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Fish & Shellfish Immunology 22 (2007) 16–26

Fish & Shellfish Immunology

www.elsevier.com/locate/fsi

Effect of sulfide on the immune response and susceptibility to *Vibrio alginolyticus* in the kuruma shrimp *Marsupenaeus japonicus*

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Received 22 February 2006; revised 14 March 2006; accepted 14 March 2006
Available online 24 March 2006

Abstract

Kuruma shrimp *Marsupenaeus japonicus* held in 34‰ seawater were injected with tryptic soy broth (TSB)-grown *Vibrio alginolyticus* (2.7×10^6 cfu shrimp^{-1}), and then placed in water containing concentrations of sulfide at 0 (control), 51, 106, 528 and 1050 μg l^{-1}, respectively. After 12–144 h, mortality of *V. alginolyticus*-injected shrimp exposed to 528 and 1102 μg l^{-1} sulfide was significantly higher than that of shrimp exposed to 51 μg l^{-1} sulfide and the control solution. In another experiment, *M. japonicus* which had been exposed to 0, 56, 112, 525 and 1076 μg l^{-1} sulfide for 6, 12, 24 and 48 h were examined for immune parameters, and phagocytic activity and clearance efficiency of *V. alginolyticus*. Sulfide concentrations at 525 μg l^{-1} or greater for 12 h resulted in decreased total haemocyte count (THC) and phenoloxidase activity, phagocytic activity and bacterial clearance efficiency, whereas a sulfide concentration at 1076 μg l^{-1} for 24 h caused a significant increase in respiratory burst and superoxide dismutase activity of *M. japonicus*. It is concluded that concentrations of sulfide at 528 μg l^{-1} or greater increased the susceptibility of *M. japonicus* against *V. alginolyticus* infection by a depression in immune ability. The increased production of superoxide anion by *M. japonicus* exposed to 525 μg l^{-1} sulfide or greater was considered to be cytotoxic to the host.

Keywords: *Marsupenaeus japonicus*; *Vibrio alginolyticus*; Sulfide; Challenge; Haemocyte count; Phenoloxidase activity; Respiratory burst; Superoxide dismutase activity; Phagocytic activity; Clearance efficiency

1. Introduction

Commercial shrimp farming mainly based on tiger shrimp *Penaeus monodon*, white shrimp *Litopenaeus vannamei* and kuruma shrimp *Marsupenaeus japonicus* has been particularly hindered by epidemic infectious viral diseases like monodon baculovirus (MBV), white spot syndrome virus (WSSV), and infectious hypodermal and hematopoietic necrosis virus (IHHNV) [1], as well as vibriosis caused by *Vibrio alginolyticus*, *Vibrio harveyi* and *Vibrio damsela* [2–4]. These diseases have also been reported to be associated with increases of *Vibrio* populations in culture pond waters [5].

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doi:10.1016/j.fsi.2006.03.008

In decapod crustaceans, three types of circulating haemocytes are recognized: hyaline, semi-granular and granular cells [6]. They are involved in recognition and release of the prophenoloxidase (proPO) activating system but also in encapsulation, coagulation and phagocytosis [7,8]. The proPO activating system is initiated by minute amounts of lipopolysaccharide or peptidoglycans from bacteria, and β-1,3-glucans from fungi through pattern recognition proteins. ProPO is converted to phenoloxidase by an endogenous trypsin-like serine proteinase, the so-called prophenoloxidase activating enzyme (ppA). Phenoloxidase catalyses hydroxylation of monophenol to diphenol, and oxidation of diphenol to quinones, subsequently leading to melanin synthesis [9,10].

Phagocytosis is generally recognized as an important way to eliminate micro-organisms or foreign materials [11]. Once a pathogen enters the haemolymph, the host's NADPH-oxidase is activated, which in turn reduces oxygen and subsequently produces several reactive oxygen intermediates (ROIs) such as superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). This process is known as the respiratory burst, and plays an important role in anti-microbial activity [12]. Several enzymes participate in controlling the levels of ROIs including NADPH-oxidase, superoxide dismutase, peroxidase and catalase. Superoxide dismutase (SOD) is an enzyme that catalyses the rapid two-step dismutation of the toxic superoxide anion to molecular oxygen and hydrogen peroxide through the alternate reduction and oxidation of the active-site metal ion [13].

Sulfide which is produced under anaerobic conditions through decomposition of organic matter and reduction of sulfate is commonly found in the bottom layer and sediment of aquatic environments. Sulfide is considered highly toxic to fish and shrimp [14]. The 96-h LC50 of sulfide is 0.340 and 0.378 mg l^{-1} in Kadal shrimp *Metapenaeus dobsoni* for the size of 25–40 mm and 20–25 mm, respectively, and 0.144, 0.281 and 0.342 mg l^{-1} in the Indian white shrimp *Fenneropenaeus indicus* for the size of 85–90, 35–40 and 20–25 mm, respectively [15]. It has been reported that *M. japonicus* lost equilibrium when exposed to 0.1–2.0 mg l^{-1} sulfide, and instantly succumbed when exposed to 4.0 mg l^{-1} sulfide [16]. However, nothing is known regarding the effect of sulfide on the disease resistance of penaeid shrimp. Accordingly, this study is aimed at examining (1) the susceptibility of *M. japonicus* to *V. alginolyticus*, and (2) the immune parameters of *M. japonicus* under stress from sulfide. For the immune parameter assays, THC (total haemocyte count), differential haemocyte count (DHC), phenoloxidase activity, respiratory burst (RB), superoxide dismutase activity, phagocytic activity and bacterial clearance efficiency were used as indicators.

2. Materials and methods

2.1. M. japonicus

M. japonicus were obtained from a commercial farm in Iilan, Taiwan, and acclimated in the laboratory for 2 weeks before experimentation. Only shrimp in the intermoult stage were used for the study. The moult stage was identified by the examination of uropoda in which partial retraction of the epidermis could be distinguished [17]. For the susceptibility experiment, there were six treatments (five challenged test groups and one unchanged control group). The test and control groups were comprised of 10 shrimp and in triplicate tanks. For the experiment of immune parameter assays, there were 25 treatments (five concentrations of sulfide at 0, 50, 100, 500 and 1000 μg l^{-1} combined with five exposure times at 0, 6, 12, 24 and 48 h). Tests were carried out in two replicate test groups consisting of four shrimp each in 20 l PVC tanks. In all tests, the shrimp were fed twice daily with a formulated shrimp diet (Tairoun Feed Company, Taipei, Taiwan). The shrimp ranged from 8.47 to 11.30 g, averaging 9.87 ± 1.03 g (mean ± SD) with no significant size difference among the treatments. During experiments, water conditions were 25 ± 1 °C, pH 7.9–8.1 and salinity at 34‰.

2.2. Culture of V. alginolyticus

A pathogenic strain of *V. alginolyticus* (CH003) isolated from diseased *L. vannamei* was used for the study [18]. Stocks were cultured on tryptic soy agar (TSA supplemented with 3% NaCl, Difco) for 24 h at 25 °C and transferred to 10 ml tryptic soy broth (TSB supplemented with 3% NaCl, Difco) for 24 h at 25 °C as stock bacterial broth. The broth cultures were centrifuged at 7155 × *g* for 15 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution at 1.35×10^8 and 3.9×10^7 colony-forming units (cfu) ml^{-1} as bacterial suspensions for the susceptibility test, and for the test of phagocytic activity and clearance efficiency of *M. japonicus* to *V. alginolyticus*, respectively.