of fish and shrimp (Leonard et al., 1985; Vargas-Albores et al., 1998; Biswas and Mandal, 1999; Homblad and Soderhall, 1999) and aim at enhancing the non-specific defence mechanisms (Song and Sung, 1990). Enhanced survival of shrimp to bacterial pathogens after challenge with immunostimulants has been reported (Itami et al., 1998; Takahashi et al., 1998; Sritunyalucksana et al., 1999), but there is little knowledge regarding cellular and humoral responses of shrimp to immunostimulants (Vargas-Albores, 1995).

In immune defence research of crustaceans, a thorough understanding of haemocyte function is important, but has not been studied in detail, particularly the capacity to generate oxidative and antioxidant responses (Roch, 1999). Furthermore, some authors have reported the importance of shrimp tissues as sites of recognition and removal of bacteria and virus (Martin et al., 1993; Jiravanichpaisal and Miyazaki, 1994; De la Peña et al., 1995).

Superoxide dismutases are one of the main antioxidant defence pathways in response to oxidative stress (Fridovich, 1995). Increased SOD activity in L. vannamei, after challenge, appeared earlier in haemocytes (6 h) than in muscle (24 h). In addition, we found that SOD activity decreased in haemocytes after 48-h exposure to immunostimulants, similar to the enzymatic activity reported in Palaemonetes argentinus shrimp related to oxidative stress (Neves et al., 2000).

The increase in RPC 48 h after exposure to immunostimulants (Fig. 2b) may be related to the increase in proteins involved in stress (Jennissen, 1995). Downs et al. (2001) reported increased levels of Mn–SOD, glutathione, heat shock proteins and ubiquitin in grass shrimp P. pugio after heat stress, specifically in response to increased protein synthesis and denaturation (Ellis, 1996), indicating that mitochondria were experiencing and responding to oxidative stress. Fridovich (1995)
reported that increased levels of Mn–SOD provided tolerance to factors that induce oxidative stress, and moreover that Mn–SOD is a specific indicator of mitochondria experiencing oxidative stress. According to these authors, increased SOD activity in *L. vannamei* haemocytes may promote the generation of other immunoproteins after 48 h of exposure to immunostimulants (Fig. 2b).

The increase in respiratory burst capacity (Sung et al., 1994; Muñoz et al., 2000) and antioxidant levels in stimulated haemocytes is considered to be a response to changes in the lipid composition of cell membranes, and to enhance the production of cell-activating factors (cytokines or chaperonins) that may improve the phagocytic capability of haemocytes (Itami et al., 1998). Therefore, increased antioxidant and immunostimulants in cells (the strongest immune response) against pathogens is expected after exposing shrimp to immunostimulants (Sung et al., 1994; Muñoz et al., 2000; Downs et al., 2001).

Haemocytes play an important role in removing foreign particles such as bacteria from haemolymph by phagocytosis (Ratner and Vinson, 1983). The decrease in RHC observed at 6 and 24 h following immersion of shrimp in the immunostimulants (Fig. 3) can be associated with oxidative stress and the possible susceptibility of shrimp to potential pathogens (Johansson and Soderhall, 1992). In addition, the use of THC as a potential indicator of immune status in shrimp has been reported (Henning et al., 1998; Le Moullac et al., 1998; Lorenzon, et al., 1999). In crustaceans, THC is a stress indicator, but varies non-specifically according to the natural rhythms of the environment, as well as chemical and physico-chemical stress. For example, shrimp show several adaptive responses to hypoxia (Hill et al., 1991). The response of the shrimp *P. stylirostris* exposed to severe hypoxia (1 mg O₂ ml⁻¹) was measured in terms of THC (Le Moullac et al., 1998). Hypoxia induced a significant decrease in THC, with a significant decrease in semi-granular and hyaline cells. In *P. monodon*, the phagocytic activity of haemocytes was less efficient in oxygen-depleted shrimp (Direkbusarakom and Danayadol, 1998). Victor et al. (1990) showed that decreased circulation of haemocytes can be a consequence of haemocyte immobilisation in the gills in response to Hg. In contrast, the increase in RHC 48 h after exposure to immunostimulants may be related to the protective effects of the shrimp immune system against potential pathogens (Chisholm and Smith, 1995).

Sung et al. (1994) and Dugger (1999) reported that twice-a-week exposure to food containing β-1,3 glucan is sufficient to maintain optimum, non-specific immune cell activation in shrimp. In the present study, only a single exposure with β-glucan was necessary to activate the haemocyte antioxidant responses, as well as RPC and RHC.

If the oxidant/antioxidant balance is an important determinant of immune cell function, including the control of signal transduction and gene expression, increased levels of antioxidants will be needed for improving immune response.

In addition, it is important to design studies focusing on which components are regulated by the differentiation and proliferation of circulating haemocytes after a challenge with immunostimulants, similar to the conditions in fish and humans, where cytokines and chaperonins have been observed to play an important role as modulators of the immune response.

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