Fish & Shellfish Immunology 29 (2010) 733–739

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Efficacy of heterologous and homologous heat shock protein 70s as protective agents to *Artemia franciscana* challenged with *Vibrio campbellii*

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ARTICLE INFO

Article history:
Received 10 April 2010
Received in revised form
28 June 2010
Accepted 2 July 2010
Available online 17 July 2010

Keywords:
Heat shock protein (Hsp) 70
DnaK
Phenoloxidase
Artemia franciscana
Vibrio campbellii

ABSTRACT

The Hsp70 class of heat shock proteins (Hsps) has been implicated at multiple points in the immune response of both vertebrates and invertebrates. This class of chaperones is highly conserved in both sequence and structure, from prokaryotes to higher eukaryotes. In view of their high degree of homology, it was assumed that these Hsp70 proteins derived either from the prokaryotes or eukaryotes would have similar functions, especially in relation to their protective ability in a challenge assay. To verify this, we compared two evolutionary diverse Hsp70s, *Artemia* Hsp70 and *Escherichia coli* Hsp70 equivalent DnaK (each overproduced in *E.coli*), for their ability to protect *Artemia* against *Vibrio* challenge. Results showed that *Artemia* fed with *E. coli* producing *Artemia* Hsp70 or DnaK proteins, as assessed by immune-probing in western blots, survived better in a *Vibrio* challenge assay. The observed effects could be due to enhancement of the *Artemia* immune system as phenoloxidase activity was found to be increased by these proteins. These two Hsp70 proteins exhibit a high degree of homology, particularly in the peptide-binding domain (the putative innate immunity-activating portion) with 59.6% identity, indicating that the observed protective capacity of homologous or heterologous Hsp70 proteins might reside within this peptide-binding domain.

1. Introduction

Diseases caused by pathogenic or opportunistic bacteria, such as *Vibrio* spp. are still considered a major constraint to the sustainable development of aquaculture worldwide [1]. Although, the use of antibiotics and disinfectants had some success in the prevention or cure of such diseases [2], such practices are undesirable as they promote the selection and dissemination of antibiotic-resistant bacteria in both the target organisms, as well as in the environment [3]. Therefore, the use of alternative control approaches is becoming increasingly important for further development of more sustainable aquaculture practices.

In recent years, heat shock proteins (Hsps) have received considerable attention owing to their multi-functional features [4,5]. They are soluble intracellular chaperones present in all organisms from prokaryotes to eukaryotes [6,7]. Most Hsps are constitutively expressed under normal physiological conditions, however, their expression is up-regulated by various physiological stressors, such as high temperature, toxins, osmotic stress, ultra-violet and gamma radiation, certain chemicals and drugs, hypoxia, glucose deprivation, and microbial infection that could potentially damage the cellular and molecular structures in the cells [8,9]. Hsps perform essential biological functions under both normal and stressful conditions such as assisting in the folding of nascent proteins, translocation of these proteins between cell organelles, assembly and disassembly of multi-subunit complexes, refolding or degradation of denatured proteins due to stresses, dissolution of pathological protein aggregates, and other processes enhancing the survival of normal and diseased cells and tissues [10].

Evidence from several studies suggested that Hsps, particularly those of the Hsp70 family, can mediate the generation of strong innate and adaptive immune responses against many diseases [6,7,11], leading to the formulation of strategies to fight infections. Recently, our laboratory has explored the hypothesis that Hsps control disease in aquaculture and found that induction of Hsp in *Artemia* (eukaryotic Hsp70) through a non-lethal heat shock is associated with protection against virulent *Vibrios* [12]. In another study [13], the same authors observed that ingestion of *Escherichia coli* overproducing DnaK (prokaryotic equivalent to Hsp70) significantly improved the survival of gnotobiotically cultured *Artemia* upon challenge with *V. campbellii*. These studies suggest that Hsp70 (either prokaryotic or eukaryotic) conferred protection to *Vibrio*-challenged *Artemia*. The Hsp70 family members derived either from the prokaryotes or eukaryotes have a high degree of sequence

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doi:10.1016/j.fsi.2010.07.011

homology (about 60%), and there is a possibility that all Hsp70 proteins, irrespective of source, might have the same basic biological activity [14], especially in relation to their protective properties against bacterial infection. However, this hypothesis remains controversial as evidence from several studies, particularly *in vitro*, suggested that Hsp of the same family, but from different species, might have markedly different activity [15–17].

This study was set up to verify the hypothesis that feeding *Artemia* with *E. coli* overproducing homologous *Artemia* Hsp70 would equally well protect *Artemia* against the pathogen *Vibrio campbellii* as through feeding *E. coli* overproducing heterologous DnaK.

2. Materials and methods

2.1. PCR amplification and cloning of Artemia Hsp70 and bacterial DnaK genes

Artemia Hsp70 cDNA, a generous gift from Dr. Thomas H. MacRae, Department of Biology, Dalhousie University, Canada, was amplified by polymerase chain reaction and cloned into the TOPO® cloning vector using a pBAD Directional TOPO® Expression Kit (Invitrogen™, Merelbeke, Belgium) according to manufacturer recommendations. PCR reaction was performed in a 50 μl reaction mixtures containing 1 μl cDNA as template, 2 mM of $MgSO_4$, 0.2 mM of dNTP mix, 1.25 unit of proofreading *pfu* DNA polymerase (Fermentas), 1 × *pfu* buffer, and 1 μM each of oligonucleotide primers *Artemia* $Hsp70_{forward}$ 5′- CACCATGGCAAAGGCACCAGCAATAGG-3′ and $Hsp70_{reverse}$ 5′- ATAGTTGGGCCACTGCCTGTTCCAG-3′. PCR conditions were as follows: denaturation at 94 °C for 5 min followed by 35 cycles of 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min followed by 10 min at 72 °C. Amplification of the appropriate 1935 base pairs fragment was verified by electrophoresis. It was then ligated into the TOPO cloning vector and transformed into One Shot TOP10 (non-pathogenic *E. coli*) cells, which were grown on Luria-Bertani (LB) agar containing 100 μg ml^{-1} ampicillin at 37 °C. A bacterial clone containing *Artemia* Hsp70 cDNA was isolated from the LB plate, labelled A_{native}, and stored in 40% glycerol at −80 °C.

Strains YS2 (which expresses the 70-kDa bacterial Hsp, DnaK, upon arabinose induction) and YS1 (which does not express DnaK upon arabinose induction) were described previously [13].

2.2. Induction of Hsp70 proteins in E. coli strains

E. coli strains YS1, YS1 and A_{native} were grown at 37 °C for 24 h on LB agar and then to log phase in LB broth by incubation at 37 °C. *V. campbellii* strain LMG 21363 stored in 40% glycerol at −80 °C were grown at 28 °C for 24 h on marine agar and then to log phase in marine broth 2216 (Difco Laboratories, Detroit, Mich.) by incubation at 28 °C. Overproduction of *Artemia* Hsp70 protein in A_{native} cells was stimulated by adding different doses of L-arabinose (0, 0.5, 1, 2, and 4 mg ml^{-1}) for a fixed time (1 h). Subsequently, L-arabinose at 0.5 mg ml^{-1}, which gave the best induction in the dose-response experiment, was tested for different time intervals (1, 2, 3 and 4 h) [18]. Maximum production of *Artemia* Hsp70 in A_{native} cells was obtained at 0.5 mg ml^{-1} L-arabinose for 4 h, as obtained for YS2 cells overproducing DnaK [13]. The respective bacteria after induction were transferred to sterile tubes, centrifuged at 2200 × *g* for 15 min at 28 °C, suspended in filtered (0.2 μm) autoclaved sea water, and fed immediately to *Artemia* larvae. Bacteria cell numbers were determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells ml^{-1}.

2.3. Artemia and axenic hatching

Axenic *Artemia* were obtained following decapsulation and hatching procedures as described by Marques et al. [19]. Briefly, *Artemia* cysts (60 mg) originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Baasrode, Belgium) were hydrated in 9 ml ofdistilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 0.33 ml NaOH (32%) and 5 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 necessary tools were previously autoclaved at 121 °C for 20 min. The decapsulation was stopped after about 2 min by adding 5 ml $Na_2S_2O_3$ (10 g l^{-1}). The aeration was then stopped and the decapsulated cysts were washed with filtered (0.2 μm) and autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were re-suspended in a 50 ml glass tube containing 30 ml of filtered and autoclaved artificial seawater and incubated for 28 h (allowing the emerged nauplii to reach stage II in which they are able to ingest bacteria) on a rotor (4/min) at 28 °C with constant illumination (approximately 2000 lux). Groups of 30 nauplii were transferred to new sterile 50 ml glass tubes that contained 30 ml of filtered and autoclaved artificial seawater. The nauplii were incubated for 6 h with YS1, YS2 and A_{native} strains at 10^7 cells ml^{-1}. They were then challenged with *V. campbellii* at 10^7 cells ml^{-1} for 36 h. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. The survival of *Artemia* was scored 36 h after the challenge. Each treatment was carried out in quintuplicate and each experiment was repeated twice to check the reproducibility.

2.4. Methods used to verify axenicity of Artemia

After hatching, the axenicity of the *Artemia* nauplli was verified by spread plating 100 μl of the hatching water on Marine agar (Difco, Detroit, USA) followed by incubating at 28 °C for 5 days [20]. Experiments started with non-sterile nauplii were discarded.

2.5. Nutritional effect of the bacterial strains

To examine the nutritional value of the induced and non-induced bacteria, axenically cultured *Artemia* larvae were fed once with approximately 10^7 cells ml^{-1} of each bacterial strains (YS1, YS2, and A_{native}) without *V. campbellii* challenge. Swimming larvae were collected after two days, counted and fixed in lugol's solution. Survival percentage was calculated as described above. Individual length was ascertained by measuring fixed larvae with a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software *Artemia* 1.0® (courtesy of Marnix Van Domme).

2.6. Protein extraction, detection and analysis

Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads 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) [21] containing protease inhibitor cocktail (Catalogue #P8465; Sigma–Aldrich, Inc.) at the highest recommended level. Subsequent to centrifugation at 2200 × *g* for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method [22] 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. 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 1x 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 monoclonal antibody 8E2/2, raised in mouse to DnaK, at the recommended dilution of 1:1000 (Stressgen Bioreagents) as primary antibody in case of YS1 and YS2 cells or with mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO) at the recommended dilution of 1:5000 in case of A_{native} cells. Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO) for all the bacterial cells. 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).

2.7. Axenic Artemia rearing set-up for phenoloxidase (PO) assay

The method for rearing axenic *Artemia* for PO assay was as described previously [23]. Briefly, *A. franciscana* cysts were decapsulated-disinfected as described in Section 2.3. After 28 h incubation at 28 °C, swimming nauplii were collected, counted volumetrically (3 replicates) and thereafter transferred to sterile glass bottles (1000 ml). The nauplii were incubated for 6 h with different induced or non-induced *E. coli* strains (as cited above) prior to *V. campbellii* (10^7 cells ml^{-1}) exposure. After 12 h exposure, 0.1 g of live nauplii was harvested (equal amount of animals for all treatments), rinsed in FASW and stored at −80 °C. The experiment was repeated to obtain three samples per treatment for PO analysis.

2.8. Preparation of crude enzyme extracts

Sampling hemolymph from the microscopic *Artemia* larvae is a difficult task. Therefore, the entire larvae were used to prepare crude enzyme extracts [23,24]. Samples were homogenized in cold homogenizing mixture of 0.85% NaCl-2.5 mM EDTA and Triton-X 1%-10 mM $CaCl_2$ (1:1) so as to prepare 10% homogenate. The homogenates were placed at 4 °C for 36 h for enzyme extraction, thereafter centrifuged at 10, 000 × *g* for 20 min at 4 °C. The supernatants were used as crude enzyme extracts for PO assay.

2.9. Assay of PO activity

The PO activity of the enzyme extracts was determined according to the procedure described by Ashida et al. [25] with some modification. The enzyme extract (100 μl) was added to the well of a 24-well microtitre plate to which 1 ml of L-DOPA (substrate) dissolved in 100 mM sodium acetate-citric acid buffer (pH 7.1) containing 10 mM $CaCl_2$ was added. The reactive mixture was incubated in dark conditions at 30 °C and monitored for changes in optical density (OD) at 490 nm for over 48 h using ELISA reader (Tecan, Männedorf, Switzerland). Background OD_{490} increment due to spontaneous non-enzymatic dopachrome production was assayed in wells without crude enzyme extract using only diluent mix and L-DOPA. Apparent PO activity was recorded as the change in absorbance over 48 h time interval and expressed in units as defined by Rojas-García et al. [23].

2.10. Sequence comparisons

Homology between the recombinant *Artemia* Hsp70 and DnaK proteins was determined by aligning the amino acid sequences of *Artemia* Hsp70 (GenBank accession no. AAL27404) and *E. coli* Dnak (GenBank accession no. NP_414555.1) using Clustal W multiple-alignment method [26].

2.11. Statistical analysis

Survival data were arcsin transformed while individual length was either logarithmic or square root transformed to satisfy normality and homocedasticity requirements as necessary. Data were then subjected to one-way analysis of variances (ANOVA) followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences (SPSS) version 14.0. to determine significant differences among treatments. Significance level was set at $P < 0.05$. The experiments for testing the nutritional value of bacterial strains and resistance to *Vibrio* challenge, as described above in different sections, were performed twice with each replicate done in quintuplicate.

3. Results

3.1. Optimizing L-arabinose dose and induction time for Artemia Hsp70 overproduction in E. coli

To optimize the Hsp70 induction by L-arabinose, an expression experiment was set up by first incubating the A_{native} cells with varying concentration of L-arabinose for 1 h, and then by running a time course of expression. It could be shown that in the absence of arabinose some induction of *Artemia* Hsp70 occurred (Fig. 1B, control). Control strains, such as, induced or non-induced YS1, do not produce a protein recognised by monoclonal antibody 8E2/2, raised in mouse to DnaK (result not shown, see Sung et al. [13]). Already with 0.5 mg ml^{-1} arabinose and 1 h induction, maximal induction could be obtained. A single polypeptide of approximately 83 kDa size was detected in the extract prepared from arabinose-induced A_{native} strain (Fig. 1B and D). Similarly, DnaK was also up-regulated in arabinose-induced YS2 strain, yielding a polypeptide of approximately 83 kDa visible on Western blot probed with antibody to DnaK (result not shown, see Sung et al. [13]). The increase in molecular mass of approximately 13 kDa in the induced A_{native} and YS2 strains, as compared with the normal mass of Hsp70 or DnaK, was because of the amino-terminal incorporation of thioredoxin encoded by the TOPO® cloning vector. Results also showed that longer induction (2 h or more) or higher arabinose concentration did not result in a substantial higher detectable amount of *Artemia* Hsp70 in the *E. coli* cells (Fig. 1B and D). For that the effect of feeding *E. coli* strains overproducing *Artemia* Hsp70 was investigated first (Tables 1 and 2) Secondly, the effect of feeding *E. coli* strains overproducing DnaK or *Artemia* Hsp70 was directly compared (Table 3). As can be seen, a significant ($P < 0.01$) increase in the survival (compared with the control) was recorded during the challenge tests when *Artemia* larvae were fed with A_{native} strain induced with L-arabinose in the range of 0.5–4.0 mg ml^{-1}, with maximum protection at the lowest L-arabinose concentration, however, not significantly ($P > 0.05$) different from the others (Table 1, Experiment 1). But in the second experimental run inducing *E. coli* with a higher concentration of arabinose (2 or 4 mg ml^{-1}) resulted in a lower *Artemia* survival with respect to the lower arabinose concentrations (0.5 and 1 mg ml^{-1}), yet the survival was still significantly ($P < 0.01$) higher than in the control (Table 1, experiment 2). Using 0.5 mg ml^{-1} arabinose, an induction period of 1 h was sufficient to confer some protection against pathogenic *V. campbellii*. The protection, however, was maximum and significantly ($P < 0.05$) higher after an induction period of 4 h (Table 2).

3.2. Survival of vibrio-challenged Artemia fed homologous or heterologous Hsp70 proteins

In the second set of *in vivo* tests, the effect of feeding induced and non-induced *E. coli* on the survival of *Artemia* either challenged or

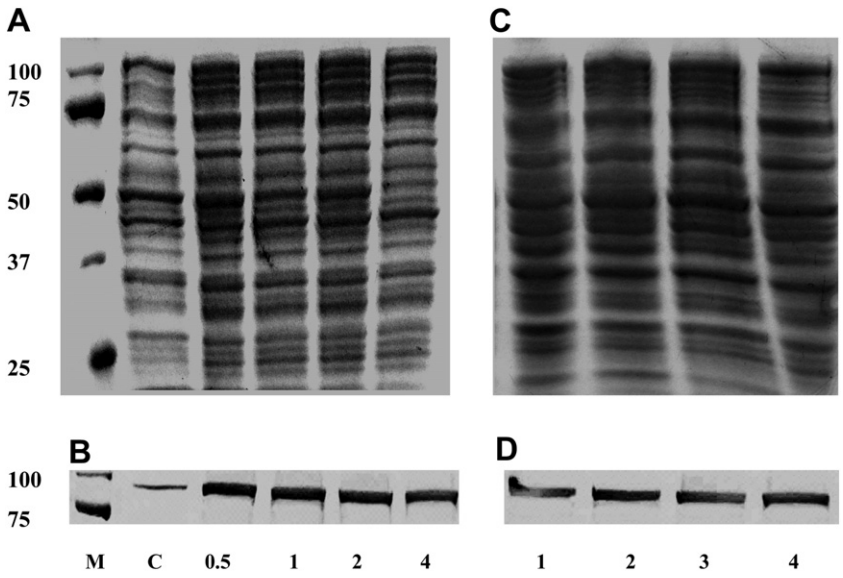

Fig. 1. Arabinose induced over-expression of *Artema* Hsp70 protein in the A_{native} strain. Protein extract from the *E. coli* strain A_{native} induced with L-arabinose was resolved in SDS-PAGE gels and then either stained with Coomassie Biosafe (A, C) or transferred to polyvinylidene fluoride membranes and probed with antibody to *Artema* Hsp70 (B, D). C, non-induced; 0.5–4.0, L-arabinose induction for 1 h, respectively, at 0.5–4.0 mg ml^{-1}; 1–4, induction with 0.5 mg ml^{-1} L-arabinose, respectively, for 1–4 h. Fifty microgram of bacterial protein was loaded in each lane. Molecular mass standards (M) in kDa are on the left.

not with *V. campbellii* was investigated. The survival of the non--challenged nauplii fed either induced or non-induced *E. coli* cells (YS1, YS2 or A_{native} cells) did not differ significantly ($P > 0.05$) among each other (Table 3). In contrast, a significant ($P < 0.001$) difference among the challenged groups was noted. Survival of *Artemia* nauplii fed either induced or non-induced strain YS1 was low upon challenge with *V. campbellii*, results similar to those obtained with non-induced YS2 and A_{native} (Table 3). However, a significantly ($P < 0.001$) higher survival was obtained when arabinose-induced YS2 or A_{native} strains were provided to challenged nauplii in comparison to challenged nauplii supplied with non-induced *E. coli* cells. Comparison between the challenged groups fed either induced YS2 or A_{native} cells showed no significant differences ($P < 0.05$). The second experiment (Table 3, Experiment 2) conducted to check the reproducibility showed similar results.

3.3. Nutritional effect of the bacterial strains

As the susceptibility of *Artemia* to pathogenic *V. campbellii* depends on the quality of feed fed [27], a test was performed by feeding induced and non-induced bacterial strains to *Artemia* in order to examine their nutritional value. *Artemia* nauplii fed with *E. coli* had significantly ($P < 0.05$) higher survival and length compared with the non-fed *Artemia* (Table 4). However, the number of live larvae and the length among the different groups fed either induced or non-induced bacterial strains was not significantly ($P < 0.05$) different.

3.4. Phenoloxidase activity

To elucidate the role Hsp70 proteins in the host defence against *V. campbellii*, apparent phenoloxidase (PO) activity was determined in the *Vibrio* challenged *Artemia*, and this showed that the PO activity varied among different treatments (Fig. 2). The level of PO activity in the group fed either arabinose-induced YS2 or A_{native} strains was significantly ($P < 0.05$) higher than those fed either non-induced YS2 or A_{native} strains. *Artemia* fed either induced or non-induced YS1 strain had a marked ($P < 0.05$) decrease in PO activity compared to those fed induced YS2 or A_{native} strains, however, they were significantly ($P < 0.05$) higher than that of the group fed non-induced YS2 strain.

3.5. Sequence comparison of Artemia and E. coli Hsp70 proteins

The amino acid sequence of *Artemia* Hsp70 protein showed a high degree of identity to that of the Hsp70 protein of *E. coli*

Table 1
Percentage survival of *Artemia* nauplii after 36 h challenge with *Vibrio campbellii* LMG21363.

L-arabinose (mg ml^{-1})	Survival (%)	
	Experiment 1	Experiment 2
0	78.0 ± 0.8^{b}	62.7 ± 4.5^{c}
0.5	86.7 ± 1.8^{a}	96.0 ± 2.7^{a}
1	86.0 ± 1.2^{a}	100.0 ± 5.3^{a}
2	86.7 ± 1.8^{a}	76.0 ± 3.4^{b}
4	84.7 ± 0.8^{a}	78.7 ± 3.9^{b}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.01$). The A_{native} cells were induced with different concentration of L-arabinose for 1 h. *Artemia* nauplii were fed once with A_{native} cells at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* LMG21363 for 36h.

Table 2
Percentage survival of *Artemia* nauplii after 36 h challenge with *Vibrio campbellii* LMG21363.

Induction time (h)	Survival (%)	
	Experiment 1	Experiment 2
0	40.0 ± 3.7^{c}	32.0 ± 4.0^{c}
1	51.3 ± 5.0^{b}	46.7 ± 3.2^{b}
2	53.3 ± 1.5^{b}	53.3 ± 3.8^{b}
3	51.3 ± 2.3^{b}	52.0 ± 5.3^{b}
4	68.0 ± 1.7^{a}	70.0 ± 3.3^{a}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.01$). The A_{native} cells were induced with L-arabinose at a concentration of 0.5 mg ml^{-1} for different durations (0, 1, 2, 3, and 4 h). *Artemia* nauplii were fed once with A_{native} cells at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* LMG21363 for 36h.

Table 3
Percentage survival of *Artemia* nauplii after 36 h challenge with *Vibrio campbellii* LMG21363.

Treatments	Survival (%)	
	Experiment 1	Experiment 2
YS1 (−)	82.7 ± 2.2 ^{ab}	78.0 ± 3.3^{c}
YS1 (−) + VC	41.3 ± 2.7^{d}	36.7 ± 2.4^{f}
YS1 (+)	81.3 ± 2.7^{a}	80.0 ± 2.4^{c}
YS1 (+) + VC	43.3 ± 3.9^{d}	38.7 ± 3.4^{f}
YS2 (−)	84.7 ± 4.9 ^{ab}	82.0 ± 4.0^{bc}
YS2 (−) + VC	50.0 ± 3.8^{d}	54.0 ± 3.7^{e}
YS2 (+)	90.7 ± 2.7 ^{ab}	90.7 ± 0.7 ^{ab}
YS2 (+) + VC	70.0 ± 3.8^{c}	68.0 ± 3.4^{d}
A_{native} (−)	90.7 ± 2.4 ^{ab}	83.3 ± 5.1^{abc}
A_{native} (−) + VC	41.3 ± 3.4^{d}	35.3 ± 3.3^{f}
A_{native} (+)	92.0 ± 2.0^{a}	92.7 ± 1.9^{a}
A_{native} (+) + VC	62.0 ± 3.7^{c}	59.3 ± 2.4^{de}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.001$). The bacterial strains YS1 (a Hsp70 non-producer), YS2 (a DnaK producer after arabinose induction), and A_{native} (an *Artemia* Hsp70 producer after arabinose induction) were either induced (+) or non-induced (−) with L-arabinose at concentration of 0.5 mg ml^{-1} for 4 h. *Artemia* larvae were fed once with the bacterial strains at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* (VC) for 36 h.

(Fig. 3). Identity between these two proteins was higher in the peptide-binding domain (59.6%), compared to the N-terminal ATPase domain (48.8%) and the C-terminal lid domain (19.4%) (Table 5). The overall identity of the two proteins was in the range of 45.8%.

4. Discussion

A substantial sequence homology exists between equivalent Hsp family members derived either from prokaryotes or eukaryotes [14,28]. Hence it was assumed that these Hsps would have a high degree of functional conservation, also with respect to their protective capacity in a *Vibrio* challenge test. In order to document this hypothesis, gnotobiotically grown *Artemia franciscana* was used to test the efficacy of prokaryotic (*E. coli* DnaK) and eukaryotic (*Artemia* Hsp70) Hsp70 proteins overproduced in *E. coli* in protecting *Artemia* nauplii against *Vibrio* challenge. Working in gnotobiotic circumstances facilitates the interpretation of the results in terms of a cause effect relationship. In this study, strain YS2 was used as the source of prokaryotic Hsp70. This strain was

Table 4
Percentage survival and individual length (mm) of *Artemia* after 48 h of feeding L-arabinose induced or non-induced *E. coli* strains.

Treatments	Experiment 1		Experiment 2	
	Survival	Individual length	Survival	Individual length
Non-fed *Artemia*	70.0 ± 1.5^{b}	0.66 ± 0.03^{b}	68.0 ± 0.8^{c}	0.66 ± 0.03^{b}
YS1 (+)	83.3 ± 2.8^{a}	0.81 ± 0.02^{a}	78.0 ± 4.3^{b}	0.79 ± 0.03^{a}
YS1 (−)	81.3 ± 4.0^{a}	0.81 ± 0.02^{a}	82.0 ± 3.1 ^{ab}	0.76 ± 0.03^{a}
YS2 (−)	88.0 ± 2.5^{a}	0.78 ± 0.03^{a}	80.7 ± 4.1 ^{ab}	0.76 ± 0.02^{a}
YS2 (+)	89.3 ± 3.7^{a}	0.76 ± 0.04^{a}	88.7 ± 3.6^{a}	0.75 ± 0.01^{a}
A_{native} (+)	85.3 ± 3.8^{a}	0.83 ± 0.02^{a}	84.0 ± 1.3 ^{ab}	0.81 ± 0.02^{a}
A_{native} (−)	82.0 ± 4.4^{a}	0.81 ± 0.03^{a}	77.3 ± 1.3^{b}	0.77 ± 0.02^{a}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.05$). The bacterial strains YS1 (a Hsp70 non-producer), YS2 (a DnaK producer after arabinose induction), and A_{native} (an *Artemia* Hsp70 producer after arabinose induction) were either non-induced (−) or induced (+) with L-arabinose at concentration of 0.5 mg ml^{-1} for 4 h. *Artemia* were fed with these bacteria strains at 10^7 cells ml^{-1} and following 2 days incubation, the number and length of the live larvae were determined. *Artemia* that were not fed was used as control.

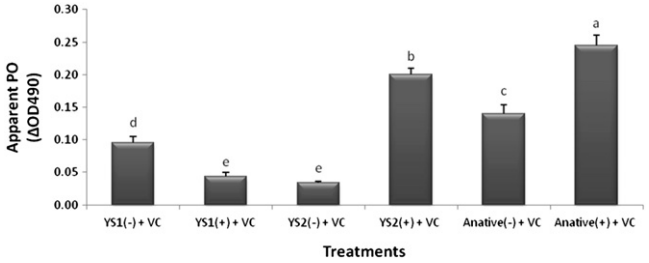

Fig. 2. Apparent PO activity *Artemia*. Data represents the mean of three independent experiments. Error bars with different alphabet letters indicate significant difference ($P < 0.05$). The bacterial strains YS1 (a Hsp70 non-producer), YS2 (a DnaK producer after arabinose induction), and A_{native} (an *Artemia* Hsp70 producer after arabinose induction) were either induced (+) or non-induced (−) with L-arabinose at concentration of 0.5 mg ml^{-1} for 4 h. *Artemia* larvae were fed once with the bacterial strains at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* (VC) for 12 h.

developed in our previous study [13] and is producing DnaK by arabinose induction. As a source of eukaryotic Hsp70 protein, the strain A_{native} was constructed in this study. This strain was developed in a similar fashion [13], except that *Artemia* Hsp70 cDNA was cloned into the same plasmid pBAD.

A problem in studying the effect of recombinant proteins produced in *E. coli* is the possibility that the observed protective effects are caused by lipopolysaccharide [29,30]. To accommodate for this possibility, the strain YS1 (non-induced or induced) carrying the empty cloning vector was used as control.

Results of our *in vivo* challenge test revealed that arabinose-induced YS2 and A_{native} strains were more potent and efficacious than their non-induced counterparts in safeguarding *Artemia* against *V. campbellii*. Furthermore, results also showed that both the induced strains conferred protection to *Vibrio*-challenged *Artemia* to a similar extent, consistent with the capacity of the YS2 or A_{native} strains to produce respective Hsp70 proteins upon arabinose addition. In contrast, the arabinose-induced or non-induced YS1 strain, a Hsp70 non-producer upon arabinose addition, was found to be the least effective of these three bacterial strains in conferring protection to *Artemia*. This strongly indicates that both *Artemia* Hsp70 and DnaK are responsible for conferring protection to the *Vibrio*-challenged *Artemia*, and that their protective capacity is caused by the recombinant intracellular Hsp70 proteins and not the result of lipopolysaccharide exposure or any other *E. coli*-bound cellular material. Furthermore, our result also showed that the nutritional value of the induced *E. coli* strains was not significantly different from that of the non-induced strains, in accordance to the findings of Sung et al. [13]. This observation further substantiates the above-mentioned conclusion that the recombinant Hsp70 proteins (and not the bacterial cells) were responsible for protecting *Artemia* against bacterial infection and that the difference in *Artemia* protection were not due to difference in the nutritional value of the non-induced versus induced strains.

In a previous study [12], it was found that *Artemia* was protected against *V. campbellii* after a non-lethal heat shock, resulting into an increased Hsp70 expression. That research suggested that these two phenomena were linked. In this paper, we extend this work basically showing that offering *Artemia* its homologous Hsp70 protein through the feed also results into a protective effect, establishing a much closer casual link between Hsp70 and protection against *V. campbellii*. Obviously, the route of exposure is completely different, raising questions about how the observed effects are brought about for instance at the immunological level. The molecular mechanisms involved in such a protective effect is not yet clearly known, however, could be attributed to strong

Fig. 3. Scheme of the three functionally distinct domains of *Artemia* Hsp70 and DnaK proteins (adapted from Todryk et al. [17] and Kabani and Martineau [47]).

induction of immunological responses in *Artemia* by these proteins as elevated activity of PO enzyme, one of the most important defence mechanisms in crustaceans [31], in groups fed arabinose-induced YS2 and A_{native} strains was recorded. The occurrence and modulation of other immunological responses such as, nitric oxide synthase, superoxide dismutase, lysozymes and others by these Hsp70 proteins might also occur in challenged *Artemia*, however, it needs to be demonstrated. In line with our results, there is evidence that Hsps modulate immune and inflammatory responses in other animals [13,32–35]. For instance, application of Hsps *in vitro* induced phagocytes and granulocytes to release reactive oxygen species, cationic peptides, lysozyme and cytokines [36,37]. Additionally, macrophages and neutrophils were stimulated to produce nitric oxide synthase [38], nitric oxide [39], tumour necrosis factor-α, interleukin (IL)-1β and IL-6 [39–41], all extracellular suppressors of infection. Hsps also activate Toll like receptors, which transduce inflammatory signals to innate immune cells and promote resistance against disease and infections [42,43]. Furthermore, exogenous administration of Hsps up-regulates two major macrophage/monocyte differentiation markers [44], all of which suppress infection.

The primary structure of the bacterial Hsp70 protein, DnaK, is very similar to its eukaryotic counterpart and consists of three functionally distinct domains: an N-terminal 44-kDa ATPase domain, an 18-kDa peptide-binding domain, and a C-terminal 10-kDa lid domain [5,45]. Several studies demonstrated that the immune stimulating functions of Hsp70 reside in the C-terminal portion (amino acid 359-610, Fig. 3), particularly in the 18-kDa peptide binding domain (amino acid 359-494) [5,46]. Comparison of the peptide sequence of the two Hsp70 proteins demonstrate a high degree of homology, particularly in the peptide-binding domain (see Table 5), suggesting that this part of the protein might be responsible for the observed effects.

Taken together, the results demonstrate that both *Artemia* Hsp70 and DnaK, in qualitative terms, appeared to be equally efficient in protecting *Artemia* in a *Vibrio* challenge assay, when offered to *Artemia* in *E. coli* overproducing cells. In quantitative terms, it is not possible to state which molecule protects *Artemia* better, as it is not possible to quantify the amount of Hsp70 or DnaK ingested by *Artemia*. The latter is due to various reasons. Firstly, different antibodies were used to detect the DnaK and Hsp70 antigens possibly resulting in a different dose-response relationship in the western blot. Secondly, the actual ingestion of *E. coli* cells was not measured. In the future, detailed research has to be focused on this aspect, also revealing the dose-response relationship at the immunological level, using for example established markers such as phenoloxidase expression or activity [31].

Table 5
Percentage of identity and similarity between different domains of *Artemia* Hsp70 and *E. coli*-equivalent Dnak proteins.

Domains	Identity (%)	Similarity (%)
ATPase domain	48.8	78.2
Peptide-binding domain	59.6	83.3
Lid domain	19.4	52.9

Acknowledgements

This study was supported by a doctoral grant to the first author from developing countries (Bijzonder Onderzoeksfonds) by Ghent University, Belgium. We thank the technical staffs of Lab of Aquaculture and *Artemia* Reference Centre, Ghent University for their assistance in carrying out various analyses during the research work. The projects entitled 'Hsp70 in gastheer-microbiele interacties (1.5.092.09.N.00)' and 'Probiont-induced functional responses in aquatic organisms (G.0.491.08.N.10)' funded by Research Foundation – Flanders, Belgium were highly acknowledged.

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