



## Detection of endogenous peroxidase and phenoloxidase in the mantle and liver of the clam *Ruditapes philippinarum*

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### ABSTRACT

Peroxidase catalyzes the oxidation of a large variety of hydrogen-donating substrates in the presence of hydrogen peroxide, while phenoloxidase (PO) catalyzes mono- and/or diphenols into quinines and melanin with oxygen as a proton acceptor. Oxidases reduce the molecular oxygen during oxidation of their substrates, and further yield the oxidized product hydrogen peroxide. In this study, the distribution of endogenous peroxidase and PO in the mantle and liver of the Clam *Ruditapes philippinarum* were investigated with diaminobenzidine (DAB) and L-Dopa method, respectively. The results indicated that peroxidase and PO distributed widely in the mantle and liver cells of *Ruditapes philippinarum*. The peroxidase and PO reactivity were detected in many epithelial cells of the gill. However, peroxidase and PO were mainly present at the edges of mantle. There were fewer granules of peroxidase and PO in the interior of the mantle. It shows that peroxidase and PO may play an important role in regulating the defence system in the mantle and liver of the Clam *Ruditapes philippinarum*.

**Key words:** peroxidase, phenoloxidase, gill, mantle, *Ruditapes philippinarum*.

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### INTRODUCTION

Oxidases, which are a widespread group of enzymes, exist in various tissues, cells and subcellular compartments. Oxidases reduce the molecular oxygen during oxidation of their substrates, and further yield the oxidized product hydrogen peroxide [1]. Free radical chemistry comprises scavenging radicals or generation of radicals. Peroxidase is one of the most important enzymes for scavenging free radicals, and catalyzes the oxidation of a large variety of hydrogen-donating substrates in the presence of hydrogen peroxide [2-6].

Based on the reduction of soluble electron acceptors or the capture of hydrogen peroxide, a variety of methods have been developed for histochemical localization of oxidases [1]. The tissues are usually stained with diaminobenzidine (DAB) to detect peroxidase activity and visualize the subcellular peroxidatic enzymes. It is found that the single membrane bounded organelles are characterized by an asymmetrical matrix capable of oxidizing DAB to an electron dense inclusion [7]. The DAB method has been used to investigate the endogenous peroxidase activity in the hamster submandibular gland and intralobular ducts with light and electron microscopy [8, 9]. In addition, the DAB technique facilitates the identification of various phagocyte types, and the distinct subcellular distribution of peroxidase is revealed in the mononuclear phagocytes [10].

Phenoloxidase (PO), which is a bifunctional copper-dependent enzyme, is produced by the hydrolysis of precursor prophenoloxidase (proPO) [11, 12]. The proPO cascade will be activated in the haemolymph of invertebrates under immune challenge or wounding. It is known that PO catalyses the oxidation of dopa rapidly to dopaquinone, which in turn polymerises non-enzymatically into insoluble melanin [13].

Since the endogenous peroxidase and PO are present widely in the tissues, the peroxidase and PO activity are usually assayed in the antioxidants studies. Moreover, the endogenous peroxidase activity was usually blocked artificially with saturating amounts of H<sub>2</sub>O<sub>2</sub> in the process of immunohistochemistry. However, there were fewer studies to investigate the distribution of endogenous peroxidase and PO in the tissues of clams. The aim of this study was to investigate the distribution of endogenous peroxidase and PO in the mantle and liver of the Clam *Ruditapes philippinarum*.

## EXPERIMENTAL SECTION

### *Experimental Animals*

The Manila clams (*Ruditapes philippinarum*) were purchased from a commercial market and acclimated in aerated seawater held at 21°C for several days prior to experiment and artificial feed was given twice a day.

### *Tissue Preparation*

The mantle and liver of clams were fixed for 12 h in a solution of 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (PBS, pH 7.4) at 4°C. Then the tissues were washed three times with PBS (pH 7.4).

### *Histochemical Localization of Peroxidase*

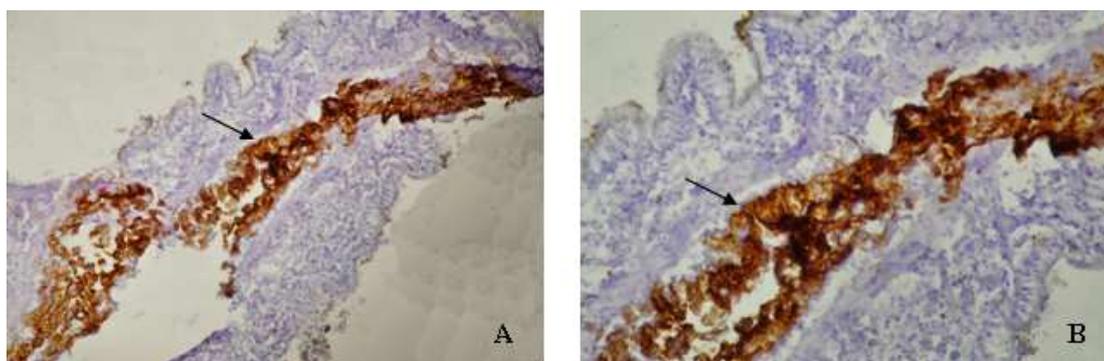
The mantle and liver of clams were sectioned at a thickness of 35 µm in a cryostat (Leica CM1850, Germany) in the transverse plane, and mounted on chromalum-gelatin-coated glass slides. The sections were rehydrated in PBS (pH 7.4), and incubated in 1.0 mmol/L 3,3'-diaminobenzidine (purchased from Sigma) and 3.0 % hydrogen peroxide (in pH 6.5, 0.1 mol/L PBS) for 2.0 hours at 37°C. A negative control was provided by incubation with PBS instead of 3,3'-diaminobenzidine. Finally, the slides were dyed with hematoxylin, dehydrated, cleared in xylene, and analyzed under light microscope.

### *Histochemical Localization of PO*

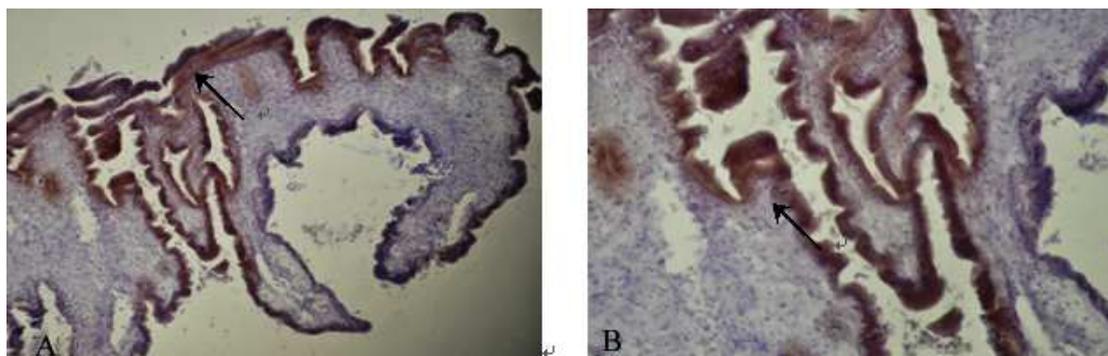
The sections of mantle and liver were rehydrated in double distilled water, and incubated in 1.0 mg/mL L-Dopa (in pH 6.0, 0.1 mol/L PBS) for 3.0 h at 37°C. A negative control was provided by incubation with PBS instead of L-Dopa. The sections were washed three times with PBS for 5 min. Finally, the slides were dyed with hematoxylin, dehydrated, cleared in xylene, and analyzed under light microscope.

## RESULTS AND DISCUSSION

In mollusc, the mantle extends from the dorsum and wraps the visceral mass and gill. The mantle is composed of three layers. The outer layer of mantle is the epidermal layer, which contains many glandular cells and plays a significant role on shell formation. The middle layer is thick and interweaved with muscle and connective tissues. However, the inner layer of mantle is the epidermis with cilia, which facilitates to obtain the water and food. In this study, Fig.1 and Fig.2 showed that the peroxidase and PO activity were mainly present at the edges of mantle, but there were fewer granules of peroxidase and PO in the interior of the mantle (Fig.1 and Fig.2).

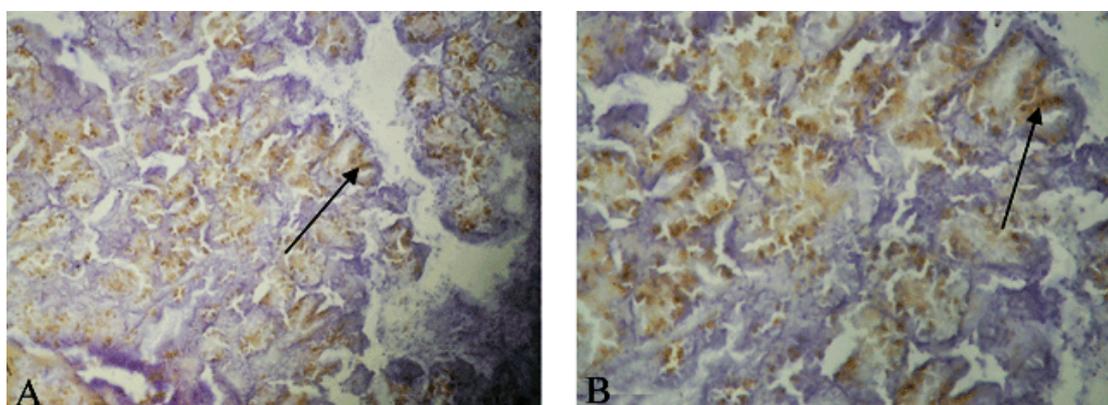


**Fig.1:** Histochemical localization of peroxidase in the mantle of the clam *Ruditapes philippinarum*. Peroxidase is present at the edges of mantle (arrow). The nucleus was dyed with hematoxylin. A: ×100, B: ×200.

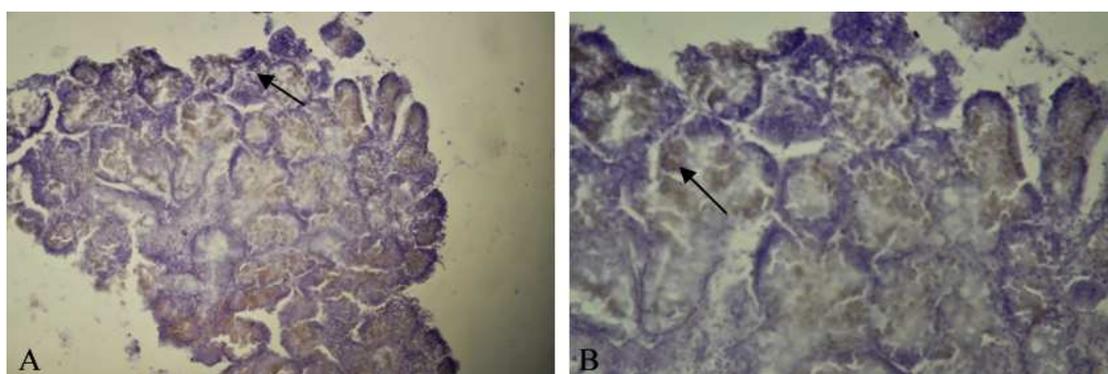


**Fig.2:** Histochemical localization of PO in the mantle of the clam *Ruditapes philippinarum*. PO is present at the edges of mantle (arrow). The nucleus was dyed with hematoxylin. A:  $\times 100$ , B:  $\times 200$ .

The liver of the Clam *Ruditapes philippinarum* has many ramose hepatopancreatic ducts, which arrive at small ducts and join with big ducts. In the principal hepatopancreatic ducts, there are many epithelial cells showing peroxidase and PO reactivity (Fig.3 and Fig.4).



**Fig.3:** Histochemical localization of peroxidase in the liver of the clam *Ruditapes philippinarum*. Peroxidase is present in the epithelial cells (arrow). The nucleus was dyed with hematoxylin. A:  $\times 100$ , B:  $\times 200$ .



**Fig.4:** Histochemical localization of PO in the liver of the clam *Ruditapes philippinarum*. PO is present in the epithelial cells (arrow). The nucleus was dyed with hematoxylin. A:  $\times 100$ , B:  $\times 200$ .

Reactive-oxygen species (ROS) mainly include superoxide radicals, hydrogen peroxide, and hydroxyl radical. ROS play a significant role on the physiology, growth and survival of aquatic organisms [14, 15]. However, the antioxidant enzymes and nonenzymatic antioxidants could eliminate ROS [16, 17]. Some antioxidant enzymes, such as peroxidase and catalase, provide the first defense system against oxidative toxicity and participate in converting  $H_2O_2$  to water and oxygen. Especially, peroxidase catalyzes the oxidation of phenolic compounds using  $H_2O_2$  or organic peroxides as the oxidizing agent [18, 19]. It has been found that peroxidase participated in regulating the defence system and protecting oral tissues from bacteria and toxic accumulation of  $H_2O_2$  [20]. Peroxidase has been widely used in clinical biochemistry and enzyme immunoassays [21, 22]. In this study, peroxidases distribute widely in the mantle and liver cells of the clam *Ruditapes philippinarum*. It shows that peroxidases play an important role in

regulating the defence system in these tissues.

PO is one of the most important and ubiquitous enzyme released by invertebrates in response to immunostimulation. Previous studies have shown the importance of the PO in invertebrate defense system [23, 24]. Using oxygen as a proton acceptor, PO catalyzes mono- and/or diphenols into quinines and melanin [25]. It has been found that PO activity was present intracellularly in blood cells and extracellularly in cell-free plasma of vertebrates and invertebrates [26-28]. Our study showed that PO distributed widely in the mantle and liver cells of the clam *Ruditapes philippinarum*. PO may participate in regulating the digestion and defense system, which needs to be further studied.

In summary, the results indicated that peroxidase and PO distributed widely in the mantle and liver cells of the clam *Ruditapes philippinarum*. Peroxidase and PO may play an important role in regulating the defence system in these tissues.

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