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Abreviations :

PO: phenoloxidase; HLS: haemocyte lysate supernatant; PPD: p-phenylenediamine; PTU : 1-phenyl-2-thiourea; CTAB: cethyltrimethylammonium bromide; MBTH: 3-methyl-2-benzothiazolinone hydrazone; Tris HCl: trizma hydrochloride ; AS: ammonium sulfate; SDS: sodium dodecyl sulfate; TEMED: N,N,N',N'-tetramethylethylenediamine; BSA: bovine serum albumin.

Abstract: Phenoloxidases (POs) play a key role in melanin production, are involved in invertebrate immune mechanisms, and have been detected in different bivalves. Recently, we identified catecholase- and laccase-like PO activities in plasma and haemocyte lysate supernatant (HLS) of the Pacific oyster *Crassostrea gigas.* To go further in our investigations, the aims of this study were *(i)* to determine the tissue distribution of PO activities in *C. gigas,* and *(ii)* to identify and characterise the different sub-classes of POs (i.e. tyrosinase, catecholase and/or laccase) involved in these oxido-reductase activities. With dopamine and p-phenylenediamine (PPD) but not with L-tyrosine used as substrates, PO-activities were detected by spectrophotometry in the gills, digestive gland, mantle, and muscle. These results suggest the presence of catecholase and laccase but not of tyrosinase activities in oyster tissues. The highest activity was recovered in the digestive gland. PO-like activities were all inhibited by 1-phenyl-2-thiourea (PTU) and by the specific laccase inhibitor, cethyltrimethylammonium bromide (CTAB). With dopamine as substrate, the catecholase inhibitor 4-hexylresorcinol (4-HR) only inhibited PO in the muscle. SDS-PAGE zymographic assays with dopamine and PPD elicited a unique ~40 kDa protein band in the muscle. In the other tissues, laccase-like activities could be related to ~10 kDa and/or ~200 kDa protein bands. The ~10 kDa protein band was also detected in plasma and HLS, confirming the presence of a laccase in the later compartments, and probably in most of the tissues of *C.gigas*. This is the first time to our knowledge that a ~10 kDa protein band is associated to a laccase-like activity in a mollusc species, contributing to the characterisation of phenoloxidase activities in marine bivalves.

Key Words: bivalve; phenoloxidase; laccase; catecholase; zymography

1. Introduction

Phenoloxidases (POs, EC 1.14.18.1) are a class of copper proteins widely distributed in bacteria, fungi, plants and animals (Cerenius et al. 2008). They play a key role in melanin production and are implicated in immune defence mechanisms in invertebrates. This class of enzymes include tyrosinases (EC 1.14.18.1), catecholases (EC 1.10.3.1) and laccases (EC 1.10.3.2), all capable of o-diphenol oxidation. However, among these three enzymes, only tyrosinases can hydroxylate monophenols (e.g. L-tyrosine), and only laccases can oxidise p-diphenols and aromatic compounds containing amine groups (e.g. p-phenylenediamine, PPD) (Thurston 1994, Solomon et al. 1996). In addition to that, a panel of inhibitors exert different actions on these three types of enzymes: while 1-phenyl-2-thiourea (PTU) inhibits the three types of PO activities (Williamson 1997, Jordan and Deaton 2005), 4-hexylresorcinol (4-HR) inhibits tyrosinase and catecholase but not laccase activities (Dawley and Flurkey 1993, Zavarzina and Zavarzin 2006) and cethyltrimethylammonium bromide (CTAB) specifically inhibits laccase activity (Walker and McCallion 1980). Recently, we conducted a study to identify PO activities present in the haemolymph of the Pacific oyster *Crassostrea gigas* (Luna-Acosta et al. 2010a). By using different PO substrates, such as L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine or PPD, and different PO inhibitors, such as PTU, 4-HR and CTAB, results suggested the presence of both catecholase- and laccase-like activities in the plasma, and the presence of a laccase-like activity in the haemocyte lysate supernatant (HLS, Luna-Acosta et al. 2010a). Our interest in *C. gigas* comes from the fact that this organism dominates over all other molluscs with respect to global distribution and aquaculture production, but suffers from massive summer mortality each year (Cheney et al. 2000). Summer mortality of *C. gigas* has been suggested to be the result of a complex interaction between the host, pathogens and environmental factors (Cheney et al. 2000). Importantly, studies in *C. gigas* have shown that PO activities, usually detected by using the o-diphenol substrates L-DOPA or dopamine, can be modulated by environmental factors, such as the presence of heavy metals or hydrocarbons (Gagnaire et al. 2004a, Bado-Nilles et al. 2008, Luna-Acosta et al. 2010b). In addition to that, a gene coding for a laccase in the haemocytes from *C. gigas* was modulated in the presence of hydrocarbons (Bado-Nilles et al. 2010). To the best of our knowledge, studies on POs in *C. gigas* have only been carried out in the haemolymphatic compartment. However, POs may be present in other body tissues in bivalves, e.g. in the prismatic shell layer (Nagai et al. 2007) or in the byssus gland (Hellio et al. 2000). A better characterisation and localisation of POs in *C. gigas* is needed to expand our knowledge on the immune defence mechanisms in this organism and therefore to a better understanding of the potential causes of summer mortality events.

In this general context, our goal was to determine the distribution and the nature of PO activities (tyrosinase, catecholase, and laccase) in different oyster body compartments, namely gills, digestive gland, mantle, muscle, plasma and HLS. PO activities were determined by spectrophotometry using different PO substrates (L-tyrosine, dopamine and PPD) and PO inhibitors (PTU, 4-HR, CTAB). Electrophoretic techniques using polyacrylamide gels are useful to detect PO enzymes and their associated molecular weights in crude extracts without the necessity of enzyme purification (Cardenas and Dankert 2000, Decker et al. 2001, Dicko et al. 2002, Perdomo-Morales et al. 2007). Hence, SDS-PAGE zymographic assays were carried out on crude and partially purified samples from the different oyster compartments. Differences between tissues, in terms of PO-like activity and molecular weight characteristics, are discussed.

2. Materials and methods

2.1. Chemicals and materials

L-tyrosine, dopamine, p-phenylenediamine (PPD), 1-phenyl-2-thiourea (PTU), 4-hexylresorcinol (4-HR), cethyltrimethylammonium bromide (CTAB), 3-methyl-2-benzothiazolinone hydrazone (MBTH), trizma hydrochloride (Tris HCl), sodium chloride (NaCl), ammonium sulfate (AS), sodium dodecyl sulfate (SDS), trizma base, glycine, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, glacial acetic acid, Coomassie brilliant blue, bovine serum albumin (BSA), copper sulfate and bicinchoninic acid were obtained from Sigma-Aldrich (France). Magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) were obtained from Acros organics (France). Acrylamide/Bis acrylamide 30% was obtained from Bio-Rad.

2.2. Oysters

Three years old Pacific oysters, *Crassostrea gigas* (n= 30; mean ± SD; weight: 75.5 ± 8.7 g; length: 9 ± 3 cm) were purchased during October-November 2008 from shellfish farms in Aytré Bay (Charente Maritime, France), on the French Atlantic coast, and were processed immediately after their arrival in the laboratory.

2.3. Collection of oyster tissues

After opening the oyster shells by cutting off the adductor muscle, a quantity (0.5-1 ml) of haemolymph was withdrawn directly from the pericardial cavity with a 1-ml syringe equipped with a needle (0.9 x 25 mm), and the haemolymph from 10 oysters was pooled to reduce inter-individual variation (Gagnaire et al. 2004b). Haemolymph samples were centrifuged (260 g, 10 min, 4°C) to separate the cellular fraction (i.e. haemocytes) from plasma, as described previously (Hellio et al. 2007). Gills, digestive gland, mantle and muscle were removed from oysters and pooled. Three replicates from 10 oysters were prepared per tissue.

Haemocytes, gills, digestive gland, mantle and muscle were homogenized at 4°C in Tris buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) adjusted to pH 7. Haemocytes were lysed using a Thomas-Potter homogenizer (IKA-Labortechnik, clearance 0.13-0.18mm) at 200 rpm for 1 min. Gills, digestive gland, mantle and muscle were homogenized, as described previously (Luna-Acosta et al. 2010b), using an Ultra Turrax (T25 basic, IKA-WERKE) at 19 000 rpm for 1 min followed by twelve up and down strokes of Thomas-Potter homogenizer at 200 rpm for 1 min (IKA-Labortechnik RW 20.n, size 0.13-0.18mm, BB). All homogenized samples were centrifuged at 10 000 g for 10 min at 4°C. The resulting haemocyte lysate supernatant (HLS) and tissue supernatants were collected for enzymatic studies.

Aliquots (100 µl) of plasma, HLS and tissue samples were stored at -80°C. Each aliquot was used only once per microplate for spectrophotometric analysis, or per gel running for zymographic studies.

2.4. Partial purification

A previous analysis, by using different concentrations of saturated ammonium sulfate (0, 30, 40, 60, 70, 80, 100%), revealed that precipitation with 60% saturated ammonium sulfate (60P-SAS) was the best condition for protein concentration to detect PO-like activity for oyster tissues, i.e. the gills, digestive gland, mantle and muscle (data not shown), and was in agreement with other studies (Cong et al. 2005, Liu et al. 2006). Therefore, proteins of collected supernatants from oyster tissues were brought to 60% saturation concentration by addition of solid ammonium sulfate at 4°C, and allowed to stand overnight. The resulting precipitate was collected by centrifugation (15 500 g for 10 min), dissolved in a small volume of Tris buffer , and dialysed at 4°C against distilled water for 12h and twice against Tris buffer for 8h. Crude plasma samples were concentrated with Centricon-5 centrifugal concentration units (AmiconTM).

2.5. Phenoloxidase assays

Phenoloxidase-like (PO-like) activity was measured spectrophotometrically by recording the formation of o-quinones, as described previously (Luna-Acosta et al. 2010a). PO assays were conducted in 96-well microplates (Nunc, France). Dopamine or p-phenylenediamine (PPD) were used as substrates, at final concentrations of 100 mM and 50 mM, respectively. Dopamine (100 mM) was prepared just before being used in Tris buffer. At 25°C, 10 µl of sample was incubated with 80 µl of dopamine and 50 µl of Tris buffer. Several control wells were systematically used: 'buffer control' containing only buffer, 'sample control' containing only sample and buffer, and 'non-enzymatic control' containing only substrate and buffer, always in a final volume reaction of 140µl. Immediately after dopamine addition, PO-like activity was monitored during 4h by using a VersaMax™ microplate reader (Molecular Devices) and by following the increase of absorbance at 490 nm. Because of solubility constraints, the protocol was slightly modified in the case of PPD: the sample was incubated with 7 µl of PPD (50 mM diluted in methanol) and 123 µl of buffer (no effect of methanol was observed on the enzymatic reactions). PO-like activity was monitored during 2h at 420 nm. For all conditions, the experiments were performed with three pooled oyster samples. Each pool was tested in triplicate wells and average rates were calculated by dividing the sum of replicate measurements from the three oyster pools, by the number of measurements, i.e. 9 (3 replicate measurements x 3 oyster pools).

For enzymatic oxidation, the results were systematically corrected for non-enzymatic autoxidation of the substrate and were expressed in specific activity (SA), i.e. in international units (IU) per mg of protein. One IU is defined as the amount of enzyme that catalyzes the appearance of 1 µmole of product per min (Fenoll et al. 2002) under the above conditions using molar extinction coefficient of dopamine and PPD reactions products of 3 300 M^{-1} cm^{-1} (Waite 1976) and 43 160 M^{-1} cm^{-1} (Eggert et al. 1996, Paranjpe et al. 2003), respectively.

2.6. Phenoloxidase inhibition assays

Working solutions of inhibitors were prepared just before being used in Tris buffer. PO inhibition assays were performed by preincubating 10 µl of the specific PO inhibitor PTU (5 mM, final concentration), the specific tyrosinase and catecholase inhibitor 4-HR (1 mM, final concentration), or the specific laccase inhibitor CTAB (1 mM, final concentration), with 10 µl of sample for 20 minutes. Then, PO assay was carried out with dopamine (100 mM, prepared in Tris buffer) or PPD (50 mM, prepared in methanol). Appropriate controls were used as described before. Experiments were performed with three pooled oyster samples. Each pool was tested in triplicate wells and average rates were calculated.

2.7. Protein assays

Protein concentrations were determined by the slightly modified Lowry method, as described previously (Smith et al. 1985), using bovin serum albumin as standard.

2.8. Gel electrophoresis and zymography

To associate PO enzyme activities with individual proteins, and estimate the molecular weights of the enzymes, SDS-PAGE and 1 D-zymography were used. Aliquots of the different oyster tissues (equivalent to 76 µg of proteins for gills, 76 µg for digestive gland, 57 µg for mantle, 40 µg for muscle, 47 µg for plasma and 1.55 µg for HLS) were mixed with sample buffer (65 mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue). Samples were then applied to 7% SDS-PAGE gels or 15% SDS-PAGE gels in non reducing conditions (i.e. without boiling samples after the addition of sample buffer) and with an upper gel of 4% using a Mini-PROTEAN III Cell (Bio-Rad). Electrophoresis was carried out according to the method of Laemmli (1970) at 110V for 2h45. Two gels containing the same samples were run and processed in parallel. For each tissue, samples previously brought to 0, 30, 40 or 60% saturation concentration by addition of solid ammonium sulfate at 4°C were runned per gel. After electrophoresis, SDS-PAGE gels were washed 2 x 10 min in distilled water and 2 x 10 min in Tris buffer.

The first SDS-PAGE gel was stained with a solution containing 100 mM L-tyrosine and 5 mM MBTH (to detect tyrosinase activity), 100 mM dopamine and 5 mM MBTH (to detect catecholase activity), or 100 mM PPD (to detect laccase activity). MBTH was used, according to the method of Dicko et al. (2002), to trap o-quinone products originating from the oxidation of phenolic compounds by phenoloxidases. All substrates were dissolved in Tris buffer. The gels were developed for 1 h, at 25°C and then rinsed with distilled water several times, dried at room temperature and photographed.

The second SDS-PAGE gels were immediately washed with distilled water and stained with Coomassie brilliant blue R-250 for visualizing total proteins. The molecular weight of PO activity bands were estimated with pre-stained molecular weight markers (Broad Range Markers, Tebu Bio, France) that were run together with samples (data not shown).

In order to test the specificity of the zymographic assay, a purified laccase from *Trametes versicolor* (20 µg) and a purified superoxide dismutase (SOD) from bovine erythrocytes (20 µg) were included in the activity gels.

2.9. Statistical analysis

All values are reported as mean ± standard deviation (SD). Statistical analysis was carried out with SYSTAT 11.0. Values were tested for normality (Shapiro test) and homogeneity of variances (Bartlett test). In some cases, logarithmic transformations (Log_{10}) were used to meet the underlying assumptions of normality and homogeneity of variances. For normal values, one-way nested ANOVA tests were used followed by a Tukey post-hoc test. For non normal values, Kruskal-Wallis tests were applied, followed by Dunn's multiple comparisons test (Zar 1984). The statistical significance was designed as being at the level of $p<0.05$.

3. Results

3.1. Spectrophotometric studies

Different PO substrates (L-tyrosine, dopamine and PPD), the common PO inhibitor, PTU, the tyrosinase and catecholase inhibitor, 4-HR, and the laccase inhibitor, CTAB, were used. When L-tyrosine was used as substrate, no PO-like activity was detected in any of the tissues that were tested, i.e. gills, digestive gland, mantle, and muscle (data not shown). When dopamine and PPD were used as substrates, PO-like activity was detected in all oyster tissues (Fig. 1). PO-like activity was inhibited by PTU. The inhibition was total in muscle with dopamine as substrate (Fig. 1g), and in digestive gland (Fig. 1d), mantle (Fig. 1f), and muscle (Fig. 1h) with PPD as substrate. PO-like activity was insensitive to 4-HR except in the muscle with dopamine as substrate (Fig. 1g). By contrast, PO-like activity was fully (or almost fully) inhibited by the laccase inhibitor CTAB (1 mM) in all the oyster tissues with both dopamine and PPD as substrates (Fig. 1).

Since fresh weight differs between the different analyzed tissues (i.e. the gills, digestive gland, mantle and muscle), tissue distribution of PO-like activity was also examined in terms of recovery of enzymatic activity (Table 1). With dopamine as substrate, the highest total PO-like activity was recovered in the digestive gland, followed by the gills, mantle and muscle (Table 1). With PPD as substrate, the total PO-like activity was considerably higher in the digestive gland compared to the other compartments.

3.2. SDS-PAGE zymographic assays

When gels were stained with L-tyrosine, no bands were observed, and this, for all the oyster tissues tested, i.e. the gills, digestive gland, mantle, muscle, plasma and HLS (data not shown). However, PO-like activity was detected in all oyster tissues that were analyzed by SDS-PAGE zymographs, with both dopamine and PPD as substrates.

PO substrates such as dopamine or PPD can oxidise non-enzymatically. This leads to different intermediary products of the melanization cascade such as quinone radicals. Unspecific reactions between quinone radicals issued from non-enzymatically oxidation reactions and radical species that could be produced by SOD-like proteins are thus likely to take place in zymographic studies (Eibl et al. 2010). However, PO-like activity was detected in the presence of laccase from *T. versicolor* but not in the presence of SOD from bovine erythrocytes (data not shown), confirming that our zymographic conditions were well adapted to discriminate between true PO-like activities and other enzymatic activities involving radical species.

With dopamine as substrate, the activity detected for the gills and the mantle corresponded to one upper band with an estimated molecular weight of ~200 kDa and to a lower band with an estimated molecular weight of ~10 kDa (Fig. 2a). In both tissues, most of the PO activity appeared in the higher molecular mass band. For the digestive gland the activity corresponded to an upper band of ~200 kDa and for the plasma and the HLS to one lower band with an estimated molecular weight of ~10 kDa (Fig. 2a). Similarly, an upper band with a molecular weight of ~200 kDa was observed in the presence of PPD for the gills and the mantle, but not for the digestive gland (Fig. 2b), and a lower band with a molecular weight of ~10 kDa was detected in the presence of PPD for the gills, digestive gland, mantle, crude plasma and crude HLS (Fig. 2b). Again, most activity in samples from the gills and the mantle was evident in higher molecular mass bands. The bands of ~10 kDa are not likely to be an artefact since they stained differentially with dopamine and PPD, depending on the tissue that was analyzed, e.g. stained with PPD but not with dopamine in the digestive gland. Contrary to the other tissues, the activity detected for the muscle, with dopamine and PPD as substrates, corresponded to a band with an estimated molecular weight of ~40 kDa.

4. Discussion

POs are of widespread occurrence in bacteria, fungi, plants, invertebrates and vertebrates (Sanchez-Ferrer et al., 1995; Cerenius et al., 2008). Despite the importance of the reactions and the functional roles associated to POs, the enzymes belonging to the PO class have not been thoroughly characterised in molluscs and especially in bivalve species. In the present study, spectrophotometric analyses were conducted to identify PO-like activity in different tissues from *C. gigas*. L-tyrosine, dopamine and PPD were used as substrates and different PO inhibitors were tested. When L-tyrosine was used as substrate, no PO-like activity was detected in any of the tissues that were tested, i.e. the gills, digestive gland, mantle, and muscle. Similar results were recently obtained with plasma and HLS (Luna-Acosta et al. 2010a). These data suggest the total absence of tyrosinase-like activity in *C. gigas*. Inhibition assays in the present study were thus only conducted with dopamine and PPD as substrates.

The choice on PO specific inhibitors was based on a previous study carried out on the PO inhibitors described in the literature (Luna-Acosta et al. 2010a). Indeed, PO substrates can chemically oxidise in the absence of PO (autoxidation) and many PO inhibitors described in the literature such as reducing agents (e.g. 2-mercaptoethanol or sodium azide) can inhibit autoxidation reactions (Luna-Acosta et al. 2010a). Such inhibitors are likely to react with the substrate and/or the quinone intermediates derived from the autoxidation reaction. Therefore, this type of inhibitors should be avoided for identifying PO activity. Among PO inhibitors described in different species, PTU was chosen because it has been described as a common inhibitor of all POs (Arias et al. 2003, Zufelato et al. 2004), 4-HR as a tyrosinase and a catecholase but not a laccase specific inhibitor (Dawley and Flurkey 1993, Zavarzina and Zavarzin 2006) and CTAB as a laccase but not a tyrosinase or a catecholase specific inhibitor (Walker and McCallion 1980, Martinez-Alvarez et al. 2008). Moreover, no inhibitory effect was observed with these chemicals in dopamine or PPD autoxidation reactions (Luna-Acosta et al. 2010a).

In the present study, PO-like activity in the muscle was completely inhibited in the presence of PTU (5 mM) and 4-HR (1 mM) with dopamine as substrate, and in the presence of PTU (5 mM) and CTAB (1 mM) with PPD as substrate (Fig. 1), suggesting the presence of catecholase and laccase in this tissue. These results are in agreement with those obtained previously with plasma (Luna-Acosta et al. 2010a). However, in the previous study, no inhibition was exerted by CTAB with plasma and with dopamine as substrate (Luna-Acosta et al. 2010a), while, in the present study, an inhibitory effect by CTAB was observed in the muscle and with dopamine as substrate. Since 4-HR and CTAB are specific catecholase and laccase inhibitors (van Doorn and Vaslier 2002), respectively, results in the muscle suggest the presence of a laccase sensitive to inhibition by 4-HR, or a catecholase sensitive to inhibition by CTAB.

Interestingly, PO-like activities in all other tissues were partially inhibited in the presence of PTU and completely inhibited in the presence of CTAB with dopamine and PPD as substrates, suggesting the presence of laccase activity in different tissues from *C. gigas* (Fig. 1). These results are in agreement with those obtained previously with HLS (Luna-Acosta et al. 2010a).

In zymographic studies, catecholase- and/or laccase- but not tyrosinase-like activities were detected in the gills, digestive gland, mantle, muscle, plasma and HLS (Fig. 2). This coincides with properties of the Asian swimming crab *Charybdis japonica* (Liu et al. 2006) and the eastern oyster *C. virginica* (Jordan and Deaton 2005), and differs from tyrosinase-type POs from other invertebrates, such as the vinegar fly *Drosophila melanogaster* (Asada et al. 1993), the bloodfluke planorb *Biomphalaria glabrata* (Bai et al. 1997), the Manila clam *Ruditapes philippinarum* (Cong et al. 2005) and the Sydney rock oyster *Saccostrea glomerata* (Aladaileh et al. 2007).

In the present study, when dopamine or PPD were used as substrates, PO-positive bands of ~10, 40 or 200 kDa were detected in *C. gigas* depending on the tissue. These tissue-dependent differences in molecular weights may be due to *(i)* the activation state of POs or *(ii)* the existence of polymeric forms of the enzyme. Indeed, the molecular weights of POs vary depending on the activation state, animal tissue and animal species that are studied (Table 2). In molluscs, the molecular weights of POs estimated by exclusion chromatography or SDS-PAGE electrophoresis are in the range of 35 to 381 kDa, and in invertebrates, POs occur as monomers, dimers, tetramers or pentamers (Jaenicke and Decker 2003). Thus, differences in molecular weights in the present study may be explained by the existence of polymeric forms of the enzyme. Generally, the molecular weight of monomeric forms is about 40 to 45 kDa, and generally each subunit possesses two copper atoms (Prota et al. 1981). In the present study, a PO-positive band of ~40 kDa was detected in the muscle of *C. gigas*, suggesting that a monomeric form of a laccase sensitive to inhibition by 4-HR, or a catecholase sensitive to inhibition by CTAB could be present in the muscle of this species. In the other analyzed tissues, PO-positive bands of ~10 or ~200 kDa were detected, and in the haemolymphatic compartments, PO-positive bands of ~10 kDa were detected. Results of the upper band of ~200 kDa are in agreement with the large range of molecular weights of PO reported for molluscs, i.e. from 35 to 381 kDa (Table 2). However, to our knowledge, this is the first time that a PO-positive band of ~10 kDa is reported in a mollusc species. A PO-positive band of ~10 kDa was detected in a non-mollusc aquatic invertebrate, the red swamp crayfish

Procambarus clarkii (Cardenas and Dankert 2000), suggesting that *C. gigas* possesses PO with characteristics (i.e. PO-like activity and molecular weight) comparable to that of arthropods.

Overall, in the present study, differences between tissues were observed in terms of *(i)* substrate affinity *(ii)* effect of PO inhibitors, and *(iii)* number and molecular weight of the bands detected by zymography. The data presented here suggest that zymography can be a useful way of characterising PO-like activities present in *C. gigas*. Interestingly, results of the present study revealed that numerous differences exist between the tissues and the haemolymphatic compartment in *C. gigas*, both in terms of PO-like activities and in terms of proteins that may be responsible for these activities. Our results indicate that at least three oligomeric forms of POs coexist in the Pacific oyster. This comparative study gives first evidences of structure-function relationships of tissue POs in *C. gigas*, contributing to the understanding of tissue-specific heterogeneity of PO activities in this marine organism. As POs are involved in immune response, tests based on modifications in oligomeric forms and functions of this class of enzymes, and more particularly of laccase, could be used as a probe to measure health conditions in this economically important species.

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References

Aladaileh, S., Rodney, P., Nair, S.V., Raftos, D.A., 2007. Characterization of phenoloxidase activity in Sidney rock oysters (*Saccostrea glomerata*). Comp. Biochem. Physiol. B 148, 470-480.

Arias, M.E., Arenas, M., Rodriguez, J., Soliveri, J., Ball, A.S., Hernandez, M., 2003. Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl. Env, Microbiol. 69, 1953-1958.

Asada, N., Fukumitsu, T., Fujimoto, K., Masuda, K., 1993. Activation of prophenoloxidase with 2-propanol and other organic compounds in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 23, 515-520.

Bado-Nilles, A., Gagnaire, B., Thomas-Guyon, H., Le Floch, S., Renault, T., 2008. Effects of 16 pure hydrocarbons and two oils on haemocyte and haemolymphatic parameters in the Pacific oyster, *Crassostrea gigas* (Thunberg). Toxicol. In Vitro 22, 1610-1617.

Bado-Nilles, A., Renault, T., Faury, N., Le Floch, S., Quentel, C., Auffret, M., Thomas-Guyon, H. 2010. *In vivo* effects of LCO soluble fraction on immune-related functions and gene transcription in the Pacific oyster, *Crassostrea gigas* (Thunberg). Aquat. Toxicol. 97, 196-203.

Bai, G., Brown, J., Watson, C., Yoshine, T., 1997. Isolation and characterization of phenoloxidase from egg masses of the gastropod mollusc, *Biomphalaria glabrata*. Comp. Biochem. Physiol. B 118, 463-469.

Cardenas, W., Dankert, J., 2000. Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish. Fish Shellfish Immunol. 10, 33-46.

Cerenius, L., Lee, B., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29, 263-271.

Cheney, D., Macdonald, E., Elston, R., 2000. Summer mortality of Pacific oysters *Crassostrea gigas* (Thunberg): Initial findings on multiple environmental stressors in Puget Sound, Washington. J. Shellfish Res. 19, 353-359.

Cong, R., Sun, W., Liu, G., Fan, T., Meng, X., Yang, L., Zhu, L., 2005. Purification and characterization of phenoloxidase from clam *Ruditapes philippinarum*. Fish Shellfish Immunol. 18, 61-70.

Dawley, R.M., Flurkey, W.H., 1993. Differentiation of tyrosinase and laccase using 4-hexylresorcinol, a tyrosinase inhibitor. Phytochemistry. 33, 281-284.

Decker, H., Ryan, M., Jaenicke, E., Terwilliger, N., 2001. SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. J. Biol. Chem. 276, 17796-17799.

Dicko, M., Hilhorst, R., Gruppen, H., Laane, C., van Berkel, W., Voragen, A., 2002. Zymography of monophenolase and o-diphenolase activities of polyphenol oxidase. Anal. Biochem. 306, 336-339.

Eggert, C., Temp, U., Eriksson, K., 1996. The lignolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. App. Env. Microbiol. 62, 1151-1158.

Eibl, J., Abdallah, Z., Ross, G., 2010. Zinc–metallothionein: a potential mediator of antioxidant defence mechanisms in response to dopamine-induced stress. Can. J. Physiol. Pharmacol. 88, 305–312.

Fan, T., Li, M., Wang, J., Yang, L., Cong, R., 2009. Purification and characterization of phenoloxidase from *Octopus ocellatus*. Acta Biochim. Biophys. Sin. 41, 865-872.

Fenoll, L., Rodriguez-Lopez, J., Garcia-Molina, F., Garcia-Canovas, F., Tudela, J., 2002. Unification for the expression of the monophenolase and diphenolase activities of tyrosinase. Int. U. Biochem. Mol. Biol. Life 54, 137-141.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004a. *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunol. 16, 501-512.

Gagnaire, B., Renault, T., Thomas-Guyon, H., 2004b. *In vitro* and *in vivo* effects of mercury on haemocytes of Pacific oyster, *Crassostrea gigas* (Thunberg): development of techniques evaluating estuarine pollution. In: Vème Congrès International de Limnologie-Océanographie, Paris, France.

Hellio, C., Bourgougnon, N., Le Gal, Y., 2000. Phenoloxidase (E.C. 1.14.18.1) from *Mytilus edulis* byssus gland: purification, partial characterization and application for screening products with potential antifouling activities. Biofouling 16, 235-244.

Hellio, C., Bado-Nilles, A., Gagnaire, B., Renault, T., Thomas-Guyon, H., 2007. Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) *in vitro*. Fish Shellfish Immunol. 22, 433-440.

Jaenicke, E., Decker, H., 2003. Tyrosinases from crustaceans form hexamers. Biochem. J. 371, 515-523.

Jordan, P., Deaton, L., 2005. Characterization of phenoloxidase from *Crassostrea virginica* hemocytes and the effect of *Perkinsus marinus* on phenoloxidase activity in the hemolymph of *Crassostrea virginica* and *Geukensia demissa*. J. Shellfish Res. 24, 477-482.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Liu, G., Yang, L., Fan, T., Cong, R., Tang, Z., Sun, W., Meng, X., Zhu, L., 2006. Purification and characterization of phenoloxidase from crab *Charybdis japonica*. Fish Shellfish Immunol. 20, 47-57.

Luna-Acosta, A., Rosenfeld, E., Amari, M., Fruitier-Arnaudin, I., Bustamante, P., Thomas-Guyon, H., 2010a. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol. 28, 719-726.

Luna-Acosta, A., Bustamante, P., Godefroy, J., Fruitier-Arnaudin, I., Thomas-Guyon, H., 2010b. Seasonal variation of pollution biomarkers to assess the impact on health status of juvenile Pacific oysters *Crassostrea gigas* exposed *in situ* Env. Sci. Pol. Res. 17, 999-1008.

Martinez-Alvarez, O., Montero, P., Gomez-Guillen, C., 2008. Evidence of an active laccase-like enzyme in deepwater pink shrimp (*Parapenaeus longirostris*). Food Chem. 108, 624-632.

Maruyama, N., Etoh, H., Sakata, K., Ina, K., 1991. Studies on phenoloxidase from *Mytilus edulis* associated with adhesion. Agric. Biol. Chem. 55, 2887-2889,

Nagai, K., Yano, M., Morimoto, K., Miyamoto, H., 2007. Tyrosinase localization in mollusc shells. Comp. Biochem. Physiol. B 146, 207-214.

Naraoka, T., Uchisawa, H., Mori, H., Matsue, H., Chiba, S., Kimura, A., 2003. Purification, characterization and molecular cloning of tyrosinase from the cephalopod mollusk, *Illex argentinus*. Eur. J. Biochem. 270, 4026–4038. Paranjpe, P., Karve, M., Padhye, S., 2003. Characterization of tyrosinase and accompanying laccase from *Amorphophallus campanulatus*. Indian J. Biochem. Biophys. 40, 40-45.

Perdomo-Morales, R., Montero-Alejo, V., Perera, E., Pardo-Ruiz, Z., Alonso-Jiménez, A., 2007. Phenoloxidase activity in the hemolymph of the spiny lobster *Panulirus argus*. Fish Shellfish Immunol. 23, 1187-1195.

Prota, G., Ortonne, J., Voulot, C., Khatchadourian, C., Nardi, G., Palumbo, A., 1981. Occurence and properties of tyrosinase in ejected ink of cephalopods. Comp. Biochem. Physiol. B 118, 463-469.

Renwrantz, L., Schmalmack, W., Redel, R., Friebel, B., Schneewei, H., 1996. Conversion of phenoloxidase and peroxidase indicators in individual haemocytes of *Mytilus edulis* specimens and isolation of phenoloxidase from haemocyte extract. Comp. Biochem. Physiol. B 165, 647-658.

Sanchez-Ferrer, A., Rodriguez-Lopez, J.N., Garcia-Canovas, F., Garcia-Carmona, F. 1995. Tyrosinase: a comprehensive review of its mechanism. Biochim. Biophys. Acta 1247, 1-11.

Smith, P., Khron, R., Hermanson, G., Mallia, A., Gartner, F., Provanzano, M., Fujimoto, E., Goeke, N., Olson, B., Goeke, N., Olson, B., Klenk, D., 1985. Measurement of a protein using bicinchoninic acid. Anal. Biochem. 150, 76-85.

Solomon, E., Sunduran, U., Machonkin, T., 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96, 2563-2606.

Thurston, C., 1994. The structure and function of fungal laccases. Soc. Gen. Microbiol. 140, 19.

van Doorn, W., Vaslier, N., 2002. Wounding-induced xylem occlusion in stems of cut chrysanthemum flowers: roles of peroxidase and cathechol oxidase. Postharvest Biol. Technol. 26, 275-284.

Waite, J., 1976. Calculating extinction coefficients for enzymatically produced o-quinones. Anal. Biochem. 75, 211-218.

Waite, J., Wilbur, K., 1976. Phenoloxidase in the periostracum of the marine bivalve *Modiolus demissus* Dillwyn. J. Exp. Zool. 195, 359-367.

Walker, J., McCallion, R., 1980. The selective inhibition of *ortho*- and *para*-diphenol oxidases. Phytochemistry 19, 373-377.

Williamson, P., 1997. Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. Front. Biosc. 2, 99-107.

Zar, J., 1984. Biostatistical analysis. Prentice-Hall, New Jersey.

Zavarzina, A.G., Zavarzin, A.A., 2006. Laccase and tyrosinase activities in lichens. Microbiology. 75, 546-556.

Zufelato, M.S., Lourenço, A.P., Simões, L.P., Jorge, J.A., Bitondi, M.M., 2004. Phenoloxidase activity in *Apis mellifera* honey bee pupae, and ecdysteroid-dependent expression of the prophenoloxidase mRNA. Insect Biochem. Mol. Biol. 34, 1257-1268.

Figure 1. Inhibition of phenoloxidase-like activity in precipitated protein fractions from the gills, digestive gland, mantle and muscle. Both dopamine (a, c, e, g) and PPD (b, d, f, h) were used as substrates. 'Control' corresponds to the condition without inhibitor. PO inhibitor concentrations correspond to final concentrations in the assay. Mean ± SD µmol.min^{-1}.mg prot^{-1}, n = 9; *statistical difference of $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively.

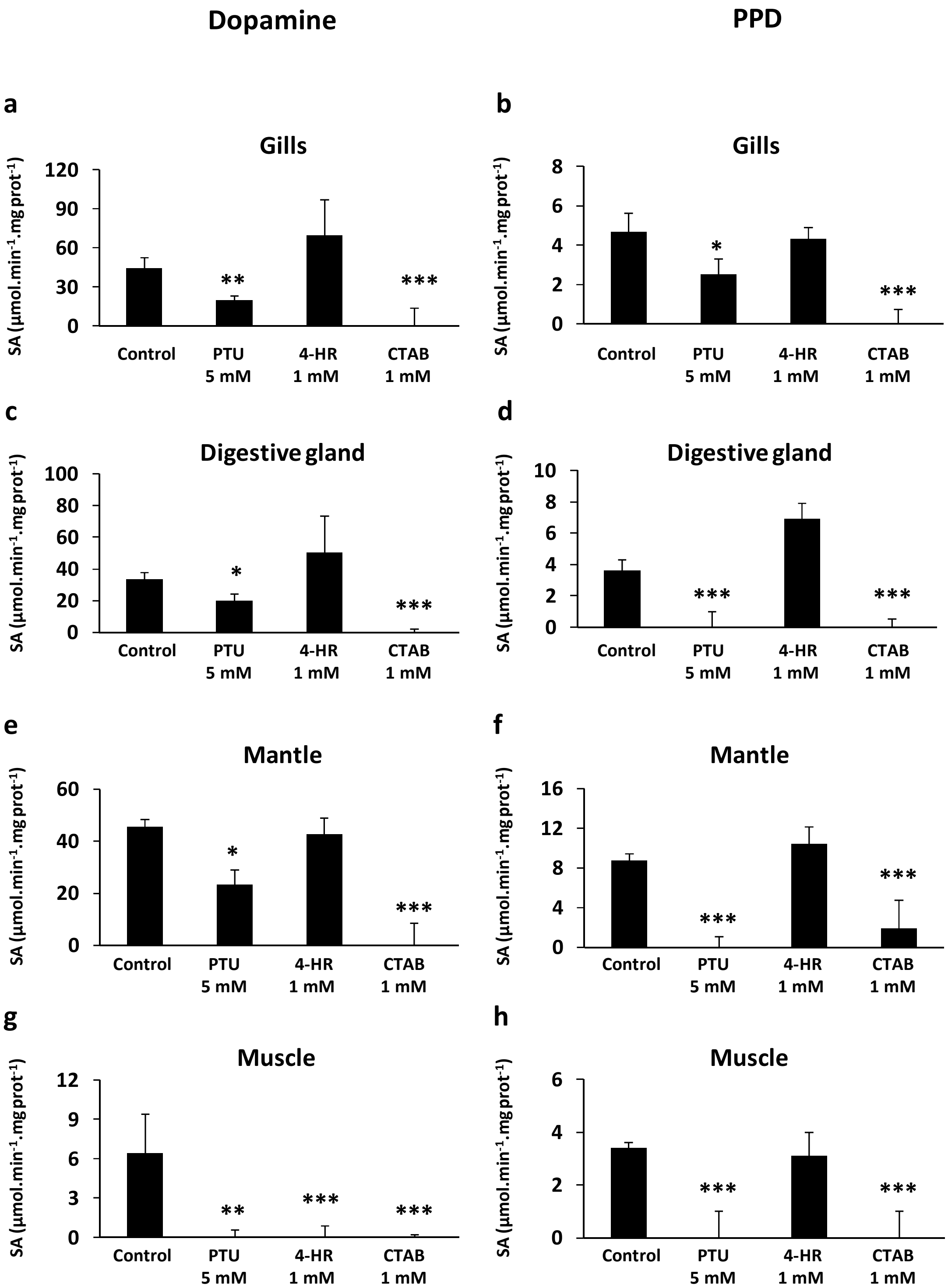

Figure 2. Zymograms for the determination of phenoloxidase activities from different *C. gigas* tissues after partial purification by precipitation with 60% of sulfate ammonium saturation for the gills, digestive gland (DG), mantle (M), muscle (Mu) and plasma or with crude sample for the haemocyte lysate sypernatant (HLS). Samples were run on 7% or 15% analytical SDS-PAGE gels and stained with (a) 100 mM dopamine and 5 mM MBTH and with (b) 100 mM PPD. Gills, DG, M, Mu, plasma and HLS were loaded at a protein concentration of 76, 76, 57, 40, 57 and 1.55 μg, respectively. The arrows indicate the bands showing PO-like activity. Their estimated molecular weights (in kDa) are indicated below the arrows.

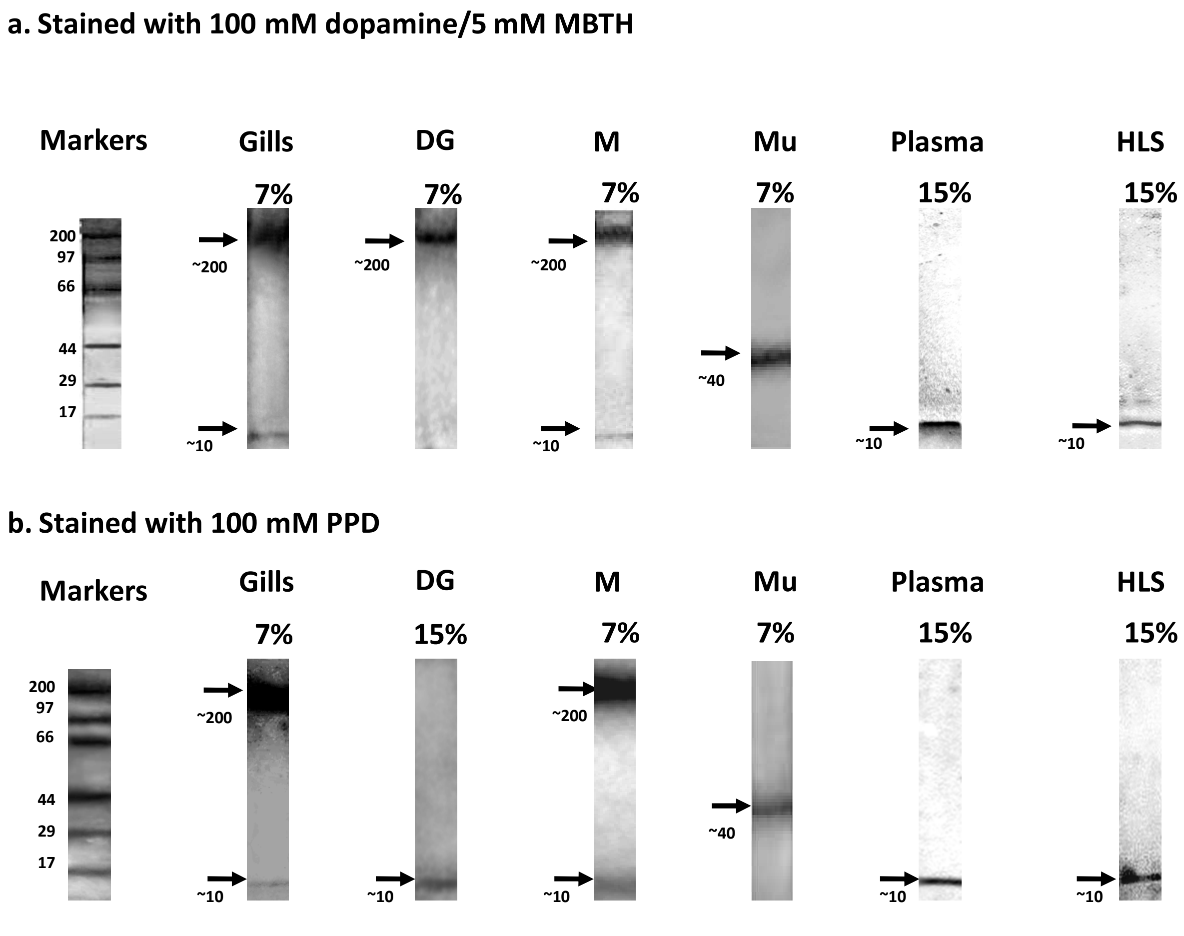

Table 1. Total phenoloxidase-like activity (Mean ± standard deviation, µmol.min^{-1}.mg prot^{-1}.g fresh weight, n=3) from different oyster body tissues with dopamine and PPD as substrates.

Body tissue	**Total phenoloxidase-like activity**	
	Dopamine	PPD
Gills	1.8 ± 0.3	0.2 ± 0.0
Digestive gland	3.0 ± 0.4	50.4 ± 4.3
Mantle	0.9 ± 0.1	0.2 ± 0.0
Muscle	0.5 ± 0.2	0.3 ± 0.1

Table 2 Phenoloxidases from molluscs: characteristics reported in the literature

Vernacular name	Scientific name	Molecular weight (kDa)	Localisation	Reference
Freshwater snail	*Biomphalaria glabrata*	35	Egg mass	Bai et al. 1997
Common octopus	*Octopus vulgaris*	205	Ink	Prota et al. 1981
Ocellated octopus	*Octopus ocellatus*	153.8	Ink	Fan et al. 2009
Argentine shortfin squid	*Illex argentinus*	127.6	Ink	Naraoka et al. 2003
Common cuttlefish	*Sepia officinalis*	125	Ink	Prota et al. 1981
European squid	*Loligo vulgaris*	135	Ink	Prota et al. 1981
Blue mussel	*Mytilus edulis*	381, 316	Haemocyte lysate supernatant (HLS)	Renwrantz et al. 1996
		49, 135, 260	Foot gland	Maruyama et al. 1991
Ribbed mussel	*Modiolus demissus*	70	Periostracum	Waite and Wilbur (1976)
Manila clam	*Ruditapes philippinarum*	76.9	Haemolymph	Cong et al. 2005
Japanese pearl oyster	*Pinctada fucata*	43, 49	Prismatic shell layer	Nagai et al. 2007
Eastern oyster	*Crassostrea virginica*	133	Haemocyte membranes supernatant (HMS)	Jordan and Deaton 2005