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*

Kartik Baruah, Jayant Ranjan, Patrick Sorgeloos, Peter Bossier*

Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, 9000 Ghent, Belgium

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

* Corresponding author. Tel.: +32 926 43754; fax: +32 926 44193.
E-mail address: peter.bossier@ugent.be (P. Bossier).

1050-4648/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
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™