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Feeding truncated heat shock protein 70s protect *Artemia franciscana* against virulent *Vibrio campbellii* challenge

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ABSTRACT

The 70 kDa heat shock proteins (Hsp70s) are highly conserved in evolution, leading to striking similarities in structure and composition between eukaryotic Hsp70s and their homologs in prokaryotes. The eukaryotic Hsp70 like the DnaK (*Escherichia coli* equivalent Hsp70) protein, consist of three functionally distinct domains: an N-terminal 44-kDa ATPase portion, an 18-kDa peptide-binding domain and a C-terminal 10-kDa fragment. Previously, the amino acid sequence of eukaryotic (the brine shrimp *Artemia franciscana*) Hsp70 and DnaK proteins were shown to share a high degree of homology, particularly in the peptide-binding domain (59.6%, the putative innate immunity-activating portion) compared to the N-terminal ATPase (48.8%) and the C-terminal lid domains (19.4%). Next to this remarkable conservation, these proteins have been shown to generate protective immunity in *Artemia* against pathogenic *Vibrio campbellii*. This study, aimed to unravel the *Vibrio*-protective domain of Hsp70s *in vivo*, demonstrated that gnotobiotically cultured *Artemia* fed with recombinant C-terminal fragment (containing the conserved peptide binding domain) of *Artemia* Hsp70 or DnaK protein were well protected against subsequent *Vibrio* challenge. In addition, the prophenoloxidase (proPO) system, at both mRNA and protein activity levels, was also markedly induced by these truncated proteins, suggesting epitope(s) responsible for priming the proPO system and presumably other immune-related genes, consequently boosting *Artemia* survival upon challenge with *V. campbellii*, might be located within this conserved region of the peptide binding domain.

1. Introduction

Heat shock proteins (Hsps), known as molecular chaperones or stress proteins, are highly conserved proteins found in all prokaryotes and eukaryotes [1,2]. Most of these proteins are constitutively expressed at a low level under physiological conditions, however, they are strongly upregulated following a wide variety of stressful stimuli in order to protect cells from lethal damage [3,4]. Inside the cell, Hsps play essential housekeeping functions in protein folding, intracellular transport processes and protein degradation, and also play key roles in antigen processing [5–7]. In general, these chaperones bind to peptide segments of non-native proteins, thus preventing protein aggregation and mediating proper folding. These proteins range in size from 27 to 110 kDa and can be classified into five families according to their molecular mass: Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps [8]. Among the Hsps, the members of the Hsp70 family represent the most conserved and best-studied class [2,4,9,10]. In eukaryotic cells, distinct members of the Hsp70 family are present in various cellular compartments including cytoplasm, endoplasmic reticulum, mitochondria and chloroplasts [11]. In contrast, in bacteria only a single form of Hsp70, referred to as DnaK protein, has been identified [5,12].

The DnaK protein, like eukaryotic Hsp70, consist of three functionally distinct domains which include an N-terminal 44-kDa ATPase portion (amino acids 1–358), followed by an 18-kDa peptide-binding domain (amino acids 359–510) and a C-terminal 10-kDa fragment (amino acids 511–609) [13]. Both eukaryotic Hsp70 and DnaK proteins, apart from their chaperone function, have also been described for initiating the innate immune responses in various animal models [2,10,14,15]. For instance, in a previous study [16], it was demonstrated that induction of Hsp in an invertebrate model system, *Artemia franciscana* (eukaryotic Hsp70) through a non-lethal heat shock was associated with protection against virulent vibrios. In another study [17], the same authors observed that ingestion of *Escherichia coli* overproducing DnaK (prokaryotic

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Hsp70) significantly improved the survival of gnotobiotically cultured *Artemia* challenged with *Vibrio campbellii*. In a further study, we extended the work of the above authors with the aim of comparing the efficacy of eukaryotic Hsp70 (that is *Artemia* Hsp70) with that of prokaryotic Hsp70 (DnaK), both of which have a high degree of amino acid sequence identity (45.8%), in conferring protection to *Artemia* against *V. campbellii* infection and the results revealed that *Artemia* Hsp70 or DnaK protein when ingested conferred protection to *Vibrio*-challenged *Artemia* to a similar extent [2]. We correlated this similar protective effect of these two proteins to the high degree of sequence identity (59.6%) between the two proteins, particularly in the peptide-binding domain (the putative innate immunity-activating portion). This finding suggests that the observed protective capacity of *Artemia* Hsp70 and DnaK proteins might reside within this peptide-binding domain. However, to our knowledge, no *in vivo* studies were carried out to determine the immunological/protective functions of this domain.

In this study, we generated two recombinant, truncated versions of the full length *Artemia* Hsp70 or DnaK protein by removing the ATPase portion (amino acid 1–358) and keeping the C-terminal portion, which contains the 18-kDa peptide-binding domain of *Artemia* Hsp70 or DnaK (amino acid 359–510). We used these truncated proteins to begin testing our hypothesis that a specific region of Hsp70/DnaK accounts for its protective ability. Here we used a model system of *Artemia*, a minute crustacean, cultured in gnotobiotic (or microbiologically controlled) conditions. The effects of the truncated proteins were compared with that of recombinant native *Artemia* Hsp70 and DnaK proteins, which were previously shown to confer protection to *Vibrio*-challenged *Artemia* [2,10].

2. Materials and methods

2.1. Gnotobiotic Artemia culture system (GART system)

The rationale for using GART system is to eliminate the possibility of microbial interference in mechanistic studies [18]. In GART system, axenic *Artemia* were obtained following decapsulation and hatching [19]. Briefly, 2.5 g of *Artemia* 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 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^{-1}. The aeration was then terminated 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 suspended in 1^{-1} 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 lux. After 28 h incubation, swimming nauplii at the stage II (at which time they ingest bacteria) were used for the *in vivo* assays. All these manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii.

2.2. Methods used to verify axenicity of Artemia

After 28 h of incubation, the axenicity of the *Artemia* nauplli 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 [19]. Experiments started with non-sterile nauplii were discarded.

2.3. Generation of recombinant native and truncated Hsp70 proteins

The full-length recombinant *Artemia* Hsp70 (A_{native}) and *E. coli* Hsp70 equivalent DnaK (E_{native}) proteins were generated from the One Shot TOP10 (non-pathogenic *E. coli*) cells as described previously [2,17]. The truncated portion of *Artemia* Hsp70 (*Artemia* Hsp359–644, indicated as A_{359}) and DnaK (*E. coli* Hsp359–638, indicated as E_{359}) were also prepared in a similar fashion as described previously for the native recombinant proteins [2,17, Fig. 1]. Briefly, *Artemia* Hsp70 cDNA encoding the C-terminal peptide-binding domain (amino acids 359–644) was amplified by polymerase chain reaction. The amplification was carried out 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 (Table 1). The PCR temperature profile was 94 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min. The obtained PCR products were then purified by using PureLink™ PCR Purification Kits (Invitrogen™, Merelbeke, Belgium) to remove primer dimers or short spurious PCR products. Amplification of the truncated fragments was verified by electrophoresis. The purified PCR product was ligated into the TOPO® cloning vector and transformed into One Shot TOP10 cells using a pBAD Directional TOPO® Expression Kit (Invitrogen™, Merelbeke, Belgium) according to manufacturer recommendations. In a similar way, DnaK cDNA from plasmid pKJE7 encoding the C-terminal peptide-binding domain (amino acids 359–638) were also amplified and cloned. The *E. coli* cells were then grown on Luria-Bertani (LB) agar containing 50 μg ml^{-1} kanamycin at 37 °C. A bacterial clone containing the gene encoding either A_{359} or E_{359} was isolated from the LB plate and stored in 40% glycerol at −80 °C. All the native and truncated recombinant proteins were expressed with additional amino acid residues i.e., with a N-terminal thioredoxin and C-terminal polyhistidine (6 × His) tags.

2.4. Induction of native and truncated Hsp70 proteins in E. coli strains

Culture of the *E. coli* strains E_{native}, A_{native}, A_{359} and E_{359} grown overnight on LB agar was re-inoculated in LB medium containing kanamycin and allowed to grow at 37 °C until log phase. For maximum induction of *Artemia* Hsp70 and DnaK in the A_{native} and E_{native} strains, respectively, a pre-optimized L-arabinose dose of 0.5 mg ml^{-1} was added to the medium and the culture was incubated for 4 h [2]. For optimizing the L-arabinose dose for maximum expression of truncated *Artemia* Hsp70 and DnaK proteins in A_{359} and E_{359} strains, respectively, the cultures were induced by the addition of L-arabinose at varying concentrations (0, 0.25, 0.5,1, 2 and 4 mg ml^{-1}) for 1 h. 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 seawater, and fed immediately to *Artemia* larvae. Bacteria were counted and adjusted to 10^7 cells ml^{-1} before feeding [2]. After feeding *Artemia*, aliquots of the A_{359} and E_{359} cells that remained were used for further analysis of SDS-PAGE and western blot as described below.

2.5. Growth of V. campbellii for challenge assay

V. campbellii strain LMG21363, stored in 40% glycerol at −80 °C, was grown at 28 °C for 24 h on marine agar and then to log phase in marine broth 2216 (Difco Laboratories, Detroit, MI. USA) by incubation at 28 °C with shaking for use in challenge experiments. Bacterial cell numbers were determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy

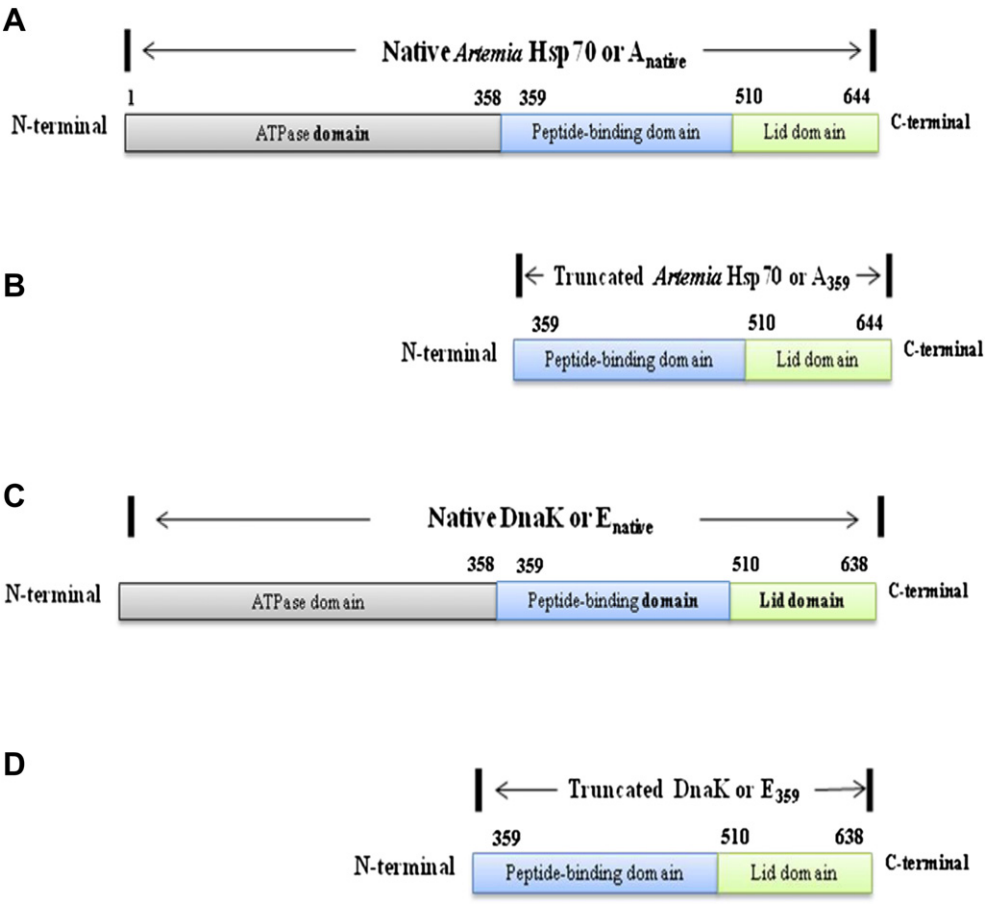

Fig. 1. Schematic diagram of *A. franciscana* Hsp70 (A), DnaK protein (C) and their respective truncated portions, A_{359} (B) and E_{359} (D) that were amplified by PCR reaction and cloned.

L'Etoile, France), with an OD of 1.000 corresponding to 1.2×10^9 cells ml^{-1}.

2.6. Assay of Artemia survival

Groups of 30 nauplii at the instar II stage were transferred to fresh sterile 40 ml glass tubes that contained 30 ml of filtered and autoclaved artificial seawater. The nauplii were incubated for 6 h with native (A_{native} or E_{native}) or truncated (A_{359} or E_{359}) Hsp70/DnaK producing *E. coli* strains at 10^7 cells ml^{-1}. They were then challenged with *V. campbellii* at 10^7 cells ml^{-1} [2]. Control *Artemia* were not challenged but otherwise treated the same as experimental animals. Each treatment was carried out in quintuplicate. The survival of *Artemia* was scored after 36 h of *V. campbellii* exposure. All manipulations were performed under a laminar flow hood.

2.7. Growth measurement of Artemia fed with A_{359} or E_{359} strain

Axenically hatched *Artemia* nauplii at stage 2 were fed once with approximately 10^7 cells ml^{-1} of A_{359} or E_{359} strain induced with different concentration of L-arabinose. The animals were then challenged with *V. campbellii* for 36 h as described above. Swimming nauplii from each replicate of a treatment were collected after 36 h, pooled and then fixed for 5 min in a fixative solution consisting of 80 ml of 100% ethyl alcohol, 15 ml of 40% formaldehyde and 5 ml of acetic acid, and transferred to 70% ethanol. The specimens, immediately after fixation, were viewed and photographed using a stereomicroscope (Olympus SZX 7, Olympus, Belgium). Subsequently, the individual lengths were measured using the software cell*D (Olympus soft imaging solutions).

Table 1
Primers used for PCR.

Primer	Sequence (5′–3′)
A_{native} (forward)	CACC ATG GCA AAG GCA CCA GCA ATA GG
E_{native} (forward)	CACC ATG GGT AAA ATA ATT GGT
A_{359} (forward)	CACC ATG TTA AAC AAG TCC ATC ACC CAG
E_{359} (forward)	CACC ATG AAA GAG CCG CGT AAA GACG
A_{native} (reverse) or A_{359} (reverse)	ATA GTT GGG CCA CTG CCT GTT CCA G
E_{native} (reverse) or E_{359} (reverse)	TTT TTT GTC TTT GAC TTC TTC

2.8. Protein extraction, detection and analysis

All the four *E. coli* strains generated 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) [20] containing protease inhibitor cocktail (Catalogue #P8465; Sigma–Aldrich, Inc., USA) in a mini beadbeater (Biospec, USA). Subsequent to centrifugation at $2200 \times g$ for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method [21] 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-polyacrylamide 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 1× phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room

temperature. Blocking was followed by incubation for 60 min with Anti-Thio™ antibody at the recommended dilution of 1:1000 (Invitrogen™, Merelbeke, Belgium). Membranes were incubated with horseradish peroxidase conjugated donkey antimouse IgG for 30 min at the recommended dilution of 1:2500 (Affinity Bio-Reagents Inc., Golden, CO, USA) for all *E. coli* cells and then rinsed three times, each time for 5 min, with washing buffer at room temperature. Detection was done with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate in association with 0.01% (v/v) H_2O_2 in 0.1 m Tris–HCl (pH 7.6).

2.9. Assay of prophenoloxidase gene expression and phenoloxidase activity

After 28 h incubation at 28 °C, swimming nauplii were collected, counted volumetrically and transferred to 500 ml sterile glass bottles. The nauplii were incubated for 6 h with arabinose-induced or non-induced *E. coli* strains, as described above, prior to *V. campbellii* challenge at 10^7 cells ml^{-1}. Each treatment was carried out in triplicate. After 6 h of *Vibrio* exposure, samples containing 0.1 g of live nauplii were harvested from all treatments, rinsed in cold distilled water, immediately frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted from *Artemia* larvae using TRIzol® (Invitrogen™, Merelbeke, Belgium) according to the manufacturer's instructions and treated with RNase-free DNase to remove DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 1 μg total RNA using the RevertAid™ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) according to the manufacturer's instructions. Prophenoloxidase (proPO) mRNA expression in *Artemia* was analyzed by RT-PCR using a pair of specific primers designed on the basis of *Artemia* proPO cDNA sequence [10]. The real time PCR amplifications were carried out in a total volume of 25 μl, containing 5.5 μl of nuclease free water, 1 μl of each primer, 12.5 μl of Maxima SYBR Green qPCR master mix (Fermentas, Cambridgeshire) and 5 μl of cDNA template. A four-step amplification protocol was used in Light-Cycler (ABI Prism SDS 7000, Applied Biosystems): denaturation (10 min at 95 °C); amplification and quantification repeated 40 times (15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C); melting curve (55–95 °C with a heating rate of 0.10 °C s^{-1} and a continuous fluorescence measurement) and cooling (4 °C). To obtain an internal control for the RT-PCR experiments, a pair of primers was designed based on the conserved sequence of the *Artemia* actin gene [10]. Master mixes were prepared in duplicate for each sample and quantitative PCR for target and reference genes was performed. Relative quantification of target gene transcripts with a chosen reference gene transcript was done following the Pfaffl method with the Relative Expression Software tool (REST©) [22]. The mathematical algorithm, which needs no calibration curve, computes an expression ratio, based on real time PCR efficiency and the crossing point deviation of the sample versus a control, as illustrated in the following formula:

$$\text{Ratio} = (E_{\text{target}})^{\Delta CT}\text{target}(\text{control} - \text{sample}) / \left(E_{\text{ref}}\right)^{\Delta CT} \times \text{ref}(\text{control} - \text{sample})$$

where CT (cycle threshold) corresponds to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. E is PCR efficiency determined by standard curve using serial dilution of cDNA. The value of E is calculated according to the equation $E = 10^{(-1/\text{slope})}$. CT values of dilution series (1×, 2×, 4×, 8× etc.) were used to calculate the slope for target and reference genes. Each dilution was tested in triplicate. ΔCT is the crossing point deviation of the sample versus a control.

Protein extracts were prepared from sampled larvae [2] and their phenoloxidase (PO) activity was determined according to Ashida et al. [23] with some modification. Equal volumes and protein amounts of each extract were added to the wells of 24-well microtitre plates to which 1 ml of the substrate L-DOPA (0.5 mM) dissolved in 100 mM sodium acetate-citric acid buffer (pH 7.1) containing 10 mM $CaCl_2$, was added. The reaction mixture was incubated in the dark at 30 °C for 48 h and OD was measured at 490 nm using an ELISA reader (Tecan, Männedorf, Switzerland). Increases in OD490 due to spontaneous non-enzymatic dopachrome production were determined in wells without protein extract. Apparent PO activity was recorded as the change in absorbance over 48 h and it was expressed in units as defined previously [2]. The protein concentration of the extracts was measured by the Bradford method.

2.10. Statistical analysis

Survival data were arcsin transformed to satisfy normality and homocedasticity requirements as necessary. Survival and PO 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 version 14.0 to determine significant differences among treatments. Results for proPO mRNA quantification are presented as fold expression relative to *Artemia* actin. The expression level in control *Artemia* fed non-induced *E. coli* strain was regarded as 1.000 and thereby the expression ratio of the treatments was expressed in relation to the control. Significant differences in expression between control and treatments were analyzed by relative expression software tool–multiple condition solver (REST–MCS) version 2 using pair wise fixed reallocation randomization test© [22]. Significance level was set at $P < 0.05$.

3. Results

3.1. PCR amplification of the native and truncated portions of Artemia Hsp70 and DnaK genes

The cDNA of the native *Artemia* Hsp70 and DnaK has a size of 1935 and 1917 base pairs, respectively and the expected size of the truncated portion of *Artemia* Hsp70 and DnaK genes was 855 and 837 base pairs, respectively. PCR reaction, using the full length cDNAs as templates and primers specific for either native or truncated Hsp70 genes, was run to amplify the genes of interest and the identities of the products were validated by their predicted sizes in the agarose gel. Fig. 2 showed that all the four genes with the expected lengths were amplified.

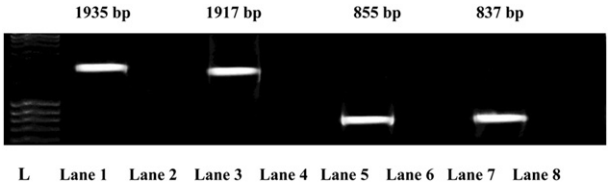

Fig. 2. Agarose gel electrophoresis of the PCR amplified native and truncated portion of *Artemia* Hsp70 and DnaK genes. L: DNA ladder, lane 1 and 2: amplified and non-amplified native *Artemia* Hsp70 gene, lane 3 and 4: amplified and non-amplified DnaK gene, lane 5 and 6: amplified and non-amplified truncated *Artemia* Hsp70 gene, and lane 7 and 8: amplified and non-amplified truncated DnaK gene. bp: base pair.

3.2. Optimization of L-arabinose dose for the induction of truncated Hsp70s in the E. coli strains

The pBAD directional TOPO® vector used for cloning in this study carries the *ara*BAD promoter which can be induced in the presence of L-arabinose [24]. A dose–response effect of L-arabinose was analyzed to optimize the induction level of the truncated Hsp70/DnaK protein by western blot. As shown in Figs. 3 and 4, detection of the truncated proteins, A_{359} and E_{359} in protein extracts from A_{359} and E_{359} strains, respectively, using Anti-Thio™ antibody revealed a single protein band of approximately 47 kDa for both the strains (Figs. 3B and 4B). It was also observed that L-arabinose induced the expression of A_{359} and E_{359} proteins in the A_{359} and E_{359} strains, respectively, in a dose-dependent fashion. Maximum increase was seen at a concentration of 4 mg ml^{-1}. The normal molecular mass of the truncated proteins should be approximately 31 kDa. However, the increase in the size of the recombinant proteins by 16 kDa, as compared with the normal mass of 31 kDa was because of the integration of thioredoxin in the N-terminal and polyhistidine tag in the C-terminal encoded by the TOPO® cloning vector.

Next, we carried out an *in vivo* test by feeding *Artemia* with A_{359} or E_{359} strain induced with different L-arabinose concentrations and scoring their survival in order to determine the optimum L-arabinose dose. Results showed that *Vibrio*-challenged *Artemia* exhibited highest survival when fed with A_{359} cells induced with 0.5 mg ml^{-1} of arabinose concentration (Table 2, Run 1). Feeding *Artemia* with A_{359} cells induced with arabinose at concentration either less than 0.25 mg ml^{-1} or higher than 1 mg ml^{-1} did not show a marked increase in their survival. In contrast, a significant decrease in the survival was noted when *Artemia* were fed with A_{359} cells induced with highest concentration of arabinose (4 mg ml^{-1}, $P < 0.05$). Similar trend was also observed when the experiment was repeated: maximum survival was observed when *Vibrio*-challenged *Artemia* were fed with 0.5 mg/ml of arabinose-induced A_{359} cells (Table 2, Run 2).

The survival of *Vibrio*-challenged *Artemia* fed with E_{359} strain induced with different arabinose concentrations differed significantly ($P < 0.001$, Table 2). A marked increase in the survival (compared with the control) was recorded when *Artemia* larvae were fed with E_{359} strain induced with L-arabinose in the range of 0.25–1 mg ml^{-1}, with maximum survival at 0.5 mg ml^{-1} L-arabinose concentration, however, not significantly ($P > 0.05$) different from the others. Feeding *Artemia* with E_{359} strain induced with higher L-arabinose concentration of 2 or 4 mg ml^{-1} resulted in a significant ($P < 0.05$) decrease in their survival. These results suggested an arabinose concentration of 0.5 mg ml^{-1} and an induction period of 1 h as the optimum conditions and were therefore chosen for inducing the *E. coli* strains for our subsequent *in vivo* experiments.

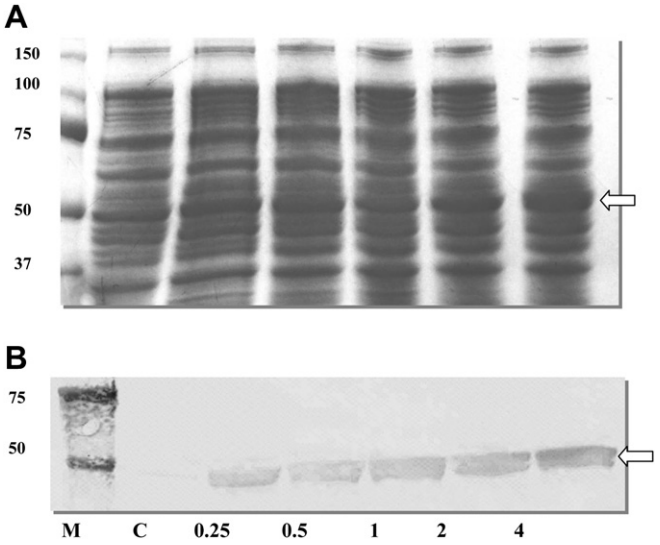

Fig. 3. Arabinose-induced overproduction of A_{359} protein in the A_{359} strain. Protein extract from the *E. coli* strain A_{359} induced with L-arabinose was resolved in SDS-PAGE gels and then either stained with Coomassie Biosafe (A) or transferred to polyvinylidene fluoride membranes and probed with anti-thio antibody (B). C, non-induced; 0.25–4.0, L-arabinose induction for 1 h, respectively, at 0.25–4.0 mg ml^{-1}. Fifteen microgram of bacterial protein was loaded in each lane. Molecular mass standards (*M*) in kDa are on the left. Labeled arrow indicates A_{359} protein.

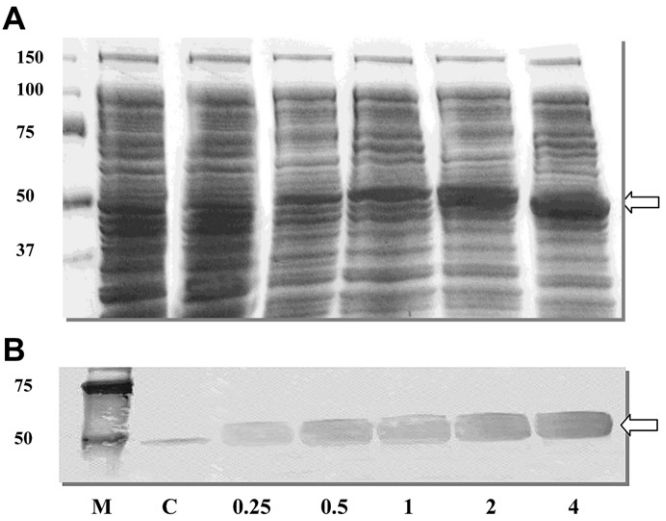

Fig. 4. Arabinose-induced over-production of E_{359} protein in the E_{359} strain. Protein extract from the *E. coli* strain E_{359} induced with L-arabinose was resolved in SDS-PAGE gels and then either stained with Coomassie Biosafe (A) or transferred to polyvinylidene fluoride membranes and probed with anti-thio antibody (B). C, non-induced; 0.25–4.0, L-arabinose induction for 1 h, respectively, at 0.25–4.0 mg ml^{-1}. Fifteen microgram of bacterial protein was loaded in each lane. Molecular mass standards (*M*) in kDa are on the left. Labeled arrow indicates E_{359} protein.

3.3. Effect of truncated Hsp70s on the growth of Artemia

The data presented above showed that L-arabinose dose-dependently increased the expression of A_{359} and E_{359} proteins in A_{359} and E_{359} strains, respectively and feeding A_{359}/E_{359} strain induced with high dose of arabinose (4 mg ml^{-1}) appeared to have detrimental effect on *Artemia*. To determine the effect of feeding

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

L-arabinose (mg ml^{-1})	Survival (%)			
	A_{359}		E_{359}	
	Run 1	Run 2	Run 1	Run 2
0	58.0 ± 3.9^{b}	54.7 ± 4.3^{bc}	50.0 ± 4.2^{b}	46.7 ± 3.3^{cd}
0.25	67.3 ± 2.9^{ab}	67.0 ± 3.2^{a}	66.0 ± 3.9^{a}	54.7 ± 3.3^{bc}
0.5	74.7 ± 4.3^{a}	71.0 ± 3.1^{a}	70.0 ± 4.3^{a}	67.3 ± 3.4^{a}
1	64.0 ± 1.9^{b}	62.0 ± 3.4^{ab}	57.3 ± 4.3^{ab}	64.0 ± 4.3^{ab}
2	58.7 ± 4.3^{b}	48.7 ± 4.8^{c}	50.0 ± 4.3^{b}	44.0 ± 2.7^{d}
4	43.3 ± 2.4^{c}	48.0 ± 3.8^{c}	48.7 ± 7.8^{b}	37.3 ± 3.2^{d}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.05$). Experiments were repeated once indicated as Run 1 and Run 2. The A_{359} or E_{359} strain was induced with different concentrations of L-arabinose for 1 h. *Artemia* nauplii were fed once with either A_{359} or E_{359} cells at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* LMG21363 for 36 h.

A_{359} or E_{359} protein above the optimum level on the growth of *Artemia*, the length of *Vibrio*-challenged or non-challenged *Artemia* fed arabinose-induced *E. coli* strains was measured and the results are shown in Table 3. The non-challenged groups fed either arabinose-induced A_{359} or E_{359} strain did not have any significant difference ($P > 0.05$) among themselves. However, the group that was fed with 4 mg ml^{-1} arabinose-induced A_{359} or E_{359} strain had comparatively lower growth than the others. Similarly, the *Vibrio*-challenged *Artemia* fed arabinose-induced A_{359} strain also did not differ significantly ($P > 0.05$) among each other. However, their growth appeared to be less when fed with A_{359} strain induced with higher arabinose concentration. When challenged with *V. campbellii*, *Artemia* fed with E_{359} strain induced with different arabinose concentrations differed significantly ($P < 0.05$). Highest and lowest growth was observed in the group fed with 0.5 and 4 mg ml^{-1}, respectively, arabinose-induced E_{359} strain. These results also indicated that feeding *Artemia* with *E. coli* strains overproducing truncated Hsp70s may be deleterious.

3.4. Protective activity of truncated Hsp70s

We previously reported that feeding *Artemia* with *E. coli* producing full length recombinant *Artemia* Hsp70 or DnaK protein induced protection to subsequent *Vibrio* challenge [2,10]. Having seen that the *E. coli* strains A_{359} and E_{359} produce the A_{359} and E_{359} proteins, respectively, in the presence of arabinose, we tested in the subsequent experiment the protective ability of truncated *Artemia* Hsp70 or DnaK protein using the *Artemia*–*V. campbellii* host–pathogen model. The results showed that in absence of *V. campbellii*, the survival of *Artemia* fed with arabinose-induced or non-induced *E. coli* strains expressing either truncated (A_{359} or E_{359}) or native Hsp70s (A_{native} or E_{native}) did not differ significantly ($P > 0.05$) among each other (Table 4). However, in the presence of *V. campbellii*, a significant ($P < 0.05$) variation in the survival of *Artemia* fed with different *E. coli* strains was noted. *Artemia* receiving arabinose-induced A_{359} and E_{359} strains had survival significantly higher by 2.2- and 1.6-fold than those receiving the non-induced A_{359} and E_{359} strains, respectively. In a similar way, a significantly ($P < 0.05$) higher survival (by 1.6-fold) was obtained when arabinose-induced A_{native} or E_{native} strain was provided to challenged nauplii in comparison to the challenged nauplii supplied with non-induced A_{native} or E_{native} strain. These results suggest that both A_{359} and E_{359} proteins conferred protection to *Artemia* against pathogenic *V. campbellii* and interestingly, this response was identical with that of the native Hsp70s.

Table 3
Individual length (μm) of *Artemia* after 36 h of feeding L-arabinose induced or non-induced *E. coli* strains.

L-arabinose (mg/ml)	Individual length (μm)			
	A_{359}		E_{359}	
	Without *V. campbellii*	With *V. campbellii*	Without *V. campbellii*	With *V. campbellii*
0	894.6 ± 10.6	842.7 ± 16.7	876.4 ± 15.6	840.0 ± 18.7^{ab}
0.25	893.1 ± 12.2	857.8 ± 16.6	840.0 ± 17.5	816.0 ± 18.3^{ab}
0.5	875.8 ± 12.0	871.7 ± 19.2	875.0 ± 17.4	870.0 ± 17.3^{a}
1	883.6 ± 17.3	851.0 ± 23.7	865.3 ± 9.6	861.0 ± 17.0^{a}
2	869.2 ± 15.5	857.5 ± 12.3	836.2 ± 18.5	833.6 ± 22.0^{ab}
4	846.4 ± 14.3	820.3 ± 20.1	803.4 ± 60.6	784.0 ± 14.5^{b}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.05$). Experiments were repeated once indicated as Run 1 and Run 2. For the treatment groups, refer to Table 1 for explanation. *Artemia* were fed with these bacterial strains at 10^7 cells ml^{-1} and after 6 h they were challenged with *V. campbellii* or not. Length of the live larvae was measured after 36 h of incubation.

Table 4
Percentage survival of *Artemia* nauplii after 36 h challenge with *Vibrio campbellii* LMG21363.

Treatments	Survival (%)	
	Run 1	Run 1
E_{native} (−)	90.0 ± 1.5^{a}	90.0 ± 2.8^{a}
E_{native} (+)	84.0 ± 3.4^{a}	90.0 ± 4.6^{a}
A_{native} (−)	88.7 ± 1.7^{a}	82.7 ± 3.0^{a}
A_{native} (+)	87.3 ± 3.4^{a}	84.0 ± 4.6^{a}
E_{359} (−)	83.3 ± 3.0^{a}	82.0 ± 1.7^{a}
E_{359} (+)	85.3 ± 2.5^{a}	87.3 ± 2.7^{a}
A_{359} (−)	90.7 ± 1.2^{a}	90.7 ± 0.7^{a}
A_{359} (+)	90.7 ± 2.5^{a}	82.0 ± 3.7^{a}
E_{native} (−) + VC	23.3 ± 2.1^{c}	20.7 ± 3.6^{e}
E_{native} (+) + VC	38.0 ± 4.2^{b}	38.0 ± 2.5^{bc}
A_{native} (−) + VC	20.7 ± 0.7^{cd}	29.3 ± 2.4^{cde}
A_{native} (+) + VC	32.7 ± 2.9^{b}	44.0 ± 2.2^{b}
E_{359} (−) + VC	20.0 ± 1.5^{cd}	23.3 ± 2.1^{de}
E_{359} (+) + VC	32.0 ± 1.3^{b}	45.3 ± 3.6^{b}
A_{359} (−) + VC	14.7 ± 2.7^{d}	26.0 ± 1.9^{de}
A_{359} (+) + VC	32.0 ± 2.3^{b}	32.0 ± 2.5^{cd}

Data are represented as mean ± standard error ($n = 5$). Different superscripts in the same column represent significance difference ($P < 0.001$). Experiments were repeated once indicated as Run 1 and Run 2. The bacterial strains E_{native} (a DnaK producer after arabinose induction), A_{native} (an *Artemia* Hsp70 producer after arabinose induction), E_{359} (a truncated DnaK producer after arabinose induction), and A_{359} (a truncated *Artemia* Hsp70 producer after arabinose induction) were either induced (+) or non-induced (−) with L-arabinose at concentration of 0.5 mg ml^{-1} for 1 h (for A_{359} and E_{359} strains) and 4 h (for A_{native} and E_{native} strains). *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. Control nauplii were not challenged with *V. campbellii* but otherwise treated the same as experimental animals.

3.5. Prophenoloxidase gene expression in Artemia in response to feeding truncated Hsp70s

The prophenoloxidase (proPO)-activating system, present in the hemolymph of crustaceans and other invertebrates, is regarded as the most important component of the innate immunity and plays a vital role in defense against pathogen [25,26]. The proPO, once being stimulated into PO, will be released from hemocytes to plasma and catalyzes the formation of melanin. Melanin binds to the surface of bacteria and increases the adhesion of hemocytes to bacteria, thus accelerating their removal by nodule formation [27,28]. The previous study had showed that the native *Artemia* Hsp70 or DnaK could induce the proPO system in *Artemia* when the latter is challenged with bacteria [10]. Here, the two Hsp70 fragments were evaluated for their ability to regulate the proPO system by measuring the proPO gene expression (Fig. 5). In the absence of *V. campbellii*, feeding *Artemia* with arabinose-induced A_{359} or E_{359} strain did not led to a significant increase in the level of proPO mRNA transcripts (Fig. 5A and B). Similar trend was observed in non-challenged *Artemia* fed with arabinose-induced A_{native} or E_{native} strain (Fig. 5C and D). However, following a challenge, there occurred a significant upregulation of the proPO gene in *Artemia* fed with arabinose-induced A_{359} (1.4-fold, $P > 0.05$), E_{359} (1.5-fold, $P < 0.01$), A_{native} (1.8-fold, $P < 0.05$) or E_{native} (2.2-fold, $P < 0.01$) strain. The expression level of proPO in *Vibrio*-challenged *Artemia* fed with non-induced A_{359}, E_{359}, A_{native} or E_{native} strain appeared to decrease by a fold of 1.4 ($P > 0.05$), 1.7 ($P < 0.01$), 1.9 ($P < 0.01$) or 1.6 ($P > 0.05$), respectively.

3.6. Phenoloxidase activity in Artemia in response to feeding truncated Hsp70s

In parallel to measure proPO gene expression, we also studied the effect of feeding arabinose-induced or non-induced *E. coli* strains to *Artemia* in the presence or absence of *V. campbellii* (Fig. 6).

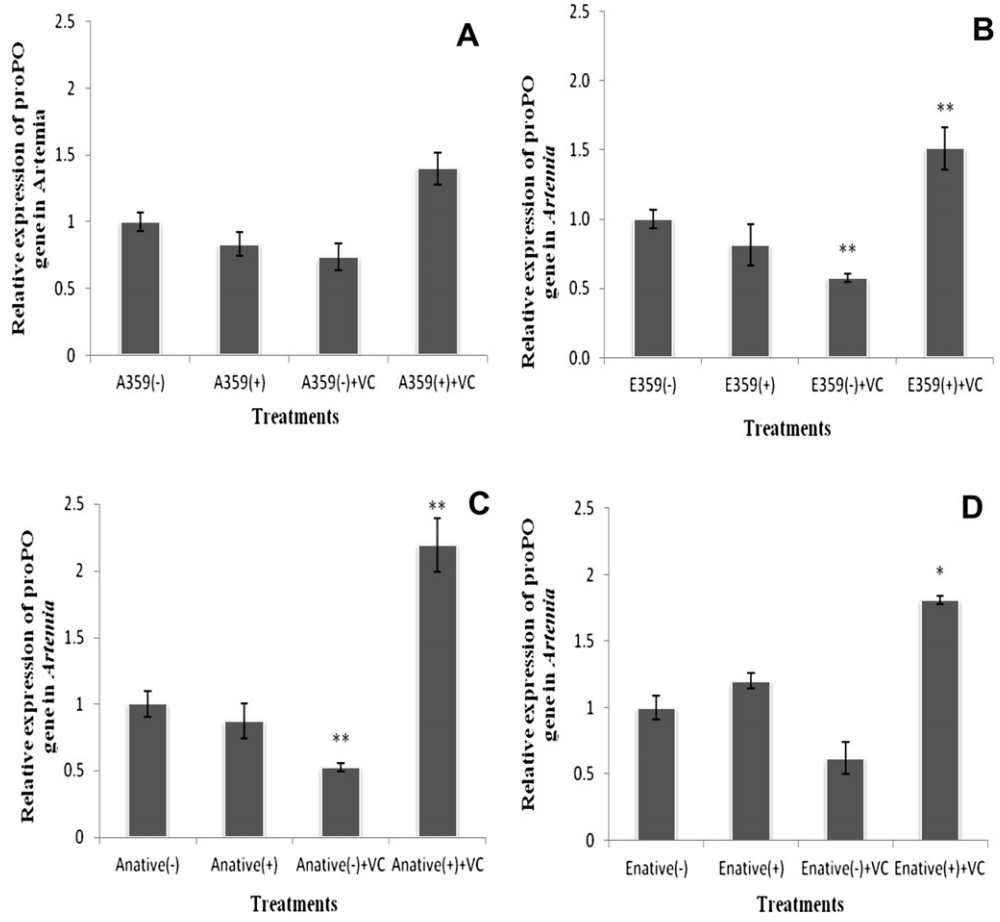

Fig. 5. Expression of proPO mRNA in *Artemia* fed either native or truncated Hsp70s. The bacterial strains (A) A_{359} (a truncated *Artemia* Hsp70 producer after arabinose induction), (B) E_{359} (a truncated DnaK producer after arabinose induction), (C) A_{native} (an *Artemia* Hsp70 producer after arabinose induction), and (D) E_{native} (a DnaK producer after arabinose induction) were either induced (+) or non-induced (−) with L-arabinose at concentration of 0.5 mg ml^{-1} for 1 h (for A_{359} and E_{359} strains) or 4 h (for A_{native} and E_{native} strains). *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 6 h. Control nauplii were not challenged with *V. campbellii* but otherwise treated the same as experimental animals. The expression of proPO mRNA in the control non-challenged *Artemia* fed non-induced *E. coli* strains was regarded as 1. Results, which are the mean of 3 replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. [22]. Bars indicate standard error. Significant differences between the treatment and control at corresponding time points are indicated by $^*(P < 0.05)$, $^{**}(P < 0.01)$.

The results showed that the activity of PO in non-challenged *Artemia* fed arabinose-induced *E. coli* strains (A_{359}, E_{359}, A_{native} or E_{native}) did not raised significantly ($P > 0.05$) compared to their respective control groups (i.e., non-challenged groups fed non-induced *E. coli* strains). Upon challenge with *V. campbellii*, the PO activity in *Artemia* fed arabinose-induced A_{359}, E_{359}, A_{native} or E_{native} strain remained significantly higher than those fed respective non-induced *E. coli* strains.

4. Discussion

Members of the Hsp70 family are widely distributed in single-cell organisms, invertebrates and vertebrates and display a high level of interspecies conservation [1]. Research into the biology of these highly conserved Hsp70s in mediating protective immune responses against infectious diseases including mycobacterial infections, such as tuberculosis, leprosy, bovine paratuberculosis and bovine tuberculosis in terrestrial animals, and vibriosis in aquaculture animals is an evolving field [1,2,15,29]. Our current study adds to the field by demonstrating *in vivo* that the C-terminus portion of *Artemia* Hsp70 and DnaK proteins, spanning amino acids 359–644 (A_{359}) and 359–638 (E_{359}), respectively, rendered substantial protection to *Artemia* against pathogenic *V. campbellii*. Interestingly, the degree of protection conveyed to *Vibrio*-challenged *Artemia* by these truncated *Artemia* Hsp70/DnaK protein was comparable to what was observed in previous study when feeding native *Artemia* Hsp70 or DnaK protein to this *V. campbellii*-challenged *Artemia* model [2]. This result suggests that the ATPase

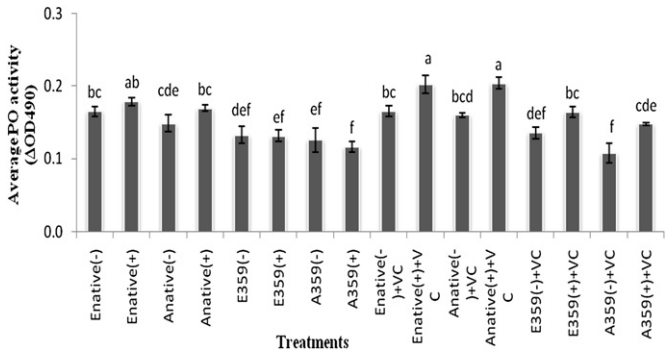

Fig. 6. PO activity in *Artemia*. For the treatment groups, refer to Table 4 for explanation. *Artemia* larvae were fed once with the bacterial strains at 10^7 cells ml^{-1} and incubated for 6 h before challenge with *V. campbellii* (VC) for 6 h. Control nauplii were not challenged with *V. campbellii* but otherwise treated the same as experimental animals. Data represent the mean of five replicates. Error bars with different alphabet letters indicate significant difference ($P < 0.05$).

domain of *Artemia* Hsp70 or DnaK protein is dispensable for Hsp70-mediated protection of *Artemia* from *V. campbellii* stress. Similarly, looking at protection against thermal stress, Li et al. [30] reported that a human Hsp70 deletion mutant (i.e., the C-terminal fragment), lacking the ATPase domain, can still protect rat-1 cells. In a subsequent study, other investigators also demonstrated that the ATPase activity of Hsp72 is not required for the anti-apoptotic effect of Hsp72 in heat-shocked rat-1 cells [31].

Several lines of studies have reported that the peptide binding domain of Hsp70 is absolutely necessary for cell protection [15,30–32]. The truncated *Artemia* Hsp70 and DnaK proteins, described in the current study, contain the peptide-binding region that spans the amino acids 359–510 and a comparison of the amino acid sequence of these truncated Hsp70s revealed that both these fragments are highly homologous particularly in the peptide-binding domain: overall sequence identity of the fragments was approximately 41.7% (sequence comparison based on using Clustal W multiple-alignment method, Thompson et al. [33]), while sequence identity in the peptide binding and lid domains was around 59.6 and 19.4%, respectively [2]. It is therefore likely that the observed *Vibrio*-protective function of the truncated Hsp70s might be located within this conserved region of the peptide binding domain.

Noteworthy, Hsp70s have been found to have a specific function in immunological processes. An increasing body of data suggests that Hsp70s play a role in inducing strong innate and adaptive immune responses in numerous infections and subsequently confer protection in experimental animal systems [10,16,34]. The exact role of Hsp70s in immunity to microbial infection is yet obscure but Hsp70 appear to be a dominant antigen for the host immune response to a variety of pathogens [35]. The invertebrates, including our model *Artemia*, are thought to rely solely on their innate immune system to combat pathogens [10,25] as they are assumed to lack immunological memory and specific form of defense like the vertebrates [36], although there have been some indications that specificity and memory might exist in invertebrates [37,38]. In our previous study, it was found that prior feeding of gnotobiotic *Artemia* with native *Artemia* Hsp70 or DnaK protein resulted in increased resistance of the shrimp against subsequent *V. campbellii* insult and this enhanced resistance to the pathogen was associated with the priming of the proPO system [10]. In this particular case, priming is considered to be a synergistic immunological response to a consecutive exposure to a "priming substance" and a pathogen, versus their single exposure [39]. Identical to the finding of our previous study [10], priming was observed to exist when *Artemia* is exposed via the gastrointestinal tract to native Hsp70 or DnaK (see Fig. 5C and D). Similarly, priming also occurred when *Artemia* was exposed to the truncated proteins (see Fig. 5A and B). So, considering all the above results, it is plausible that an epitope exists within the conserved region of the peptide binding domain of *Artemia* Hsp70 or DnaK protein which might account for priming the proPO system and presumably other immune-related genes of *Artemia*, resulting in an increased resistance against pathogenic *V. campbellii*.

The overall results of our present *in vivo* study are consistent with the findings of previous *in vitro* and *in vivo* studies [30,40,41]. For instance, Wang et al. [42] reported that the C-terminus portion of *Mycobacterium tuberculosis* Hsp70 (amino acids 359–610) was capable of inducing cytokine production in human monocytes, whereas the N-terminus portion of *M. tuberculosis* Hsp70 did not have this capacity. This same group of investigators subsequently found a 20-mer epitope within the C-terminus portion of *M. tuberculosis* Hsp70 (amino acids 407–426) having the ability to modulate cytokine production and maturation of dendritic cells [32]. In another recent study, Wheeler et al. [15] found that the C-terminus of human Hsp72, which represents the conserved substrate or peptide binding domain of full length Hsp72, seemed to be primarily responsible for activating the cells of macrophage and monocytes. Collectively, all these data indicate that the immunological active part of the Hsp70 molecule is located in the peptide-binding domain of *Artemia* Hsp70 or DnaK protein.

An important issue surrounding our current study, as well as any study on the immunological properties of Hsp70s, is that of endotoxin (lipopolysaccharide) contamination functioning as a confounding variable. Indeed, previous studies have suggested that the putative biological signaling properties of recombinant Hsps are merely artifacts of endotoxin contamination [43]. Our current studies have taken steps to address this highly important issue of endotoxin contamination. These include keeping experimental controls directly addressing endotoxin contamination i.e., by feeding *Artemia* arabinose-induced or non-induced *E. coli* strain YS1, an Hsp70 non-producer upon arabinose addition (data not shown, see [2,10]). Two of these previous studies support direct protective activity of Hsp70s, rather than endotoxin contamination. Thus, we contend that the data reported in the present study reflect the direct protective effects of the truncated Hsp70s.

Another novel important finding of this study is that the truncated Hsp70s are toxic or detrimental to *Artemia* when fed beyond a certain threshold level. Investigating induced thermotolerance and its relation to Hsp70, Krebs and Feber [44] reported that small to moderate increases in Hsp70 levels enhanced inducible thermotolerance in *Drosophila*, whereas large increases in Hsp70 levels actually decreased thermotolerance. The same authors in another study demonstrated that *Drosophila* larvae transformed with extra copies of the Hsp70 gene have greater larva-to-adult mortality and slower development than do control larvae [45]. The negative effects of overfeeding truncated Hsp70s could be attributed to two possible reasons: First, Hsp70s at high concentration could directly interfere with ongoing processes (especially protein metabolism) in the cell [46]. Second, the presence of Hsp70s in excess amount and their metabolism could consume an intolerably large fraction of a cell's or organism's nutrient and energy stores, and/or occupy a large fraction of the synthetic/catabolic apparatus that the processing of other essential biomolecules suffers [47,48]. In this study, we did not quantify the dose of each truncated Hsp70 that was actually fed to the animal. However, it is clear that the prescribed dose of these truncated Hsp70s (A_{359} and E_{359} strains induced with 0.5 mg ml^{-1} arabinose) was far below (by 8-folds) the toxic dose (A_{359} and E_{359} strains induced with 4 mg ml^{-1} arabinose). This allows a good safety margin for the therapeutic/prophylactic use of these proteins in shrimp production sector.

In summary, we have demonstrated that the C-terminus domain of Hsp70 (derived from either *Artemia* or *E. coli*), which represents the highly conserved peptide binding domain of native Hsp70, at moderate level seemed to be primarily responsible for priming the proPO system and subsequently confer resistance to *Artemia* against *V. campbellii* challenge. These findings also evoke an important hypothesis that this conserved domain of Hsp70 or DnaK protein could also induce other genes responsible for inducing immune responses in *Artemia* and generating protection against *V. campbellii*. Further studies should be carried out to verify this assumption.

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