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A novel heat-shock protein inducer triggers heat shock protein 70 production and protects *Artemia franciscana* nauplii against abiotic stressors

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ABSTRACT

Induction of heat shock protein 70 (Hsp 70) is important in the tolerance to subsequent deleterious environmental stresses. It would therefore be of great benefit to develop non-toxic Hsp70-inducing compounds that are able to induce Hsp70 in advance, for animals which are subjected to various environmental stresses. This study aimed to investigate whether Pro-Tex®, a soluble version of Tex-OE® a chaperone-stimulating factor isolated from the prickly pear cactus (*Opuntia ficus indica*), could manipulate Hsp70 expression in a gnotobiotically cultured brine shrimp *Artemia franciscana* and subsequently protect against abiotic stressors. Results showed that Tex-OE® enhanced Hsp70 expression in a dose- and time-dependent manner in *Artemia*. In addition, pretreatment of *Artemia* with Tex-OE® (152 ppb) for 1 h protected the shrimp against thermal challenge. Interestingly, the expression level of Hsp70 coincided well with the extent of protection against thermal challenge, suggesting that the protective effect of the compound is mediated by Hsp70 induction. Results also demonstrated that Tex-OE® can function in synergy with a non-lethal heat shock (37 °C for 30 min followed by 6 h recovery) conferring maximum protection to *Artemia* against thermal and hypersalinity stresses at either optimal (152 ppb) or sub-optimal (76 ppb) dose. From these results, it is suggested that Tex-OE® is a potential inducer of Hsp70 and in the presence or absence of a bona fide stress, it could be an ideal candidate for use as an anti-stressor during various aquaculture practices.

1. Introduction

Shrimp production by aquaculture is a high value activity worldwide. Recently it was estimated to have reached about three million metric tons which are valued in excess of US$ 12 billion (FAO, 2010). Farming of shrimp has been carried out in either extensive or intensive/semi-intensive culture system. However, under the latter culture systems, environmental conditions can degrade rapidly causing significant stress to the shrimp (Capy et al., 2000). The consequence of such stress includes decreased immune defense and increased susceptibility to pathogens (Horowitz and Horowitz, 2001; Le Moullac and Haffner, 2000). In shrimp production systems, many potential pathogens, such as bacteria, fungi and viruses, co-exist with the shrimp without causing a negative impact on production (de la Vega et al., 2004, 2006; Vidal et al., 2001). However, some quiescent bacterial or viral infections may develop into acute diseases if shrimp become stressed and this has repeatedly led to significant industry losses (Hall and de la Vega, 2004; Vidal et al., 2001). Therefore, management of such stress is of great relevance in aquaculture due to its negative impact on the welfare and economic production of shrimps and other aquaculture species.

Accumulating evidence over the past decades suggested that sudden exposure of cells, tissues and organisms to sub-lethal heat stress (temperature well above the ambient condition but still within the physiological range of the organism) activated the production of an array of endogenous proteins known as heat shock proteins (Hsps) (de la Vega et al., 2006; DuBeau et al., 1998; Rahman et al., 2004). Functionally, these Hsps, mainly the 70 kDa Hsp (Hsp70), are involved in the cross protection or cross-tolerance in animals and plants, i.e. a general stress response and a transient increase in the resistance to a second heterologous physiological and environmental insult (Sabehat et al., 1998). The protective function of the Hsp70 is documented to be due to its chaperone activity maintaining protein homeostasis by protecting nascent polypeptides from misfolding, facilitating co- and post-translational folding, assisting in assembly and disassembly of macromolecular complexes, and regulating translocation (Bukau et al., 2006; Morimoto, 2008; Ron and Walter, 2007). In a variety of experimental models an early peak of Hsp70 has been shown to confer thermal resistance (Frankenberg et al., 2000; Lei et al., 2005; Periago et al., 2002; Sejerkilde et al., 2003), protect against osmotic stress (DuBeau et al., 1998; Neta et al., 2005; Todgham et al., 2005), prevent oxidative toxicity and damage (Arieli et al., 2003; Collins and Clegg, 2004; Todgham et al., 2005) and improve desiccation tolerance (Ma et al., 2005). These observations clearly illustrated that Hsp70 protects multiple organisms against a further and eventually, more severe environmental insults.

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Besides, by heat shock, Hsp70 expression is also up-regulated by various physiological perturbations such as oxidative stress, nutritional deficiencies, ultraviolet radiation, chemicals, viral infection and anoxia (Pockley, 2003; Rahman et al., 2004). In an aquaculture system, however, sub-lethal heat shock is possibly not the best way to enhance Hsp production because acute temperature shifts are often detrimental, adversely affecting physiological balance and causing significant mortality. It would therefore be useful and beneficial to find less traumatic approaches for up-regulation of Hsp expression in aquatic organisms

Recently, Pro-Tex®, which contains the active molecule Tex-OE®, a patented extract from the skin of the prickly pear fruit, *Opuntia ficus indica*, has been reported as a non-stressful effector that induces high levels of endogenous or host-derived Hsps in animal tissues (Roberts et al., 2010). In this study, we investigated whether Tex-OE® (hereinafter mentioned as Hspi, for "HSP inducer") could manipulate the expression of stress protein in a gnotobiotically cultured brine shrimp *Artemia franciscana* and subsequently protect against abiotic stressors. The brine shrimp was chosen as an experimental organism in this study because it represents an ideal animal model to study crustacean stress response studies due to its ability to tolerate environmental perturbations (Clegg et al., 2000). The possibility to culture this animal under axenic/gnotobiotic conditions also eliminates the possibility of microbial interference in mechanistic studies (Baruah et al., 2011).

Herein we present findings demonstrating that pretreatment of *Artemia* with Hspi confers successful protection against abiotic stressors and that protection by Hspi is associated with the induction of endogenous Hsp70.

2. Materials and methods

2.1. Axenic hatching of Artemia

Axenic *Artemia* were obtained following decapsulation and hatching (Baruah et al., 2011). Briefly, 1.5 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) was hydrated in 89 mL of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 3.3 mL NaOH (32%) and 50 mL NaOCl (50%). During the reaction, 0.22 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 filtered (0.2 mm) and autoclaved artificial seawater containing 35 g/L of instant ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were suspended in 1-L glass bottles containing filtered and autoclaved artificial seawater and placed in rectangular tank containing water maintained at 28 °C using a thermostatic heater for incubation for 28 h with constant illumination of approximately 2000 lx. After 28 h incubation, swimming nauplii at stage II were collected, counted volumetrically and thereafter transferred to 250-mL sterile glass bottles containing filtered and autoclaved artificial seawater. Air passed through 0.2 μm air filters, was continuously provided to all the glass bottles by a compressed air pump. The nauplii were treated at the indicated concentrations (see below) with Hspi, NLHS or a combination of both Hspi and NLHS prior to thermal or osmotic challenge. All these manipulations were performed under a laminar flow hood.

2.2. In vivo pre-treatment of Artemia with Hspi

The product Pro-Tex® (containing the active compound Tex-OE®), supported in food grade ethanol, was kindly provided by Bradan Ltd Campbeltown. It was stored at room temperature until use. Prior to its use, the dry weight content of the Pro-Tex® solution was determined by drying 5 mL of the product at 80 °C to a constant weight. It was observed that 5 mL of the solution contain 18.8 mg of the compound (3.76 g/L). This amount does not represent the concentration of the active compound. The company guarantees constant concentration of active compound between batches of product as verified by an undisclosed procedure. Yet the dry weight content may vary.

In total, four separate studies were performed. In the first study, a dose response relationship of Hspi was determined. For that, the nauplii were pretreated for a fixed time (1 h) with increasing concentrations of Hspi (7.6, 15.2, 76 and 152 ppb) or with ethanol alone as negative control. The final ethanol concentration (31.6 ppm) in the negative control or Hspi treatments corresponds to the amount added in the treatment with highest Hspi concentration. A control was also maintained without the addition of Hspi and ethanol. In addition, *Artemia* that were given only non-lethal heat shock (NLHS) at 37 °C for 30 min following by 6 h recovery at 28 °C served as a positive control since it was known to induce Hsp70 and cross protect against severe stress (Sung et al., 2007). Immediately after preconditioning, the *Artemia* were subjected to lethal heat shock by immersing the *Artemia* rearing tubes for 20 min in a water bath preheated to 41 °C ($\Delta t = 5$ °C/min). Thermal-shocked *Artemia* were slowly brought back to a water temperature of 28 °C at a Δt rate of 0.5 °C/min. Thermotolerance was determined by counting the live nauplii 12 h after thermal challenge. The best dose (with the higher *Artemia* survival) was selected to perform the subsequent experiments.

The second study involved testing the effect of pretreating *Artemia* with two Hspi doses, one which gave the best protection in the dose–response experiment and the other which did not, for different time intervals (1, 2 and 4 h) in the thermal stress test. The non-effective or sub-optimal dose was chosen, assuming that longer pretreatment of *Artemia* with this dose would provide protection against lethal heat shock.

In the third and fourth studies, the synergistic effect of a combined Hspi and NLHS was determined. Therefore, the nauplii were pretreated with Hspi either in the presence or absence of a bona fide stress (i.e., a NLHS at 37 °C for 30 min). Subsequently, it was verified whether such pretreatment can protect against subsequent thermal (41 °C for 20 min) and hyper osmotic (100 ppt salinity) shocks. Each experiment involved the following groups: 76 ppb Hspi for 1 h [Hspi (76 ppb)], a co-treatment consisting of 76 ppb Hspi for 1 h and a NLHS as described above [Hspi (76 ppb) + NLHS], 152 ppb Hspi for 1 h [Hspi (152 ppb)], and a co-treatment consisting of 152 ppb Hspi for 1 h and a NLHS as described above [Hspi (152 ppb) + NLHS]. The three control groups as maintained in the first and second studies were also included.

2.3. Protein extraction and Hsp70 detection

Artemia nauplii from each treatment were collected separately on 50 μm sieves and rinsed with ice-cold distilled water. Samples containing 0.1 g of live nauplii 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), and supplemented with protease inhibitor cocktail (Catalogue# P8340, Sigma-Aldrich, Inc. USA) as recommended by the manufacturer. Subsequent to centrifugation at 2200 × *g* for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. Supernatant samples were then combined with loading buffer, vortexed, heated at 95 °C for 5 min and electrophoresed in 10% SDS-PAGE gels, with each lane receiving equivalent amounts of protein (25 μg). Gels were either stained with Coomassie Biosafe (BioRad Laboratories) or transferred to polyvinylidene fluoride membranes (BioRad Immun-Blot™ PVDF) for antibody probing. Membranes were incubated with blocking buffer [50 ml of 1× phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature and then with mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO), which recognizes both constitutive and inducible Hsp70 (Sung et al., 2007), at the recommended dilution of 1:5000. Horseradish peroxidase conjugated donkey anti-mouse IgG