

## Differential tissue distribution and specificity of phenoloxidasés from the Pacific oyster *Crassostrea gigas*

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1 **Differential tissue distribution and specificity of phenoloxidasés from the Pacific oyster**  
2 ***Crassostrea gigas***

3

4 Andrea Luna-Acosta\*, Hélène Thomas-Guyon, Myriam Amari, Eric Rosenfeld, Paco  
5 Bustamante, Ingrid Fruitier-Arnaudin\*

6

7 Littoral Environnement et Sociétés (LIENSs), UMR 6250, CNRS-Université de La Rochelle,  
8 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France

9

10 \* Corresponding authors: A. Luna-Acosta / I. Fruitier-Arnaudin

11 Littoral Environnement et Sociétés (LIENSs)

12 UMR 6250, CNRS-Université de La Rochelle

13 2 rue Olympe de Gouges

14 F-17042 La Rochelle Cedex 01, France

15 e-mail : [aluna1508@yahoo.com](mailto:aluna1508@yahoo.com) / [ifruitie@univ-lr.fr](mailto:ifruitie@univ-lr.fr)

16 tel : +33 (0)5 46 50 76 48 / +33 (0)5 46 45 85 62

17 fax : +33 (0)5 46 50 76 63

18

19 Abreviations :

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

25

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

47

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

49

## 50 **1. Introduction**

51 Phenoloxidases (POs, EC 1.14.18.1) are a class of copper proteins widely distributed in  
52 bacteria, fungi, plants and animals (Cerenius et al. 2008). They play a key role in melanin  
53 production and are implicated in immune defence mechanisms in invertebrates. This class of  
54 enzymes include tyrosinases (EC 1.14.18.1), catecholases (EC 1.10.3.1) and laccases (EC  
55 1.10.3.2), all capable of o-diphenol oxidation. However, among these three enzymes, only  
56 tyrosinases can hydroxylate monophenols (e.g. L-tyrosine), and only laccases can oxidise p-  
57 diphenols and aromatic compounds containing amine groups (e.g. p-phenylenediamine, PPD)  
58 (Thurston 1994, Solomon et al. 1996). In addition to that, a panel of inhibitors exert different  
59 actions on these three types of enzymes: while 1-phenyl-2-thiourea (PTU) inhibits the three  
60 types of PO activities (Williamson 1997, Jordan and Deaton 2005), 4-hexylresorcinol (4-HR)  
61 inhibits tyrosinase and catecholase but not laccase activities (Dawley and Flurkey 1993,  
62 Zavarzina and Zavarzin 2006) and cethyltrimethylammonium bromide (CTAB) specifically  
63 inhibits laccase activity (Walker and McCallion 1980). Recently, we conducted a study to  
64 identify PO activities present in the haemolymph of the Pacific oyster *Crassostrea gigas*  
65 (Luna-Acosta et al. 2010a). By using different PO substrates, such as L-tyrosine, L-3,4-  
66 dihydroxyphenylalanine (L-DOPA), dopamine or PPD, and different PO inhibitors, such as  
67 PTU, 4-HR and CTAB, results suggested the presence of both catecholase- and laccase-like  
68 activities in the plasma, and the presence of a laccase-like activity in the haemocyte lysate  
69 supernatant (HLS, Luna-Acosta et al. 2010a). Our interest in *C. gigas* comes from the fact  
70 that this organism dominates over all other molluscs with respect to global distribution and  
71 aquaculture production, but suffers from massive summer mortality each year (Cheney et al.  
72 2000). Summer mortality of *C. gigas* has been suggested to be the result of a complex  
73 interaction between the host, pathogens and environmental factors (Cheney et al. 2000).  
74 Importantly, studies in *C. gigas* have shown that PO activities, usually detected by using the

75 o-diphenol substrates L-DOPA or dopamine, can be modulated by environmental factors,  
76 such as the presence of heavy metals or hydrocarbons (Gagnaire et al. 2004a, Bado-Nilles et  
77 al. 2008, Luna-Acosta et al. 2010b). In addition to that, a gene coding for a laccase in the  
78 haemocytes from *C. gigas* was modulated in the presence of hydrocarbons (Bado-Nilles et al.  
79 2010). To the best of our knowledge, studies on POs in *C. gigas* have only been carried out in  
80 the haemolymphatic compartment. However, POs may be present in other body tissues in  
81 bivalves, e.g. in the prismatic shell layer (Nagai et al. 2007) or in the byssus gland (Hellio et  
82 al. 2000). A better characterisation and localisation of POs in *C. gigas* is needed to expand  
83 our knowledge on the immune defence mechanisms in this organism and therefore to a better  
84 understanding of the potential causes of summer mortality events.

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

96

## 97 **2. Materials and methods**

### 98 *2.1. Chemicals and materials*

99 L-tyrosine, dopamine, p-phenylenediamine (PPD), 1-phenyl-2-thiourea (PTU), 4-  
100 hexylresorcinol (4-HR), cethyltrimethylammonium bromide (CTAB), 3-methyl-2-  
101 benzothiazolinone hydrazone (MBTH), trizma hydrochloride (Tris HCl), sodium chloride  
102 (NaCl), ammonium sulfate (AS), sodium dodecyl sulfate (SDS), trizma base, glycine,  
103 N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, glacial acetic acid,  
104 Coomassie brilliant blue, bovine serum albumin (BSA), copper sulfate and bicinchoninic acid  
105 were obtained from Sigma-Aldrich (France). Magnesium chloride ( $MgCl_2$ ) and calcium  
106 chloride ( $CaCl_2$ ) were obtained from Acros organics (France). Acrylamide/Bis acrylamide  
107 30% was obtained from Bio-Rad.

108

## 109 2.2. *Oysters*

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

114

## 115 2.3. *Collection of oyster tissues*

116 After opening the oyster shells by cutting off the adductor muscle, a quantity (0.5-1 ml) of  
117 haemolymph was withdrawn directly from the pericardial cavity with a 1-ml syringe equipped  
118 with a needle (0.9 x 25 mm), and the haemolymph from 10 oysters was pooled to reduce  
119 inter-individual variation (Gagnaire et al. 2004b). Haemolymph samples were centrifuged  
120 (260 g, 10 min, 4°C) to separate the cellular fraction (i.e. haemocytes) from plasma, as  
121 described previously (Hellio et al. 2007). Gills, digestive gland, mantle and muscle were  
122 removed from oysters and pooled. Three replicates from 10 oysters were prepared per tissue.

123 Haemocytes, gills, digestive gland, mantle and muscle were homogenized at 4°C in Tris  
124 buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>) adjusted to pH 7.  
125 Haemocytes were lysed using a Thomas-Potter homogenizer (IKA-Labortechnik, clearance  
126 0.13-0.18mm) at 200 rpm for 1 min. Gills, digestive gland, mantle and muscle were  
127 homogenized, as described previously (Luna-Acosta et al. 2010b), using an Ultra Turrax (T25  
128 basic, IKA-WERKE) at 19 000 rpm for 1 min followed by twelve up and down strokes of  
129 Thomas-Potter homogenizer at 200 rpm for 1 min (IKA-Labortechnik RW 20.n, size 0.13-  
130 0.18mm, BB). All homogenized samples were centrifuged at 10 000 g for 10 min at 4°C. The  
131 resulting haemocyte lysate supernatant (HLS) and tissue supernatants were collected for  
132 enzymatic studies.

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

136

#### 137 *2.4. Partial purification*

138 A previous analysis, by using different concentrations of saturated ammonium sulfate (0, 30,  
139 40, 60, 70, 80, 100%), revealed that precipitation with 60% saturated ammonium sulfate (60P-  
140 SAS) was the best condition for protein concentration to detect PO-like activity for oyster  
141 tissues, i.e. the gills, digestive gland, mantle and muscle (data not shown), and was in  
142 agreement with other studies (Cong et al. 2005, Liu et al. 2006). Therefore, proteins of  
143 collected supernatants from oyster tissues were brought to 60% saturation concentration by  
144 addition of solid ammonium sulfate at 4°C, and allowed to stand overnight. The resulting  
145 precipitate was collected by centrifugation (15 500 g for 10 min), dissolved in a small volume  
146 of Tris buffer , and dialysed at 4°C against distilled water for 12h and twice against Tris

147 buffer for 8h. Crude plasma samples were concentrated with Centricon-5 centrifugal  
148 concentration units (Amicon™).

149

### 150 2.5. Phenoloxidase assays

151 Phenoloxidase-like (PO-like) activity was measured spectrophotometrically by recording the  
152 formation of o-quinones, as described previously (Luna-Acosta et al. 2010a). PO assays were  
153 conducted in 96-well microplates (Nunc, France). Dopamine or p-phenylenediamine (PPD)  
154 were used as substrates, at final concentrations of 100 mM and 50 mM, respectively.  
155 Dopamine (100 mM) was prepared just before being used in Tris buffer. At 25°C, 10 µl of  
156 sample was incubated with 80 µl of dopamine and 50 µl of Tris buffer. Several control wells  
157 were systematically used: ‘buffer control’ containing only buffer, ‘sample control’ containing  
158 only sample and buffer, and ‘non-enzymatic control’ containing only substrate and buffer,  
159 always in a final volume reaction of 140µl. Immediately after dopamine addition, PO-like  
160 activity was monitored during 4h by using a VersaMax™ microplate reader (Molecular  
161 Devices) and by following the increase of absorbance at 490 nm. Because of solubility  
162 constraints, the protocol was slightly modified in the case of PPD: the sample was incubated  
163 with 7 µl of PPD (50 mM diluted in methanol) and 123 µl of buffer (no effect of methanol  
164 was observed on the enzymatic reactions). PO-like activity was monitored during 2h at 420  
165 nm. For all conditions, the experiments were performed with three pooled oyster samples.  
166 Each pool was tested in triplicate wells and average rates were calculated by dividing the sum  
167 of replicate measurements from the three oyster pools, by the number of measurements, i.e. 9  
168 (3 replicate measurements x 3 oyster pools).

169 For enzymatic oxidation, the results were systematically corrected for non-enzymatic  
170 autoxidation of the substrate and were expressed in specific activity (SA), i.e. in international  
171 units (IU) per mg of protein. One IU is defined as the amount of enzyme that catalyzes the



172 appearance of 1  $\mu$ mole of product per min (Fenoll et al. 2002) under the above conditions  
173 using molar extinction coefficient of dopamine and PPD reactions products of 3 300  $M^{-1} cm^{-1}$   
174 (Waite 1976) and 43 160  $M^{-1} cm^{-1}$  (Eggert et al. 1996, Paranjpe et al. 2003), respectively.

175

#### 176 *2.6. Phenoloxidase inhibition assays*

177 Working solutions of inhibitors were prepared just before being used in Tris buffer. PO  
178 inhibition assays were performed by preincubating 10  $\mu$ l of the specific PO inhibitor PTU  
179 (5 mM, final concentration), the specific tyrosinase and catecholase inhibitor 4-HR (1 mM,  
180 final concentration), or the specific laccase inhibitor CTAB (1 mM, final concentration), with  
181 10  $\mu$ l of sample for 20 minutes. Then, PO assay was carried out with dopamine (100 mM,  
182 prepared in Tris buffer) or PPD (50 mM, prepared in methanol). Appropriate controls were  
183 used as described before. Experiments were performed with three pooled oyster samples.  
184 Each pool was tested in triplicate wells and average rates were calculated.

185

#### 186 *2.7. Protein assays*

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

189

#### 190 *2.8. Gel electrophoresis and zymography*

191 To associate PO enzyme activities with individual proteins, and estimate the molecular  
192 weights of the enzymes, SDS-PAGE and 1 D-zymography were used. Aliquots of the  
193 different oyster tissues (equivalent to 76  $\mu$ g of proteins for gills, 76  $\mu$ g for digestive gland, 57  
194  $\mu$ g for mantle, 40  $\mu$ g for muscle, 47  $\mu$ g for plasma and 1.55  $\mu$ g for HLS) were mixed with  
195 sample buffer (65 mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue).  
196 Samples were then applied to 7% SDS-PAGE gels or 15% SDS-PAGE gels in non reducing

197 conditions (i.e. without boiling samples after the addition of sample buffer) and with an upper  
198 gel of 4% using a Mini-PROTEAN III Cell (Bio-Rad). Electrophoresis was carried out  
199 according to the method of Laemmli (1970) at 110V for 2h45. Two gels containing the same  
200 samples were run and processed in parallel. For each tissue, samples previously brought to 0,  
201 30, 40 or 60% saturation concentration by addition of solid ammonium sulfate at 4°C were  
202 runned per gel. After electrophoresis, SDS-PAGE gels were washed 2 x 10 min in distilled  
203 water and 2 x 10 min in Tris buffer.

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

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

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

218

### 219 2.9. Statistical analysis

220 All values are reported as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out  
221 with SYSTAT 11.0. Values were tested for normality (Shapiro test) and homogeneity of

222 variances (Bartlett test). In some cases, logarithmic transformations ( $\text{Log}_{10}$ ) were used to meet  
223 the underlying assumptions of normality and homogeneity of variances. For normal values,  
224 one-way nested ANOVA tests were used followed by a Tukey post-hoc test. For non normal  
225 values, Kruskal-Wallis tests were applied, followed by Dunn's multiple comparisons test (Zar  
226 1984). The statistical significance was designed as being at the level of  $p < 0.05$ .

227

### 228 **3. Results**

#### 229 *3.1. Spectrophotometric studies*

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

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

### 247 3.2. SDS-PAGE zymographic assays

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

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

261 With dopamine as substrate, the activity detected for the gills and the mantle corresponded to  
262 one upper band with an estimated molecular weight of ~200 kDa and to a lower band with an  
263 estimated molecular weight of ~10 kDa (Fig. 2a). In both tissues, most of the PO activity  
264 appeared in the higher molecular mass band. For the digestive gland the activity corresponded  
265 to an upper band of ~200 kDa and for the plasma and the HLS to one lower band with an  
266 estimated molecular weight of ~10 kDa (Fig. 2a). Similarly, an upper band with a molecular  
267 weight of ~200 kDa was observed in the presence of PPD for the gills and the mantle, but not  
268 for the digestive gland (Fig. 2b), and a lower band with a molecular weight of ~10 kDa was  
269 detected in the presence of PPD for the gills, digestive gland, mantle, crude plasma and crude  
270 HLS (Fig. 2b). Again, most activity in samples from the gills and the mantle was evident in  
271 higher molecular mass bands. The bands of ~10 kDa are not likely to be an artefact since they

272 stained differentially with dopamine and PPD, depending on the tissue that was analyzed, e.g.  
273 stained with PPD but not with dopamine in the digestive gland. Contrary to the other tissues,  
274 the activity detected for the muscle, with dopamine and PPD as substrates, corresponded to a  
275 band with an estimated molecular weight of ~40 kDa.

276

#### 277 **4. Discussion**

278 POs are of widespread occurrence in bacteria, fungi, plants, invertebrates and vertebrates  
279 (Sanchez-Ferrer et al., 1995; Cerenius et al., 2008). Despite the importance of the reactions  
280 and the functional roles associated to POs, the enzymes belonging to the PO class have not  
281 been thoroughly characterised in molluscs and especially in bivalve species. In the present  
282 study, spectrophotometric analyses were conducted to identify PO-like activity in different  
283 tissues from *C. gigas*. L-tyrosine, dopamine and PPD were used as substrates and different  
284 PO inhibitors were tested. When L-tyrosine was used as substrate, no PO-like activity was  
285 detected in any of the tissues that were tested, i.e. the gills, digestive gland, mantle, and  
286 muscle. Similar results were recently obtained with plasma and HLS (Luna-Acosta et al.  
287 2010a). These data suggest the total absence of tyrosinase-like activity in *C. gigas*. Inhibition  
288 assays in the present study were thus only conducted with dopamine and PPD as substrates.

289 The choice on PO specific inhibitors was based on a previous study carried out on the PO  
290 inhibitors described in the literature (Luna-Acosta et al. 2010a). Indeed, PO substrates can  
291 chemically oxidise in the absence of PO (autoxidation) and many PO inhibitors described in  
292 the literature such as reducing agents (e.g. 2-mercaptoethanol or sodium azide) can inhibit  
293 autoxidation reactions (Luna-Acosta et al. 2010a). Such inhibitors are likely to react with the  
294 substrate and/or the quinone intermediates derived from the autoxidation reaction. Therefore,  
295 this type of inhibitors should be avoided for identifying PO activity. Among PO inhibitors  
296 described in different species, PTU was chosen because it has been described as a common

297 inhibitor of all POs (Arias et al. 2003, Zufelato et al. 2004), 4-HR as a tyrosinase and a  
298 catecholase but not a laccase specific inhibitor (Dawley and Flurkey 1993, Zavarzina and  
299 Zavarzin 2006) and CTAB as a laccase but not a tyrosinase or a catecholase specific inhibitor  
300 (Walker and McCallion 1980, Martinez-Alvarez et al. 2008). Moreover, no inhibitory effect  
301 was observed with these chemicals in dopamine or PPD autoxidation reactions (Luna-Acosta  
302 et al. 2010a).

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

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

319 In zymographic studies, catecholase- and/or laccase- but not tyrosinase-like activities were  
320 detected in the gills, digestive gland, mantle, muscle, plasma and HLS (Fig. 2). This coincides  
321 with properties of the Asian swimming crab *Charybdis japonica* (Liu et al. 2006) and the

322 eastern oyster *C. virginica* (Jordan and Deaton 2005), and differs from tyrosinase-type POs  
323 from other invertebrates, such as the vinegar fly *Drosophila melanogaster* (Asada et al. 1993),  
324 the bloodfluke planorb *Biomphalaria glabrata* (Bai et al. 1997), the Manila clam *Ruditapes*  
325 *philippinarum* (Cong et al. 2005) and the Sydney rock oyster *Saccostrea glomerata*  
326 (Aladaileh et al. 2007).

327 In the present study, when dopamine or PPD were used as substrates, PO-positive bands of  
328 ~10, 40 or 200 kDa were detected in *C. gigas* depending on the tissue. These tissue-dependent  
329 differences in molecular weights may be due to (i) the activation state of POs or (ii) the  
330 existence of polymeric forms of the enzyme. Indeed, the molecular weights of POs vary  
331 depending on the activation state, animal tissue and animal species that are studied (Table 2).  
332 In molluscs, the molecular weights of POs estimated by exclusion chromatography or SDS-  
333 PAGE electrophoresis are in the range of 35 to 381 kDa, and in invertebrates, POs occur as  
334 monomers, dimers, tetramers or pentamers (Jaenicke and Decker 2003). Thus, differences in  
335 molecular weights in the present study may be explained by the existence of polymeric forms  
336 of the enzyme. Generally, the molecular weight of monomeric forms is about 40 to 45 kDa,  
337 and generally each subunit possesses two copper atoms (Prota et al. 1981). In the present  
338 study, a PO-positive band of ~40 kDa was detected in the muscle of *C. gigas*, suggesting that  
339 a monomeric form of a laccase sensitive to inhibition by 4-HR, or a catecholase sensitive to  
340 inhibition by CTAB could be present in the muscle of this species. In the other analyzed  
341 tissues, PO-positive bands of ~10 or ~200 kDa were detected, and in the haemolymphatic  
342 compartments, PO-positive bands of ~10 kDa were detected. Results of the upper band of  
343 ~200 kDa are in agreement with the large range of molecular weights of PO reported for  
344 molluscs, i.e. from 35 to 381 kDa (Table 2). However, to our knowledge, this is the first time  
345 that a PO-positive band of ~10 kDa is reported in a mollusc species. A PO-positive band of  
346 ~10 kDa was detected in a non-mollusc aquatic invertebrate, the red swamp crayfish

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

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

363

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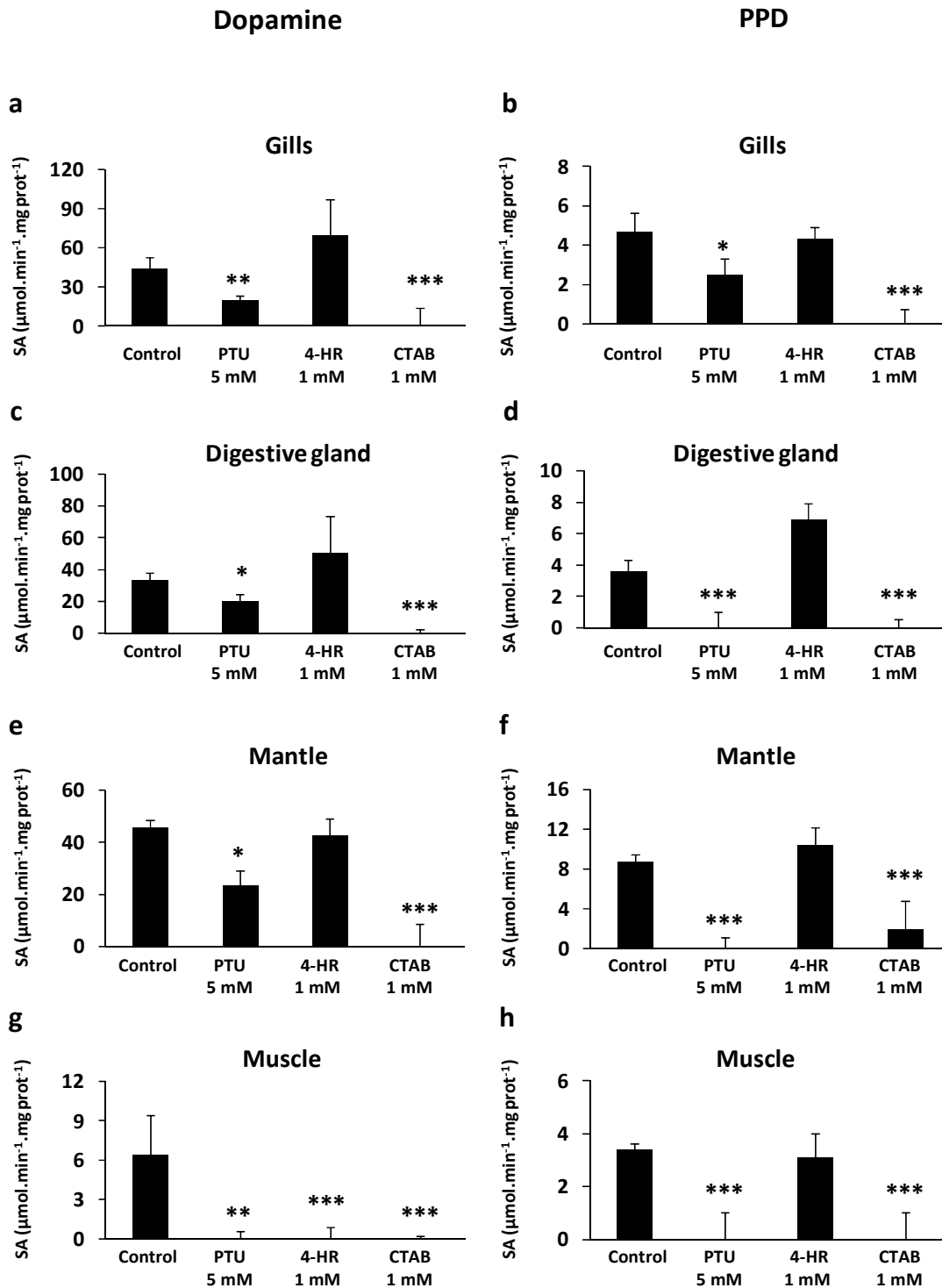
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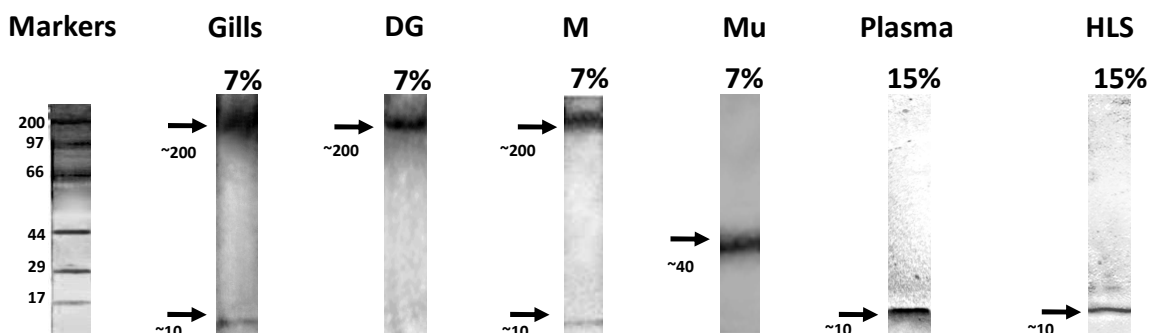
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497

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

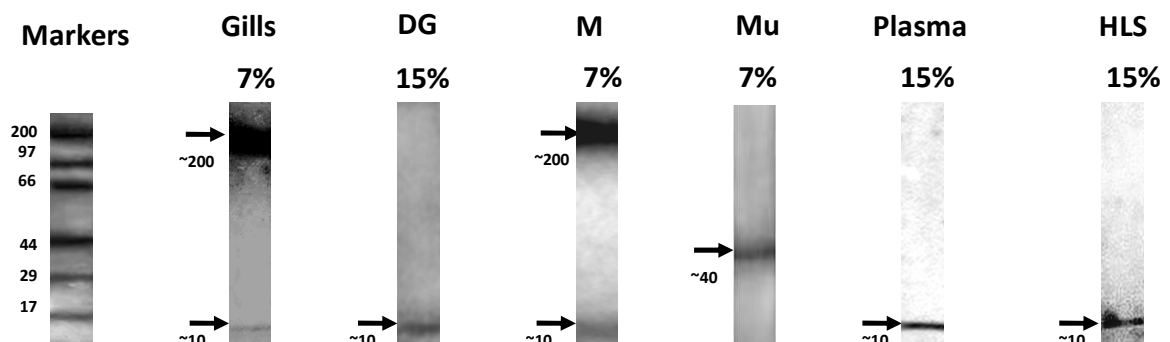


504 **Figure 2. Zymograms for the determination of phenoloxidase activities from different *C.***  
 505 ***gigas* tissues after partial purification by precipitation with 60% of sulfate ammonium**  
 506 **saturation for the gills, digestive gland (DG), mantle (M), muscle (Mu) and plasma or**  
 507 **with crude sample for the haemocyte lysate supernatant (HLS). Samples were run on 7%**  
 508 **or 15% analytical SDS-PAGE gels and stained with (a) 100 mM dopamine and 5 mM MBTH**  
 509 **and with (b) 100 mM PPD. Gills, DG, M, Mu, plasma and HLS were loaded at a protein**  
 510 **concentration of 76, 76, 57, 40, 57 and 1.55  $\mu$ g, respectively. The arrows indicate the bands**  
 511 **showing PO-like activity. Their estimated molecular weights (in kDa) are indicated below the**  
 512 **arrows.**  
 513

**a. Stained with 100 mM dopamine/5 mM MBTH**



**b. Stained with 100 mM PPD**



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517 **Table 1. Total phenoloxidase-like activity (Mean  $\pm$  standard deviation,  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$**   
518  **$\text{prot}^{-1}\cdot\text{g}$  fresh weight, n=3) from different oyster body tissues with dopamine and PPD as**  
519 **substrates.**

520

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

521

522 **Table 2 Phenoloxidases from molluscs: characteristics reported in the literature**

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

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