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Cloning and functional characterisation of a peroxiredoxin 1 (NKEF A) cDNA from Atlantic salmon (*Salmo salar*) and its expression in fish infected with *Neoparamoeba perurans*

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ABSTRACT

Peroxiredoxin 1 (Prx 1), also known as natural killer enhancing factor A (NKEF A), has been implicated in the immune response of both mammals and fish. Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, is a significant problem for the Atlantic salmon (*Salmo salar* L.) aquaculture industry based in Tasmania, Australia. Here we have cloned and functionally characterized a Prx 1 open reading frame (ORF) from Atlantic salmon liver and shown that Prx 1 gene expression was down-regulated in the gills of Atlantic salmon displaying symptoms of AGD. The Prx 1 ORF encoded all of the residues and motifs characteristic of typical 2-Cys Prx proteins from eukaryotes and the recombinant protein expressed in *Escherichia coli* catalyzed thioredoxin (Trx)-dependent reduction of H_2O_2, cumene hydroperoxide (CuOOH) and t-butyl hydroperoxide (t-bOOH) with K_m values of 122, 77 and 91 μM, respectively, confirming that it was a genuine 2-Cys Prx. The recombinant protein also displayed a double displacement reaction mechanism and a catalytic efficiency (k_{cat}/K_m) with H_2O_2 of 1.5×10^5 M^{-1} s^{-1} which was consistent with previous reports for the 2-Cys Prx family of proteins. This is the first time that a Prx 1 protein has been functionally characterized from any fish species and it paves the way for further investigation of this important 2-Cys Prx family member in fish.

1. Introduction

In mammals, peroxiredoxin (Prx) proteins have an established role as antioxidant enzymes and there is growing evidence that they are also involved in H_2O_2-mediated cell signaling [1,2]. The mammalian Prx proteins are classified as either 1-Cys or 2-Cys depending on the number of catalytic Cys residues they contain and the 2-Cys Prx proteins are further subdivided into typical or atypical depending on whether they form an intermolecular (typical) or an intramolecular (atypical) disulphide bond between the two Cys residues during the catalytic cycle [3]. The typical 2-Cys Prx include Prx 1 and 2 which are also referred to as natural killer enhancing factor (NKEF) A and B, respectively. The name NKEF was coined by Shau et al. [4] when they discovered a protein from human red blood cells which could enhance the cytotoxicity of natural killer cells. Later it was discovered that NKEF proteins were thioredoxin-dependent peroxidases and their name was changed accordingly [2]. Later still it was discovered that not all thioredoxin peroxidases used thioredoxin as the electron donor and their name was changed to peroxiredoxin. Peroxiredoxins have been extensively studied in mammals but comparatively little is known about them in fish and in the fish literature they are mostly still referred to as NKEF.

Studies with fish have shown that NKEF A and/or B gene expression was up-regulated in spleen of common carp, channel catfish, turbot and spotted green pufferfish challenged with immunostimulants (alginate, scleroglucan or lipopolysaccharide) or the pathogenic bacterium *Vibrio anguillarum* whereas it was down-regulated in a number of other tissues including kidney, heart, brain, gill, skin and gonad [5–8]. In contrast, NKEF B gene expression was up-regulated in liver, spleen, kidney, brain, heart, gill, skeletal muscle and intestine of ayu (*Plecoglossus altivelis*) following infection with the pathogenic bacterium *Aeromonas hydrophila* but not following infection with the non-pathogenic bacterium *Vibrio alginolyticus* [9]. In another example, NKEF gene expression was up-regulated up to 25-fold in peripheral blood leucocytes of rainbow trout (*Oncorhynchus mykiss*) infected with viral haemorrhagic septicaemia virus [10]. Thus, it appears that NKEF gene expression varies depending on the tissue or cell type, the fish species and the stimulus.

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Atlantic salmon (*Salmo salar* L.) is an important aquaculture species in Tasmania (Australia) where it was first introduced from the Northern Hemisphere in 1985 [11]. The main threat to the industry is amoebic gill disease (AGD) caused by the parasite *Neoparamoeba perurans* [12]. It is believed that the warmer sea temperatures off Tasmania, as compared with in the Atlantic Ocean, are at least part of the explanation for the persistence of *N. perurans* and AGD in Atlantic salmon aquaculture in Tasmania [13]. Currently, the only effective treatment for AGD is freshwater bathing which is expensive, accounting for 10–20% of production costs [11,13]. A previous study showed that glutathione peroxidase and thioredoxin gene expression were down-regulated in the gills of Atlantic salmon displaying advanced symptoms of AGD compared with naïve individuals [14]. Based on these results, the authors hypothesized that oxidative stress may be an important factor contributing to the death of AGD-affected Atlantic salmon. To investigate this further, we cloned a putative Prx 1 open reading frame (ORF) from Atlantic salmon liver and performed functional characterization of the corresponding protein recombinantly expressed in *Escherichia coli* and then we investigated Prx gene expression in the gills of Atlantic salmon infected with *N. perurans*.

2. Materials and methods

2.1. Fish sampling, RNA extraction and first strand cDNA synthesis

Atlantic salmon (*S. salar*) liver and gill samples were obtained from the study described by Bridle et al. [15]. The samples were taken at 0 and 14 days post-infection with *N. perurans* and stored in RNA*later*™ (Qiagen). Total RNA was extracted from approximately 30 mg of tissue using the RiboPure™ kit from Ambion® and first strand cDNA was synthesized from 1 μg of this RNA using the SuperScript® III RNase H$^-$ Reverse Transcriptase kit from Invitrogen™ with 1 nmole oligo dT$_{(12\text{-}18)}$ primers per reaction.

2.2. Cloning the Atlantic salmon Prx 1 open reading frame

The primers TPx forward and TPx reverse (Table 1) were designed based on the conserved regions surrounding the two catalytic Cys residues identified in a multiple sequence alignment of fish NKEF A and B proteins downloaded from the GenBank database. These primers were then used to perform polymerase chain reaction (PCR) using the cDNA synthesized from 1 μg Atlantic salmon liver RNA and the GoTaq® DNA Polymerase kit (Promega). The PCR cycling conditions were an initial step of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 49 °C for 40 s and 72 °C for 1 min followed by a final step of 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified PCR products were then sequenced and the sequences used to search the Consortium for Genomics Research on All Salmon Project (cGRASP) database (http://web.uvic.ca/grasp/). The sequence retrieved from the database was then used to design the Salmon TPxp forward and Salmon TPxp reverse primers (Table 1) to amplify the complete open reading frame (ORF) and to incorporate *Nco*I and *Bam*HI restriction enzyme sites (in underlined italics in Table 1) in the PCR products to facilitate their subsequent cloning. PCR was performed as described above and the resulting products were ligated into the pGEM®-T Easy vector (Promega). The ligation mixture was then used to transform *E. coli* DH5α cells. Successfully transformed cells were identified by their ability to form colonies in the presence of ampicillin (100 μg ml^{-1}). Colonies containing inserts were provisionally identified by blue/white colony screening and their identity was confirmed by colony PCR and nucleotide sequencing.

Table 1
Primers used for cDNA cloning and qPCR. The bases in underlined italics indicate the *Nco*I and *Bam*HI restriction enzyme sites incorporated into the forward and reverse salmon TPxp primers, respectively, to facilitate the cloning of the Atlantic salmon ORF into the pET-30a expression vector.

Primer name	Primer purpose (fragment size)	Primer sequence
TPx forward	cDNA cloning (404 bp)	5′-TATGTCGTGTTATTCTTCATCC–3′
TPx reverse	cDNA cloning (404 bp)	5′-ACTTCTCCATGTTTGTCAGTG-3′
Salmon TPxp forward	cDNA cloning (610 bp)	5′-*CCATG*GCTGCAGGTAAAGCACG–3′
Salmon TPxp reverse	cDNA cloning (610 bp)	5′-*GGATCC*TTACTGCTGCTTGGAGAAG-3′
Sa RT forward	qPCR (155 bp)	5′-GGTCATTGGTGCCTCTATC–3′
Sa RT reverse	qPCR (155 bp)	5′-CCTCATCCTCCTTCAACAC–3′

2.3. Subcloning the Atlantic salmon Prx 1 ORF into the pET-30a expression vector

Plasmid DNA was isolated from the *E. coli* DH5α cells containing the pGEM®-T Easy-Prx 1 construct using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) and the purified DNA was digested with *Nco*I and *Bam*HI before the Prx 1 ORF was ligated into the pET-30a expression vector (Novagen) digested with the same enzymes. The pET-30a expression vector fuses a His-tag to the N-terminal end of expressed proteins to facilitate their subsequent purification by Ni-affinity chromatography. The pET-30a-Prx 1 construct was then used to transform *E. coli* BL21 cells (Novagen) and successfully transformed cells were identified by their ability to form colonies in the presence of kanamycin (34 μg ml^{-1}). The presence of the Prx 1 ORF in the correct orientation and reading frame for protein synthesis was confirmed by colony PCR and nucleotide sequencing.

2.4. Recombinant expression of the Atlantic salmon Prx 1 ORF

E. coli BL21 cells containing the Atlantic salmon Prx 1 ORF in the pET-30a expression vector were induced for protein expression by incubation with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) according to the pET System Manual (Novagen). Protein expression was optimized by varying the duration of the induction time and by testing two different temperatures (25 °C or 37 °C). Aliquots of induced and uninduced cultures were removed at various times and the cells were pelleted by centrifugation before being washed twice with 20 mM Tris HCl (pH 8.0) and stored at −80 °C for subsequent analysis.

2.5. Ni-affinity purification of the recombinant Atlantic salmon Prx 1 protein

E. coli BL21 cells expressing the His-tagged Atlantic salmon Prx 1 protein were lysed in binding buffer (20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.5) by passage through an EmulsiFlex-C5 homogenizer (Avestin) at 10,000 Psi and the cell lysate was centrifuged at 10,000 *g* for 10 min before being filtered through a 0.22 μm Millipore® filter and applied to a 5 ml HisTrap™ HP nickel-Sepharose metal affinity column (GE Healthcare) at a flow rate of 2.5 ml min^{-1}. After washing the column with 50 column volumes of wash buffer (20 mM Tris–HCl, 0.5 M NaCl, 30 mM imidazole, pH 7.5), at a flow rate of 2 ml min^{-1}, the His-tagged recombinant Prx 1 protein was eluted from the column by the addition of elution buffer (20 mM Tris–HCl, 0.5 M NaCl, 500 mM