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Review

Recent findings on phenoloxidases in bivalves

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ABSTRACT

The production of melanin is a complex process involving biochemical cascades, such as the pro-phenoloxidase (proPO) system, and enzymes, such as phenoloxidases (POs). Different studies have shown a strong correlation between the decrease in PO activities and the occurrence of diseases in bivalve invertebrates, leading to mortalities in the host. Results of these studies suggest that POs could play a fundamental role in defense mechanisms in bivalves. This article reviews the fundamental knowledge on the proPO system in bivalves and the methods used to assess PO activities. Finally, this is the first report on the major findings of laboratory and field studies that indicate that a type of PO in bivalves, the laccase enzyme, is inducible and involved in the 1) immune 2) antioxidant and 3) detoxification roles in bivalves, and might be an ecological potential biomarker of environmental stress.

1. Introduction

Phenoloxidases (POs, EC 1.14.18.1) are the key enzymes of an ubiquitous biochemical cascade responsible for the production of melanin. This biochemical cascade plays a role in immune defenses, wound healing, encapsulation and melanisation, as well as in self/non-self recognition mechanisms in invertebrates (Cerenius et al., 2008). *In vitro* studies have shown a bactericidal effect of PO activities in invertebrates, such as the tobacco hornworm *Manduca sexta* (Zhao et al., 2007), the mealworm beetle *Tenebrio molitor* (Kan et al., 2008), the freshwater crayfish *Pacifastacus leniusculus* (Cerenius et al., 2010), the Pacific oyster *Crassostrea gigas* (Luna-Acosta et al., 2011a), the scallop *Chlamys farreri* (Zhou et al., 2012), the Manila clam *Venerupis philippinarum* (Le Bris et al., 2013), the bivalve mollusc *Scrobicularia plana* (Buffet et al., 2014) and the smooth venus clam *Callista chione* (Matozzo and Bailo, 2015). These results suggest that POs have a protective role on bacterial infection. Moreover, different studies have shown a strong correlation between the decrease in PO activities in the Sydney rock oyster *Saccostrea glomerata* and the occurrence of Queensland disease (QX disease), caused by the parasite *Marteilia sydneyi* and leading to mortality in the host (for review, see Raftos et al., 2014). These results suggest that POs have a protective role on parasite infection. It has also been suggested that PO activities may be involved in antiviral defenses in invertebrates (Terenius et al., 2007; Xing et al., 2008; Renault et al., 2011). Recently, studies investigated the immune responses of phenoloxidase (increased expression and spatio-temporal evolution of enzymes' activities post-infection) in the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis* in order to better understand the combined effect of temperature and two *V. tapetis* strains in the Manila clam *Venerupis philippinarum* (Richard et al., 2015; Le Bris et al., 2015).

Noxious effects in immune defense mechanisms, such as POs, due to the presence of stressors in the environment, such as chemical contaminants, can induce the occurrence and/or increase of diseases. Unfortunately, development of human activities leads to chronic pollution of the environment by a mixture of inorganic (metals) and organic (pesticides, hydrocarbons, phytosanitaries) contaminants. The use of biomarkers allows observation of the impact of chemical contaminants on coastal species while the physiological dysfunctions caused by the exposure to chemical contaminants. These dysfunctions can be early diagnosed, by studying biomarker responses at the gene or protein level (Amiard-Triquet et al., 2006). Early diagnosis means observing dysfunctions before any tissue damage or any damage on main biological functions is detected. Biomarkers can be classified in three types: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Manahan, 2003). Biomarkers of exposure indicate the presence of a contaminant inside the organism, due to an interaction of this contaminant with biological molecules. Biomarkers of effect indicate the toxic or sublethal effect of the contaminant on the organism.

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However, the definition of these classes varies depending on different authors. So, certain ecotoxicologists prefer the terminology proposed by De Lafontaine et al. (2000) and that will be used in this manuscript, contrasting biomarkers of defense and biomarkers of damage.

Biomarkers of defense correspond to mechanisms that allow the organism to survive in a deteriorated environment, and consequently, have a positive effect on their health. The energy allocated to these defense mechanisms or "cost of tolerance" to the presence of the contaminant in the organism will be retrieved from the energy allocated to the basal metabolism, growth and/or reproduction (fitness), which can impair the organism in the long term (Holloway et al., 1990). Some examples of biomarkers of defense are: phase II enzymes (glutathion-S-transferase, MXSM), metallothioneins, antioxidant defense enzymes and molecules (superoxide dismutase, catalase, glutathion peroxidase, glutathion), heat shock proteins, etc. (Amiard-Triquet et al., 2006).

Biomarkers of damage correspond to biological damages in the organism leading to an impairment in the fitness of the organism. Some examples of biomarkers of damage are: acetylcholinesterase, vitellogenin, lysosomal membrane stability, lipid peroxidation, DNA damages and adducts, molecular markers (cortisol, retinol), inmunological markers (% of phagocytosis), histological markers (histopathological index), etc. (Amiard-Triquet et al., 2006).

Among proteins that can be modulated by the presence of contaminants in the environment, different authors have shown that PO activities in bivalve molluscs can be modulated by inorganic (Gagnaire et al., 2004; Chakraborty et al., 2010; Cong et al., 2013; Haberkorn et al., 2014; Buffet et al., 2013; Buffet et al., 2014) and organic contaminants (Bado-Nilles et al., 2009a, 2009b; Bado-Nilles et al., 2010; Bianchi et al., 2014; Díaz-Resendiz et al., 2014; Luna-Acosta et al., 2011b; Milinkovitch et al., 2015a; Breitwieser et al., 2016). In addition, a gene coding for a laccase-type PO has been identified in *C. gigas* (Renault et al., 2011) and modulation of the expression of this gene has been observed, following exposure of *C. gigas* to hydrocarbons (Bado-Nilles et al., 2010) and to the pesticide diuron (Luna-Acosta et al., 2012). This type of noxious effects in immune defense mechanisms can induce in the organism the occurrence and/or increase of diseases.

The present work aims to review and discuss published data on PO activity assessment, gene expression and potential roles in bivalve molluscs. To date, most of the work published on PO activity in marine bivalves is focused on the roles of POs in immune defense mechanisms. However, recent studies suggest an ecological relevance of these POs, an especially of laccases, as a novel biomarker for marine environmental pollution studies. In this context, we will analyze data relative to studies on POs, by exposing new hypotheses regarding their potential roles in these invertebrate organisms.

2. Molecular and biochemical aspects

The first studies on POs were carried out in fungi, more than a century ago [for review, see (Mayer, 2006)]. However, isolation experiments of proteins started only 42 years later, revealing different functional and structural properties of POs (Mayer, 2006).

POs are copper-binding metalloproteins. As oxidoreductases, they catalyze the oxidation of phenolic compounds in the presence of oxygen (O_2). One of the main difficulties concerning the enzymatic group of POs are approximations found in the literature, since the terms "tyrosinases", "phenoloxidases" and "catecholases" are often used as synonyms (Solomon et al., 1996; Claus and Decker, 2006) and tyrosinases and POs possess the same EC number.

However, based on their activity, there are three distinct types of enzymes in the group of POs (Walker and Ferrar, 1998): 1) tyrosinases (EC 1.14.18.1), 2) catecholases (EC 1.10.3.1) and 3) laccases (EC 1.10.3.2). All three groups catalyze the oxidation of o-diphenols. This means that the three groups possess catecholase (or diphenoloxidase) activity. However, only tyrosinases catalyze the orhtohydroxylation of monophenols. This means that only tyrosinases possess a cresolase (or monophenoloxidase) activity. And, on the other hand, only laccases catalyze the oxidation of m- and p-diphenols and other compounds, such as aromatic amines. This means that only laccases possess a laccase activity (Fig. 1). Therefore, «tyrosinases», «phenoloxidases» and «catecholases» do not necessarily possess the same type of enzyme activities and therefore these terms should not be used as synonyms, to avoid ambiguity. (See Figs. 2 and 3.)

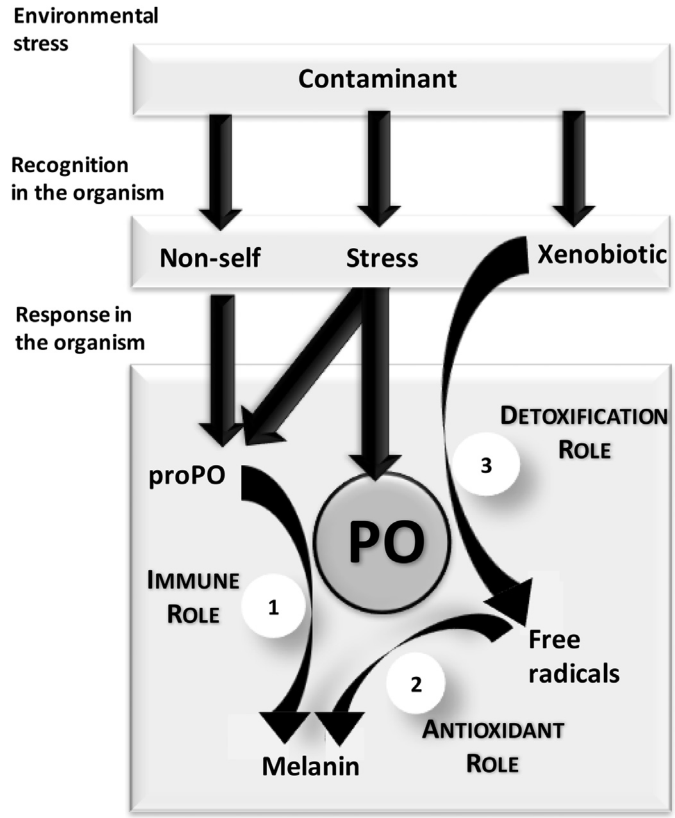

Fig. 1. Potential roles of POs in bivalve invertebrates. Contaminants present in the environment can induce a stress and be recognized as a foreign biological or chemical agent, or as other type of stressor. Therefore, bivalves will activate different defense mechanisms to ensure the maintenance of their homeostasis. In this context, POs could play an immune, antioxidant and/or detoxification role.

PO size and form vary depending on the phylum and the organism: in vertebrates, tyrosinases are dimeres while in invertebrates, POs are monomeres or oligomeres (from dimeres to pentameres; Renwrantz et al., 1996). The molecular weight of POs has been determined with chromatographic exclusion techniques or with electrophoresis under denaturating conditions. The molecular weight varies from 10 to 400 kDa and each monomer is generally 40 to 45 kDa. In bivalves, the molecular weight of proteins possessing PO activity varies from 10 to 381 kDa (Table 1).

In one hand, tyrosinases are transmembrane proteins in humans and mice; they containing a signal peptide and a transmembrane region, e.g. Decker et al. (2007). On the other hand, the presence of a signal peptide is unlikely in arthropods and insects (Cerenius et al., 2008). In fact, only the active site is likely to be conserved in the genes of mammals, invertebrates, fungi and bacteria (Decker and Terwillinger, 2000). Generally, the active site possesses two copper-binding regions called CuA and CuB. In these regions, each copper atom is coordinated by three histidine residues (Decker and Terwillinger, 2000). The gene coding for a laccase 1 identified in *C. gigas* showed 3 amino acid regions sharing a high homology with conserved Cu-oxidase domains (Renault et al., 2011) and the gene coding for a tyrosinase 1 identified in *C. gigas* showed a typical copper-binding domains and a signal peptide (Huan et al., 2013). A gene coding for a tyrosinase has also been reported in