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Note

Identification of antioxidant enzyme genes of the Indian edible oyster, *Crassostrea madrasensis* (Preston) through polymerase chain reaction

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ABSTRACT

When an organism is exposed to stress by way of environmental fluctuations or pathogenic attack, reactive oxygen species (ROS), which cause severe oxidative damage to the cells and hamper the cellular as well as membrane functions, are produced. In order to counter these effects, the cells activate the production of antioxidant enzymes which play pivotal role in removing ROS and maintaining the homeostasis within the cells. *Crassostrea madrasensis* is a promising bivalve species, living in intertidal region amidst a variety of stressors. In the present study, the RNA was isolated from gills and cDNA synthesised by Reverse Transcription PCR (RT- PCR) and amplification of these c DNA were carried out using a combination of different primers designed for super oxide dismutase (Cu/Zn-SOD), catalase (CAT) and glutathione peroxidase (GPX).. The PCR generated amplicons of 464 bp, 171 bp and 147 bp of Cu/Zn-SOD, CAT and GPX respectively were purified and sequenced. Similarity search in NCBI - BLAST confirmed these sequences as the respective antioxidant enzyme genes. The amino acid profile of Cu/Zn-SOD deduced from the sequences representing original reading frame (ORF) on InterProScan analysis was found to contain the characteristic Cu and Zn binding domains. PCR with the SOD specific primer pair resulted in the amplification of ORF of SOD with both genomic DNA and C-DNA as templates. This indicates the intronless nature of SOD gene, an adaptation to initiate fast expression at times of stress, as observed in certain other stress related genes such as the inducible form of heat shock protein 70 (Hsp 70). This is the first report on the molecular detection and identification of antioxidant enzyme genes of Indian edible oyster.

Keywords: Antioxidant enzyme genes, Catalase, *Crassostrea madrasensis,* Glutathione peroxidase, Super oxide dismutase

Oxygen is a vital factor for the existence of life and it is an important element for all flora and fauna of both terrestrial and aquatic origin. Mussels and oysters are sedentary and sessile bivalves seen growing attached on a suitable substratum. They primarily live in the intertidal zone of aquatic habitat. Owing to the characteristics of the habitat, these bivalves are constantly exposed to varying levels of oxygen, temperature and salinity. Hence suitable physiological mechanisms mediated through biochemical pathways are essential to maintain the cellular homeostasis. Reactive oxygen species (ROS) are produced in abundance when the animals are exposed to oxygen related stress, such as hypoxia. ROS molecules, such as super oxide anion radical (O_2-), hydroxyl radical (HO) and hydrogen peroxide (H_2O_2), produced during oxidation can destroy tissues and cells (Mi Seon Park *et al*., 2009). Prolonged hypoxia can result in mass mortality in oysters and a control system is essential to survive the situation (Boyd and Burnett, 1999). Antioxidant enzymes play a pivotal role in maintenance of homeostasis within the cells and in cell mediated antioxidant defense by removing ROS (Rudneva, 1999). Among the antioxidant enzymes, super oxide dismutase (SOD) is the key defense molecule which fights ROS, followed by others such as catalase (CAT) and glutathione peroxidase (GPX) playing vital roles in the detoxification pathways. Molecular detection and characterisation of antioxidant enzyme genes are helpful to detect cellular stress and also for bio-monitoring of the environment. The copper/zinc superoxide dismutase (Cu/Zn-SOD) gene in pacific oyster *Crassostrea gigas* was characterised by Boutet, *et al*. (2004). Cloning and mRNA expression of CAT and GPX in *Crassostrea gigas* was reported by Pil Gue Jo *et al*. (2008). The potential benefits of these genes are widely explored in biomonitoring and in selective breeding programs (Huvet *et al*., 2004; Timothy J. Green *et al*., 2009).

The Indian edible oyster *Crassostrea madrasensis* is one of the most important and promising economic oyster species being farmed for human consumption, and is frequently exposed to multiple stressors in the wild and captive set up. Compared to the European counter parts, reports on the functional genes, such as antioxidant enzymes, are totally lacking in this species which are predominantly inhabiting the Indian waters. So far no studies on the molecular biology of these genes have been carried out. We report, for the first time, the molecular

identification of three antioxidant genes *viz*., SOD, CAT and GPX in *C. madrasensis* by polymerase chain reaction (PCR).

The oysters were collected from the Satar Island,located in Vypeen Island near Kochi. The gill tissues were collected and stored in RNA later (Sigma). The total RNA was isolated from gill tissue using RNA isolation kit (Macherey Nagel), and used to reverse transcribe cDNA by Reverse Transcription PCR (RT- PCR), using cDNA kit (Fermentas). The synthesised c DNA was used to amplify the antioxidant enzyme gene segments SOD, CAT and GPX, using gene specific primers designed and custom synthesised. The primers were designed from the DNA sequences of *Crassostrea gigas* deposited in NCBI with the following accession numbers AJ496219 for SOD, EF687775 for CAT and EF692639 for GPX, using the software Beacon Designer 7. Different primer pairs were tested with varying PCR parameters to optimise the protocol. The sequences of the optimised primers are as follows: cmSOD forward primer (5'- ATG TCA TCT GCT CTG AAG GC-3'), cmSOD reverse primer (5' -TGG TGA TAC CGA TCA CTC CA- 3'), cmGPX forward primer (5' -GTC TAC CAG GCA TTC GCT TCA -3'), cmGPX reverse primer (5'- GCT TTT TGT CGC ATA AAG A -3'), cm CAT forward primer (5' AAC TAC TTC GCT GAG GTG-3'), cm CAT reverse primer (5'-GGT CTT GGC TTT GTA TGG-3'). The standardised PCR parameters consisted of an initial denaturation at 94 ^{0}C for 3 min followed by 30 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 56 ^{0}C for 30 seconds and extension at 72 ^{0}C for 45 seconds with a final extension at 72 ^{0}C for 5 min. The PCR products were electrophoretically separated in a 1.5% agarose gel, containing ethidium bromide along with known molecular weight marker.

The PCR products were gel eluted using Qiagen gel extraction system and sequenced in both directions. The sequences were used for similarity search by NCBI-BLAST and to deduce amino acid sequence using Six Frame translational software available with Biology workbench (http://www.workbench.sdsc.edu/). Further search to find out the characteristic Cu-Zn SOD signatures located within amino acid sequence was done with InterProScan analysis software (http://www.ebi.ac.uk/InterProScan).

PCR amplification of the c DNA template, with the gene specific primers resulted in amplicons of expected size (Fig. 1). Amplification of the Indian edible oyster genes with primers designed from the DNA sequence data of European oyster indicating the presence of conserved domains. Amplicons of expected size without non-specific amplifications were obtained in PCR using the primers designed from the sequence information of European oyster. The amplicons were of 464 bp, 171 bp and 147 bp for

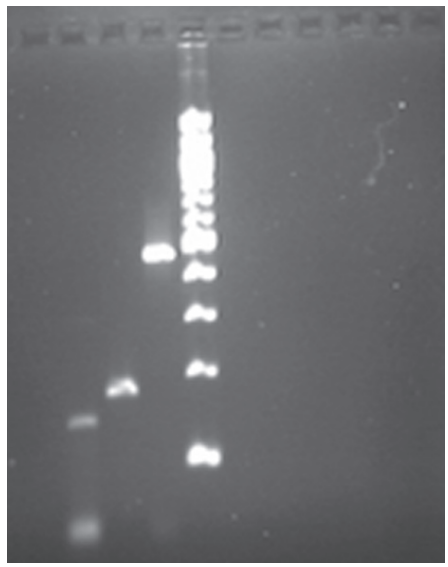

Fig.1. PCR products of SOD, CAT and GPX resolved in agarose gel electrophoresis. Lane 1. GPX, Lane 2. CAT, Lane 3. SOD, Lane 4. Molecular weight marker

super oxide dismutase (Cu/Zn-SOD), catalase (CAT) and glutathione peroxidase (GPX) respectively. BLAST similarity search of the sequences of these amplified gene segments in NCBI showed matching sequences with the antioxidant enzyme gene sequences of other bivalve species available in the data base. Cu/Zn-SOD of *C. madrasensis* has shown 93% identity with *Crassostrea ariakensis*, 92% with *C.hongkongenesis* and 90% with *C.gigas*. CAT sequences have 96% identity with *Crassostrea hongkongenesis* and 100% with *C. gigas*. GPX sequences have shown 100% similarity with *C. gigas*. All these genes are having direct role in the physiology of the animals at times of oxidative stress caused by temperature variations, hypoxia and under pathogenic attack.

The original reading frame (ORF) of the SOD, GPX and CAT were amplified in PCR and the sequences representing the partial segment of ORF were used to deduce the amino acid sequence (Fig. 2, 3, 4). Further analysis with the software InterProscan showed characteristic Cu / Zn SOD signature [GA]-[IMFAT]-H-[LIVF]-H-{S}-x-[GP]-[SDG]-x-[STAGDE]. Amplification trials with cDNA as well as genomic DNA as template has resulted in SOD amplicons with same size. This is a direct evidence for the intronless nature of SOD in *C. madrasensis* as has been reported for *C. gigas* (Boutet, *et al*., 2004), an adaptation to initiate fast expression at times of stress. This is a feature observed in certain other stress related genes, such as the inducible form of heat shock proteins (Boutet *et al*., 2004).

The objective of the present study was to identify presence of the antioxidant enzyme genes in the Indian edible oyster inhabiting the intertidal region, where the environmental parameters fluctuate widely. The antioxidant

Fig. 2. Partial nucleotide sequence of the ORF and the deduced amino acid sequence of SOD in *C. madrasensis*. Cu/Zn SOD family signature is underlined

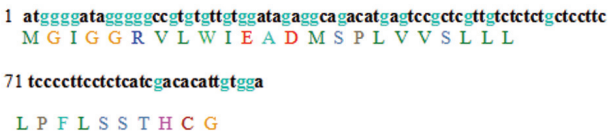

Fig. 3. Partial nucleotide sequence of the ORF and the deduced amino acid sequence of GPX in *C. madrasensis*

Fig. 4. Partial nucleotide sequence of the ORF and the deduced amino acid sequence of CAT in *C. madrasensis*

defense mechanism shall be essentially active in bivalves, as the marine habitat in which they live, is constantly exposed to varying physical parameters, like temperature and salinity as well as heavy metal and other toxic substances, leading to the production of ROS. The study has succeeded in identifying three antioxidant enzyme genes and their expression in *C. madrasensis.*

This is the first report on the detection and sequencing of antioxidant enzyme genes in *C. madrasensis* . Relative expression studies of these genes in different habitats can open up the potential use of these genes in biomonitoring of marine habitats. Such expression studies can also be used to monitor heavy metal pollution in marine environment as these genes are responding readily to such xenobiotic insults (Yan Fang *et al.,* 2010). Molecular biological approach will be a ready substitute for the conventional enzyme assays to monitor pollution. Transcriptomic expression of the genes can be used as biomarkers in marker assisted selective breeding programs (Huvet *et al*., 2004; Timothy J. Green *et al*., 2009). Studies aimed to completely characterise the full length c DNA of these genes, along with the relative expression analysis, are currently going on in our laboratory.

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