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Immobilization antigen vaccine adjuvanted by parasitic heat shock protein 70C confers high protection in fish against cryptocaryonosis

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ABSTRACT

The immobilization antigen (iAg) has been demonstrated as a protective immunogen against *Cryptocaryon irritans* infection. In this study, C-terminal domain of heat shock protein 70 cloned from *C. irritans* (Hsp70C) was tested for its immuno-stimulatory effects. The iAg and Hsp70C cDNAs were constructed independently in secretory forms and were encapsulated in chitosan nanoparticles. In the first immunization trial, grouper fingerlings orally intubated with iAg and iAg:Hsp70C presented 96% and 100% relative percent survival (RPS), respectively, after a lethal challenge. In the second trial, both iAg and iAg:Hsp70C groups showed 100% RPS and the skin trophont burden was significantly lowered. The iAg:Hsp70C still provides a significantly high protection of 51% RPS at 49 days post immunization, when an even more serious lethal infection occurs. RT-qPCR results showed that Hsp70C could up-regulate the expression of i) T cell markers: Cluster of Differentiation 8 alpha (CD8α) and CD4, ii) cytokine genes: Interferon gamma (IFNγ), Tumor Necrosis Factor alpha (TNFα) and Interleukin 12 p40 (IL-12/P40), iii) antibody genes: Immunoglobulin M heavy chain (IgM_H) and IgT_H, and iv) major histocompatibility complex (MHC-I & MHC-II), in the spleen of iAg:Hsp70C group. Furthermore, significantly high levels of iAg-specific IgM was detected in skin mucus which efficiently immobilized live theronts in iAg- and iAg:Hsp70C-immunized fish at 5 weeks post immunization. Hsp70C significantly increased the number of nonspecific $CD8^+$ skin leucocytes which exerted cytotoxicity against theronts, although cytotoxic activity showed no difference among the various groups. Because of this complementary cooperation of cellular and humoral immune responses, Hsp70C enhances the efficacy of iAg vaccine and constrains *C. irritans* infection. In view of the severe loss caused by cryptocaryonosis, application of this parasitic vaccine in farmed and ornamental fish, is worthy to be considered.

1. Introduction

Cryptocaryon irritans is a ciliated protozoan that causes white spot disease in marine fish. It is widely distributed geographically [1]. It has been the subject of research because it causes devastating infections in aquariums and farms. *C. irritans* has low host specificity and it can infect multiple fish species, including both temperate and tropical marine fish [2]. Although there is no official record on the annual economic loss attributed to marine white spot, infections by *C. irritans* causes significant economic impact on both food and aquarium fish production [3].

The orange spotted grouper *Epinephelus coioides* is a commercially important culture fish worldwide and it is one of the primary hosts of *C. irritans* [3,4]. Earlier outbreaks of *C. irritans* reported more than 50% mortality of tank cultured juvenile brown spotted grouper in Kuwait [5], and infected a range of wild fishes in Australia [6] and cage cultured fishes in Japan [7]. Hence, appropriate treatment procedures are necessary to eradicate this parasite from both aquaria system and commercial fish farms. A number of chemicals have been used to control *C. irritans* in aquarium scales, which are

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not suitable for commercial fish farms due to their high concentration use, environmental pollution problems and also the chemical residues in fish flesh would be unsafe for human consumption [8,9].

To date, several vaccine candidates have been developed and evaluated against *C. irritans*. Sub-lethal parasite exposure and immunization with parasitic theront lysates are the credible vaccine measures for *Cryptocaryon* infection in fish [10–13]. However, neither the parasitic theronts have successfully been grown under culture conditions nor the methods for its cryopreservation have been well developed [12,14]. In *Ichthyopthyrius multifillis*, the freshwater counterpart of *C. irritans*, it has been discovered that immobilization antigens (iAg), play an important role in induction of immunity [15]. In our previous work, iAg was originally sequenced from Chiayi (middle Taiwan) isolate sourced from sea bream, *Sparus sarba*, in 2000 [16]. The iAg cDNA vaccine prepared from the Chiayi isolate was proved as immunogenic [17].

Hsp70 plays crucial roles in integrating innate and adaptive immunity against infectious diseases [18]. Application of Hsp70 as adjuvant in several infection models revealed a strong cellular immunity [19,20]. Wang et al. recorded two major fragments of Hsp70: a 44-kDa N-terminal ATPase domain and a 28-kDa C-terminal domain that contains the 18-kDa peptide binding region [21]. It is suggested that the C-terminal region of Hsp70 is sufficient to be used as vaccine adjuvant [22,23]. In the present studies, we have cloned a truncated parasitic Hsp70 and the C-terminal region was constructed into a plasmid vector ahead with grouper IgT signal peptide. Similarly, iAg was constructed. The iAg and Hsp70C cDNA plasmids were encapsulated in chitosan nano particles using the associative feature of cationic chitosan and anionic DNA [24–26]. We report the benefits of parasitic Hsp70C when co-administered with iAg to fish. More importantly, we provide detailed evidence of the humoral and cellular immune responses in immunized fish against *C. irritans*.

2. Materials and methods

2.1. Cloning of parasitic heat shock protein 70

Theronts of *C. irritans* were incubated at 37 °C for 80 min and total RNA was extracted by Trizol (Invitrogen). DNA was prepared by SuperScript™ III One-Step RT-PCR system according to manufacturer's instructions. Primers of Hsp70 were designed based on the available parasitic Hsp70 sequence of *Tetrahymena thermophila* (GenBank: AAK29100.1). A 1.3 Kb DNA fragment including the complete C-terminal region was obtained by PCR using primer set (Hsp70-F & Hsp70-R, Table S1) with 58 °C annealing temperature (Tm), and PCR product was sequenced. The protein binding domain containing C-terminal region of 6810 bp named as Hsp70C was selected for further experiments.

2.2. Sequence analysis and multiple alignment of parasitic Hsp70C

The nucleotide and deduced protein sequences of Hsp70C were analyzed using BLAST programs at NCBI. Putative motifs and domains were detected using Motif Scan [24] at www.expasy.org. Parasitic HSP70 sequences were searched from NCBI database and aligned using ClustalW online at http://www.ebi.ac.uk/index.html. The deduced amino acid sequence of Hsp70C was aligned with other protozoan parasites such as *T. thermophila* heat-shock protein 70 (GenBank: AAK29100.1), *Paramecium caudatum* HSP70C-1 (ADU54551.1) and *Plasmodium vivax* heat shock 70 kDa protein (XP_001614972.1) and also with grouper *E. coioides* Hsp70 (FJ600726.1), and shrimp *Penaeus monodon* Hsp70 mRNA (AF474375.1).

2.3. Construction of DNA vaccine by overlapping PCR

To generate a DNA construct encoding the secreted form of Hsp70C protein, the signal peptide sequence of grouper immunoglobulin T heavy chain (IgT_H) was amplified by PCR #1. The template was the cDNA clone containing 60 bp signal peptide sequence of IgT_H (GenBank: GU182366.1). The primers used were SP1 and Hsp70C-SP2 (Table S1), with a PCR Tm of 57 °C. In PCR #2, the template was pcDNA3.1-Hsp70C, the primers were Hsp70C-SP3 and Hsp70C-SP4 at a Tm of 58 °C and the elution product was ~700 bp. Next, an overlapping PCR (PCR #3) was conducted by combining 0.5 μL products each from PCR #1 & PCR #2 as template by using primers SP1 and Hsp70C-SP4. The resultant product of ~760 bp was then cloned into pcDNA3.1 at the *BamHI* and *EcoRI* sites, and verified by restriction analysis and nucleotide sequencing.

Similar approach was used for iAg DNA construct. For this, PCR #1 was the same as above, but the reverse primer was iAg-SP2. For PCR #2, the template was pcDNA3.1-iAg, the primers were iAg-SP3 and iAg-SP4 and the eluted DNA band was ~950 bp. PCR #3 was conducted by using primers SP1 and iAg-SP4 at the same PCR condition as that of PCR #2. The final product of ~1 Kb was then cloned into pcDNA3.1 at the *BamHI* and *EcoRI* sites, and was verified by restriction analysis and nucleotide sequencing.

2.4. Nano-encapsulation of iAg vaccine and Hsp70C constructs

Preparation of chitosan nanoparticles and chitosan/DNA complex followed the procedure of Huang et al., 2009 [24]. The chitosan/DNA complexes (~100 nm in diameter) were prepared according to the method reported by Sato et al., 1996 [27]. Chitosan (85% deacetylation degree, 800 kDa) from crab shells was purchased from Sigma (St. Louis, MO). Briefly, 1% (w/v) chitosan was dissolved in an acetic acid solution (167 mM) under gentle heating to form a chitosan stock solution; the pH of the stock solution was adjusted to 5.5. The chitosan nanoparticles were prepared by ionic gelation. In brief, 20 mL of chitosan solution was gently added into 8 ml of tripolyphosphate (TPP) solution (0.84 mg/ml). This solution was subjected immediately to ultrasonication (29W, 4 min) at room temperature, followed by centrifugation at 12,000 g for 45 min to remove pelleted particles. The clear supernatant contained chitosan nanoparticles. Ten mL of plasmid DNA solution (5 mg/ml) was added into 115 mL chitosan solution to make a final concentration of 0.43 mg/mL.

2.5. Immunization in grouper fingerlings

Grouper (*E. coioides*) fish without any clinical symptoms of *C. irritans* infection were bought from Merit Ocean Biotech Inc. Tainan, Taiwan. They were acclimated 2 weeks prior to the following experiments. All the experimental fish were treated in strict accordance with the 'recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health'. The protocol was approved by 'the Committee on the Ethics of Animal Experiments of the National Taiwan University' (Permit Number: NTU-101-EL-10). Dissection experiments were performed under benzocaine anesthesia (0.065 g benzocaine/L sea water). Fish were maintained in 32 ppt sea water at 27 °C with continuous aeration. Immunizations were done in two trials. For trial I, grouper fingerlings of average 2 g in body weight (BW) were divided into four groups and held in separate tanks. The fish were immunized as follows: i) the first group of fish was kept untreated, ii) the second group was immunized with iAg DNA at a concentration of 20 μg/g fish BW in 60 μL volume, iii) the third group was immunized with iAg and Hsp70C each at a dose of 20 μg/g fish BW, and iv) the fourth group was immunized with Hsp70C alone at a dose of 20 μg/g fish BW. All the immunizations in this study were carried out through oral