MOLECULAR GENETIC PROFILING OF FUNCTIONAL GENES OF PEARL OYSTER, *PINCTADA FUCATA*

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By

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Declaration

I do hereby declare that the thesis entitled **"Molecular genetic profiling of functional genes of pearl oyster, *Pinctada fucata*"** is the authentic and bonafide record of the research work carried out by me under the guidance of Dr. P.C.Thomas, Principal Scientist, Central Marine Fisheries Research Institute, Cochin, in partial fulfilment for the award of Ph.D. degree under the Faculty of Marine Sciences of Cochin University of Science and Technology, Cochin and no part thereof has been previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

ANJU ANTONY MOONJELY

Place: Cochin

Date: 25/08/2014

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Certificate

This is to certify that this thesis entitled, **"Molecular genetic profiling of functional genes of pearl oyster, *Pinctada fucata*"** is an authentic record of original and bonafide research work carried out by **Mrs. Anju Antony Moonjely (Reg. No. 3779)** at Central Marine Fisheries Research Institute, Cochin, under my supervision and guidance in partial fulfilment of the requirement for the award of the degree of **Doctor of Philosophy** in the Faculty of Marine Sciences, **Cochin University of Science and Technology, Cochin, Kerala**. It is also certified that no part of the work presented in this thesis has been submitted earlier for the award of any degree, diploma or any other similar title.

Place: Cochin
Date: 25/08/2014

P.C.Thomas
Supervising Guide

Dedicated to my Parents

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Abbreviation

AiGal	-	*Argopecten irradians* galectin
BLAST	-	Basic local alignment search tool
CA	-	carbonic anhydrase
cDNA	-	complementary DNA
CRD	-	carbohydrate recognition domain
Cu/Zn SOD	-	copper zinc superoxide dismutase
CvGal	-	*Crassostrea virginica* galectin
DNA	-	deoxyribonucleic acid
Ec-SOD	-	extracellular superoxide dismutase
EMBL	-	European Molcular Biology Laboratory
EST	-	expressed sequence tag
Fe-SOD	-	iron- superoxide dismutase
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
GM	-	glucanase motif
GNBP	-	gram-negative bacteria binding proteins
GPX	-	glutathione peroxidase
GST	-	glutathione- S-transferase
LGBP	-	lipopolysaccharide and β-1, 3-glucan binding protein
LPS	-	lipopolysaccharides
Mn-SOD	-	Manganese superoxide dismutase
NCBI	-	National centre for biotechnology information
Ni-SOD	-	Nikal- superoxide dismutase
nm	-	Nanometre

NSMP	-	nacreous shell matrix protein
ORF	-	open reading frame
PAMP	-	pathogen-associated molecular patterns
PBS	-	phosphate buffered saline
PCR	-	polymeras chain reaction
PG	-	peptidoglycan
ProPO	-	prophenoloxidase
PRP	-	pattern recognition receptor protein
PsBM	-	polysaccharide binding motif
RACE	-	Random amplification of the cDNA ends
RNA	-	ribonucleic acid
ROS	-	reactive oxygen species
RT-PCR	-	Reverse transcriptase polymeras chain reaction
Se-GPX	-	Selenium dependent glutathione peroxidise
SOD	-	superoxide dismutase
TBE	-	Tris boric acid Ethylene diamine tetra acetic acid
TLR	-	Toll-like receptor
UTR	-	untranslated region
βGRM	-	β-1,3-linkage recognition motif

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Chapter **1**

GENERAL INTRODUCTION

1.1. Pearl Oyster Aquaculture

Pearls have been known to the mankind since the beginning of civilization. They are highly esteemed as gems for their beauty and splendor. Pearls are formed when an irritant like a grain of sand is swept into the pearl oyster and is lodged within it where it gets coated by a micro thin layer of nacre, a silvery substance. India has the highest demand for pearls for setting in jewelry, and is particularly famous for its pearl oyster resources which yield superb pearls. The pearl oyster fisheries of India are located mainly in two areas: the Gulf of Mannar off Tuticorin coast and the Gulf of Kutch on the northwest coast. Habitats of these pearl oysters are different viz., at depths up to 23 meters in the Gulf of Mannar and in the intertidal zone in the Gulf of Kutch, and hence have different environments. These bivalves form large beds on hard substrata in the Gulf of Mannar, while they are sparsely distributed in the Gulf of Kutch. The pearl oyster resources of these two areas have been exploited for pearls until the early 1960's.

In October 1972 the Central Marine Fisheries Research Institute started a pearl culture research project at Tuticorin. Success came in July 1973 when a perfectly spherical pearl was produced. This breakthrough was achieved by introducing a graft of the oyster mantle in the gonad of an adult specimen together

with a shell bead nucleus. The CMFRI also succeeded in artificially spawning *Pinctada fucata*, rearing of larvae and producing seed in the laboratory by hatchery techniques. This breakthrough is very important in the light of the difficulty in obtaining sustained supplies of oysters from natural banks for culture purposes. Recently the CMFRI also produced seed of the black-lip pearl oyster, *Pinctada margaritifera* which produces the highly valuable black pearl. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this species. The hatchery is one of the most important source of sustained supply of pearl oysters.

The pearling industry is among the most potentially profitable aquaculture businesses. For example, hundred grams of gem pearls could be valued at more than US$ 2,600 (Anon, 2007). This is valued hundred times than the other aquaculture commodities on a weight basis. Globally, value of cultured pearl produced was estimated at around three billion US dollars (Anonymous, 2014). It has increased by approximately 40% compared to 2003 (Kremkow, 2005). Cultured pearl is contributed primarily by four pearl producing molluscs: (1) freshwater mussels; (2) the black-lip pearl oyster, *Pinctada margaritifera* ; (3) the silver or gold-lip pearl oyster, *P. maxima*; and (4) the Akoya pearl oyster, *Pinctada fucata*.

1.2. Biology of Pearl Oyster

1.2.1 Taxonomy

The true pearl oyster is a bivalve mollusc which belongs to the genus Pinctada (Roding) under the family Pteriidae, order Pterioida. Six species of pearl oysters, *Pinctada fucata* (Gould), *P. margaritifera* (Linnaeus), *P. chemnitzii* (Philippi), *P.*

sugillata (Reeve), *P. anomioides* (Reeve) and *P. atropurpurea* (Dunker) occur along the Indian coasts. The taxonomic position of *Pinctada fucata* used for the present work is described below.

The taxonomic position of *Pinctada fucata*

Phyllum Mollusca

Class Bivalvia

Order Pterioida

Family Pteriidae

Genus Pinctada Roding, 1798

Species Fucata (Hynd 1955)

1.2.2 Morphological Features of *Pinctada fucata* (Gould)

The hinge is fairly long and its ratio to the broadest width of the shell is about 0.85 and to the dorsoventral measurement is about 0.76. The left valve is deeper than the right. Hinge teeth are present in both valves, one each at the anterior and posterior ends of the ligament. The anterior ear is larger compared to other species, and the byssal notch at the junction of the body of the shell and the ear is slit-like. The posterior ear is fairly well developed. The outer surface of the shell valves is reddish or yellowish-brown with radiating rays of lighter colour. The thick nacreous layer on the inner surface has a bright golden-yellow metallic luster. Pictorial representation of the anatomy of *P. fucata* is given in Fig. 1.1.

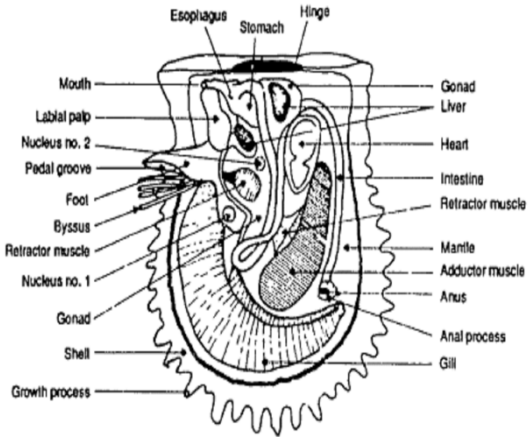

Fig.1.1 Anatomy of pearl oyster (adapted from George, 1967)

1.3 Life Cycle of Pearl Oyster

Pearl oysters start life as males and change into females after 2-3 years. Each female can release millions of eggs into the water, which are fertilized externally by sperm from the male. (Fig.1.2.). Eggs hatch and the oyster pass through various larval phases during which they remain swimming freely in the water. Length of the larval life varies with the temperature, food and other environmental factors. Larva can swim weakly by the use of highly developed velum, although they are generally transported by current and tides. Just before setting, the larva develops a pair of eye spots and a foot. This period is probably most critical in an oyster's life. Oyster shells are most common culth material, although the larvae will attach to almost any clean, hard surface. At between 25 and 35 days of age, the larvae starts spending more time crawling on the bottom and finally metamorphose into the juvenile pearl oyster that attaches itself to the substrate (Galtsoff, 1964)

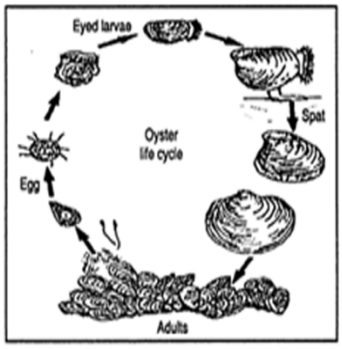

Fig. 1.2 Life cycle of pearl oyster

1.4 Pearl Formation

Natural pearls are formed as a reaction to an irritant in the internal part of a mollusc (Kunz & Stevenson, 1908; Strack, 2006; Streeter, 1886; Ward, 1995). The irritant may be small particles or parasites trapped or mantle scratches due to friction or predator attack (Strack, 2006; Ward, 1995). However, pearls will not be formed without the existence of epithelial cells from the nacre secreting mantle tissue (Simkiss & Wada, 1980). The epithelial cells begin to proliferate and form a 'pearl-sac' to cover the irritant (Taylor & Strack, 2008). The pearl-sac then begins to deposit minerals (nacre) around the irritant as a kind of internal defense mechanism resulting in the pearl (Dakin, 1913; Kunz & Stevenson, 1908). Pearl is produced by the process of bio-mineralization of calcium carbonate crystals. As the deposition continues and the resulting pearl grows. Shape of the irritant is usually irregular and this irregularity causes pearls to grow asymmetrically in shape (baroque type).

1.4.1 Cultured Pearls

Cultured pearls are divided in two types: bead nucleated and tissue nucleated (also called non-nucleated pearls) (Scarratt *et al.*, 2000). Principally, bead nucleated pearls are pearls generated from nuclei and mantle tissue while tissue nucleated

pearls are generated from mantle tissue only. Bead nucleated pearls consist of blisters (Mabe or half pearls), flat or coin pearls (not common) and round pearls (Fiske & Shepherd, 2007; Kennedy, 1998), while tissue nucleated pearls include several types of freshwater cultured pearls and keshi.

1.4.1.1 Mabe Pearl

Mabe pearl is an image pearl produced by placing a hemispherical object or a miniature image against the side of the oyster shell interior. The major advantages of Indian marine Mabes over the ones produced in freshwater mussels are the much shorter gestation period (2 months as compared to 18 months) and the superior quality of the nacre. The process of Mabe pearl formation begins with gluing a special plastic form (nucleus) on the interior of the pearl oyster shell (nucleus size from 8mm to 15mm in diameter). Once glued into the shell, the presence of the nucleus stimulates the pearl oyster to protect itself against the irritation by covering it with nacre, thus forming a Mabe pearl. Once implanted with Mabe pearl nuclei, the pearl oyster should be returned to the farm as soon as possible to minimize stress. There are varieties of ways to hold pearl oysters on a farm. The simplest way is to keep pearl oysters in plastic mesh trays, in bags on a rack or directly on the bottom in a calm, protected area.

1.4.1.2 Cultured Round Pearl

The second type of bead-nucleated pearls is the cultured round pearl which has greater value. Production of round pearls requires a round nucleus to be implanted with a piece of mantle (nacre secreting) tissue from a donor oyster into the

gonad of a recipient oyster. This process is known as 'pearl implantation' or 'grafting' or 'seeding'.

1.5 Immune/Defense Mechanisms of Pearl Oyster

The reason for high mortality in pearl oyster is related to ocean pollution, disease outbreaks and stock degeneration (Richard & Jackie, 1995; Potasman *et al.* 2002). As secondary filter feeders, the bivalves concentrate bacteria, viruses, pesticides, industrial wastes, toxic metals and petroleum derivatives, making them susceptible to disease problems. In order to control the disease and enhance the yields and quality of seawater pearl, it is necessary to carryout research on the innate immune defense mechanisms of the pearl oysters. Although, pathology information has been accumulating, research on bivalve immune systems and the underlying molecular mechanisms are still at an early stage. As an invertebrate, the bivalves rely exclusively on an innate, nonlymphoid system of immune reactions. The internal defense of bivalve is mediated by both cellular and humoral components. The former includes phagocytosis or encapsulation with subsequent pathogen destruction via enzyme activity and oxygen metabolite release, while the latter includes various reactions mediated by a series of molecules. One strategy to combat the problem is to identify defence related/disease resistance genes and use them for genetic improvement of cultured stocks. There are different types of molecules involved in the disease resistance viz. antioxidants, antimicrobial peptides, pattern recognition receptor proteins (PRPs) etc. which are genetically controlled. Among these, the antioxidant enzyme genes and PRP genes have been taken for the present study.

Stress induces the organisms to release reactive oxygen species (ROS), which are natural products of oxidative metabolism and are essential for eliminating invaders (Alves de Almeida *et al.*, 2007). However, excessive ROS can damage a number of cellular macromolecules (Hartog, *et al.*, 2003). Therefore, aerobic organisms requires an effective defense system against reactive oxygen species, which are produced following single electron reductions of molecular oxygen. Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids. (Sies, 1997; Vertuani, *et al.*, 2004). In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell. (Sies, 1997; Davies, 1995). Function of the antioxidant system is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

The innate immune system is designed to recognize molecules shared by groups of related microbes that are essential for the survival of those organisms and are not found associated with mammalian cells. These unique microbial molecules are called pathogen-associated molecular patterns (PAMP), and include lipopolysaccharides (LPS) from the gram negative cell wall, peptidoglycan (PG) and lipotechoic acids from the gram positive cell wall. In order to recognize PAMPs, various body cells have a variety of corresponding receptors called pattern recognition receptor proteins (PRPs) which are capable of binding specifically to the conserved portions of these molecules (Raetz *et al.*, 1991; Ulevitch and Tobias, 1995). Once an invading pathogen gain entry into the body of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses.

1.6 Biomineralization

Biominerlization is the formation of minerals by organisms, an extremely wide spread biological process found in organisms from all kingdoms. More than 70 mineral types are known (Mann, 2001). The first recognized scientific studies on biominerals were undertaken by zoologist more than a century ago, describing the various forms of endo and exo-skeletons in vertebrates and invertebrates respectively (Bouligand, 2004). Originally these investigations were descriptive, based primarily on typical crystallography and histology. As a result two major biominerals were catalogued; carbonates in invertebrates and calcium phosphates and carbonates in vertebrates. Furthermore, these biominerals were described with additional orders of complexity, being crystal polymorphism and numerous microstructures in which these polymorphs are arranged (Mann, 2001). Subsequently, the scientific field matured to include collaborations with chemists from which a third order of complexity emerged; the organic matrix component (Crenshaw, 1972; Marin and Luquet, 2004). It has long been theorized that this matrix is the keystone to the unique crystal structures found in biominerals, and through various demineralization processes the organic matrix was extracted and analyzed, the components of which are a mixture of proteins, glycoproteins and polysaccharides (Cariolou and Morse, 1988). Isolation of these components has proven difficult as many matrix characteristics are not amenable to classical fractionation techniques (Miyashita *et al.* 2000). The recent evolution of molecular-oriented biomineralization research could be used to elucidate the biologically controlled mineralization process.

The pearl oyster shell consists of two mineralized layers- an inner nacreous layer made from aragonite and an outer prismatic layer made from calcite. The mantle epithelium is the organ responsible for shell formation which takes place in the fluid-filled extrapallial space between the mantle and the shell. While the nacreous layer is formed by epithelial cell secretion at the mantle center, the outer prismatic layer is formed by secretion at the mantle edge (Sudo *et al.* 1997; Wada, 1999; Takeuchi and Endo, 2006). Secretion of organic and inorganic materials from mantle epithelia that condition the composition of the extrapallial fluid is an essential factor for the regulation of shell calcification. More specifically, a macromolecular complex called the organic matrix has been thought to be the controller of shell formation (Lowenstam and Weiner, 1989; Mann, 2001). Organic matrices are of two types, soluble and insoluble, the insoluble matrix provides the framework and mechanical properties for biomineralization, while the soluble matrix is involved in mineral nucleation and growth (Mann, 2001), thereby, possibly controlling calcium carbonate polymorphism (Belcher *et al.* 1996; Falini *et al.* 1996; Feng *et al.* 2000).

1.6.1 Genetic basis of Pearl Formation

Biomineralization processes leading to the formation of pearl in molluscs is controlled by specialized proteins. These proteins are regulated by specific genes encoding them. There is a paucity of sufficient information on the functional genes involved in pearl formation; therefore, an attempt was made to characterize some of them. In the present study, Mabe pearl was taken for gene expression study instead of round pearl because of the short gestation time for pearl formation.

Therefore, the present study was directed primarily towards the identification and characterization of functional genes involved in the defense and pearl formation in pearl oyster, *P. fucata*. Secondly, to evaluate the expression of these genes.

Objectives of the study

a) Identification and characterization of the following functional genes of pearl oyster.

   i. Antioxidant genes: superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione- S-transferase (GST).

   ii. Pattern recognition receptor proteins (PRP) genes: F-type lectin, galectin and lipopolysaccharide and β-1, 3-glucan binding protein (LGBP).

   iii. Pearl forming genes: nacrein, prismalin-14 and N19.

b) Expression analysis of the above defense related and pearl forming genes.

   i. Gene expression of antioxidant genes and PRP genes following exposure to lipopolysaccharide

   ii. Expression of pearl forming genes following Mabe implantation.

Chapter 2

REVIEW OF LITERATURE

2.1 Immunity of Pearl Oyster

The sedentary nature and filter-feeding habit of the bivalve mollusks result in the accumulation of large number of bacteria and chemical pollutants, which are sources of nourishment and challenge to their immune system (Bernal-Hernandez *et al.*, 2010). Immune systems protect multicellular organisms from foreign invaders. Two types of immune systems are in vogue in the animal kingdom, one providing innate (natural) immunity and the other providing adaptive (acquired) immunity. Pearl oysters like other invertebrates lack the adaptive immune system, and relies exclusively on the innate immune response to combat invading organism (Iwanaga and Lee, 2005). Both the cellular and humoral components of the innate immune system are present in them, and are operated in a coordinated way (Galloway and Depledge, 2001).

Cellular response is carried out by the circulating haemocytes that can kill microbes through phagocytosis and cytotoxic reactions. The cytotoxic reactions consists of release of lysosomal enzymes and anti-microbial peptides, and the respiratory burst resulting in the production of oxygen metabolites, like superoxide anion, hydrogen peroxide, and intermediate compounds with high bactericidal activity (Pruzzo *et al.*, 2005). The various reactive oxygen species produced from the respiratory burst can be damaging to cellular components, and therefore, all aerobic

organisms have evolved defenses mechanisms like antioxidant enzymes against the reactive oxygen species.

The two categories of humoral components taken up for the present study are antioxidant enzymes and pattern recognition receptor proteins of *Pinctada fucata*.

2.1.1 Antioxidant Enzymes

Oxygen is essential for most life forms. The full reduction of oxygen to H_2O by cytochrome oxidase is a key step in the mechanism of aerobic ATP formation. However, the partial reduction of oxygen results in the formation of various reactive oxygen species that can be damaging to cellular components. For example, about 1-4% of all oxygen consumed by cells is converted into superoxide radicals (O_2^-) by the "leaky" mitochondrial respiratory chain. Other relevant cellular sources of O_2^- include the activities of soluble oxidases (e.g. xanthine oxidase, aldehyde oxidase), NADPH oxidase at the plasma membrane of phagocytes, and the autoxidation of small molecules. Another reactive oxygen species is hydrogen peroxide (H_2O_2) which is formed by means of O_2^- dismutation (either spontaneous or catalyzed by superoxide dismutase) or by mixed function oxidase systems during the detoxification of xenobiotics. The O_2- and H_2O_2 have low oxidative toxicity themselves but they are readily converted into hydroxyl radicals ($^{\cdot}OH$) via the Haber-Weiss reaction which is catalyzed by iron or copper ions (Cadenas, 1995; Fridovich, 1998). Furthermore, the reaction of O_2^- with nitric oxide ($NO^{\cdot}$) leads to the formation peroxynitrite (ONO_2^-), a nonradical oxidant species (Inoue *et al.*, 1999). Both $^{\cdot}OH$ and ONO_2^- are highly reactive species and cause serious damage to cellular

macromolecules including lipid peroxidation, protein oxidation, and DNA damage (Cadenas, 1995).

Antioxidant enzyme systems have been evolved in all aerobic organisms as defenses against reactive oxygen species, and it include SODs, GPX, GSTs etc. Various isoforms of the superoxide dismutase like Mn-SOD (mitochondrial isoform); CuZn-SOD (cytosolic isoform) and Fe-SOD (bacterial SOD) have been reported. All SOD isoforms catalyze the dismutation of O_2^- into O_2 and H_2O_2. Glutathione peroxidase (GPX), a peroxisomal enzyme like the catalases has a major role in the decomposition of H_2O_2 (forming H_2O and O_2) but uses glutathione (GSH) as its co-substrate (Ahmad, 1995; Fridovich, 1998; Hermes-Lima *et al.*, 1998). Other peroxidases also have relevant roles for cellular H_2O_2 detoxification including ascorbate peroxidase and cytochrome C peroxidase (Ahmad, 1995; Campos *et al.*, 1999). Glutathione-S-transferases (GST) also play roles in antioxidant defense; they catalyze the conjugation of GSH to xenobiotics and also display selenium-independent GPX activity toward organic hydroperoxides (Habig and Jakoby, 1981; Hermes-Lima *et al.*, 1998).

The present study was focused mainly on three antioxidant genes viz. Superoxide dismutase (SOD), Glutathione peroxidase (GPX) and Glutathione-S-transferases (GST), and the published information on them are presented hereunder.

2.1.1.1 Superoxide Dismutase

Superoxide dismutase (SOD) enzymes are widely observed in biology: even in organisms lacking other antioxidant enzymes, such as catalase or peroxidase, and is expressed without exception (Asada *et al.*, 1977). Superoxide dismutase catalyze

the reaction of superoxide ions and 2 protons to form hydrogen peroxide and oxygen dioxide (Fridovich, 1995). SODs are classified into four groups. Copper/zinc superoxide dismutase (Cu/ZnSOD) is found only in the cytosol of animal cells, while homologs of this enzyme are found in both the cytosol and chloroplast in plants and algae (Fridovich, 1995). Manganese superoxide dismutase (MnSOD) is localized only to the mitochondria in any eukaryotic cell (Fridovich, 1995). MnSODs described are homologous to Fe-dependent bacterial SODs (FeSODs) (Fridovich, 1995). Extracellular copper zinc superoxide dismutases (EC-SOD), as the name indicate, are secreted by many types of cells (Weisiger and Fridovich, 1973; Marklund, 1982; Mantonavi and Dejana, 1989), and NiSOD has recently been purified from several aerobic soil bacteria of Streptomyces (Wuerges *et al.*, 2004). SODs accumulate in response to stress and are one of the main antioxidant defenses. Increased SOD levels have been linked to increased longevity and tolerance to ischemic or reperfusion events and factors that induce oxidative stress (Fridovich, 1995). SODs indicate that the cell is responding to an oxidative stress; MnSOD specifically shows that the mitochondria are experiencing an oxidative stress (Fridovich, 1995). Both the transcription and enzyme activity of Cu/Zn SOD are sensitive to stresses such as heavy metals, heat shock, anoxic stress and tributyltin exposure (Monari *et al.*, 2005; Kim *et al.*, 2007; Wang *et al.*, 2008; Zhang *et al.*, 2009). Cu/Zn SOD has been identified in many organism, including humans (Hallewell *et al.*, 1985), *Xenopus laevis* (Schinina *et al.*, 1989), chicken (Stanton *et al.*, 1996) and duck *Anas platyrhynchos domestica* (Liu *et al.*, 2002). Cu/Zn SOD genes have been cloned from several animals including black porgy, *Acanthopagrus schlegeli* (Lin *et al.*, 2000), red seabream, *Pagrus major* (Ken *et al.*, 2002), grouper,

Epinephelus malbaricus (Ken *et al.*, 2003), Pacific oyster, *Crassostrea gigas* (Boutet *et al.*, 2004), clam, *Ruditapes decussatus* (Geret *et al.*, 2004), prawn, *Macrobrachium rosenbergii* (Cheng *et al.*, 2006), and the abalones *Haliotis discus discus* (Kim *et al.*, 2007) and *Haliotis diversicolor supertexta* (Zhang *et al.*, 2009). So far, no examination of Cu/Zn SOD from pearl oysters has been made. The present study is the first report of the characterization of Cu/Zn SOD in pearl oyster *P. fucata*.

2.1.1.2 Glutathione Peroxidases

Glutathione peroxidase (GPX) is one of the most important endogenous antioxidant proteins and has a wide distribution in every oxygen-consuming living organism (Liu *et al.*, 2010). It catalyses the degradation of peroxides, which may include hydrogen peroxide, organic peroxide or lipid peroxides. Specificity of the enzyme for peroxides is low and the specificity for glutathione is high (Halliwell and Gutteridge, 1999). Glutathione peroxidase competes with catalase for hydrogen peroxide, or lipid peroxides. There are two types of glutathione peroxidase based on the metal cofactor present: seleniumin independent GPX and selenium dependent GPX. Selenium independent peroxidase has a preference for organic peroxides. Four other selenium-independent GPX, GPX-5 GPX-6, GPX-7 and GPX-8 are also present in mammalian tissues. Selenium dependent peroxidase is found in the cytosol and exhibits a high capacity to decompose hydrogen peroxide. Currently, four major selenium-dependent GPX (Se-GPX) have been identified in mammals: cellular GPX (GPX1) (Rotruck *et al.*, 1973), gastrointestinal GPX (GPX2) (Chu *et al.*, 1993), extracellular GPX (GPX3) (Takahashi and Cohen, 1986), and phospholipid hydroperoxide GPX (GPX4) (Ursini *et al.*, 1985). Shan *et al.* (2011) observed GPX mRNA expression in Japanese scallop, *Mizuhopecten yessoensis* after exposure to *V.*

anguillarum, increased from the initial days and then decreasing the level. The expression of GPX3 mRNA in *Chlamys farreri* have been reported to be significantly up-regulated after challenge (Mu *et al.,* 2010).

2.1.1.3 Glutathione S-transferases

Glutathione S-transferases (GSTs) comprise a large superfamily of enzymes whose soluble members primarily function as detoxification enzymes, facilitating the conjugation of a diverse array of hydrophobic electrophilic xenobiotics by the nucleophilic attack of glutathione (Sheehan *et al*., 2001). GSTs have long been known as important components of cellular defense mechanisms in mammalian systems (Hayes *et al*., 2005), and recent studies are revealing their significance in mediating allelochemical tolerance in invertebrate-host interactions (reviewed in Li *et al*., 2007). Glutathione acts as a non-specific reducing agent, thought to keep proteins in their reduced thiol form, thereby preventing the cysteine residues from oxidizing and crosslinking with one another to form disulphide bridges. Transferases are defined as enzymes that catalyse the transfer of one functional group from one molecule (donor) to another (acceptor). Glutathione-S-transferases are a complex group of enzymes which mediate the conjugation of compounds with glutathione. The soluble GSTs in metazoans are divided into eight classes (alpha, kappa, mu, pi, sigma, theta, omega, and zeta) based on sequence identity, immunological and kinetic properties (Sheehan *et al*., 2001; Hayes *et al*., 2005; Nebert and Vasiliou, 2004). While the majority of GST enzymatic characterization has been focused on mammalian forms, non-vertebrate models offer an exciting opportunity to examine the evolution of GSTs and their adaptive responses to environmental chemicals

including natural products. Zhao *et al* (2010) observed GST mRNA expression in Crab, *Eriocheir sinensis*.

Antioxidant defense activity has been identified in many organisms, including *Bathymodiolus azoricus* (Company *et al*., 2004), *Crassostrea gigas* (Jo *et al*., 2008), *Perna perna* (Almeida *et al*., 2004) and *Mytilus galloprovincialis* (Viarengo, 1990). Antioxidant system in relation to Cd contamination in a variety of fish species has been studied such as tilapia, *Oreochromis mossambicus* (Almeida *et al*., 2002) and *Oreochromis mossambicus* (Siraj Basha and Usha Rani, 2003), brown trout, *Salmo trutta* (Hansen *et al*., 2007), sea bass, *Dicentrarchus labrax* (Romeo *et al*., 2000) and rock bream, *Oplegnathus fasciatus* (Cho *et al*., 2006). So far, no examination of antioxidant defense activity in pearl oyster has been made.

2.1.2 Pattern Recognition Receptor Proteins (PRPs)

The first step in innate immune defense is the recognition of pathogens; this occurs via recognition of microbial cell wall components such as lipopolysaccharides (LPS), β-1,3- glucans and peptidoglycans by the proteins in the haemolymph. These pattern recognition proteins (PRPs) detect the "pathogen associated molecular patterns" (PAMPs) which lead to the signal transduction of immune response cascades facilitating the release of defence molecules.

It has been reported that pattern recognition molecules act as biosensors in the activation of innate immune responses in both vertebrates and invertebrates (Fabrick *et al*., 2003). Recently, several invertebrate PRPs have been isolated and characterized including LPS and/or beta-1,3-glucan binding proteins, peptidoglycan recognition proteins, gram-negative bacteria binding proteins (GNBP), C-type

lectins, galactoside binding lectins (galectins), thio-ester containing proteins, fibrinogen like domain immunolectins, scavenger receptors and hemolin (Christophides *et al.*, 2004; Dziarski, 2004). It has been reported that common motifs such as bacterial glucanase- like motif (Cerenius *et al.*, 1994; Kim *et al.*, 2000; Ochiai and Ashida 2000) bacteriophage lysozyme-like motif (Yoshida *et al.*, 1996) and immunoglobulin-like motif (Fearon and Locksley, 1996) exist in part of their primary structure. Their function in the immune response has also been studied. Lectins are the best characterized PRPs, and consist of a wide range of carbohydrate binding proteins of non-immune origin (Sharon and Lis, 2004; Vasta and Ahmed, 2008). Moreover, they present great structural diversity and a variety of carbohydrate affinity (Naganuma *et al.*, 2006). Animal lectins can be classified into several families, including C-, F-, P-, and I-type lectins, galectin, pentraxin, and others, based on their primary structure, structural fold, cation requirement etc. (Vasta *et al* 2004b; Honda *et al.*, 2000).

Lipopolysaccharide (LPS) represents a major pathogen-associated molecular pattern (PAMP) from the outer membrane of Gram-negative bacteria, and is a potent immune activator closely associated with many infectious and inflammatory diseases (Raetz *et al.*, 1991; Ulevitch and Tobias, 1995). LPS recognition by polymorpho nuclear neutrophils is one of the first steps of innate immunity. Neutrophil activation by LPS involves both the GPI-anchored molecule CD14 and the Toll-like receptor-4 (TLR-4), with occasional participation of TLR-2 binding to lipoproteins. This activation results in neutrophil degranulation and the activation of the oxidative burst (Remer *et al.*, 2003). Since LPS is a powerful immune activator and may be fatal, the

response to LPS must be tightly regulated to maintain the immune response at an appropriate level (Liew *et al.*, 2005).

Studies on Drosophila demonstrated that the GNBP (also known as LGBP) functions as a recognition receptor for LPS and beta-1,3-glucan (Kim *et al.*, 2000). The prophenoloxidase (proPO) activating system is an important non-self recognition system in invertebrates which can be activated by LPS or peptidoglycan from bacteria and beta-1,3-glucans from fungi (Ashida *et al.*, 1983; Smith *et al.*, 1984; Hoffmann *et al.*, 1996). LPS binding molecules are of particular importance because LPS binding results in markedly different functional outcomes. Some of the LPS binding molecules have opsonic effect (Cerenius *et al.*, 1994; Jomori and Natori, 1992), can degranulate blood cells (Barracco *et al.*, 1991), can participate in haemocyte nodule formation (Koizumi *et al.*, 1999), and can clear bacteria from the circulation (Koizumi *et al.*, 1997).

2.1.2.1 F-type lectin

The F-type domain is found in a range of invertebrate species, often within lineage-specific protein contexts. Presences of lectins have been shown in marine bivalves such as mussels, oyster and clams. The role of lectin induced agglutination of bacteria act as a molecular bridge between the surface of bacteria and haemocytes (Pruzzo *et al.*, 2005). Sugar binding has been demonstrated in an F-type lectin from the Japanese horseshoe crab *Tachypleus tridentatus*. Like many invertebrates, the horseshoe crabs (which are related to arachnids) have an open circulatory system:

hemolymph fluid, containing solutes and haemocytes, bathes the internal organs. The vast majority of horseshoe crab haemocytes are a single type of granular cell, which mounts an innate immune response upon recognition of bacterial lipopolysaccharide. Tachylectin-4 is an oligomeric haemocyte lectin consisting of a single F-type domain with an N-terminal extension, which exhibits Ca^{2+} dependent binding to terminal fucose. Tachylectin-4 recognizes bacterial lipopolysaccharide, probably through binding to fucose-like sugars such as colitose, a 3-deoxy form of fucose, and abequose, the D-isomer of colitose. Furrowed and CG9095 from *Drosophila melanogaster* contain single F-type domains within an architecture that also includes a C-type lectin-like domain and a number of complement control repeats. These proteins, which have homologues in other insects (eg. bee and mosquito), are the only F-type proteins known to have transmembrane domains. Signaling by these receptors is influenced by O-fucosylation, but the F-type domains in the receptors do not contain complete His/Arg/Arg triads (the C-type lectin-like domain in these receptors is also unlikely to bind sugar). It has been stated that F-type domains are not present in the nematode worm *Caenorhabditis elegans*, but a highly divergent F-type domain, which is very unlikely to bind fucose, is present in the CG9095 orthologue, C54G4.4. The divergence of F-type domains between CG9095 proteins in different species is suggestive of a non-sugar-binding function of the F-type domain in these receptors. In the sea urchin *Strongylocentrotus purpuratus*, an invertebrate which is relatively closely related to chordate organisms, single F-type

domains are present in complex protein architectures, as well as in a simple single-domain fucolectin. The complex proteins include CRL, which is involved in the complement system, and a protein containing a CCP and an EGF domain, both of which have very similar F-type domains, and a protein containing scavenger receptor Cys-rich, Kringle and other domains. F-type domains in the sea urchin are distinctive due to a number of insertions and the absence of the fucose-binding His residue and adjacent cysteines. F-type lectins were first identified and characterized in European eel, *Anguilla anguilla agglutinin* (AAA) that has been used extensively as a reagent in blood typing and histochemistry. F-type lectins have been identified and described as immune recognition molecules in Japanese horseshoe crab (*Tachypleus tridentatus*) (Salto *et al*., 1997), Japanese eel (*Anguilla Japonica*) (Honda *et al*., 2000), striped bass (*Morone saxatilis*) (Odom and Vasta, 2006), gilthead bream (*Sparus aurata*) (Cammarata *et al*., 2012), sea bass (*Dicentrarchus labrax*) (Salerno *et al*., 2009) and pearl oyster (*Pinctada martensii*) (Chen *et al*., 2011). In *Crassostrea* species, F-lectin is the main functional domain of bindin for recognition during fertilization (Moy and Vacquier, 2008).

2.1.2.2 Galectin

Galectins are a family of β-galactoside-binding lectins sharing conserved carbohydrate recognition domains (CRD) (Barondes *et al*., 1994), and they have been found in a wide variety of eukaryotic organisms ranging from fungi to mammals (Houzelstein *et al*., 2004; Vasta *et al*., 2004b). In mammals, fifteen kinds of galectin have been identified so far, which are subdivided into three groups: single

CRD galectin, galectin with tandem-repeat CRDs and chimera galectins (Hirabayashi and Kasai, 1993; Houzelstein *et al.*, 2004). In case of invertebrates, especially mollusks, there are several galectins with unique quadruple-CRD (Song *et al.*, 2010; Tasumi and Vasta, 2007; Zhang *et al.*, 2011a). All these CRDs share the highly conserved residues responsible for recognizing the β-galactoside ligand via specific hydrogen bonds or conferring binding affinity through hydrophobic stacking with the galactose moiety (Leonidas *et al.*, 1998; Nagae *et al.*, 2009). In the view of functional points, galectins are proposed to mediate diverse biological processes such as infection defense, cell activation, differentiation, cytokine secretion and apoptosis (Brewer *et al.*, 2002; Toscano *et al.*, 2007; Vasta, 2009; Vasta *et al.*, 2004a). More notably, they are involved in host–pathogen interactions of innate immunity by recognizing exogenous ligands, known as pathogen associated molecular patterns (PAMPs) on the surface of virus, bacteria, fungi or protozoa, and subsequently promote a firmer adhesion of the microbes to the cell surface for further clearance (Mey *et al.*, 1996; Moody *et al.*, 2000; Ouellet *et al.*, 2005; Pelletier *et al.*, 2003; Rabinovich and Gruppi, 2005; Tasumi and Vasta, 2007). However, in invertebrates, our knowledge of the biological roles of galectins in innate immunity is very limited and fragmentary (Vasta *et al.*, 2004b). A multidomain galectin of eastern oyster *Crassostrea virginica* (CvGal) is responsible for recognizing the protozoan parasite *Perkinsus marinus* (Tasumi and Vasta, 2007). Another multidomain galectin, AiGal from bay scallop *Argopecten irradians*, was also characterized and shown to be involved in innate immune responses (Song *et al.*, 2011). Galectin has been identified and characterized in Pacific oyster, *Crassostrea gigas* (Yamaura *et al.*, 2008), Manila clam, *Venerupis philippinarum* (Kim *et al.*, 2008), abalone, *Haliotis*

discus hannai (EF392832), freshwater snail, *Biomphalaria glabrata* (Yoshino *et al.*, 2008) and pearl oyster *Pinctada fucata* (Zhang *et al.*, 2011a).

2.1.2.3 Lipopolysaccaride and β, 1,3-glucan Binding Protein (LGBP)

LGBP plays the vital role in defense mechanism of invertebrates immune system. Structural studies of LGBP proteins showed the active site was occupied with (Arg–Gly–Asp) amino acids, which are responsible for the pattern recognition mechanism throughout adverse conditions. Basically LGBP is a glycosylated protein, which has the ability to bind with the glycosylated substrates like carbohydrate moieties. LGBP is one important member of the PRPs in invertebrate and displays various biological functions. For example, in the horseshoe crab *Tachypleus tridentatus*, LPS and β-1,3-glucan both bind specifically to LGBP, and then the coagulation cascade is activated (Muta *et al.*, 1991; Seki *et al.*, 1994). In addition, the opsonic effect (Cerenius *et al.*, 1994) and degranulation of blood cells by the β-1,3-glucan-binding protein in the freshwater crayfish *Pacifastacus leniusculus*, the opsonic effect of the LPS binding protein in the cockroach *Periplaneta americana* (Jomori and Natori, 1992), and the haemocyte nodule formation by the LPS binding protein in the silkworm *Bombyx mori* (Johansson, 1999) have already been reported as special biological properties of pattern recognition receptors. Several LGBPs have been cloned and characterized from Chinese shrimp *Fenneropenaeus chinensis* (Liu *et al.*, 2009; Du *et al.*, 2007), kuruma shrimp *Marsupenaeus japonicas* (Lin *et al.*, 2008), white shrimp *Litopenaeus vannamei* (Cheng *et al.*, 2005), tobacco hornworm *Manduca sexta* (Yu *et al.*, 2002), earthworm *Eisenia foetida* (Bilei *et al.*, 2001) and freshwater crayfish *P. leniusculus* (Lee *et al.*, 2000), and demonstrated that LGBP played an important function in the innate immune recognition. Recently, the LGBP

from zhikong scallop *Chlamys farreri* (Su *et al.*, 2004), pearl oyster *Pinctada fucata* (Zhang *et al.*, 2010) and disk abalone *Haliotis discus discus* (Nikapitiya *et al.*, 2008) were characterized and their expression pattern in different tissues and stress condition also were investigated.

2.2 Pearl Formation and Relevant Genes

Pearls are formed as a reaction to an irritant particle in the internal part of a mollusc. The epithelial cells begin to proliferate and form a 'pearl-sac' to cover the irritant. The pearl-sac then begins to deposit minerals (nacre) around the irritant. Pearl is therefore, produced by the process of bio-mineralization of calcium carbonate crystals around the irritant. As the deposition continues and the resulting pearl grows. Pearl has a "prismatic-layer"over the nucleus and an outer "nacreous-layer" over that.

Like the mineral deposit on the pearl, oyster shall also consists of two distinct structures: inner nacreous layers composed of aragonite and outer prismatic layers composed of calcite (Marin *et al.*, 2008). Pearl oysters initiate shell formation with amorphous calcium carbonate, which is transformed into either calcite or aragonite (Weiss *et al.*, 2002; Gerhke *et al.*, 2005). These transformation processes are thought to be regulated by proteins secreted from epithelial cells in outer mantle tissues (Marin *et al.*, 2008; Sarashina and Endo, 2006). These proteins form a biomineral framework and regulate the nucleation and growth of calcium carbonate. The differences in the composition of proteins secreted from the outer mantle tissues generate the calcite and aragonite polymorphs of calcium carbonate (Marin *et al.*,

2008; Evans, 2008). Nacreous and prismatic layers are formed in different regions of the outer mantle. The ventral part of the mantle (mantle edge) forms the prismatic layers, whereas the dorsal part of the mantle (pallium) forms the nacreous layers (Fig. 2.1). In pearl oyster culture, grafts from recipient pallia are transplanted with nuclei into the gonad of mother oysters. Formation of the nacreous and prismatic layers of the shell and pearl surfaces is closely related to the gene expression patterns of the shell matrix proteins (Takeuchi and Endo, 2006; Inoue *et al.*, 2010a, b) and the protein secretion properties. There are few studies on the gene expression patterns of the shell matrix proteins in the outer mantle epithelial cells of the pearl sac (Inoue *et al.*, 2010a; Wang *et al.*, 2009).

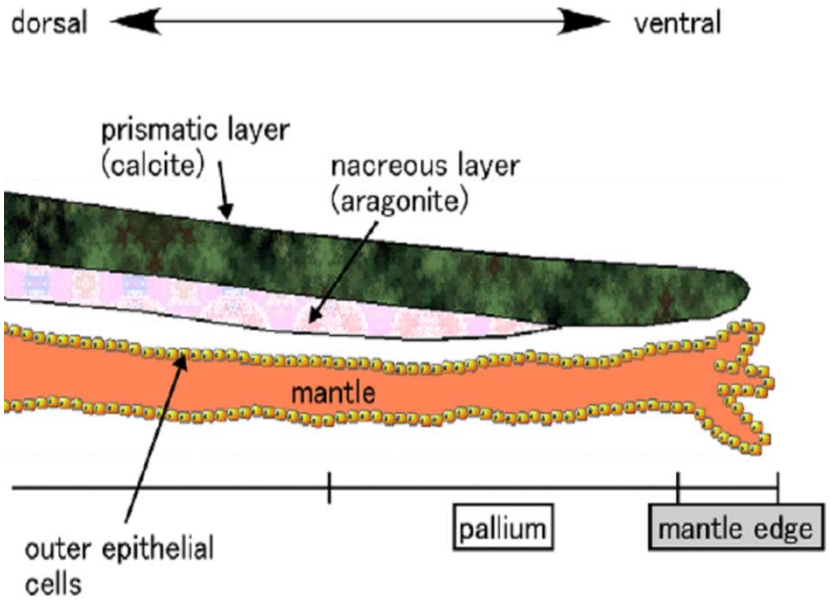

Fig.2.1 Nacreous layer and prismatic layer-producing tissue (Kinoshita *et al.*, 2011).

Secretion of organic and inorganic material from mantle epithelia that condition the composition of the extrapallial fluid is the essential factor for the regulation of shell calcification. More specifically, a macromolecular complex called the organic matrix has been thought to be the controller of shell formation (Lowenstam and Weiner, 1989;

Mann, 2001). The major components of soluble organic matrices are aspartic acid-rich calcium binding proteins (Weiner & Hood, 1975; Weiner, 1979; Wheeler *et al.*, 1981; Wheeler & Sikes, 1984; Cariolou & Morse,1988). Many matrix proteins are involved in pearl formation, and several have been identified in *P. fucata*: Nacrein (Miyamoto *et al.*, 1996), MSI60 (Sudo *et al.*, 1997), N16/pearlin (Samata *et al.*, 1999), P10 (Zhang *et al.*, 2006), N19 (Yano *et al.*, 2007), Pif80 (Suzuki *et al.*, 2009) and Pif97 (Suzuki *et al.*, 2009) are involved in the nacreous layer, while MSI31 (Sudo *et al.*, 1997), MSI7 (Zhang *et al.*, 2003), prismalin-14 (Suzuki *et al.*, 2004), aspein (Tsukamoto *et al.*, 2004), KRMP family (Zhang *et al.*, 2006) prisilkin-39 (Kong *et al.*, 2009), and the prismin family (Takagi and Miyashita, 2010) are involved in the prismatic layer. In addition, the shematrin family (Yano *et al.*, 2007) of proteins are involved in the formation of both nacreous and prismatic layers. One point worth mention is that, in the inner surface of the shell there is a section that connects the nacreous and prismatic layers, known as the 'aragonitic line', where a new biomineralizing conditions arise that result in a complete change in the mineralogy, shape, size, and growth modalities of biocrystals (Cuif and Dauphin, 1996; Saruwatari *et al.*, 2009; Dauphin *et al.*, 2008). However, little is known about the formation of the sharp boundary between the calcitic and aragonitic domains. No proteins are yet known to control the structure of the 'aragonitic line'(Fang *et al.*, 2011). Shell matrix proteins are secreted by the mantle tissue that covers the inner surface of the shell (Marin *et al.*, 2008; Weiss *et al.*, 2006; Wilt *et al.*, 2003; Cariolou and Morse, 1988; Weiner, 1979) and these proteins are likely to control crystal growth, so the mantle must have a key role in this process.

The process of biominerlization has been reported in previous studies. Kinoshita *et al.* (2011) conducted an expressed sequence tag (EST) analysis of *P.*

fucata genes expressed in the pallial mantle, which forms the nacreous layer, and in the mantle edge, which forms the prismatic layer. That study generated 29,682 unique sequences, from which the authors succeeded in identifying multiple genes that may play roles in pearl formation. Takeuchi *et al.* (2012) sequenced the genome of the pearl oyster *P. fucata*, and this provides a basic platform for further studies of the biosynthesis of pearl. Inoue *et al.* (2011) reported that gene expression patterns change before and after pearl sac formation and this may be regulated by the donor oyster.

Of the many genes that may be involved in the pearl formation, focus of the present study is on three of them viz., nacrein, prismalin- 14 and N-19. The major concepts concerning the genes associated with biominerlization of *Pinctada fucata* described below are related to the research conducted in this study.

2.2.1 Nacrein

Nacrein is a water-soluble protein present in the nacreous layer of the pearl oyster *Pinctada fucata* (Miyamoto *et al*., 1996). Nacrein has a domain similar to the carbonic anhydrase (CA), and showed a clear enzymatic CA activity. One unique characteristic of nacrein in many animal phyla is that a Gly-X-Asn repeat sequence, which probably binds calcium ions, is inserted into the CA homologous domain. The nacrein gene is also found in *Pinctada maxima* (Kono *et al*., 2000), which is a congeneric species of *P. fucata*. Miyamoto *et al.* (2003) suggested that nacrein is conserved and plays an important role in the shell formation in the nacreous layer-producing species of bivalve and the gastropod. The result of *in situ* hybridization analysis presented by Miyamoto *et al.* (2005) indicates that nacrein is highly expressed in the outer epithelial cells of both the mantle edge and pallial, which form

the lining of the shell and are involved in the transport of calcium into the mineralizing site and in the secretion of some proteins into the shell. This suggests that nacrein is important for shell formation in both the calcitic prismatic layer and the aragonitic nacreous layer. Inoue *et al.* (2011) who found that highest nacrein gene expression of the implanted oyster mantle in *P. fucata* was at the initial days compared to other time intervals.

2.2.2 Prismalin-14

Prismalin-14 is secreted from the mantle epithelial cells to extrapallial fluid and taken up into the prismatic layer. Suzuki *et al.* (2004) purified a matrix protein, prismalin-14, from the acid-insoluble fraction of the prismatic layer of the Japanese pearl oyster, *Pinctada fucata*. The whole sequence of prismalin-14 consists of 105 amino acid residues and includes four structurally characteristic regions; a repeated sequence of Pro-Ile-Tyr-Arg, a Gly/Tyr-rich region and N- and C-terminal Asp-rich regions. Prismalin-14 showed an inhibitory activity on calcium carbonate precipitation and a calcium-binding ability *in vitro*. Prismalin-14 is an acidic protein (pI = 3.8) and has an inhibitory activity on calcification, suggesting that prismalin-14 is associated with calcification of the prismatic layer. Suzuki and Nagasawa (2007) observed that the central part of prismalin-14 including the GY-rich region may bind to chitin, and N- and C-terminal Asp-rich regions possibly bind to calcium carbonate crystals or serve as a nucleation site. The calcium carbonate crystals then grow to form the prismatic layer of the shell. Inoue *et al.* (2011) also found that highest prismalin-14 gene expression of the implanted oyster mantle in *P. fucata* was at the

initial days compared to other time intervals. It indicates that pismalin-14 is the key component of pearl biomineralization.

2.2.3 N19

A novel 19 kDa protein was isolated from the nacreous layer of the pearl oyster *P. fucata*, and was named N19. This is one of the predominant proteins found in the water-insoluble fraction of the nacreous layer. The N19 transcript was more abundant in the pallial region of the mantle, suggesting that the N19 protein is predominantly expressed in the pallial region and translocated into the nacreous layer, since N19 may play a role in calcification of the shell. Previous study of nacreous shell matrix proteins (NSMP) in *P. fucata* by Wang *et al.* (2009) suggested that the N19 gene was the only one that exhibited a high expression level in pearl sac, which was even higher than that in mantle pallial. It indicates that N19 plays important roles in governing both pearl production and nacreous shell growth, and the N19 gene should be in a deeper association with the pearl formation than any other known NSMP genes. Liu *et al.* (2012) suggested that significant expression of N19 gene in the pearl sac was detected by day 30–35 after nucleus implantation. Current knowledge about the changes at the gene expression level of N19 is very limited.

Chapter **3**

MATERIALS AND METHODS

Molecular identification, characterization and expression analysis of genes encoding antioxidant enzymes (SOD, GPX and GST), pattern recognition receptor proteins (F-type lectin, galectin and LGBP) and pearl forming genes (nacrein, prismalin-14 and N19) of Indian pearl oyster, *Pinctada fucata* were carried out. For this a partial segment of the target genes were initially amplified from the cDNAs developed through RT-PCR of the mRNA. Random amplification of the cDNA ends (RACE) of these segments were carried out to develop the full length sequence of the genes.

From this, the amino acid sequence being coded by the open reading frame (ORF) region were deduced using Transeq software of European Molcular Biology Laboratory (EMBL), and used for the identification of characteristic domains and binding sites of the proteins through InterProscan analysis.

NCBI-BLAST search of the sequence of the partially amplified gene/RACE products and amino acid sequences were carried out to confirm their identity. The bioinformatics procedures like multiple sequence alignment comparison and phylogenetic analysis of the nucleiotide and amino acid sequences using the tool like Bio Edit, MEGA etc. were also carried out.

Expression studies of these genes following their induction using appropriate agents were also carried out.The antioxidant genes and pattern recognition receptor protein genes were induced for expression through challenge trials by exposing the experimental animals to bacterial lipopolysaccharides. In case of pearl forming genes, their expressions were studied following Mabe implantation.

Fig.3.1 Indian pearl oyster, *Pinctada fucata*

3.1 Collection of Experimental Animals and Acclimatization in the Laboratory Conditions

Adult individuals of *P. fucata* (4.5 to 5.5 cm shell length and 20-30 g body weight) were collected from the Pearl Farm in Tuticurin, and maintained at 25^0C in tanks containing static aerated seawater (0.5 L/oyster, 25 ppt salinity) in the laboratory. The seawater was changed every day, and the pearl oysters were fed with *Isochrysis galbana* twice daily. After two weeks of acclimatation, they were used for the trials.

RNA was collected from the haemolymph and mantle tissues of the animals to develop cDNA for the gene characterization. Part of the animals were used for expression studies.

Fig.3.2. Acclimatation of the experimental animals in the laboratory conditions

3.2 Induction of Immune Related Gene by Lipopolysaccharide (LPS)

For stimulation of the immune related functional genes, 50 µl of LPS (*E.coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in phosphate buffered saline (PBS) at concentration of 10 µg ml^{-1} was injected into the adductor muscles of each of the animals. The control groups were injected with 50 µl of PBS only. At each time point following the injection (0, 4, 8, 12, 24 and 36 h), haemolymph was collected from both the control and the LPS stimulation groups. Haemolymph sample collection from the adductor muscles were carried out using a syringe, and immediately centrifuged at 5000×g at 4^0C for 10 min to harvest the haemocytes. Five control and five LPS injected individuals were sampled at each time point. The haemocyte pellets were immediately used for RNA extraction.

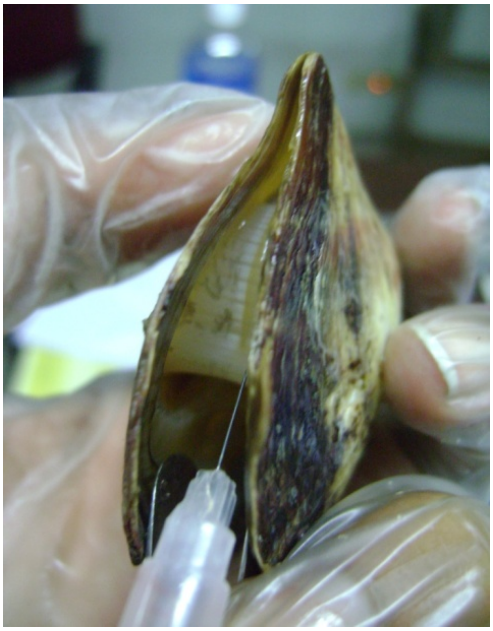

Fig 3.3 Injection of lipopolysaccharide

3.3 Mabe Implantation

The process of Mabe pearl formation begins when a base image made from shell powder and resin/carved shell/special plastic image (8 to 10 mm diameter) is glued on the interior of the pearl oyster shell. Once glued onto the shell, the oyster attempt to protect itself against the irritation by covering the implanted object with nacre, thus forming a Mabe pearl. For this study, the pearl oysters were opened using the speculam (a flat edge opening tool) that is inserted on the same side of the oyster as the byssus threads, putting less stress on the adductor muscle. Using a cotton swab, that part of the shell where the image to be implanted is first dried. A drop of glue is put on the flat side of the base image, and it is pressed firmly against top shell beneath the mantle tissue and held for 10 seconds. The implanted oysters were placed in tanks with fresh seawater and the water replaced every day. At each pre-determined day post implantation (day 10, 30, and 40) five oysters were harvested. Mantles surrounding the Mabe were carefully isolated and used for RNA isolation.

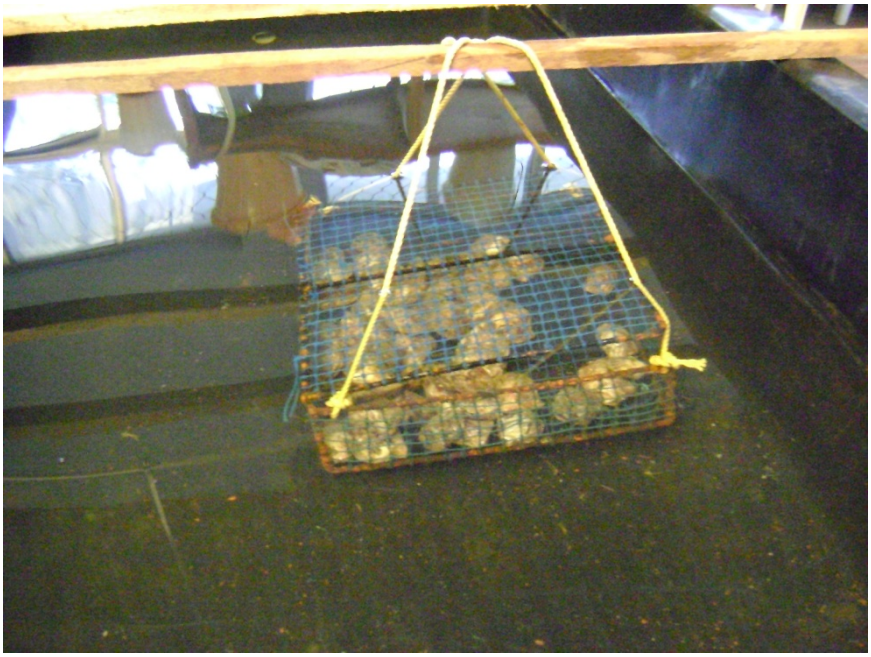

Fig.3.4 Mabe implanted oysters kept in laboratory conditions

3.4 RNA Isolation

Total RNA were isolated from Haemocytes and mantle surrounding the Mabe using the RNA isolation kit (NucleoSpin RNA II reagent-MACHEREY-NAGEL GmbH & Co, Germany) as per the manufacturer's instructions and stored at -80^{0}C until further use. Integrity of the isolated nucleic acid was checked by 0.8% agarose gel electrophoresis and followed by ethidium bromide staining and the gels were documented using a Gel documentation system (Bio-rad).

3.5 Quantification of Nucleic Acid

The total RNA isolated were quantified spectrophotometrically using biophotometer by mearuring the optical density at 260 nm. The concentration of the RNA was adjusted as per the requirements of the downstream applications such as polymeras chain reaction (PCR), cDNA synthesis and random amplified cDNA ends (RACE).

3.6 cDNA Synthesis

After ensuring the integrity of RNA using gel electrophoresis and quantification by spectrophotometer, the total RNA was used to synthesize the cDNA. One micrograms of total RNA was used to synthesize the cDNA by following the instructions given by the manufacturer of cDNA synthesis kit (Fermentas Inc). The total RNA was initially incubated with oligo dT. The preparation was mixed with RNAse inhibitor, dNTP mix, reaction buffer and reverse transcriptase provided in the kit. The cDNA synthesis was carried out at 42^0C for 60 minutes and the reaction was terminated by incubating at 70^0C for 5 minutes. The cDNA thus prepared were either used immediately or stored at -20^0C till further use.

3.7 Polymerase Chain Reaction (PCR)

3.7.1 Primer Selection and Design

The general strategy followed for the PCR amplification of the genes under study was the initial amplification of the conserved region of genes using gene specific primers from closely related species. The resultant amplicons were sequenced, and sequences of all the amplicons thus obtained were subjected to homology search in NCBI-BLAST. New primers were again designed from the conserved region of the sequences which has shown similarity with the targeted genes in the data base.

3.8 PCR Amplification of Antioxidant Enzyme Genes, PRP Genes and Pearl Forming Genes

3.8.1 Superoxide Dismutase (SOD)

The following primers were used for the amplification of SOD (Boutet *et al.*, 2004)

Forward Primer : 5` ATGTCATCTGCTCTGAAGGCCGT 3`

Reverse Primer : 5` CTACTTGGTGATACCGATCACTCCACA 3`

The PCR condition standardized : The reaction volume of 25 ml consisted of 2.5 ml of 10X PCR buffer, 0.5 ml of dNTP (10 mM), 1 ml of each primer (10 mM), 18.7 ml of PCR-grade water, 0.3 ml (1 U) of Taq polymerase (Sigma Aldrich) and 1 ml of cDNA.

The thermal profile of PCR were as follows.

1 Initial Denaturation	-	94^0C for 5 min
2 Cyclic Denaturation	-	94 ^{0}C for 30 s
3 Annealing	-	60^0C for 30 s
4 Extension	-	72^0C for 45 s
5 Repeating step 2-4	-	for 30 cycles
6 Final extension	-	72^0C for 10 min

3.8.2 Glutathione Peroxidase (GPX)

The following primers were designed and used for the amplification

Forward Primer	:	5` AATACGGCGTCCTTATCAG 3`
Reverse Primer	:	5` TCTCTAGTCCCTTTGTTTGG 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation	-	94^0C for 5 min
2 Cyclic Denaturation	-	94 ^{0}C for 30 s
3 Annealing	-	54^0C for 30 s
4 Extension	-	72^0C for 45 s
5 Repeating step 2-4	-	for 30 cycles
6 Final extension	-	72^0C for 10 min

3.8.3 Glutathione-S-transferase (GST)

The following primers were designed and used for the amplification

Forward Primer : 5` GATGATGAGGATGTATAAC 3`

Reverse Primer : 5` TTATGTAAGTCTTCTTCAC 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94^0C for 30 s

3 Annealing - 59^0Cfor 30 s

4 Extension - 72^0C for 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.8.4 F-type Lectin

The following primers were designed and used for the amplification

Forward Primer : 5` ATGTATTTATTAACTGTGCTTCTTT 3`

Reverse Primer : 5` CTACCTTCCCATGACTTCGACCTCGC 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - $94\ ^0$C for 30 s

3 Annealing - 60^0C for 30 s

4 Extension - 72^0C for 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.8.5 Galectin

The following primers were designed and used for the amplification

Forward Primer : 5` TCCAGGCAAGGCTATCAG 3`

Reverse Primer : 5` TAACGCAATGTCGCTGTC 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94 ^{0}C for 30 s

3 Annealing - 55^0Cfor 30 s

4 Extension - 72^0Cfor 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.8.6 Lipopolysaccharide and β-1, 3-glucan Binding Protein (LGBP)

The following primers were designed and used for the amplification

Forward Primer : 5` GACAACAACACGGACAAC 3`

Reverse Primer : 5` GGCTGACTGAATTGGATTG 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94 ^{0}C for 30 s

3 Annealing - 55^0C for 30 s

4 Extension - 72^0C for 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.8.7 Nacrein

The following primers were designed and used for the amplification

Forward Primer : 5` ATAGGAGTATATGTGAAG 3`

Reverse Primer : 5` CGTATGTATAGTAATGTC 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94 ^{0}C for 30 s

3 Annealing - 51^0C for 30 s

4 Extension - 72^0C for 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0Cfor 10 min

3.8.8 Prismalin-14

The following primers were designed and used for the amplification

Forward Primer : 5` CGATCTCTGCTAGTCCTCC 3`

Reverse Primer : 5` TAGCCGTTGTCGTCATCT 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94^0C for 30 s

3 Annealing - 56^0C for 30 s

4 Extension - 72^0C for 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.8.9 N19

The following primers were designed and used for the amplification

Forward Primer : 5` TTGTTTCTTGGCTCTTTGTC 3`

Reverse Primer : 5` CAGCAACGGTACTGGTAC 3`

The thermal profile of PCR was as follows.

1 Initial Denaturation - 94^0C for 5 min

2 Cyclic Denaturation - 94 ^{0}C for 30 s

3 Annealing - 56^0C for 30 s

4 Extension - 72^0Cfor 45 s

5 Repeating step 2-4 - for 30 cycles

6 Final extension - 72^0C for 10 min

3.9 Electrophoresis, Elution, Molecular Cloning and Sequencing of PCR Products

3.9.1 Agarose Gel Elactrophoresis

Electrophoresis with 1.5% agarose gel in 1X Tris Boricacid EDTA (TBE) buffer with ethidium bromide (µg/ml) was used to check the PCR products. Samples mixed with sample dye were loaded and the electrophoresis was carried out in a sub marine electrophoresis unit (Hoefer Inc.) with a constant voltage of 80V. DNA molecular markers (100 bp and 1kb ladder) were run parallel to the samples. The gels were documented in gel documentation system.

3.9.2 Elution of PCR Products, Molecular Cloning and Sequencing

The amplicons were gel eluted using the GenElute^{TM} Gel extraction Kit and the eluted products were ligated into the pJET vector (Fermentas, EU), and used to transform *E.coli* TOP10 cells. The positive clones were selected using antibiotic selection (Amp). The colony PCR was conducted to screen the presence of recombinant plasmid and the positive clones were used to isolate plasmids using mini prep plasmid kit (Fermentas). The isolated plasmids were sequenced at the sequencing facility of M/S Sigenome, Kochi.

3.10 Random Amplification of cDNA ends (RACE)

The technique of random amplification of cDNA ends (RACE) was used to generate the full length sequence of the genes under study. SMARTer^{TM} RACE cDNA amplification kit of M/S Clontech was used for doing RACE. The basic protocol involved the isolation of total RNA from the Haemocytes using the procedure followed for RNA isolation. The RACE-Ready cDNA of both 5` and 3` region was synthesized separately with the following constituents.

5`RACE	**3`RACE**
2.75µl RNA (1µg)	2.75µl RNA (1µg)
1.0µl 5`-CDS primer A	1.0µl 5`-CDS primer A
	1.0 µl sterile waters

The above preparations were incubated at 72^0C for 3 minutes and then cooled to 42^0C for 2 minutes. The incubation was performed in PCR thermo cycler. Denaturation of the secondary structures as well as the binding of oligo (dT) at the 3` end took place during this step.The entire mix was centrifuged for 10 seconds at

12000 rpm. One microliter of SMARTer II A oligo provided in the kit was added to 5` reaction and the binding of smarter oligo at the 5` end took place.The RACE-Ready cDNA of both 5` and 3`reaction mix were added to 5.25 µl of the following constituents.

Ingredients of RACE master mix with the following constituents were prepared fresh for both 3` and 5`RACE and kept at room temperature.

2.0µl 5X first-strand buffer

1.0µl DTT (20mM)

1.0µl dNTP Mix (10mM)

To this mix was added 0.25 µl of RNase inhibitor (40U/µl) and 1.0µl SMARTScribe reverse transcriptase, and the volume was made to 5.25µl.

The 4 µl of RACE-Ready cDNA of both 5` and 3`reaction mix were added separately with 5.25 µl constituents and making up to the volume to 10 µl. The preparation was incubated at 42^0C for 90 minutes in a thermo cycler followed by heating at 70^0C for 10 minutes. Then first strand cDNA of 5` and 3`region were synthesized.

The gene specific amplification using the prepared RACE ready cDNA was carried out with the enzyme Advantage polymerase from M/S Clontech along with gene specific primers (GSP) indicated in table (3.1). Forward and reverse primers (gene specific) were used for 3` and 5` RACE respectively. Universal primer mix provided in the RACE kit was used in both 5` and 3` reactions. The constituents of PCR mix were as follows.

34.5µl PCR grade water

5.0µl 10X Advantage 2 PCR buffer

1.0µl dNTP Mix

1.0µl 50X Advantages 2 polymerase mix

2.5µl RACE-Ready cDNA (5` or 3`)

0.2µl (10µM) of gene specific primer (GSP-forward for 3` and reverse for 5`)

5.0µl of UPM (universal primer mix)

The thermal parameters followed were

94^0C for 30sec

58^0C for 30 sec

72^0C for 3 minutes

The number of cycles were 28 followed by a final extension for 10 minutes at 72^0C. The RACE products were checked with 1.5% agarose gel electrophoresis and documented as described. The amplicons were gel eluted and cloned as described earlier and sequenced.

Table.3.1 Gene specific primers used for the RACE- PCR

RACE-Primer	Primer Sequence (5`-3`)
SOD-GSP1 (for 3')	GGTGATCCTGGAGCCTCTTGG
SOD-GSP2 (for 5')	AATCAGCATCACCGACAA
GPX-GSP1 (for 3')	CTCGTTCATAGCCCTCATTTCCC
GPX-GSP2 (for 5')	CCCGTAACTCGGAGTGATATTTC
GST-GSP1 (for 3')	GGACTCGTTCACCACTATATCG
GST-GSP2 (for 5')	GGTGTGATAACCGTCATAGC
F-lectin-GSP1 (for 3')	GGTGATCCTGGAGCCTCTTGG
F-lectin-GSP2 (for 5')	AATCAGCATCACCGACAA
Galectin-GSP1 (for 3')	AGGCACACCGCTCGTTAC
Galectin-GSP2 (for 5')	CCTGGTCAACTAGGAGACATCAAC
LGBP-GSP1 (for 3')	ATGCTATTTCCAGTTTCGCTTCC
LGBP-GSP2 (for 5')	CAAGACCCAATGACGAGTGTAC
Nacrein-GSP1(for 3')	CGATGTTGATTGGAGATTGT
Nacrein -GSP2 (for 5')	GTGCTTATAGAAACGATA
Prismalin-GSP1 (for 3')	GAAGAAACCGTTGTCATC
Prismalin-GSP2 (for 5')	GGAGGATATGGTGGGTTC
UPM-Mix	AAGCAGTGGTATCAACGCAGAGT–CTAATACGACTCACTATAGGGC

3.11 Semi-quantitative PCR

Time dependant expression of the genes under study following their induction was quantified. Semi-quantitative PCR using the cDNA synthesized from challenged/implanted and control pearl oysters at defined time points were used for this. Primers for semi-quantitative PCR were designed from the cDNA sequence of the respective genes. The GAPDH was amplified in PCR reaction as a control. The cycle numbers at half-maximal amplification were used for subsequent quantitative analysis of gene expression. The PCR cycles were optimized in such a way that the target gene and house-keeping gene amplification were in logarithmic phase.

Table.3.2 Primers used for the semiquantitative PCR

primer	sequence (5`- 3`)	cycles
SOD-F SOD-R	AATCAGCATCACCGACAA TTGGTGATACCGATCACTCCACA	30
GPX-FP GPX-RP	GGCAGGCTTGTCATTCTC GGCAGGCTTGTCATTCTC	28
GST-F GST-R	GGCAGGCTTGTCATTCTC GGCAGGCTTGTCATTCTC	28
F-type lectin-FP F-type lectin-RP	TGGATGGTATAAGTAAT TCTGTTCGTTATTCTGAT	28
Galectin –FP Galectin -FP	AGATTTCCCCTTCAGTCCTTTC TGAAGAAATTGCATTCATGGAC	30
LGBP-F LGBP-R	CACACAGCAAGCCCCTGATCC CCTCCTCCGCCAGTTTGAGATG	30
Nacrein -FP Nacrein -RP	CTTCATTGCATGTGGAATTGGA TCGGTTCTGATGATTGGTCACT	28
Prismalin-14-FP Prismalin-14-RP	TGCGATCTCTGCTAGTCCTCCTTG AGTAGCCGTTGTCGTCATCTCCTC	28
N19-FP N19-RP	TGGCAACAAAGCAGTCATAACCG GGCGTCGTTGTAGCATTGAAGG	28
GAPDH-FP GAPDH-RP	TATTTCTGCACCGTCTGCTG ATCTTGGCGAGTGGAGCTAA	25

The other conditions for semiquantitative PCR were similar to that of conventional PCR. On completion of the reaction, the PCR products were electrophoresed using 2.0% agarose gel and the band intensity was quantified using imageJ analysis software.

3.12 Software's and Data Bases used for Analysis

The following online databases and software's were used in the study for designing primers, aligning and comparing the sequences, for similarity searches, translating the sequences and to identify characteristic domains.

3.12.1 Software used for Primer Design

http://www.premierbiosoft.com/molecularbeacons

The sequences from closely related species available in the databases/ literature/generated through this study were fed into this software as input data and searched for suitable primers. The software suggested a sets of primers from which the suitable primer pairs with optimum Tm value and GC content were short listed for custom synthesis. The primer pairs thus synthesized were used for standardization of PCR and the best ones were selected for further use.

3.12.2 Software used for Sequence Data Analysis

http://www.mbio.ncsu.edu/bioedit/bioedit.html

http://www.appliedbiosystems.com/absite/us/en/home/support/software/dna sequencing/sequencing-analysis.html

http://www.dnabaser.com/

These three important free softwares with a variety of application for analyzing the DNA sequences were downloaded. These softwares were used to check the quality of sequences, for editing the sequences obtained through double pass sequencing. All the sequence data obtained were analyzed using these three

softwares and suitable alignments were generated using the options for reverse complementing and generation of 'Cap contig' for finalizing the sequences.

3.12.3 Data base for Similarity Search

http://blast.ncbi.nlm.nih.gov/Blast.cgi

This is an important data base used for homology search or similarity analysis of the sequences. The data base provided information on both nucleotide and amino acid sequences of various flora and fauna. The sequences generated from this study after necessary editing and translation were deposited in this data base using special software **sequin,** upon which suitable accession numbers were allotted by the site to view the sequences deposited.

3.12.4 Online Software for Sequence Alignment

http://www.ebi.ac.uk/clustalw/index.html

With this software ClastalW analysis to align the sequences were carried out. The parallel sequences of both strands to be aligned was fed into the online software and the alignment was obtained. Variations like substitution, additions and deletions in the gene sequences were detected using this software.

3.12.5 Software for Deducing Amino Acid Sequences

http://www.ebi.ac.uk/Tools/st/emboss_transeq/

This software was used in this study to translate the coding nucleotides into amino acids. The nucleotide sequence data were fed into the online software and the amino acid sequences in all the six frames were predicted.

3.12.6 Online Software for the Detection of Signal Peptide

http://www.cbs.dtu.dk/services/SignalP/

This online software was used to detect signal peptide if any in the coding region. The complete coding regions were uploaded for analysis to detect signal peptide sequences.

3.12.7 Online Software for Identifying the Characteristic Domains and Family Signature within the Amino Acid Sequences

http://www.ebi.ac.uk/InterProScan/

This is an important software used to identify the gene specific domains. The gene specific domains within all the genes under study were detected using this software.

3.12.8 Software for Phylogenetic Analysis (MEGA, version 5.05)

The phylogenetic trees were constructed using the neighbour-joining method in the program MEGA version 5.05. The sequences generated were uploaded along with the related sequences from other species and phylogenetic trees were constructed.

3.12.9 Software for Quantification of Relative Gene Expression in the Semi-Quantitative PCR

3.12.9.1 ImageJ

The imageJ software was used to quantify the intensity of bands from documented gels for semi quantitative estimation of gene expressions, generating values for plotting the standard curve and statistical analysis.

3.12.9.2 Statistical analysis

Statistical analysis of relative gene expressions on exposure to LPS and Mabe implantation were carried out using SPSS programme 13.0 (SPSS Inc, Chicago, USA). Analysis were carried out using the data from five individual samples (n=5), and the means of all parameters were examined for significance by analysis of variance (ANOVA) followed by Duncan multiple test.

Chapter 4
RESULTS

Result of the molecular identification, characterization and expression analysis of genes encoding antioxidant genes, pattern recognition receptor protein genes and the genes involved in the pearl formation in *Pinctada fucata* was carried out as part of the present study are presented below.

4.1 Molecular Identification and Characterization of Functional Genes

4.1.1 Antioxidant Genes

Molecular identification and characterization of three antioxidant enzyme genes viz. SOD, GPX and GST were carried out. The initial partial characterization was followed by RACE to develop the full length nucleotide sequence. The amino acid sequence of the proteins being encoded by them were deduced, and their charactacteristic features were worked out as detailed below.

4.1.1.1 Superoxide Dismutase (SOD)

Polymerase Chain Reaction (PCR) carried out to amplify the SOD gene using the primers designed from the conserved region gave positive amplicon. A single PCR product of 471 bp was visualized by agarose gel electrophoresis. This product was purified and sequenced. This partial cDNA sequence provided the necessary information to obtain an additional 368 bp sequence by 3`RACE, and 87 bp sequence by 5`RACE. Finally, the full-length sequence information of the SOD cDNA was obtained by overlapping the three cDNA sequences. The nucleotide sequence and the deduced amino acid sequence are shown in Fig.4.1. The full-length SOD cDNA is comprised of 924 bp,

containing 87 bp in the 5`-terminal untranslated region (UTR), 471 bp in the ORF, 366 bp in 3`-terminal UTR with a poly(A) tail of 30 bp and a putative polyadenylation consensus signal (AATAAA). Deduction of amino acid sequence from the complete coding region using online software Transeq of European Molecular Biology Laboratory (EMBL) revealed that 156 amino acids are encoded by the ORF. Analysis of the amino acid sequence using InterProscan online software to find out specific domains revealed that it is a Cu/Zn SOD with copper/zinc binding domains within the SOD sequence. The SOD cDNA sequence and its deduced amino acid sequence were submitted to the NCBIGenBank under accession no (JX013537). No signal peptide was identified in the deduced amino acid sequence of Cu/Zn SOD by the signal P program, indicated that this SOD was cytoplasmic Cu/Zn SOD.

In order to determine the variation and similarity, BLAST searches were conducted in NCBI. The BLAST search showed that the deduced amino acid sequence of SOD has extremely high identity with the Cu/Zn SOD of *Crassostea hongkongensis, C. gigas* and *Mytilus chilensis* (99%). Similarly, it was found to have high identity with Cu/Zn SOD of *Crassostrea ariakensis* (98%) and *Haliotis discus discus* (97%).

Multiple alignment of the deduced amino acid sequences (Fig.4.2) with other closely related cytoplasmic Cu/Zn SOD sequences showed the presence of three cysteines (Cys 9, Cys 60 and Cys 149) in the mature SOD. The Cys 60 and Cys 149 were conserved in all Cu/Zn SODs and it was believed that they form an intramolecular disulfide bond. The other amino acids which were required for binding of copper (His-49, -51, -66, and -123) and zinc (His-66, -74 and -83 and Asp-86) were also conserved. Two Cu/Zn SOD family signature sequences were found in the deduced amino acid sequence of SOD; signature 1 (consensus sequences: [GA]-[IMFAT]-H-[LIVF]-H-{S}-

x-[GP]-[SDG]-x-[STAGDE]. and signature 2 (consensus sequences: G-[GNHD]-[SGA]-[GR]-x-R-x-[SGAWRV]-C-x(2)-[IV]).

Phylogenetic relationships of Cu/Zn SOD from pearl oyster and other invertebrates and vertebrates were estimated. Cu/Zn SOD of *Candida albicans* was used as the out-group. As shown in Fig.4.3, Cu/Zn SOD of *P. fucata* formed a single cluster with the Cu/Zn SODs from oyster *C. gigas* and mussel *M. edulis* indicated closer evolutionary relationship than that with other aquatic invertebrates. Vertebrates were evolutionarily distinctly separated.

Fig.4.1 Nucleotide sequence of SOD cDNA from *P. fucata* and its deduced amino acid sequence. Two Cu/Zn SOD family signatures are underlined (_). The start codon is in bold and termination codon is indicated with asterisk (*). The amino acids required for binding of copper (His-49 -51, -66, and -123) and zinc (His-66, -74, and -83 and Asp-86) are shaded. Polyadenylation consensus signal sequences are shaded black with white lettering. Two cysteines (Cys60 and Cys149) predicted to be engaged in the disulfide bond formation were boxed.

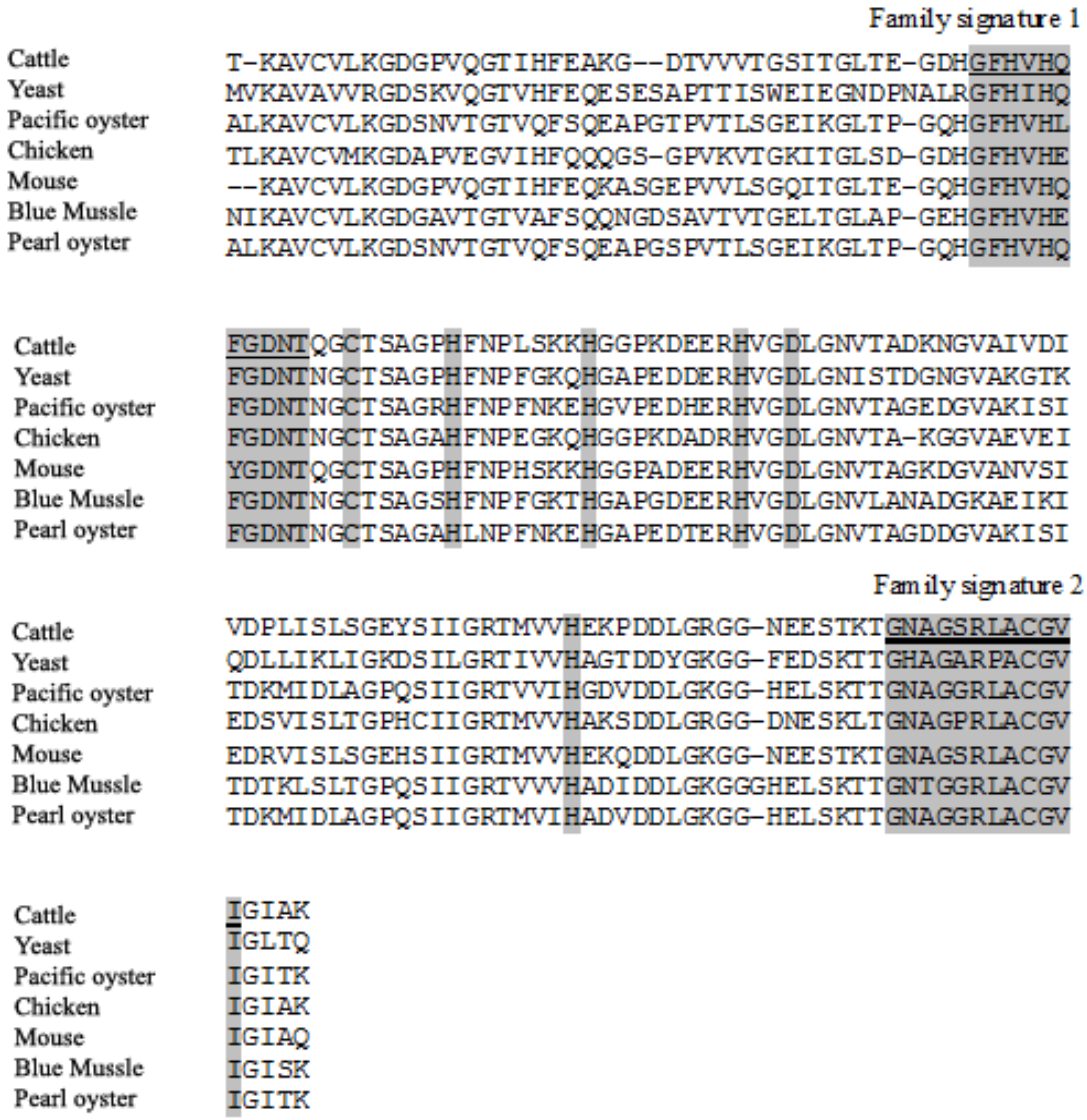

Fig.4.2 Alignment of the deduced amino acid sequence of SOD of different species. The characteristic conserved regions are shaded.

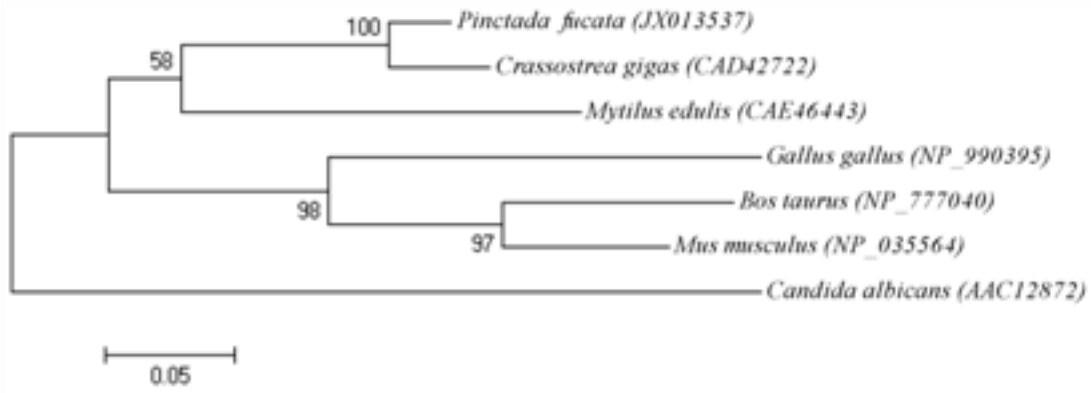

Fig. 4.3 Phylogentic tree using SOD amino acid sequences from pearl oyster *P. fucata* and other species. Note: Numbers represent the bootstrap values. GenBank accession numbers are shown next to each species.

4.1.1.2 Glutathione Peroxidase (GPX)

PCR amplification of the GPX gene segment using the cDNA and primer designed from the conserved regions successfully produced amplicon (<450 bp) as visualized through agarose gel electerophoresis. Optimization of PCR parameters helped to get specific amplicon free from nonspecific PCR product. The PCR product was sequenced for identification and characterization of the gene. Sequencing of the purified amplicon in both directions gave the sequence information of 436 bp length. The sequences were aligned by using Bio Edit software. The nucleotide sequence obtained were subjected to similarity search in NCBI using BLAST and confirmed to be GPX gene fragment.

RACE-PCR using the above 436 bp product revealed the sequence of a 1201 bp long segment of the GPX gene which was used for translation to amino acids in Transq (EMBL). It consisted of the ORF region and part of UTR (Fig.4.4). The ORF encodes a polypeptide of 198 amino acids. No signal peptide was present in the GPX sequence. The active site of the enzyme contains a selenocysteine encoded by a TGA, which is the main characteristic feature of the selanoprotein family. Se-GPX

contains a characteristic GPX signature sequence motif 2, consisting of 8 amino acids at ^{75}LGFPCNQF^{82}. Also, it showed an extra active site motif located at ^{163}WNFEKF^{168} and glutamine-Gln (Q_{81},Q_{85}), tryptophan-Trp (W_{153},W_{163}) residues, which was responsible for fixation of selenium. Additionally, Se-GPX showed two arginine residues at R_{101} and R_{199} for directing donor glutathione substrate towards the catalytic center. A potential N-glycosylation site $_{87}$NCTN$_{90}$ was identified using the PROSITE program, which has been observed in other GPX amino acid sequences.

ClustalW pairwise amino acid sequence alignment was performed to determine the identity percentage (%) of Se-GPX with other species Se-GPXs. BLAST analysis showed that the amino acid sequence of Se-GPX deduced in the present study has extremely high identity with that of *Pinctada fucata* reported from China. Multiple alignment of Se-GPX sequence of *Pinctada fucata* with that of other species are shown in Fig.4.5. The results showed that characteristic selenocysteine residue, ^{75}LGFPCNQF^{82} signature motif2, ^{163}WNFEKF^{168} active site, functional residues of Glutamine-Gln (Q_{81},Q_{85}), tryptophan-Trp (W_{153},W_{163}) residues were aligned across all the selected species. The Se-GPx sequence resolved in this study revealed the main characteristic motifs and functional amino acid residues of the Se-GPx protein family. As there is a general lack of sequence information on these genes from other bivalves, further phylogenetic analysis were not possible.

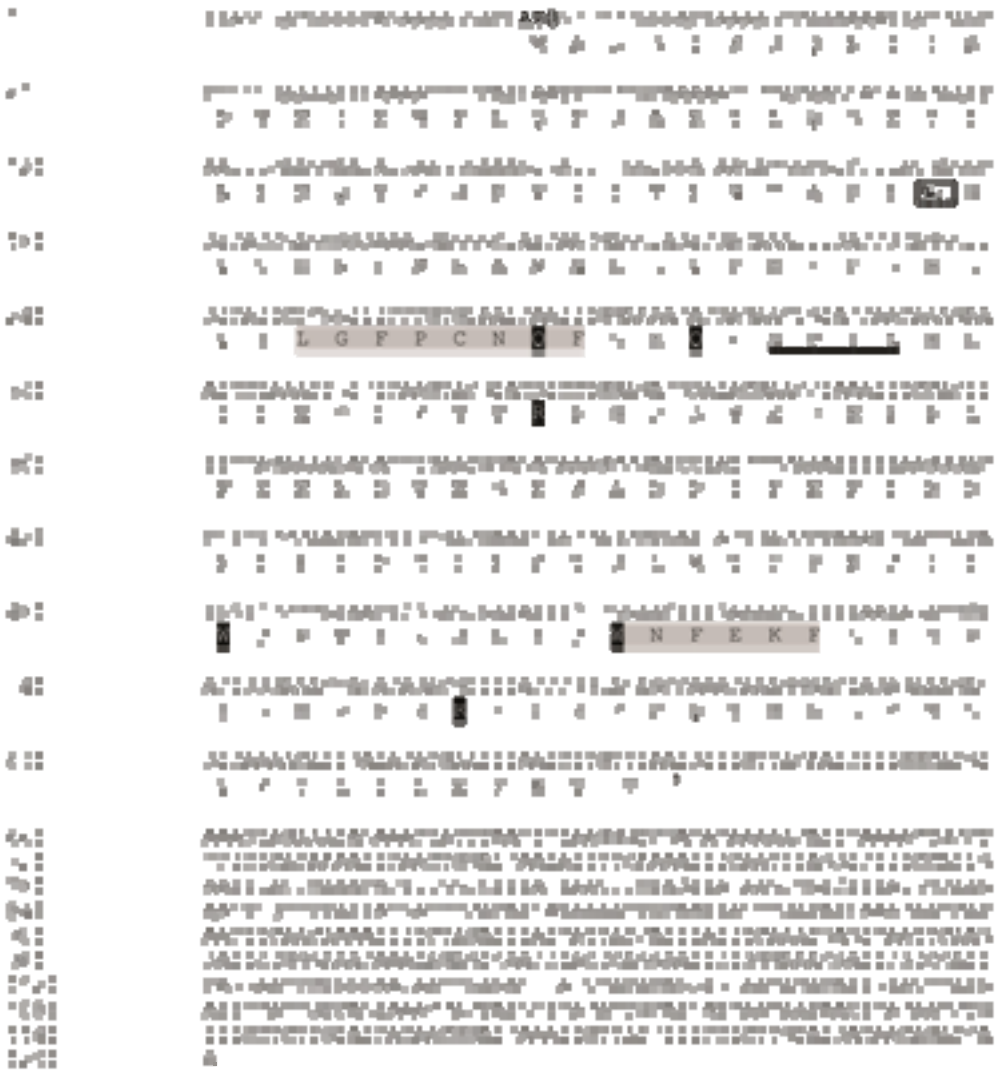

Fig.4.4 Nucleotide sequence of GPX cDNA from *P. fucata* and its deduced amino acid sequence. GPX signature sequence motif 2, consisting of 8 aminoacids at ^{75}LGFPCNQF^{82}and activesite motif located at ^{163}WNFEKF^{168}are shaded gray with black lettering. Glutamine-Gln(Q_{81},Q_{85}), tryptophan-Trp (W_{153},W_{163}) residues, which are responsible for fixation of selenium are shaded black with white lettering. Arginine residues, R_{101} and R_{199} for directing donor glutathione substrate towards the catalytic center. Selenocysteine encoded by a TGA were boxed.The start codon is in bold and termination codon is indicated with asterisk (*).

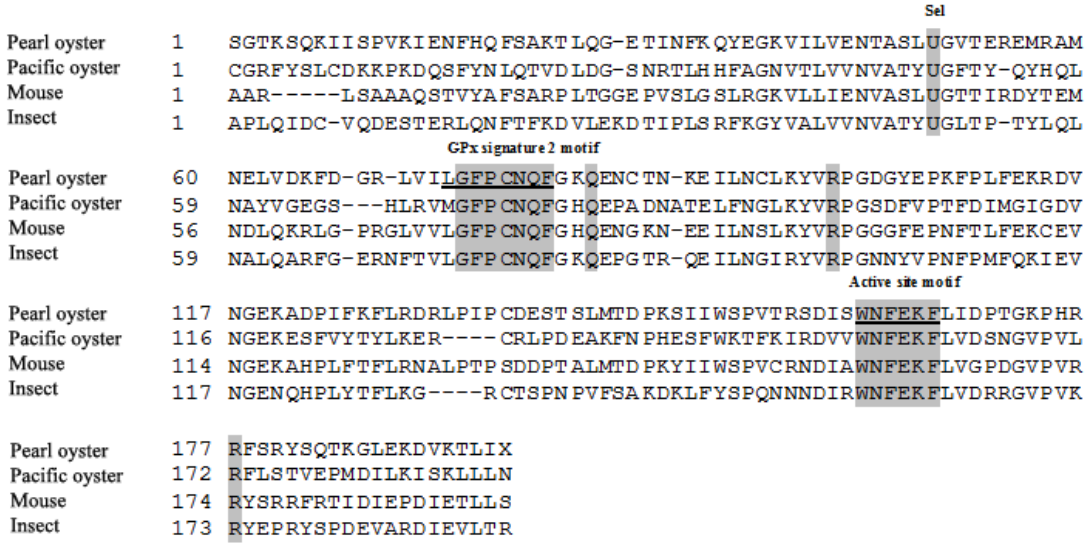

Fig.4.5 Alignment of the deduced amino acid sequence of GPX of different species. The characteristic conserved regions are shaded.

4.1.1.3 Glutathione-s-transferase (GST)

In case of GST, the initial PCR of the cDNA using GST specific primers resulted in the amplification of a partial segment of 596 bp which was sequenced. RACE-PCR carried out revealed the complete ORF region and the UTR comprising of 1652 bp (Fig.4.6). Deduced amino acid sequence encoded by it on analysis with signalP program revealed no signal peptides. NCBI BLAST analysis revealed that the gene sequence identified in this study belong to omega GST. Conserved domain search at NCBI revealed that the omega GST has spatially distinct domain: a small thioredoxin-like N-terminal domain and a larger helices rich C-terminal domain. The N and C-terminal domains in the position between 18-96 and 101-237 were identified by Prosite software. The GST active site was composed of a specific glutathione binding site (G-site) common to all GSTs, and a nonspecific substrate binding site (H-site) which varied between different classes and isotypes. Residues from the N-terminal thioredoxin domain form the G-site while the H-site was

comprised mainly of residues from the C-terminal alpha helical domain. The residues of G-site (Cys-28, Pro-29, Phen-30, Ala-31, Leu-52, Lys-55, Leu-67, Val-68, Glu-80 and Ser-81) were conserved. The composition of cysteine-containing tetramer (C-P-F-A) is marked on figure 4.6. The H-site (Gly-116, Thr-119, Gly-120, Tyr-123, Glu-124, Trp-174, Arg-177 and His-221) were also identified.

BLAST analysis showed that the deduced amino acid sequence of omega GST has extremely high identity with the omega GST of *Pinctada fucata* reported from China. Multiple alignment of omega GST sequence with other species (Fig.4.7) showed that the characteristic cysteine-containing tetramer (C-P-F-A) and G-site (Cys-28, Pro-29, Phen-30, Ala-31, Leu-52, Lys-55, Leu-67, Val-68, Glu-80 and Ser-81) were aligned in all the species used for alignment.

Phylogenetic analysis using omega GST from pearl oyster, other invertebrates and vertebrates was carried out with cyanobacteria as the out-group (fig.4.7). The GSTs of *P. fucata* and abalone *H. discus discus* only formed a single cluster while other species did not form part of it.

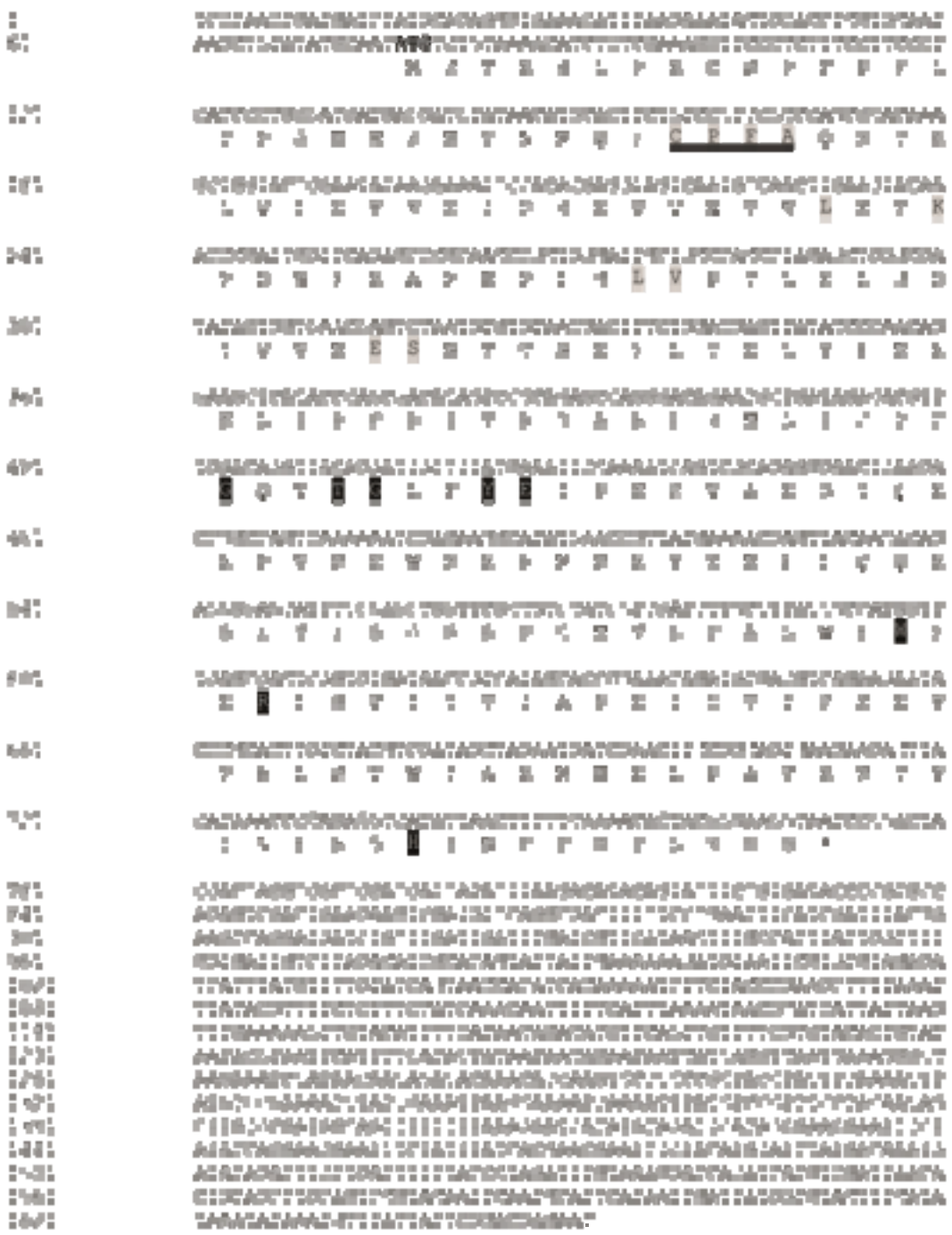

Fig.4.6 Nucleotide sequence of GST cDNA from *P. fucata* and its deduced amino acid sequence. Cysteine-containing tetramer (C-P-F-A) is underlined.The amino acids required for G-site (Cys-28, Pro-29, Phen-30, Ala-31, Leu-52, Lys-55, Leu-67, Val-68, Glu-80 and Ser-81) are shaded gray with white lettering. The residues for H-site (Gly-116, Thr-119, Gly-120, Tyr-123, Glu-124, Trp-174, Arg-177 and His-221) are shaded black with white lettering. The start codon is in bold and termination codon is indicated with asterisk (*).

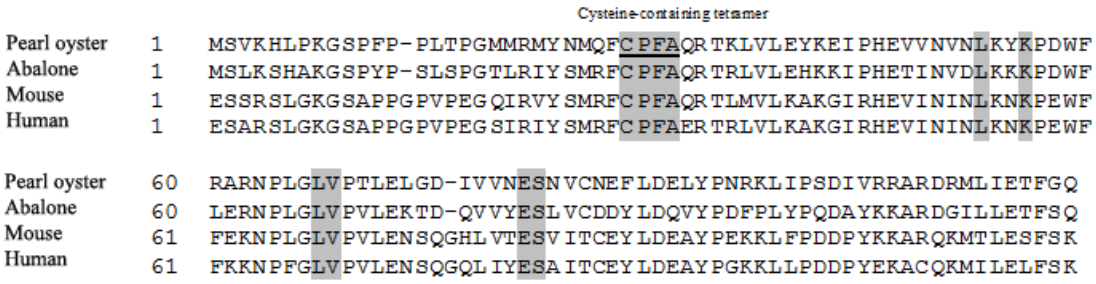

Fig.4.7 Alignment of the deduced amino acid sequence of GST of different species. The characteristic conserved regions are shaded.

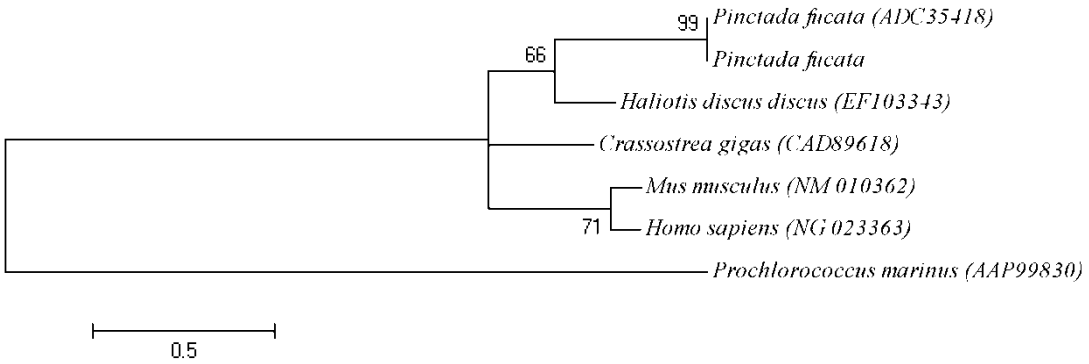

Fig.4.8 Phylogentic analysis using GST amino acid sequences from pearl oyster *P. fucata* and other species. Note: Numbers represent the bootstrap values. GenBank accession numbers are shown next to each species.

4.1.2 Pattern Recognition Receptor Protein Genes

The three pattern recognition receptor protein genes namely F-type lectin, galectin and LGBP were taken up for molecular identification and characterization. Full length chacterization with sequence information of complete coding region, deduced amino acid sequence, identification of binding sites of putative proteins were worked out for F-type lectin. In case of galectin and LGBP genes, characterization of partial segment was only achieved. Detailed account of the results is presented below:

4.1.2.1 F-type lectin

Polymerase Chain Reaction (PCR) carried out to amplify the F-type lectin gene using the primers designed from the conserved region gave positive amplicon. A single PCR product of the expected size (<600 bp) was obtained. Purification followed by sequencing of this product revealed the nucleotide sequence of 588 bp length. This partial sequence provided the necessary information to obtain an additional 257 bp sequence by 3`RACE, and 46 bp sequence by 5`RACE. Finally, the full-length sequence information of the F-type lectin cDNA was obtained by overlapping these three sequences (588+257+46). The nucleotide sequence is shown in Fig.4.9. The full-length F-type lectin cDNA is of 891 bp, consisting of a 46 bp 5`-terminal untranslated region (UTR), 588 bp open reading frame (ORF), 257 bp 3`-terminal UTR with a poly(A) tail of 21 bp, and a putative polyadenylation consensus signal (AATAAA). The ORF encodes a polypeptide of 196 amino acids (Fig.4.9). A signal peptide was identified at the N-terminus of the deduced polypeptide by signal P program and its cleavage site was located between the positions of Gly^{19} and Tyr^{20}. The cDNA sequence of F-type lectin and its deduced amino acid sequence were submitted to the NCBI GenBank under accession no. JX103557.

In order to determine the variation and similarity, BLAST searches were conducted in NCBI. The BLAST analysis showed that the deduced amino acid sequence of F-type lectin has 100% identity with F-type lectin of *Pinctada martensii,* whereas, it has 71% identity with *Xenopus laevis*, *Morone saxatilis*, and *Anolis carolinensis*. F-type lectin of *P. fucata* was found to possess only one domain, which is similar to the F-lectin of *Pinctada martensii.*

Multiple alignment of deduced amino acid sequences (Fig.4.10) with other closely related F-type lecin sequences showed that seven cysteines (Cys 12, Cys 92, Cys 124, Cys 125, Cys 147, Cys 164 and Cys 188) are present in the mature F-type lectin and six of them (Cys 92, Cys 124, Cys 125, Cys 147, Cys 164 and Cys 188) were conserved. Conserved domain search at NCBI revealed that the F-type lectin domain extends from Lys^{55} to Val^{192}. The first carbohydrate binding residue was serine.

Phylogenetic relationships of F-type lectin from pearl oysters and other invertebrates and vertebrates were estimated. F-lectin of *Drosophila melanogaster* was used as the out-group. As shown in Fig.4.11, F-type lectin of congenic species of pearl oysters *Pinctada fucata* (present study) and *Pinctada martensii* formed one distinct cluster, supported by high brootstrap values (NJ-100%) indicating their close genetic relationship, whereas, the F-lectins from *Xenopus laevis*, *Morone saxatilis* and *Anguilla japonica* formed another distinct clade. Interestingly, the F-lectin from the edible oyster *Crassostrea gigas* formed a third cluster along with other vertebrate species *Lateolabrax japonicas*, and *Anolis carolinensis* though with low broodstrap values.

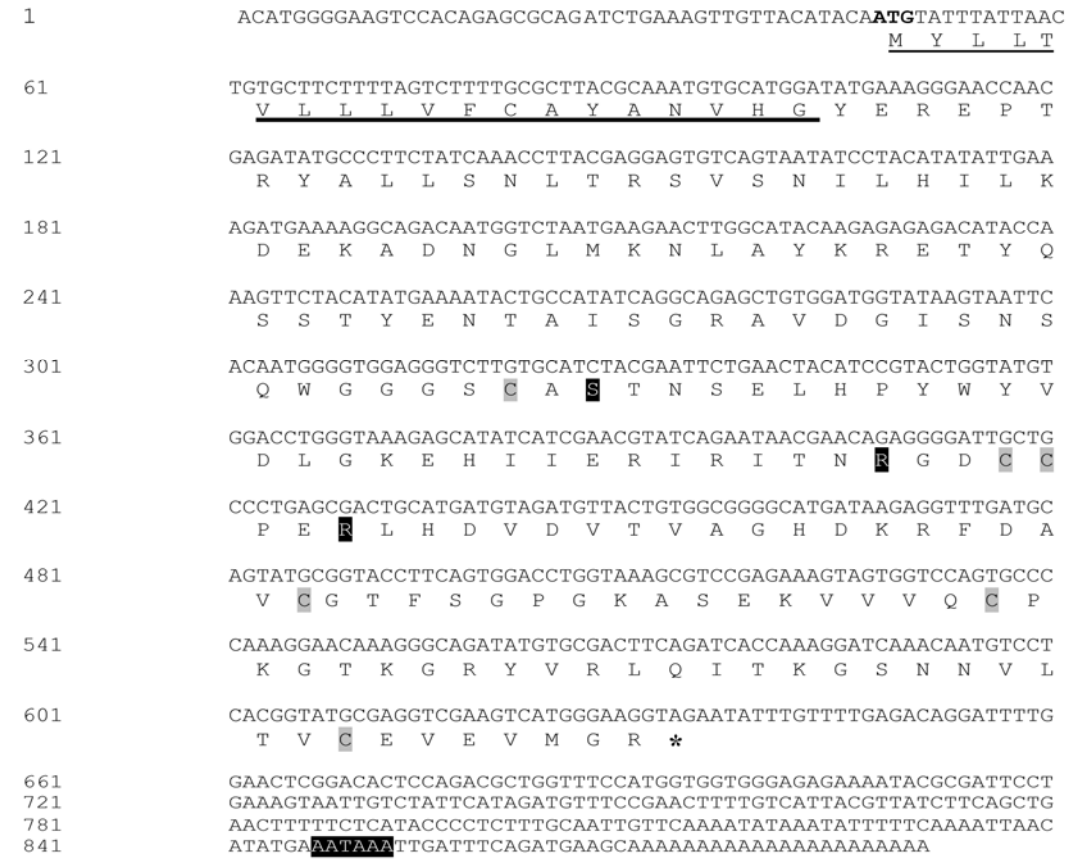

Fig.4.9 Nucleotide sequence and deduced amino acid sequences of F-type lectin of *Pinctada fucata*. Nucleotide numbers are shown on the left. The predicted signal peptide sequence is underlined. Six conserved cysteine residues are highlighted in grey, and three residues for sugar binding are highlighted in black. Polyadenylation consensus signal sequences are shaded. The start codon is in bold and the termination codon is indicated with asterisk (*).

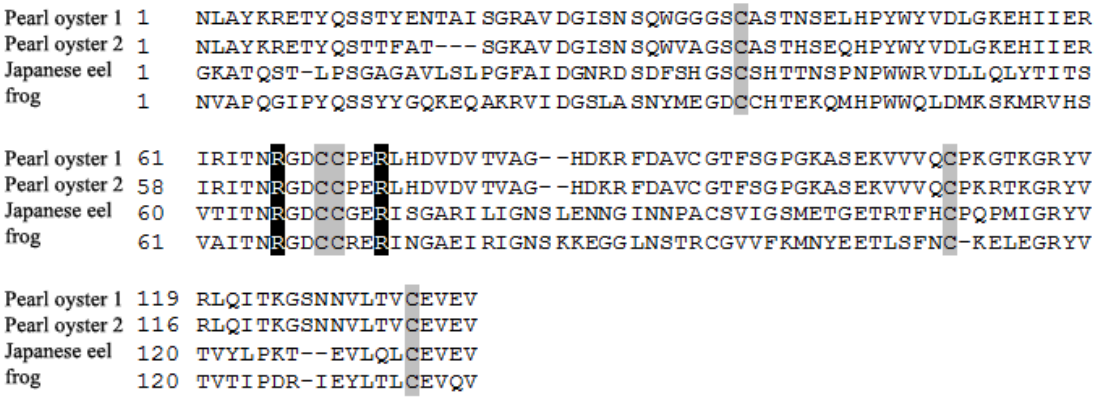

Fig.4.10 Alignment of the deduced amino acid sequence of F-type lectin of different species. The characteristic conserved regions are shaded.

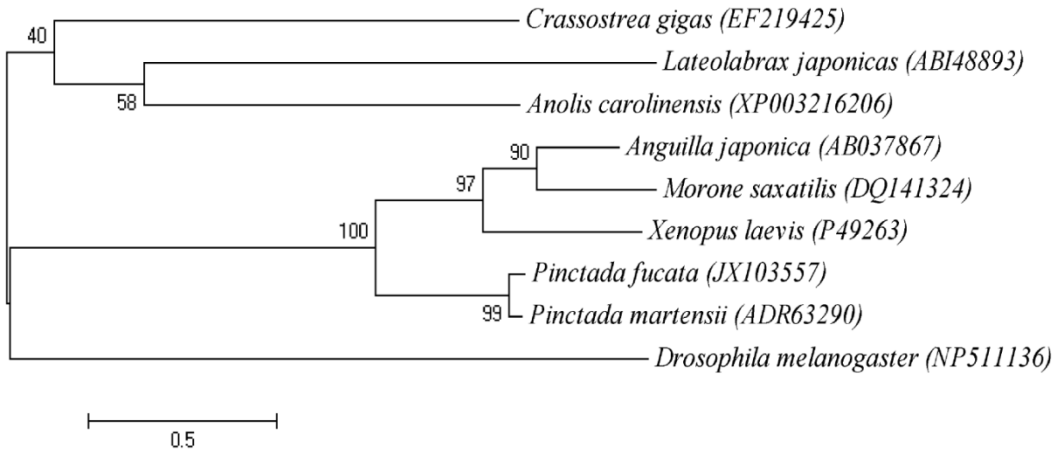

Fig.4.11 Phylogenetic analysis using amino acid sequence of F-type lectin from pearl oyster *Pinctada fucata* and other species. Note: Numbers represent the bootstrap values. GenBank accession numbers are shown next to each species.

4.1.2.2 Galectin

Amplification of the galectin gene segment of *Pinctada fucata* through PCR using cDNA of the target gene and primer designed from the conserved regions successfully produced amplicon (<950 bp) as visualized through agarose gel electerophoresis. Optimization of PCR parameters helped to get specific amplicons free from nonspecific PCR product. For the identification and characterization of gene, the PCR products were sequenced. Sequencing of the purified amplicon in both directions gave the sequence information of 925 bp length. The sequence were aligned by using Bio Edit software. The nucleotide sequence obtained were subjected to similarity search in NCBI-BLAST and confirmed to be the galectin gene fragment.

RACE-PCR using the primers designed from the above partial sequence information revealed sequence of 1750 bp portion of the galectin gene, comprising of the ORF region and part of UTR. The ORF sequence was used for prediction of the

amino acids being coded, using Transq (EMBL). The ORF encodes a polypeptide of 551 amino acids. The nucleiotide sequences of galectin and the amino acid sequences encoded by them are presented in Fig.4.12. No signal peptide was present in the galectin sequence. Conserved domain search at NCBI revealed that it possess four galectin specific domains, each of which are indicated in Fig.4.12. The alignment of galectin sequence of *P. fucata* with that of various vertebrate and invertebrate species (Fig.4.13) revealed that 7 amino acid residues were observed to be conserved in all the four galectin specific domains viz. His44, Asn46, Arg48, Asn61, Trp68, Glu71, and Arg73; the numbers corresponding to the bovine galectin-1 and each of these four galectin domains are connected by a unique 11 amino acid residues, underlined in Fig.4.12.

BLAST analysis showed that the deduced amino acid sequence of galectin deduced in the present study has high identity with the galectin of *Pinctada fucata* reported from China. Multiple alignment of amino acid sequences of galectin of *P. fucata* with other selected species are presented in Fig.4.13. The results indicated that the seven characteristic residues in all the four galectin domains were conserved across these species.

Phylogenetic analysis of galectin (Fig.4.14) of congenic species of pearl oysters *Pinctada fucata* (present study) and *Pinctada fucata* (AC036044) formed one distinct cluster, supported by high brootstrap values (NJ-100%) indicated their close genetic relationship, whereas, the galectin from *Crassostrea virginica*, *Agropecten irradians* and *Xenopus laevis* formed distinct clades. *Drosophila melanogaster* was used as the out-group.

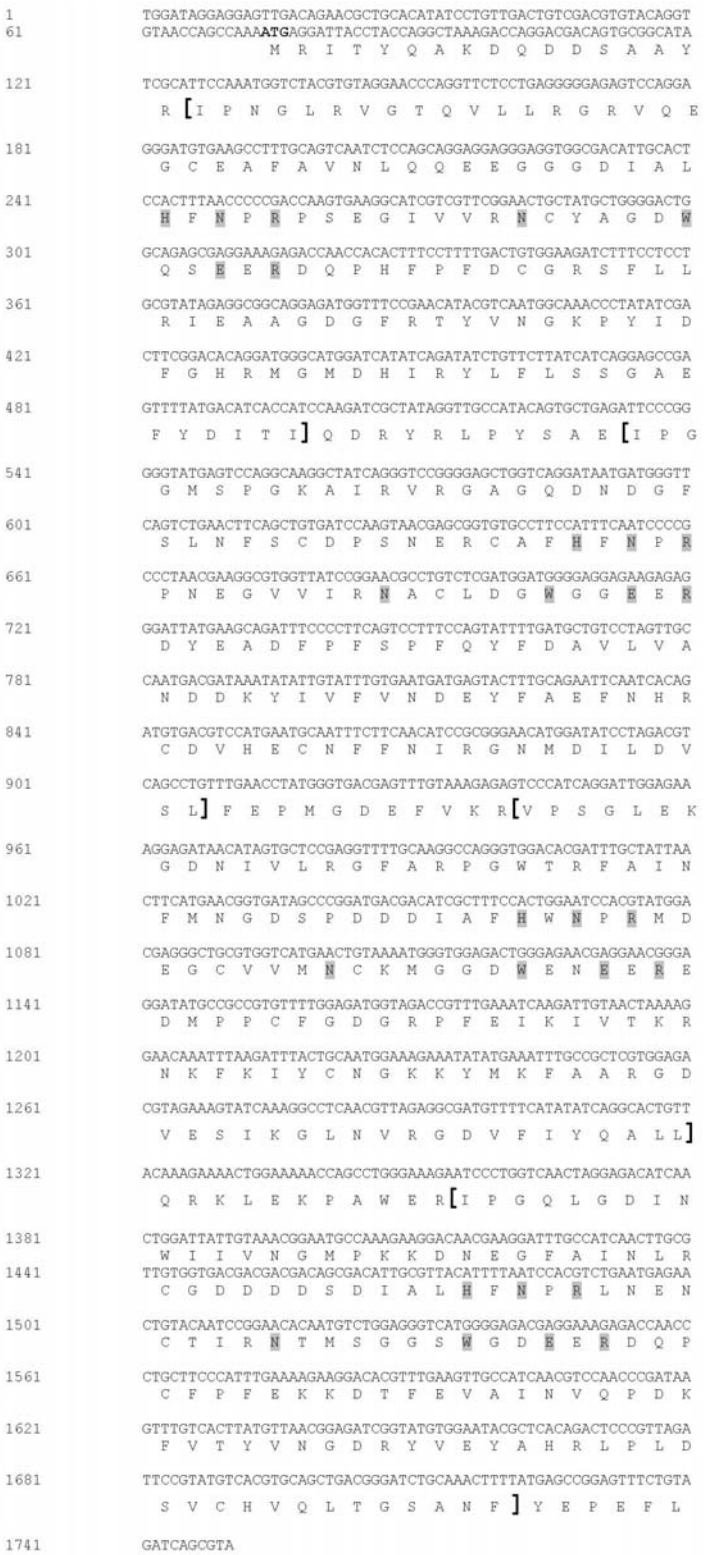

Fig.4.12 Nucleotide and deduced amino acid sequences of galectin of *Pinctada fucata*. Nucleotide numbers are shown on the left. Seven conserved residues in four galectin domaine are highlighted in grey. Four galectin domaines are in brackets. The start codon is in bold and the termination codon is indicated with asterisk (*).

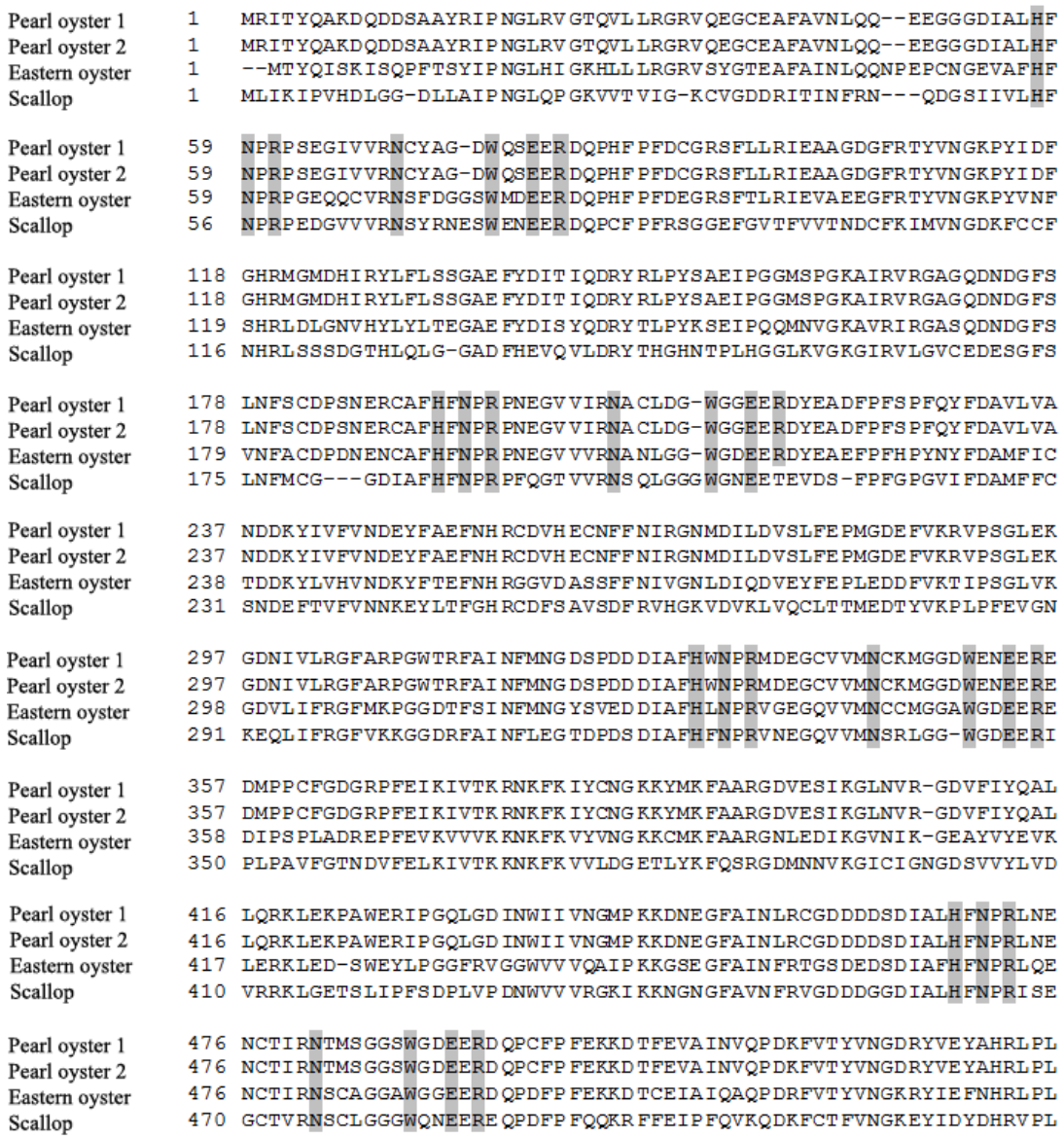

Fig.4.13 Alignment of the deduced amino acid sequence of galectin of different species. The characteristic conserved residues within four domaines are shaded.

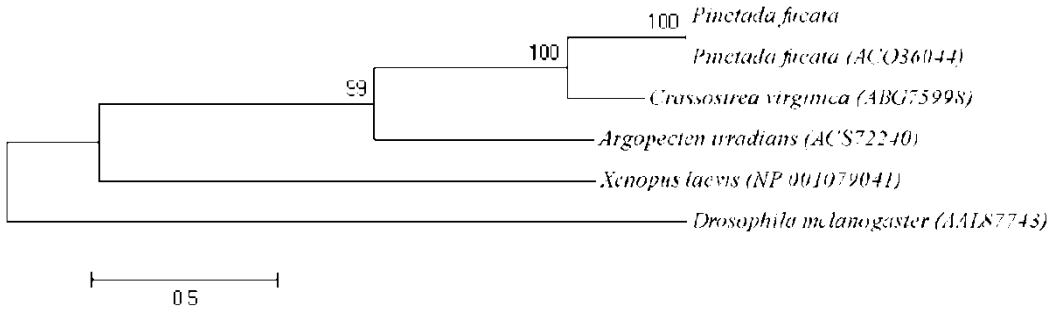

Fig.4.14 Phylogenetic analysis using amino acid sequences of Galectin from pearl oyster *Pinctada fucata* and other species. Note: Numbers represent the bootstrap values. GenBank accession numbers are shown next to each species.

4.1.2.3 Lipopolysaccharide and β-1, 3-Glucan Binding Protein (LGBP)

In case of LGBP, the initial PCR of the cDNA using LGBP specific primers resulted in the amplification of a partial segment of 628 bp, which was sequenced. This partial sequence was used to design new primers and carry out 3` and 5` RACE. RACE-PCR product of LGBP on cloning and sequencing revealed additional sequence data of 1112 bp making up the total 1740 bp. This is a partial segment of the ORF, and it encodes 563 amino acids of the LGBP. The partial coding sequences, the amino acid sequences being encoded by it and its characteristics are presented in Fig.4.15. Glycoside hydrolase family 16 were identified in the sequence (277-480). Furthermore, *P. fucata* LGBP contained a β-1,3-glucanase site with active residues of W (Trp), E (Glu), I (Ile) and D (Asp) at positions of 365, 370, 371 and 372, respectively. Threonine-rich region (T-96, T-132) and a glycine-rich region (G-141, G-199) were also found. The LGBP sequence contained a protein kinase C phosphorylation site ($_{321}$SAK$_{323}$) and a modified cell adhesive site ($_{345}$KGD$_{347}$). Moreover, a polysaccharide binding motif (340-357), a LPS-binding site (380-396) and a β-1,3-linkage recognition motif of polysaccharides (428-446) were also found

in the LGBP sequence. Two potential "N-linked glycosylation" sites consisting of 3 amino acids, each were identified at positions N-255, R-256, S257 and N-441, I-442, S-443.

BLAST analysis showed that the deduced amino acid sequence of LGBP has high identity with the LGBP of *Pinctada fucata* reported from China. Multiple alignment of LGBP sequence with other species (Fig.4.16) showed that the potential N-linked glycosylation sites, protein kinase C phosphorylation site ($_{321}$SAK$_{323}$), modified cell adhesive site ($_{345}$KGD$_{347}$), LPS-binding site, polysaccharide binding motif and β-1,3-linkage recognition motif of polysaccharides are aligned in all the species used for alignment.

Phylogenetic tree using LGBP sequence of the pearl oyster and other species was constructed (Fig.4.17), in which the congenic species of pearl oysters *Pinctada fucata* (present study) and *Pinctada fucata* (ACN76701) formed one distinct cluster, supported by high brootstrap values (NJ-100%) indicated their close genetic relationship, whereas LGBP from other species formed separate clades. *Thermotoga neapolitana* was used as the out-group.

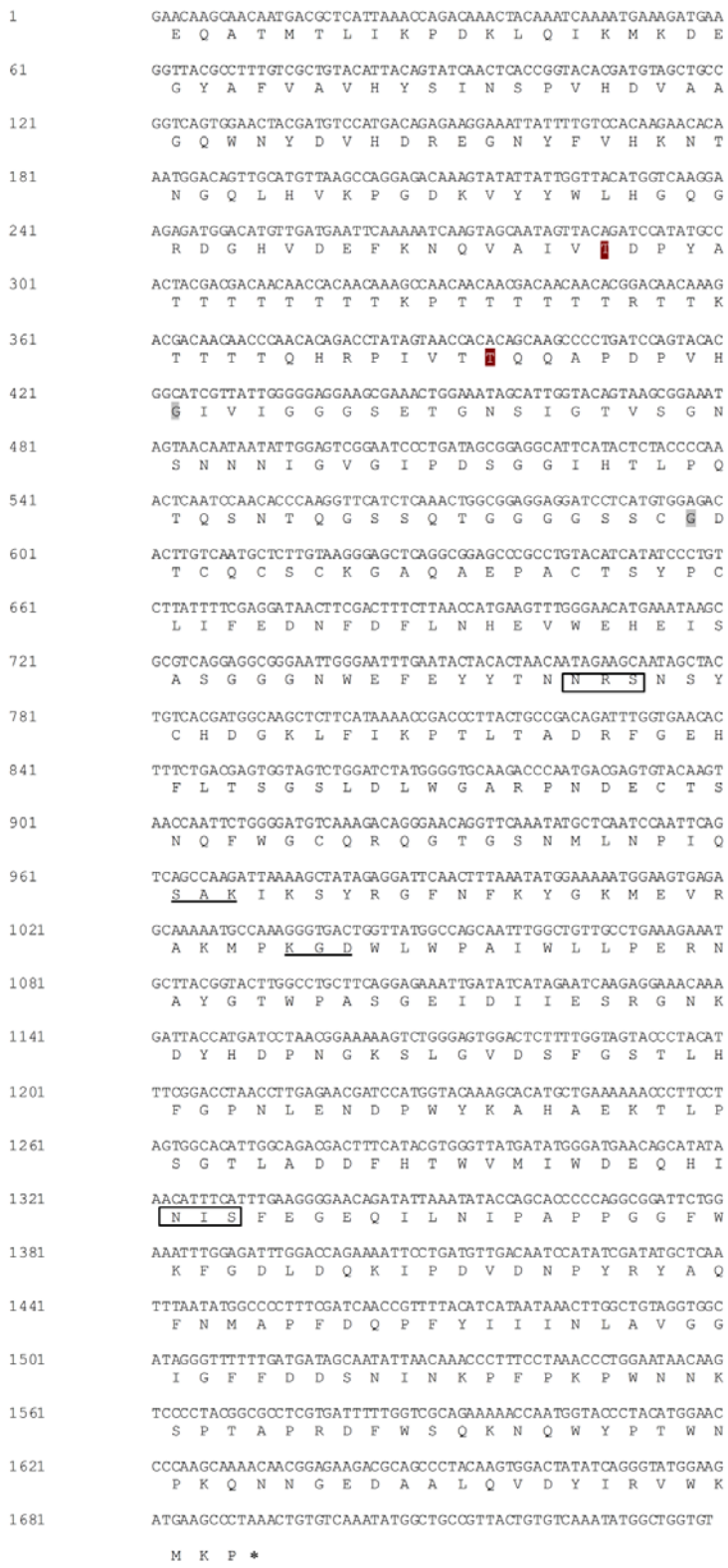

Fig4.15 Nucleotide and deduced amino acid sequences of LGBP of *Pinctada fucata*. Nucleotide numbers are shown on the left. Threonine-rich region (T-96, T-132) and a glycine-rich region (G-141, G-199) are shaded black with white lettering and grey with black lettering. Two potential N-linked glycosylation sites are boxed. The amino acid residues of protein kinase C phosphorylation site ($_{321}$SAK$_{323}$) and a modified cell adhesive site ($_{345}$KGD$_{347}$) are underlined. The termination codon is indicated with asterisk (*).

```
Pearl oyster 1    1   ACTSYPCLIFEDNFDFLNHEVWEHEISASGGGNWEFEYYTNNRSNSYCHDGKLFIKPTLT
Pearl oyster 2    1   ACTSYPCLIFEDNFDFLNHEVWEHEISASGGGNWEFEYYTNNRSNSYCHDGKLFIKPTLT
Pacific oyster    1   SCTSYPCLIFEDNFDFLNFETWTHDLTASGGGNWEFEYYTNNRTNSYTKDGKLFIKPTLT
Abalone           1   ECTEYPCLMFHDNFDTLDFKVWEHELTAGGGGNWEFQFYTNNRTNSYVRDGVLYIKPTLT

Pearl oyster 1   61   ADRFGEHFLTSGSLDLWGARPNDECTSNQFWGCQRQGTGSNMLNPIQSAKIKSYRGFNFK
Pearl oyster 2   61   ADRFGEHFLTSGSLDLWGARPNDECTSNQFWGCQRQGTGSNMLNPIQSAKIKSYRGFNFK
Pacific oyster   61   ADNYGEHFLSSGTLDLWGGEPNSLCTSNQFWGCSRQGSPEHIVNPIQSARLRSDKAFNFK
Abalone          61   ADQFGEAFLTSGKLELWGGGPHDTCTGNAFYGCERTGSNANIINPIQSARLRSSRGLNFK
                              PsBM                          GM
Pearl oyster 1  121   YGKMEVRAKMPKGDWLWPAIWLLPERNAYGTWPASGEIDIIESRGNKDYHDPNGKSLGVD
Pearl oyster 2  121   YGKMEVRAKMPKGDWLWPAIWLLPERNAYGTWPASGEIDIIESRGNKDYHDPNGKSLGVD
Pacific oyster  121   YGKMEVRAKMPKGDWIWPAIWLLPHRNAYGGWPASGEIDVVESRGNTDYHDENGRSQGVD
Abalone         121   YGKVEISAQLPMGDWLWPAIWMLPTYTECGRWPASGEIDIMESRGNRHYYDANGRSVGVD
                                                          βGRM
Pearl oyster 1  181   SFGSTLHFGPNLENDPWYKAHAEKTLPSGTLADDFHTWVMIWDEQHINISFEGEQILNIP
Pearl oyster 2  181   SFGSTLHFGPNLENDPWYKAHAEKTLPSGTLADDFHTWVMIWDEQHINISFEGEQILNIP
Pacific oyster  181   SFGSTLHFGPVYGYDPYEKAHGEMTIPSGTLNDDFHIWTLEWDEEHIKVSFEGQEVMNVS
Abalone         181   AYGSTLHFGPDYFNNGWSRAHQSWVKETGTYGDEFHTYGVEWDEDAITFSFDGRQTLRVT

Pearl oyster 1  241   APPGGFWKFGDLDQKIPDVDNPYRYAQFNMAPFDQPFYIIINLAVGGIGFFDDSNINKPF
Pearl oyster 2  241   APPGGFWKFGDLDQKIPDVDNPYRYAQFNMAPFDQPFYIIINLAVGGIGFFDDSNINKPF
Pacific oyster  241   PPPEGFWKLGELDKTN--INNPYKYTNNKMAPFDQEFFIILNVAVGGVGFFPDKFRNSPY
Abalone         241   PGETGFWGFGEFNKTG--YENPWKDAP-KIAPFDKEFYIILNVAVGGFNYFDDSYNNTAY

Pearl oyster 1  301   PKPWNNKSPTAPRDFWSQKNQWYPTWNPKQNNGEDAALQVDYIRVWKMKP
Pearl oyster 2  301   PKPWNNKSPTAPRDFWSQKNQWYPTWNPKQNNGEDAALQVDYIRVWKMKP
Pacific oyster  299   PKPWNDKSEFTARDFWNHKSQWYPTWNPDQNDGEQAAMQVDYIRVWKMKP
Abalone         298   PKPWTDAT---ASQFWAAKDQWYPTWNPDVRGGEDAALKIDSVKVWKLK-
```

Fig.4.16 Alignment of the deduced amino acid sequence of LGBP of different species. The characteristic conserved residues such as polysaccharide binding motif (PsBM), glucanase motif (GM) and β-1,3-linkage recognition motif (βGRM) are shaded.

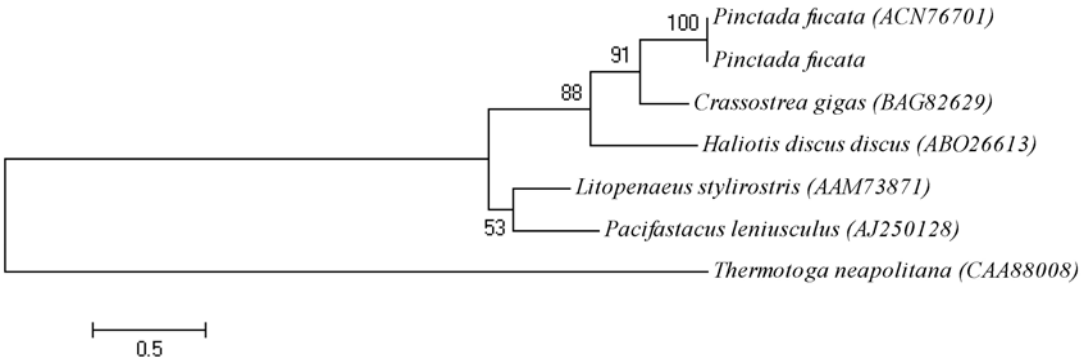

Fig.4.17 phylogenetic analysis using amino acid sequence of LGBP from pearl oyster *Pinctada fucata* and other species. Note: Numbers represent the bootstrap values. GenBank accession numbers are shown next to each species.

4.1.3 Pearl Forming Genes

The pearl formation genes of *P. fucata* taken up for molecular identification and characterization in this study were nacrein, prismalin-14 and N-19. PCR amplification of a segment of each of these gene was initially carried out using the cDNA synthesized from the total RNA. These gene segments were sequenced and the sequence data were used for RACE to develop the nucleotide sequence on its 3' and 5' regions. The amino acid sequence of the proteins being encoded by these gene segments were deduced and their charactacteristic features worked out.

4.1.3.1 Nacrein

PCR of the nacrein gene of *Pinctada fucata* using the cDNA and primers designed from the conserved region successfully amplified a segment of the gene (<1000 bp) as visualized through agarose gel electerophoresis. Optimization of the PCR parameters helped to get specific amplicon free from nonspecific PCR product. For the identification and characterization of gene, the PCR product was purified and sequenced. Sequencing of the amplicon in both directions gave the sequence information of 984 bp length. Similarity search of the sequence was carried out in the NCBI data base. The sequences were aligned by using Bio Edit software. The nucleotide sequence obtained on similarity search in NCBI using BLAST confirmed it to be a segment of the nacrein gene. This partial nucleotide sequence showed 100% homology with the nacrein gene sequence of *P. fucata* available in the NCBI GenBank.

RACE-PCR using the primers designed from the above partial sequence information revealed the sequence of a 1444 bp portion of the nacrein gene, comprising of part of the ORF region and part of UTR. The ORF sequence was used

for prediction of the amino acids being coded by it, using Transq (EMBL). The ORF was found to encode a polypeptide of 438 amino acids.

BLAST analysis showed that the deduced amino acid of nacrein deduced in the present study has high identity with the nacrein of *Pinctada fucata* reported from Japan. Alignment of the amino acid sequences of nacrein of *P. fucata* (present study) with that from Japan revealed that the characteristic features of both of them were observed to be similar.

Nucleotide and deduced amino acid sequences of nacrein are presented in Fig.4.18. Thirty six residues of the active site (R-46, S-72, Q-107, N-108, R-109, A-110, P-111, E-112, E-114, H-136, N-137, H-139, H-141, E-151, H-152, E-162, H-164, V-166, L-182, F-192, V-194, G-196, Y-370, Y-372, L-376, T-377, T-378, P-379, P-380, T-382, S-384, V-385, W-387, V-389, T-421, R-423) and three zinc binding histidine residues (H-141, H-152, H-164) were also identified in the sequence.

```
1            TTGTTATTCCGCTGTGTTATGGCGCCTCCATGTTTAAACATGACCACTACATGGACAATG
                V  I  P  L  C  Y  G  A  S  M  F  K  H  D  H  Y  M  D  N

61           GTGTGAGGTATCCTAATGGTGACGGAATCTGTAAACAATTGAATGAAACCAAATGTGATG
              G  V  R  Y  P  N  G  D  G  I  C  K  Q  L  N  E  T  K  C  D

121          CAGGGTTTAGCTATGATAGGAGTATATGTGAAGGTCCTCATTATTGGCACACCATATCGA
              A  G  F  S  Y  D  R  S  I  C  E  G  P  H  Y  W  H  T  I  S

181          AATGCTTCATTGCATGTGGAATTGGACAGAGACAATCTCCAATCAACATCGTTTCTTATG
              K  C  F  I  A  C  G  I  G  Q  R  Q  S  P  I  N  I  V  S  Y

241          ATGCTAAATTTCGTCAGCGTTTGCCAAAATTGAAATTCAAGCCACATATGGAGAAATTAA
              D  A  K  F  R  Q  R  L  P  K  L  K  F  K  P  H  M  E  K  L

301          AAACAGAAGTGACCAATCATCAGAACCGAGCTCCAGAGTTCGAGCCAGAGGATGGGGAAA
              K  T  E  V  T  N  H  Q  N  R  A  P  E  F  E  P  E  D  G  E

361          ATCTGTACGTGAAGCTAAATAACCTAGTGGACGGTCATTATAAATTCCATAATCTTCACG
              N  L  Y  V  K  L  N  N  L  V  D  G  H  Y  K  F  H  N  L  H

421          TTCATAATGGTAGAACCAGACGTAAGGGATCAGAACACAGTGTTAACGGTCGTTTCACAC
              V  H  N  G  R  T  R  R  K  G  S  E  H  S  V  N  G  R  F  T

481          CTATGGAGGCTCATTTGGTTTTCCATCATGATGATCAAACACACTTTGAACCTACACGCA
              P  M  E  A  H  L  V  F  H  H  D  D  Q  T  H  F  E  P  T  R

541          CTAAGCTGGGAGGAGCATTCCCTGGTCATAACGATTTTGTCGTCGTTGGAGTTTTTCTTG
              T  K  L  G  G  A  F  P  G  H  N  D  F  V  V  V  G  V  F  L

601          AGGTCGGAGATGACGGCTTTGGCGACGAACCGGATGACGAAGAATGTAAACACATCTTAA
              E  V  G  D  D  G  F  G  D  E  P  D  D  E  E  C  K  H  I  L

661          AGGGACATCACCCTGATAATAACGAGAACGGCAATGGAGACAATGGCAATAACGGCTACA
              K  G  H  H  P  D  N  N  E  N  G  N  G  D  N  G  N  N  G  Y

721          ATGGGGACAACGGTAACAATGGTGACAACGGCAATAACAGCTACAATGGGGACAACGGTA
              N  G  D  N  G  N  N  G  D  N  G  N  N  S  Y  N  G  D  N  G

781          ACAATGGTGTCAACGGCAATAACGGCTACAATGGGGACAACGGTAACAATGGAGACAACG
              N  N  G  V  N  G  N  N  G  Y  N  G  D  N  G  N  N  G  D  N

841          GCAATAACGGCTACAATGGGGACAACGGTAACAATGGTGACAACGGCAATAACGGTGAAA
              G  N  N  G  Y  N  G  D  N  G  N  N  G  D  N  G  N  N  G  E

901          ACGGCAATAACGGTGAAAACGGCAATAACGGTGAAAATGGTCACAAACACGGATGTCGGG
              N  G  N  N  G  E  N  G  N  N  G  E  N  G  H  K  H  G  C  R

961          TAAAGAAAGCAAAGCATCTCAGTAGGATCCTGGAATGTGCTTATAGAAACGATAAGGTCA
              V  K  K  A  K  H  L  S  R  I  L  E  C  A  Y  R  N  D  K  V

1021         GAGAGTTCAAGAAAGTTGGAGAAGAGGAAGGGTTAGATGTTCATCTAACACCGGAGATGG
              R  E  F  K  K  V  G  E  E  E  G  L  D  V  H  L  T  P  E  M

1081         CTTTGCCGCCACTGAAGTACAGACATTACTATACATACGAGGGATCCCTGACCACTCCCC
              A  L  P  P  L  K  Y  R  H  Y  Y  T  Y  E  G  S  L  T  T  P

1141         CGTGTACAGAGTCTGTCCTCTGGGTTGTTCAAAAATGCCATGTGCAGGTGTCAAGAAGGG
              P  C  T  E  S  V  L  W  V  V  Q  K  C  H  V  Q  V  S  R  R

1201         TTCTTCATGCATTACGAAATGTTGAAGGATATAAAGATGGTACCACACTAAGAAAGTATG
              V  L  H  A  L  R  N  V  E  G  Y  K  D  G  T  T  L  R  K  Y

1261         GAACTAGACGTCCAACGCAAAAGAATAAAGTTACTGTGTACAAAAGCTTCAAATAGTTGA
              G  T  R  R  P  T  Q  K  N  K  V  T  V  Y  K  S  F  K  *

1321         CATAGTTTTTGTTCTTTTCCTTATAGAGACATGTAACACAGCCAATTATGTTTCATATGT
1381         AATCCATGTAAAATACAGGATCTTTACATAAATATTCATGTGAAACAAGCACGAACATTA
1441         AAGG
```

Fig.4.18 Nucleotide and deduced amino acid sequences of nacrein of *Pinctada fucata*. Nucleotide numbers are shown on the left. Thirty six residues of the active site (R-46, S-72, Q-107, N-108, R-109, A-110, P-111, E-112, E-114, H-136, N-137, H-139, H-141, E-151, H-152, E-162, H-164, V-166, L-182, F-192, V-194, G-196, Y-370, Y-372, L-376, T-377, T-378, P-379, P-380, T-382, S-384, V-385, W-387, V-389, T-421, R-423) are shaded. Three zinc binding histidine residues are boxed. Termination codon is indicated with asterisk (*).

4.1.3.2 Prismalin-14

PCR amplification of the prismalin-14 gene segment of *Pinctada fucata* using cDNA of the target gene and the primer designed from the conserved regions successfully produced amplicon (<200 bp) as visualized through agarose gel electerophoresis. Specific amplicon free from nonspecific products could be amplified through optimization of the PCR parameters. For the identification and characterization of the gene, the PCR product was sequenced. Sequencing of the purified amplicon in both directions gave the sequence information of 195 bp length. The sequences were aligned by using Bio Edit software. The nucleotide sequence obtained were subjected to similarity search in NCBI-BLAST and confirmed to be the prismalin-14 gene fragment.

Sequence of 400 bp portion of the prismalin-14 gene was further resolved through RACE-PCR using the primers designed from the sequence of the initial PCR product, and this segment was found to be comprising of the ORF region and part of UTR of the prismalin-14 gene. The ORF sequence was used for prediction of the amino acids being coded, using Transq (EMBL). The ORF encoded a polypeptide of 121 amino acids. The coding sequences of prismalin-14 and the amino acid sequences encoded by them are presented in Fig.4.19. BLAST analysis showed that the deduced amino acid sequence prismalin-14 deduced in the present study has high identity with the prismalin-14 of *Pinctada fucata* reported from Japan. A signal peptide was identified at the N-terminus of the deduced polypeptide by signal P program and its cleavage site is located between the positions of Gly^{17} and Tyr^{18}.

```
1   CCATTCATCAGCAACAACAATGCGATCTCTGCTAGTCCTCCTTGCCTTAGCTGCTTGTGC
                      M  R  S  L  L  V  L  L  A  L  A  A  C  A

61  TAGTGCACAGTACTTCTTCCGTGGTGGGGACGATGACAACGGTTTCTTCGGAGGAGATGA
      S  A  Q  Y  F  F  R  G  G  D  D  D  N  G  F  F  G  G  D  D

121 CGACAACGGCTACTTCGGTTATTTCCCGCGTTTCTCCTATCCCATTTACCGTCCAATCTA
      D  N  G  Y  F  G  Y  F  P  R  F  S  Y  P  I  Y  R  P  I  Y

181 CCGCCCAATTTACTATCCTCAAATCATAAGACCATTCTACGGCTATGGGTATGGCGGATT
      R  P  I  Y  Y  P  Q  I  I  R  P  F  Y  G  Y  G  Y  G  G  F

241 TAACGGTGGTTACGGAGGACTAGGATTATATGGAGGATATGGTGGGTTCGGAGGCTATGG
      N  G  G  Y  G  G  L  G  L  Y  G  G  Y  G  G  F  G  G  Y  G

301 GTACAGGCCATTCTCTTACGGATACAATCCATTCTCTTATGGATACTACGGATTTGGTGA
      Y  R  P  F  S  Y  G  Y  N  P  F  S  Y  G  Y  Y  G  F  G  D

361 CGATGATGGCGGATTTGATGATTAAGGTATTTACTTCTAT
      D  D  G  G  F  D  D  *
```

Fig.4.19 Nucleotide and deduced amino acid sequences of prismalin-14 of *Pinctada fucata*. Nucleotide numbers are shown on the left.The predicted signal peptide sequence is underlined.The start codon is in bold and the termination codon is indicated with asterisk (*).

4.1.3.3 N19

Amplification of the N19 gene segment of *Pinctada fucata* through PCR using cDNA of the target gene and primer designed from the conserved regions successfully produced an amplicon (<600 bp) as visualized through agarose gel electerophoresis. Optimization of PCR parameters helped to get specific amplicons free from nonspecific PCR product. Purification of this PCR product followed by sequencing revealed that it consists of 557 nucleotides (Fig.4.20). The sequence was aligned by using Bio Edit software. The nucleotide sequence obtained on similarity search in NCBI-BLAST confirmed to be N19 gene. Deduction of amino acid sequence using online software Transeq of European Molecular Biology Laboratory (EMBL) revealed that 185 amino acids were encoded by the ORF. The nucleotide and amino acid sequence of N19 has shown to be matching with only *P. fucata*. RACE-PCR did not successfully work in case of N19 gene.

```
1           TTGTTTCTTGGCTCTTTGTCCTCGTTATAATCCAGCTTGAGGCGTGGCCATGCGATGACA
              V  S  W  L  F  V  L  V  I  I  Q  L  E  A  W  P  C  D  D

61          AGTCAACACCGTACAATGATGATGGAAATGGCGACATGGCGTTCTTGGACAGGCACATAC
             K  S  T  P  Y  N  D  D  G  N  G  D  M  A  F  L  D  R  H  I

121         TCAGGTGTTATTCTAAAGGCATGGCCAGATTTCAACTCGATCTAAGGACCGGTCAAATTC
             L  R  C  Y  S  K  G  M  A  R  F  Q  L  D  L  R  T  G  Q  I

181         GATACGAATATTTTTGCTGTGAATTGCCGAAGAACACCATTCAGTTTTCGTTCAGTACAG
             R  Y  E  Y  F  C  C  E  L  P  K  N  T  I  Q  F  S  F  S  T

241         CTTTCACTTTTGATGGGTATGGCGACGTTGCCTATCTGGACCAACAGACTGTGAACTGTG
             A  F  T  F  D  G  Y  G  D  V  A  Y  L  D  Q  Q  T  V  N  C

301         GCAACAAAGCAGTCATAACCGGTTTTAATTTGCAGAGAAATCTGGATGGAACGAAGGTTA
             G  N  K  A  V  I  T  G  F  N  L  Q  R  N  L  D  G  T  K  V

361         GATACAACGTGACTTGCCGGGAGTTTATTAATATCAGTTACCTTCAATGCTACAACGACG
             R  Y  N  V  T  C  R  E  F  I  N  I  S  Y  L  Q  C  Y  N  D

421         CAACCTCATGGCAGTCTGACCAAAATGGCGACGTACAGGCCCTGGCTAAACATAATGTCA
             A  T  S  W  Q  S  D  Q  N  G  D  V  Q  A  L  A  K  H  N  V

481         AGTGCGCAAGCGGATATTTCGTCAACAAGTTCTCATTACAGGCAGATTACAATTGGTATC
             K  C  A  S  G  Y  F  V  N  K  F  S  L  Q  A  D  Y  N  W  Y

541         AGATCAGGTACCAGTAC
             Q  I  R  Y  Q  Y
```

Fig.4.20 Nucleotide and deduced amino acid sequences of N19 of *Pinctada fucata*. Nucleotide numbers are shown on the left.

4.2 Gene Expression Studies

Expression levels of the defense related genes and pearl forming genes of *Pinctada fucata* following gene induction through exposure to lipopolysaccharide or Mabe implantation, were estimated through semi-quantitative RT-PCR, along with that of the untreated controls. The relative gene expression levels are presented below.

4.2.1 Expression of Defense Related Genes

Live adult individuals of *P. fucata* acclimatized for two weeks in the laboratory at 25^0C in tanks containing static aerated seawater (0.5 L/oyster, 25 ppt salinity) were used as controls as well as for exposure to lipopolysaccharide. Test animals which received LPS dissolved in PBS is showed enhanced expression levels of antioxidant genes and PRP genes. The expression levels at different time points

following LPS treatments were tested for significance the differences. Expression in control animals administrated with PBS alone showed slight variations attributable to the effect of PBS/ physiological variations. Since variations among the controls were statistically non-significant, it could be ignored. Statistical analysis to evaluate significance of the enhanced gene expression levels over that of the control at different time intervals were also carried out before arriving at conclusion. Expression levels of the different genes under study are presented below.

4.2.1.1 Antioxidant Gene Expression

Cu/Zn-Superoxide dismutase (Cu/Zn SOD)

Semi-quantitative PCR estimation of the mRNA levels revealed that while a certain basel level of expression of SOD gene was noticed in the control animals, LPS stimulation significantly increased SOD mRNA levels in the haemocytes in the treated animals in a time-dependent manner. These differences in the expression levels at all the time points were statistically significant. Quantification of expression was made by measuring the intensity of the amplified PCR product in the agarose gel, using the imageJ software. After 4 hours of exposure the level showed a significant increase, which further increased and reached the maximum at 8 h post treatment and then dropped to basal levels by 36 h (Fig.4.21). Comparison of the gene expression in LPS treated and the control at different time intervals were made, and the statistical analysis showed that the expression level at 4 and 8 hours after LPS treatment are significantly different ($P<0.05$) from that of the control(Fig.4.21). At the maximum, the expression of SOD showed a 48% increase over the control.

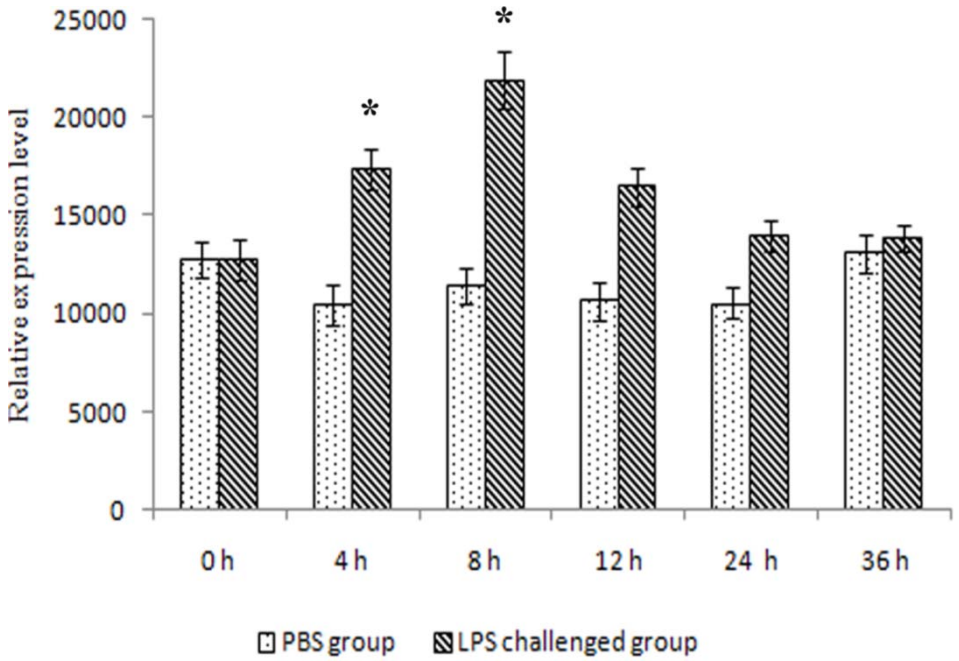

Fig.4.21 Expression level of Cu/Zn SOD mRNA in haemolymph of the control and LPS challenged pearl oysters. Vertical bars represent the mean ± S.E (N=5). Significant differences ($P<0.05$) between control and LPS treated are indicated with the asterisk (*).

Glutathione peroxidase and glutathione-S-transferse (GPX and GST)

Both the GPX and GST involved in degrading the peroxides that was produced as a result of the breakdown of reactive oxygen species by the SOD. Following LPS stimulation, the pattern of expression of GPX and GST genes were similar to that of SOD - mRNA levels of both genes increased. The difference in expression levels at all the time points were compared, and found to be statistically significant. Expression levels of both these genes in the LPS challenged specimens showed significant difference from that of the control, reaching the maximum at 8 h, and thereafter, decreasing slowly to control levels. At the maximum (8 h), the relative mRNA expression of both GPX and GST was 34% more than the control. The time dependent variation in the expression of GPX and GST following LPS stimulation were represented in graph (Fig.4.22 and Fig.4.23). The statistical analysis showed that the gene expression level at 8 h after LPS are significantly different ($P<0.05$) from control.

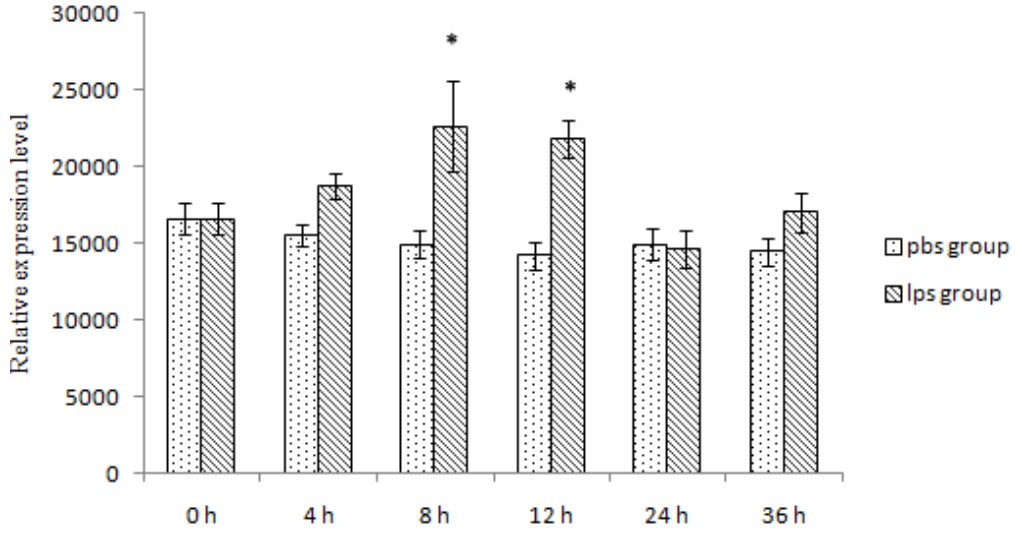

Fig.4.22 Expression level of GPX mRNA in haemolymph of the control and LPS challenged pearl oyster. Vertical bars represent the mean ± S.E (N=5). Significant differences (P<0.05) between control and LPS treated are indicated with the asterisk (*).

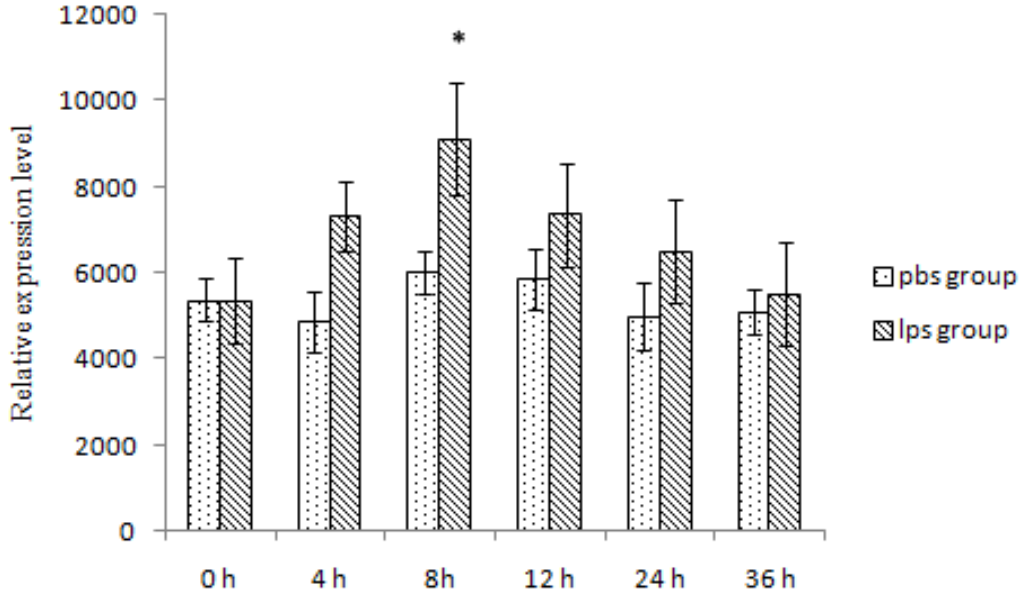

Fig.4.23 Expression level of GST mRNA in haemolymph of the control and LPS challenged pearl oyster. Vertical bars represent the mean ± S.E (N=5). Significant differences (P<0.05) between control and LPS treated are indicated with the asterisk (*).

4.2.1.2 Pattern Recognition Receptor Protein Genes (PRP)

Pattern recognition receptor protein (PRP) genes are involved in the recognition of different immune modulators, and they are capable of binding specifically to the conserved portion of microbial cell wall component.

F-type lectin

In the present study, LPS stimulation substantially increased the expression of F-type lectin in the haemocytes (Fig.4.24). The mRNA level of F-type lectin in the challenged animals reached the maximum at 4 h and then gradually decreased over the time. However, the level of expression at each time points were significantly different from each other. Though, the level of F-type lectin in the challenged animals were higher than the control at all the time points, it was significantly higer at 4 hr and 8 hr only. At the maximum (4 hr) the relative mRNA expression of F-type lectin showed a 50% increase over the control. The graphical representation of the expression level quantified using the imageJ software is presented in graph (Fig.4.24). Result of the statistical analysis of the expression level at different time intervals following exposure to LPS, using SPSS software is also presented in the graph. The statistical analysis showed that the gene expression level at 4 and 8 hours after LPS are significantly different ($P<0.05$) from control.

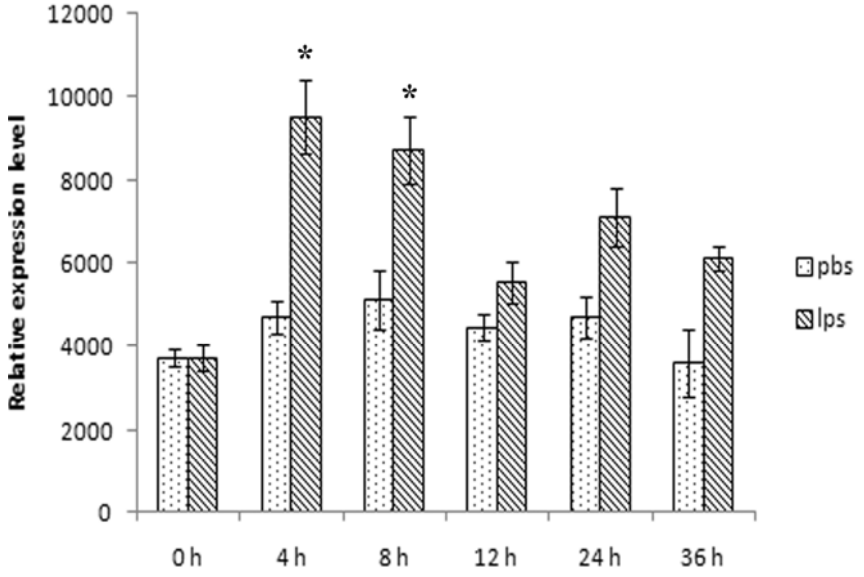

Fig.4.24 Expression level of F-type lectin mRNA in haemolymph of the control and LPS challenged pearl oyster. Vertical bars represent the mean ± S.E (N=5). Significant differences ($P<0.05$) between control and LPS treated are indicated with the asterisk (*).

Galectin

Galectin is important for recognizing β-galactoside ligand of the pathogen by their conserved carbohydrate recognition domains (CRD) and play crucial role in innate immunity. Expression of the galectin gene on exposure of the animal to LPS were higher than the control, from 4 to 36 h post challenge. Graphical representation of the expression level quantified using the imageJ software (Fig.4.25) shows that it reached the maximum at 8 h post treatment, and then dropped to basal level at 36 h. Statistical analysis of the gene expression levels in LPS treated and control animas at different time intervals, using SPSS software showed that the expression level at 8 hours post exposure was only significantly different ($P<0.05$) from the control. However, the expression levels at different time points, when compared without considering the control were significantly different.

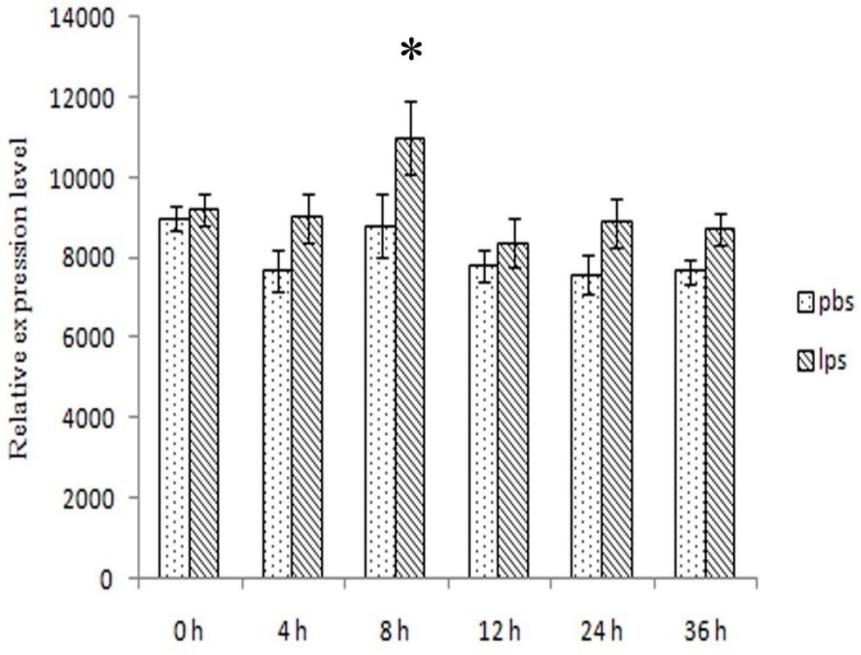

Fig.4.25 Expression level of galectin mRNA in haemolymph of the control and LPS challenged pearl oyster. Vertical bars represent the mean ± S.E (N=5). Significant differences ($P<0.05$) between control and LPS treated are indicated with the asterisk (*).

Lipopolysaccharide and β-1, 3-glucan binding protein (LGBP)

In the present study, LPS stimulation significantly increased the mRNA expression of LGBP in the Haemocytes in a time-dependent manner (Fig.4.26). The level of LGBP mRNA in the challenged animals increased significantly compared to the control, and reached maximal levels at 8 h and then gradually decreased over the time. At the maximum the mRNA level of LGBP was 24% more over the control. The graphical representation of the expression level at different time intervals following exposure to LPS, quantified using the imageJ software is presented in Fig.4.26 along with the result of the statistical analysis using SPSS software. The statistical analysis showed that the gene expression level at 8 hours after LPS were significantly different ($P<0.05$) from the control. However the LPS treated samples at different time intervals were showing statistically significant differences when compared without considering the control.

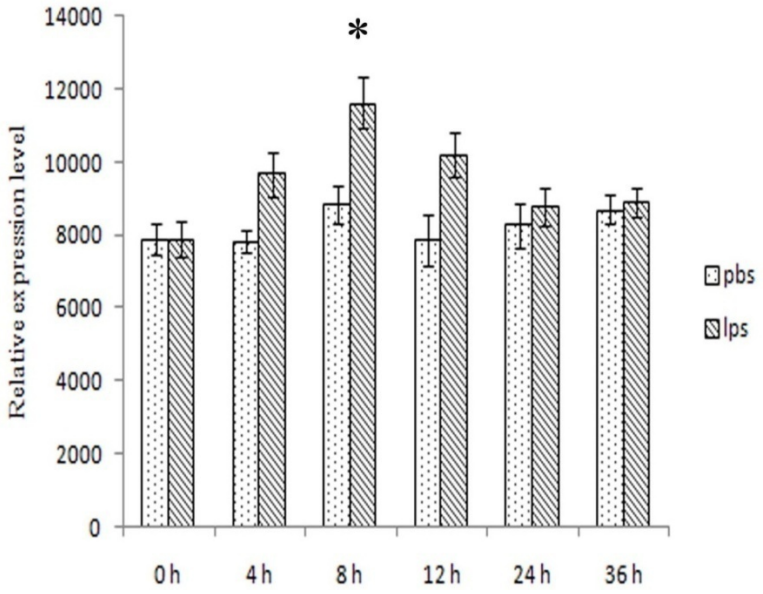

Fig.4.26 Expression level of LGBP mRNA in haemolymph of the control and LPS challenged pearl oyster. Vertical bars represent the mean ± S.E (N=5). Significant differences ($P<0.05$) between control and LPS treated are indicated with the asterisk (*).

4.2.2 Expression of Pearl Forming Genes

Expression levels of the pearl forming genes in *Pinctada fucata* following Mabe implantation estimated through semi-quantitative RT-PCR along with that of the controls are presented below.

Acclimation of oyster at the optimum salinity under the controlled condition was carried out to understand the normal gene expression, which would serve as the baseline to compare the gene expressions following implantation. At each pre-determined day (10, 30 & 40) following implantation, five oysters were harvested. Mantle surrounding the Mabe were carefully isolated and subjected to transcriptome analysis. The gene coding for GAPDH RNA was used as housekeeping gene, as its expression is not effected by environmental stressors, and therefore, could serve as an internal control in the relative gene expression studies following implantation. The expression levels of the four pearl forming genes at different time intervals are presented below.

Nacrein

Expression of the nacrein gene after Mabe implantation have shown initial up regulation. As shown in fig.4.27 endogenous expression of nacrein gene increased in the implanted animals. The increased level of nacrein mRNA in the experimental oysters was significantly higher than the control up to the 30th day. The increase was 53% more than the control at day 10. On the 30th day the gene expression was marginally reduced than the 10th day level. However, there was a steep decrease on day 40 compared to the earlier days, but was still higher than the control. Fluctuations in the control animals during the period was only very marginal. Graphical representation of the expression level quantified using the imageJ

software, which detects the intensity of the amplified gene product in the agarose gel are presented in Fig.4.27. Statistical analysis of the gene expression levels at different days following Mabe implantation, using SPSS software showed that the gene expression level at day 10 and 30 were significantly higher ($P<0.05$) than the control (Fig.4.27).

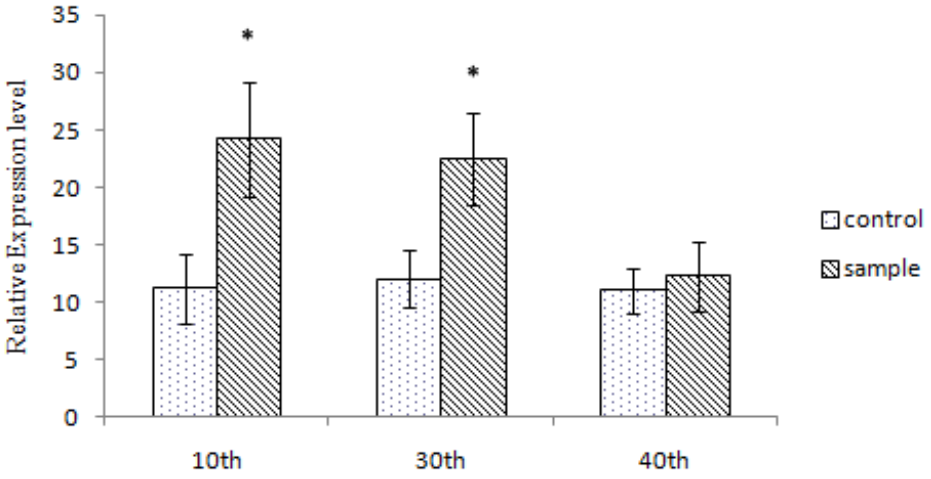

Fig.4.27 Expression level of Nacrein gene in mantle surrounding the Mabe quantified by semi-quantitative PCR. Vertical bars represent the mean ±S.E (N=5). Significant differences ($P<0.05$) are indicated with the asterisk (*).

Prismalin-14

As shown in Fig.4.28 prismalin-14 gene expression increased in the implanted animals. The level of prismalin-14 mRNA in the experimental oysters was significantly higher in the initial period itself than the control, and it was sustained up to 30 days. The increase was 35% more than the control on day 10. On the 30th day the gene expression was marginally less than the 10th day level. This level was sustained on the 40th day also. Fluctuations in the control during the period was only very marginal. The graphical representation of the expression level quantified using the imageJ software which detected the intensity of the amplified gene product in the

agarose gel is presented in fig.4.28. Statistical analysis of the gene expression levels at different days following Mabe implantation using SPSS software showed that the gene expression level at day 10 post exposure was significantly higher ($P<0.05$) than the control (Fig.4. 28).

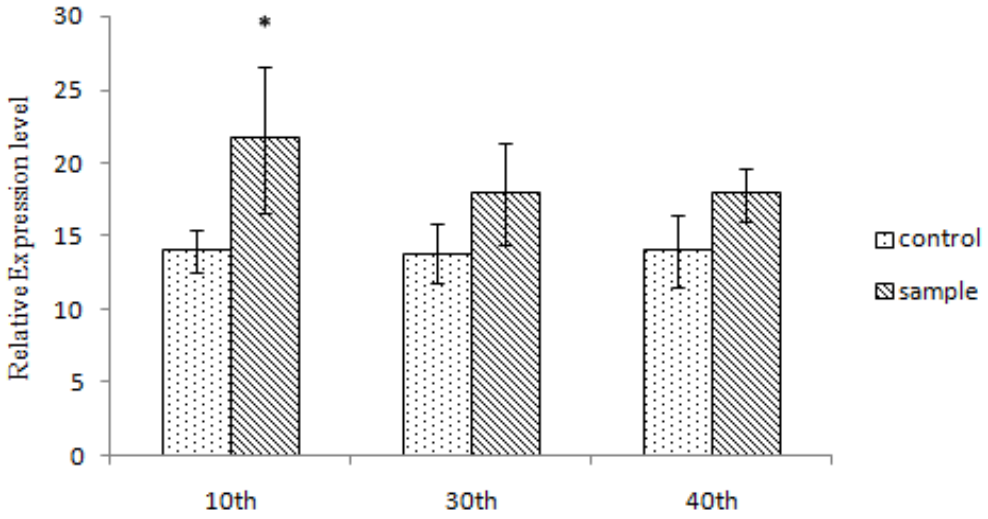

Fig.4.28 Expression level of Prismalin-14 gene in mantle surrounding the Mabe measured by semi-quantitative PCR. Vertical bars represent the mean ±S.E (N=5). Significant differences ($P<0.05$) are indicated with the asterisk (*).

N19

Marginal increase in the expression of N19 gene was observed at day 10 compared to the control as shown in fig.4.29. However, at day 30 gene expression of N19 increased further, and was significantly higher than the control. At 40th day, though there was a slight decrease, compared to 30th day, the level was higher than that on the 10th day. Fluctuations in the control animals during different time intervals was very marginal. The gene expression at different days estimated through imageJ software are graphicaly represented in fig.4.29. The statistical analysis using SPSS software showed that the gene expression level at 30th day after Mabe implantation was significantly different ($P<0.05$) from control.

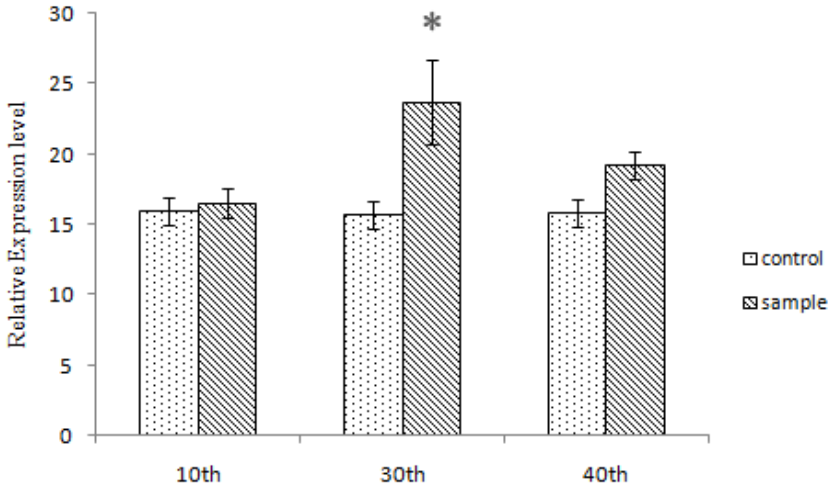

Fig.4.29 Expression level of N19 gene in mantle surrounding the Mabe measured by semi-quantitative PCR. Vertical bars represent the mean ±S.E (N=5). Significant differences ($P<0.05$) are indicated with the asterisk (*).

Chapter 5

DISCUSSION

As secondary filter feeders, pearl oysters are exposed to various kinds of stressors like bacteria, viruses, pesticides, industrial wastes, toxic metals and petroleum derivatives, making them vulnerable to diseases. Environmental changes and ambient stress also affect non-specific immunity, making the organisms susceptible to infections. One of the strategies to combat these problems is to get insight into the disease resistance genes, and use them for disease control and health management. Similarly, although it is known that formation of pearl in molluscs is mediated by specialized proteins which are in turn regulated by specific genes encoding them, there is a paucity of sufficient information on these genes. Therefore, work on these aspects were carried out in the Indian pearl oyster *P. fucata* in the present study viz, molecular characterization and expression analysis of the genes involved in disease resistance and pearl formation, and the outcome are discussed hereunder.

5.1 Molecular Identification and Characterization of Functional Genes

5.1.1 Antioxidant Enzyme Genes

Oxygen is a vital factor for the existence of life and it is an important element for all flora and fauna of terrestrial and aquatic origin. Reactive oxygen species (ROS) are produced in abundance when the animals are exposed to pathogenic attack and other abiotic stressors. Excessive ROS can damage a number of cellular macromolecules (Hartog *et al.*, 2003). Consequently, organisms contain a complex

network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids. (Sies, 1997; Vertuani *et al.*, 2004). Super oxide dismutase (SOD) is the key defence molecule which fight ROS followed by others such as glutathione peroxidise (GPX) and glutathione-S-transferase playing vital roles in the detoxification pathways. Molecular detection and characterization of antioxidant enzyme genes are helpful for detecting cellular stress and also for bio-monitoring of environment. Though, study of Cu/Zn SOD gene including cloning have been carried out in several animals including Pacific oyster, *Crassostrea gigas* (Boutet *et al.*, 2004), clam, *Ruditapes decussatus* (Geret *et al.*, 2004), prawn, *Macrobrachium rosenbergii* (Cheng *et al.*, 2006), and the abalones *Haliotis discus discus* (Kim *et al.*, 2007) and *Haliotis diversicolor supertexta* (Zhang *et al.*, 2009), no such study in pearl oysters has been made so far. The present study, for the first time, report the molecular identification, complete characterization and expression analysis of the gene encoding the most prominent antioxidant enzyme viz., superoxide dismutase (SOD). However, the cloning and mRNA expression of other antioxidant enzyme genes viz. GPX and GST in pearl oyster *P. fucata* from China have been reported by Jiang *et al* (2014). In the present study we also have carried out the PCR amplification and partial characterization of the GPX and GST genes in Indian pearl oyster *P. fucata.*

5.1.1.1 Superoxide Dismutase (SOD)

SOD is the first line of defence against superoxide radicals which exist in the haemocytes. In the present study, the full length nucleotide sequence of the cytoplasmic SOD of the pearl oyster, *Pinctada fucata,* and the amino acid sequence encoded by it were elucidated and reported. This is the first report of the kind from

this species. Out of the 924 bp long cDNA of SOD, the ORF consisted of only 471 bp encoding 156 amino acids. The InterProscan analysis of the amino acid sequence have revealed that it is a Cu/Zn SOD with copper/zinc binding domains within the sequence. There was no signal peptide indicating that it should be a member of the cytoplasmic SOD family. Multiple alignment of the deduced amino acid sequence of SOD with other cytoplasmic SODs revealed that two cysteines (Cys 60 and Cys 149) were conserved in all SODs, which were predicted to form one disulphide bond. Ni *et al.* (2007) have reported that Cu/Zn SODs can be discriminated from other types of SODs by their copper and zinc binding amino acids which are highly conserved. In the present study, it was found that *P. fucata* SOD contains copper and zinc binding regions, suggesting that pearl oyster SOD is a Cu/Zn SOD. Several reports have indicated that copper and zinc ions have critical functions in the quaternary structure and kinetic properties of Cu/Zn SOD (Assfalg *et al.,* 2003; Cioni *et al.,* 2003; Lynch and Colon 2006). Matsuo *et al.* (1997) found that limiting the dietary copper and zinc decreased SOD activity in the onion fly, *Delia antique*. The two Cu/Zn SOD family signature sequences of *P. fucata* SOD gene found in this study (signature 1 $_{47}$GFHVHQFGDNT$_{57}$ and signature 2 $_{141}$GNAGGRLACGVI$_{152}$) are similar to the family signature sequence reported from *Chlamys farreri* and *Crassostrea gigas* (Ni *et al.*, 2007; Boutet *et al.*, 2004). Multiple alignments and phylogenetic analysis of Cu/Zn SOD genes of *P. fucata* with other molluscs and vertebrates have shown that these family signatures are conserved across the species (*Crassostrea gigas* CAD42722, *Mytilus edulis* CAE46443, *Candida albicans* AAC12872, *Gallus gallus* NP_990395, *Bos taurus* NP_777040, *Mus musculus* NP_035564).

BLAST analysis carried out using the deduced amino acid sequence with that of other species revealed extremely high identity (99%) with *C. hongkongensis, C. gigas* and *M. chilensis*.

Close evolutionary relationship between *P. fucata* and other aquatic invertebrates was revealed from the phylogenetic tree constructed using SOD amino acid sequence of *P. fucata* and other invertebrates as well as vertebrates. Vertebrates were evolutionarily distinctly separated.

5.1.1.2 Glutathione Peroxidase (GPX)

GPX enzyme constitute the second line of defence against lipid peroxidation, preventing the breakdown of lipid hydroperoxide which would otherwise lead to the formation of new radicals (Lawrence and Burk, 1976; Ren *et al*., 1997; Bao and Williamson, 2000; Cossio-Bayugar *et al*., 2005). In the present study the nucleiotide sequence of GPX gene and the amino acids encoded by it were elucidated and reported. The amino acid sequence deduced from the ORF region was found to have many charactacteristic features. Absence of a signal peptide suggests that the glutathione peroxidase of *P. fucata* should be classified as a cytoplasmic GPX. The present study has shown that the structurally important amino acid residue viz., selenocysteine (Sel), the GPX signature motif, and the active site motif are highly conserved in the deduced amino acid. Similar pattern have been reported in *Haliotis discus discus* and *Chlamys farreri* by De Zoysa *et al.* (2008) and Mu *et al.* (2010). Though, generally the active site of Se-GPX and other selenoproteins is based on a selenium moiety (generally a single covalently bound atom of selenium) present as the amino acid selenocysteine, Gamble *et al.* (1997) reported an extension of the genetic code to include the codon TGA as the 21st codon specifying the presence of

the selenocysteine in the polypeptide structure of selenoproteins (selenium dependent proteins). In the present study, it was found that *P. fucata* GPX contains a TGA codon corresponding to Sel residue, suggesting that pearl oyster GPX is a selenium dependent protein and could be considered as a member of the selenoprotein family. The deduced amino acid sequence displays two important motifs, namely the Se-GPx signature 2 at ^{75}LGFPCNQF^{82} and the active site at ^{163}WNFEKF^{168}, which contain Gln(Q) and Trp(W). Depege *et al.* (1998) had reported that these residues are important for the catalytic activity of GPX. Earlier workers have identified the amino acids important for different functional roles of Selenium dependent glutathione peroxidase (Se-GPX) in other species. It has been reported that two residues, Gln (Q_{85}) and Trp (W_{153}), are involved in the fixation of selenium (Ladenstein *et al.*, 1986). Also, two arginine residues (R_{101} and R_{199}), contribute to the electrostatic architecture that directs the glutathione donor substrate toward the catalytic center (Aumann *et al.*, 1997). The present study have also identified Gln_{85}, Trp_{153}, Arg_{101}, and Arg_{199} amino acid residues in the *P. fucata* Se-GPX, supporting the view that they are involved in the functional and biochemical activities. Multiple alignments analysis carried out in the present study revealed that the selenocysteine residue, the GPX signature motif and the active site motif are highly conserved in the species such as pacific oyster, *Crassostrea gigas* (EF692639), mouse, *Mus musculus* (NM_008160) and insect, *Ixodes scapularis* (AAY66814). On account of these characteristic features, the *P. fucata* GPX can be considered as belonging to the selenium dependent glutathione peroxidase family.

5.1.1.3 Glutathione-S-transferase

Glutathione-S-transferases (GST) are the major phase II detoxifcation enzymes that play central roles in the defense against various environment toxicants. In the present study, the nucleiotide sequence of GST and the amino acid sequence encoded by it have been elucidated. The full ORF sequence was found to be consisting of 231 amino acids. There was no signal peptide indicating that it should be a member of the cytoplasmic GST family. Previous reports suggest that GST commonly possess two binding domains. Mannervik and Danieison, (1988); Ivarsson *et al.* (2003) and Sheehan *et al.*(2001) reported that the N-terminal domain contains a GSH-binding site (G-site) that is well conserved among different classes of GSTs, and the C-terminal domain contains a hydrophobic substrate binding site (H-site) that varies widely in different classes, resulting in different substrate specificities. Conserved domain search using the amino acid sequence of *P. fucata* GST deduced in the present study revealed that it has also a G-site in its N-terminal region and an H-site in the C-terminus. GST genes are ubiquitous in almost all the existing organisms from bacteria to humans. Earlier workers have identified the co-presence of GST isoforms in a number of species, but with similar enzymatic features and physiological functions. In the case of disk abalone, *Haliotis discus discus,* six GST genes including one mu class GST, three sigma class GSTs and two omega class GST have been cloned and characterized, exhibiting protective roles against several organic pollutants (Wan *et al.*, 2008a,b; Wan *et al.*, 2009). Multiple alignments and phylogenetic analysis of *P. fucata* GST genes with other molluscs and vertebrates showed that they share some conserved domains (Fig.4.7) and that the *P. fucata* GST gene identified belongs to the omega class of the GST gene family (Fig.4.8).Wan *et*

al. (2009) found that two omega GSTs in abalone have two differences in the G-site, Tyr30 and Thr67 in HdGST01 (*Haliotis discus discus* glutathione-S-transferace 01) and Phe30 and Val68 in HdGSTO2 (*Haliotis discus discus* glutathione-S-transferace 02) sequence. In the present study, similar G-site pattern of HdGSTO2 have been identified in GST sequence of *P. fucata*. The compositions of the cysteine-containing tetramers are as divergent as C–P–Y–C, C–P–F–A, C–P–Y–A, C–P–Y–S, C–P–W–A, etc (Wan *et al.,* 2009). In this study it was found that *P. fucata* GST shared the same active tetramer of C–P–F–A with abalone HdGSTO2, human GSTO1 and pig omega GST.

5.1.2 Pattern Recognition Receptor Protein Genes

Microorganisms have highly conserved and widely distributed signature molecules in their cell walls such as LPSs. They are recognized by the molecules of the immune or defense systems that have been named as "pattern recognition molecules" (Medzhitov and Janeway, 1997). They are also referred to as "pattern recognition receptor proteins" (PRPs). To date, a number of LPS-recognition/binding protein molecules have been isolated and characterized from several animals, and these pattern recognition molecules have been shown to be involved in the innate immune system of both invertebrates and vertebrates (Medzhitov and Janeway, 1997; Carroll and Prodeus, 1998; Hoffamnn *et al.*, 1999).Three types of pattern recognition receptor proteins of *P. fucata* viz, F-type lectin, galectin and LGBP were taken for this study.

5.1.2.1 F-type lectin

F-type lectin is a PRP which participate in the innate immune defense system. They play important roles in the immune response of both invertebrates and

vertebrates either by recognizing the exposed glycans of potential pathogens or by their immunoregulatory roles through the binding to carbohydrates on the surfaces of immune competent cells (Kuhlman *et al.*, 1987; Cooper *et al.*, 1994; Tino and Wright, 1996; Goldstein *et al.*, 1980; Rabinovich *et al.*, 2007). In the present study, the full length nucleotide and the amino acid sequence encoded by the F-type lectin gene of the pearl oyster, *Pinctada fucata,* were elucidated. This study is the first report of full length F-type lectin in pearl oyster *P. fucata*. The ORF of F-type lectin consisted of 588 bp nucleotides encoding a putative peptide of 196 amino acids. A signal peptide was identified at the N-terminus of the deduced polypeptide. There are seven cysteines (Cys 12, Cys 92, Cys 124, Cys 125, Cys 147, Cys 164 and Cys 188) in the mature F-type lectin, and multiple alignment of deduced amino acid sequences with other closely related F-type lecin sequences showed that six of them (Cys 92, Cys 124,Cys 125, Cys 147, Cys 164 and Cys 188) are conserved. Similar pattern have been reported in *P. martensii* (Chen *et al.*, 2011). It is believed that these are involved in an internal disulfide bond. Conserved domain search in the NCBI revealed that the F-type lectin domain extends from Lys^{55} to Val^{192}. Several reports have indicated that the first carbohydrate binding residue in F-type lectin is a His residue in European eel (*Anguilla anguilla*), Japanese eel (*Anguilla japonica*) etc followed by 26 other residues and the RGD (Arginine, glycine, Aspartic acid) motif. (Bianchet *et al.*, 2002; Cammarata *et al.*, 2012; Pierschbacher *et al.,* 1985; Salerno *et al.*, 2009). However, the present study has indicated that in *P. fucata*, the first carbohydrate binding residue is a serine followed by 26 other residues and the RGD motif, which is similar to that reported in *P. martensii*. Pierschbacher *et al.* (1985) has reported that RGD motif is conserved in cell adhesion proteins. Many of the F-

type lectin contains multiple domains: the zebrafish FBPL-I contains two F-type lectin domain (NCRD and CCRD), the Pacific oyster bindin protein contains one to five F-type lectin domains, and the F-type lectin domain of *Xenopus* pentraxin is combined with other domains. The *P. fucata* F-type lectin possesses only one domain which is similar to fucolectin of *A. japonica* reported by Honda *et al.* (2000) and F-type lectin of *P. martensii* reported by Chen *et al.* (2011). Multiple alignment of *P. fucata* F-type lectin sequence of the present study also was in agreement. To evaluate the molecular evolutionary relationships of F-type lectin of *P. fucata*, a phylogenetic tree was constructed. While the F-type lectin of *P. fucata* of the present study and that from *P. martensii* formed one cluster, other molluscs formed different clusters indicating relationship of varying degrees with each other.

5.1.2.2 Galectin

Galectins have been reported to bind glycans on the cell-surface and extracellular matrix of potentially pathogenic microorganisms, thereby functioning as recognition molecule and effectors in innate immunity (Vasta, 2009). In the present study, the nucleiotide sequence of galectin from the *P. fucata* and the amino acid sequence encoded by them were cloned and sequenced. The nucleiotide sequence of galectin gene of *P. fucata* decipherd in the present study was 1750 bp long of which the ORF region consisted of 1727 bp nucleotides encoding a putative peptide of 551 amino acids. The amino acid sequence deduced from the ORF region has many charactacteristic features.

Sequence of these amino acids were highly homologous to bivalve galectins reported in the literature for *P. fucata* from China, *Crassostrea virginica* and *Argopectin irradians*. Bivalve galectins have been reported to possess four

carbohydrate recognition domains (CRDs) with conserved carbohydrate binding sites in each CRD, whereas, tandem-repeat galectins contain two CRDs covalently linked through a unique link peptide (Vasta *et al.*, 2004b; Leffler *et al.*, 2004). In the present study it was found that 7 amino acid residues are conserved in each of the four galectin specific domains, and each of these four domains are connected by unique 11 amino acid residues. Recently, galectin cDNAs were cloned and characterized from Eastern oyster (Tasumi and Vasta, 2007), Pacific oyster (Yamaura *et al.*, 2008), Manila clam (Kim *et al.*, 2008), abalone (EF392832), freshwater snail (Yoshino *et al.*, 2008), and bay scallop (Song *et al.*, 2010). Four CRDs have only been found in molluscan galectins, including *Crassostrea virginica*, *Argopectin irradians*, *P. fucata* from China and *P. fucata* of the present study. Hirabayashi and Kasai (1993) have classified the mammalian galectins, based on their CRD organization, into three subfamilies as proto-, chimera and tandem-repeat types. Since the tandem-repeat galectins could simultaneously bind to multivalent ligands and greatly enhance the binding activity (Arata *et al.*, 1997), the joining of more number of different CRDs in the same galectin could open up the possibility to specifically crosslink more distinct types of ligands, as opposed to homo-functional crosslinking by multimeric prototype galectins (Cooper, 2002).The previous studies demonstrated that galectins might be secreted via a "nonclassical" secretory pathway, which involves direct translocation of proteins across the inner plasma membrane followed by membrane blabbing and secretion (Nickel, 2003). The present study indicated that *P. fucata* galectin did not contain typical signal sequence, supporting the "nonclassical" secretory pathway.

From multiple alignment of *P. fucata* galectin CRDs with other known galectin CRDs, Hirabayashi *et al.* (2002) showed that *P. fucata* galectin contained many evolutionally conserved structural features in each CRD, such as the typical motifs H-NPR and WG-ER. Multiple alignment of *P. fucata* galectin sequence of the present study also was in agreement. To evaluate the molecular evolutionary relationships of *P. fucata* galectin, a phylogenetic tree was constructed. Galectin of *P. fucata* of the present study and that from China formed one cluster, while the other molluscs formed different clusters indicating relationship of varying degrees with each other.

5.1.2.3 LGBP

LGBP is a glycosylated protein, which has the ability to bind with the glycosylated substrates like carbohydrate moieties. In the present study, the nucleiotide sequence of LGBP gene from the *P. fucata* and the amino acid sequence encoded by them were cloned and sequenced. The nucleiotide sequence of LGBP gene of *P. fucata* decipherd in the present study was of 1740 bp long. The amino acid sequence deduced from the ORF region has many charactacteristic features. The ORF of LGBP consisted of 1692 bp nucleotides encoding a putative peptide of 563 amino acids.

The amino acid sequence of LGBP gene of the pearl oyster in the present study contained a potential recognition motif for β1,3-linkage of polysaccharides. Similar pattern have been identified in the sequence of *P. fucata* from China. The amino acid sequence of this motif was slightly modified in the blue shrimp *Penaeus stylirostris* (Roux *et al.*, 2002), white shrimp *L. vennamei* (Cheng *et al.*, 2005) and crayfish *P. leniusculus* (Lee *et al.*, 2000). A LPS-binding site also was observed in

the pearl oyster LGBP, which was similar to the zhikong scallop LGBP (Su *et al.*, 2004). The carbohydrate recognition domains in the scallop might facilitate the interaction of immunocytes with the pathogen and subsequently induce cellular defenses (Su *et al.*, 2004). It is generally believed that the invertebrate LGBP genes might have evolved from bacterial glucanase and lost the glucanase activity during evolution, but retained the glucan-binding properties and therefore, played a role in innate immune response (Lee *et al.*, 2000). It was found that LGBP of *P. fucata* contains a threonine-rich region and a glycine-rich region. Similar pattern have been reported in the *P. fucata* of China by Zhang *et al.* (2010). Johansson (1999) suggested that the RGD (Arg–Gly–Asp) motif reported in other crustacean LGPBs (Du *et al.*, 2007; Lin *et al.*, 2008; Padhi and Verghese 2008) are putative cell adhesive sites. However, there is no RGD motif in zhikong scallop LGBP (Su *et al.*, 2004) and disk abalone HdPRP (Nikapitiya *et al.*, 2008), and the *P. fucata* (Zhang *et al.*, 2010). But, a modified form viz. KGD (Lysine, Glycine, Aspartic acid) have been reported in the molluscs. It is generally believed that the invertebrate LGBP genes might have evolved from bacterial glucanase and lost the glucanase activity during evolution, but retained the glucan-binding properties and therefore, played a role in innate immune response (Lee *et al.*, 2000). However, recently, Pauchet and coworkers identified a 40 kDa β-1,3-glucan-binding protein from midgut of lepidopteran, which was similar to previously characterized epidopteran βGRPs, and demonstrated that this βGRP was an active β-1,3-glucanase and was mainly expressed in midgut, and this type of PRP was named as catalytic PRP (Pauchet *et al.*, 2009). At present, a similar glucanase domain with the conserved active sites has also been observed in the kuruma shrimp LGBP (Lin *et al.*, 2008), earthworm LGBP

(Bilei *et al*., 2001), zhikong scallop LGBP (Su *et al*., 2004) and disk abalone HdPRP (Nikapitiya *et al*., 2008). In the present study, it was revealed that the *P. fucata* LGBP also had a glucanase motif with the conserved active sites, similar to the lepidopteran βGRP (Pauchet *et al*., 2009). The *P. fucata* LGBP might also be an active glucanase. Multiple alignments of *P. fucata* LGBP genes with other species showed that they share some conserved motifs (Fig.4.16). To evaluate the molecular evolutionary relationships of *P. fucata* LGBP, a phylogenetic tree was constructed. LGBP of *P. fucata* of the present study and that from China formed one cluster, while the other groups formed different clusters indicating relationship of varying degrees with each other.

5.1.3 Genes Involved in the Pearl Formation

Identification of pearl forming genes and their expression pattern during pearl formation are valuable, both scientifically and economically, as it can be used for improving the quality and productivity of pearls. The present study was focused on the identification and characterization of three genes involved in pearl formation, and study of their expression pattern during Mabe pearl formation.

5.1.3.1 Nacrein

Pearl is produced by the process of bio-mineralization of calcium carbonate crystals. Formation of the nacreous layer on the pearl surfaces is closely related to the expression patterns of the gene involved in the matrix proteins (Takeuchi and Endo, 2006; Inoue *et al*., 2010a, b). Nacrein constitutes a significant portion of the soluble matrix proteins of the nacreous layer of the pearl. It is widely believed that aspartic acid-rich calcium binding proteins are responsible for biomineralization in mollusk (Weiner and Hood, 1975; Weiner, 1979; Wheeler *et al*., 1981; Wheeler and

Sikes, 1984; Cariolou and Morse, 1988). There are only a few studies on the gene expression patterns of the matrix proteins in the mantle epithelial cells of the pearl sac (Inoue *et al.* 2010a; Wang *et al.*, 2009). In the present study attempt was made to characterize the nacrein gene. The partial ORF cDNA of nacrein was cloned from *P. fucata*. The amino acid sequence of *P. fucata* nacrein deduced in the present study was highly homologous to that from Japan. Boskey (1992) reported that the regularly spaced carboxyl groups of Asp or Glu side chains on the surface of such calcium binding protein participate directly in calcium binding, either alone or in concert with other Asp or Glu in the polypeptide, such as osteopontin. Nacrein has an acidic Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) domain, which suggests that nacrein is a calcium binding protein. Probably, nacrein is the major aspartic acid rich calcium binding protein in the nacreous layer. The Gly-Xaa-Yaa (Yaa = any amino acid) repeats like those in the collagenous domain are also found in a structural protein of the inner ear, which produces calcium carbonate crystals (Davis *et al.*, 1995). Two different forms of calcium carbonate crystal exist in a shell: one is calcite in the prismatic layer and another is aragonite in the nacreous layer. The major difference between these forms is the position of the carbonate groups. Addadi,Weiner and colleagues (Addadi and Weiner, 1985; Addadi *et al.*, 1989) have already demonstrated that acidic proteins from shells regulate biological calcite growth. Moreover, Falini *et al.* (1996) and Belcher *et al.* (1996) showed that acidic macromolecules extracted from the prismatic and nacreous shell layers induced calcite and aragonite formation respectively, *in vitro*. These results suggest that the macromolecules modulate the stereochemical position of carbonate groups in the process of biological calcium carbonate crystallization. The mantle of mollusc shellfish is known to contain

carbonic anhydrase (CA) activity (Freeman and Wilbur 1948). However, the exact nature of acidic proteins and CA activity in the formation of prismatic or nacreous layer is not well understood. Analysis of the nacrein amino acid sequence deduced from the DNA sequence in the present study showed that the presence of Gly-Xaa-Asn repeats (Xaa = Asp, Asn, or rarely Glu) may be the sites for interaction with calcium. A search of the Swissprot protein data base by Miyamoto *et al.* (1996) revealed homology between nacrein and CA. The CA domain of nacrein exhibited greatest identity to human CAII (Montgomery *et al.*, 1987; Murakami *et al.*, 1987). In particular, among the 36 residues of the active site (Tashian, 1989), 63% identity to human CAII was observed. Furthermore, nacrein retained the three histidines involving zinc-binding residues at positions 141, 152, and 164 (Murakami *et al.*, 1987). In the present study the characteristic thirty six residues of the active site and three zinc binding histidine residues (H-141, H-152, H-164) were identified in the nacrein sequence which are similar to the previous report.

5.1.3.2 Prismalin-14

Prismalin-14 is the second macromolecule so far characterized from the prismatic layer of *P. fucata*. Prismalin-14 is an acidic protein and is associated with calcification of the prismatic layer. In the present study, the full ORF cDNA of a prismalin-14 was cloned from *P. fucata*. Prismalin-14 have been reported to have a peculiar amino acid composition comprising only 11 kinds of amino acids with high proportions of glycine (27.6%) and tyrosine (20.0%) residues. According to a previous report (Wada, 1966), high contents of glycine (23.0%) and tyrosine (12.3%) were also found in the crude matrix proteins from the prismatic layer of *P. fucata*. In the present study, it was found that the deduced amino acid of prismalin-14 also

comprises of 11 kinds of amino acids with high content of glycine and tyrosine residues. A signal peptide was identified at the N-terminus of the deduced polypeptide. Previous reports suggest that acidic amino acid residues are located near both the N- and C-termini. This acidic nature of prismalin-14 is similar to that of many other macromolecules isolated and characterized from various biominerals and is supposed to be essential for direct interactions with calcium ions and/or the surface of calcium carbonate crystals (Fujisawa *et al.*, 1996; Gilbert *et al.*, 2000). Prismalin-14 showed an ability to bind calcium ions and showed inhibitory activity on calcium carbonate crystallization by Suzuki *et al.* (2004). Tkatchenko *et al.* (2001) and Whitbread *et al.* (1991) reported that prismalin-14 has a Gly/Tyr-rich region. The amino acid sequence of this region is similar to that of keratin, produced by vertebrates to some extent, and prismalin-14 shares common functions with keratin as an extracellular matrix protein, although the functions of other parts of prismalin-14 are completely different. This region may also form the structural motif termed as the glycine loop. The glycine loop may contribute to the elasticity and flexibility of the molecular conformation (Steinert *et al.*, 1984; Zhang *et al.*, 2003).

5.1.3.3 N19

N19 is one of the predominant proteins found in the water-insoluble fraction of the nacreous layer. This 19 kDa protein was first isolated by Yano *et al.* (2007) from the nacreous layer of the pearl oyster *Pinctada fucata*, and was named N19. They suggested that N19 is localized in the nacre, and plays a negative regulatory role in calcification in the pearl oyster. N19 play important roles in governing both pearl production and nacreous shell growth, and the N19 gene should be in a deeper association with the pearl formation than any other known nacreous shell matrix

protein genes (Yano *et al.*, 2007; Wang *et al.*, 2009). In the present study, partial ORF region of N19 gene was identified and confirmed by similarity search in NCBI BLAST analysis. The primer was designed for the expression study and to investigate their role during pearl formation. The present study revealed that N19 gene was constitutively expressed in mantle tissue which indicates its role in pearl formation. The amplification of the target genes with the cDNA synthesized from the total RNA indicates the expression of the candidate genes in Indian Pearl oyster.

5.2 Gene Expression

5.2.1 Antioxidant Genes

While the reactive oxygen species (ROS) produced by the respiratory burst of haemocytes can kill the invading pathogen, excess of it may cause the degradation of the host's biomolecules (Mates *et al.*, 2000). As a safeguard against the accumulation of ROS, several enzymatic or nonenzymatic antioxidants do exist in the body which play important role in maintaining normal cellular physiology (Mates *et al.*, 1999). In order to study the time dependent expression level of three antioxidant genes in the candidate species viz., *P. fucata*, LPS challenge followed by semiquantitative PCR analysis of the genes was carried out. LPS, the principal component of the cell wall of gram-negative bacteria, acts as a powerful stimulator of innate immunity (Ulevitch and Tobias, 1995; Lemaitre, 1996). Some marine invertebrates such as the Atlantic horseshoe crab and the giant African snail are highly responsive to LPS (Biswas and Mandal, 1999; Iwanaga, 2002). In the present study semi-quantitative PCR estimation revealed that LPS stimulation significantly increased mRNA expression of SOD, GPX and GST in the haemocytes in a time-dependent manner.

SOD is the first line of defense against superoxide radicals generated in haemocytes. Previous reports show that pathogens or environmental pollutants can induce expression of cytoplasmic Cu/Zn SODs in invertebrates. Various expression patterns of cytoplasmic Cu/Zn SOD have been reported which might be depending on the species and the challenging condition. In all cases significantly higher levels of expression were detected. For example, in giant freshwater prawn *Macrobrachium rosenbergii,* the cytoplasmic Cu/Zn SOD expression in haemocytes was upregulated after injection with bacterial suspension of *Lactococcus garvieae* (Cheng *et al.*, 2006). In bumble bee *Bombus ignites*, the cytoplasmic Cu/Zn SOD expression was upregulated after LPS injection (Choi *et al.*, 2006). In oyster *C. gigas*, mRNA expression of cytoplasmic Cu/Zn SOD increased after 7 days exposure to hydrocarbons (Boutet *et al.*, 2004). The present study revealed that SOD mRNA expression got up-regulated over the time, and showed a significant increase 4 h after exposure to LPS, compared to the control. The level further increased to reach the maximum at 8 h post treatment and then dropped to basal levels at 36 h.

GPX enzymes constitute the second line of defense against lipid peroxidation, preventing the breakdown of lipid hydroperoxide which would otherwise initiate new radicals (Lawrence and Burk, 1976; Ren *et al.*, 1997; Bao and Williamson, 2000; Cossio-Bayugar, *et al.*, 2005). In the present study, GPX mRNA expression had increased from 4 hour onwards, compared to the control, and reached maximal levels at 8 h, when it was significant. It is likely that these temporal changes in GPX expression correspond with increased ROS production, and the ensuring detoxification of the ROS might have resulted in the decrease of GPX-mRNA levels from 8 h to 12 h. Upregulation of GPX following exposure to inducing agents have

been reported in other invertebrates. Shan *et al.* (2011) showed that GPX mRNA expression in Japanese scallop, *Mizuhopecten yessoensis* after exposure to *V. anguillarum*, increased from 3 h onwards and reached maximal levels at 12 h before declining to the basal levels. Mu *et al.* (2010) also had reported significant up-regulation of GPX resulting in increased mRNA expression in scallop *Chlamys farreri* after challenge with *L. anguillarum*.

GSTs are a group of multifunctional enzymes that reduce organic hydroperoxides, or catalyse the conjugation of glutathione to a large variety of electrophilic alkylating compounds, including hydroxyl alkenals, thereby protecting the cell against their potential toxicity (Forman *et al.*, 2009). The increased GST mRNA levels from 4 h post LPS challenge and reaching the maximum at 8 h in the present study is indicative of the role of GST in the immune response of pearl oysters. Similar observations were reported by Park *et al.* (2009) while searching for biomarkers. The authors showed increasing mRNA expression of the pi, rho, and sigma class GSTs in Antarctic bivalves, *Laternula elliptica* following exposure to PCB. Zhao *et al.* (2010) observed that GST mRNA expression in crab, *Eriocheir sinensis* was significantly upregulated at 6 h after *Aeromonas hydrophila* challenge and dropped to basal levels at 12 h, suggesting that GST is important in initiating the host response in acute infection.

The present study showed that messenger RNA levels of GPX and GST increased almost parallel to that of SOD reaching a maximum at 8 h, and thereafter decreasing slowly to control levels. At the maximum, the relative mRNA expression of SOD, GPX and GST increased to 48%, 34% and 34% over the control, respectively. The mRNA expression levels of all three antioxidant enzymes peaked

at 8 hours post challenge; SOD attained higher expression level earlier than GPX and GST, evidenced by the significance of SOD increase at 4 hours post challenge. The high level of GPX expression lasted longer (12 h) when the decline was slow. Assuming that mRNA expression reflects the timing of enzymatic action, it can be suggested that during respiratory burst, SOD is the first enzyme to react which is followed in short succession by GST and GPX. There is, however, a broad time window in which all three enzymes act in concert eliminating the different molecular forms of reactive oxygen species.

In conclusion, antioxidant genes are constitutively expressed but can also be induced, enabling them to play critical roles in the detoxification of ROS during the respiratory burst. As filter feeders, pearl oysters are exposed constantly to various pathogenic bacteria naturally present in the microflora of coastal environments, and the basel level of expression noticed in the unchallenged animals indicate their preparedness to face them. Under experimental conditions elevated levels of expressed antioxidant genes peaked at 8 hours after challenge. Under aqua-cultural conditions elevated levels of antioxidant enzymes can be of diagnostic value, as a measure of stress on the pearl oyster in the ponds.

5.2.2 Pattern Recognition Receptor Protein Genes (PRP)

A critical step in the immune response is the identification of an invading organism as foreign. This recognition step involves interactions between microbial structural motifs and host receptors. This immune recognition process is being carried out by the innate immune receptors called PRPs. In oysters, haemocytes are responsible for cell-mediated defense. A major defense exhibited by haemocytes involves the direct phagocytosis of antigens. During phagocytosis, the haemocyte

recognizes and binds to an antigen by the presence of specific lectins either in the haemolymph or in the membrane of the haemocyte (Ford and Tripp, 1996). Though these lectins cannot destroy foreign matter, they are involved in the recognition by different immune modulators leading to its destruction.

In order to study the time dependent expression level of three PRP genes, LPS challenge followed by semiquantitative PCR analysis of the candidate genes was carried out, and it was observed that the mRNA level of F-type lectin in the challenged animals increased, compared to the control, and reached maximal level at 4 h and then gradually decreased over the time. This may be due to the combined effect of progressive recognition of the bacterial LPS and the clearance of LPS by the activated cellular or humoral immune responses concurrently taking place. In the previous reports, various expression patterns of F-type lectin occurring in other invertebrate animals have been reported. In Japanese sea perch (*Lateolabrax japonicus*), upregulation of mRNA expression of F-type lectin at 4 h after LPS stimulation was reported, and from the 6 h the expression level decreased (Qiu *et al.*, 2011). In *P. martensii*, the expression level of F-type lectin significantly increased at 3 h post challenge and then decreased gradually over the time (Chen *et al.*, 2011). The basal level of expression in unchallenged animals and substiantial increase of expression following challenge may indicate that it is a constitutive as well as inducible acute-phase protein, and maintains the animal in a constant state of readiness by providing immediate detection of an invading infectious threat.

Among the PRPs, galectin is important as it is used for recognizing β-galactoside ligand of the pathogens by their conserved carbohydrate recognition domains (CRD) (Barondes *et al.*, 1994), and play crucial roles in the innate

immunity. Up-regulation of invertebrate galectin induced by bacteria, virus, fungi or parasites has been reported in oyster (Zhang *et al*., 2011a), amphioxus (Yu *et al*., 2007) and clam (Kim *et al*., 2008). Lectin–glycan interactions are ubiquitous and essential to biological systems. In bay scallop (*Argopecten irradians*), up-regulation of AiGal2 leading to increased the mRNA expression level after *Vibrio anguillarum* or *Micrococcus luteus* challenge was reported by Song *et al*. (2011). The present study has also revealed that the galectin mRNA expression increased following LPS challenge, and reached maximal levels at 8 h, and then dropped by 36 h. This is in agreement with the report of Zhang *et al*. (2011b) who found that the expression of galectin in *P. fucata* was significantly up-regulated between 8 h and 12 h after bacterial challenge. Fermino *et al*. (2011) reported that glycan-binding protein, galectin (Gal 3) secreted by activated macrophages and mast cells at inflammation sites play an important role in inflammatory diseases caused by bacteria and their products such as lipopolysaccharides (LPS). Infection by gram negative bacteria result in LPS-galectin interactions, and the galectin could serve as a sensor to detect small amounts of LPS and allow it to efficiently activate recruited neutrophils. These results suggest that galectin is involved in immune defense against a broad- spectrum of bacteria and their products.

LGBP is another important member of the PRPs in invertebrate which displays various biological functions. In scallop *Chlamys farreri,* LGBP gene expression was up-regulated initially after stimulation by *V. anguilarum* and subsequently reduced to the normal level (Su *et al*., 2004). Zhang *et al*. (2010) reported that LGBP gene expression was up-regulated at 8h and 12 h after bacterial and LPS stimulation in *P. fucata*. LPS could up-regulate the mRNA level of LGBPs

in several marine invertebrates including kuruma shrimp (Lin *et al.*, 2008), crayfish (Lee *et al.*, 2000) and disk abalone (Nikapitiya *et al.*, 2008). In the present study, the expression of LGBP gene was significantly increased at 8 h after LPS stimulation and then declined gradually.

In conclusion, the PRP genes are not only constitutively expressed genes but can also be induced by LPS, enabling them to play critical role in innate immune defense of pearl oyster, *P. fucata*. The overall data in the present study revealed that when a foreign object enters the body, the PRP gene shows an increasingly higher level of transcription. At the maximum the relative mRNA expression of F-type lectin, galectin and LGBP increased to 50%, 30% and 24% over the control, respectively. This is to be expected since it has been suggested that PRP functions by recognizing PAMP and may activate different immune genes to defend against these pathogens. Hence, information on this PRP in *P. fucata* may be useful in studies of PRPs in other marine invertebrates.

5.2.3 Expression of pearl forming genes following Mabe implantation

In the present study time-dependent expression analyses was carried out in three genes viz., nacrein, prismalin-14, and N19 which are reported to be involved in the bio-mineralization process of pearl formation.

Miyamoto *et al.* (1996) who purified a matrix protein from the nacreous layers of the pearl from the pearl oyster *P. fucata* named it as nacrein, and reported that it is involved in pearl formation. Nacrein has been identified in various kinds of molluscs (Norizuki and Samata, 2008) and appears to be conserved in the families Pteriidae and Turbindae (Miyamoto *et al.*, 2005).

In the present study the expression of nacrein gene (as indicated by the nacrein mRNA level) have shown an up-regulation soon after Mabe implantation. The increase was 53% more than the control at day 10. The increased level of nacrein mRNA in the experimental oysters was significantly higher than the control up to 30 days. The expression on the 30th day was only marginally lower than the 10th day level. Steep decrease in the nacrein level in the present study started only from the 40thday of Mabe implantation. Inoue *et al.* (2011) has reported an early expression of nacrein gene in *P. fucata* (15 days) following implantation.

Miyamoto *et al.* (1996) showed that nacrein contains a carbonic anhydrase (CA) domain and a number of characteristic Gly-Xaa-Asn repeats. They suggested that the CA domain plays a role in calcium carbonate crystal formation of the nacreous layer, and proposed a model for the nacreous layer formation controlled by nacrein. Medakovic (2000) described high CA activity preceding biomineralization in *Mytilus edulis*.

Although there is increasing agreement that the CA domain is involved in the biomineralization of shells, the underlying mechanism is not fully understood. It is thought that the carbonic anhydrase (CA) domain catalyzes bicarbonate-formation and supplies bicarbonate ions to form $CaCO_3$ crystals (Miyamoto *et al.*, 1996, 2005). The enhanced expression of nacrein observed in the present study probably indicate the high CA activity during the biomineralization process taking place during the Mabe pearl formation following the implantation, taking the cue from Medakovic (2000) who observed increased CA activity in *Mytilus edulis* during the process of biomineralization.

Pearl has an inner "prismatic-layer and an outer "nacreous-layer"over the nucleus. Generally, in the early stages of pearl formation, the prismatic layer is first formed over the nucleus, followed by the nacreous layer formed on the prismatic layer (Wada, 1962; Hongyan *et al.* 2007). Inoue *et al.* (2011) reported that changes in gene expression are closely related to the layer types (nacreous layer or prismatic layer) on the nucleus. Nacrein presumably participates in the formation of both the nacreous and prismatic layers (Kono *et al.*, 2000), and hence its early upregulation which is sustained for longer period.

Prismalin-14 is a matrix protein capable of interacting with the calcium carbonate and is associated with calcification of the prismatic layer. Suzuki *et al.* (2004) suggested that prismalin-14 is the main one involved in the prismatic layer. Suzuki and Nagasawa (2007) suggested that Prismalin-14 bind to chitin through its chitin-binding site and participate in calcium carbonate crystal formation through an acidic region on the protein surface. In the present study it can be seen that (Fig.4.28) endogenous mRNA expression increases significantly in the initial days. The expression at day 10 was higher compared to the day 30 and 40 expression. This suggests that the prismalin-14 participate in the early phase of pearl formation shortly after Mabe implantation when the prismatic layer is being formed. The present result is in agreement with the report of Inoue *et al.* (2011) who found that highest prismalin-14 gene expression of the round pearl implanted into the oyster mantle was at day 10. This might indicate that prismalin-14 is the key component of pearl biominerlization.

Gene expression pattern for nacrein and prismalin-14 in the early phase show a common trend with highest mRNA levels observed at day 10. Since nacrein participates in the formation of both the nacreous and prismatic layers (Kono *et al.*, 2000), its enhanced expression is sustained during the nacrein layer formation also, unlike prismalin -14. Inoue *et al.* (2010) reported that the quality of pearl is determined by the ratio of the thickness of the lower prismatic layer to that of the upper nacreous layer, and suggested that inhibition of the prismatic layer in the early stages of pearl formation will improve pearl quality. It is well known that the nacreous pearl is of higher quality than the prismatic pearl.

Wang *et al.* (2009) observed that N19 gene is the only gene that exhibited a high expression level in the pearl sac, which was even higher than that in the pallial mantle, indicating that N19 plays an important role in governing both pearl production and nacreous shell growth. He has suggested that the N19 protein have a more profound association with pearl formation than any other known nacreous shell matrix protein (NSMP). It was reported that N19 protein plays an important role in $CaCO_3$ precipitation (Yano *et al.*, 2007).

In the present study only a slight increase in the expression of N19 gene was observed at day 10 in the implanted animals compared to control, whereas, at day 30 gene expression of N19 was significantly higher than on day 10, indicating its late action. On the 40^{th} day there was only a slight decrease in expression when compared to day 30. This indicates that the expression of the N19 gene for participation in pearl formation starts 10 days after implantation. The peak of mRNA expression, and therefore, the main activity occurs by 30^{th} day, which is in agreement with a recent study of nacreous shell matrix proteins in *P. fucata* by Liu *et al.* (2012) suggesting

that significant expression of N19 gene in the pearl sac occurs by day 30–35 after implantation.

The expression of nacreous layer forming genes in the present study support the view by Liu *et al.* (2012) that temporal expression of various matrix protein genes are involved in the nacreous layer deposition , and is crucial for pearl development.

In conclusion, the present study revealed the relative expression of three genes involved in pearl formation. The general trend was an up-regulation between day 10 and day 30 post Mabe implantation. Expression levels of nacrein, prismalin-14 and N19 showed an up-regulation with variations at different time points. Nacrein and prismalin-14 had an early start of expression, and reached highest level at day 10 after implantation, and then gradually decreased until day 40. On the other hand expression level of N19 was highest at day 30. Based on the knowledge on the time dependent expression of different genes involved in pearl formation, suitable genetic intervention strategies like promoting nacreous layer forming genes and silencing prismatic layer forming genes during pearl formation could be developed for stimulating the production of high quality nacreous pearls.

Chapter **6**

SUMMARY AND CONCLUSION

There are a number of genes involved in the regulation of functional process in marine bivalves. In the case of pearl oyster, some of these genes have major role in the immune/defence function and biomineralization process involved in the pearl formation in them. As secondary filter feeders, pearl oysters are exposed to various kinds of stressors like bacteria, viruses, pesticides, industrial wastes, toxic metals and petroleum derivatives, making susceptible to diseases. Environmental changes and ambient stress also affect non-specific immunity, making the organisms vulnerable to infections. These stressors can trigger various cellular responses in the animals in their efforts to counteract the ill effects of the stress on them. These include the expression of defence related genes which encode factors such as antioxidant genes, pattern recognition receptor proteins etc. One of the strategies to combat these problems is to get insight into the disease resistance genes, and use them for disease control and health management. Similarly, although it is known that formation of pearl in molluscs is mediated by specialized proteins which are in turn regulated by specific genes encoding them, there is a paucity of sufficient information on these genes.

In view of the above facts, studies on the defence related and pearl forming genes of the pearl oyster assumes importance from the point of view of both sustainable fishery management and aquaculture. At present, there is total lack of sufficient knowledge on the functional genes and their expressions in the Indian pearl

oyster *Pinctada fucata*. Hence this work was taken up to identify and characterize the defence related and pearl forming genes, and study their expression through molecular means, in the Indian pearl oyster *Pinctada fucata* which are economically important for aquaculture at the southeast coast of India.

The present study has successfully carried out the molecular identification, characterization and expression analysis of defence related antioxidant enzyme genes and pattern recognition proteins genes which play vital role in the defence against biotic and abiotic stressors. Antioxidant enzyme genes viz., Cu/Zn superoxide dismutase (Cu/Zn SOD), glutathione peroxidise (GPX) and glutathione-S-transferase (GST) were studied. Concerted approaches using the various molecular tools like polymerase chain reaction (PCR), random amplification of cDNA ends (RACE), molecular cloning and sequencing have resulted in the identification and characterization of full length sequences (924 bp) of the Cu/Zn SOD, most important antioxidant enzyme gene. BLAST search in NCBI confirmed the identity of the gene as Cu/Zn SOD. The presence of the characteristic amino acid sequences such as copper/zinc binding residues, family signature sequences and signal peptides were found out. Multiple sequence alignment comparison and phylogenetic analysis of the nucleotide and amino acid sequences using bioinformatics tools like BioEdit, MEGA etc revealed that the sequences were found to contain regions of diversity as well as homogeneity. Close evolutionary relationship between *P. fucata* and other aquatic invertebrates was revealed from the phylogenetic tree constructed using SOD amino acid sequence of *P. fucata* and other invertebrates as well as vertebrates.

As the pearl oysters are more prone to oxidative stress owing to the nature of their habitat, the study was extended towards other antioxidant enzyme genes like

glutathione peroxidise (GPX) and glutathione-S-transferase (GST) in them. The molecular identification and characterization of complete coding sequences of both the genes were carried out. Sequence information of 1201 bp and 1652 bp long segments of the GPX and GST genes were generated and the homology search confirmed their identity. Amino acid sequence deduced from the complete coding region revealed the characteristic features of the enzymes. The active site of the GPX enzyme was found to contain a selenocysteine encoded by a TGA, which is the main characteristic feature of the selanoprotein family. This Se-GPX contains a characteristic GPX signature sequence motif and glutamine & tryptophan residues, which are responsible for fixation of selenium. The main characteristic feature of GST is that it formed by cysteine-containing tetramer and residues of G-site & H-site. Homology search of the sequences of these antioxidant enzyme genes with the available sequences of NCBI database was carried out. Sequences of both of these genes were found to show homology with GPX and GST sequences of *P. fucata* from Japan deposited in NCBI.

Pattern recognition protein genes act as biosensors in the activation of innate immune responses in both vertebrates and invertebrates. Three pattern recognition receptor proteins of *P. fucata* viz., F-type lectin, galectin and LGBP were taken for the study. Molecular identification and characterization of full-length sequence of F-type lectin gene with a size of 891 bp was carried out using molecular tools. The NCBI domain search revealed that it possesses only one carbohydrate recognition domain. The presence of the characteristic amino acid sequences such as six conserved cysteine residues and three residues for sugar binding have confirmed the identity of the F-type lectin gene. Phylogenetic analysis of the amino acid sequences

of the F-type lectin placed *P. fucata* of the present study and *P. martensii* in one cluster, while the other molluscs formed different clusters indicating relationship of varying degrees with each other.

Molecular identification and the partial characterization of other PRP genes of importance viz., galectin, and LGBP, were also carried out in the present study. Galectin with 1750 bp and LGBP with 1740 bp size got amplified from *P. fucata* and subsequent sequencing confirmed their identity. The main characteristic feature of galectin is that it possessed four carbohydrate recognition domains (CRDs) with 7 conserved carbohydrate binding sites in each CRDs. The amino acid sequence of LGBP gene of the pearl oyster in the present study contained a protein kinase C phosphorylation site, a modified cell adhesive site, polysaccharide binding motif, LPS-binding site and a β-1, 3-linkage recognition motif. Two potential "N-linked glycosylation" sites were also identified in the sequence. The bioinformatics procedures like multiple alignment comparison and phylogenetic analysis of nucleotide and amino acid sequences using the tools like BioEdit, MEGA etc. were carried out during the study. The sequences of both of these genes were found to show homology with galectin and LGBP sequences of *P. fucata* from China deposited in NCBI.

Pearl forming genes taken up for molecular identification and characterization in this study are nacrein, prismalin-14 and N-19. Initial PCR using the cDNA and nacrein gene specific primers resulted in the amplification of a 984 bp gene segment which on sequencing confirmed to be of nacrein gene. RACE-PCR successfully amplified the 3` and 5` regions, and revealed the sequence of 1444 bp segment of the nacrein gene. The amino acid sequences deduced from these ORF revealed thirty six

residues of the active site and three zinc binding histidine residues in them. In case of prismalin-14, RACE-PCR product on cloning and sequencing revealed the sequence of a 400 bp segment of the gene, which consisted of the ORF region and part of UTR. The nucleotide sequences of prismalin-14 have shown 100% homology with the prismalin-14 gene sequence of *P. fucata* available in the NCBI GenBank. The ORF encodes a polypeptide of 121 amino acids. A signal peptide was identified at the N-terminus of the deduced polypeptide by signal P program. Molecular identification of the gene coding for pearl forming gene N19 was also carried out in this study. RACE-PCR did not successfully work in N19 gene.

Relative gene expression studies on antioxidant genes and PRP genes were carried out by exposing acclimated pearl oysters to lipopolysaccharide (LPS) for inducing the gene expression. Following LPS injection, mRNA levels of GPX and GST increased almost parallel to SOD mRNA levels, reaching the maximum at 8 h, and thereafter, decreased slowly to the level of the control. In the case of PRP genes, mRNA level of F-type lectin in the challenged animals increased significantly with respect to the control and reached maximal levels at 4 h and then gradually decreased over the time. The mRNA expression of galectin and LGBP increased substantially with respect to the control and reached maximal levels at 8 h and then dropped to reach below the control level by 36 h.

The time-dependent expression analyses of gene involved in the biomineralization process of pearl formation viz, nacrein, prismalin-14 and N19 were carried out. The expression studies on pearl forming genes were carried out following the implantation of Mabe into acclimated pearl oyster. Expression levels of nacrein, prismalin-14 and N19 showed an up-regulation with variations at different

time points. Expression of nacrein reached highest level at day 10 after the implantation, and then gradually decreased until day 40. In the case of prismalin-14 gene expression, a significant increase in the experimental oysters at day 10 compared to the day 30 and 40. Slight increase in the expression of N19 gene was observed at day 10 in the implanted animals compared to control, whereas, at day 30 gene expression of N19 was significantly higher than on day 10, indicating its late action. On the 40^{th} day there was only a slight decrease in expression when compared to day 30. Based on the time dependent expression of different genes involved in pearl formation, suitable strategies can be developed. Promoting nacreous layer forming genes and silencing prismatic layer forming genes during pearl formation could stimulate the production of high quality nacreous pearl.

The general trend of expression of the genes under study was an up-regulation following induction. These genes could be used as biomarkers for monitoring the aquatic environmental health and also used as biomarker for selective breeding to develop strains with enhanced stress tolerance and disease resistance. The knowledge on these genes could also be used to produce genetically engineered oysters for pearl formation and with enhanced disease resistance.

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Sl no	Title	Status
Journal Articles		
1	**A. Anju**, J. Jeswin, P.C Thomas and K.K Vijayan. 2013. Molecular cloning and characterization of F-type lectin from pearl oyster *Pinctada fucata*. **Fish and shellfish Immunology**. 35: 170-174.	Published
2	**A. Anju**, J. Jeswin, P.C. Thomas, M.P. Paulton and K.K. Vijayan. 2013. Molecular cloning, characterization and expression analysis of cytoplasmic Cu/Zn-Superoxid Dismutase (SOD) from pearl oyster *Pinctada fucata*. **Fish and Shellfish Immunology**. 34: 946-950.	Published
3	**A. Anju**, J. Jeswin, P.C. Thomas, M.P. Paulton and K.K. Vijayan. 2014. mRNA expression studies on antioxidant genes of pearl oyster *Pinctada fucata* in response to lipopolysaccharide (LPS) challenge. **Indian Journal of Fisheries**.	Under review
4	**A. Anju**, J. Jeswin, P.C. Thomas, M.P. Paulton and K.K. Vijayan. Messenger RNA expression of pattern recognition receptor proteins (PRP) in the pearl oyster *Pinctada fucata* in response to LPS stimulation. **Indian Journal of Fisheries**.	Under review
Papers in International Conferences		
5	**Anju Antony,** Jeswin Joseph, M.P Paulton, P.C Thomas and K.K Vijayan (2011). Expression analysis of genes involved in the biomineralization of mabe pearl. Asia Pacific Aquaculture Conference organized by World Aquaculture Society and College of Fisheries, Kerala Agricultural University, India. January 17-21, 2011.	Presented
6	**Anju Antony,** Jeswin Joseph, M.P Paulton and P.C Thomas and K.K Vijayan (2011). Expression analysis of antioxidant genes involved in the innate immunity of pearl oyster *Pinctada fucata*. Indian Fisheries Forum organized by Central Marine Fisheries Research Institute, India. December 19-23, 2011.	Presented

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Short communication

Molecular cloning, characterization and expression analysis of F-type lectin from pearl oyster *Pinctada fucata*

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ABSTRACT

F-type lectin is an important type of pattern recognition receptor that can recognize and bind carbohydrate moieties on the surface of potential pathogens through its carbohydrate recognition domains (CRDs). This paper reports the cloning of an F-type lectin (designated as pfF-type lectin) from the pearl oyster (*Pinctada fucata*) using rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of this pfF-type lectin contains an open reading frame (ORF) of 588 bp coding for196 amino acids. A signal peptide at the N-terminus of the deduced polypeptide was predicted by the signal P program and the cleavage site is located between the positions of $Gly^{19}$and Tyr^{20}. Conserved domain search at NCBI revealed the pfF-type lectin domain extends from $Lys^{55}$to Val^{192}. Semi-quantitative analysis in adult tissues showed that the pfF-type lectin mRNA was abundantly expressed in haemocytes and gill and rarely expressed in other tissues tested. After challenge with lipopolysaccharide (LPS), expression of pfF-type lectin mRNA in haemocytes was increased, reaching the highest level at 4 h, then dropping to basal levels at 36 h. These results suggest that F-type lectin play a critical role in the innate immune system of the pearl oyster *P. fucata*.

1. Introduction

The innate immune system is the first line of defense that recognizes conserved molecular patterns present on microorganisms. Microorganisms have β-1,3-glucan (bG), lipopolysaccharide (LPS) and peptidoglycan (PG) on their surface, known as pathogen-associated molecular patterns (PAMP) and can be recognized by pattern recognition receptor (PRP) of the host [1]. Once invading pathogens gain entry into the body of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses. Animal lectins can be classified into several families, including C-, F-, P-, and I-type lectins, galectin, pentraxin, and others, based on their primary structure, structural fold, cation requirement etc [2,3]. F-lectin is a type of PRP that can recognize and bind carbohydrate moieties on the surface of potential pathogens through its carbohydrate recognition domains (CRDs). The F-type domain is found in invertebrate species, often within lineage-specific protein contexts.

F-type lectins were first identified and characterized in European eel, *Anguilla anguilla agglutinin* (AAA) that has been used extensively as a reagent in blood typing and histochemistry. F-type lectins have been identified and described as immune recognition molecules in Japanese horseshoe crab (*Tachypleus tridentatus*) [4], Japanese eel (*Anguilla Japonica*) [3], striped bass (*Morone saxatilis*) [5], gilthead bream (*Sparus aurata*) [6], sea bass (*Dicentrarchus labrax*) [7] and pearl oyster (*Pinctada martensii*) [8]. In *Crassostrea* species, F-lectin is the main functional domain of binding for recognition of the egg surface during fertilization [9].

Pearl oyster *Pinctada fucata* is distributed over South coast of India and is the most important bivalve mollusk for seawater pearl production in India. In 1972 the Central Marine Fisheries Research Institute started pearl culture research at natural pearl oyster beds in Tuticorin. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this bivalve species. Recent decline in pearl production is mainly due to mortality of pearl oyster. The reason for high mortality is related to ocean pollution, disease outbreaks and stock degeneration [10,11]. In order to control disease and enhance the yields and quality of seawater pearl, it is necessary to research the innate immune defense mechanisms of pearl oyster, which lack the adaptive immune system. One strategy to combat disease problem is to identify disease resistance genes and use them for genetic improvement of cultured stock. Therefore, the aims of the present study were (1) to determine the nucleotide sequence of F-type lectin from the pearl oyster *P. fucata* and compare its deduced amino acid sequence with the other known F-type lectins; (2) to

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examine the expression of pfF-type lectin in various tissues; and (3) to evaluate its expression after pearl oysters were challenged by LPS.

2. Materials and methods

2.1. Experimental animal

Live individuals of adult *P. fucata* (about 4.5–5.5 cm in shell length and body weight 20–30 g) were collected from the Pearl oyster farm in Tuticurin, and maintained at 25 °C in tanks containing static aerated seawater (0.5 L/oyster) in the laboratory. The seawater was changed every day and the pearl oysters were fed with *Isochrysis galbana* twice daily. Animals were kept 2 weeks for acclimatization before they were used.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from the haemocytes of the adductor muscles using NucleoSpin RNA II reagent (MACHEREY-NAGEL GmbH & Co, Germany) as per the manufacturer's instructions and stored at −80 °C until further use. cDNA was synthesized with iScript cDNA synthesis (Bio-rad) in accordance with the manufacturer's protocols. Finally, the synthesized cDNA was diluted 10 fold (total 200 μL) before being stored at −20 °C. Primers were designed using Beacon designer (Bio-rad version 7.51) from the sequence information of pearl oyster, *P. martensii* [8] available in the data base (GenBank accession HQ199600). Polymerase chain reactions (PCR) were carried out using sense and antisense primers to get open reading frame (ORF) of F-type lectin. The reaction volume of 25 μl consisted of 2.5 μl of 10 × PCR buffer, 0.5 μl of dNTP (10 mM), 1 μl of each primer (10 mM), 18.7 μl of PCR-grade water, 0.3 μl (1U) of Taq polymerase (Sigma Aldrich) and 1 μl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 35cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and the final extension step at 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The PCR product was then eluted and cloned into the pJET vector (fermentas, EU), and used for transformation of competent *Escherichia coli* TOP10 cells. Positive clones were identified as white colonies on LB medium and were used for sequencing in both directions.

The full-length F-type lectin cDNA of *P. fucata* was obtained by the reverse-transcription polymerase chain reaction (RT-PCR) and RACE methods. The 5′ region of the transcript was obtained in 5′-RACE reactions using the SMARTScribe™ Reverse Transcriptase (Clontech) according to the manufacturer's instructions. The primers consisted of GSP1 with the universal primer mix (UPM) (Table 1) for RACE to derive the 5′-terminal untranslated region (UTR). For 3′-RACE, the primers of GSP2 with universal Primer mix (UPM) (Table 1) were used for amplification of the target cDNA. The PCR fragments were subjected to electrophoresis on 1.5% agarose gel to determine length differences. The amplified cDNA fragments were cloned into the pJET vector (Fermentas, EU) following the manufacturer's instructions. Recombinant clones were identified as white colonies on LB (Luria broth) medium and confirmed by colony PCR. Plasmids containing the inserted fragment were used as a template for DNA sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)
For conventional PCR	ATGTATTTATTAACTGTGCTTCTTT
Sense primer	CTACCTTCCCATGACTTCGACCTCGC
Antisense primer	
For RACE PCR	ACATTGTTTGATCCTTTG
GSP1	TGTGGATGGTATAAGTAAT
GSP2	CTAATACGACTCACTATAGGGC
UPM	AAGCAGTGGTATCAACGCAGAGT
For Semi-quantitative PCR	TGGATGGTATAAGTAAT
F-type lectin-F	TCTGTTCGTTATTCTGAT
F-type lectin-R	TATTTCTGCACCGTCTGCTG
GAPDH-F	ATCTTGGCGAGTGGAGCTAA
GAPDH-R	

2.3. Homology analysis

The sequence was analyzed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment was generated using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). Signal peptide prediction was performed by SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [12].

2.4. Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of the selected F-type lectin (Fig. 2) using the neighbour-joining method with MEGA, version 4.1 [13]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.5. Immune challenge

For stimulation with LPS, animals were injected with 50 μl of LPS (*E. coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in PBS (LPS 10 μg ml $^{-1}$) into the adductor muscles of each pearl oyster. The control groups were injected with 50 μl of PBS. At each time point (0, 4, 8, 12, 24 and 36 h), haemolymph was collected from the control group and the LPS stimulation group. Haemolymph samples were withdrawn from the adductor muscles using a syringe and immediately centrifuged at 5000 × g at 4 °C for 10 min to harvest the haemocytes. At each sampling time, five control and five LPS injected individuals were sampled. The haemocyte pellets were immediately used for RNA extraction. The tissues including adductor muscle, gill filaments, mantle, digestive gland, gonad, heart and haemocytes were collected from five healthy individuals to investigate the tissue-specific expression of pfF-type lectin.

2.6. Semi-quantitative PCR

Semi-quantitative PCR was conducted to determine the relative expression of pfF-type lectin in *P. fucata*. The differential expression patterns of the pfF-type lectin in the challenged oysters at different time intervals and the control were quantified based on the gel band intensity using ImageJ analysis software [14]. Primers for semi-quantitative PCR were designed from the known sequences of the *P. fucata* as shown in Table 1. The PCR condition for pfF-type lectin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows: initial denaturation at 94 °C for 3 min, then different cycles of amplification of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. The GAPDH was amplified in PCR reaction as a loading control. The products were analyzed on 2.0% agarose gel. The cycle numbers at half-maximal amplification were used for subsequent quantitative analysis of gene expression, and they are 28 cycles for pfF-type lectin, 25 cycles for GAPDH. The PCR cycles had been optimized so that the target gene and house- keeping gene amplification were at logarithmic phase.

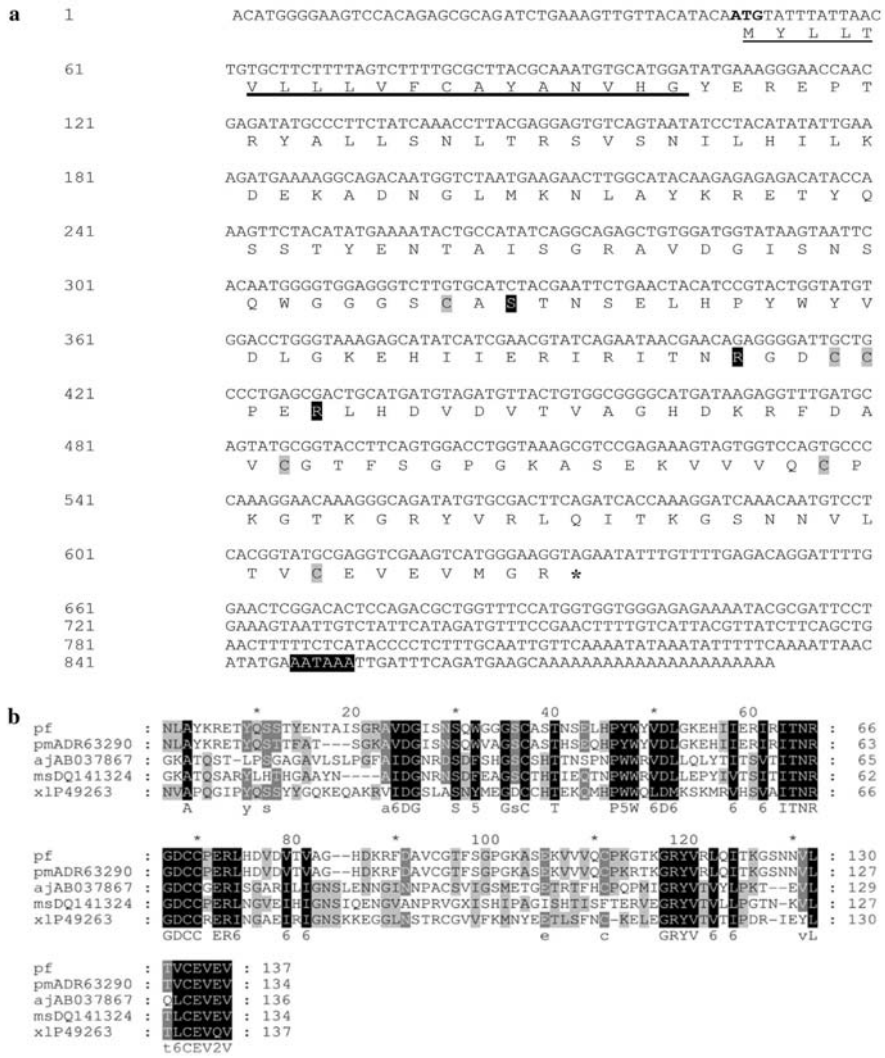

Fig. 1. a) Nucleotide and deduced amino acid sequences of pfF-type lectin. Nucleotide numbers are shown on the left. The start codon is in bold and the termination codon is indicated with asterisk (*). The predicted signal peptide sequence is underlined. Six conserved cysteine residues are highlighted in grey, and three residues for sugar binding are highlighted in black. b) Multiple alignment of the CRD of pfF-type lectin with other members of this family. Amino acid numbers (excluding gap) are shown on the right. Invariant residues are shaded black, conserved residues in ≥75 of sequences are shaded grey with white lettering and those in ≥60 of sequences are shaded grey with black lettering. Pf: *Pinctada fucata*; pmADR63290: *Pinctada martensii*; ajAB037867: *Anguilla japonica*; msDQ141324: *Morone saxatilis*; xlP49263: *Xenopus laevis*.

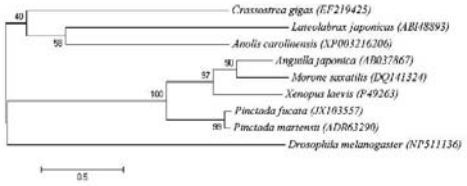

Fig. 2. Phylogenetic analysis of pfF-type lectin from pearl oyster *Pinctada fucata* and other species. The bar (0.5) shows genetic distance and GenBank accession numbers are shown next to each species.

2.7. Statistical analysis

Multiple comparisons using Duncan's test were made to check the differences between the gene expression in the control and challenged oysters using SPSS13.0 software.

3. Result and discussion

3.1. cDNA cloning of F-type lectin

RT-PCR was used to clone ORF fragment of F-type lectin using total RNA extracted from haemocytes of *P. fucata*. A single PCR product of the expected size (588 bp) was obtained. This partial sequence provided the necessary information to obtain an additional 257 bp sequence by 3′RACE, and an additional 46 bp sequence by 5′RACE. Finally, the full-length sequence information of the F-type lectin cDNA was obtained by overlapping the three cDNA sequences. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1 a The full-length pfF-type lectin cDNA is comprised of 891 bp, containing 46 bp in the 5′-terminal untranslated region (UTR), 588 bp in the open reading frame (ORF), 257 bp in 3′-terminal UTR with a poly(A) tail of 21 bp, and a putative polyadenylation consensus signal (AATAAA). The ORF encodes a polypeptide of 196 amino acids. The F-type lectin cDNA sequence and its deduced amino acid sequence were submitted to the NCBI GenBank under accession no. JX103557.

3.2. Sequence alignment and genetic distance analysis among F-type lectin

A signal peptide was identified at the N-terminus of the deduced polypeptide by signal P program and its cleavage site is located between the positions of $Gly^{19}$and Tyr^{20}. Multiple alignment of deduced amino acid sequences (Fig. 1 b) with other closely related F-type lectin sequences showed that seven cysteines (Cys 12, Cys 92, Cys 124, Cys 125, Cys 147, Cys 164 and Cys 188) are present in the mature F-type lectin and six of them (Cys 92, Cys 124, Cys 147, Cys 164 and Cys 188) are conserved, as in *P. martensii*. It is believed that these are involved in internal disulfide bonds. Based upon these predictions the calculated molecular mass of the mature protein is 19.5 kDa. This value does not include other post-translational modification, i.e., glycosylations. Conserved domain search at NCBI revealed that the pfF-type lectin domain extends from $Lys^{55}$to Val^{192}. Several reports have indicated that the first carbohydrate-binding residue in F-type lectin is His residue followed by 26 other residues and the RGD motif [6,7,15]. However, this study has indicated that in *P. fucata*, first carbohydrate binding residue is a serine as has been reported in *P. martensii* by Chen et al. [8]. RGD motif is conserved in cell adhesion proteins [16].

BLAST analysis shows that the deduced amino acid sequence of pfF-type lectin has extremely high identity with F-type lectin *P. martensii* (100%). Similarly, it has 71% identity with *Xenopus laevis*, *M. saxatilis*, and *Anolis carolinensis*. Two carbohydrate recognition domains (N-CRD and C-CRD) are present in *M. saxatilis* [5], *D. labrax* [7] and *S. aurata* [6]. However, pfF-type lectin possesses only one domain, which is similar to pmF-lectin of *P. martensii*. The difference of the two-domain versa the one-domain F-type lectin in *P. fucata* is due to a number of insertions and the absence of the fucose-binding His residue like, *P. martensii*.

Phylogenetic relationships of F-type lectin from pearl oysters and other invertebrates and vertebrates were estimated. F-lectin of *Drosophila melanogaster* was used as the out-group. As shown in Fig. 2, F-type lectin of congenic species of pearl oysters *P. fucata* (present study) and *P. martensii* formed one distinct cluster, supported by high brootstrap values (NJ-100%) indicating their close genetic relationship, where as F-lectins from *X. laevis*, *M. saxatilis* and *A. japonica* formed another distinct clade. Interestingly the F-lectin from the edible oyster *Crassostrea gigas* formed a third cluster along with other vertebrate species *Lateolabrax japonicas*, and *A. carolinensis* though with low broodstrap values. PfF-type lectin is very different from the F-lectin in *C. gigas*, it is much shorter and the only good alignment is the center of the F-lectin domain. The study has revealed the closer evolutionary relationship of *P. fucata* with *P. martensii*.

3.3. Tissue distribution of pfF-type lectin mRNA and expression pattern after LPS challenge

RT-PCR was carried out to analyze the distribution of pfF-type lectin mRNA in the adult tissues of the pearl oyster. RT-PCR analyses showed that pfF-type lectin mRNA is abundantly expressed in the gill (4 fold to the least) and heamocytes (5 fold to the least), moderately expressed in the adductor muscle, mantle, gonad, heart and digestive gland (Fig. 3). Haemocytes are considered as the most suitable tissue to analyze the pearl oysters immune function. This is in agreement with the report of Kuchel et al. [17] who found haemocytes defense enzyme expression in *Pinctada impricata*. As

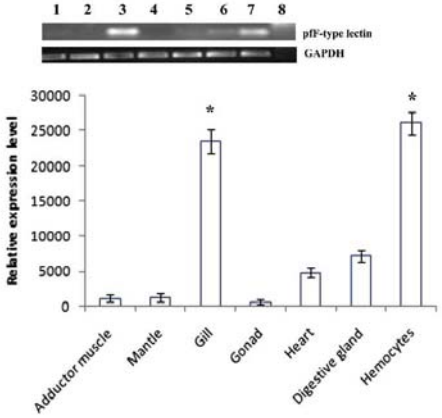

Fig. 3. Distribution of pfF-type lectin mRNA in different adult tissues of pearl oyster. Expression analysis of pfF-type lectin mRNA in different adult tissues of pearl oyster by RT-PCR.GAPDH was used as an internal control. Lane 1: adductor muscle. Lane 2:mantle.Lane 3: gill. Lane 4: gonad.Lane5:heart. Lane 6: digestive gland. Lane7: haemocytes. Lane 8: 100 bp ladder. The values are showed as mean ± S.E. ($N = 5$). Significant differences ($P < 0.05$) are indicated with the asterisk (*).

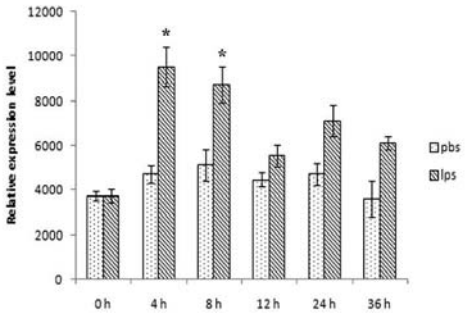

Fig. 4. Temporal expression pattern analysis of pfF-type lectin mRNA in haemolymph of the pearl oyster challenged with LPS. Vertical bars represent the mean ± S.E. ($N = 5$). Significant differences ($P < 0.05$) are indicated with the asterisk (*).

histological studies have revealed the presence of large amount of haemocytes in bivalve gill tissues [18–20], a high expression level in gill is more likely associated with haemocyte abundance.

In order to investigate the immunological functions of pfF-type lectin in pearl oyster, *P. fucata* were measured by RT-PCR following injection with lipopolysaccarides. LPS stimulation significantly increased pfF-type lectin mRNA expression in the haemocytes in a time-dependent manner (Fig. 4). In the present study, mRNA level of pfF-type lectin in the challenged animals increased significantly with respect to the control and reached maximal levels at 4 h and then gradually decreased over time. At the maximum the relative mRNA expression of pfF-type lectin increased to 2.5-fold over control. This may be due to the combined effect of progressive recognition of the bacterial LPS and the clearance of LPS by activating cellular or humoral immune responses. The high level of expression in haemocytes and gill suggest that pfF-type lectin could be involved in the innate immune response. In previous report, various expression patterns of F-type lectin occur in other invertebrate animals. In Japanese sea perch (*Lateolabrax japonicus*), F-type lectin mRNA expression was up-regulated at 4 h after LPS stimulation and from the 6 h the expression level became to decrease [21]. In *P. martensii*, the expression level of F-type lectin was significantly increased at 3 h post challenge and then the expression level decreased gradually over time [8]. As shown in Fig. 4, early response of F-type lectin obtained in the study indicated that it is a constitutive and inducible acute-phase protein and maintains the animal in a constant state of readiness by providing immediate detection of an invading infectious threat.

In conclusion, the full-length cDNA of this pfF-type lectin contains an open reading frame (ORF) of 588 bp coding for196 amino acids. Semi-quantitative analysis in adult tissues showed that the pfF-type lectin mRNA was abundantly expressed in haemocytes and gill and rarely expressed in other tissues tested. After challenge with lipopolysaccharide (LPS), expression of pfF-type lectin mRNA in haemocytes was increased, reaching the highest level at 4 h, then dropping to basal levels at 36 h. These results suggest that F-type lectin play a critical role in the innate immune system of the pearl oyster *P. fucata*.

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Short communication

Molecular cloning, characterization and expression analysis of cytoplasmic Cu/Zn-superoxid dismutase (SOD) from pearl oyster *Pinctada fucata*

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ABSTRACT

Because of its capacity to rapidly convert superoxide to hydrogen peroxide, superoxide dismutase (SOD) is crucial in both intracellular signalling and regulation of oxidative stress. In this paper we report the cloning of a Cu/Zn SOD (designated as pfSOD) from the pearl oyster (*Pinctada fucata*) using rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of this Cu/Zn SOD contains an open reading frame (ORF) of 471 bp coding for 156 amino acids. No signal peptide was identified at the N-terminal amino acid sequence of Cu/Zn SOD indicating that this pfSOD encodes a cytoplasmic Cu/Zn SOD. This is supported by the presence of conserved amino acids required for binding copper and zinc. Semi-quantitative analysis in adult tissues showed that the pfSOD mRNA was abundantly expressed in haemocytes and gill and scarcely expressed in other tissues tested. After challenge with lipopolysaccharide (LPS), expression of pfSOD mRNA in haemocytes was increased, reaching the highest level at 8 h, then dropping to basal levels at 36 h. These results suggest that Cu/Zn SOD might be used as a bioindicator of the aquatic environmental pollution and cellular stress in pearl oyster.

1. Introduction

The superoxide dismutases (SODs) are the first and most important of the antioxidant enzyme defense systems against reactive oxygen species, particularly the breakdown of superoxide anion into oxygen and hydrogen peroxide that prevents generation of highly toxic hydroxyl radicals. Superoxide dismutase (EC 1.15.1.1) is divided into four distinct groups according to their metal content: iron SOD (FeSOD), manganese SOD (MnSOD), copper/zinc SOD (Cu/Zn SOD), and nickel SOD (NiSOD). MnSOD and Cu/Zn SOD are found in both prokaryotes and eukaryotes, FeSOD is found in prokaryotes and plants [1], and NiSOD has recently been purified from several aerobic soil bacteria of Streptomyces [2]. SOD is one of the sensitive biomarker to indicate organisms being under stress [3,4].

Cu/Zn SOD is very important because of its physiological function and therapeutic potential. This enzyme requires Cu and Zn for its biological activity; the loss of Cu results in its complete inactivation, and is the cause of multiple diseases in human and animals [5–9]. There are two types of Cu/Zn SOD, extracellular Cu/Zn SOD with an N-terminal signal peptide for secretion, and cytoplasmic Cu/Zn SOD without signal peptide [10–13].

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Both the transcription and enzyme activity of Cu/Zn SOD are sensitive to stresses such as exposure to heavy metals or biocides, like tributyltin, heat shock, and anoxia [3,4,14,15]. Cu/Zn SOD genes have been cloned from several aquatic species including frog, *Xenopus laevis* [16], grouper, *Epinephelus malbaricus* [17], Pacific oyster, *Crassostrea gigas* [18] and the abalones *Haliotis discus discus* [4]. So far, the Cu/Zn SOD from pearl oysters has not been elucidated. The present study is the first report of the characterization of Cu/Zn SOD in pearl oyster *Pinctada fucata*.

Pearl oyster, *P. fucata* is distributed along the South coast of India and is the most important bivalve mollusc for seawater pearl production in India. In 1972, the Central Marine Fisheries Research Institute started pearl culture research at natural pearl oyster beds in Tuticorin. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this bivalve species. Recent decline in pearl production is mainly due to mortality of pearl oyster. The cause for high mortality is related to ocean pollution, disease outbreaks and stock degeneration [19,20]. In order to control disease and enhance the yields and quality of seawater pearls, it is necessary to study the innate immune defense mechanisms of pearl oysters, which lack the adaptive immune system. One major strategy to combat disease problem is to identify disease resistance genes and employ them for genetic improvement of cultured stock. Therefore, the aims of

the present study were (1) to determine the nucleotide sequence of Cu/Zn SOD from the pearl oyster *P. fucata* and compare its deduced amino acid sequence to other known Cu/Zn SOD proteins; (2) to examine the expression of pfSOD in various tissues; and (3) to evaluate pfSOD expression after LPS challenge.

2. Materials & methods

2.1. Animal culture

Live individuals of adult *P. fucata* (about 4.5–5.5 cm in shell length and body weight 20–30 g) were collected from the Pearl Farm in Tuticurin, and maintained at 25 °C in tanks containing static aerated seawater (0.5 L/oyster) in the laboratory. The seawater was changed every day and the pearl oysters were fed with *Isochrysis galbana* twice daily. Animals were kept 2 weeks for acclimatization before they were used.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from the haemocytes of the adductor muscles using NucleoSpin RNA II reagent (MACHEREY-NAGEL GmbH & Co, Germany) as per the manufacturer's instructions and stored at −80 °C until further use. cDNA was synthesized with iScript cDNA synthesis (Bio-rad) in accordance with the manufacturer's protocols. Finally, synthesized cDNA was diluted 10 fold (total 200 µl) and stored at −20 °C. Primers were designed using Beacon designer (Bio-rad) from the sequence information of Pacific oyster *C. gigas* available in the data base (GenBank accession AJ496219). Polymerase chain reactions (PCR) were carried out using sense and antisense primers (Table 1) to obtain the open reading frame (ORF) of pfSOD. The reaction volume of 25 µl consisted of 2.5 µl of 10× PCR buffer, 0.5 µl of dNTP (10 mM), 1 µl of each primer (10 mM), 18.7 µl of PCR-grade water, 0.3 µl (1 U) of Taq polymerase (Sigma Aldrich) and 1 µl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and the final extension step of 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (EB). The PCR product was then eluted and cloned into the pJET vector (Fermentas, EU) and transformed into competent *Escherichia coli* TOP10 cells. Positive clones were identified as white colonies on LB (Luria broth) agar and were used for sequencing in both directions.

The full-length Cu/Zn SOD cDNA of *P. fucata* was obtained by the reverse-transcription polymerase chain reaction (RT-PCR) and RACE methods. The 5′ region of the transcript was obtained in 5′-RACE reactions using the SMARTScribe™ Reverse Transcriptase (Clontech) according to the manufacturer's instructions. The primers were the pfSOD-specific antisense primer GSP1in combination with the universal primer mix (UPM) (Table 1) for RACE to derive the 5′-terminal untranslated region (UTR). For 3′-RACE, the pfSOD-specific sense primerGSP2and the universal primer mix (UPM) (Table 1) were used for amplification of the target cDNA. The PCR fragments were subjected to electrophoresis on 1.5% agarose gels to determine length differences. The amplified cDNA fragments were cloned into the pJET vector (Fermentas, EU) following the manufacturer's instructions. Recombinant clones were identified as white colonies on LB (Luria broth) agar and confirmed by colony PCR. Plasmids containing the inserted fragment were used as a template for DNA sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)
For conventional PCR	
Sense primer	ATGTCATCTGCTCTGAAGGCCGT
Antisense primer	CTACTTGGTGATACCGATCACTCCACA
For RACE PCR	
GSP1	GGTGATCCTGGAGCCTCTTGG
GSP2	AATCAGCATCACCGACAA
UPM mix	AAGCAGTGGTATCAACGCAGAGT–CTAATACGACTCACTATAGGGC
For RT-PCR	
pfSOD-F	AATCAGCATCACCGACAA
pfSOD-R	TTGGTGATACCGATCACTCCACA

2.3. Homology analysis

The sequence was analysed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment was generated using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). Signal peptide prediction was performed by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [21] Protein family signatures were identified using InterPro program (http://www.ebi.ac.uk/InterProScan/).

2.4. Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of the selected Cu/Zn SODs (Fig. 2) using the WAG + G method with MEGA, version 5 [22]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.5. Immune challenge

For stimulation with LPS, animals were injected with 50 µl of LPS (*E. coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in PBS (LPS 10 µg ml $^{-1}$) into the adductor muscles of each pearl oyster. The control groups were injected with 50 µl of PBS. At each time point (0, 4, 8, 12, 24 and 36 h), haemolymph was collected from the control group and the LPS stimulation group. Haemolymph samples were withdrawn from the adductor muscles using a syringe and immediately centrifuged at 5000× *g* at 4 °C for 10 min to harvest the haemocytes. At each time point, five control and five LPS injected individuals were sampled. The haemocyte pellets were immediately used for RNA extraction. The tissues including adductor muscle, gill filaments, mantle, digestive gland, gonad, heart and haemocytes were collected from five healthy individuals to investigate the tissue-specific expression of pfSOD.

2.6. Semi-quantitative PCR

Semi-quantitative PCR was conducted to determine the relative expression of pfSOD in *P. fucata*. At defined time points pfSOD in the challenged oysters and vehicle controls were processed and quantified based on the gel band intensity using ImageJ analysis software [23]. Primers for semi-quantitative PCR were designed from the pfSOD cDNA sequence and are shown in Table 1. The PCR condition for pfSOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: initial denaturation at 94 °C for 3 min, then different cycles of amplification of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The GAPDH was amplified in PCR reaction as a loading control. The products were analysed on 2.0% agarose gel containing ethidium bromide. The cycle numbers at half-maximal amplification were used for subsequent quantitative analysis of gene expression. The PCR cycles, 28 cycles for pfSOD and 25 cycles for GAPDH were optimized as such that the target gene and house-keeping gene amplification were in logarithmic phase.

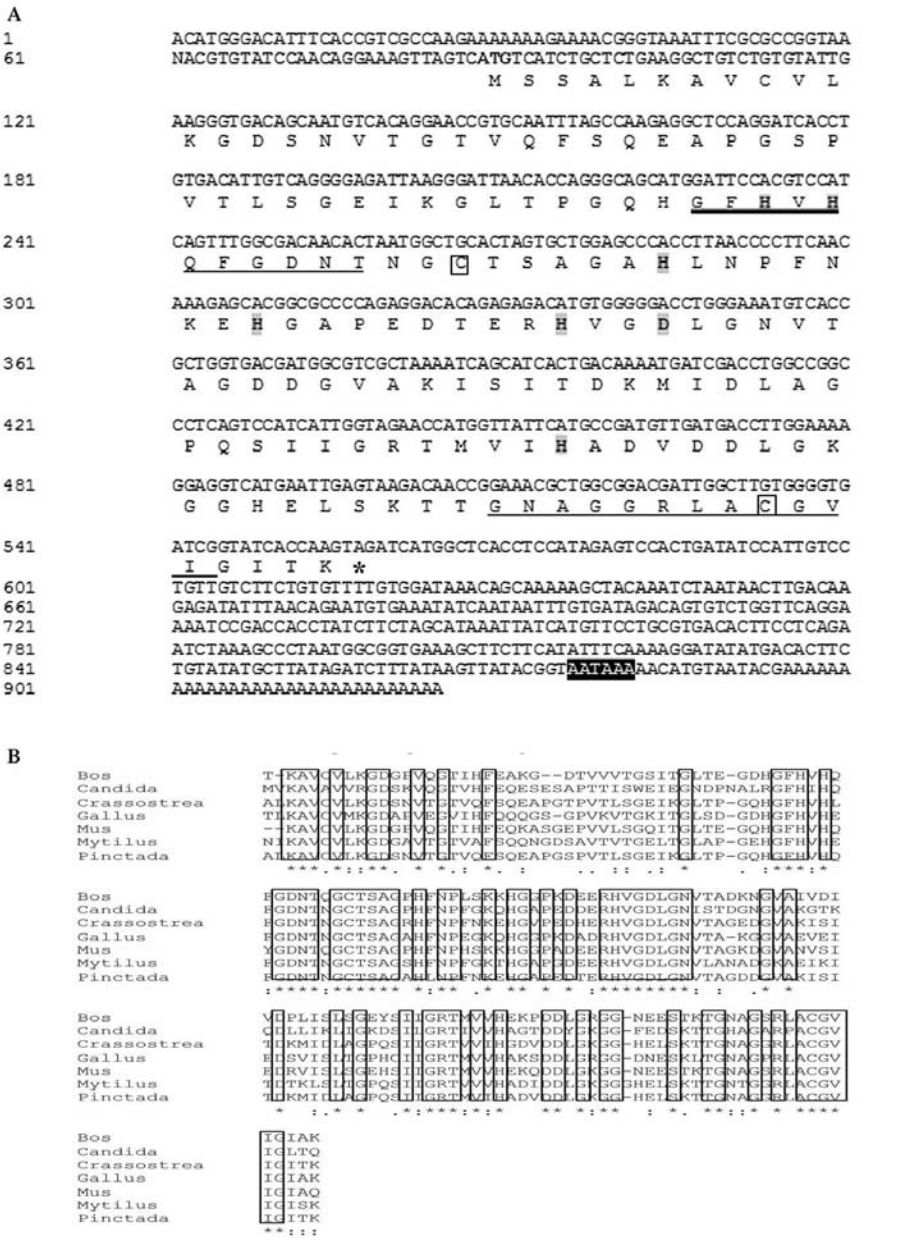

Fig. 1. (A) Nucleotide sequence of pfSOD cDNA from *P. fucata* and its deduced amino acid sequence. Two Cu/Zn SOD family signatures are underlined (_). The start codon is in bold and termination codon is indicated with asterisk (□). The amino acids required for binding of copper (His-49, -51, -66, and -123) and zinc (His-66, -74, and -83 and Asp-86) are shaded. Two cysteines (Cys 60 and Cys 149) predicted to be engaged in the disulfide bond formation were boxed. (B) The alignment of deduced amino acid sequence of pfSOD. Conserved regions are represented in box.

2.7. Statistical analysis

Multiple comparisons using Duncan's test were made to check the differences between the gene expression in the control and challenged oysters using SPSS13.0 software.

3. Results and discussion

RT-PCR was used to clone the open reading frame of SOD using total RNA extracted from haemocytes of *P. fucata*. A single PCR product of 471 bp was obtained. The size of this segment correlated well with SOD genes from other species. This partial cDNA sequence provided the necessary information to obtain an additional 368 bp sequence by 3′RACE, and an additional 87 bp sequence by 5′RACE. Finally, the full-length sequence information of the Cu/Zn SOD cDNA was obtained by overlapping the three cDNA sequences. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1A. The full-length SOD cDNA is comprised of 924 bp, containing 87 bp in the 5′-terminal untranslated region (UTR), 471 bp in the ORF, 366 bp in 3′-terminal UTR with a poly(A) tail of 30 bp and a putative polyadenylation consensus signal (AATAAA). The ORF encodes a polypeptide of 156 amino acids. The SOD cDNA sequence and its deduced amino acid sequence were submitted to the NCBI GenBank under accession no (JX013537). No signal peptide was identified in the deduced amino acid sequence of Cu/Zn SOD by the signal P program, indicating that this pfSOD is a cytoplasmic Cu/Zn SOD.

Multiple alignment of the deduced amino acid sequences (Fig. 1B) with other closely related cytoplasmic Cu/Zn SOD sequences showed that three cysteines (Cys 9, Cys 60 and Cys 149) are present in the mature pfSOD. Cys 60 and Cys 149 are conserved in all Cu/Zn SODs and it is believed that those form an intramolecular disulfide bond. The amino acids required for binding of copper (His-49, -51, -66, and -123) and zinc (His-66, -74 and -83 and Asp-86) are also conserved. Two Cu/Zn SOD family signature sequences were found in the deduced amino acid sequence of pfSOD; signature 1 (consensus sequences: [GA]-[IMFAT]-H-[LIVF]-H-{S}-x-[GP]-[SDG]-x-[STAGDE].) and signature 2 (consensus sequences: G-[GNHD]-[SGA]-[GR]-x-R-x-[SGAWRV]-C-x(2)-[IV]). These family signature sequences are conserved in all Cu/Zn SODs. Several reports have shown that copper and zinc ions have critical functions in stabilizing the quaternary structure and therefore in the kinetic properties of Cu/Zn SOD [24–26]. BLAST analysis shows that the deduced amino acid sequence of pfSOD has extremely high identity with the Cu/Zn SOD of *Crassostrea hongkongensis*, *C. gigas* and *Mytilus chilensis* (99%). Similarly, it has high identity with Cu/Zn SOD of *Candida ariakensis* (98%) and *H. discus discus* (97%).

Phylogenetic relationships of Cu/Zn SOD from pearl oyster and other invertebrates and vertebrates were estimated. Cu/Zn SOD of

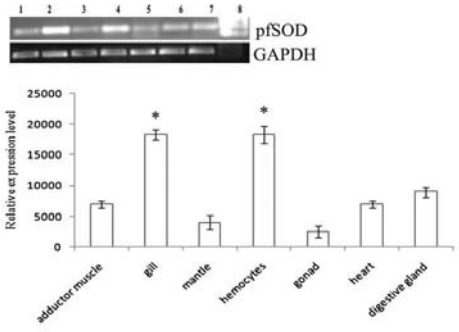

Fig. 3. Distribution of Cu/Zn SOD mRNA in different adult tissues of pearl oyster. Expression analysis of Cu/Zn SOD mRNA in different adult tissues of pearl oyster by RT-PCR.GAPDH was used as an internal control. Lane 1: adductor muscle. Lane 2: gill. Lane 3: mantle. Lane 4: haemocytes. Lane 5: gonad. Lane 6: heart. Lane 7: digestive gland. Lane 8: 100 bp ladder. The values are showed as mean ± S.E ($N = 5$).

Candida albicans was used as the out-group. As shown in Fig. 2, Cu/Zn SODs of pfSOD formed a separate cluster with cytoplasmic Cu/Zn SODs from oyster *C. gigas* and mussel *Mytilus edulis* indicative of the closer evolutionary relationship of *P. fucata* with other aquatic invertebrates. Vertebrates are evolutionarily distinctly separated.

RT-PCR was carried out to analyse the distribution of pfSOD mRNA in the adult tissues of the pearl oyster. RT-PCR analyses revealed that pfSOD mRNA is abundantly expressed in the gill and haemocytes. Levels are up to 3 fold higher than the moderately expressed pfSOD in the adductor muscle, mantle, gonad, heart and digestive gland (Fig. 3). Hence, haemocytes are considered as the most suitable tissue to analyse the pearl oysters immune function. This is in agreement with the report by Kuchel et al. [27] who found haemocytes defense enzyme expression in *Pinctada imbricata*. As histological studies have revealed the presence of a large amount of haemocytes in bivalve gill tissues [28–30], a high expression level in gill is more likely associated with haemocyte abundance.

In order to investigate the immunological function of pfSOD in pearl oyster, *P. fucata* we determined the levels of pfSOD cDNA in haemocytes after challenge with lipopolysaccharides (LPS). LPS stimulation significantly increased pfSOD mRNA expression in the haemocytes in a time-dependent manner (Fig. 4). Over time pfSOD mRNA expression reached a significant increase 4 h after exposure to LPS. pfSOD mRNA levels further increased to reach a maximum at 8 h post treatment and then dropped to basal levels at 36 h. At the maximum the relative mRNA expression of pfSOD increased to

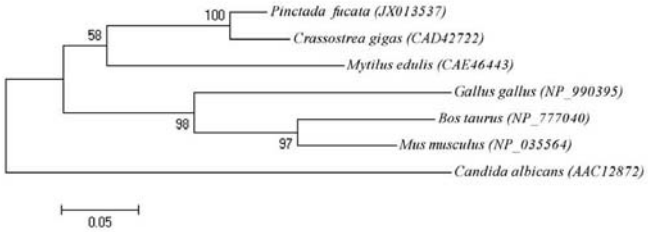

Fig. 2. Neighbour-joining phylogentic tree of pfSOD amino acid sequences from 7 species. Note: Numbers represent the bootstrap values. The amino acid sequences for the phylogenetic tree are shown in Fig. 1B.

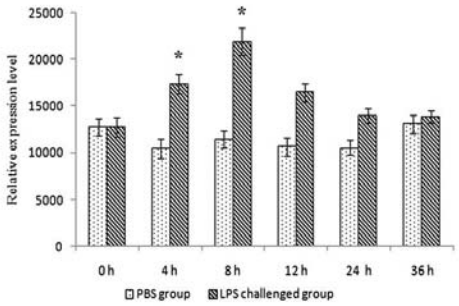

Fig. 4. Temporal expression pattern analysis of Cu/Zn SOD mRNA in haemolymph of the pearl oyster challenged with LPS. Vertical bars represent the mean ± S.E ($N = 5$). Significant differences ($P < 0.05$) are indicated with the asterisk (*).

2-fold over control. The high level of expression in haemocytes and gill suggest that pfSOD could be involved in the innate immune response.

In conclusion, the full-length cDNA of Cu/Zn pfSOD contains an open reading frame (ORF) of 471 bp coding for 156 amino acids. Semi-quantitative analysis in adult tissues showed that the pfSOD mRNA was abundantly expressed in haemocytes and gill. After challenge with lipopolysaccharide (LPS), expression of pfSOD mRNA in haemocytes was increased, reaching the highest level at 8 h, then dropping to basal levels at 36 h. These results suggest that Cu/Zn SOD could be used as a bioindicator of the aquatic environmental pollution and cellular stress in pearl oyster.

Acknowledgements

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EXPRESSION ANALYSIS OF GENES INVOLVED IN THE BIOMINERLIZATION OF *MABE* PEARL

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ABSTRACT

There are specialized proteins responsible for the biominerlization processes leading to the formation of pearl in molluscs. These proteins are regulated by the genes encoding them, and paucity of sufficient information on these genes is a major gap in our knowledge on pearl formation genomics. Expression analysis of four genes involved in biominerlization process of mabe pearl formation viz. nacrein, prismalin-14, calreticulin and N19 were carried out in the present study. Though the expression of these genes in the mantle surrounding the mabe showed differential expression levels, the general trend was an up-regulation between 10th to 30th days after implantation except calreticulin. Based on the time trend in the expression of different genes involved in pearl formation, suitable strategy may be developed to produce high quality pearls.

INTRODUCTION

- Pearls have been admired and adored as a symbol of beauty, power and love.
- Pearl is produced by the process of bio-mineralization of Calcium Carbonate crystals (HCO_3^-). The HCO_3 in the form of Argonite crystals is embedded within the organic matrix protein.
- A mabe pearl is a dome shaped or image pearl produced by placing a hemispherical object or a miniature image against the side of the oyster shell interior on which the organic matrix is secreted.
- The main advantage of Indian marine mabes over the ones produced by freshwater mussels is the very short gestation period (2 months as compared to 18 months) apart from the superior quality of the nacre of Indian marine pearl oyster, *Pinctada fucata*.
- Cultured mabes are used for such things as rings and earrings, rather than for stringing on necklaces. They tend to be very beautiful with high luster and orient, but are priced much lower than round pearls.
- Mabe pearl culture technology has been successfully developed by Central Marine Fisheries Research Institute during the early 1980s.
- There are specialized proteins responsible for the biominerlization processes in mollusc shell.
- These proteins are regulated by the genes encoding them, and paucity of sufficient information on these genes is a major gap in our knowledge on pearl formation genomics.
- The present study aims to elucidate the functional genes involved in the pearl formation.

METHODOLOGY

- Live specimens of *Pinctada fucata*, 4.5-5 cm in length were subjected to mebe implantation, and were individually placed in rearing tanks.
- Oysters were harvested on pre-determined days (10th, 30th and 40th) post implantation.
- Mantle surrounding the mabe was carefully dissected from the implanted and control animals.
- Total RNA were extracted from the tissues using standardized procedure (sigma kit), and its quantity was determined from optical density at 260 nm (OD260 nm) using spectrophotometer.
- Total RNA was used to synthesize single-strand cDNA in accordance with the manufacturer's recommendation (Biorad).
- Primers for the four genes under study, nacrein, prismalin-14, calreticulin and N19 were designed and custom synthesized.
- The cDNA of these genes were amplified with the above primers, and their expression level at different intervals was determined by semiquantitative PCR analysis.
- Housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was selected as references for calculation of relative expression levels of target genes.

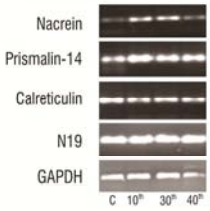

Fig. 1 Time course study of gene expression after PCR amplification with 35 cycles

FINDINGS

- The diffential expression patterns of the four genes viz. nacrein, prismalin-14, calreticulin and N19 in the implanted oysters at different time intervals and control were quantified based on the gel band intensity using Image J analysis software.
- In general, mRNAs of all the four genes were detected in target tissues with different levels of expression.
- Expression levels of nacrein, prismalin-14 and N19 showed a predominant up-regulation with slight variations among the different time periods.
- Expression pattern of nacrein and Prismalin-14 were similar, and reached highest expression on the 10th day after incubation and then decreased by 40th day (Fig.2).
- The expression of N19 showed a marked up-regulation on 30th day and then decreased by 40th day to the 10th day level.
- Expression level of calreticulin in the implanted oysters was lower than the controls, though the difference was only marginal.

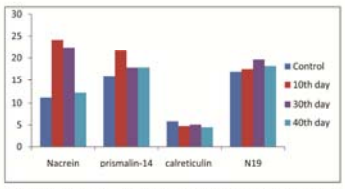

Fig.2 Gene expression profile of mabe pearl in *Pinctada fucata*

It is well known that nacreous pearl is high quality pearl than the prismtic pearls. Based on the time trend in the expression of different genes involved in pearl formation, suitable strategy may be developed to produce high quality pearls

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Expression analysis of antioxidant genes involved in the innate immunity of pearl oyster *Pinctada fucata*

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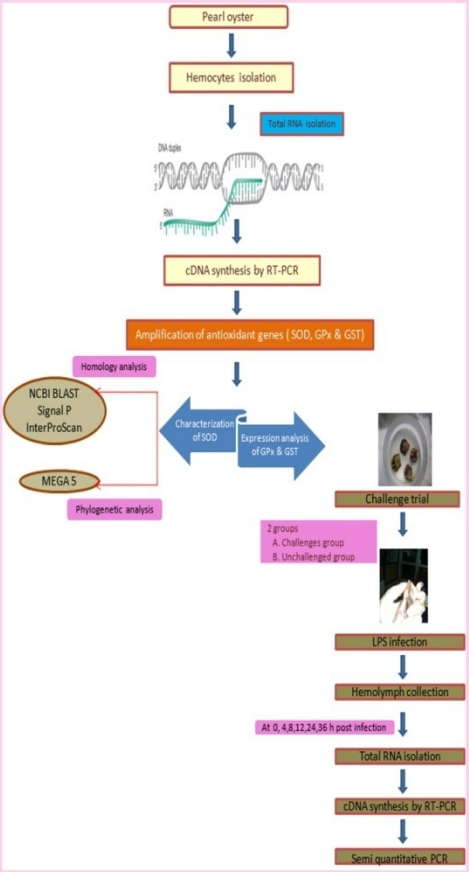
INTRODUCTION

Pearl oyster *Pinctada fucata* is distributed over South coast of India and is the most important bivalve mollusk for seawater pearl production in India. Decline in pearl production is mainly due to mortality of pearl oyster. The reason for high mortality is related to ocean pollution, disease outbreaks and stock degeneration (Richard & Jackie 1995; Potasman 2002). In order to control disease and enhance the yields and quality of seawater pearl, it is necessary to study the innate immune defense mechanisms of pearl oyster, which lacks the adaptive immune system. One strategy is to identify disease resistance genes and use them for genetic improvement of cultured stocks and this in context the present study was taken to study the characterization and expression level of antioxidant genes in pearl oyster, *Pinctada fucata.*

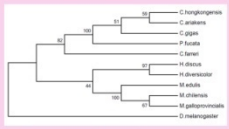
MATERIALS AND METHODS



RESULT & DISCUSSION

The PCR product obtained was 471bp, and its nucleotide sequence showed high identities with other known SODs, especially the SODs from molluscs. No signal peptide was identified in the deduced amino acid sequence of Cu Zn SOD by the signal P program, indicating that pfSOD was a cytoplasmic Cu, Zn SOD. Cytoplasmic Cu/Zn SOD family signatures were identified using InterProScan program.

Phylogenetic tree was constructed based on the aminoacid sequences of selected Cu, Zn SODs with the neighbour-joining method.



Semiquantitative PCR was employed to quantify the expression levels of antioxidant genes GPx and GST showed a marked up-regulation with slight variations among the different time periods.

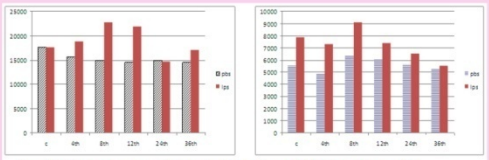


Fig a & b. The gene expression levels of GPx (a) and GST (b) gene transcript challenged by LPS

CONCLUSION

The present study may support the proper use of antioxidant genes as biomarkers and pave the way for investigation of the defense mechanism of the pearl oyster.

National Center for Biotechnology Information
List of sequences submitted to NCBI

Sl.No	Title	GenBank accession no	Authers
1	*Pinctada fucata* voucher CMFRI-MBTD-TPF1 F-type lectin 1 mRNA, complete cds	JX103557.2	Anju, A., Jeswin, J., Thomas, P.C. and Vijayan, K.K.
2	*Pinctada fucata* voucher CMFRI-MBTD-TPFSOD01 superoxide dismutase (Cu/Zn SOD) mRNA, complete cds	JX013537.3	Anju, A., Jeswin, J., Thomas, P.C., Paulton, M.P. and Vijayan, K.K.
3	*Pinctada fucata* voucher CMFRI-MBTD-KOPF4 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708020.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
4	*Pinctada fucata* voucher CMFRI-MBTD-KOPF3 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708019.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
5	*Pinctada fucata* voucher CMFRI-MBTD-KOPF2 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708018.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
6	*Pinctada fucata* voucher CMFRI-MBTD-KOPF1 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708017.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
7	*Pinctada fucata* voucher CMFRI-MBTD-TUPF8 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708016.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
8	*Pinctada fucata* voucher CMFRI-MBTD-TUPF7 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708015.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
9	*Pinctada fucata* voucher CMFRI-MBTD-TUPF6 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708014.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
10	*Pinctada fucata* voucher CMFRI-MBTD-TUPF5 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708013.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
11	*Pinctada fucata* voucher CMFRI-MBTD-TUPF4 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708012.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
12	*Pinctada fucata* voucher CMFRI-MBTD-TUPF3 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708011.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
13	*Pinctada fucata* voucher CMFRI-MBTD-TUPF2 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708010.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
14	*Pinctada fucata* voucher CMFRI-MBTD-TUPF1 cytochrome b (cytb) gene, partial cds; mitochondrial	KC708009.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
15	*Pinctada fucata* voucher CMFRI-MBTD-KOPF4 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811441.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
16	*Pinctada fucata* voucher CMFRI-MBTD-KOPF3 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811440.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
17	*Pinctada fucata* voucher CMFRI-MBTD-KOPF2 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811439.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
18	*Pinctada fucata* voucher CMFRI-MBTD-KOPF1 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811438.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
19	*Pinctada fucata* voucher CMFRI-MBTD-TUPF8 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811437.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
20	*Pinctada fucata* voucher CMFRI-MBTD-TUPF7 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811436.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
21	*Pinctada fucata* voucher CMFRI-MBTD-TUPF6 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811435.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
22	*Pinctada fucata* voucher CMFRI-MBTD-TUPF5 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811434.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
23	*Pinctada fucata* voucher CMFRI-MBTD-TUPF4 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811433.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
24	*Pinctada fucata* voucher CMFRI-MBTD-TUPF3 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811432.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
25	*Pinctada fucata* voucher CMFRI-MBTD-TUPF2 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811431.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.
26	*Pinctada fucata* voucher CMFRI-MBTD-TUPF1 ATP synthase subunit 6 (atp6) gene, partial cds; mitochondrial	KC811430.1	Anju, A., Thomas, P.C., Reynold, P. and Vijayan, K.K.