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Characterization of a laccase-like activity in the hemolymph of the abalone *Haliotis tuberculata*

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ABSTRACT

Along French coasts, the abalone *Haliotis tuberculata* is affected by mass mortality events caused by both immune depression and the pathogen *Vibrio harveyi*. During this immune depression various immune parameters have been monitored including phenoloxidase (PO) activity, which significantly decreases. Nevertheless the basal PO activity level, to date, has not been characterized biochemically and the PO subclass involved has not been identified. The aim of this study was to use various substrates and inhibitors, specific to one or more PO subclasses, to biochemically characterize enzymatic activity. Among specific and non-specific substrates, PO had the highest affinity for dopamine ($K_m = 1.92$, $V_{max} = 0.086$). Tyrosinase-specific substrates were not oxidized whereas both laccase-specific substrates tested were oxidized. Results obtained with the different inhibitors tested are in agreement with the results obtained with substrates. In fact, the laccase-specific CTAB totally inhibited PO activity and the IC50 for this inhibitor was the lowest of any tested. The molecular weight has been estimated between 83 and 106 kDa.

1. Introduction

In 1960, *Haliotis tuberculata* mass mortalities were recorded along the French coasts of Brittany but these mortalities were explained by extreme cold temperatures during winter (Nicolas et al., 2002). Since late 2000, this species has also been affected by mass mortality events in both wild and farmed populations along the French coasts of Brittany and Normandy (Huchette and Clavier, 2004). These mass mortality events, occurring at the end of the summer, are attributable to the pathogenic agent *Vibrio harveyi* (junior synonym: *Vibrio carchariae*) (Nicolas et al., 2002), but it is not the only cause. Mortality events occur only if the presence of the bacterium coincides with high temperature and with immune depression in the abalone (Travers et al., 2008, 2009). Furthermore, immature abalone are not affected by the vibriosis because the immune depression ties in with the spawning period (Travers et al., 2008). In fact, there is an energetic trade-off between gametogenesis and the immune system, involving reallocation of energy from immune defenses to reproduction. Other causes of mass mortality events have been studied in abalone worldwide: *Candidatus Xenohaliotis californiensis* in *Haliotis cracherodii* in the USA (Friedman and Finley, 2003) and *Haplosporidium montforti* in *H. tuberculata* in Europe (Balseiro et al., 2006); *Vibrio fluvialis* II in *Haliotis discus hannai* in China (Li et al., 1998); and *Pseudoklossia haliotis* sp. in *Haliotis* spp. in the USA (Friedman et al., 1995). Several studies have monitored the immune system of infected abalone through different immune parameters, such as total hemocyte count, phagocytosis or production of reactive oxygen species (ROS) (Hooper et al., 2007). Another immune parameter recorded in abalone is the phenoloxidase (PO) cascade, which is modulated when exposed to various biotic or abiotic stresses. These modulations can be caused by intrinsic biotic factors such as gonad maturation and spawning events, which significantly decreased PO activity (Travers et al., 2008). Other studies focus on abiotic factors, and experiments by Cheng et al. (2004e) showed that when the abalone, *Haliotis diversicolor supertexta*, were exposed hypoxic conditions, PO activity significantly increased. Otherwise, it has been reported that salinities ranging from 30 to 35‰ and temperatures from 24 to 30 °C are optimal for this species growth, but when the animals were transferred from 28 °C to 20 °C sea water, PO activity increased whereas it decreased when the animals were transferred to sea water at 24 or 32 °C (Cheng et al., 2004c). There was also a decrease of PO activity when the animals were transferred from 30‰ salinity sea water to 20, 25 or 35‰ salinity sea water (Cheng et al., 2004d). In contrast, PO activity significantly increased in the animals exposed to sea water with nitrite concentrations from 0.96 to 10.16 $mg.mL^{-1}$ (Cheng et al., 2004a) and ammonia concentrations from 1.08 to 10.30 $mg.mL^{-1}$ (Cheng et al., 2004b). The effects of these compounds were studied because nitrite is an intermediate product of bacterial nitrification of ammonia. These PO responses were measured in order to evaluate the impact of abiotic factors on the susceptibility of the abalone to *Vibrio parahaemolyticus*. Mottin et al. (2010) studied another abiotic factor, showing that PO activity increased in a dose dependent manner in *H. tuberculata* hemocytes exposed to zinc. Most often, these factors, whether biotic or abiotic, are considered separately, despite the fact

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that mortality events in *H. tuberculata* are associated with a combination of biotic and abiotic factors (Travers et al., 2008, 2009). PO has also been explored at the genetic level to understand its role against the bacterium *V. harveyi*: the pathogen implied in mortality events in *H. tuberculata*. Partial silencing of two proPO genes resulted in a significant and strong PO activity reduction and a very high mortality in the shrimp *Penaeus monodon* after *V. harveyi* challenge (Amparyup et al., 2009).

These different studies underline the importance of PO activity to evaluate the immune capacities of animals facing biotic or abiotic stresses; nevertheless, no study has focused on characterizing PO activity in the abalone to better understand its biochemical role and functioning. Previous studies showed that PO activity in the circulating hemolymph of the clam, *Venerupis philippinarum*, was mainly a laccase-like activity but there was also smaller-scale tyrosinase activity (Le Bris et al., 2013). Thus, the aim of this study was to biochemically characterize the PO activity in the circulating hemolymph of *H. tuberculata* using various substrates and inhibitors, in order to determine which PO subclass is present from among the tyrosinases, catecholoxydases and laccases. The optimal pH was also determined and a partial purification of the enzyme was carried out to estimate its molecular weight.

2. Materials and methods

2.1. Live material

Fifty-six adult abalone, *H. tuberculata*, 57.59 ± 3.19 mm shell length, were transferred from the hatchery at France Haliotis (Plouguerneau, France) to the LEMAR laboratory (University of Western Brittany) in March 2012. These abalone were maintained in aerated aquaria (100 L) at room temperature (16 °C) and acclimated for 5 days before sampling.

2.2. Preparation of plasma samples

Just before sampling, the animals were removed from their aquaria. Hemolymph of the 56 abalone was withdrawn from the cephalic arterial sinus in the anterior muscle, using a needle (25 G 0.5 × 16 mm) fitted onto a 2 mL sterile syringe. The 85 mL haemolymph collected from all abalone was pooled and centrifuged at 785 *g* for 10 min at 4 °C, to separate the cellular fraction (hemocytes) from the plasma (haemolymph, or HL). The resulting supernatant (i.e., plasma) was stored at −80 °C until use in the assays. PO auto activation has been previously tested and after freezing at −80 °C, PO was activated in HL (data not shown).

2.3. Chemicals

All chemicals were purchased from Sigma-Aldrich (France); except for BioRad Protein Assay Dye Reagent Concentrate used for total protein concentration, obtained from BioRad France; and Sephacryl S-100 chromatography media, obtained from GE Healthcare.

2.4. Protein determination

The total protein content of *H. tuberculata* plasma was determined by the method of Bradford (1976) using BioRad Protein Assay Dye Reagent Concentrate (BioRad France) and bovine serum albumin (BSA) as the protein standard.

2.5. Phenoloxidase activity assays

PO activity was assayed spectrophotometrically in 96-well microplates (Greiner 96-F-bottom), by recording the formation of quinones according to the protocol of Le Bris et al. (2013). Briefly, 50 μL Tris–HCl buffer (0.10 M, pH 8.2) was added to 50 μL of HL, and this mixture was incubated for 10 min at 25 °C. Then 100 μL substrate, prepared at several concentrations, was added to each well. The plate was quickly mixed and placed in a microplate spectrophotometer (POLARstar OMEGA—BMG Labtech) to measure absorbance (A) at 25 °C immediately after the substrates were added. PO activity was then monitored for 30 min following the increase of absorbance at the substrate-specific wavelength. At the same time, the spontaneous oxidation of each substrate was measured by replacing HL with pure water in blank runs, and the values obtained were subtracted from test values. Buffer controls (containing 50 μL HL, 50 μL pure water and 100 μL substrate) and substrate controls (containing 50 μL HL, 50 μL Tris–HCl buffer and 100 μL pure water) were also carried out. For each treatment, controls, samples and blanks were tested in triplicate. PO activity was estimated based on the increment in the rate of absorbance (A) per minute obtained from the slope of the linear region from the plot of absorbance on time. The PO specific activities were expressed in arbitrary unit (U) per milligram of total protein. One U is defined as the quantity of enzyme that, in optimum conditions of pH, temperature and substrate saturation, produces 1 μmol of product per minute. PO specific activity values were obtained as follows:

$$\text{PO specific activity} = \left(\Delta A.\text{min}^{-1} \times \text{dilution factor}\right)/\text{Total protein concentration}.$$

2.5.1. Substrates

A number of different potential substrates were tested. These substrates could be tyrosinase-specific (monophenols), laccase-specific (metoxiphenols, p-diphenols and non-phenolic substrates) or o-diphenols, which may or may not be oxidized directly by each PO subclass.

L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine were used as substrates common to all three PO subclasses. L-tyrosine and p-hydroxyphenyl propionic acid (PHPPA) were used as a tyrosinase-specific substrates. P-phenylenediamine (PPD) and 1,4-benzenediol (hydroquinone) were used as laccase-specific substrates. Substrate solutions were prepared in pure water just before use except for PPD which was prepared in methanol. Each time it was possible, the Michaelis–Menten constant (K_m) and the maximum reaction velocity (V_{max}) were obtained from the Lineweaver–Burk equation, a linear transformation of the Michaelis equation. An Eadie–Hoftsee plot was also made to calculate the inhibition constant K_i when there was an excess-substrate inhibition.

2.5.2. Phenoloxidase inhibition assay

A PO inhibition assay was performed by preincubating 50 μL PO inhibitor (prepared at various concentrations) in Tris–HCl buffer with 50 μL HL for 10 min at 25 °C. A PO assay was then carried out with 100 μL L-DOPA (0.04 M) dissolved in pure water.

Kojic acid, citric acid and ethylenediaminetetraacetic acid (EDTA) were tested as potential common PO inhibitors. 4-Hexylresorcinol (4-Hr) and salicylhydroxamic acid (SHAM) were tested as potential tyrosinase- and catecholase-specific inhibitors. Hexadecyltrimethylammonium bromide (CTAB) was tested as a potential laccase inhibitor. Dose–response curves were plotted and calculations were performed to identify the concentration corresponding to the maximum of inhibition and the concentration causing 50% inhibition (IC50).

2.6. Partial purification

4.75 mL hemolymph supernatant from the 85 mL pooled was desalted and concentrated using Centricon devices with ultracel YM-10 membrane (10,000 NMWL, Amicon™), resulting in 0.9 mL of concentrated HL. This solution was loaded onto a Sephacryl S-100 column (86 mL—fractionation range from10 to 100 kDa) and eluted with