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

2

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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 O₂ 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: O₂ oxidoreductase, EC
62 1.14.18.1), catecholases (*o*-diphenol: O₂ oxidoreductase, EC 1.10.3.1), and laccases (*p*-
63 diphenol: O₂ oxidoreductase, benzenediol: O₂ 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 ± 8.7 g; length: 9 ± 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 μ 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 MgCl₂ and 10 mM CaCl₂) 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 μ l) were stored at -80°C. Each aliquot was used only once.

116

117

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 (NaN₃), diethyldithiocarbamate (DETC), 1-
125 phenyl-2-thiourea (PTU), trizma hydrochloride (Tris HCl), sodium chloride (NaCl),
126 ammonium sulphate ((NH₄)₂SO₄) and catalase from bovine liver were obtained from Sigma-
127 Aldrich (France). 2-mercaptoethanol (2-ME) was obtained from MERCK (France).
128 Magnesium chloride (MgCl₂) and calcium chloride (CaCl₂) 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 MgCl₂ and 10 mM CaCl₂) 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 μ l
146 of sample were incubated with 7 μ l of PPD and 123 μ 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 μ 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 μ l of PO inhibitor (prepared at
160 various concentrations in Tris buffer, Fig. 3) with 10 μ 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).

166

167

168 2.7. *Hydrogen peroxide scavenging by exogenous catalase*

169 Plasma (10 μ l) was preincubated at 25°C for 30 minutes in the presence of 10 μ l of catalase
170 from bovine liver at 1000 U/ml [25]. The total scavenging of H₂O₂ 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 μ 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.

179

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.

183

184 2.9. *Measurements of oxygen uptake*

185 Oxygen uptake was followed with a Clark-type oxygen electrode (Hansatech, DW1) in a
186 700- μ 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 μ l of buffer was distributed in the chamber.
189 In the second unit ('sample control'), 250 μ l of plasma and 450 μ l of buffer were distributed.
190 In the third unit ('non-enzymatic control'), 700 μ l of substrate (L-DOPA 10 mM or dopamine
191 100 mM) were distributed. In the fourth unit, 250 μ l of plasma and 450 μ l of substrate were
192 distributed. With PPD (50 mM) as substrate, the same protocol was adopted with slight

193 modifications, i.e. 500 μ l of the sample were incubated with 35 μ l of PPD and 165 μ 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- μ 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 μ 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. O₂ 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 O₂ 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 H₂O₂ 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 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 O₂. 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), 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 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], NaN_3 [35], ammonium tetramolybdate [41], SHAM [35],
325 kojic acid [35] and CTAB [42-44]. We did not use *N*-hydroxyglycine because, at μM
326 concentrations, *N*-hydroxyglycine was shown to bleach solutions of substrates oxidized either
327 chemically or enzymatically by laccase [45]. For 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

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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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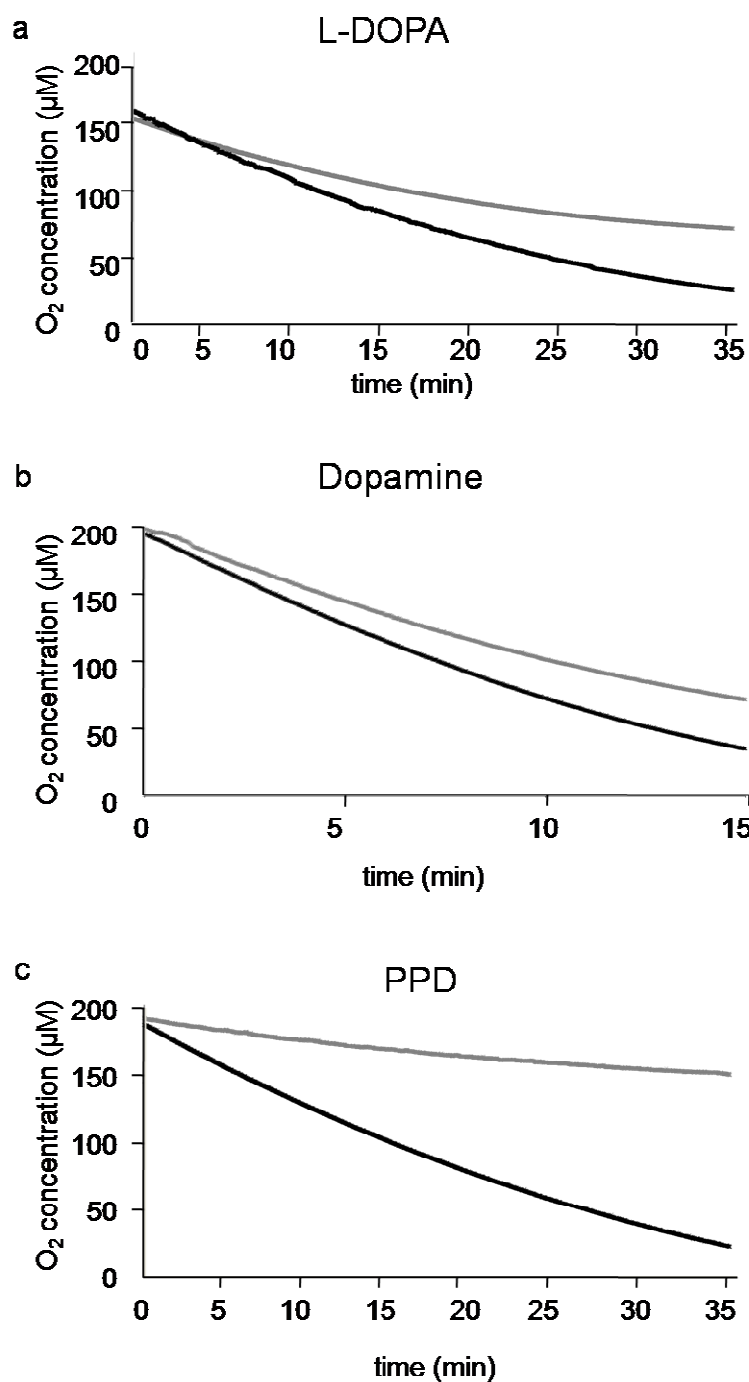
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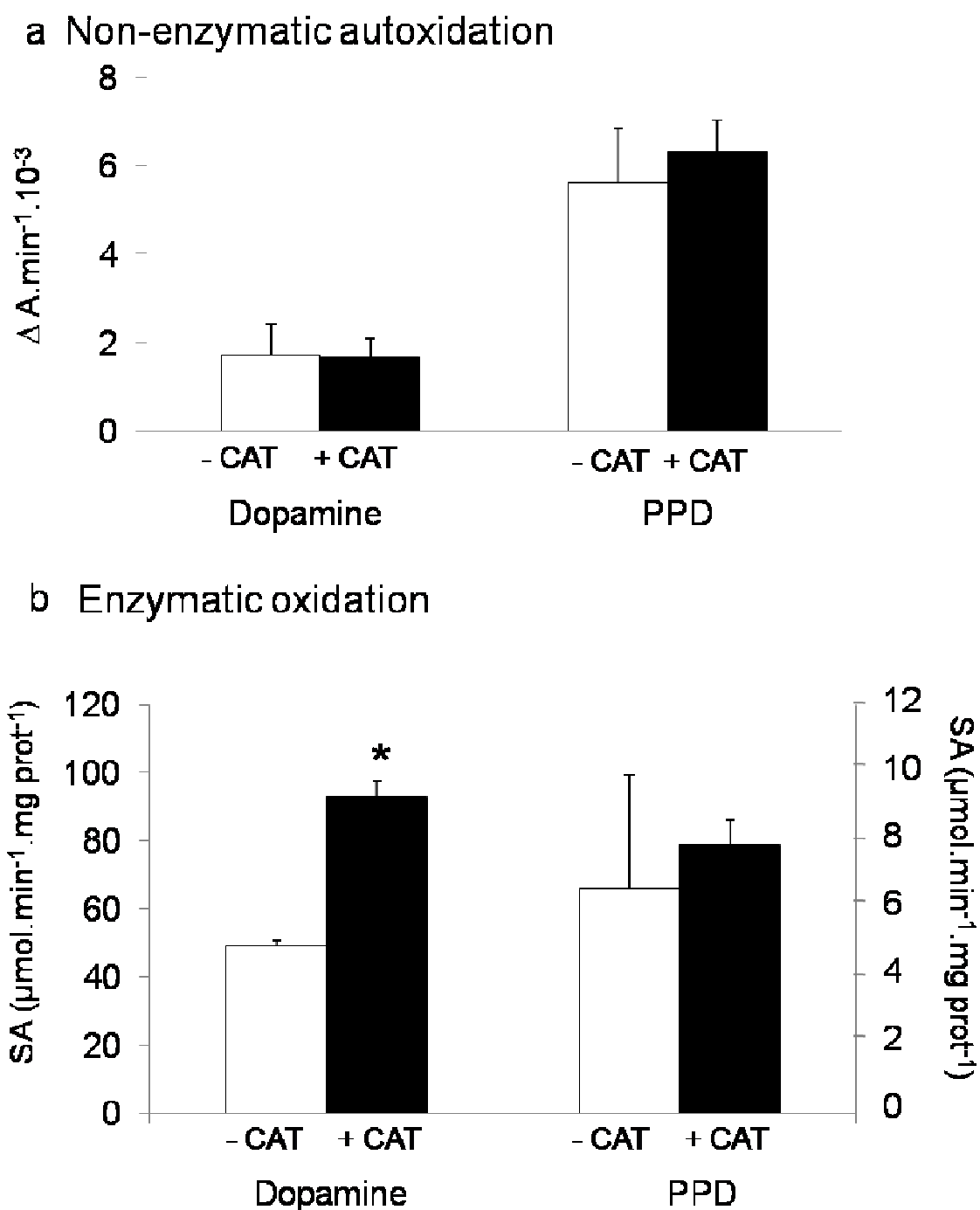
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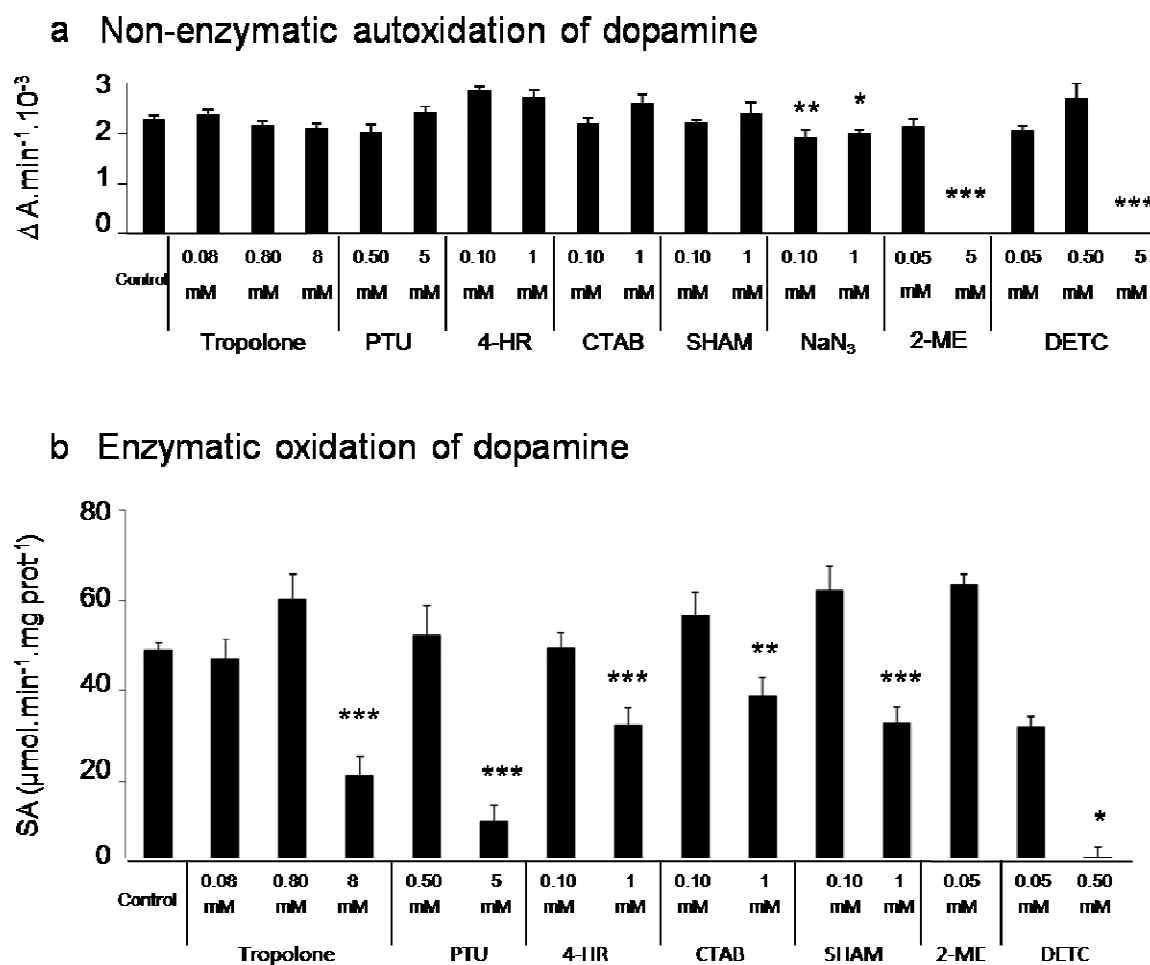
540 **Fig. 1** Oxygen uptake during oxidation of PO substrates. Non-enzymatic (- plasma, gray
541 lines) and enzymatic (+ plasma, black lines) oxidation reactions were followed using
542 oxygraphy with the substrates: (a) L-DOPA 10 mM, (b) dopamine 100 mM, and (c) PPD 50
543 mM. Experiments were repeated 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 $p < 0.05$.



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.

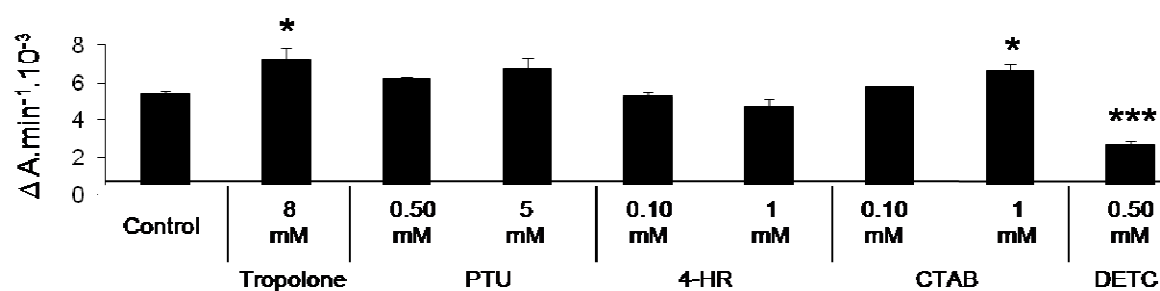


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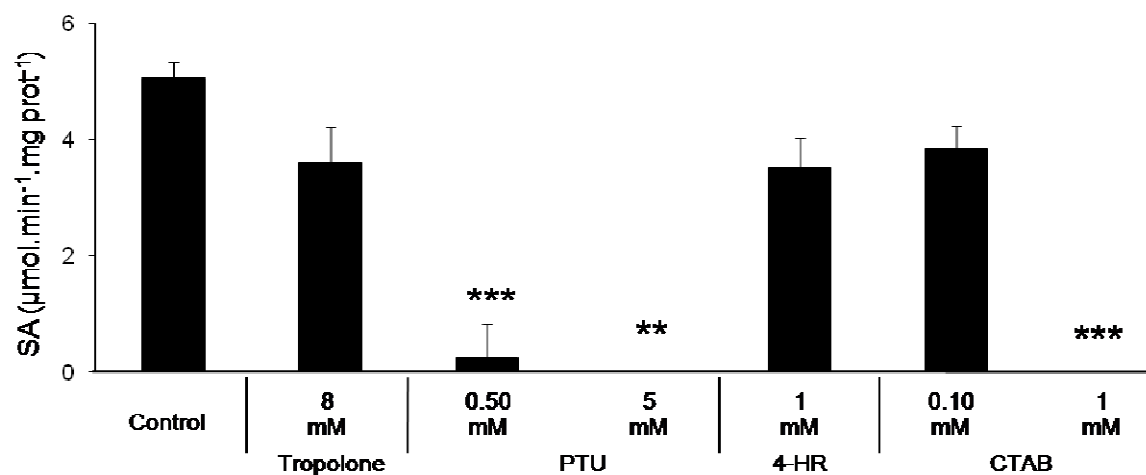
577

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



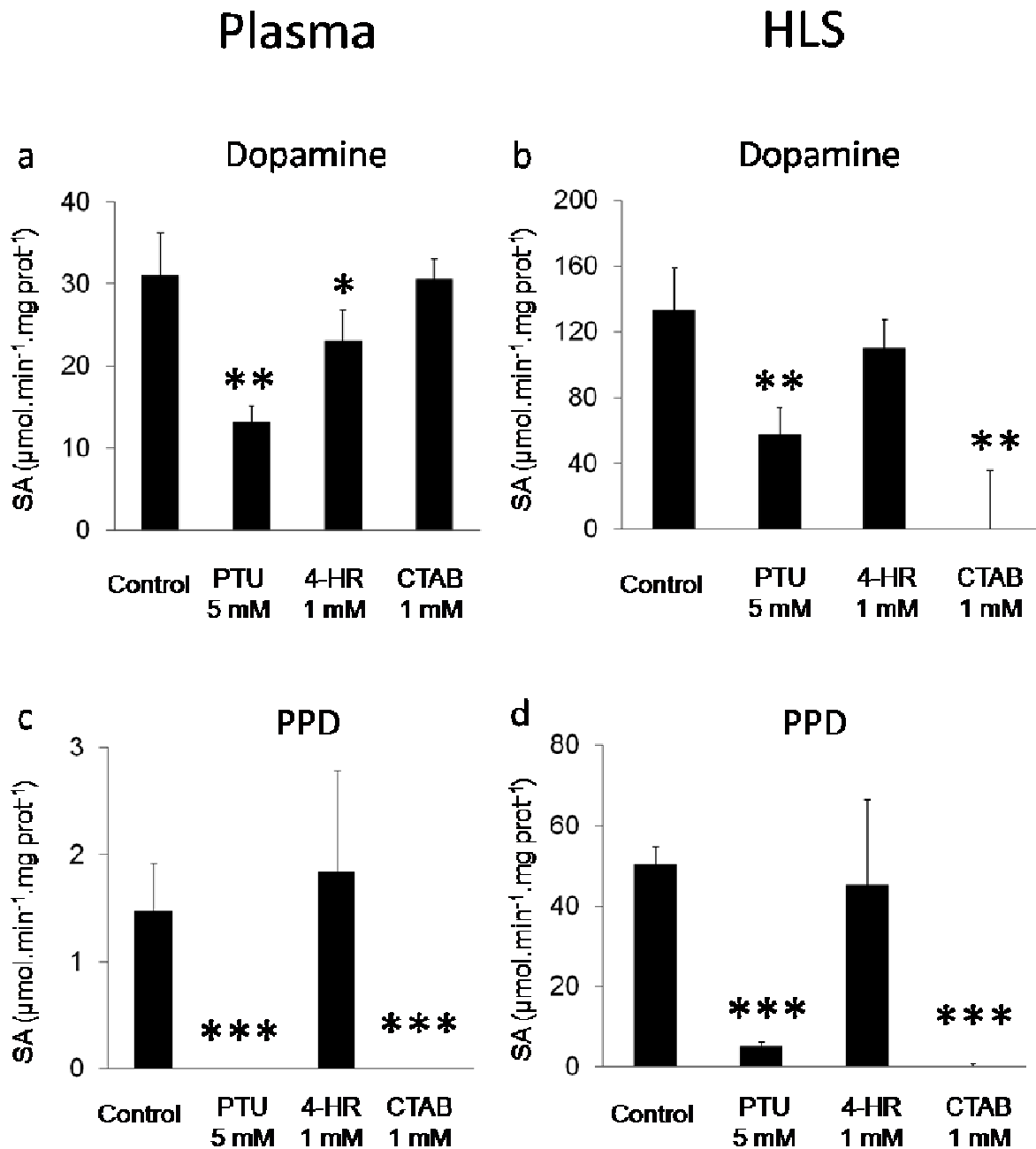
b Enzymatic oxidation of PPD



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 prot^{-1} , $n = 9$, *statistical difference for $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, respectively.



591 **Table 1**

592 Phenoloxidase-like inhibitors and modes of action. DETC: diethyldithiocarbamate; PTU: 1-phenyl-2-thiourea; 2-ME: 2-mercaptoethanol; NaN₃: sodium azide; 4-HR: 4-
 593 Hexylresorcinol; SHAM: salicylhydroxamic acid; CTAB: cetyl trimethyl ammonium bromide.

594

595

| Inhibitor | Mode of action | Reference |
|-----------------------------------|--|------------------|
| DETC ^{1,2,3} | Copper chelation (competitive inhibition) | [16, 55] |
| PTU ^{1,2,3} | Copper chelation (competitive inhibition): sulphur binds to copper at the active site of the enzyme, blocking accessibility of the substrate | [39, 56] |
| Tropolone ^{1,2,3} | Substrate of peroxidases and inhibitor of POs (copper chelation) | [35, 56] |
| 2-ME ^{1,2,3} | 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 ₃ ^{1,2,3} | Metal chelator: inhibitor of all types of POs | [35, 59] |
| 4-HR ^{1,2} | Fixation on the active site: competitive inhibitor of tyrosinases and catecholases but not of laccases | [59, 60] |
| SHAM ^{1,2} | 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 ^{1,2,3} | Competitive or mixed-type inhibitor of POs | [35, 37, 41, 46] |
| CTAB ³ | Cationic detergent: competitive or non competitive inhibitor of laccases, but not of other POs | [42-44] |

596

597 ¹ Tyrosinase inhibitor598 ² Catecholase inhibitor599 ³ 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 | λ (nm) ⁴ | Final substrate concentrations tested (mM) | Substrate saturating concentration (mM) | Km_{app} (mM) | Vm_{app} ($\Delta A \text{ min}^{-1} \cdot 10^{-3}$) |
|--------------------------------------|----------------|-----------------------------|--|---|-----------------|--|
| Monophenol ¹ | 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 ^{1,2,3} | 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 ³ | Syringaldazine | 525 | 0.01, 0.1, 1 | \emptyset | \emptyset | \emptyset |
| Non-phenolic substrates ³ | ABTS | 420 | 1, 2, 3, 4, 5 | \emptyset | \emptyset | \emptyset |
| | PPD | 420 | 5, 10, 25, 50, 100 | 50 | 45 | 0.59 |

604

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