
Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro

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Abstract:

The prophenoloxidase (ProPO) system is the origin of melanin production and is considered to be an innate defence mechanism in invertebrates. In different bivalve species, phenoloxidase (PO) is present in the haemolymph as an inactive form of ProPO. The present study focuses on the Pacific adult oyster, *Crassostrea gigas*, an economically important bivalve species along French coasts. The results indicate that many factors may inhibit the PO-like activity. These include: phenylthiourea (PTU), sodium diethylthiocarbamate (DETC), β -mercaptoethanol and tropolone, which repressed the spontaneous PO activity. The activation of PO-like activity in *C. gigas* acellular fraction by lipopolysaccharide (LPS) involved participation of other factors, including at least one serine protease. PO was present as proPO in the acellular fraction of haemolymph and haemocytes of *C. gigas* and could be activated by an exogenous protease (trypsin-*N*-tosyl-L-phenylalanine chloromethyl ketone) when used at 1 g L^{-1} . Treatment of acellular fractions with other modulators/activators namely LPS (1 g L^{-1}), zymosan (0.6 g L^{-1}) or laminarin (0.6 g L^{-1}) also increased PO-like activity but to a less important way. The study demonstrated the evidence of a true phenoloxidase activity in Pacific oyster, *C. gigas* (Thunberg). The activation of a proPO system by non-self molecules suggests the role played by PO in vivo in the internal defence mechanisms. Understanding the activation of the ProPO system could enable the evaluation of the health of oyster stocks.

Keywords: *Crassostrea gigas*; Defence; Immunity; Oyster; Phenoloxidase; Prophenoloxidase.

1. Introduction

In invertebrates, haemocytes and some proteolytic pathways (coagulation, melanin synthesis and opsonisation) are involved in defence reactions toward pathogens. The prophenoloxidase (ProPO) system, at the origin of melanin production, has been suggested as an innate defence mechanism in invertebrates (1). In arthropods, it has been demonstrated that phenoloxidase (PO) is present in the haemolymph as an inactive form of ProPO. ProPO is cleaved by proteolysis via an endogenous activator system or exogenous agents, to PO, the enzymatically active form (2). PO is copper-dependent and catalyses the synthesis of o-diphenols from monophenols by ortho-hydroxylation, which are then dehydrogenated into o-quinones (3-5). Non-enzymatic polymerisation of o-diphenols leads to the production of melanin, a common response of invertebrates to the entry of an infectious agent. The major function of the PO cascade is the deposition of the pigment, melanin derived from tyrosine-based substrates such as L-dopa. Components of the PO cascade can also opsonize pathogens for phagocytosis, while others appear to be fungistatic or cytotoxic (6). The activation of the enzymatic system by bacterial or fungal components is an additional clue of the involvement of this enzyme in invertebrate immune defence mechanisms. Many components of the system have recently been isolated in invertebrates, particularly from aquatic arthropods (7-9).

Phenoloxidase-like activity has also been detected in the haemolymph of molluscs such as *Mya arenaria* Linne, *Mytilus edulis*, *Patella vulgata* Linne, *Ruditapes philippinarum*, *Chamelea gallina*, *Tapes decussatus* and *Hyriopsis cumingii* (1, 10-17) but until now, only few studies have focused on the adult Pacific oyster *Crassostrea gigas* (16-18). Moreover, in all these studies, no real PO activity was demonstrated. PO activity was followed using the classical spectrophotometric technique based on L-Dopa hydrolysis. However, several molecules are able to hydrolyse L-Dopa. In these conditions, it appears necessary to demonstrate that the activity detected is a real PO activity. Understanding the activation of the pro-PO system in *C. gigas* oysters could be used as a probe for the evaluation of their health and could provide rapid inexpensive tests for environmental stress (6). This is of particular importance as the aquaculture of oysters contributes significantly to the economic development of many tropical, subtropical and temperate countries (19). France is currently one of the leading European shellfish producing countries, harvesting annually more than 150 000 metric tons of *C. gigas* (20). But aquaculture is threatened by the repeated appearance of new diseases, non-infectious and infectious. Difficulties in controlling these diseases come mainly from the differences in susceptibility of the animals according to their developmental stage (from larvae to adults) and from the diversity of pathogens (parasites, viruses, fungi and bacteria) that affect them (21-26). Antibiotics have been used intensively as preventive and curative measures but such practices are now questioned due to the appearance of drug-resistant bacteria and their harmful effects on the environment (21). As an outcome, alternative treatments as well as animal health monitoring have to be established as preventing measures. Investigation of the innate immune systems may give new insights into the management and control of diseases in aquaculture (27-30).

In this study, *in vitro* experiments on the modulation of the PO-like activity have been conducted in the cellular and the acellular fraction of haemolymph, in adult Pacific oyster, (*C. gigas*). The specific inhibitors of PO activity were tested phenylthiourea, β -mercaptoethanol, sodium diethyldithiocarbamate and tropolone. Some activators of PO activity, LPS, laminarin, zymosan, trypsin were used to modulate activation of oyster proPO system *in vitro*. This allowed the demonstration of a true phenoloxidase activity and the process of PO activation in *C. gigas* to be characterised.

2. Material and methods

2.1. Animals

One year old Pacific oysters, *Crassostrea gigas* were purchased during January-May 2002 from shellfish farms in Aytré Bay (Charente Maritime, France), on the French Atlantic coast and were processed immediately after their arrival in the laboratory.

2.2. Circulating haemolymph collection

After opening the oyster shells by cutting off the adductor muscle, haemolymph was withdrawn directly from the pericardial cavity with a 1-mL syringe equipped with a needle (0.9 x 25 mm). A quantity (0.5 - 1 mL) of haemolymph was withdrawn and the haemolymph from 30 oysters was pooled to reduce inter-individual variation.

2.3. Detection of phenoloxidase-like (PO-like) activity

Haemolymph samples were centrifuged (260 g, 10 min, 4°C), and PO-like activity was analysed in the acellular fraction. The cellular pellet and the haemocyte lysate supernatants of *C. gigas* were also used as sources of PO activity after 24 h incubation.

The cellular pellet was resuspended in cacodylate buffer (CAC: 10 mM sodium cacodylate, 100 mM trisodium citrate, 0.45 M NaCl, 10 mM CaCl₂, 26 mM MgCl₂, pH=7.0) and agitated. This preparation was then centrifuged (40 000 g, 25 minutes, 4°C, Microfuge 12, Beckman), and the haemocyte lysate supernatants (HLS) removed. PO-like activity was analysed in the cellular pellet and the HLS too.

The detection of PO-like activity in haemolymph samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-dopa) transformation to dopachromes (14). Transformation was monitored by spectrophotometry at 490 nm. Samples were distributed in 96 well microplates (Nunc, France). Various well-known modulators of PO activity (all purchased from Sigma, France) were tested at concentrations ranging from 0.2 g.L⁻¹ to 10 g.L⁻¹: LPS, laminarin, zymosan and purified trypsin. Phenylthiourea (10 mM), sodium diethylthiocarbamate (DETC) (10 mM), β-mercaptoethanol (10 mM) and tropolone (16 mM) were also tested for their effects on PO-like activity (all these compounds were purchased from Sigma France). Sixty μL of cacodylate buffer (CAC: 10 mM sodium cacodylate, 100 mM trisodium citrate, 0.45 M NaCl, 10 mM CaCl₂, 26 mM MgCl₂, pH=7.0), 20 μL of a PO activity modulator, 20 μL of L-dopa (Sigma) and 20 μL of acellular fraction of haemolymph were added in each well. The assay was run at 20°C. Control wells and negative control contained only 120 μL of CAC buffer and 100 μL of CAC buffer plus 20 μL of L-dopa, respectively.

Each sample tested was tested in triplicate well and A₄₉₀ was measured after a 21 h incubation period (17, 18, 20).

2.4. Statistical analysis

Data were expressed as mean values ± SD. Each experiment involved three replicates. The values presented were the averages of nine replicates. The data were statistically analysed using XLStat Pro version 7.5.2 soft ware. Normality was tested using the Anderson-Darling test (31). One way analysis of variance (ANOVA) was applied to analyse the differences among treatments and control. P values lower than 0.05 were used to identify significant differences. Where significant differences occurred, an *a posteriori* Student-Newman-Keuls (SNK) test was used.

3. Results

PO-like activity was detected in the acellular fraction of the haemolymph of adult *C. gigas*. The absorbance reached a maximum after 21 hours of incubation (Figure 1). Oxidation of L-dopa was significantly increased after incubation of the acellular fraction of oyster haemolymph with Trypsin at 0.5 g.L⁻¹ or 1 g.L⁻¹. From 2 g.L⁻¹, PO-like activity was significantly lower than in the control and was not detected at Trypsin concentration of 10 g.L⁻¹ after 21 h incubation (Figure 2).

A significant stimulation of PO-like activity was induced with zymosan at various concentrations. A maximum effect was observed at 10g.L⁻¹ but below 1 g.L⁻¹, highest stimulation was with a zymosan concentration of 0.6 g.L⁻¹ (Figure 3). Stimulation was not dose-dependent.

Incubation of the acellular haemolymph of *C. gigas* with laminarin at concentrations ranging from 0.2 g.L⁻¹ to 1 g.L⁻¹ stimulated PO-like activity. At 0.5 and 0.6 g.L⁻¹, laminarin induced the highest PO-like activity (Figure 4). Stimulation was not dose-dependent.

PO-like activity in the acellular fraction was significantly enhanced with LPS concentrations of 0.5 g.L⁻¹, 0.9 g.L⁻¹, 1 g.L⁻¹ (Figure 5).

As shown as Table I, in the cellular pellet and haemocyte lysate supernatant, PO activity is low compared to the acellular fraction. For the acellular fraction, the addition of trypsin enhanced significantly the PO-like activity for incubation from 4h to 24h. At the opposite, for the cellular pellet and the haemocyte lysate supernatant, the PO-like was always low despite addition of trypsin or not.

Phenylthiourea, a copper binding compound significantly inhibited PO-like activity compared to the control. PTU, β -mercaptoethanol and DETC completely abolished PO-like activity ($p < 0.05$). Tropolone, a specific inhibitor of PO and a potential substrate for peroxidase repressed the spontaneous activity and abolished the stimulating effect of LPS (Table II).

4. Discussion

The present study confirmed that a PO-like activity can be detected *in vitro* in the acellular fraction of the haemolymph from adult Pacific oysters, *C. gigas*. Maximal absorbance was observed in all samples after a 21 h incubation period with the substrate (L-dopa). The present study establishes also the effect of various factors on the phenoloxidase-like activity, in cellular fraction of the Pacific adult oyster *C. gigas* haemolymph. Some molecules (tropolone, DETC, β -mercaptoethanol and PTU) inhibit the PO-like activity. This may be due to copper chelation by PTU, DETC and β -mercaptoethanol, as copper is essential to the functionality of the phenoloxidase. The activity estimated at 490 nm was not due to a peroxidase-like activity but definitely to a phenoloxidase activity, as tropolone, a specific inhibitor of phenoloxidase but potential substrate of peroxidase, suppressed the activating effects of the LPS and also the oxidation of L-dopa.

The stimulation of PO activity by Trypsin, a serine protease, suggests that PO may be present in the acellular haemolymph in the form of a pro-enzyme and required a proteolytic cleavage for activation. Furthermore, this result suggests that endogenous serine-proteases may enhance the proteolytic cleavage of the pro-enzyme. The cleavage of inactive proPO into active PO may be earlier in *C. gigas* than in mussels since Coles and Pipe (10) showed that the activation of the proPO system in *Mytilus edulis* occurred after a 96 h incubation with L-dopa.

The PO like activity in *C. gigas* is sensitive to *in vitro* activation by LPS, zymosan or laminarin. Increase of the enzymatic activity does not appear to be dependent on the concentrations of the exogenous molecules used. A maximum of PO activity was obtained during the incubation of the acellular haemolymph with concentrations of Trypsin at 0.5 g.L⁻¹ or 1 g.L⁻¹. In contrast, in the shrimp *Penaeus paulensis*, Trypsin is used at a concentration of 1 g.L⁻¹ and in the mussel *Perna viridis* at 0.7 g.L⁻¹ (11, 24). It has been shown that in the mussel *P. viridis*, the pro-PO system is 1000 times more sensitive to LPS than to laminarin (11). Trypsin enhances of PO activity in a more efficient way than LPS or β -glucans (laminarin and zymosan) in Pacific oyster, *C. gigas*.

Increase of the enzymatic PO activity by exogenous molecules in this study suggests that the pro-PO system play a role in non-self recognition and defence reactions in the adult *C. gigas*. The mechanism of the activation of pro-PO system via exogenous molecules was studied before. Some proteins from serum capable of reacting with β -glucans (laminarin and zymosan) and

initiating the activation of the pro-PO system have been identified in insects and shellfish (8, 33-37).

Although PO is widely distributed throughout the animal kingdom, this study unambiguously demonstrated its presence as a proenzyme (proPO) in acellular fraction of haemolymph of *C. gigas*. Microbial substances enhanced the enzyme activity *in vitro* suggesting its potential role in host defence. The activation responses of this proenzyme in *C. gigas* to exogenous proteases, microbial cell wall components, and its susceptibility to protease inhibitors *in vitro* was similar to the proPO activation system of arthropods. The similarities in activation responses of haemolymph proPO system in animals belonging to two different phyla tend to imply a unifying biochemical mechanism for immune recognition among invertebrates.

In aquaculture, animals are submitted to stressful environmental and ecological conditions and this have been linked to high-density animal populations, pollution and nutritional imbalances. As ProPO and PO are involved in the immune response, a biochemical test on PO or Pro-PO activities could be used as a probe to measure health conditions of animals.

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Figure 1: *In vitro* PO-like activity (A_{490}) detected in acellular fraction of *C. gigas* haemolymph after 24 h incubation. Values are averages of 9 replicates. *= significant differences $p < 0.05$.

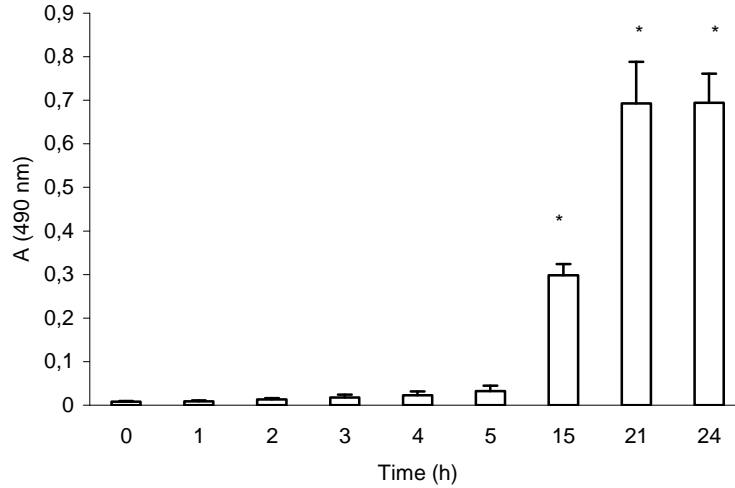


Figure 2: *In vitro* PO-like activity (A_{490}) stimulated with Trypsin detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, Trypsin was replaced by CAC buffer. Values are averages of nine replicates. *= significant differences $p < 0.05$.

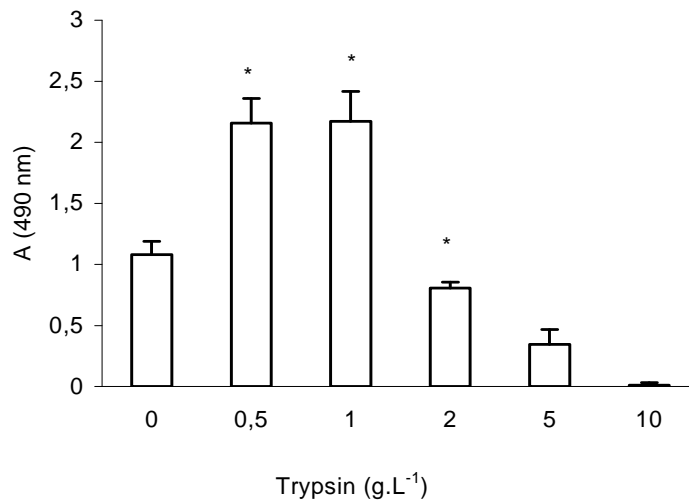


Figure 3: *In vitro* PO-like activity (A_{490}) with zymosan detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, zymosan was replaced by CAC buffer. Values are averages of nine replicates. *= significant differences $p < 0.05$.

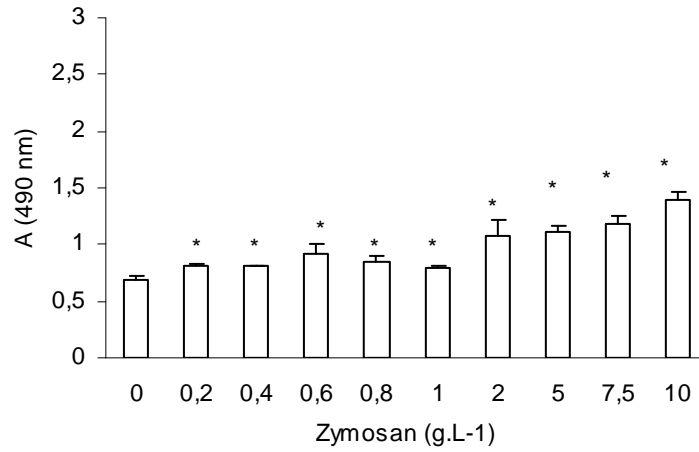


Figure 4: *In vitro* PO-like activity (A_{490}) with laminarin detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. For the control, laminarin was replaced by CAC buffer. Values are averages of nine replicates. *= significant differences $p < 0.05$.

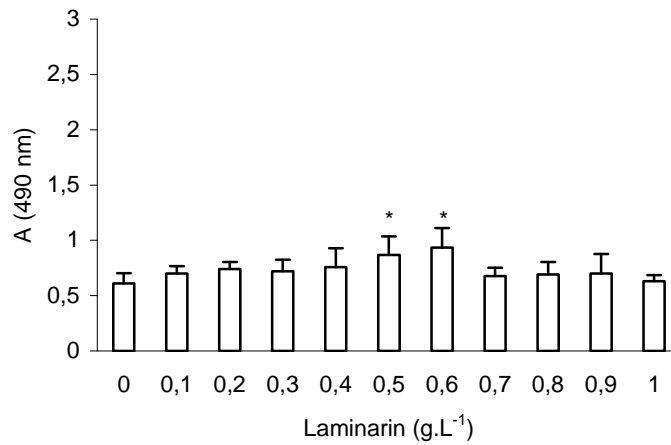


Figure 5: *In vitro* PO-like activity (A_{490}) with LPS detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. In control, LPS was replaced by CAC buffer. Values are averages of nine replicates. *= differences $p < 0.05$.

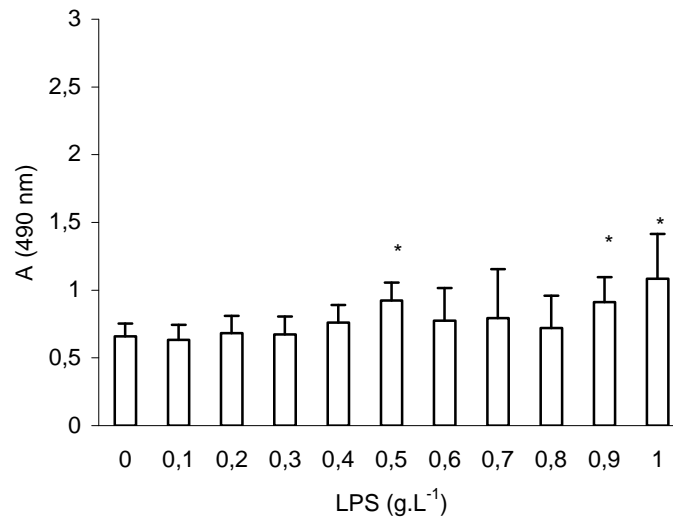


Table I: *In vitro* PO-like activity (A_{490}) with (stimulated activity) or without (spontaneous activity) Trypsin in acellular fraction, supernatant and cellular pellet of *C. gigas* haemolymph after 24 h incubation. Control was pre-incubated in CAC buffer for 20min before reaction with L-dopa. Values are averages \pm SD of 3 replicates.

Fraction analysed		0h	2h	4h	15h	21h	24h
Acellularfraction	No trypsin	0,033 \pm 0	0,055 \pm 0	0,104 \pm 0,038	0,235 \pm 0,082	0,541 \pm 0,102	0,684 \pm 0,091
	Trypsin	-0,0006 \pm 0,016	0,034 \pm 0,0182	0,198 \pm 0,053	0,849 \pm 0,126	0,831 \pm 0,062	0,925 \pm 0,128
Haemocyte lysate supernatant	No trypsin	0,003 \pm 0,002	0,005 \pm 0,004	0,006 \pm 0,005	0,002 \pm 0,005	0,053 \pm 0,017	0,052 \pm 0,029
	Trypsin	0,002 \pm 0,011	0,004 \pm 0,007	0,004 \pm 0,008	0,004 \pm 0,007	0,030 \pm 0,006	0,025 \pm 0,005
Cellular pellet	No trypsin	-0,001 \pm 0	0,009 \pm 0,015	0,006 \pm 0,014	0,006 \pm 0,045	0	0,019 \pm ,002
	Trypsin	0,003 \pm 0,006	0,008 \pm 0,011	0,008 \pm 0,011	0,004 \pm 0,007	0,036 \pm 0,015	0,025 \pm 0,010

Table II: *In vitro* PO-like activity (A_{490}) with different inhibitor agents detected in acellular fraction of *C. gigas* haemolymph after 21 h incubation. Control was pre-incubated in CAC buffer for 20min before reaction with L-dopa. Values are averages of three replicates. *** = Significant differences $p < 0.001$.

Treatment	PO-like activity (A_{490})	Significance
Control	0,693 \pm 0,095	
PTU (10 mM)	0,001 \pm 0,001	***
DETC (10 mM)	0,001 \pm 0,001	***
β -mercaptoethanol (10 mM)	0,001 \pm 0,001	***
Tropolone (16 mM)	0,000 \pm 0,000	***