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Reactive oxygen species generated by a heat shock protein (Hsp) inducing product contributes to Hsp70 production and Hsp70-mediated protective immunity in *Artemia franciscana* against pathogenic vibrios

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ABSTRACT

The cytoprotective role of heat shock protein (Hsp70) described in a variety of animal disease models, including vibriosis in farmed aquatic animals, suggests that new protective strategies relying upon the use of compounds that selectively turn on Hsp genes could be developed. The product Tex-OE® (hereafter referred to as Hspi), an extract from the skin of the prickly pear fruit, *Opuntia ficus indica*, was previously shown to trigger Hsp70 synthesis in a non-stressful situation in a variety of animals, including in a gnotobiotically (germ-free) cultured brine shrimp *Artemia franciscana* model system. This model system offers great potential for carrying out high-throughput, live-animal screens of compounds that have health benefit effects. By using this model system, we aimed to disclose the underlying cause behind the induction of Hsp70 by Hspi in the shrimp host, and to determine whether the product affects the shrimp in inducing resistance towards pathogenic vibrios. We provide unequivocal evidences indicating that during the pretreatment period with Hspi, there is an initial release of reactive oxygen species (hydrogen peroxide and/or superoxide anion), generated by the added product, in the rearing water and associated with the host. The reactive molecules generated are the triggering factors responsible for causing Hsp70 induction within *Artemia*. We have also shown that Hspi acts prophylactically at an optimum dose regimen to confer protection against pathogenic vibrios. This salutary effect was associated with upregulation of two important immune genes, prophenoloxidase and transglutaminase of the innate immune system. These findings suggest that inducers of stress protein (e.g. Hsp70) are potentially important modulator of immune responses and might be exploited to confer protection to cultured shrimp against *Vibrio* infection.

1. Introduction

Vibriosis is one of the most prevalent bacterial diseases in the farming of marine fish and shellfish, impeding both economic and social development in many countries (Defoirdt et al., 2007; FAO, 2010). Besides affecting a broad range of marine taxa, several *Vibrio* species (for e.g., *V. parahaemolyticus* and *V. vulnificus*) that are found in association with marine animals are also zoonotic pathogens of humans (Lowry and Smith, 2007). It is generally accepted that novel anti-infective strategies for effective control of bacterial infections in farmed aquatic animals are greatly warranted.

One potential new approach is offered by the discovery of molecular chaperones or heat shock proteins (Hsp), and their cytoprotective potential (Almeida et al., 2011; Baruah et al., 2011; Powers et al., 2010). Hsps, mainly the 70 kDa Hsp (Hsp70), are highly conserved proteins of which expression is either constitutive or inducible under different conditions. The constitutive form has a crucial function as molecular chaperone and is involved in protein biogenesis and protein homeostasis in the cells (Muchowski and Wacker, 2005; Young et al., 2004). These functions include the folding of nascent polypeptides, assembly/disassembly of multi-subunit oligomers, translocation of proteins across intracellular membranes, process of endocytosis, regulation of apoptosis and cytoskeletal organization (Ohtsuka et al., 2007). When induced by environmental stress, Hsp70s work to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins and inhibit protein aggregation, thus protecting cells from harmful environmental stresses (Parsell and Lindquist, 1993). Besides these, Hsp70s are also implicated in eliciting immune responses against many (bacterial) diseases as

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demonstrated in a wide variety of experimental models *in vitro* and *in vivo* (Chen and Cao, 2010; Johnson and Fleshner, 2006; Tsan and Gao, 2009). So far, non-lethal heat shock (NLHS), preconditioning stress and transfection of Hsp genes have been used to induce Hsp70 production within organisms (Roberts et al., 2010). Recently, some compounds have been reported to have the ability of inducing Hsps within the host (Ohtsuka et al., 2005; Soti et al., 2005; Westerheide and Morimoto, 2005). Identification of chaperone inducers that are clinically safe would be of great benefit for the prevention or treatment of diseases, such as vibriosis.

We have recently reported that the Tex-OE® (hereinafter referred to as Hspi, for "Hsp inducer"), a patented natural product containing stimulating compounds derived from the skin of the prickly pear fruit *Opuntia ficus indica*, enhances the production of Hsp70 in animal tissues without any adverse effect (Baruah et al., 2012; Roberts et al., 2010; Sung et al., 2012). Previous studies have reported that certain plant-based compounds auto-oxidize in aqueous solutions, resulting in the release of reactive oxygen species (ROS) (see review, Akagawa et al., 2003). In this study, we used an invertebrate model organism brine shrimp (*Artemia franciscana*) cultured under gnotobiotic conditions (i.e., a germ-free system, where the microbial communities can subsequently be controlled, Marques et al., 2006) to determine whether Hspi releases ROS, and if the generation of ROS is the underlying cause behind Hsp70 induction by Hspi. Previously, Hsp70 (recombinant) was shown to induce protective effects in animal models against pathogenic agents (Baruah et al., 2013) by chaperoning with antigenic proteins, presenting these antigens to the immune cells and subsequently activating the immune responses (Asea et al., 2000). We therefore further investigated whether Hspi-mediated Hsp70 has a protective effect on *Vibrio*-induced mortality in the brine shrimp larvae and if this effect was associated with the regulation of two major genes of the innate immune system prophenoloxidase (*proPO*) and transglutaminase (*tgase*) immune genes, known for their critical role in dealing with a pathogen attack (Cerenius et al., 2008; Liu et al., 2007).

2. Materials and methods

2.1. Gnotobiotic brine shrimp rearing system

The gnotobiotic system was developed by hatching brine shrimp cysts axenically (germ-free) following decapsulation and hatching procedures as described previously (Baruah et al., 2009). Briefly, 1.5 g of brine shrimp cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 ml of distilled water for 1 h. Sterile cysts and larvae were obtained via decapsulation using 3.3 ml NaOH (32%) and 50 ml NaOCl (50%). During the reaction, 0.2-μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were autoclaved at 121 °C for 20 min. The decapsulation was stopped after about 2 min by adding 50 ml $Na_2S_2O_3$ at 10 g/l. The aeration was then terminated and the decapsulated cysts were washed with sterile artificial seawater containing 3.5% of Instant Ocean® synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts, suspended in 1-l glass bottles containing sterile artificial seawater, were incubated for 28 h for hatching at 28 °C with constant illumination of approximately 27 $\mu E/m^2$ s. After 28 h incubation, hatched larvae at developmental stage instar II (in which their mouth opens to ingest particles) were collected, counted volumetrically and thereafter transferred to 500-ml sterile glass bottles containing sterile seawater. Air passed through 0.2-μm air filters, was continuously provided to all glass bottles by an air pump. The larvae were treated as indicated below with Hspi. All these manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and larvae.

2.2. Methods used to verify axenicity of the brine shrimp larvae

After 28 h of incubation, the axenicity of the brine shrimp larvae was verified both by spread plating (100 ml) and by adding (500 μl) hatching water on Marine Agar and Marine Broth (Difco, Detroit, USA), respectively followed by incubating at 28 °C for 5 days (Baruah et al., 2009). Experiments started with non-sterile larvae were discarded.

2.3. The Hsp-inducing product

Tex-OE® (named Hspi) powder was kindly provided by Bradan Ltd Campbeltown. It was dissolved in sterile distilled water at 2 g/l. A fresh stock was prepared for each experiment.

2.4. Brine shrimp larvae survival studies – in vivo pretreatment of larvae with Hspi

In total, two separate survival studies were conducted. In the first study, a dose response relationship of Hspi was determined. To this end, brine shrimp larvae were pretreated with increasing concentrations of Hspi (2.5, 5, 10 and 50 mg/l) for a period of 2 h at 28 °C. The larvae were washed repeatedly with sterile artificial seawater and allowed to recover for 2 h at 28 °C. Following pretreatment, groups of 20 larvae from the 500-ml glass bottles were transferred to sterile 40-ml glass tubes that contained 10 ml of sterile artificial seawater (3.5% salinity) and then challenged with either *V. campbellii* or *V. harveyi* at 10^7 cells/ml (Baruah et al., 2010). The survival of brine shrimp larvae was scored 48 h after the challenge. As control groups, non-pretreated larvae that were not challenged with *Vibrio* (non-pretreated control) or challenged (control) were used. Each treatment and control was performed in quintuplicate and each survival experiment was repeated once to check the reproducibility.

2.5. Brine shrimp larvae survival studies – in vivo pretreatment of larvae with Hspi and/or antioxidant enzymes

In the second study, gnotobiotically cultured brine shrimp larvae were pretreated with an optimized dose of Hspi (dose which gave maximum protection to *Vibrio*-challenged larvae in the dose–response assay), a mixture of antioxidant enzymes catalase (10 mg/l, Sigma–Aldrich®, Belgium) and superoxide dismutase (SOD, 75 units, Sigma–Aldrich®, Belgium) or a combination of Hspi and enzymes mixture in a similar fashion as described above. The shrimp larvae in the control group did not undergo any pretreatment. Following pretreatment, the required amount of live larvae (0.1 g) from all groups were collected separately on 50-μm sieves, rinsed in distilled water, immediately frozen in liquid nitrogen and stored at −80 °C for Hsp70 analysis (see below). The remaining larvae were counted, distributed into sterile 40-ml glass tubes and then challenged with *V. campbellii* or *V. harveyi* as described in the dose–response study. Controls were maintained as described in the Figure legend. Survival of the larvae was scored after 48 h of challenge.

2.6. Protein extraction and Hsp70 detection

The brine shrimp larvae samples were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Clegg et al., 2000), supplemented with protease inhibitor cocktail (Sigma–Aldrich®, USA) as recommended by the manufacturer. Subsequent