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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

was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO). Detection was done with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) in association with 0.01% (v/v) H_2O_2 in 0.1 m Tris–HCl (pH 7.6).

2.4. Assay of Artemia survival challenged with thermal or hyperosmotic shock

After the pretreatments, groups of 30 nauplii from the 250-mL glass bottles were transferred to sterile 50-mL glass tubes that contained 30 mL of filtered and autoclaved artificial seawater (35 ppt salinity), challenged with thermal shock at 41 °C for 20 min ($\Delta t = 5$ °C/min) and then transferred to 28 °C. Protection against thermal shock was determined 12 h after heat shock by counting the live nauplii.

The osmotic shock was administered by transferring the nauplii, as described above, to sterile 50-mL glass tubes that contained 30 mL of filtered and autoclaved artificial seawater with a salinity of 100 ppt. The survival of *Artemia* was scored 36 h after the shock. In all the experiments, 5 replicates were kept for each treatment and each experiment was carried out twice to check the reproducibility.

The axenicity of *Artemia* nauplii used in survival assays was verified by spread plating 100 mL of the hatching water on Marine agar (Difco, Detroit, USA) followed by incubating at 28 °C for 5 days (Baruah et al., 2010). Experiment results obtained with non-axenic nauplii were discarded.

2.5. Statistical analysis

Survival data were arcsin transformed to satisfy normality and homoscedasticity requirements as necessary. Data were then subjected to one-way analysis of variances followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences (SPSS) version 16.0 to determine significant differences among treatments. Significance level was set at $P<0.05$.

3. Results

3.1. In vivo induction of Hsp70 by Hspi

In the initial study, in order to determine whether Hspi has an inductive effect on Hsps in vivo, we analyzed Hsp70 expression by SDS-PAGE and Western blot in *Artemia* after 1-h treatment with different doses of Hspi. As shown in Fig. 1A, there was a constitutive expression of Hsp70 in the control and the expression was almost the same to that in the negative control. However, Hspi treatment at concentration as low as 7.6 ppb markedly increased Hsp70 expression compared with the controls. The maximal effect was observed to occur with a dose of 152 ppb. The Hsp70 expression level in the (positive control) group exposed to NLHS at 37 °C was almost comparable to that by Hspi at highest concentration (152 ppb).

In the subsequent study, we chose two Hspi doses from the dose–response experiment and then manipulated the exposure time (1, 2 and 4 h) in order to investigate whether increased exposure time could enhance the expression of Hsp70. The results showed that the Hspi (either 76 or 152 ppb) time-dependently induced Hsp70 expression in *Artemia* (Fig. 1B). Hspi at high dose (152 ppb) for all the exposure times enhanced Hsp70 expression more strongly than those of low dose (76 ppb) for the corresponding exposure time.

3.2. Protective effect of Hspi against thermal stress

As Hspi at different doses and exposure time enhanced Hsp70 expression differently (Fig. 1), we then determined the appropriate dose and exposure time at which Hspi could possibly confer protection to *Artemia* against thermal stress. As shown in Fig. 2, *Artemia* given a pretreatment with Hspi at 152 ppb concentration were best protected from thermal stress, with survival augmented by 2.5-fold as compared to the control animals. In addition, survival of these pretreated *Artemia* was the same as those of *Artemia* treated with a NLHS (37 °C for 30 min, positive control). Interestingly, the maximum protection also corresponded well with the maximum induction of Hsp70 at this dose of Hspi (152 ppb), indicating it to be the optimum dose among the doses tested. In contrast, *Artemia* pretreated with Hspi in doses ranging from 7.6 to 76 ppb could not induce protection against thermal stress.

Next, we carried out time–response study under the same conditions as described in Fig. 2 in order to determine the exposure time inducing maximum thermotolerance. *Artemia* pretreated with suboptimal dose of Hspi (76 ppb) for different time intervals did not exhibit significant ($P>0.05$) improvement in the survival compared with all the three controls. Instead, prolonged exposure (2 or 4 h) with this dose further significantly ($P<0.001$) reduced the survival of *Artemia* (Fig. 3). In contrast, the survival of *Artemia* pretreated with optimal

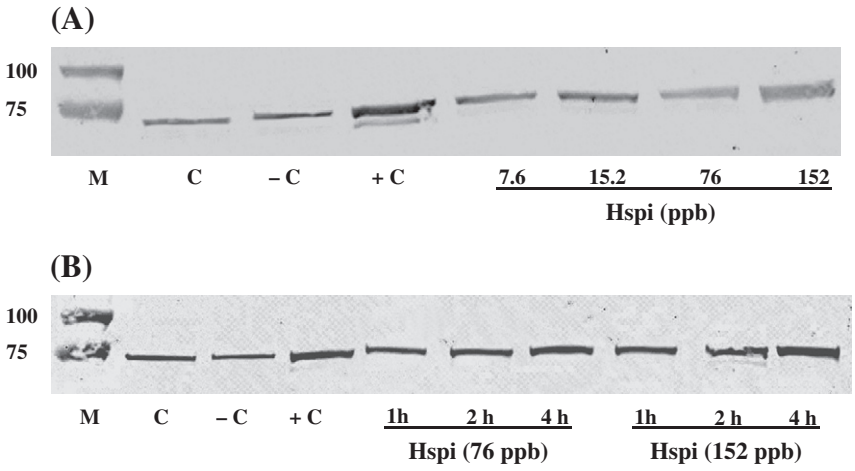

Fig. 1. Induction of Hsp70 in *Artemia*. (A) *Artemia* were pretreated with different doses of Hspi (7.6, 15.2, 76 or 152 ppb) or with absolute ethanol alone (31.6 ppm) as negative control (−C) for 1 h. (B) *Artemia* were pretreated with two different doses of Hspi (76 or 152 ppb) for different time periods (1, 2 or 4 h) or with absolute ethanol alone (31.6 ppm) as negative control for 1 h. *Artemia* not pretreated with Hspi and/or ethanol served as control (C), whereas those given only NLHS at 37 °C for 30 min following by 6 h recovery at 28 °C served as a positive control (+C). Protein extracted from different groups was resolved in SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes and probed with antibody to *Artemia* Hsp70. Twenty five micrograms of *Artemia* protein was loaded in each lane. Molecular mass standards (M) in kilodaltons were on the left. The unexpected appearance of an extra band in the positive control (+C) in A could be due to non-specific binding or a handling error. Appearance of such dual bands was not observed in our previous studies (Baruah et al., 2010; Sung et al., 2007).

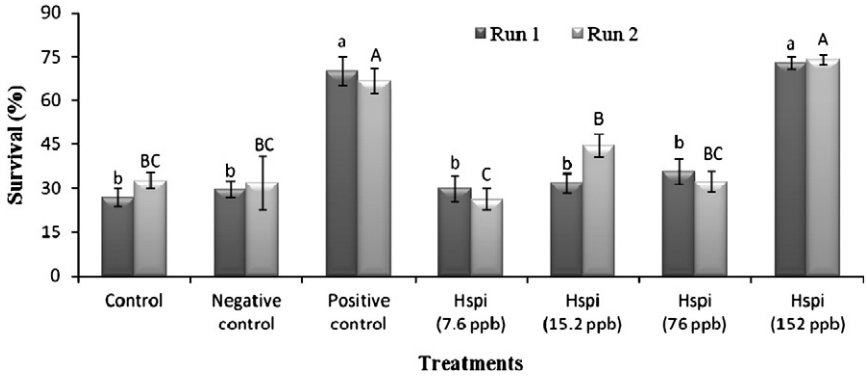

Fig. 2. Survival of Hspi pretreated-*Artemia* nauplii after challenged with thermal shock. Experiments were repeated once indicated as Run 1 and Run 2. Data in each experiment represents the mean of five replicates. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference ($P<0.05$). *Artemia* were pretreated with different doses of Hspi (7.6, 15.2, 76 or 152 ppb) or with absolute ethanol alone (31.6 ppm) as negative control for 1 h followed by exposure to lethal heat shock at 41 °C for 20 min. *Artemia* not pretreated with Hspi and/or ethanol served as control, whereas those given only NLHS at 37 °C for 30 min following by 6 h recovery at 28 °C served as a positive control. Survival was scored after 12 h.

dose of Hspi (152 ppb) for a period of 1 to 4 h significantly ($P<0.001$) increased survival, being comparable with that of the positive control. Based on the dose– and time–response studies, a Hspi dose of 152 ppb, treated for a period of 1 h was chosen as optimum condition for *Artemia* under our experimental conditions.

3.3. Synergistic effects of Hspi on the induction of Hsp70

To investigate the synergistic effects of Hspi and a NLHS, we incubated *Artemia* with a sub-optimal (76 ppb) or an optimal (152 ppb) dose of Hspi for 1 h at 28 °C in the presence or absence of a classic stress inducer, i.e., 30-min NLHS at 37 °C as described in the Materials and methods. The results showed that Hspi (76 or 152 ppb) could enhance Hsp70 expression by itself. However, in the presence of NLHS, the Hsp70 enhancing effect of Hspi was found to be more prominent as indicated by high intensity of the bands (Fig. 4). But surprisingly, the immunoreactive band of Hsp70 in groups treated with a combination of both Hspi and NLHS did not appear to be markedly increased from that of NLHS treatment under our experimental conditions.

3.4. Protective effects of Hspi in the presence or absence of a bona fide stress

The possibility that Hspi in the presence of a bona fide stress might protect *Artemia* against thermal stress to a greater extent was then monitored. As expected, we found maximum survival (73.3%) in the group pretreated with sub-optimal Hspi (76 ppb) in the presence of NLHS but surprisingly not in group pretreated with optimal Hspi (152 ppb) in combination with NLHS (Fig. 5). The latter group, however, had survival (58.9%) which was significantly ($P<0.001$) higher than that of the control groups (no ethanol, 32.5%; only ethanol, 30%) and also that of the 76 ppb Hspi pretreated group (30%). Results also showed no significant differences ($P>0.05$) in the survival among the groups pretreated with NLHS, 152 ppb Hspi, or combination of both. In the second experimental run, which was conducted to check the reproducibility, almost similar result was obtained. The Hspi (76 ppb) pretreated *Artemia* in the presence of NLHS showed maximum survival, however, did not vary significantly ($P>0.05$) from the groups pretreated with NLHS, 152 ppb Hspi or a combination of both (Fig. 5).

To further confirm the protective effect of Hspi in the presence or absence of a bona fide stress, we exposed the pretreated *Artemia* to another stressor, hypersalinity stress (a sudden increase from 37 to 100 g/L salt). Exposure of *Artemia* to NLHS or pretreatment with 152 ppb Hspi resulted in a significant increase in the survival as shown in Fig. 6. The survival increased from 45% in the ethanol-treated control group to 58% in the *Artemia* exposed to NLHS and to 60% in the *Artemia* pretreated with 152 ppb Hspi. This represents an approximate 15% increase in survival on pretreatment of the *Artemia* with 152 ppb Hspi. Such increased survival was not recorded at an Hspi dose of 76 ppb (48%). However, pretreatment of *Artemia* with this dose of Hspi in the presence of NLHS resulted in a significant increase in the survival (61%). Results also showed that the Hspi (152 ppb) pretreated group in the presence of NLHS had the maximum survival (66%), however, was not significantly ($P>0.05$) different from the groups pretreated with NLHS, 152 ppb Hspi or combination of both.

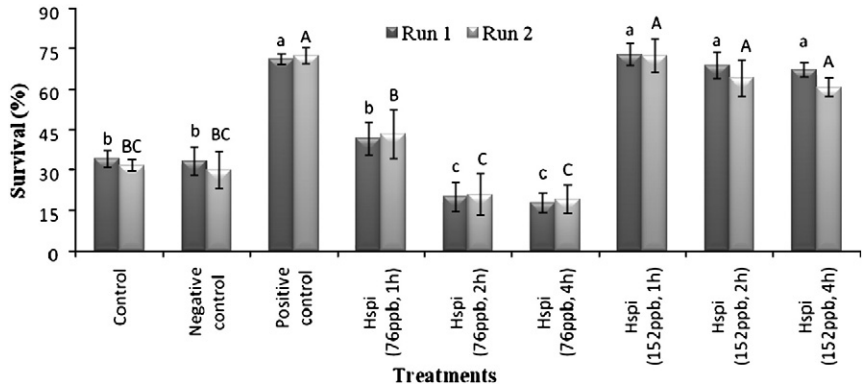

Fig. 3. Survival of Hspi pretreated-*Artemia* nauplii after challenged with thermal shock. Experiments were repeated once indicated as Run 1 and Run 2. Data in each experiment represents the mean of five replicates. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference ($P<0.05$). *Artemia* were pretreated with two different doses of Hspi (76 or 152 ppb) for different time periods (1, 2 or 4 h) or with absolute ethanol alone (31.6 ppm) as negative control for 1 h followed by exposure to lethal heat shock at 41 °C for 20 min. *Artemia* not pretreated with Hspi and/or ethanol served as control, whereas those given only NLHS at 37 °C for 30 min following by 6 h recovery at 28 °C served as positive control. Survival was scored after 12 h.

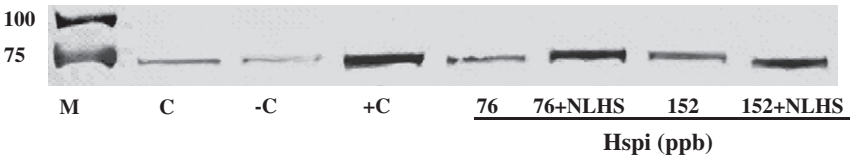

Fig. 4. Synergistic effect of Hspi and NLHS on the expression of Hsp70 in *Artemia* nauplii. Results are shown for animals given the following treatments: 76 ppb Hspi for 1 h (76), a cotreatment consisting of 76 ppb Hspi for 1 h and a NLHS as described above (76 + NLHS), 152 ppb Hspi for 1 h (152), and a cotreatment consisting of 152 ppb Hspi for 1 h and a NLHS as described above (152 + NLHS). *Artemia* not pretreated with Hspi and/or ethanol served as control (C), those pretreated with ethanol alone served as negative control (−C) and those given only non-lethal heat shock at 37 °C for 30 min following by 6 h recovery at 28 °C served as a positive control (+C). Protein (25 μg) extracted from different treatment groups was resolved by in SDS-PAGE gel and electroblotted on then transferred to polyvinylidene fluoride membranes as described for Fig. 1 and probed with antibody to *Artemia* Hsp70. Twenty five micrograms of *Artemia* protein was loaded in each lane. Molecular mass standards (M) in kilodaltons were on the left.

4. Discussion

The production of Hsps in fish and shellfish has been shown to have profound protective effects under detrimental conditions (Baruah et al., 2010, 2011; Iwama et al., 1998; Roberts et al., 2010; Sung et al., 2007, 2008). These observations justify attempts to characterize novel Hsp inducers, which could be used to increase environmental stress resistance of different aquaculture species.

In this in vivo study, we examined the effect of an Hsp stimulating factor Tex-OE® (or Hspi) on the modulation of Hsp70 in *Artemia*. Our results demonstrated that Hspi evokes expression of Hsp70 in a concentration- and time-dependent manner in *Artemia*. In addition, Hspi exhibited Hsp70 induction similar to those observed for NLHS. These results evidently suggest that the compound is a potent enhancer of Hsp70. The mechanistic detail for the induction of Hsp70 by this compound was not determined in this study, because the active compound is not disclosed by the manufacturing company. Our finding is similar to the results of a previous report (Sung et al., in press), showing that exposure of common carp *Cyprinus carpio* fingerlings to Tex-OE® before exposure to acute ammonia stress rapidly enhances the level of Hsps.

Previous studies have suggested that Hsp70 induction is an important component of cross-tolerance in aquatic animals as well as in other model organisms (de la Vega et al., 2006; Roberts et al., 2010; Sung et al., 2007; Todgham et al., 2005). Since Hspi was found to induce Hsp70 in *Artemia*, we investigated whether pre-exposure to Hspi could protect the brine shrimp against thermal challenge. As expected, prior treatment with 152 ppb of Hspi for an hour was shown to significantly increase the survival of *Artemia* challenged with thermal shock. Interestingly, the extent of the protective effect of Hspi on the thermal-stressed *Artemia* coincided well with the Hsp70 expression level. These results suggest that Hspi can protect *Artemia* against thermal challenge and that the protective effect of this compound is mediated by the induction of Hsp70. In agreement with our results, it has recently been shown by Sung et al. (in press) that pre-exposure to Tex-OE® protect common carp *C. carpio* fingerlings against acute ammonia stress and this development of tolerance was correlated with endogenous Hsp70 synthesis. Additionally, the protective effect of Tex-OE® has also been well documented in other fish models (for review, see Roberts et al., 2010).

In this study, although Hsp70 induced by Hspi appeared to confer effective protection against thermal stress, however, the induction of a constellation of stress proteins (like Hsp27, Hsp40, Hsp90 etc.) by this compound and their collective involvement in protection cannot be excluded (Brown et al., 1992; Kong et al., 2011; Miller and McLennan, 1988a; Sreedhar and Csermely, 2004). Further studies are required to substantiate this assumption by developing or using antibodies specific against these stress proteins or by using other approaches, such as radiolabeling with $NaH^{14}CO_3$ and autoradiography or two-dimensional gel electrophoresis as previously described by Miller and McLennan (1988b) in cysts and nauplius larvae of *Artemia* exposed to different temperatures.

A very important point that needs to be considered before using any type of anti-stress products on aquaculture species is that they should not be toxic or deleterious. In this study, lengthening the exposure duration of the optimized dose of Hspi (152 ppb) from 1 to 4 h had no apparent effect on the survival of *Artemia* compared to those pretreated with only NLHS (Fig. 1B). Therefore at this point we would like to suggest that Hspi did not appear to be deleterious to *Artemia* at the indicated concentrations and exposure duration. However, this suggestion needs to be confirmed by carrying out cytotoxicity assays as described previously for various chaperone inducing compounds (Liu et al., 2008; Yan et al., 2004).

A feature of modulators of the heat shock response is that certain inducers can have combinatorial synergy with heat stress (Lee et al.,

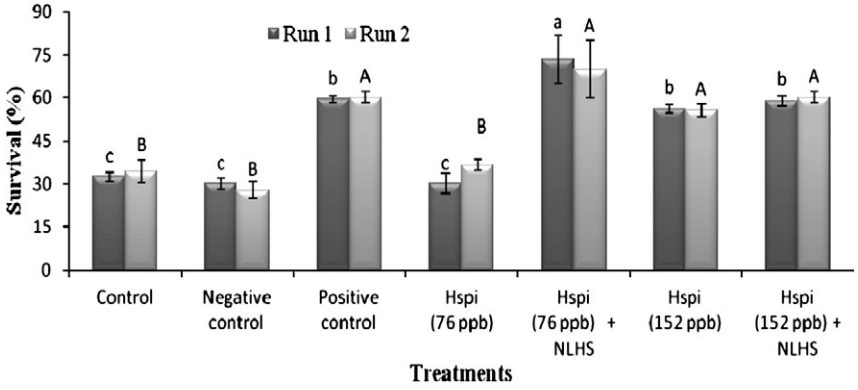

Fig. 5. Synergistic effect of Hspi and NLHS on the survival of *Artemia* nauplii challenged with thermal shock. Results are shown for animals given the following treatments: 76 ppb Hspi for 1 h [Hspi (76 ppb)], a cotreatment consisting of 76 ppb Hspi for 1 h and a NLHS as described above [Hspi (76 ppb) + NLHS], 152 ppb Hspi for 1 h [Hspi (152 ppb)], and a cotreatment consisting of 152 ppb Hspi for 1 h and a NLHS as described above [Hspi (152 ppb) + NLHS]. *Artemia* not pretreated with Hspi and/or ethanol served as control, those pretreated with ethanol alone served as negative control and those given only non-lethal heat shock at 37 °C for 30 min following by 6 h recovery at 28 °C served as a positive control. Survival was scored after 12 h. Experiments were repeated once indicated as Run 1 and Run 2. Data in each experiment represents the mean of five replicates. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference ($P<0.05$).

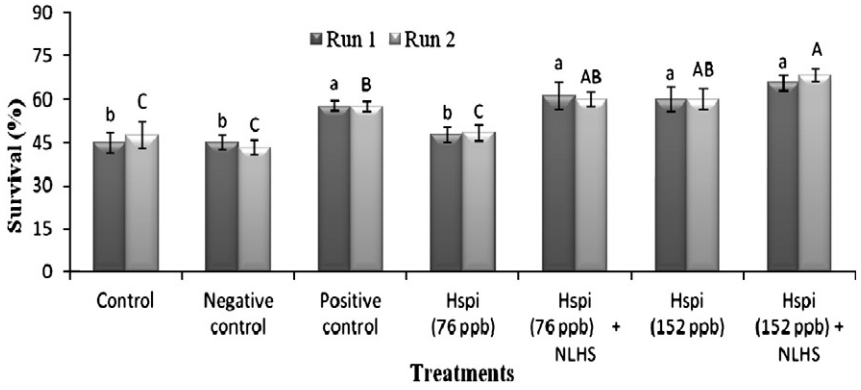

Fig. 6. Synergistic effect of Hspi and NLHS on the survival of *Artemia* nauplii challenged with hypersalinity shock. For the treatment groups, refer to Fig. 5 for explanation. Experiments were repeated once indicated as Run 1 and Run 2. Data in each experiment represents the mean of five replicates. Error bars with different alphabet letters (small and capital letters for Run 1 and Run 2, respectively) indicate significant difference ($P<0.05$).

1995; Vigh et al., 1997). For example, a chaperone inducer celastrol activate heat shock gene transcription synergistically with sub-optimal levels of heat shock and exhibits cytoprotection against subsequent exposures to other forms of lethal stress (Westerheide et al., 2004). In another study, Lee et al. (1995) demonstrated that pre-exposure of HeLa cells to an anti-inflammatory drug indomethacin, 40 °C, or 41 °C by itself had a modest effect on cell survival against lethal challenge temperature of 44.5 °C (2–15% survival). However, indomethacin together with either 40 °C or 41 °C had a strongly protective effect (12–60% survival). Similar to these observations, in this study, we observed that the combination of sub-optimal Hspi (76 ppb) level and NLHS resulted in the cross protection against thermal stress well above that observed for the other treatment groups. These results reveal that in the presence of a bona fide stress, Hspi could evoke its protective effect in thermally-challenged *Artemia* at concentration below its threshold level that actually is required for protection against thermal stress. Furthermore, our results also demonstrated that Hspi can function in synergy with NLHS conferring maximum cross protection to *Artemia* against hypersalinity stress at either optimal or sub-optimal dose. This cross protective effect might be closely linked to the expression of Hsp70. The mechanism by which Hsps cross protect against stress has yet to be determined but the increment of intracellular level of Hsp70 may mediate tolerance by preventing protein denaturation, refolding damaged proteins or ensuring degradation of irreversibly damaged proteins, thus preventing accumulation of abnormal proteins and their aggregates (Hartl and Hayer-Hartl, 2009; Mchaourab et al., 2009). These processes are crucial in maintaining a normal cellular homeostasis during stress. Additionally, the specific cellular mechanisms by which Hsp70 might allow tolerance to osmotic shock also remain unclear, however, moderation of the effects of cell dehydration on protein stability, folding and solubility is a likely candidate (DuBeau et al., 1998; Sheikh-Hamad et al., 1994).

In conclusion, overall results provide strong evidences that Tex-OE® is a potential inducer of Hsp70 and in the presence or absence of a bona fide stress, it can induce tolerance against subsequent stressors. This suggests that this product, pending thorough verification, could be used as an anti-stress agent for various aquaculture practices such as handling and movement of fish/crustaceans in day to day farming operations, or in anticipation of stressful conditions such as high water temperature, anoxic water conditions, poor water quality, presence of nitrite, chlorine or ammonia in water.

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