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Research paper

Insights into the multifunctional role of natural killer enhancing factor-A (NKEF-A/Prx1) in big-belly seahorse (*Hippocampus abdominalis*): DNA protection, insulin reduction, H_2O_2 scavenging, and immune modulation activity

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ABSTRACT

Natural killer enhancing factor A (NKEF-A), also known as peroxiredoxin 1 (Prx1), is a well-known antioxidant involved in innate immunity. Although NKEF-A/Prx1 has been studied in different fish species, the present study broadens the knowledge of *NKEF-A* gene in terms of molecular structure, function, and immune responses in fish species. *Hippocampus abdominalis* NKEF-A (*HaNKEF-A*) cDNA encoded a putative protein of 198 amino acids containing a thioredoxin_2 domain, VCP motifs, and three conserved cysteine residues including peroxidatic and resolving cysteines. Amino acid sequence comparison and phylogenetic breakdown showed the higher sequence identity and closer evolutionary position of *HaNKEF-A* to those of other fish counterparts. A recombinant protein of *HaNKEF-A* was shown to i) protect supercoiled DNA against mixed catalyzed oxidation, ii) reduce insulin disulfide bonds, and iii) scavenge extracellular H_2O_2. Results of in vitro assays demonstrated the concentration dependent antioxidant function of recombinant *HaNKEF-A*. In addition, qPCR assessments revealed that the *HaNKEF-A* transcripts were constitutively expressed in fourteen tissues with the highest expression in liver. As an innate immune response, *HaNKEF-A* transcripts were up-regulated in liver post injection of LPS, *Edwardsiella tarda*, *Streptococcus iniae*, and polyinosinic-polycytidylic acid. Thus, *HaNKEF-A* can safeguards big-belly seahorse from oxidative damage and pathogenic infections. This study provides insight into the functions of NKEF-A/Prx1 in fish species.

1. Introduction

Vulnerability toward oxidative stress may cause complications for living beings in their natural habitat. Particularly, marine aquatic ecosystems are confronted by hazardous substances and pathogenic organisms which can lead to the generation of intracellular reactive oxygen species (ROS, which includes superoxide anions, superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Wang et al., 2006)). These ROS also can be produced naturally as byproducts of aerobic metabolism in somatic cells (Castex et al., 2010). When eliminating foreign invaders during pathogenic infection, aerobic host cells generate large amounts of ROS, which activate phagocytosis; this important process is known as respiratory burst activity (Ekanayake et al., 2008). ROS like H_2O_2 are critical in cell proliferation, cell differentiation, cascading intracellular signals (Aguirre et al., 2005; Ermak and Davies, 2002; Yu, 1994), and provoking immune responses (Bogdan et al., 2000; Roch, 1999). However, excessive production, continuous exposure, or accumulation of free radicals in host cells can cause cellular death through the breaking of DNA strands, peroxidation of lipids, and oxidation of proteins (Nikapitiya et al., 2009). In order to defend host cells against oxidative stress and maintain ROS homeostasis, aerobic cells have established antioxidant enzymes like catalase, superoxide dismutases, glutathione peroxidases, and peroxiredoxins (Prxs).

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; BLAST, Basic Local Alignment Search Tool; CDS, coding sequence; CFU, colony forming units; CDD, conserved domain search program; Cys, cysteines; DTT, dithiothreitol; LPS, lipopolysaccharide; MCO, metal-catalyzed oxidation; MEGA, molecular Evolutionary Genetics Analysis; NCBI, National Center for Biotechnology Information; NKEF-A, natural killer enhancing factor A; Prx, peroxiredoxin; PBS, phosphate buffered saline; poly I:C, polyinosinic-polycytidylic acid; p.i, post injection; qPCR, quantitative real time PCR; ROS, reactive oxygen species; rMBP, recombinant maltose binding protein; SD, standard deviation; UTR, untranslated regions

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Peroxiredoxins are a family of antioxidant enzymes that can protect cells from oxidative damage by catalyzing the reduction of cellular peroxides; they are also termed thioredoxin peroxidases (Wood et al., 2003b). Prxs contain catalytically active redox cysteines (Cys) at N- and/or C-terminals (Robinson et al., 2010), and can be classified into three sub classes as typical 2-Cys Prxs (Prx1, Prx2, Prx3, Prx4), atypical 2-Cys Prx (Prx5), and 1-Cys Prx (Prx6). The typical 2-Cys Prxs are the largest class of Prxs that possess two catalytically active two redox cysteines with the peroxidatic cysteine at N-terminal and the resolving cysteine at C-terminal (Hofmann et al., 2002; Ren et al., 2014). The first evidence of natural killer enhancing factor (NKEF) has been reported by Shau et al. (1993) and was described as a cytosolic protein that enhances the natural killer cytotoxic activity (Shau et al., 1993). Apart from cytotoxic activity, NKEF has shown a potential antioxidant function similar to that of peroxiredoxin members (Sauri et al., 1995). Hence, natural killer enhancing factor A (NKEF-A) is also referred as peroxiredoxin 1 (Wood et al., 2003b). It protects the host cells from oxidative damages caused by various factors such as hydrogen peroxide, alkyl hydroperoxide, and heavy metals (Kim et al., 1997). Previous studies have reported that the NKEF protein has potential to be involved in in vitro antiviral activity (Geiben-Lynn et al., 2003), cell proliferation, differentiation (Huang et al., 2009), and apoptosis (Geiben-Lynn et al., 2003; Sauri et al., 1996). Several teleostean studies have shown the significant up-regulation of *NKEF-A* transcripts upon bacterial or viral immunostimulant challenges (Esteban et al., 2013; Kim et al., 2011; Loo et al., 2012; Wang et al., 2015), revealing their roles in host acute phase responses. However, collective studies on the structural, functional, and transcriptional responses of NKEF-A from teleosts are limited.

The aim of this study was to characterize the *NKEF-A* gene from big-belly seahorse (*Hippocampus abdominalis*), an important aquaculture species that has been used for traditional medicine in the Republic of Korea. Variable stresses due to unfavorable stress conditions and pathogenic attacks (Balcazar et al., 2010; Vincent and Clifton-Hadley, 1989) lead to the suppression of immunity in big-belly seahorse. Development of resistance against stress conditions and infectious diseases may improve seahorse production efficiency. However, the cellular and molecular processes involved in antioxidant and immune defense mechanisms in this seahorse are barely understood. To have a better understanding of the antioxidant and immune defense mechanisms of seahorses, we analyzed big-belly seahorse *NKEF-A* (*HaNKEF-A*) molecular, functional, and mRNA expression characteristics under both aseptic and septic conditions.

2. Methodology

2.1. Transcriptomic database construction and sequence identification of HaNKEF-A

The big-belly seahorse transcriptomic database was constructed by the 454 GS-FLX™ sequencing technique as reported in our previous study (Perera et al., 2016). A putative *HaNKEF-A* cDNA contig was recognized and isolated from the seahorse transcriptomic database and deposited in GenBank under the accession number of KY640300. It was homology screened with the known NKEF-A/Prx1 counterparts available at the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Sequence analysis and structure characterization

DNAssist (version 2.2) was used to acquire the putative coding sequence (CDS) of *HaNKEF-A* cDNA and derive the corresponding protein sequence. The putative protein sequence of *HaNKEF-A* was subjected to a series of bioinformatics tools to identify its characteristic features. A functional domain search was accomplished using the conserved domain search program (CDD; http://www.ncbi.nlm.nih.gov/cdd) at NCBI. The availability of signal sequence was assured by the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/) in ExPASy tool. Conserved cysteine residues were predicted using the Cys finder (http://clavius.bc.edu/~clotelab/DiANNA/). The pairwise sequence alignment was carried out by the EMBOSS Needle program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) to determine the identity and similarity between homolog counterparts. Multiple sequence alignment was employed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was assembled using the maximum likelihood method available in the Molecular Evolutionary Genetics Analysis (MEGA v 5.0) program (Tamura et al., 2011), with bootstrap values calculated for 5000 replications to estimate the robustness of internal branches.

2.3. Cloning and purification of recombinant HaNKEF-A (rHaNKEF-A) protein

The cDNA fragment encoding the CDS of the *HaNKEF-A* gene was cloned into pMAL-c5X vector (New England Biolabs, USA), as previously described in Godahewa et al. (2015). Gene specific enzymes (Supplementary Table 1) were used to employ restriction digestion at *Eco*RV and *Eco*RI sites. Recombinant construct was transformed into *Escherichia coli* DH5α competent cells, and positive clones were confirmed through restriction digestion followed by sequence verification (Macrogen, Korea). Finally, sequence verified recombinant construct was transformed into *E. coli* ER2523 (New England Biolabs, UK) competent cells for protein expression.

The rHaNKEF-A fusion protein expression and purification were carried out as described in our previous study (Godahewa et al., 2016b), following the instructions of the pMAL Protein Fusion and Purification System (New England Biolabs, USA). As a control protein, recombinant maltose binding protein (rMBP) was also expressed and purified. Concentrations of both rHaNKEF-A and rMBP proteins were determined by Bradford assay (Bradford, 1976). The successive induction and purification were affirmed by 12% SDS-PAGE along with a protein marker (Enzynomics, Korea).

2.4. DNA protection activity by metal-catalyzed oxidation (MCO) assay

The MCO assay was carried out to assess the supercoiled DNA protection activity of rHaNKEF-A. This assay was performed as described by De Zoysa et al. (2008) with slight modifications. The 100 μL of total reaction mixture contained MCO system (4 mM dithiothreitol; DTT, 30 μM $FeCl_3$ and H_2O) and different concentrations of purified rHaNKEF-A (0–100 μg/mL) or control protein (rMBP). Samples were incubated for 2 h at 37 °C, mixed with 1500 ng of pUC19 DNA, and further incubated for 2 h at 37 °C. In order to terminate the MCO reaction, samples were PCR purified using a PCR purification kit (Bioneer, Korea) according to the vendor's protocol, and the samples were analyzed on 1% agarose gel stained with ethidium bromide.

2.5. Insulin reduction assay

In order to detect the antioxidant activity of rHaNKEF-A, an insulin disulfide reduction assay was carried out as described in Wang et al. (2015) with slight modifications. Briefly, reaction mixtures included 4 mM EDTA (pH 7.0), 2 mg/mL insulin, 25 μg/mL rHaNKEF-A, 50 μg/mL rHaNKEF-A, 100 μg/mL rHaNKEF-A, and 200 μg/mL rHaNKEF-A or 200 μg/mL rMBP (control protein). In order to commence the reaction, 2 mM of DTT was added, and phosphate buffered saline (PBS) was used to top up the reaction mixture up to 250 μL. Samples were incubated at 25 °C using the microplate spectrophotometer (Thermo Scientific™, Multiskan™ GO, USA) for 100 min, and the absorbance at 650 nm was measured at 10 min intervals.