

Peroxiredoxin 6 from cuttlefish (*Sepiella maindroni*): molecular characterisation during development and the response to thermal stress

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Abstract The aim of this work was to investigate the function of the peroxiredoxin family member Peroxiredoxin 6 in *Sepiella maindroni* (*Smprx6*) during embryonic development and the response to thermal stress. The 1064 bp full-length coding sequence of *Smprx6* includes an open reading frame of 660 bp encoding a polypeptide of 219 amino acids with a predicted molecular mass of 24.4 kDa. The deduced protein sequence shares significant homology with other reported Prx6 enzymes. Phylogenetic analysis classified SmPrx6 into the 1-Cys-Prx cluster, and quantitative real-time PCR (qPCR) demonstrated constitutive mRNA expression in all tested tissues and during five different embryonic developmental stages, as previously documented in other cephalopods. Thermal stress induced *Smprx6* expression, which peaked at 6 h then returned to control levels. Recombinant SmPrx6 was active at different concentrations and was thiol-dependant. The results suggest that SmPrx6 plays a major role in the development of *Sepiella maindroni* and its response to oxidative and thermal stress.

Keywords *Sepiella maindroni* · Peroxiredoxin 6 (Prx6) · Embryonic development · Thermal stress · Phylogenetic analysis · qPCR

Introduction

Cephalopods have high rates of growth and food conversion, which for aquaculture results in a short culture cycle, a high ratio of production to biomass, and high cost-effectiveness. However, at present, only small-scale culturing of a limited number of species is possible (Castellanos-Martínez and Gestal 2013). Furthermore, most reported culturing procedures rely on captured wild individuals (Vidal et al. 2014). Our research group previously developed a system for breeding *Sepiella maindroni*, a commercially important fishery species accounting for a large proportion of cuttlefish aquaculture in China, by creating artificial culture cycles capable of rearing at least six generations in captivity (Wei-Wei and Chun-Lin 2009). However, despite the

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development of an artificial rearing method, *S. maindroni* suffers from the predicament of a high summer mortality. Cephalopods are susceptible to environmental changes, especially water temperature fluctuations, but if they survive such changes, they readily adapt to new environmental conditions (Gestal and Castellanos-Martinez 2015). Thermal fluctuations in a marine environment can induce physiological changes through oxidative stress-related mechanisms that generate reactive oxygen species (ROS) to modulate gene expression (Bogdan et al. 2000).

Although low concentrations of ROS are essential for normal physiological signalling (Poynton and Hampton 2014), excess ROS and oxidative stress can lead to cell damage, mutations, and even death (Zheng et al. 2010). Antioxidants are known to play a critical role in regulating ROS levels by detoxifying deleterious oxygen intermediates without disrupting normal cellular functions (Wood et al. 2003b). Peroxiredoxins (Prx) are a ubiquitous family of antioxidant enzymes that prevent oxidative damage (Robinson et al. 2010) and serve as effective medicinal agents for treating ROS-related diseases (Poynton and Hampton 2014). The Prx family has six isoforms classified into two sub-groups, including five 2-Cys-Prxs (Prx1 to Prx5) and one 1-Cys Prx (Prx6). The 2-Cys Prxs are divided into ‘typical’ and ‘atypical’ classes, depending on whether the conserved cysteine residues form intermolecular or intramolecular disulfide bridges, respectively (Wood et al. 2003b).

Prx6 is a bifunctional enzyme possessing both peroxidase and phospholipase A2 (PLA2) activities (Lee et al. 2015). Prx6 has been studied in various aquatic organisms including rock cod (*Trematomus bernacchii*) (Tolomeo et al. 2016), Japanese eel (*Anguilla japonica*) (Priyathilaka et al. 2016), turbot (*Scophthalmus maximus*) (Zheng et al. 2010), Chinese mitten crab (*Eriocheir sinensis*) (Mu et al. 2009), bivalve (*Laternula elliptica*) (David et al. 2007), disk abalone (*Haliotis discus discus*) (Nikapitiya et al. 2009), oyster (David et al. 2007), black tiger shrimp (*Penaeus monodon*) (Qiu et al. 2010) and Chinese shrimp (*Fenneropenaeus chinensis*) (Zhang et al. 2007). Prx6 has a single conserved catalytic cysteine residue in the N-terminal catalytic motif (PVCTTE) that is responsible for the peroxidase activity (Manevich and Fisher 2005). Evidence suggests Prx6 is distributed in all major tissues that are sensitive to oxidative stress (Manevich and Fisher 2005), and its expression is induced by stimuli such as bacteria (Zheng et al. 2010), viruses (Nikapitiya et al. 2009), pathogen-associated molecular patterns (PAMPs) (De Zoysa et al. 2012), chemicals (Wang et al. 2008), thermal stress (Park et al. 2008; Tolomeo et al. 2016) and environmental pollutants (David et al. 2007).

Antioxidant enzymes operating within the oxidant system of *S. maindroni* are significantly affected by thermal stimulation, particularly those related to scavenging ROS, as demonstrated by cDNA microarray studies based on an EST library (in review). A better understanding of the mechanisms underpinning the responses to oxidative stress could help to improve squid rearing and cephalopod aquaculture. In this study, we identified a peroxiredoxin 6 homolog in *S. maindroni* (*Smprx6*), measured *Smprx6* mRNA levels in the liver during oxidative and thermal stress, and produced and characterised the recombinant protein.

Materials and methods

Animals, embryos, acute thermal temperature challenge and sample collection

S. maindroni weighing 158.2 ± 2.8 g were collected in Ningbo, China, and acclimated in a blue plastic cylinder tank (diameter 1 m, depth 0.6 m) with a salinity of 28–30‰ at 22 ± 2 °C for 1 week before processing. Cuttlefish (120; length = 3.1 ± 0.5 cm) were randomly divided into six groups containing 20 individuals for the thermal stress experiment. After acclimation for 2 days, three groups were exposed to thermal stress conditions by gradually increasing the temperature by 1 °C per h to 32 °C, which is the thermal limit for survival based on breeding experiments (Jiang et al. 2010). Additionally, three control groups were maintained at 22 °C, which is the normal temperature for rearing in seawater. After treatment, five individuals were randomly sampled at 0, 0.5, 2, 6, 12, 24, 48 and 72 h, and the liver was collected and preserved in RNAlater (TaKaRa, Tokyo, Japan) for RNA extraction. Eight tissues (muscle, ink sac, liver, pancreas, ovary, testis, stomach, and gill) were collected from *S. maindroni* adults, and total RNA was extracted using RNAlater Plus (TaKaRa, Tokyo, Japan) according to the manufacturer’s protocol.

Several clutches of fertilised eggs were collected and acclimated at 20 ± 1 °C. Embryonic development of *S. maindroni* can be divided into 12 stages (Jiang et al. 2010), with the early stages combined into five major developmental phases: (I) the zygote stage; (II) the pre-embryonic stage, which occurs over an extremely short



MEGA 6.0. Tertiary structures of *Smprx6* were predicted using the SWISSMODEL prediction algorithm (<http://swissmodel.expasy.org>).

Transcriptional analysis of SmPrx6 by quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using an ABI 7300 (Applied Biosystems, Foster City, USA). Reactions were carried out in a final volume of 25 μL containing 12.5 μL of SYBR Green Real-time PCR Master Mix (TaKaRa, Tokyo, Japan), 9.5 μL of water, 0.5 μL of each primer (P5 and P6 for SmPrx6, and P7 and P8 for β -actin; see Table 1), and 2 μL of cDNA. Reactions were incubated at 95 $^{\circ}\text{C}$ for 3 s, followed by 40 cycles at 95 $^{\circ}\text{C}$ for 5 s, 56 $^{\circ}\text{C}$ for 5 s, and 72 $^{\circ}\text{C}$ for 20 s, and a final extension was performed at 72 $^{\circ}\text{C}$ for 1 min. Reactions were performed in a 96-well plate, each sample was repeated in triplicate, and an internal control gene (β -actin) was included. Dissociation curve analysis of amplification products was performed at the end of each reaction to confirm that only one PCR product was amplified and detected. PCR data were analysed using the ABI 7300 SDS software (Applied Biosystems, Foster City, USA). To maintain consistency, the baseline was set automatically by the software. The comparative C_t method was used to analyse the relative expression levels of *Smprx6*. C_t values for the amplified target products and the internal control (β -actin) were determined for each sample, and the difference in C_t for the target and internal control was calculated to normalise the differences in template and the efficiency of RT-PCR. Liver from squid in the control group was used as the reference sample for calibration. ΔC_t values for each sample were subtracted from the ΔC_t of the calibrator to generate $\Delta\Delta C_t$ values. The expression level of *Smprx6* was calculated using the $2^{-\Delta\Delta C_t}$ method, and values represent the n-fold difference compared with the calibrator (Livak and Schmittgen 2001).

Expression of recombinant SmPrx6

A PCR fragment encoding the mature SmPrx6 polypeptide was amplified with gene-specific primers P7 and P8 (Table 1) and cloned into the pMD18-T simple vector (TaKaRa, Tokyo, Japan), digested with restriction enzymes *NdeI* and *XhoI* (New England Biolabs, MA, USA), then subcloned into the *NdeI* and *XhoI* sites of the expression vector pET-21a(+) (Novagen, Shanghai, China). The recombinant plasmid was transformed into *E. coli* Origami (DE3) (Novagen, Shanghai, China) and subjected to DNA sequencing. The parent vector without the inserted fragment was used as a negative control. After sequencing to confirm in-frame insertion, positive transformants and the negative control were incubated in LB medium containing 50 $\mu\text{g}/\text{mL}$ ampicillin at 37 $^{\circ}\text{C}$ with shaking at 220 rpm. When the culture reached an OD_{600} of 0.5–0.7, IPTG was added to a final concentration of 1 mM and culturing continued for an additional 4 h under the same conditions.

Cells were harvested by centrifugation at $8000\times g$ for 2 min and suspended in 50 mM Tris containing 5 mM EDTA, 50 mM NaCl, and 5% glycerol (pH 7.9). After sonication for 60 s on ice, the rSmPrx6 and negative control samples were purified using a HisTrap Chelating Column (Amersham Biosciences, Stockholm, Sweden) according to the manufacturer's instructions. Purified recombinant proteins were analysed by 15% SDS-PAGE to check the molecular mass and purity. Assaying of the antioxidant activity was performed using the Total Antioxidant Capacity Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China) using the 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) ABTS method (Miller et al. 1993). Peroxidase activity was measured based on the change in absorbance at 420 nm that accompanies the breakdown of H_2O_2 . One unit of activity was defined as the amount of enzyme catalysing the breakdown of 1 mg of substrate by 1 g of fresh tissue in the reaction system at 37 $^{\circ}\text{C}$. Peroxidase activity was calculated using the formula supplied with the Peroxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All data were expressed as the mean \pm standard deviation ($n = 5$), and mean values were subjected to one-way analysis of variance (ANOVA). SPSS20 software was used to determine statistical significance ($p < 0.05$), and Origin 8.0 was used to prepare figures.

Results

Cloning and analysis of *SmPrx6*

The complete cDNA sequence of *Smprx6* was obtained by overlapping EST GT618012 with amplified fragments. The sequence has been deposited in Genbank under accession number HQ662844. The full-length mRNA of *Smprx6* is 1064 bp and includes an open reading frame of 660 bp encoding a polypeptide of 219 amino acids with a predicted molecular mass of 24.4 kDa and a theoretical isoelectric point of 6.17 (Fig. 1). The deduced amino acid sequence of *Smprx6* is shown in Fig. 1. The protein includes a typical Prx domain (from Leu4 to Ser160) and an alkyl hydroperoxide reductase (AhpC) domain (from Leu4 to Ser140) as identified by the SMART program. A signal peptide was not predicted by SignalP.

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1 GGGGGTTCACATGTCAAACTTTAAAAGCGGACATGCCGATTGTCACCTCATTCTGTT
  1                               M A N L G D
61 TCTTTTTTCCCCAGTCGTCAGATTGTAAATTCGATTGCAAGGATGGCTAACCTTGAGAT
  7 I F P N F D A D T T A G H F N F H E F I
121 ATTTCCAAACTTTGATGCTGACACCACAGCGGCCATTTCAACTTTCATGAATTCATC
27 N N S W A I L F S H P A D Y T P V C T T
181 AATAATCTTGGGCCATCCTCTCTCATCCTGCTGATTACACACCTGTCTGCACAACG
47 E L T R V V Q L I P E F K K R G V K L C
241 GAGCTGACGAGAGTTGTCCAGTTGATTCCAGAATTCAAGAAAAGGGGAGTAAACTGTGT
67 A L S C D D V E S H H G W I K D I I A Y
301 GCCTGTGATGTGATGATGTTGAAAGTCATCATGGTTGGATAAAAAGATATTATTGCTTAC
87 G K L A E F P Y P I I S D K T R K L A V
361 GGCAAACCTTGCTGAGTTTCCATATCCAATCATCTCTGACAAAACCCGAAAGCTCGCAGTC
107 S L G M V D P D E K D L A G M P L T A R
421 AGTCTGGGTATGGTGGATCCTGATGAAAAGGACCTGGCTGGAATGCCCTAACTGCACGC
127 A V F I I G P D K K L K L S I L Y P A T
481 GCGGTTTTTCATCATTGGCCCTGACAAGAAATTTAAAAGTGGGACTCCTCTATCCAGCAACC
147 T G R N F D E I L R V I D S L Q L T A K
541 ACTGGGAGAAATTTGATGAAATTTGAGAGTGATTGATCCCTCCAATTAACGGCTAAG
167 H K V A T P V D W K V G D K C M V V P S
601 CACAAGGTGGCCACTCCTGTCGACTGGAAGTTGGTGATAAATGCATGGTTGTCCCTCC
187 V K V E D E K E I F P K G V E T I P V P
661 GTCAAAGTTGAAGACGAAAAGAAATCTTCCCAAGGGGGTTGAAACAATCCCAGTGCCT
207 S G K S Y L R M T P H P E *
721 TCAGGAAAATCCTATCTTCGTATGACACCCCATCCAGAATAAAGAAATGCAGAAAGGGCA
781 GGA CTGCTGACCTTTTTTGCTTTTGACCTCTTTGAGATAAATTCAAAGTGGGGGGGCAG
841 GGGGCTACTCTAAGAACAAAATACTGCTGTAATTAATTGTAAGAATGCCAGTCGTGTTTG
901 AAATCTATTTTTTTTTTCTACTCATAACGAATTGTTAAAAATCTGATTGTATAAATCATT
961 TTGAAAGTAAAAACAGTTTTGTATTTGAGTCGTTCTACCGACTGTAATTTGGATGAAGGG
1021 ATGAATTTGATTCAATAAAGATTTCTAAAGGAAAAAAAAAAAAA

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Fig. 1 Nucleotide and deduced amino acid sequences of *SmPrx6*. The asterisk (*) indicates the stop codon. The polyadenylation signal is shown in *bold italics*. The active cysteine residue is in *bold* and is *underlined*. The catalytic motif is *shaded*. The conserved positively charged His 36 and Arg 126 are enclosed by *solid lines*



Multiple sequence alignment and phylogenetic analysis

The deduced amino acid sequence of *Smprx6* shares significant homology with other reported Prx6 proteins, with 71% identity with *Saccostrea glomerata* (ACX50969), and 70.9% identity with *Haliotis discus discus* (ABO26614). Multiple alignments revealed that Cys44 is conserved in all analysed Prx VI sequences. Moreover, the predicted SmPrdx6 protein has a unique cysteine residue (C44) in the peroxidase motif (42PVCTTE47) and a catalytic serine residue (S29) in the lipase motif (27NNSWA31) at the N-terminus (Fig. 2). Both of them are essential amino acid residues in the active sites that mediate peroxidase and PLA2 activity, respectively.

A phylogenetic tree was constructed based on Prx amino acid sequences (Fig. 3). Three distinct groups (1-Cys, 2-Cys, and an atypical 2-Cys subgroup) formed distinct clusters, with all Prx5 proteins part of the atypical 2-Cys subgroup, and Prx1, Prx2, Prx3, and Prx4 clustered in the 2-Cys subgroup. The *Smprx6* sequence and other Prx6 sequences from invertebrates were placed in the 1-Cys subgroup.

Expression of *Smprx6* in different tissues

To determine tissue-specific mRNA expression profiles of *Smprx6*, quantitative RT-PCR was carried out using various tissues from unchallenged (control) cuttlefish. Expression levels were normalised against β -actin mRNA levels which were tested in different tissues and following treatments to provide an internal control. The results revealed no significant differences in cycle threshold (C_t) values for β -actin in different experiments (Fig. 4; $p > 0.05$). The expression level of *Smprx6* in the ink sac was defined as 1, and expression was 460.71-fold higher in the pancreas, followed by liver (148.76-fold), gill (90.15-fold), ovary (26.6-fold), testis (11.53-fold), muscle (7.10-fold), and stomach (1.01-fold).

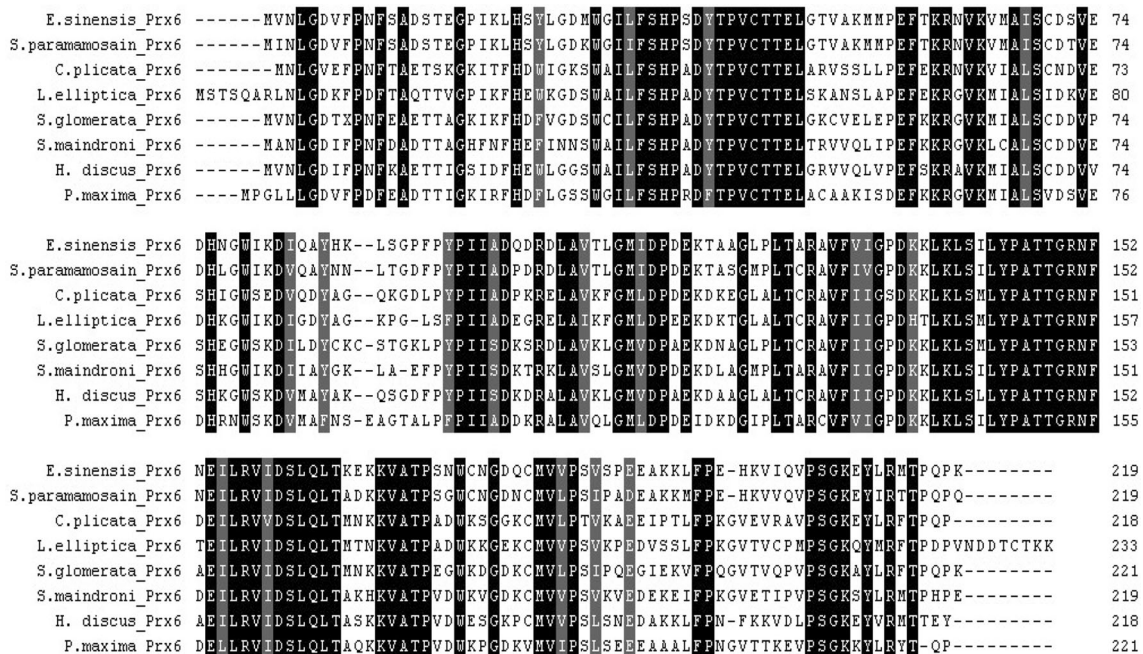


Fig. 2 Multiple alignments of the deduced amino acid sequence of SmPrx6 with other Prx6 proteins deposited in GenBank. *Black shading* indicates conserved amino acid residues. *Gaps* are indicated by *dashes* to improve the alignment. Abbreviations are as follows ACX50969, *Saccostrea glomerata*_Prx6; ABO26614, *Haliotis discus discus*_Prx6; ACF35639, *Eriocheir sinensis*_Prx6; ADN06076, *Cristaria plicata*_Prx6; ACJ53746, *Scylla paramamosain*_Prx6; ADJ57694, *Psetta maxima*_Prx6; ACE76885, *Laternula elliptica*_Prx6. Prx6 from *S. maindroni* is designated as *Smprx6*



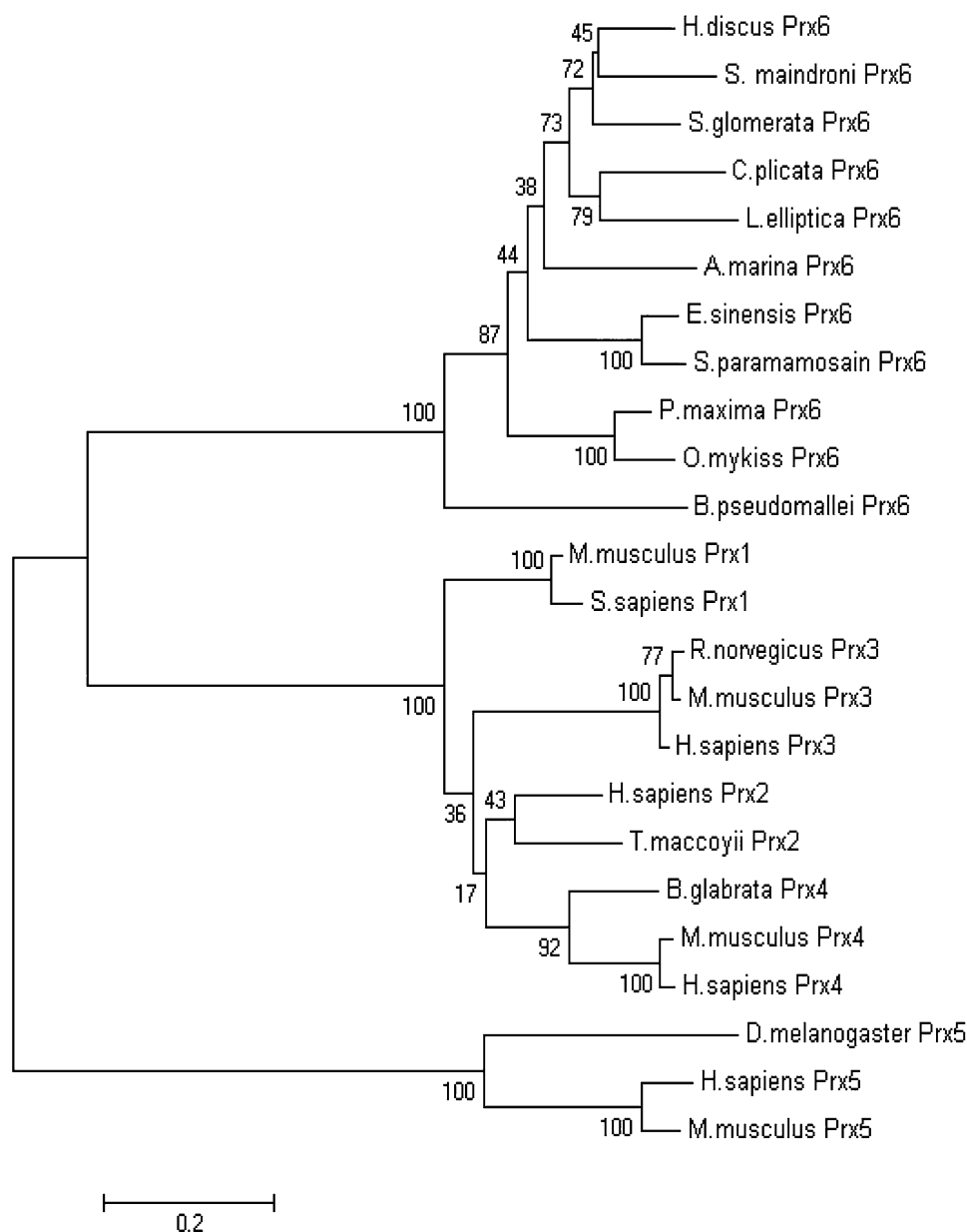


Fig. 3 Consensus neighbour-joining phylogenetic tree based on Prx sequences from the following species: CAM16508, *Mus musculus*_Prx1; NP_859048, *Homo sapiens*_Prx1; NP_005800, *Homo sapiens*_Prx2; ABW88997, *Thunnus maccoyii*_Prx2; AAH60567, *Rattus norvegicus*_Prx3; AAH05626, *Mus musculus*_Prx3; CAG29340, *Homo sapiens*_Prx3; NP_058044, *Mus musculus*_Prx4; ACI42880, *Biomphalaria glabrata*_Prx4; CAG46506, *Homo sapiens*_Prx4; AAF55497, *Drosophila melanogaster*_Prx5; CAG33484, *Homo sapiens*_Prx5; AAG13450, *Mus musculus*_Prx6; ACX50969, *Saccostrea glomerata*_Prx6; ABO26614, *Haliotis discus*_Prx6; ACF35639, *Eriocheir sinensis*_Prx6; ADN06076, *Cristaria plicata*_Prx6; AAY96294, *Arenicola marina*_Prx6; ACJ53746, *Scylla paramamosain*_Prx6; ADJ57694, *Psetta maxima*_Prx6; NP_001158604, *Onchorynchus mykiss*_Prx6; ACE76885, *Laternula elliptica*_Prx6

Expression of *Smprx6* during embryonic development

Smprx6 mRNA levels in embryos were measured by quantitative RT-PCR and transcripts were detected in all stages of development, with maximum expression during the pre-embryonic stage (1369.11-fold), but expression then decreased markedly during the organogenesis stage (15.2-fold), and only trace amounts were detected in the pre-hatching stage ($p < 0.05$; Fig. 5).



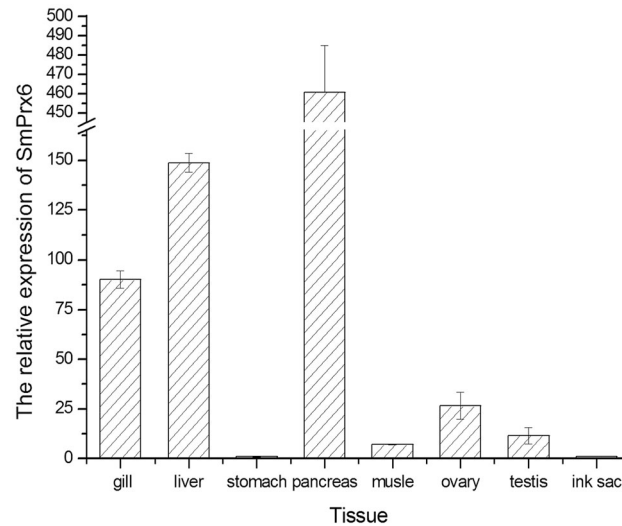


Fig. 4 Tissue distribution of *Smprx6* transcripts measured by SYBR Green RT-PCR. Muscle, ink sac, liver, pancreas, ovary, testis, gastric and gill were collected from three individual cuttlefish. Vertical bars represent the mean \pm SD ($p < 0.05$)

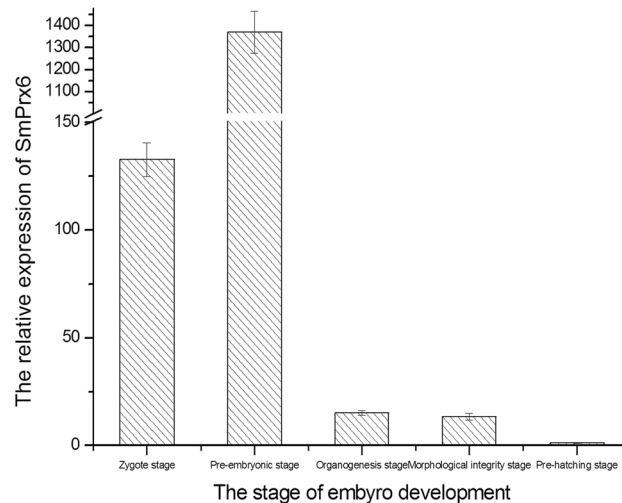


Fig. 5 *Smprx6* mRNA expression in embryos measured by SYBR Green RT-PCR. The β -actin gene was used as an internal control for all samples. Vertical bars represent the mean \pm SD, and bars with different letters are significantly different ($p < 0.05$)

Expression of SmPrx6 in liver after thermal treatment

To investigate *Smprx6* expression following an external temperature stimulus, *S. maindroni* were exposed to 32 °C for 0.5, 2, 6, 12, 24, 48 and 72 h, while controls were maintained at 22 °C. Expression of *Smprx6* was assayed by quantitative real-time RT-PCR with β -actin as an internal control. At 1 h after thermal stimulation, *Smprx6* mRNA levels were slightly up-regulated, but the difference from the control group was not significant ($p > 0.05$). *Smprx6* mRNA levels then increased rapidly and peaked at 6 h, representing an increase of 3.72-fold ($p < 0.05$), before declining and eventually returning to control levels (Fig. 6).

Expression, purification and characterisation of recombinant SmPrx6

The recombinant plasmid pET-21a–SmPrx6 was transformed into *E. coli* Origami (DE3) as described above, and rSmPrx6 was expressed and purified under native conditions. SDS-PAGE confirmed the presence of an overexpressed protein in the whole cell lysate following IPTG induction, and the purified protein ran as a



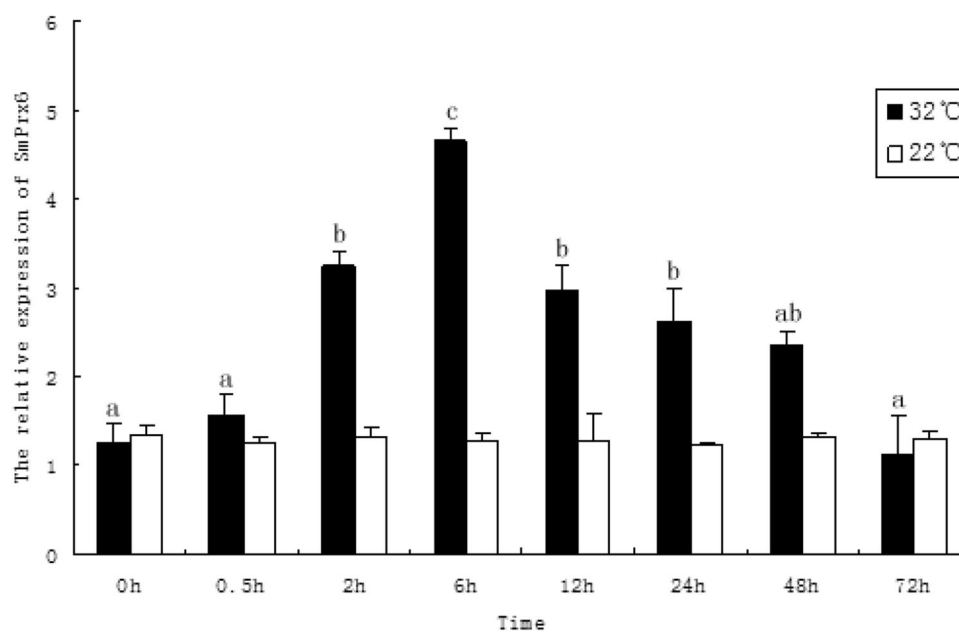


Fig. 6 Temporal expression profile of *Smprx6* transcripts in liver following thermal stress measured by SYBR Green RT-PCR. *S. maindroni* adults were incubated at 32 °C for 0, 0.5, 2, 6, 12, 24, 48 and 72 h. The control group was maintained at 22 °C. Vertical bars represent the mean \pm SD, and bars with different letters are significantly different ($p < 0.05$)

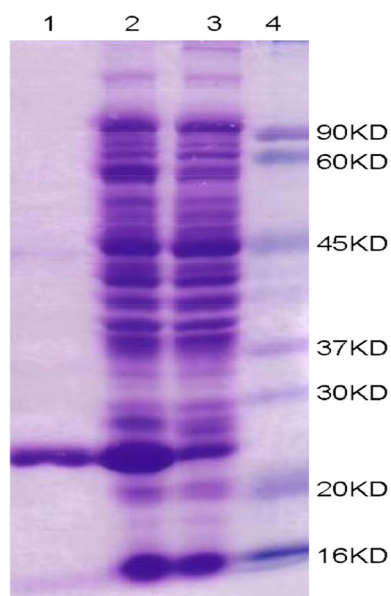


Fig. 7 SDS-PAGE of SmPrx6. Lane 1 purified rSmPrx6; lane 2 expression of rSmPrx6 after IPTG induction; lane 3 uninduced control without IPTG induction; lane 4 protein molecular standards

signal band with a molecular mass (~ 25 kDa) comparable to that predicted for rSmPrx6 (Fig. 7). The purified rSmPrx6 enzyme displayed antioxidant activity of 6.17 U/mg and POD activity of 10.17 U/mg.

Discussion

SmPrx6 shares common structural features with other invertebrate and vertebrate Prx6 proteins. The phylogenetic tree showed that Smprx6 falls into the 1-cys-Prx cluster, members of which contain a consensus

sequence with a conserved cysteine residue (Cys 44) in the N terminus, and a AhpC domain with a peroxidase catalytic motif (PVCTT). In mammals, Cys47, His39 and Arg132 form the catalytic triad responsible for peroxidase activity, with Cys47 bound to His39 via hydrogen bonding, and electrostatically activated by Arg132 (Manevich and Fisher 2005). Interestingly, a catalytic triad (Cys44, His36 and Arg 126) is also present in squid and other mollusks (Nikapitiya et al. 2009). The catalytic motif responsible for PLA2 activity (27NNSWA31) was observed in the deduced amino acid sequence, with Ser29 the catalytic nucleophile forming part of the NNSWG consensus sequence responsible for the PLA2 hydrolase activity (Lee et al. 2015). Ser is replaced with a Met, Arg or Gly in *E. sinensis* (Mu et al. 2009), *Xenopus laevis* (Genbank accession no. NP_001084316) and *Suberites domuncula* (Genbank accession no. CAC38779), respectively. The effect this replacement has on the catalytic activity warrants further study.

Smprx6 was constitutively transcribed in all examined tissues, suggesting it is ubiquitously expressed in squid, and levels were highest in pancreas and liver, which is different from other aquatic animals. Ploch et al. (1999) reported that liver cells from a variety of fish species are relatively resistant to DNA modification by ROS generated by various compounds. Based on the observed high Prx6 expression levels in pancreas and liver, we propose that Prx6 in squid possesses other activities such as the PLA2 activity described above. Indeed, ubiquitous expression of EsPrx6 mRNA in different tissues has been linked to an involvement in numerous physiological functions including basal metabolism in *E. sinensis* (Mu et al. 2009). In channel catfish, Prx6 is expressed in liver, head, kidney, intestine, and skin, but not spleen (Yeh and Klesius 2007). However, Zheng and colleagues reported that turbot Prx6 is more highly expressed in blood and heart than liver in unchallenged fish, in contrast with rock bream and channel catfish that display tissue-specific expression profiles (Zheng et al. 2010). This apparent variation in the transcription of Prx6 in different organisms may be due to the expression of multiple isoforms of Prx family members in different organs (Huang et al. 2011).

The consumption of yolk stores during a specific period of development involves governing regulatory signals and controlling ROS by-products (Shafer et al. 2011). Most antioxidant enzymes such as catalase and glutathione peroxidase are expressed at high levels during yolk consumption. Understanding the sub-cellular management of metabolism before, during, and after yolk consumption is, therefore, an intriguing area of investigation. As observed in many other organisms, SmPrx6 mRNA was detected throughout embryonic development. Expression was highest during the pre-embryonic stage (II), which was 1358.26-fold higher than the pre-hatching stage ($p < 0.05$), and expression then declined gradually from stage III to stage V. The early embryonic period can be considered a time of substantial proliferative stress because early-stage embryogenesis involves massive cellular proliferation over a very short time. Correspondingly, SmPrx6 has been implicated in complex cellular processes including cell proliferation (Wood et al. 2003a), differentiation (Rabilloud et al. 1995) and apoptosis (Zhou et al. 2000; Bernier-Villamor et al. 2004), with the highest expression in early-stage embryos. Our present results indicate that SmPrx6 plays a major role in embryonic development in *S. maindroni*, especially during the early stages of tissue differentiation.

Oxidative stress often accompanies thermal stress. Although low levels of ROS modulate a variety of cellular functions such as cell proliferation, high concentrations of ROS cause cell damage or apoptosis (Park et al. 2008). Among the antioxidant systems that protect organisms from excessive ROS, the Prx family of antioxidant enzymes has received much attention during the past decade. The significant upregulation of Prx transcript levels after a high-temperature stimulus indicates the induction of gene expression (Park et al. 2005). Expression of SmPrx6 increased rapidly in *S. maindroni* and peaked at 6 h post-stimulus (3.72-fold; $p < 0.05$) before declining and eventually returning to control levels. This indicates that SmPrx6 may play a major role as an antioxidant protein to protect organisms from excessive ROS following thermal stress (Tolomeo et al. 2016).

Cephalopods are highly adaptable marine organisms, and can adjust their biology and life cycles in response to the prevailing environmental and ecosystem conditions. These organisms can presumably avoid increased levels of oxidative damage normally during short-term thermal stress, possibly by altering metabolic processes.

In conclusion, we identified and characterised Prx6 from *S. maindroni*, and detected *Smprx6* mRNA transcripts in all tested tissues and developmental stages. The observed upregulation of Prx6 expression following a high-temperature stimulus indicates that its peroxidase activity may be important for the response



to oxidative stress. Therefore, Prx6 may play a major role in maintaining proteins in a reduced state by scavenging toxic ROS.

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