

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

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21

22 **Abstract:** Phenoloxidasases (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:** phenoloxidasase; 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 melanization 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 (*Mytillus edulis*, *Mytillus*  
72 *galloprovincialis*, *Perna viridis*), clams (*Ruditapes decussatus*), scallops (*Nodipecten*  
73 *subnodosus*) and oysters (*Crassostrea gigas* *Crassostrea virginica*, *Saccostrea glomerata*) [11-  
74 17]. 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.

92

## 93 2. Materials and methods

94

### 95 2.1. Oysters

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

100

### 101 2.2. Collection of plasma

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103 hemolymph was withdrawn directly from the pericardial cavity with a 1-ml syringe equipped  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

108

### 109 2.3. Hemocyte lysate supernatant

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

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#### 118 2.4. Chemicals

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

130

#### 131 2.5. Phenoloxidase assays

132 Phenoloxidase-like (PO-like) activity has been reported to be higher in plasma than in HLS  
133 from *C. gigas* [23]. Therefore, constitutive PO-like activity was first analyzed in crude  
134 plasma. PO-like activity was measured spectrophotometrically by recording the formation of  
135 *o*-quinones. The method of Asokan et al. [5] was used with some modifications. Working  
136 solutions of substrates were prepared just before use in Tris buffer (0.1 M Tris HCl, 0.45 M  
137 NaCl, 26 mM MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>) adjusted to pH 7, except for PPD which was  
138 prepared in methanol. The latter did not affect PO-like activities in the conditions tested (data  
139 not shown). Samples were distributed in 96-well microplates (Nunc, France). Ten microliters  
140 of sample were incubated with 80 µl of substrate and 50 µl of Tris buffer at 25°C. Several  
141 control wells were systematically used: 'buffer control' containing only buffer, 'sample  
142 control' containing only sample and buffer, and 'non-enzymatic control' containing only

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

154 Apparent Michaelis-Menten constants ( $Km_{app}$ ) and maximum velocities ( $Vm_{app}$ ) were  
155 estimated from double reciprocal plots (Lineweaver-Burk) of velocity vs substrate  
156 concentration.

157

## 158 2.6. Phenoloxidase inhibition assay

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

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167

### 168 2.7. *Hydrogen peroxide scavenging by exogenous catalase*

169 Plasma (10  $\mu$ l) was preincubated at 25°C for 30 minutes in the presence of 10  $\mu$ l of catalase  
170 from bovine liver at 1000 U/ml [25]. The total scavenging of H<sub>2</sub>O<sub>2</sub> was verified using the  
171 Catalase kit CAT-100 (Sigma) and specifications included (data not shown). Then, PO assay  
172 was carried out with dopamine (100 mM) or PPD (50 mM). The effect of catalase on non-  
173 enzymatic autoxidation was also followed by incubating (25°C, 30 min) the substrates  
174 (dopamine or PPD at 100 mM or 50 mM, respectively) in the presence of 10  $\mu$ l of catalase.  
175 Enzymatic oxidation (in the presence of catalase) was systematically corrected for  
176 autoxidation of the substrate (in the presence of catalase). All the experiments were performed  
177 with three oyster pools. Each pool was tested in triplicate wells and average rates were  
178 calculated.

### 180 2.8. *Protein determination*

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

### 184 2.9. *Measurements of oxygen uptake*

185 Oxygen uptake was followed with a Clark-type oxygen electrode (Hansatech, DW1) in a  
186 700- $\mu$ l closed chamber thermostatted at 25°C with continuous stirring [27]. In a typical  
187 experiment, oxygen uptakes were recorded simultaneously using four separate electrode units.  
188 In the first unit ('buffer control'), a volume of 700  $\mu$ l of buffer was distributed in the chamber.  
189 In the second unit ('sample control'), 250  $\mu$ l of plasma and 450  $\mu$ l of buffer were distributed.  
190 In the third unit ('non-enzymatic control'), 700  $\mu$ l of substrate (L-DOPA 10 mM or dopamine  
191 100 mM) were distributed. In the fourth unit, 250  $\mu$ l of plasma and 450  $\mu$ l of substrate were  
192 distributed. With PPD (50 mM) as substrate, the same protocol was adopted with slight

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

195

#### 196 *2.10. Preparation of protein fractions from plasma and hemocyte lysate supernatant*

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

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

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

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

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

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

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

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

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

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

207 for PO-like activity.

208

#### 209 *2.11. Statistical analysis*

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

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

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

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

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

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

216

217

## 218 3. Results

219

### 220 3.1. Substrate specificity of PO-like activity in plasma

221 Enzymatic oxidation results were systematically corrected for non-enzymatic autoxidation.  
222 Table 2 shows that no PO-like activity was detected in the presence of PHPPA, L-tyrosine, 4-  
223 HA, DHPPA, syringaldazine and ABTS. Conversely, PO-like activity was detected using L-  
224 DOPA, dopamine and PPD, with final concentrations of substrate saturation being equal to 10  
225 mM, 100 mM and 50 mM, respectively.  $K_{m_{app}}$  values for L-DOPA, dopamine and PPD were  
226 7, 51, and 45 mM, respectively (Table 2).  $K_{m_{app}}$  for L-DOPA was thus 6 to 7 times lower than  
227  $K_{m_{app}}$  for dopamine and PPD.  $V_{m_{app}}$  values for L-DOPA, dopamine and PPD were 0.45, 0.51  
228 and  $0.59 \Delta A \cdot \text{min}^{-1} \cdot 10^{-3}$ , respectively (Table 2). Thus,  $V_{m_{app}}$  value obtained with PPD was 1.15  
229 to 1.31 times higher than values obtained with L-DOPA and dopamine.

230

### 231 3.2. O<sub>2</sub> requirements of PO-like activity

232 Using oxygraphy, we easily confirmed the non-enzymatic autoxidation of L-DOPA,  
233 dopamine, and to a lesser extent, of PPD (Fig. 1). Most importantly, we found that O<sub>2</sub> uptake  
234 was higher in the presence of plasma, independently of the substrate, confirming the presence  
235 of at least one PO-type oxidase in plasma.

236

### 237 3.3. Effect of catalase

238 Exogenous catalase was used to scavenge the H<sub>2</sub>O<sub>2</sub> potentially involved in peroxidase-  
239 dependent oxidation reactions. Fig. 2a shows that catalase did not affect autoxidations of  
240 dopamine and PPD. Most importantly, catalase did not inhibit oxidations of both substrates in  
241 the presence of plasma (Fig. 2b). Fig. 2b also shows that catalase induced a two-fold increase  
242 of PO-like activity with dopamine as substrate.

### 243 3.4. Effect of various PO inhibitors

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

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

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

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

252 the presence of PO inhibitors). Fig. 3b shows that enzymatic oxidation was strongly inhibited  
253 by 0.5 mM DETC and 5 mM PTU (94 and 77% inhibition, respectively), and also

254 significantly inhibited by 8 mM tropolone, 1 mM SHAM, and 1 mM CTAB (56, 33, and 21%  
255 inhibition, respectively). The catecholase inhibitor 4-HR (1 mM) exerted 34 % inhibition.

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

257 DETC (0.5 mM, Fig. 4a). Therefore, DETC was not used for further studies. Tropolone (8  
258 mM) and the laccase inhibitor CTAB (1 mM) only slightly interfered (stimulation) with the

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

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

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

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

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

264

### 265 3.5. PO-like activity in protein fractions

266 Independently of the substrate, specific PO-like activity was considerably higher in hemocyte

267 lysate supernatant (HLS) than in plasma (Fig. 5). Moreover, the results obtained with

268 precipitated protein fractions confirm that the activities measured derived from a protein  
269 source. Results with precipitated protein fractions from plasma are summarized in Fig. 5a,c.  
270 With dopamine as substrate (Fig. 5a), the PO inhibitor PTU (5 mM) and the catecholase  
271 inhibitor 4-HR (1 mM), inhibited PO-like activity by 57 and 26%, respectively. In contrast to  
272 the results obtained with crude plasma, the laccase inhibitor CTAB (1 mM) did not exert  
273 inhibition in precipitated protein fractions from plasma. With PPD as substrate (Fig. 5c), PTU  
274 and CTAB exerted 100% inhibition of PO-like activity.

275 Results with precipitated protein fractions from HLS are summarized in Fig. 5b,d. With  
276 dopamine as substrate (Fig. 5b), PTU and CTAB inhibited PO-like activity by 57 and 100%,  
277 respectively. Interestingly, with PPD as substrate (Fig. 5d), PTU and CTAB exerted 90 and  
278 100% inhibition, respectively.

279

#### 280 **4. Discussion**

281

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

287 Oxidation catalyzed by POs requires O<sub>2</sub>. However, PO substrates are also readily autoxidized  
288 in contact with air [15, 29]. Therefore, a special attention should be paid to substrate  
289 autoxidations before studying PO activity. Using both spectrophotometry and oxygraphy, we  
290 confirmed that L-DOPA, dopamine, and to a lesser extent PPD, could be readily autoxidized.  
291 These non-enzymatic oxidation reactions probably involve quinone redox cycling leading to

292 the formation of different types and quantities of oxygen radicals and quinone-derived  
293 products [30].

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

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

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

334 Several difficulties are encountered when identification of a PO-like activity is performed in a  
335 non purified or in a partially purified tissue homogenate because substrates used by PO may  
336 be used by (i) peroxidases (ii) hemocyanins, (iii) cytochrome oxidases (EC 1.9.3.1) and (iv)  
337 ceruloplasmines or ferroxidases (EC 1.16.3.1). Oxygraphic data showed the involvement of  
338 true oxidase activities in plasma (Fig. 1). The involvement of peroxidases [47] was excluded  
339 since exogenous catalase did not inhibit dopamine and PPD oxidation activities. It should be  
340 noted that, with dopamine as substrate, catalase induced a two-fold increase of PO-like

341 activity. This could be explained by the generation of  $H_2O_2$  as an auto-inhibitor of PO during  
342 dopamine oxidation [48]. Hemocyanins, cytochrome oxidases, and ceruloplasmins are absent  
343 in the plasma and in the HLS obtained from *C. gigas* [49-52]. Therefore, only PO-like activity  
344 was detected in crude plasma.

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

359 It is important to notice that, with dopamine as substrate, CTAB inhibited 21% of PO-like  
360 activity in crude plasma samples, suggesting the presence of a laccase in the plasma of *C.*  
361 *gigas*. However, this inhibitory effect was suppressed in precipitated protein fractions. Thus,  
362 results with crude plasma suggest that (i) a parasitic reaction (even minor) is measured in  
363 parallel with the enzymatic dopamine oxidation and that (ii) this parasitic reaction is  
364 suppressed when proteins are precipitated. This confirms the interest of this purification step  
365 for identification of PO-like activity.

366 POs are an important component in immune defence mechanisms in bivalves. For example,  
367 the importance of phenoloxidase activity in the resistance to *M. sydneyi* has been reported in  
368 *S. glomerata* [17]. Besides, the presence of laccases has previously been evoked in molluscs  
369 [16, 53]. Moreover, a gene encoding a laccase was recently identified from Pacific oyster, *C.*  
370 *gigas*, hemocytes (Faury and Renault, pers. comm.) and its total sequence deposited in  
371 GenBank under accession n° NCBI ID: EU678320. This gene was shown to be over-  
372 expressed in the presence of polyaromatic hydrocarbons, suggesting a potential use of laccase  
373 as a biomarker of pollution exposure [54]. In this context, the present study demonstrates, for  
374 the first time through the use of a panel of POs substrates and inhibitors, that a laccase-like  
375 activity is present in a mollusc species, the Pacific oyster, *C. gigas*. A better characterization  
376 of laccase and/or catecholase systems would help to extend our knowledge on immune  
377 defence mechanisms in *C. gigas*, and thus, would improve our ability to monitor and manage  
378 the production and survival of this important species.

379

### 380 **Acknowledgments**

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384 organiques dans le bassin de Marennes-Oléron, toxicité des produits de dégradation sur  
385 l'huître creuse).

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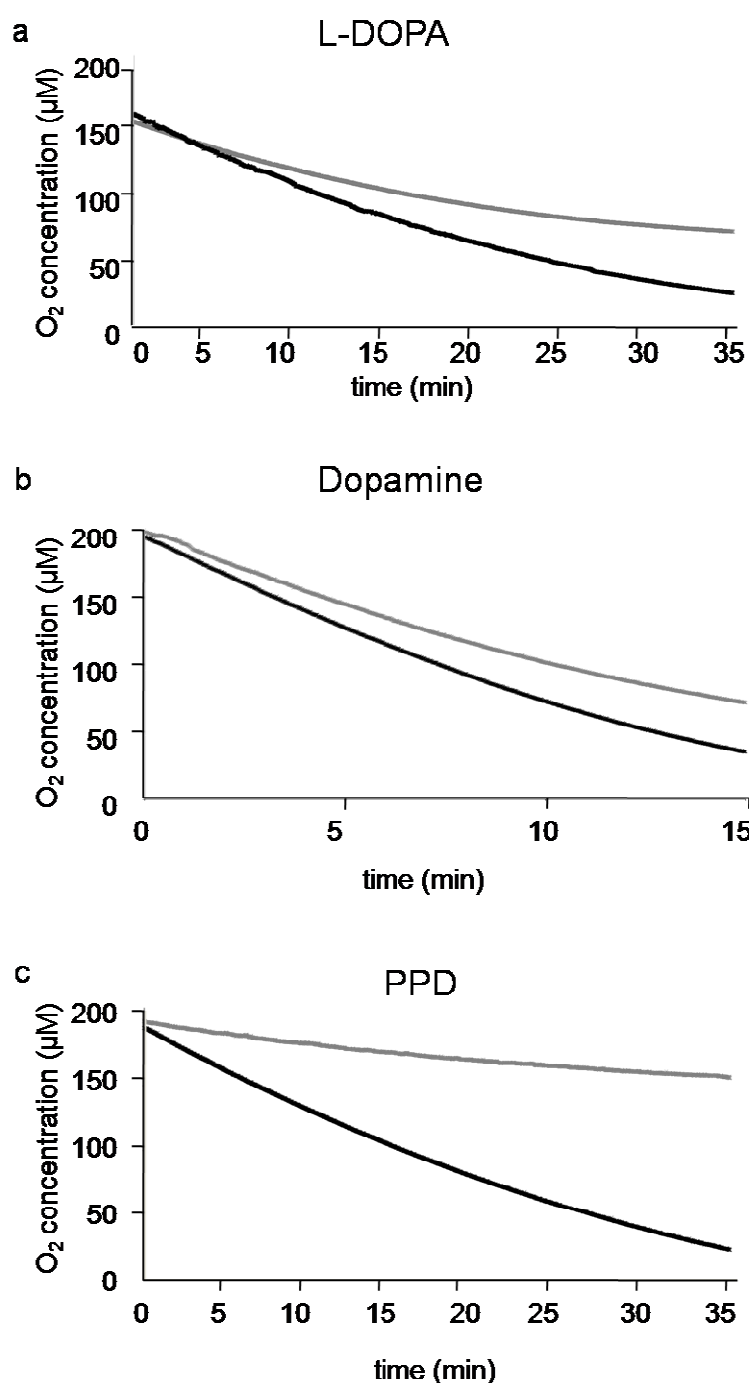
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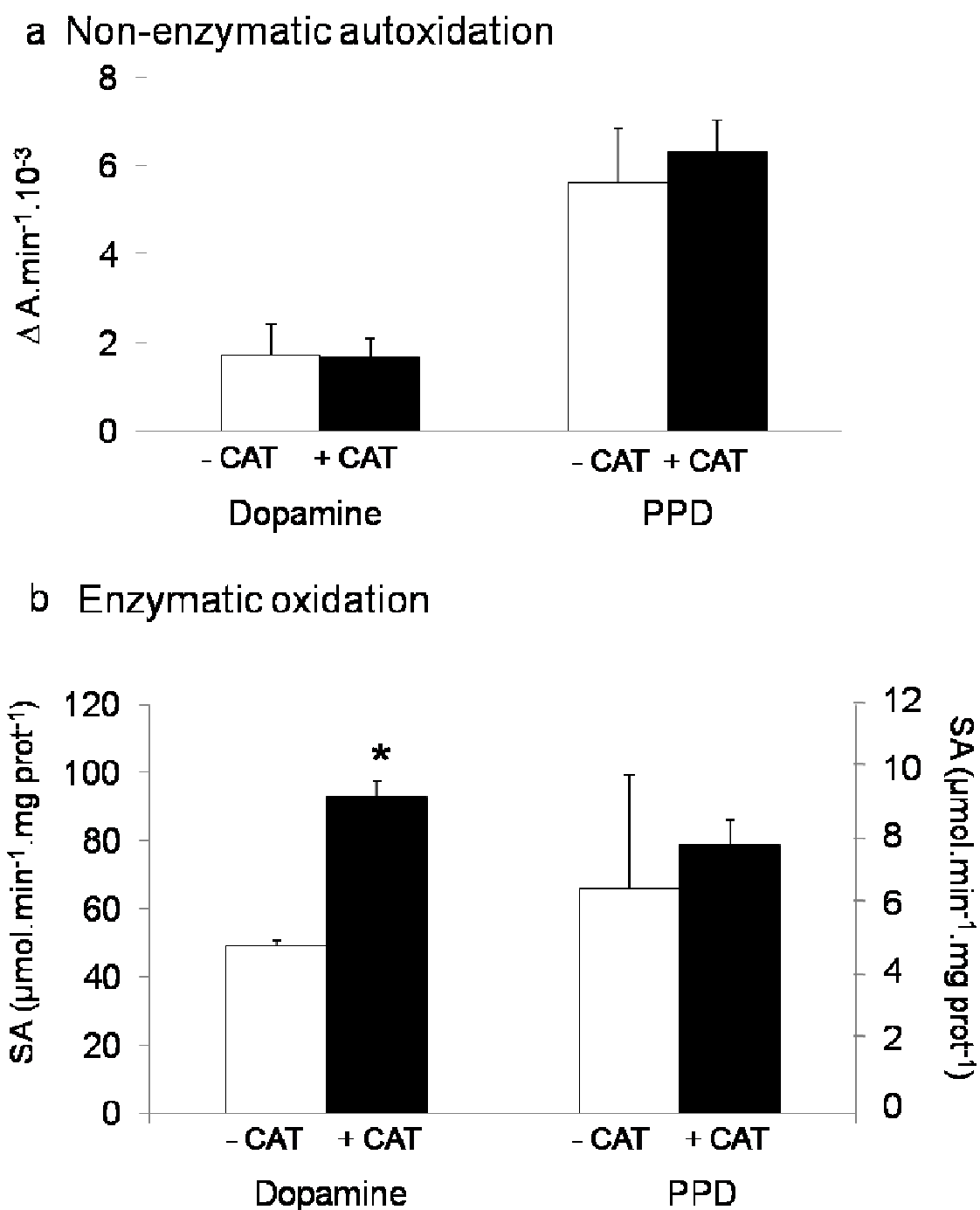
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538  
539

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

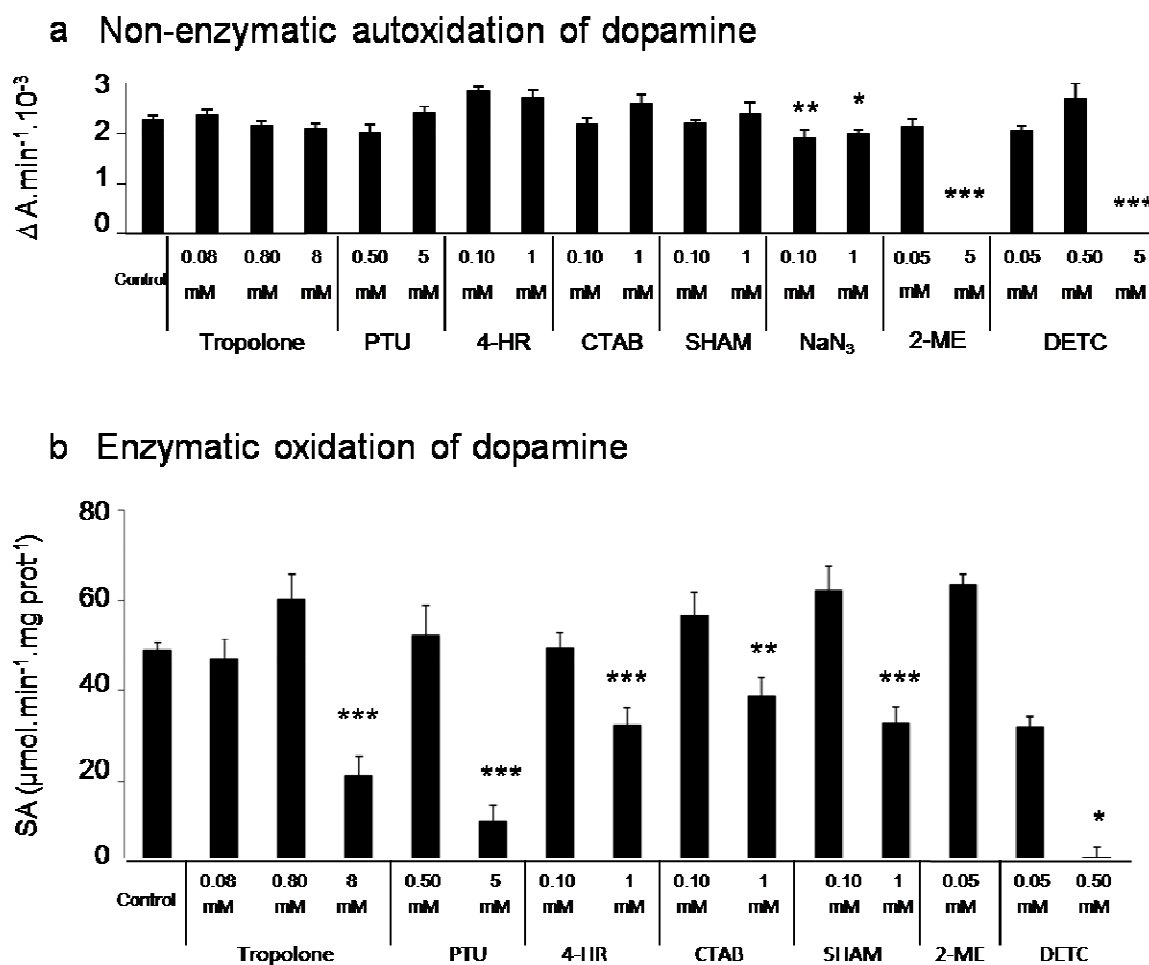


565 **Fig. 2** Effect of catalase on autoxidation (a) and PO-like activity (b). Both dopamine and PPD  
 566 were used as substrates in the presence (+ CAT) or in the absence (- CAT) of catalase. Left y  
 567 axis corresponds to results obtained with dopamine +/- CAT and right y axis corresponds to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



570

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

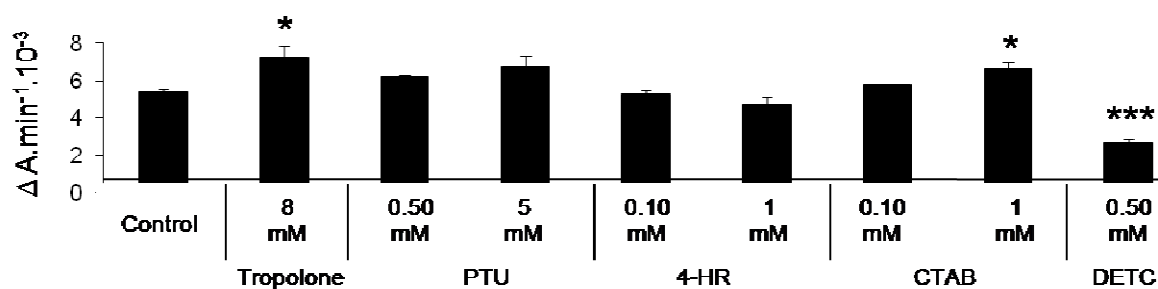


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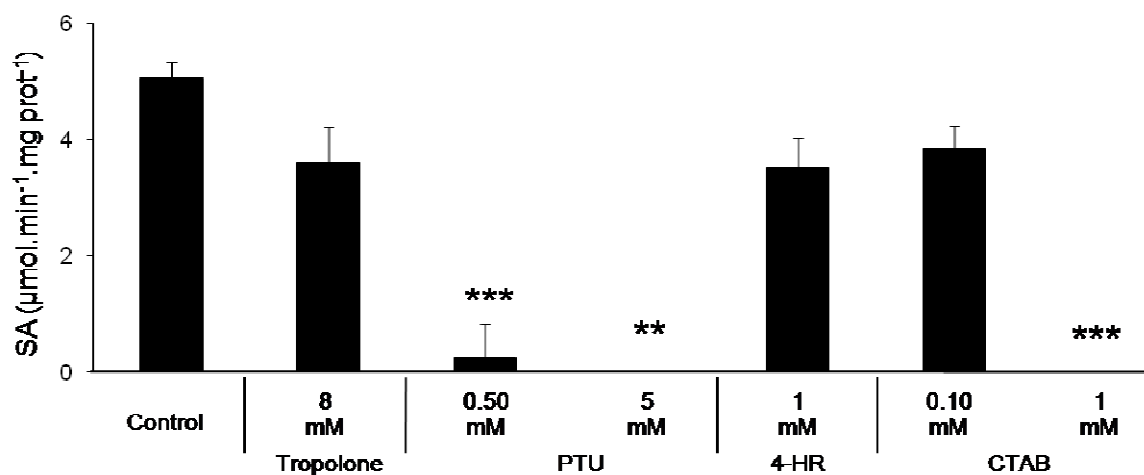
577

578 **Fig. 4** Effect of inhibitors on autoxidation and enzymatic oxidation of PPD. (a) Non-  
 579 enzymatic autoxidation. (b) Enzymatic oxidation. 'Control' corresponds to the condition  
 580 without inhibitor. PO inhibitor concentrations correspond to final concentrations in the assay.  
 581 Mean  $\pm$  SD  $\mu\text{mol}\cdot\text{min}^{-1}\text{ mg prot}^{-1}$ ,  $n = 9$ , \*statistical difference for  $p < 0.05$ , \*\* $p < 0.01$  and  
 582 \*\*\* $p < 0.001$ , respectively.

### a Non-enzymatic autoxidation of PPD



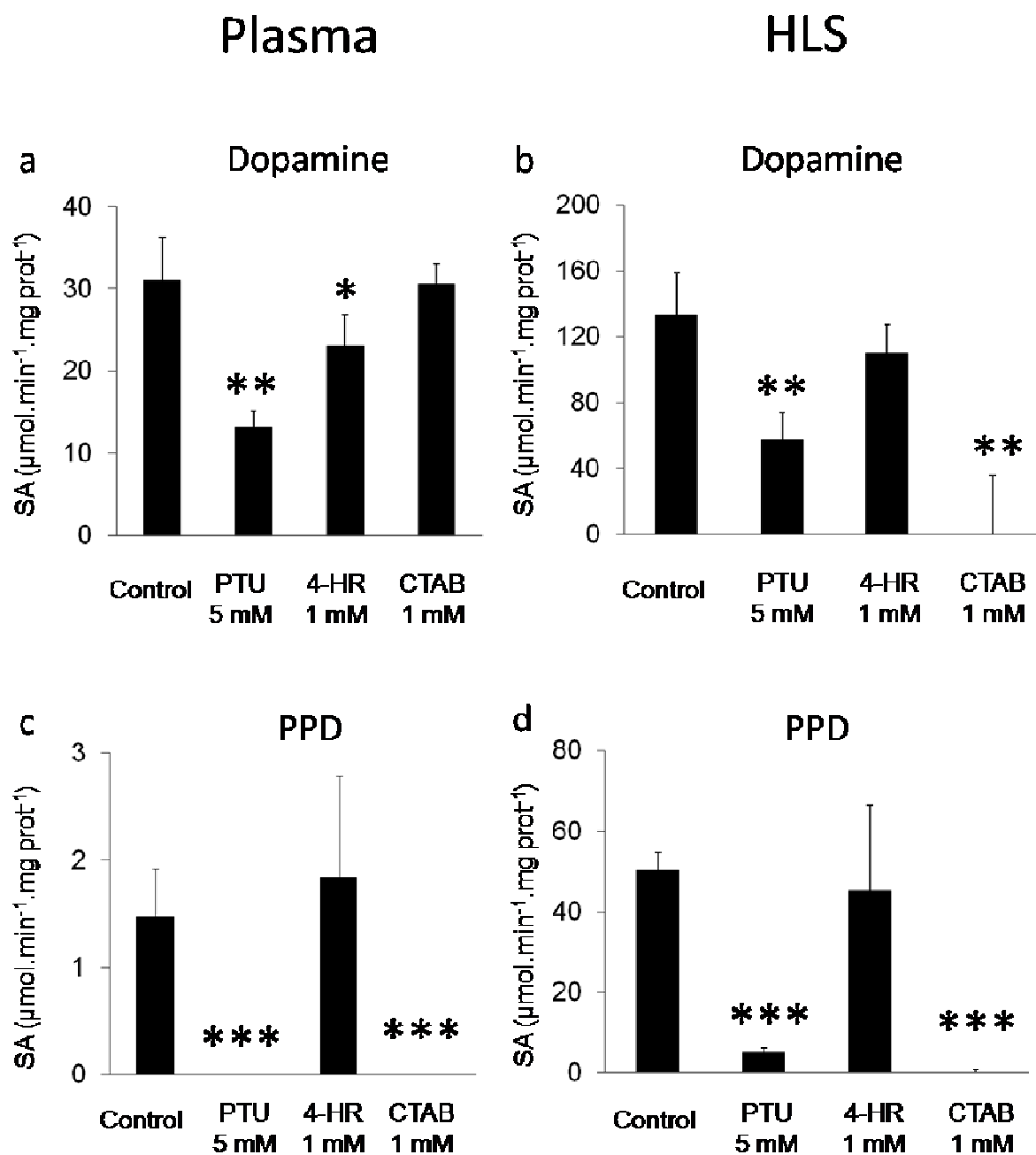
### b Enzymatic oxidation of PPD



583

584

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



590

591 **Table 1**

592 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-HR: 4-  
 593 Hexylresorcinol; SHAM: salicylhydroxamic acid; CTAB: cetyl trimethyl ammonium bromide.

594

595

Inhibitor	Mode of action	Reference
DETC <sup>1,2,3</sup>	Copper chelation (competitive inhibition)	[16, 55]
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, 56]
Tropolone <sup>1,2,3</sup>	Substrate of peroxidases and inhibitor of POs (copper chelation)	[35, 56]
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	[57, 58]
NaN <sub>3</sub> <sup>1,2,3</sup>	Metal chelator: inhibitor of all types of POs	[35, 59]
4-HR <sup>1,2</sup>	Fixation on the active site: competitive inhibitor of tyrosinases and catecholases but not of laccases	[59, 60]
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, 60]
Kojic acid <sup>1,2,3</sup>	Competitive or mixed-type inhibitor of POs	[35, 37, 41, 46]
CTAB <sup>3</sup>	Cationic detergent: competitive or non competitive inhibitor of laccases, but not of other POs	[42-44]

596

597 <sup>1</sup> Tyrosinase inhibitor598 <sup>2</sup> Catecholase inhibitor599 <sup>3</sup> Laccase inhibitor

600 **Table 2**

601 Identification of phenoloxidase-like activity in plasma of *Crassostrea gigas* by using a panel of substrates.  $\emptyset$ , no  
 602 PO-like activity detected.

603

Type of substrate	Substrate	$\lambda$ (nm) <sup>4</sup>	Final substrate concentrations tested (mM)	Substrate saturating concentration (mM)	$Km_{app}$ (mM)	$Vm_{app}$ ( $\Delta A \text{ min}^{-1} \cdot 10^{-3}$ )
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	$\emptyset$	$\emptyset$	$\emptyset$
<i>o</i> -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

604

605 <sup>1</sup> Tyrosinase substrate, in Tris buffer606 <sup>2</sup> Catecholase substrate, in Tris buffer607 <sup>3</sup> Laccase substrate, in methanol608 <sup>4</sup> Wavelengths used to measure by spectrophotometry the formation of each *o*-quinone derivative