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Identification of a Cytoplasmic Manganese Superoxide Dismutase (cMnSOD) in the Red Swamp Crawfish, *Procambarus clarkii*: cDNA Cloning and Tissue Expression

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A cytoplasmic manganese superoxide dismutase (cMnSOD) cDNA was cloned from the hepatopancreas of the red swamp crawfish, *Procambarus clarkii*. An initial cDNA fragment was identified by using degenerate primers, and the complete sequence was obtained by using RACE methodology. The full sequence comprises 1140 bp, with an open reading frame of 858 bp encoding a protein of 286 amino acids. Sequence analysis showed that this protein is highly homologous to previously obtained crustacean cMnSODs. Phylogenetic analysis clusters it with all known cMnSODs and in a group distinct from mitochondrial MnSODs. cMnSOD transcripts were detected in the gills, tail muscle, green glands, and hepatopancreas. The data provide additional evidence for the hypothesis that cMnSOD replaced CuZnSOD in crustaceans that use haemocyanin as the respiratory pigment.

Key words: antioxidant, cytoplasmic MnSOD, cDNA, hepatopancreas, *Procambarus clarkii*

INTRODUCTION

Molecular oxygen serves a vital role as the final electron acceptor in the process of mitochondrial ATP production in eukaryotic organisms. However, the byproducts from oxygen chemistry can be quite destructive if not protected against. Production of reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) occurs via a variety of mechanisms within the cytosol and cellular organelles (Machlin and Bendich, 1987; Fridovich, 1989; Sies, 1997; Li and Shah, 2004). These superoxide and hydroxyl radicals, which in turn can generate more potent oxidants, are quite reactive and are responsible for the damaging effects of oxidative stress (Fridovich , 1997; Pryor et al., 2006). Prime targets for free radical reactions include almost all major structural organic biomolecules such as proteins, nucleic acids, and membrane lipids (Machlin and Bendich, 1987; Fridovich, 1989). Evolutionary driving forces necessitated the appearance of several cellular adaptations to deal with the potential toxic side effects of oxygen chemistry. Superoxide dismutase and catalase are prime examples of such enzymatic antioxidant defense mechanisms that aim to minimize the damaging effects of ROS; the former converts superoxide radicals to hydrogen peroxide (H_2O_2) and water, and the latter transforms H_2O_2 to water and oxygen (Fridovich, 1975; Scandalios, 2005).

Superoxide dismutases (SODs) are quite ubiquitous in aerobic organisms. They function as essential antioxidant metallo-enzymes and are defined by the metal ion in the catalytic site (Fridovich, 1975; Fink and Scandialos, 2002). Previous research recognized that multicellular aerobic animals possess two types of SODs: a cytoplasmic CuZnSOD and a mitochondrial MnSOD (mtMnSOD). The amino acid sequences of CuZnSODs are closely related to each other, as are the mtMnSODs, but the two forms of SODs show no similarity to each other (Fridovich, 1989; Zelko et al., 2002); mtMnSODs are furthermore characterized by the presence of a mitochondrial transit peptide that is cleaved after translocation into the mitochondria. Continued research on the importance of these enzymes resulted in the discovery of additional members of the SOD family. Examples include an extracellular CuZnSOD in mammals (Marklund et al., 1982) and invertebrates (Brouwer et al., 2003; Lina et al., 2008); iron-based SODs (FeSOD) in some prokaryotes, protista, and higher plant chloroplasts (Wright, 2003; Scandalios, 2005); and a nickel-based SOD enzyme in a select group of prokaryotes (Youn et al., 1996).

Within crustaceans, the tissue distribution pattern of SODs appears to have evolved slightly differently depending on the respiratory pigment present (Brouwer et al., 1997). Those that use copper-based haemocyanin for oxygen transport have been shown to be lacking the typical CuZnSOD in their cytoplasm. Instead, reports indicate that it has been replaced by a novel cytoplasmic manganese-based SOD (cMnSOD; Brouwer et al., 2003; Cheng et al., 2006; Gomer-Anduro et al., 2006). The aim of the present study was to verify the presence of a cMnSOD in *Procambarus clarkii*, a freshwater crustacean with haemocyanin as its respiratory pigment. Brouwer et al. (2003) hinted at indirect evidence for the presence of this enzyme in this animal. This paper presents the nucleotide sequence of this cMnSOD and

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discusses this finding by comparing it with SODs known from other decapod crustaceans.

MATERIALS AND METHODS

RNA isolation and reverse transcription (RT)

Red swamp crawfish (*Procambarus clarkii*) were obtained from local suppliers. After immobilization of the animals on ice, the hepatopancreas and other organs were quickly isolated and flash frozen using liquid nitrogen. RNA was isolated from powdered tissue using the Tri Reagent Protocol (Sigma, USA) and stored at –80°C. A small amount of RNA (1–5 μg) was converted to cDNA using MMLV Reverse Transcriptase and OligodT 15 primer, according to the manufacturer protocol (Promega, USA). The final reaction was diluted to 100 μl with nuclease-free H_2O.

PCR, 3'RACE, and 5'RACE

A multiple alignment and comparison of cMnSOD nucleotide sequences from other crustaceans was performed, and degenerate primers DGN-L1 (5'-CMATCTTCTGGACYAACAT-3') and DGN-R (5'-GCMCKVAGGTTCTTRTACTG-3') were designed based on highly conserved nucleotides regions. An initial putative cMnSOD amplicon was amplified in 25-μl reaction media, using 5 μl of RT product, DGN-L1 and DGN-R primers, Promega's MasterMix adjusted to 2 mM $MgCl_2$, and the following PCR-touchdown conditions: 94°C for 2 min (one cycle); 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min (8 cycles, with a decrease in annealing temperature by 2°C every cycle); 94°C for 1 min, 44°C for 1 min, and 72°C for 1 min (10 cycles); and 94°C for 1 min and 46°C for 1 min (22 cycles). The PCR was terminated with a 7 min extension at 72°C. This amplification yielded a single electrophoretic amplicon that was gel extracted, cloned, and submitted for sequencing.

Following the confirmation of a 399-bp cDNA sequence, the 3'-terminal end was obtained by using similar PCR reaction conditions, a newly constructed gene-specific left primer (GSP-L1: 5'-AACTTCAAGGACAAGTTCTCTGC-3') and an anchored OligodT18 primer. PCR conditions were similar to those described above, and a major electrophoretic amplicon of the correct expected size was extracted, cloned, and sequenced. Additional upstream sequence information was obtained by using a degenerate upstream primer (DGN-L2: 5'-ARGTDGATCAGGTTAAAAAGAATCAG-3') and a gene specific right primer (GSP-R1: 5'-GCAGAGAACTTGTCCTTGAAGTT-3'), resulting in an additional 350 bp of upstream sequence information. The final 5'-end sequence was pursued with the 5'RACE protocol and cloning kit from Invitrogen, USA. Briefly, 2.5 μg of total RNA were reverse transcribed by using a gene-specific primer (GSP-RT). The first-strand cDNA was tailed at the 5'-end by the terminal transferase TdT and dCTP, after which a diluted fraction was used for PCR. The first amplification round used primers GSP-R1 and AAP, while the second PCR run used primers GSP-R2 and AUAP. Primers AAP and AUAP were provided in the 5'RACE cloning kit. PCR conditions for both runs were as above, except that all annealing temperatures were at 55°C for the first run and 57°C for the second run. In both instances, a predominant single amplicon was observed after agarose electrophoresis. A schematic outline of the protocol is depicted in Fig. 1.

Cloning and sequencing

All PCR experiments were analyzed on a 1.5% agarose gel using SybrSafe Green (Invitrogen). PCR amplicons of the expected size were excised and gel extracted by using a Gel Extraction Kit (Qiagen, USA), and 1 μl was ligated into pGEM T-Easy vector (Promega), transformed into TOP-10 cells (Invitrogen), and plated onto AMP/XGal/IPTC plates. Recombinant cells were selected by blue/white screening and used in 5-ml overnight cultures. Plasmids were isolated and purified by using Qiagen's Mini Prep Protocol, and samples were submitted for sequencing to the DNA Sequencing Facility of the University of Chicago Cancer Research Center.

Analysis and phylogenetics

All analysis procedures were executed with programs available via Internet. Primers were selected by means of Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), while alignments were executed with the CLUSTALW2 (Larkin et al., 2007) or MultAlin (Corpet, 1988) interface sites. Nucleotide sequences and subsequent translations were analyzed by means of the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or EXPASY (http://www.expasy.ch/tools/) sites. Phylogenetic relationships among the chosen sequences were determined by using the Phylogeny.fr website (http://www.phylogeny.fr/), which connects various bioinformatics programs to reconstruct robust phylogenetic trees from a set of sequences (DeRepper et al., 2008), based on a new confidence index (Anisimova and Gascuel, 2006). The default settings were used, which included alignment with Muscle, curation via Gblocks, and phylogenetic tree construction by means of PhyML (maximum likelihood). Statistical tests for branch support were conducted via a Chi2-based parametric approximate likelihood-ratio test (aLRT) using the WAG substitution model.

Tissue expression of cMnSOD mRNA

Tissue samples from gills, green glands, tail muscle, and hepatopancreas were isolated from ice-immobilized animals and flash frozen in liquid nitrogen. RNA was isolated via the Trizol method, and one microgram of RNA was used for RT with MMLV reverse transcriptase and OligodT15 (Promega). The final RT solution of 20 μl was diluted to 100 μl with nuclease free water at the end of the RT procedure. Amplifications of GAPDH and cMnSOD were done with 2.5 μl of diluted RT product in total reaction volumes of 25 μl using the following conditions: 94°C for 2 min; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (30 cycles); 72°C for 5 min. Left and right primers used were 5'-CTGTTGCGAAAGTCCTTCAT-3' and 5'-ATGGCAGCTTTGATATCGTC-3' for GAPDH (GenBank AB094145) and 5'-GAAATGGCGGAGTATGTGTC-3' and 5'-GCAGAGAACTTGTCCTTGAAGTT-3' for cMnSOD. PCR product (10 μl) was visualized by using SybrSafe Green following agarose (2.0%) electrophoresis and digitized with a Kodak digital camera. Data were analyzed with ImageJ software from NIH and subjected to ANOVA and Student's t-test. A P value<0.05 was considered to be significant.

RESULTS AND DISCUSSION

Excluding the primers, the initial amplification with the degenerate primer pair yielded a 399-bp sequence. A translated BLAST screening of this sequenced fragment

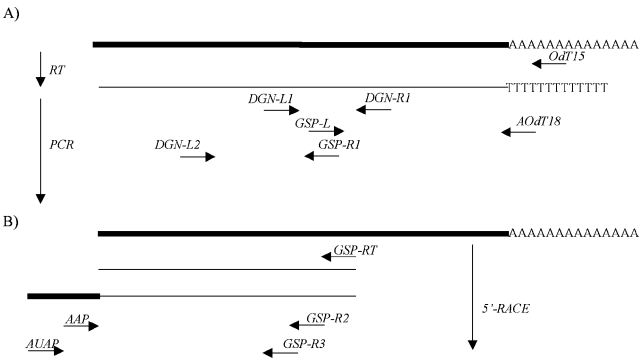

Fig. 1. Diagram showing the work flow during **(A)** RT-PCR and **(B)** 5'RACE protocols and the relative positions of the specific primers used. OdT, OligodT primers; DGN, degenerate primers; GSP, gene-specific primers. AAP and AUAP are 5'-RACE primers (Invitrogen). Additional details are provided in Materials and Methods.

indicated it to be highly similar to the cMnSOD of the giant freshwater prawn, *Macrobrachium rosenbergii* (GenBank AAY79405). The 3' end of the *P. clarkii* cMnSOD sequence was obtained by using gene-specific left primer GSP-L and an anchored OligodT18 right primer, while part of the upstream sequence information was obtained with degenerate upstream primer DGN-L2 and gene-specific right primer GSP-R1. The final 5'-end sequence was pursued according to a 5' RACE procedure, using a newly designed gene-specific right primers and 5' primers included with the kit (Fig. 1).

The complete *P. clarkii* cMnSOD cDNA sequence of 1140 bp (Fig. 2) was obtained by overlapping and merging the respective fragments. The cDNA consists of a 30-bp 5'-untranslated region (UTR), an 858-bp open reading frame encoding a 286 amino acid polypeptide, and a 250-bp 3'-UTR with polyA tail. By using ProtParam (http://us.expasy.org/tools/protparam.html), the molecular mass of this 286 amino acid protein was calculated to be 31,360 kDa, with an estimated pI of 5.44. The manganese superoxide dismutase signature DVWE-HAYY was observed from residues 243 to 250. The cDNA sequence and deduced amino acid sequence has been submitted to the NCBI GenBank (EU254488).

The predicted amino acid sequence of this crawfish cMnSOD is highly similar to the deduced cMnSOD sequences from the giant freshwater prawn, *M. rosenbergii* (GenBank AAY79405); the black tiger shrimp, *Penaeus monodon* (GenBank AAW50395); the white shrimp, *Litopenaeus vannamei* (GenBank AAY57407); and the blue crab, *Callinectus sapidus* (GenBank AAF74771). The percentage identities are 81%, 79%, 79%, and 78%, respectively, and the CLUSTAL alignment of the different crustacean sequences is shown in Fig. 3. All sequences show conserved motifs and the presence of four invariant amino acids (His-110, His-158, Asp-243, and His-247), respective binding sites responsible for manganese coordination.

A phylogenetic tree of the amino acid sequences of mitochondrial and cytoplasmic MnSODs, using baker's yeast as an outgroup, is shown in Fig. 4. The phylogram reveals an obvious clustering of the two forms into two groups with robust separate branches. The presently deduced SOD protein from *P. clarkii* falls into the same subgroup as the cMnSODs previously sequenced from other crustaceans, providing strong support that the cDNA

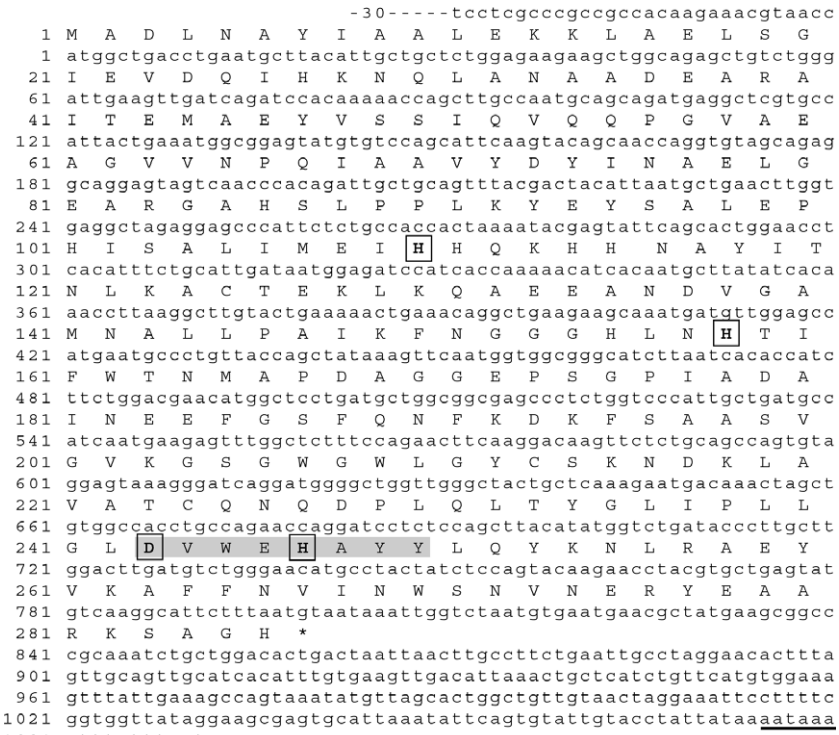

Fig. 2. Full nucleotide and deduced amino acid sequences of MnSOD from *P. clarkii*. The start codon is located at position 1, and the asterisk indicates the stop codon. The MnSOD signature is shaded gray (positions 243–250), and the putative manganese coordination sites are indicated by the boxed amino acids. The polyadenylation signal (aataa) is underlined.

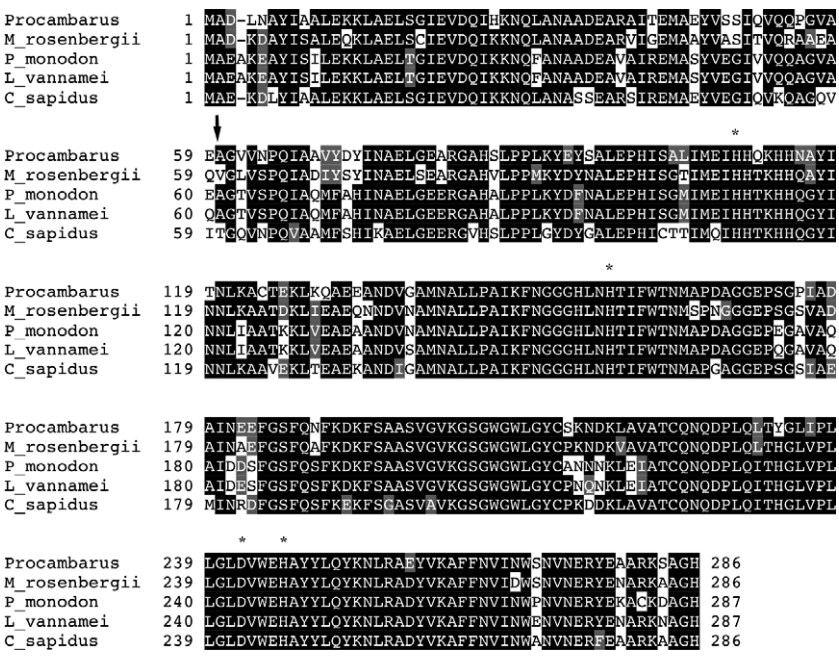

Fig. 3. Multiple alignment of the deduced amino acid sequence of cMnSOD from *P. clarkii* with complete cMnSOD sequences from other crustaceans: *M. rosenbergii* (AAY79405, giant freshwater prawn), *P. monodon* (AAW50395, black tiger shrimp), *L. vannamei* (AAY57407, white shrimp) and *C. sapidus* (AAF74771, blue crab). The arrow indicates the putative N terminus of the mature protein. The amino acids responsible for the metal coordination are indicated with an asterisk. Identical amino acids are shaded in black while the intensity of gray shading indicates the degree of similarity between the residues.

sequence is indeed a cytoplasmic form of MnSOD. The fact that the vertebrate (mouse) mtMnSOD is more closely related to the crustacean mtMnSOD furthermore suggests that the divergence of cytoplasmic and mitochondrial MnSODs is quite old. As Brouwer et al. (2003) argued, this sort of phylogeny strongly suggests that both forms of MnSOD arose via a gene duplication that occurred after the divergence of plants and animals but prior to the divergence of the protostomes and deuterostomes, placing the duplication roughly at the time of origin of the phylum Arthropoda.

Using a similar approach with degenerate primers designed from crustacean CuZnSODs, we were unable to obtain any PCR products from the hepatopancreatic cDNA pool of copper-exposed crawfish (data not shown). Although far from a definitive answer, this supports notion that the cytosolic CuZnSOD has been replaced by a cMnSOD in these haemocyanin-based crustaceans as well (Brouwer et al., 1997, 2003). Confirmation that this family of proteins is retained in the cytosol comes from the work by Brouwer et al. (1997) on crab cMnSOD. The purified protein indicated that residues 1–59 constitute a putative leader sequence, of unknown function, that is not retained in the mature protein. The known cMnSODs, including the present one, have this added a conserved N-terminal extension of around 60 amino acids but lack a mitochondrial targeting sequence present in the mtSODs. In contrast to mtMnSOD, which forms homotetrameric proteins (Zelko et al., 2002), cMnSOD exists in a monomer-dimer equilibrium attributed to the substitution of critical lysine residues, postulated to form important interactions with other subunits at the dimer-dimer interface of mtMnSODs (Brouwer et al., 2003).

A survey of the expression of the message for this protein in different tissues was executed with a left primer designed from the first 180-bp region, encoding the unique sequence not present in mtMnSODs, thus excluding the possibility of inadvertent amplification of mitochondrial MnSOD. Using GAPDH as internal control, the data indicate that cMnSOD mRNA is present in all tissues examined (gills, tail muscle, green gland and hepatopancreas) (Fig. 5). Similar data were obtained for the white shrimp, *L. vannamei* (Gomez-Andura et al., 2006; Wang et al., 2007), and the giant freshwater prawn, *M. rosenbergii* (Cheng et al., 2006), suggesting a ubiquitous tissue presence for this essential enzyme. Although we did notice an indication of differential expression in the tissues examined, a more controlled study will be needed to identify the factors that influence the expression pattern. Temporal up and down regulation of the cMnSOD transcripts has been reported in *C. sapidus* (Brouwer et al., 2004), *M. rosenbergii* (Cheng et al., 2006), and *L. vannamei* (Wang et al., 2008) and indicate that the expression of cMnSOD is susceptible to both environmental and biological challenges.

In conclusion, the high similarity between crawfish cMnSOD and crab cMnSOD, and the absence of critical lysine residues suggest that this crawfish MnSOD is most likely synthesized as a precursor molecule, becoming a dimeric cytosolic protein after posttranslational modification. The message for this enzyme is present in several tissues, but the tissue-specific response to environmental challenges requires further evaluation. The appearance of this cMnSOD in *P. clarkii* provides additional support for the hypothesis that haemocyanin-dependant crustaceans circumvented an intracellular copper-utilization conflict by replacing a copper-dependent SOD with a manganese-based SOD.

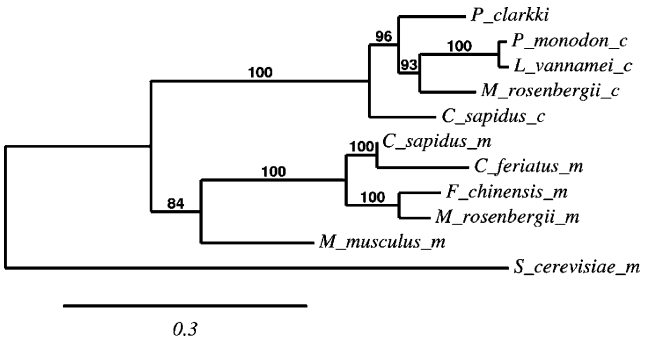

Fig. 4. Phylogenetic tree of MnSODs, reconstructed as described in Materials and Methods. The letter _m or _c at the end of a species name refers to the mitochondrial or cytoplasmic form. Species and accession numbers were taken from the NCBI Entrez Protein Database: *P_monodon* _c (AAW50395, black tiger shrimp), *L_vannamei*_c (AAY57407, white shrimp), *M_rosenbergii*_m (AAZ81617, giant freshwater prawn), *C_sapidus*_c (AAF74771, blue crab), *C_sapidus*_m (AAF74770), *C_feriatus*_m (AAD01640, crucifix crab), *F_chinensis*_m (ABB05539, Chinese shrimp), *M_rosenbergii*_c (AAY79405), *M_ musculus*_m (NP_038699, house mouse). MnSOD from baker's yeast (S_cerevisiae, CAA26092) was used as an outgroup. Numbers above branches indicate support in percent for the adjacent node among 100 trees calculated. The scale indicates the number of substitutions per site.

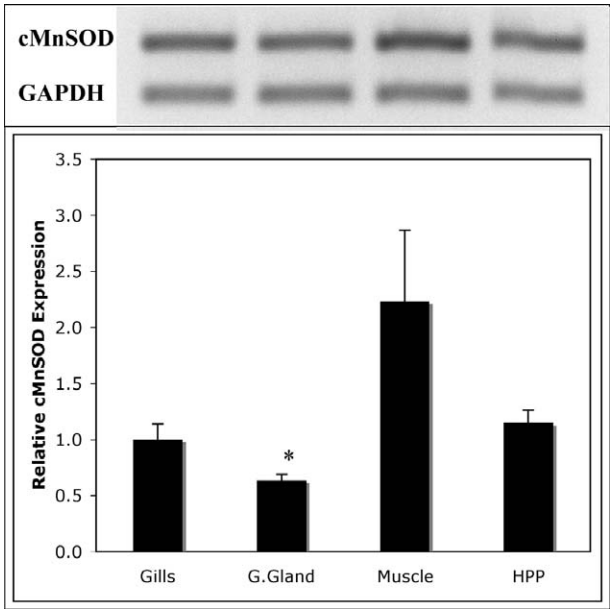

Fig. 5. Tissue expression of cMnSOD in the red swamp crawfish, *P. clarkii*. Pixel densities of cMnSOD and GAPDH (internal control) PCR bands were analyzed with ImageJ software. At the top is an inverted gray image from a representative PCR experiment. The vertical bars in the graph show the mean±S.E.M. of the ratio of cMnSOD to GAPDH from three different animals. *, Significant difference from hepatopancreas tissue expression ($P<0.05$); G.Gland, green gland; HPP, hepatopancreas.

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