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### ► To cite this version:

Andrea Luna Acosta, Eric Rosenfeld, Myriam Amari, Ingrid Fruitier-Aranaudin, Paco Bustamante, et al.. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. *Fish and Shellfish Immunology*, 2010, 28 (4), pp.719-726. 10.1016/j.fsi.2010.01.008 . hal-00477727v1

**HAL Id: hal-00477727**

**<https://hal.science/hal-00477727v1>**

Submitted on 30 Apr 2010 (v1), last revised 3 May 2010 (v2)

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1           **First evidence of laccase activity in the Pacific oyster *Crassostrea gigas***

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22 **Abstract:** Phenoloxidases (POs) are a family of enzymes including tyrosinases, catecholases  
23 and laccases, which play an important role in immune defence mechanisms in various  
24 invertebrates. The aim of this study was to thoroughly identify the PO-like activity present in  
25 the hemolymph of the Pacific oyster *Crassostrea gigas*, by using different substrates (i.e.  
26 dopamine and p-phenylenediamine, PPD) and different PO inhibitors. In order to go deeper in  
27 this analysis, we considered separately plasma and hemocyte lysate supernatant (HLS). In  
28 crude plasma, oxygraphic assays confirmed the presence of true oxidase activities. Moreover,  
29 the involvement of peroxidase(s) was excluded. In contrast to other molluscs, no tyrosinase-  
30 like activity was detected. With dopamine as substrate, PO-like activity was inhibited by the  
31 PO inhibitors tropolone, phenylthiourea (PTU), salicylhydroxamic acid and diethyldithio-  
32 carbamic acid, by a specific inhibitor of tyrosinases and catecholases, i.e. 4-hexylresorcinol  
33 (4-HR), and by a specific inhibitor of laccases, i.e. cetyltrimethylammonium bromide  
34 (CTAB). With PPD as substrate, PO-like activity was inhibited by PTU and CTAB. In  
35 precipitated protein fractions from plasma, and with dopamine and PPD as substrates, PTU  
36 and 4-HR, and PTU and CTAB inhibited PO-like activity, respectively. In precipitated protein  
37 fractions from hemocyte lysate supernatant, PTU and CTAB inhibited PO-like activity,  
38 independently of the substrate. Taken together, these results suggest the presence of both  
39 catecholase- and laccase-like activities in plasma, and the presence of a laccase-like activity in  
40 HLS. To the best of our knowledge, this is the first time that a laccase-like activity is  
41 identified in a mollusc by using specific substrates and inhibitors for laccase, opening new  
42 perspectives for studying the implication of this enzyme in immune defence mechanisms of  
43 molluscs of high economic value such as *C. gigas*.

44

45 **Key Words:** phenoloxidase; catecholase; melanin; mollusc; bivalve; hemolymph; hemocyte;  
46 plasma

## 47 **1. Introduction**

48

49 Phenoloxidases are a family of copper proteins, widely distributed in microorganisms, plants  
50 and animals [1, 2]. They are the rate limiting enzymes in enzymatic browning in fruits and  
51 vegetables, and in melanisation in animals. Melanin production starts with the oxidation of  
52 phenols and the concomitant reduction of O<sub>2</sub> to water. This reaction is catalysed by POs and  
53 yields to corresponding quinones, which are then polymerized by non-enzymatic reactions  
54 toward the formation of melanin [3]. Melanin and intermediates are toxic substances with  
55 fungistatic, bacteriostatic and antiviral properties [4]. In invertebrates, PO enzymes are also  
56 involved in many cellular defence responses, such as self/non-self recognition, phagocytosis  
57 and nodule and capsule formation [4, 5]. Interestingly, similarities of the PO system have  
58 been drawn with other cascades involved in defence such as the *Drosophila*-Toll cascade and  
59 the mammalian complement and blood clotting [6].

60 A major constraint when studying POs is the ambiguity of nomenclature existing in the  
61 literature. POs include tyrosinases (monophenol, o-diphenol: O<sub>2</sub> oxidoreductase, EC  
62 1.14.18.1), catecholases (o-diphenol: O<sub>2</sub> oxidoreductase, EC 1.10.3.1), and laccases (p-  
63 diphenol: O<sub>2</sub> oxidoreductase, benzenediol: O<sub>2</sub> oxidoreductase, EC 1.10.3.2). However,  
64 tyrosinases and POs, and tyrosinases and catecholases have been used in the literature as  
65 synonyms [7, 8], and tyrosinases and POs are given the same EC number even if they are not  
66 obviously the same. POs are capable of o-diphenol oxidation. However, among these three  
67 enzymes, only tyrosinases can hydroxylize monophenols (e.g. L-tyrosine) and only laccases  
68 can oxidise p-diphenols and aromatic amines (e.g. p-phenylenediamine) [9, 10]. In addition to  
69 that, various compounds have been described as inhibitors of these three types of POs with  
70 their respective specificity (Table 1).

71 POs have been detected in different bivalve species, such as mussels (*Mytilus edulis*, *M.*  
72 *galloprovincialis*, *Perna viridis*), clams (*Ruditapes decussatus*), scallops (*Nodipecten*  
73 *subnodosus*) and oysters (*Crassostrea gigas*, *C. virginica*, *Saccostrea glomerata*) [11-17].  
74 Among bivalves, the Pacific oyster *C. gigas* (Thunberg, 1753) is an ecologically and  
75 economically important species that dominates over all other molluscs with respect to global  
76 world distribution and aquaculture production [18]. However, massive summer mortalities in  
77 *C. gigas* have become a widespread concern in the world in recent decades [19]. Among the  
78 different factors suspected to be responsible of these mortalities, impairment of immune  
79 defence functions, elicited by environmental factors, is considered to be of major importance  
80 [20]. The increasing interest for PO comes from its apparent role in immune defence  
81 mechanisms in oysters, e.g. in the resistance of *S. glomerata* to *Marteilia sydneyi* [17].  
82 Moreover, ecotoxicological studies have shown that PO in *C. gigas* may be modulated by the  
83 presence of heavy metals or polyaromatic hydrocarbons [21, 22]. To the best of our  
84 knowledge, studies on PO in *C. gigas* have been carried out by using the non specific o-  
85 diphenol substrate L-3,4-dihydroxyphenylalanine (L-DOPA).

86 In this general context, the purpose of our work was to thoroughly identify the PO-like  
87 activity that has been previously detected in *C. gigas*. We compared PO activity in plasma  
88 from *C. gigas* in the presence of several tyrosinase, catecholase and laccase substrates and  
89 inhibitors. Furthermore, we measured oxygen uptakes during enzymatic and non-enzymatic  
90 oxidation reactions. Finally, partial purification of proteins from plasma and hemocyte lysate  
91 supernatant was used to identify PO-like activities in the hemolymph.

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## 96 **2. Materials and methods**

97

### 98 *2.1. Oysters*

99 One hundred 3 years old *C. gigas* (mean  $\pm$  SD; weight:  $75.5 \pm 8.7$  g; length:  $9 \pm 3$  cm) were  
100 purchased during October-November 2008 from shellfish farms in Aytré Bay (Charente  
101 Maritime, France), on the French Atlantic coast, and were processed immediately after their  
102 arrival in the laboratory.

103

### 104 *2.2. Collection of plasma*

105 After opening the oyster shells by cutting off the adductor muscle, a quantity (0.5-1 ml) of  
106 hemolymph was withdrawn directly from the pericardial cavity with a 1-ml syringe equipped  
107 with a needle (0.9 x 25 mm), and the hemolymph from 10 oysters was pooled to reduce inter-  
108 individual variation [21]. Hemolymph samples were centrifuged (260 g, 10 min, 4°C) to  
109 separate the cellular fraction (i.e., hemocytes) from plasma [23]. Aliquots (100  $\mu$ l) were  
110 stored at -80°C. Each aliquot was used only once.

111

### 112 *2.3. Hemocyte lysate supernatant*

113 Hemocytes were homogenized at 4°C in Tris buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM  
114 MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>) adjusted to pH 7. They were lysed using an Ultra-Turrax (T25  
115 basic, IKA-WERKE) at 19 000 rpm for 30 sec and a Thomas-Potter homogenizer (IKA-  
116 Labortechnik, clearance 0.13-0.18mm) at 200 rpm for 1 min, and centrifuged at 10 000g for  
117 10 min at 4°C. The resulting hemocyte lysate supernatant (HLS) was collected for enzymatic  
118 studies. Aliquots (100  $\mu$ l) were stored at -80°C. Each aliquot was used only once.

119

120

#### 121 2.4. Chemicals

122 L-tyrosine, p-hydroxyphenyl propionic acid (PHPPA), 4-hydroxyanisole (4-HA), L-3,4-  
123 dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenyl propionic acid (DHPPA), catechol,  
124 dopamine, p-phenylenediamine (PPD), 4-Hydroxy-3,5-dimethoxybenzaldehyde azine  
125 (syringaldazine), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt  
126 (ABTS), tropolone, 4-hexylresorcinol (4-HR), cethyltrimethylammonium bromide (CTAB),  
127 salicylhydroxamic acid (SHAM), sodium azide ( $\text{NaN}_3$ ), diethyldithiocarbamate (DETC), 1-  
128 phenyl-2-thiourea (PTU), trizma hydrochloride (Tris HCl), sodium chloride (NaCl),  
129 ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$ ) and catalase from bovine liver were obtained from Sigma-  
130 Aldrich (France). 2-mercaptoethanol (2-ME) was obtained from MERCK (France).  
131 Magnesium chloride ( $\text{MgCl}_2$ ) and calcium chloride ( $\text{CaCl}_2$ ) were obtained from Acros  
132 (France).

133

#### 134 2.5. Phenoloxidase assays

135 Phenoloxidase-like (PO-like) activity has been reported to be higher in plasma than in HLS  
136 from *C. gigas* [23]. Therefore, constitutive PO-like activity was first analysed in crude  
137 plasma. PO-like activity was measured spectrophotometrically by recording the formation of  
138 o-quinones. The method of Asokan *et al* [5] was used with some modifications. Working  
139 solutions of substrates were prepared just before use in Tris buffer (0.1 M Tris HCl, 0.45 M  
140 NaCl, 26 mM  $\text{MgCl}_2$  and 10 mM  $\text{CaCl}_2$ ) adjusted to pH 7, except for PPD which was  
141 prepared in methanol. The latter did not affect PO-like activities in the conditions tested (data  
142 not shown). Samples were distributed in 96-well microplates (Nunc, France). Ten  $\mu\text{l}$  of  
143 sample were incubated with 80  $\mu\text{l}$  of substrate and 50  $\mu\text{l}$  of Tris buffer at 25°C. Several  
144 control wells were systematically used: 'buffer control' containing only buffer, 'sample  
145 control' containing only sample and buffer, and 'non-enzymatic control' containing only

146 substrate and buffer. Immediately after substrate addition, PO-like activity was monitored  
147 during 4h by following the increase of absorbance at a specific wavelength (Table 2).  
148 Because of solubility constraints, in the case of PPD, the protocol was slightly modified: 10  $\mu$ l  
149 of sample were incubated with 7  $\mu$ l of PPD and 123  $\mu$ l of buffer and PO-like activity was  
150 monitored during 2h. For all conditions, experiments were performed with three oyster pools.  
151 Each pool was tested in triplicate wells and average rates were calculated. For non-enzymatic  
152 oxidation, results were expressed as the mean value of the increment of absorbance per  
153 minute ( $\Delta A \cdot \text{min}^{-1}$ ). For enzymatic oxidation, results were systematically corrected for non-  
154 enzymatic autoxidation of the substrate. Specific activities (SA) were expressed in  
155 international units (IU) per mg of total protein. One IU is defined as the amount of enzyme  
156 that catalyzes the appearance of 1  $\mu$ mole of product per min [24].

157 Apparent Michaelis-Menten constants ( $K_{m_{app}}$ ) and maximum velocities ( $V_{m_{app}}$ ) were  
158 estimated from double reciprocal plots (Lineweaver-Burk) of velocity vs substrate  
159 concentration.

160

#### 161 *2.6. Phenoloxidase inhibition assay*

162 PO inhibition assay was performed by preincubating 10  $\mu$ l of PO inhibitor (prepared at  
163 various concentrations in Tris buffer, Fig. 3) with 10  $\mu$ l of sample for 20 minutes, at 25°C.  
164 Then, PO assay was carried out with dopamine or PPD, at final concentrations of 100  $\mu$ M  
165 and 50  $\mu$ M, respectively. Experiments were performed with three oyster pools. Each pool  
166 was tested in triplicate wells and average rates were calculated. Enzymatic oxidation (in the  
167 presence of PO inhibitor) was systematically corrected for non-enzymatic autoxidation of the  
168 substrate (in the presence of PO inhibitor).

169

170



171 *2.7. Hydrogen peroxide scavenging by exogenous catalase*

172 Plasma (10  $\mu$ l) was preincubated at 25°C for 30 minutes in the presence of 10  $\mu$ l of catalase  
173 from bovine liver at 1 000 U/ml [25]. The total scavenging of H<sub>2</sub>O<sub>2</sub> was verified using the  
174 Catalase kit CAT-100 (Sigma) and specifications included (data not shown). Then, PO assay  
175 was carried out with dopamine (100 mM) or PPD (50 mM). The effect of catalase on non-  
176 enzymatic autoxidation was also followed by incubating (25°C, 30 min) the substrates  
177 (dopamine or PPD at 100 mM or 50 mM, respectively) in the presence of 10  $\mu$ l of catalase.  
178 Enzymatic oxidation (in the presence of catalase) was systematically corrected for  
179 autoxidation of the substrate (in the presence of catalase). All the experiments were performed  
180 with three oyster pools. Each pool was tested in triplicate wells and average rates were  
181 calculated.

182

183 *2.8. Protein determination*

184 Protein concentration was determined by the slightly modified Lowry method, as described  
185 previously [26]. Serum albumin (Sigma-Aldrich, France) was used as standard.

186

187 *2.9. Measurements of oxygen uptake*

188 Oxygen uptake was followed with a Clark-type oxygen electrode (Hansatech, DW1) in a  
189 700- $\mu$ l closed chamber thermostatted at 25°C with continuous stirring [27]. In a typical  
190 experiment, oxygen uptakes were recorded simultaneously using four separate electrode units.  
191 In the first unit ('buffer control'), a volume of 700  $\mu$ l of buffer was distributed in the chamber.  
192 In the second unit ('sample control'), 250  $\mu$ l of plasma and 450  $\mu$ l of buffer were distributed.  
193 In the third unit ('non-enzymatic control'), 700  $\mu$ l of substrate (L-DOPA 10 mM or dopamine  
194 100 mM) were distributed. In the fourth unit, 250  $\mu$ l of plasma and 450  $\mu$ l of substrate were  
195 distributed. With PPD (50 mM) as substrate, the same protocol was adopted with slight

196 modifications, i.e. 500  $\mu$ l of the sample were incubated with 35  $\mu$ l of PPD and 165  $\mu$ l of  
197 buffer. All the experiments were carried out with three oyster pools.

198

#### 199 *2.10. Preparation of protein fractions from plasma and hemocyte lysate supernatant*

200 Plasma and HLS were precipitated overnight with 60% saturated  $(\text{NH}_4)_2\text{SO}_4$  solution at 4°C.

201 After centrifugation at 10 000 g for 10 min at 4°C, the precipitate was dissolved in 1 ml and

202 dialyzed against Tris buffer. Partially purified fractions from plasma and hemocyte lysate

203 supernatant were filtered through a 0.22  $\mu$ m sterile filter (Millipore membrane-Millipore Co.,

204 Bedford, MA, USA), in order to eliminate the natural bacterial flora of samples. In order to

205 make certain the absence of bacteria after this treatment, the samples were incubated with 4.0

206 ml of Zobell medium (4 g peptone, 1 g yeast extract, 0.1 g ferric phosphate, 30 g sea salt per

207 liter) and grown at 25°C with shaking to allow potential bacterial growth. Then,  $A_{620\text{nm}}$

208 readings were carried out at 0, 5 and 6 h, which evidenced the absence of bacterial growth

209 (data not shown). Aliquots (100  $\mu$ l) of the dialyzates were stored at -80°C before being tested

210 for PO-like activity.

211

#### 212 *2.11. Statistical analysis*

213 All values are reported as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out

214 with SYSTAT 11.0. Values were tested for normality (Shapiro test) and homogeneity of

215 variances (Bartlett test). For normal values, an ANOVA test was used to analyse the results,

216 followed by a Dunnett post-hoc test. For non normal values, a Kruskal-Wallis test was used,

217 followed by a Dunn's multiple comparisons test [28]. Statistical significance was designed as

218 being at the level of  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ .

219

220

## 221 3. Results

222

### 223 3.1. Substrate specificity of PO-like activity in plasma

224 Enzymatic oxidation results were systematically corrected for non-enzymatic autoxidation.

225 Table 2 shows that no PO-like activity was detected in the presence of PHPPA, L-tyrosine, 4-

226 HA, DHPPA, syringaldazine and ABTS. Conversely, PO-like activity was detected using L-

227 DOPA, dopamine and PPD, with final concentrations of substrate saturation being equal to 10

228 mM, 100 mM and 50 mM, respectively.  $K_{m_{app}}$  values for L-DOPA, dopamine and PPD were

229 7, 51, and 45 mM, respectively (Table 2).  $K_{m_{app}}$  for L-DOPA was thus 6 to 7 times lower

230 than  $K_{m_{app}}$  for dopamine and PPD.  $V_{m_{app}}$  values for L-DOPA, dopamine and PPD were 0.45,

231 0.51 and 0.59  $\Delta A \cdot \text{min}^{-1} \cdot 10^{-3}$ , respectively (Table 2). Thus,  $V_{m_{app}}$  value obtained with PPD

232 was 1.15 to 1.31 times higher than values obtained with L-DOPA and dopamine.

233

### 234 3.2. $O_2$ requirements of PO-like activity

235 Using oxygraphy, we easily confirmed the non-enzymatic autoxidation of L-DOPA,

236 dopamine, and to a lesser extent, of PPD (Fig. 1). Most importantly, we found that  $O_2$  uptake

237 was higher in the presence of plasma, independently of the substrate, confirming the presence

238 of at least one PO-type oxidase in plasma.

239

### 240 3.3. Effect of catalase

241 Exogenous catalase was used to scavenge the  $H_2O_2$  potentially involved in peroxidase-

242 dependent oxidation reactions. Fig. 2a shows that catalase did not affect autoxidations of

243 dopamine and PPD. Most importantly, catalase did not inhibit oxidations of both substrates in

244 the presence of plasma (Fig. 2b). Fig. 2b also shows that catalase induced a two-fold increase

245 of PO-like activity with dopamine as substrate.

246

247 *3.4. Effect of various PO inhibitors*

248 The next step in the identification of PO-like activity in plasma from *C. gigas* consisted on  
249 studying the effect of different PO inhibitors with dopamine and PPD as substrates.

250 Results with dopamine are summarized in Fig. 3. Since many inhibitors are reducing agents,  
251 we systematically examined the effects of PO inhibitors on the non-enzymatic autoxidation.

252 Autoxidation was reduced by using  $\text{NaN}_3$  at 0.1 and 1 mM, and suppressed with 2-ME and  
253 DETC at 5 mM (Fig. 3a). These compounds were therefore not used at these concentrations

254 for further studies. Moreover, enzymatic oxidation (in the presence of plasma and PO  
255 inhibitors) was systematically corrected for non-enzymatic autoxidation of the substrate (in

256 the presence of PO inhibitors). Fig. 3b shows that enzymatic oxidation was strongly inhibited

257 by 0.5 mM DETC and 5 mM PTU (94 and 77% inhibition, respectively), and also

258 significantly inhibited by 8 mM tropolone, 1 mM SHAM, and 1 mM CTAB (56, 33, and 21%

259 inhibition, respectively). The catecholase inhibitor 4-HR (1 mM) exerted 34 % inhibition.

260 Results with PPD as substrate are summarized in Fig. 4. Autoxidation was suppressed by

261 DETC (0.5 mM, Fig. 4a). Therefore, DETC was not used for further studies. Tropolone (8

262 mM) and the laccase inhibitor CTAB (1 mM) only slightly interfered (stimulation) with the

263 autoxidation of PPD. Since CTAB is the better documented inhibitor of laccase, we decided

264 to maintain it in the study. Interestingly, Fig. 4b shows that enzymatic oxidation was strongly

265 inhibited by CTAB (1 mM). Moreover, the PO inhibitor PTU (0.5 and 5 mM) exerted 100%

266 inhibition. Taken together, these results confirm the presence of a PO-like activity in *C. gigas*

267 and suggest the presence of a catecholase-like and/or a laccase-like activity in plasma.

268

269

270

### 271 3.5. PO-like activity in protein fractions

272 Independently of the substrate, specific PO-like activity was considerably higher in hemocyte  
273 lysate supernatant (HLS) than in plasma (Fig. 5). Moreover, the results obtained with  
274 precipitated protein fractions confirm that the activities measured derived from a protein  
275 source. Results with precipitated protein fractions from plasma are summarized in Fig. 5a and  
276 5c. With dopamine as substrate (Fig. 5a), the PO inhibitor PTU (5 mM) and the catecholase  
277 inhibitor 4-HR (1 mM), inhibited PO-like activity by 57 and 26%, respectively. In contrast to  
278 the results obtained with crude plasma, the laccase inhibitor CTAB (1 mM) did not exert  
279 inhibition in precipitated protein fractions from plasma. With PPD as substrate (Fig. 5c), PTU  
280 and CTAB exerted 100% inhibition of PO-like activity.

281 Results with precipitated protein fractions from HLS are summarized in Fig. 5b and 5d. With  
282 dopamine as substrate (Fig. 5b), PTU and CTAB inhibited PO-like activity by 57 and 100%,  
283 respectively. Interestingly, with PPD as substrate (Fig. 5d), PTU and CTAB exerted 90 and  
284 100% inhibition, respectively.

285

## 286 4. Discussion

287

288 Most studies on PO from *C. gigas* have been performed with L-DOPA. However, this  
289 common substrate for the three classes of POs, i.e. tyrosinases, catecholases and laccases, was  
290 not appropriate to discriminate between these three classes of POs. Therefore, in the present  
291 work, various concentrations of different substrates were used for identifying the endogenous  
292 PO-like activity in hemolymph from this bivalve.

293 Oxidation catalyzed by POs requires O<sub>2</sub>. However, PO substrates are also readily autoxidized  
294 in contact with air [15, 29]. Therefore, a special attention should be paid to substrate  
295 autoxidations before studying PO activity. Using both spectrophotometry and oxygraphy, we

296 confirmed that L-DOPA, dopamine, and to a lesser extent PPD, could be readily autoxidized.  
297 These non-enzymatic oxidation reactions probably involve quinone redox cycling leading to  
298 the formation of different types and quantities of oxygen radicals and quinone-derived  
299 products [30].

300 Another constraint for studying PO is the possible interference between PO inhibitors and  
301 non-enzymatic autoxidation. For instance, the PO inhibitor 2-ME is also a well-known  
302 reducing agent (Table 1), that may react with the substrate and/or the quinone intermediates  
303 derived from the autoxidation reaction. We systematically examined the effects of various PO  
304 inhibitors on substrate autoxidations. We found that 2-ME (5 mM),  $\text{NaN}_3$  (0.1-1 mM) and  
305 DETC (5 mM) interfered with dopamine autoxidation, and that DETC (0.5 mM) interfered  
306 with PPD autoxidation. 2-ME probably acts as a reducing agent while  $\text{NaN}_3$  and DETC might  
307 possibly act as direct free radical scavengers [31, 32]. These inhibitors (at the concentrations  
308 used) should therefore be avoided for identifying PO activity.

309 We focused on PO-like activity from crude plasma. By using both spectrophotometry and  
310 oxygraphy, PO-like activity was detected in the presence of o-diphenols (L-DOPA,  
311 dopamine), suggesting the presence of a catecholase- or laccase-like activity (Table 2, Fig. 1).  
312 Interestingly, the  $K_{m_{app}}$  value for L-DOPA calculated in the current study was similar to  
313 values previously described in hemocytes of *Saccostrea glomerata* and *Crassostrea virginica*  
314 [16, 33]. Importantly, results with the laccase substrate PPD suggest the presence of a laccase-  
315 like activity never reported before in this organism. However, at this stage, it remains  
316 uncertain whether the dopamine oxidation activity is the result of the functioning of a mixture  
317 of laccase and catecholase or of a single laccase. We next attempted to clarify this issue using  
318 moderate concentrations of PO inhibitors. With dopamine as substrate, PO-like activity was  
319 partially inhibited by the catecholase inhibitor 4-HR and the laccase inhibitor CTAB. With

320 PPD as substrate, PO-like activity was fully inhibited by CTAB. These data suggest that both  
321 catecholase and laccase are present in the plasma of *C. gigas*.

322 Most of the PO inhibitors listed in Table 1 are copper chelators and constitute therefore  
323 potential catecholase and laccase inhibitors [34-37]. Accordingly, we found that PO-like  
324 activity from plasma was inhibited by PTU, DETC, and to a lesser extent, by SHAM and  
325 tropolone. PTU was previously described as an inhibitor of tyrosinases and catecholases [38]  
326 but also as an inhibitor of laccases [25, 39]. It contains a sulphur compound that binds copper  
327 at the active site of catecholase [40]. We found that PTU strongly inhibited dopamine and  
328 PPD oxidation suggesting that it can inhibit both catecholase and laccase. To the best of our  
329 knowledge, the following chemical products have been reported in the literature as laccase  
330 inhibitors : N-hydroxyglycine [35],  $\text{NaN}_3$  [35], ammonium tetramolybdate [41], SHAM [35],  
331 kojic acid [35] and CTAB [42-44]. We did not use N-hydroxyglycine because, at  $\mu\text{M}$   
332 concentrations, N-hydroxyglycine was shown to bleach solutions of substrates oxidized either  
333 chemically or enzymatically by laccase [45]. For  $\text{NaN}_3$  (Fig. 2) and ammonium  
334 tetramolybdate (data not shown), an effect was observed on the autoxidation of, at least, one  
335 laccase substrate. SHAM and kojic acid are PO inhibitors but not laccase specific [37, 46].  
336 Therefore, although CTAB is also known as a cationic detergent, it appeared to be the most  
337 pertinent laccase inhibitor. Indeed, CTAB was the only molecule reported as a specific  
338 inhibitor of laccase but not other phenoloxidases [42-44], and we confirmed that it did not  
339 affect autoxidation of laccase substrates.

340 Several difficulties are encountered when identification of a PO-like activity is performed in a  
341 non purified or in a partially purified tissue homogenate because substrates used by PO may  
342 be used by (i) peroxidases (ii) hemocyanins, (iii) cytochrome oxidases (EC 1.9.3.1) and (iv)  
343 ceruloplasmines or ferroxidases (EC 1.16.3.1). Oxygraphic data showed the involvement of  
344 true oxidase activities in plasma (Fig. 1). The involvement of peroxidases [47] was excluded

345 since exogenous catalase did not inhibit dopamine and PPD oxidation activities. It should be  
346 noted that, with dopamine as substrate, catalase induced a two-fold increase of PO-like  
347 activity. This could be explained by the generation of H<sub>2</sub>O<sub>2</sub> as an autoinhibitor of PO during  
348 dopamine oxidation [48]. Hemocyanins, cytochrome oxidases, and ceruloplasmins are absent  
349 in the plasma and in the HLS obtained from *C. gigas* [49-52]. Therefore, only PO-like activity  
350 was detected in crude plasma.

351 In order to confirm that PO-like activity observed in crude plasma was unambiguously due to  
352 a protein source, the next step was to partially purify fractions from plasma. Our data obtained  
353 with precipitated protein fractions confirmed that the signal measured was from a protein  
354 source (Fig. 5). The results obtained with dopamine and PPD as substrates and with PTU (5  
355 mM), 4-HR (1 mM) and CTAB (1 mM) as inhibitors confirmed the presence of a catecholase-  
356 like and a laccase-like activity in plasma (Fig. 5a and 5c). Precipitated protein fractions from  
357 HLS were tested for PO-like activity with the aim to localize endogenous PO-like activity in  
358 hemolymph from *C. gigas*. Independently of the substrate, specific PO-like activity was  
359 considerably higher in hemocyte lysate supernatant (HLS) than in plasma (Fig. 5). In addition  
360 to that, we found that catecholase-like activity was absent in the HLS while a high laccase-  
361 like activity was detected in this fraction (Fig. 5b and 5c). Therefore, the type of PO-like  
362 activity that can be detected depends on the hemolymphatic compartment that is studied, i.e.  
363 (i) two types of PO-like activity can be detected in plasma (catecholase and laccase), and (ii)  
364 one type of PO-like activity can be detected in HLS (laccase).

365 It is important to notice that, with dopamine as substrate, CTAB inhibited 21% of PO-like  
366 activity in crude plasma samples, suggesting the presence of a laccase in the plasma of *C.*  
367 *gigas*. However, this inhibitory effect was suppressed in precipitated protein fractions. Thus,  
368 results with crude plasma suggest that (i) a parasitic reaction (even minor) is measured in  
369 parallel with the enzymatic dopamine oxidation and that (ii) this parasitic reaction is



370 suppressed when proteins are precipitated. This confirms the interest of this purification step  
371 for identification of PO-like activity.

372 POs are an important component in immune defence mechanisms in bivalves. For example,  
373 the importance of phenoloxidase activity in the resistance to *M. sydneyi* has been reported in  
374 *S. glomerata* [17]. Besides, the presence of laccases has previously been evoked in molluscs  
375 [16, 53]. Moreover, a gene encoding a laccase was recently identified from Pacific oyster, *C.*  
376 *gigas*, hemocytes (Faury and Renault, pers. comm.) and its total sequence deposited in  
377 GenBank under accession n° NCBI ID: EU678320. This gene was shown to be over-  
378 expressed in the presence of polyaromatic hydrocarbons, suggesting a potential use of laccase  
379 as a biomarker of pollution exposure [54]. In this context, the present study demonstrates, for  
380 the first time through the use of a panel of POs substrates and inhibitors, that a laccase-like  
381 activity is present in a mollusc species, the Pacific oyster, *C. gigas*. A better characterization  
382 of laccase and/or catecholase systems would help to extend our knowledge on immune  
383 defence mechanisms in *C. gigas*, and thus, would improve our ability to monitor and manage  
384 the production and survival of this important species.

385

### 386 **Acknowledgments**

387 This study was supported by a PhD grant from the Conseil Général of the Charente-Maritime  
388 for A. Luna-Acosta. The Conseil Régional de Poitou-Charentes is acknowledged for financial  
389 support through the research project 'POLERON' (Modifications chimiques de polluants  
390 organiques dans le bassin de Marennes-Oléron, toxicité des produits de dégradation sur  
391 l'huître creuse).

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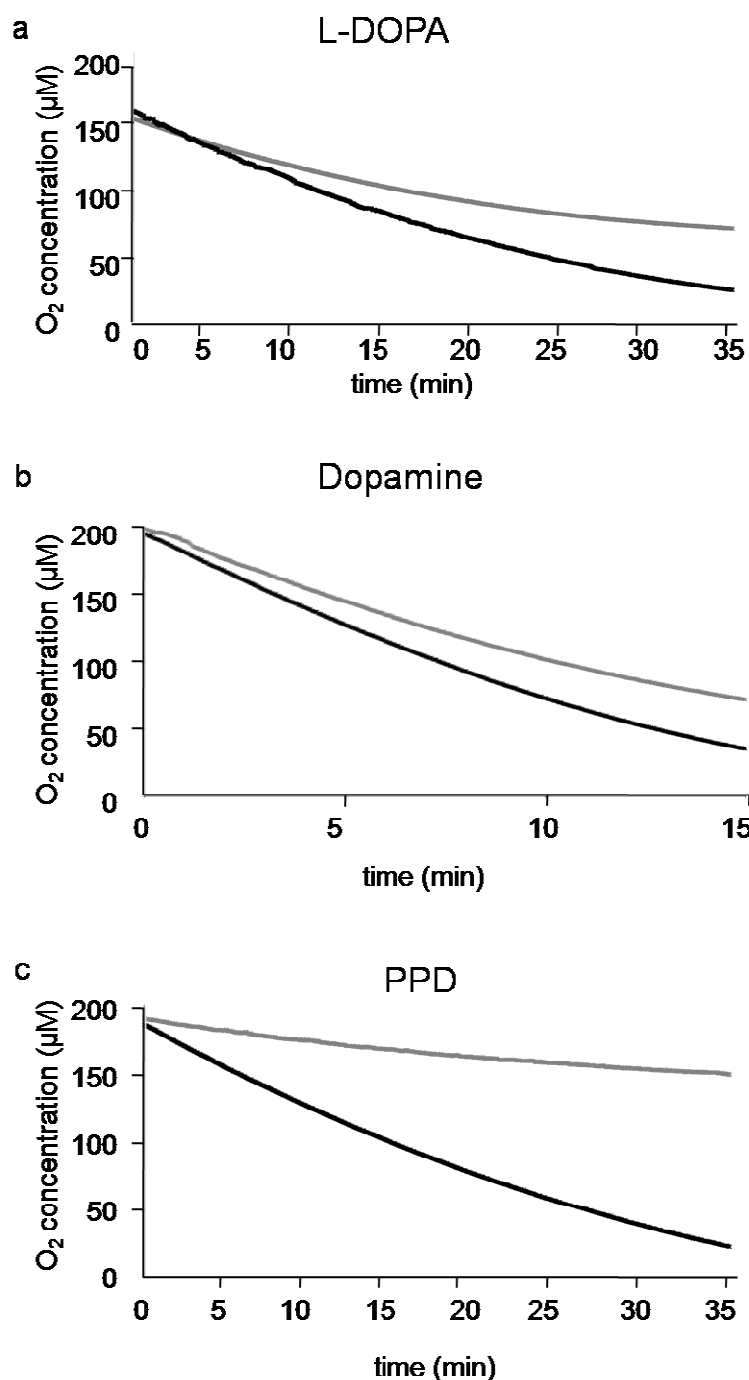
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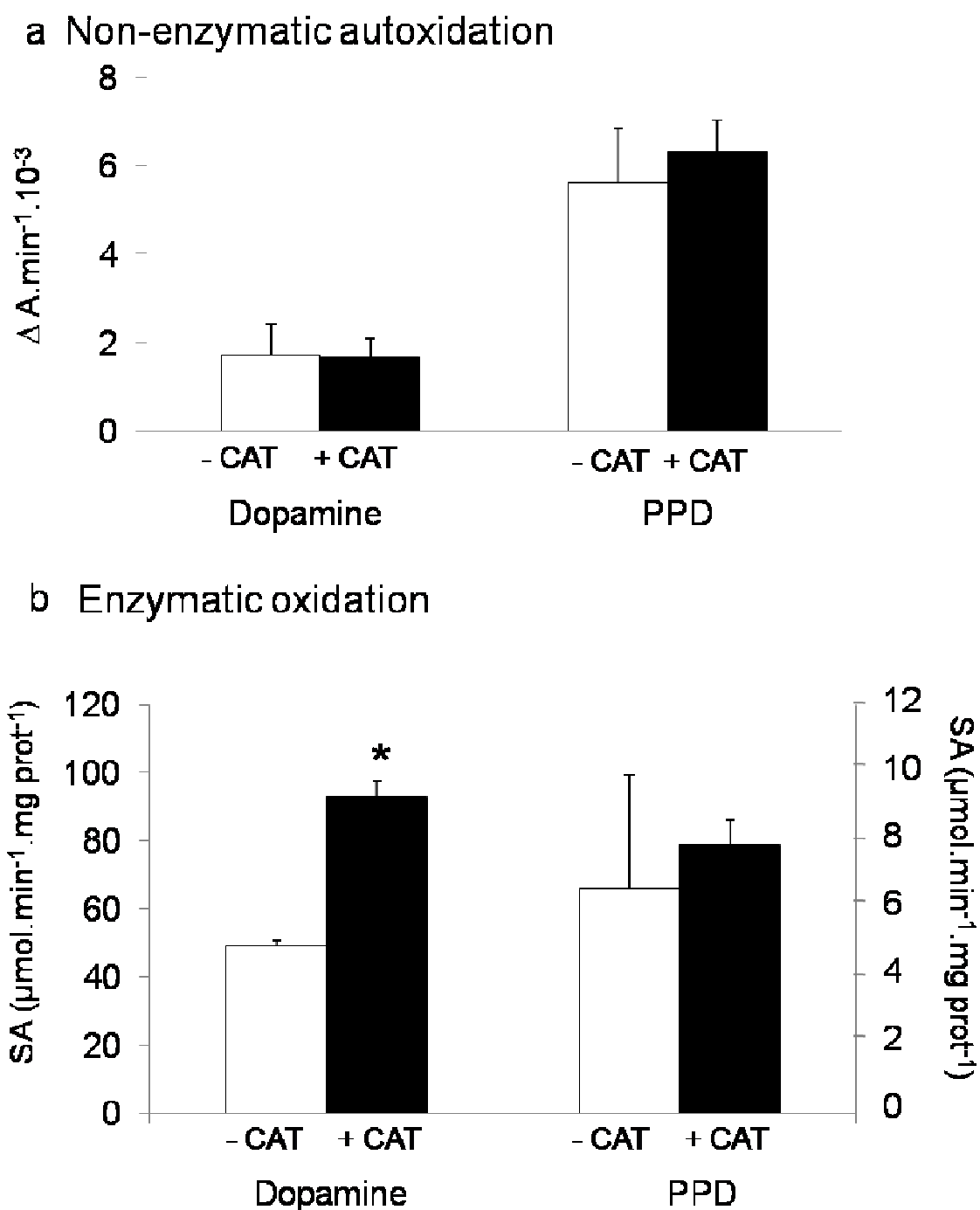
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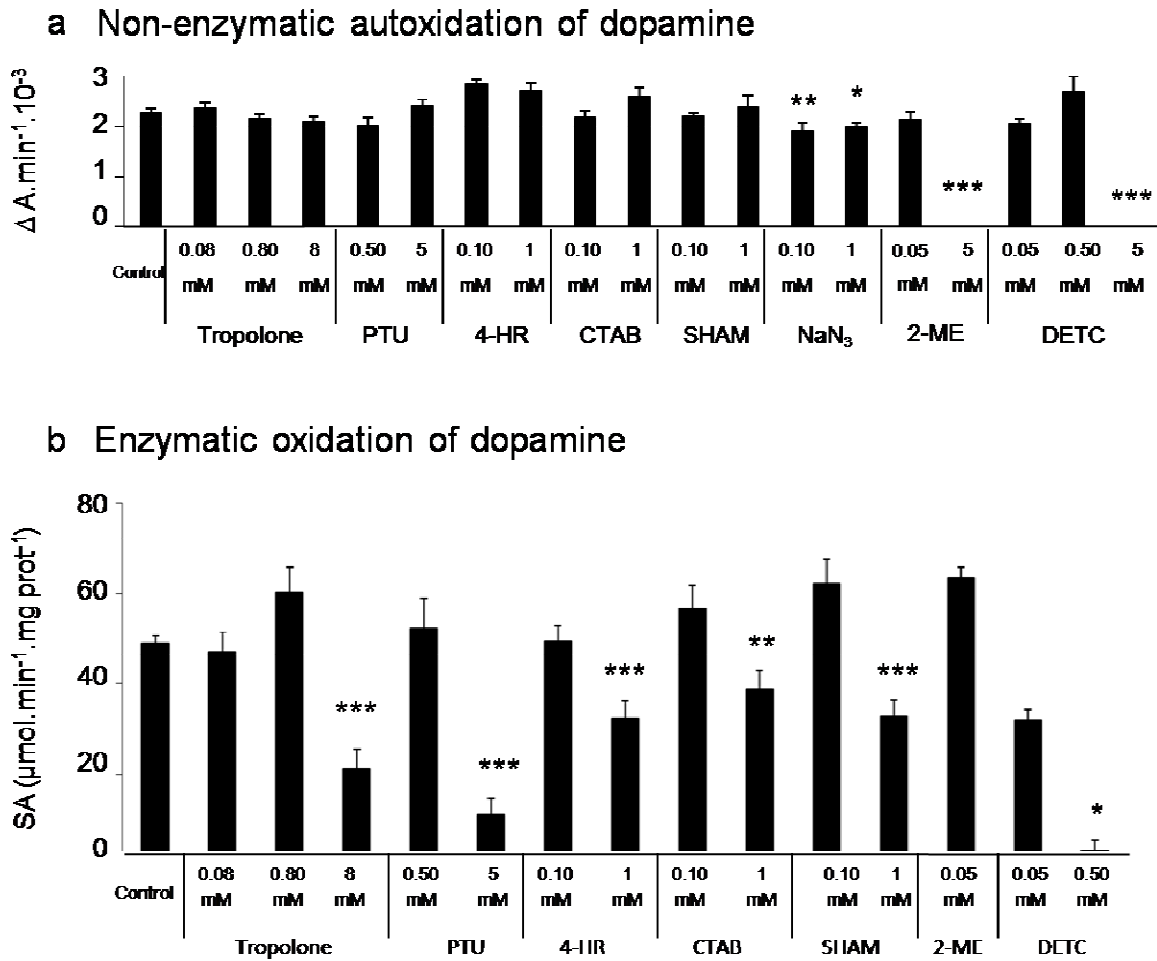
546 **Figure 1 Oxygen uptake during oxidation of PO substrates.** Non-enzymatic (- plasma,  
547 gray lines) and enzymatic (+ plasma, black lines) oxidation reactions were followed using  
548 oxygraphy with the substrates: **a.** L-DOPA 10 mM, **b.** dopamine 100 mM, and **c.** PPD 50  
549 mM. Experiments were repeated three times for each substrate. For clarity, only one typical  
550 experiment is shown. No oxygen uptake was observed in 'buffer' and 'sample' controls (data  
551 not shown).



571 **Figure 2 Effect of catalase on autoxidation (a) and PO-like activity (b).** Both dopamine  
 572 and PPD were used as substrates in the presence (+ CAT) or in the absence (- CAT) of  
 573 catalase. Left y axis corresponds to results obtained with dopamine +/- CAT and right y axis  
 574 corresponds to results obtained with PPD +/- CAT. Mean  $\pm$  SD  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot}^{-1}$ , n=9, \*=  
 575 statistical difference for  $p<0.05$ .



577 **Figure 3 Effect of inhibitors on autoxidation and enzymatic oxidation of dopamine. a.**  
 578 Non-enzymatic autoxidation (without plasma). **b.** Enzymatic oxidation (with plasma).  
 579 'Control' corresponds to the condition without inhibitor. PO inhibitor concentrations  
 580 correspond to final concentrations in the assay. Mean  $\pm$  SD  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot}^{-1}$ , n=9, \*, \*\*, \*\*\*  
 581 \*\*\* = statistical difference for p<0.05, p<0.01 and p<0.001, respectively.



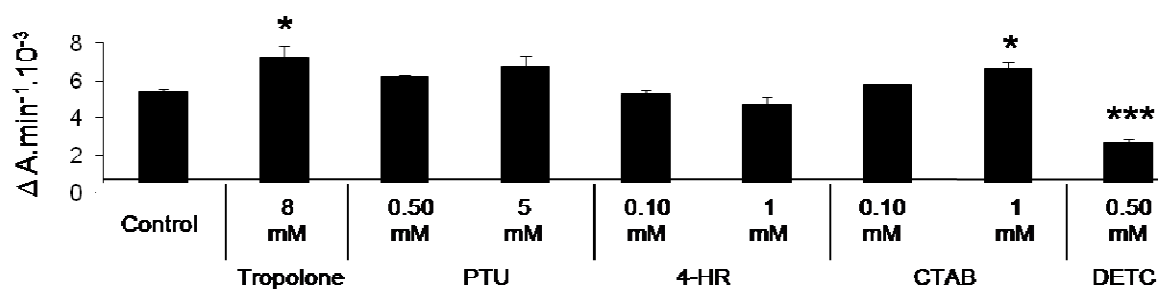
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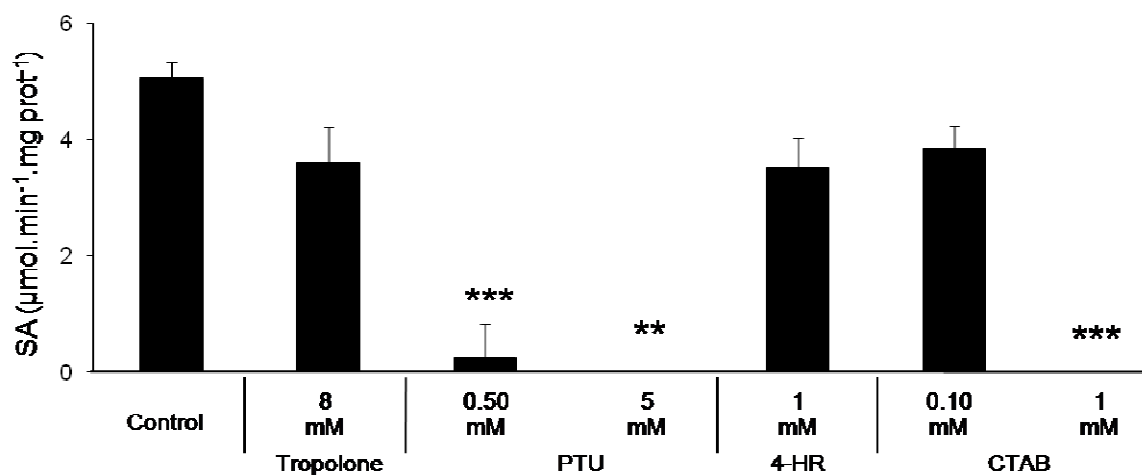


584 **Figure 4 Effect of inhibitors on autoxidation and enzymatic oxidation of PPD. a.** Non-  
 585 enzymatic autoxidation. **b.** Enzymatic oxidation. ‘Control’ corresponds to the condition  
 586 without inhibitor. PO inhibitor concentrations correspond to final concentrations in the assay.  
 587 Mean  $\pm$  SD  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot}^{-1}$ , n=9, \*, \*\*, \*\*\* = statistical difference for  $p<0.05$ ,  $p<0.01$   
 588 and  $p<0.001$ , respectively.

### a Non-enzymatic autoxidation of PPD



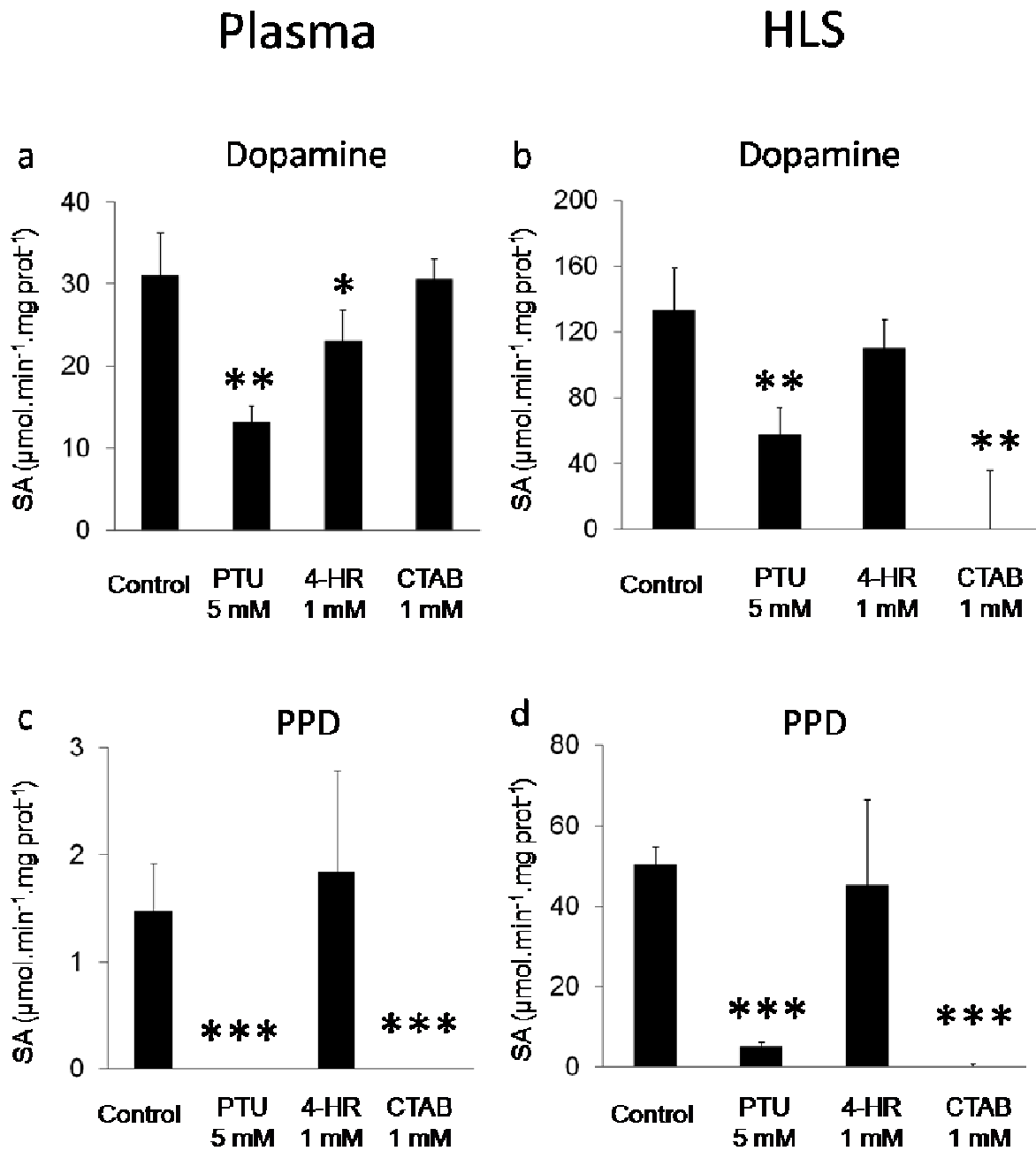
### b Enzymatic oxidation of PPD



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590

591 **Figure 5 Inhibition of phenoloxidase-like activity in precipitated protein fractions from**  
 592 **plasma and hemocyte lysate supernatant (HLS).** Both dopamine (a, b) and PPD (c, d) were  
 593 used as substrates. 'Control' corresponds to the condition without inhibitor. PO inhibitor  
 594 concentrations correspond to final concentrations in the assay. Mean  $\pm$  SD  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$   
 595  $\text{prot}^{-1}$ , n=9, \*, \*\*, \*\*\* = statistical difference for  $p<0.05$ ,  $p<0.01$  and  $p<0.001$ , respectively.



596

597

598 **Table 1 Phenoloxidase-like inhibitors and modes of action.** DETC: diethyldithiocarbamate; PTU: 1-phenyl-2-thiourea; 2-ME: 2-mercaptoethanol; NaN<sub>3</sub>: sodium azide; 4-  
599 HR: 4-Hexylresorcinol; SHAM: salicylhydroxamic acid; CTAB: cetyl trimethyl ammonium bromide. .

600

601

Inhibitor	Mode of action	Reference
DETC <sup>1,2,3</sup>	Copper chelation (competitive inhibition)	33, 49
PTU <sup>1,2,3</sup>	Copper chelation (competitive inhibition): sulphur binds to copper at the active site of the enzyme, blocking accessibility of the substrate	39, 50
Tropolone <sup>1,2,3</sup>	Substrate of peroxidases and inhibitor of PO (copper chelation)	35, 50
2-ME <sup>1,2,3</sup>	Reducing agent: sulphur containing compounds are quinone chelators, blocking their participation in secondary reactions of melanization and/or acting directly with the enzyme	51, 52
NaN <sub>3</sub> <sup>1,2,3</sup>	Metal chelator: inhibitor of all types of PO	35, 53
4-HR <sup>1,2</sup>	Fixation on the active site: competitive inhibitor of tyrosinases and catecholases but not of laccases	53, 54
SHAM <sup>1,2</sup>	Metal chelator described as an inhibitor of alternative oxidases in plants: competitive inhibitor of tyrosinases and catecholases but not of laccases	35, 54
CTAB <sup>3</sup>	Cationic detergent: competitive inhibitor of laccases but not of other POs	55-57

602

603 <sup>1</sup> Tyrosinase inhibitor604 <sup>2</sup> Catecholase inhibitor605 <sup>3</sup> Laccase inhibitor

606

607 **Table 2 Identification of phenoxidase-like activity in plasma of *Crassostrea gigas* by using a panel of**  
 608 **substrates.  $\emptyset$ : no PO-like activity detected.**  
 609

Type of substrate	Substrate	$\lambda$ (nm) <sup>4</sup>	Final substrate concentrations tested (mM)	Substrate saturating concentration (mM)	K <sub>m,app</sub> (mM)	V <sub>m,app</sub> ( $\Delta A \cdot \text{min}^{-1} \cdot 10^{-3}$ )
monophenol <sup>1</sup>	L-tyrosine	490	4, 6, 8, 10, 20	$\emptyset$	$\emptyset$	$\emptyset$
	4-HA	490	4, 6, 8, 10, 20	$\emptyset$	$\emptyset$	$\emptyset$
	PHPPA	490	4, 6, 8, 10, 20	$\emptyset$	$\emptyset$	$\emptyset$
o-diphenol <sup>1,2,3</sup>	L-DOPA	490	4, 6, 8, 10, 20	8	7	0.45
	Dopamine	490	10, 25, 50, 100, 200	100	51	0.51
	DHPPA	400	4, 6, 8, 10, 20	$\emptyset$	$\emptyset$	$\emptyset$
metoxi phenol <sup>3</sup>	Syringaldazine	525	0.01, 0.1, 1	$\emptyset$	$\emptyset$	$\emptyset$
non phenolic substrates <sup>3</sup>	ABTS	420	1, 2, 3, 4, 5	$\emptyset$	$\emptyset$	$\emptyset$
	PPD	420	5, 10, 25, 50, 100	50	45	0.59

610

611 <sup>1</sup>Tyrosinase substrate, in Tris buffer612 <sup>2</sup>Catecholase substrate, in Tris buffer613 <sup>3</sup>Laccase substrate, in methanol614 <sup>4</sup>Wavelengths used to measure by spectrophotometry the formation of each o-quinone derivative