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

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

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

1. Introduction

Phenoloxidases are a family of copper proteins, widely distributed in microorganisms, plants and animals [1, 2]. They are the rate limiting enzymes in enzymatic browning in fruits and vegetables, and in melanization in animals. Melanin production starts with the oxidation of phenols and the concomitant reduction of O_2 to water. This reaction is catalysed by POs and yields to corresponding quinones, which are then polymerized by non-enzymatic reactions toward the formation of melanin [3]. Melanin and intermediates are toxic substances with fungistatic, bacteriostatic and antiviral properties [4]. In invertebrates, PO enzymes are also involved in many cellular defence responses, such as self/non-self recognition, phagocytosis and nodule and capsule formation [4, 5]. Interestingly, similarities of the PO system have been drawn with other cascades involved in defence such as the *Drosophila*-Toll cascade and the mammalian complement and blood clotting [6].

A major constraint when studying POs is the ambiguity of nomenclature existing in the literature. POs include tyrosinases (monophenol, *o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1), catecholases (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1), and laccases (*p*-diphenol: O_2 oxidoreductase, benzenediol: O_2 oxidoreductase, EC 1.10.3.2). However, tyrosinases and POs, and tyrosinases and catecholases have been used in the literature as synonyms [7, 8], and tyrosinases and POs are given the same EC number even if they are not obviously the same. POs are capable of *o*-diphenol oxidation. However, among these three enzymes, only tyrosinases can hydroxylize monophenols (e.g. L-tyrosine) and only laccases can oxidise *p*-diphenols and aromatic amines (e.g. *p*-phenylenediamine) [9, 10]. In addition to that, various compounds have been described as inhibitors of these three types of POs with their respective specificity (Table 1).

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

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

2. Materials and methods

2.1. Oysters

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

2.2. Collection of plasma

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

2.3. Hemocyte lysate supernatant

Hemocytes were homogenized at 4°C in Tris buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) adjusted to pH 7. They were lysed using an Ultra-Turrax (T25 basic, IKA-WERKE) at 19 000 rpm for 30 sec and a Thomas-Potter homogenizer (IKA-Labortechnik, clearance 0.13-0.18mm) at 200 rpm for 1 min, and centrifuged at 10 000 x g for 10 min at 4°C. The resulting hemocyte lysate supernatant (HLS) was collected for enzymatic studies. Aliquots (100 µl) were stored at -80°C. Each aliquot was used only once.

2.4. Chemicals

L-tyrosine, *p*-hydroxyphenyl propionic acid (PHPPA), 4-hydroxyanisole (4-HA), L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenyl propionic acid (DHPPA), catechol, dopamine, *p*-phenylenediamine (PPD), 4-Hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tropolone, 4-hexylresorcinol (4-HR), cethyltrimethylammonium bromide (CTAB), salicylhydroxamic acid (SHAM), sodium azide (NaN_3), diethyldithiocarbamate (DETC), 1-phenyl-2-thiourea (PTU), trizma hydrochloride (Tris HCl), sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) and catalase from bovine liver were obtained from Sigma-Aldrich (France). 2-mercaptoethanol (2-ME) was obtained from MERCK (France). Magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) were obtained from Acros (France).

2.5. Phenoloxidase assays

Phenoloxidase-like (PO-like) activity has been reported to be higher in plasma than in HLS from *C. gigas* [23]. Therefore, constitutive PO-like activity was first analyzed in crude plasma. PO-like activity was measured spectrophotometrically by recording the formation of *o*-quinones. The method of Asokan et al. [5] was used with some modifications. Working solutions of substrates were prepared just before use in Tris buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) adjusted to pH 7, except for PPD which was prepared in methanol. The latter did not affect PO-like activities in the conditions tested (data not shown). Samples were distributed in 96-well microplates (Nunc, France). Ten microliters of sample were incubated with 80 µl of substrate and 50 µl of Tris buffer at 25°C. Several control wells were systematically used: ‘buffer control’ containing only buffer, ‘sample control’ containing only sample and buffer, and ‘non-enzymatic control’ containing only substrate and buffer. Immediately after substrate addition, PO-like activity was monitored during 4h by following the increase of absorbance at a specific wavelength (Table 2). Because of solubility constraints, in the case of PPD, the protocol was slightly modified: 10 µl of sample were incubated with 7 µl of PPD and 123 µl of buffer and PO-like activity was monitored during 2h. For all conditions, experiments were performed with three oyster pools. Each pool was tested in triplicate wells and average rates were calculated. For non-enzymatic oxidation, results were expressed as the mean value of the increment of absorbance per minute ($\Delta A\ min^{-1}$). For enzymatic oxidation, results were systematically corrected for non-enzymatic autoxidation of the substrate. Specific activities (SA) were expressed in international units (IU) per mg of total protein. One IU is defined as the amount of enzyme that catalyzes the appearance of 1 µmole of product per min [24].

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

2.6. Phenoloxidase inhibition assay

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

2.7. Hydrogen peroxide scavenging by exogenous catalase

Plasma (10 µl) was preincubated at 25°C for 30 minutes in the presence of 10 µl of catalase from bovine liver at 1000 U/ml [25]. The total scavenging of H_2O_2 was verified using the Catalase kit CAT-100 (Sigma) and specifications included (data not shown). Then, PO assay was carried out with dopamine (100 mM) or PPD (50 mM). The effect of catalase on non-enzymatic autoxidation was also followed by incubating (25°C, 30 min) the substrates (dopamine or PPD at 100 mM or 50 mM, respectively) in the presence of 10 µl of catalase. Enzymatic oxidation (in the presence of catalase) was systematically corrected for autoxidation of the substrate (in the presence of catalase). All the experiments were performed with three oyster pools. Each pool was tested in triplicate wells and average rates were calculated.

2.8. Protein determination

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

2.9. Measurements of oxygen uptake

Oxygen uptake was followed with a Clark-type oxygen electrode (Hansatech, DW1) in a 700-µl closed chamber thermostatted at 25°C with continuous stirring [27]. In a typical experiment, oxygen uptakes were recorded simultaneously using four separate electrode units. In the first unit (‘buffer control’), a volume of 700 µl of buffer was distributed in the chamber. In the second unit (‘sample control’), 250 µl of plasma and 450 µl of buffer were distributed. In the third unit (‘non-enzymatic control’), 700 µl of substrate (L-DOPA 10 mM or dopamine 100 mM) were distributed. In the fourth unit, 250 µl of plasma and 450 µl of substrate were distributed. With PPD (50 mM) as substrate, the same protocol was adopted with slight modifications, i.e. 500 µl of the sample were incubated with 35 µl of PPD and 165 µl of buffer. All the experiments were carried out with three oyster pools.

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

Plasma and HLS were precipitated overnight with 60% saturated $(NH_4)_2SO_4$ solution at 4°C. After centrifugation at 10 000 x g for 10 min at 4°C, the precipitate was dissolved in 1 ml and dialyzed against Tris buffer. Partially purified fractions from plasma and hemocyte lysate supernatant were filtered through a 0.22-µm sterile filter (Millipore membrane-Millipore Co., Bedford, MA, USA), in order to eliminate the natural bacterial flora of samples. In order to make certain the absence of bacteria after this treatment, the samples were incubated with 4.0 ml of Zobell medium (4 g peptone, 1 g yeast extract, 0.1 g ferric phosphate, 30 g sea salt per liter) and grown at 25°C with shaking to allow potential bacterial growth. Then, A_{620nm} readings were carried out at 0, 5 and 6 h, which evidenced the absence of bacterial growth (data not shown). Aliquots (100 µl) of the dialyzates were stored at -80°C before being tested for PO-like activity.

2.11. Statistical analysis

All values are reported as mean ± standard deviation (SD). Statistical analysis was carried out with SYSTAT 11.0. Values were tested for normality (Shapiro test) and homogeneity of variances (Bartlett test). For normal values, an ANOVA test was used to analyse the results, followed by a Dunnett post-hoc test. For non normal values, a Kruskal-Wallis test was used, followed by a Dunn's multiple comparisons test [28]. Statistical significance was designed as being at the level of $p < 0.05$, $p < 0.01$ or $p < 0.001$.

3. Results

3.1. Substrate specificity of PO-like activity in plasma

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

3.2. O_2 *requirements of PO-like activity*

Using oxygraphy, we easily confirmed the non-enzymatic autoxidation of L-DOPA, dopamine, and to a lesser extent, of PPD (Fig. 1). Most importantly, we found that O_2 uptake was higher in the presence of plasma, independently of the substrate, confirming the presence of at least one PO-type oxidase in plasma.

3.3. Effect of catalase

Exogenous catalase was used to scavenge the H_2O_2 potentially involved in peroxidase-dependent oxidation reactions. Fig. 2a shows that catalase did not affect autoxidations of dopamine and PPD. Most importantly, catalase did not inhibit oxidations of both substrates in the presence of plasma (Fig. 2b). Fig. 2b also shows that catalase induced a two-fold increase of PO-like activity with dopamine as substrate.

3.4. Effect of various PO inhibitors

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

Results with dopamine are summarized in Fig. 3. Since many inhibitors are reducing agents, we systematically examined the effects of PO inhibitors on the non-enzymatic autoxidation. Autoxidation was reduced by using NaN_3 at 0.1 and 1 mM, and suppressed with 2-ME and DETC at 5 mM (Fig. 3a). These compounds were therefore not used at these concentrations for further studies. Moreover, enzymatic oxidation (in the presence of plasma and PO inhibitors) was systematically corrected for non-enzymatic autoxidation of the substrate (in the presence of PO inhibitors). Fig. 3b shows that enzymatic oxidation was strongly inhibited by 0.5 mM DETC and 5 mM PTU (94 and 77% inhibition, respectively), and also significantly inhibited by 8 mM tropolone, 1 mM SHAM, and 1 mM CTAB (56, 33, and 21% inhibition, respectively). The catecholase inhibitor 4-HR (1 mM) exerted 34 % inhibition.

Results with PPD as substrate are summarized in Fig. 4. Autoxidation was suppressed by DETC (0.5 mM, Fig. 4a). Therefore, DETC was not used for further studies. Tropolone (8 mM) and the laccase inhibitor CTAB (1 mM) only slightly interfered (stimulation) with the autoxidation of PPD. Since CTAB is the better documented inhibitor of laccase, we decided to maintain it in the study. Interestingly, Fig. 4b shows that enzymatic oxidation was strongly inhibited by CTAB (1 mM). Moreover, the PO inhibitor PTU (0.5 and 5 mM) exerted 100% inhibition. Taken together, these results confirm the presence of a PO-like activity in *C. gigas* and suggest the presence of a catecholase-like and/or a laccase-like activity in plasma.

3.5. PO-like activity in protein fractions

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

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

4. Discussion

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

Oxidation catalyzed by POs requires O_2. However, PO substrates are also readily autoxidized in contact with air [15, 29]. Therefore, a special attention should be paid to substrate autoxidations before studying PO activity. Using both spectrophotometry and oxygraphy, we confirmed that L-DOPA, dopamine, and to a lesser extent PPD, could be readily autoxidized. These non-enzymatic oxidation reactions probably involve quinone redox cycling leading to the formation of different types and quantities of oxygen radicals and quinone-derived products [30].

Another constraint for studying PO is the possible interference between PO inhibitors and non-enzymatic autoxidation. For instance, the PO inhibitor 2-ME is also a well-known reducing agent (Table 1), that may react with the substrate and/or the quinone intermediates derived from the autoxidation reaction. We systematically examined the effects of various PO inhibitors on substrate autoxidations. We found that 2-ME (5 mM), NaN_3 (0.1-1 mM) and DETC (5 mM) interfered with dopamine autoxidation, and that DETC (0.5 mM) interfered with PPD autoxidation. 2-ME probably acts as a reducing agent while NaN_3 and DETC might possibly act as direct free radical scavengers [31, 32]. These inhibitors (at the concentrations used) should therefore be avoided for identifying PO activity.

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

Most of the PO inhibitors listed in Table 1 are copper chelators and constitute therefore potential catecholase and laccase inhibitors [34-37]. Accordingly, we found that PO-like activity from plasma was inhibited by PTU, DETC, and to a lesser extent, by SHAM and tropolone. PTU was previously described as an inhibitor of tyrosinases and catecholases [38] but also as an inhibitor of laccases [25, 39]. It contains a sulphur compound that binds copper at the active site of catecholase [40]. We found that PTU strongly inhibited dopamine and PPD oxidation suggesting that it can inhibit both catecholase and laccase. To the best of our knowledge, the following chemical products have been reported in the literature as laccase inhibitors : *N*-hydroxyglycine [35], NaN_3 [35], ammonium tetramolybdate [41], SHAM [35], kojic acid [35] and CTAB [42-44]. We did not use *N*-hydroxyglycine because, at µM concentrations, *N*-hydroxyglycine was shown to bleach solutions of substrates oxidized either chemically or enzymatically by laccase [45]. For NaN_3 (Fig. 2) and ammonium tetramolybdate (data not shown), an effect was observed on the autoxidation of, at least, one laccase substrate. SHAM and kojic acid are PO inhibitors but not laccase specific [37, 46]. Therefore, although CTAB is also known as a cationic detergent, it appeared to be the most pertinent laccase inhibitor. Indeed, CTAB was the only molecule reported as a specific inhibitor of laccase but not other phenoloxidases [42-44]**,** and we confirmed that it did not affect autoxidation of laccase substrates.

Several difficulties are encountered when identification of a PO-like activity is performed in a non purified or in a partially purified tissue homogenate because substrates used by PO may be used by (i) peroxidases (ii) hemocyanins, (iii) cytochrome oxidases (EC 1.9.3.1) and (iv) ceruloplasmines or ferroxidases (EC 1.16.3.1). Oxygraphic data showed the involvement of true oxidase activities in plasma (Fig. 1). The involvement of peroxidases [47] was excluded since exogenous catalase did not inhibit dopamine and PPD oxidation activities. It should be noted that, with dopamine as substrate, catalase induced a two-fold increase of PO-like activity. This could be explained by the generation of H_2O_2 as an auto-inhibitor of PO during dopamine oxidation [48]. Hemocyanins, cytochrome oxidases, and ceruloplasmins are absent in the plasma and in the HLS obtained from *C. gigas* [49-52]. Therefore, only PO-like activity was detected in crude plasma.

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

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

POs are an important component in immune defence mechanisms in bivalves. For example, the importance of phenoloxidase activity in the resistance to *M. sydneyi* has been reported in *S. glomerata* [17]. Besides, the presence of laccases has previously been evoked in molluscs [16, 53]. Moreover, a gene encoding a laccase was recently identified from Pacific oyster, *C. gigas*, hemocytes (Faury and Renault, pers. comm.) and its total sequence deposited in GenBank under accession nº NCBI ID: EU678320. This gene was shown to be over-expressed in the presence of polyaromatic hydrocarbons, suggesting a potential use of laccase as a biomarker of pollution exposure [54]. In this context, the present study demonstrates, for the first time through the use of a panel of POs substrates and inhibitors, that a laccase-like activity is present in a mollusc species, the Pacific oyster, *C. gigas.* A better characterization of laccase and/or catecholase systems would help to extend our knowledge on immune defence mechanisms in *C. gigas,* and thus, would improve our ability to monitor and manage the production and survival of this important species.

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

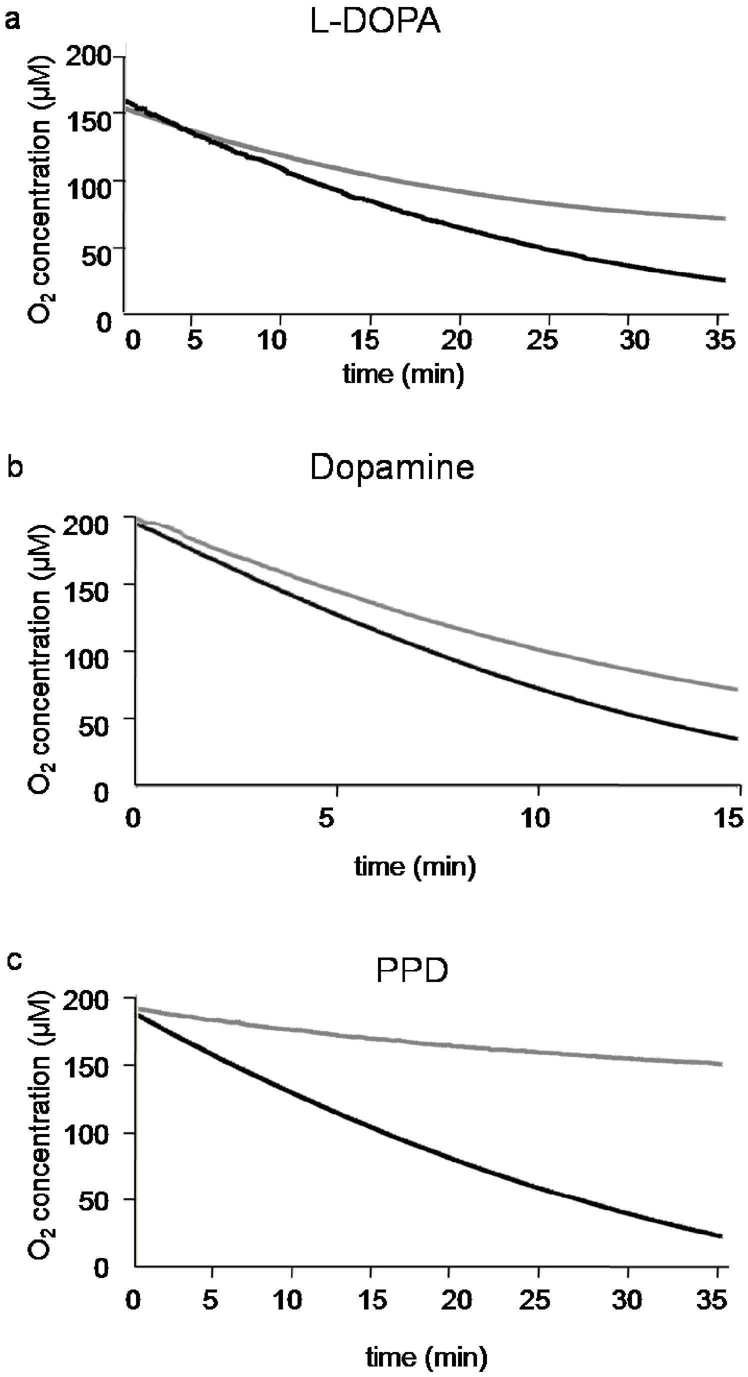
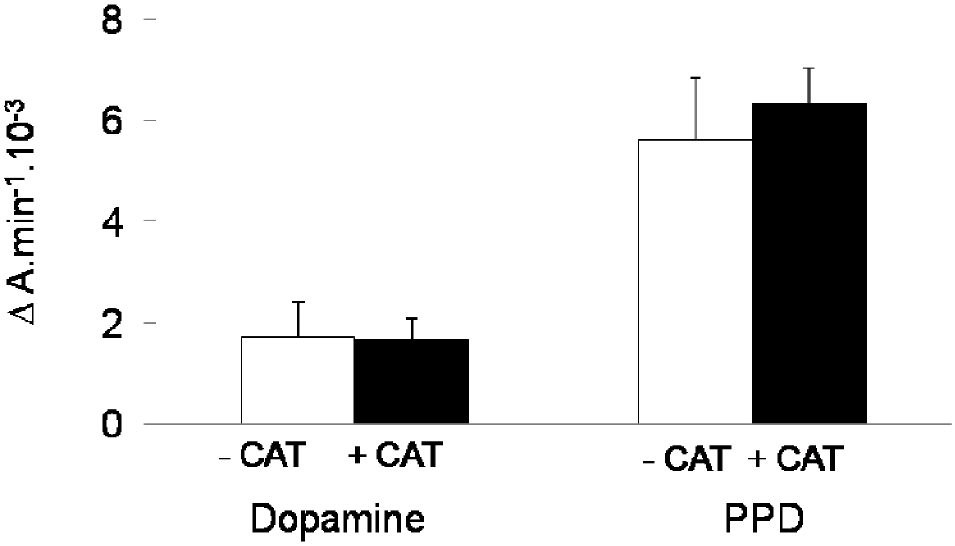


Fig. 2 Effect of catalase on autoxidation (a) and PO-like activity (b). Both dopamine and PPD were used as substrates in the presence (+ CAT) or in the absence (- CAT) of catalase. Left y axis corresponds to results obtained with dopamine +/- CAT and right y axis corresponds to results obtained with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

a Non-enzymatic autoxidation



b Enzymatic oxidation

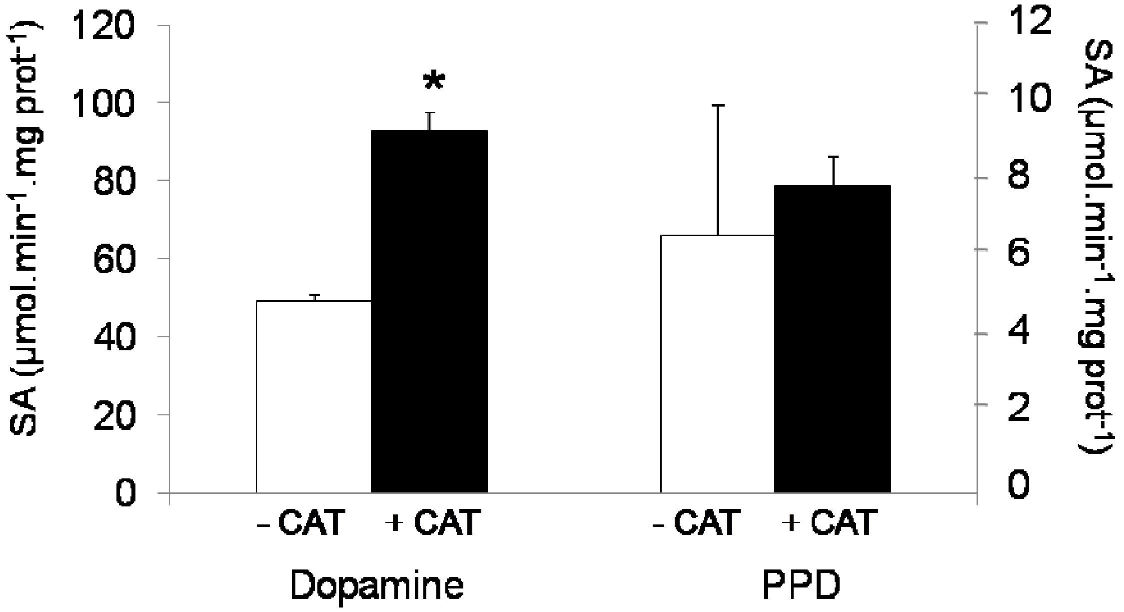
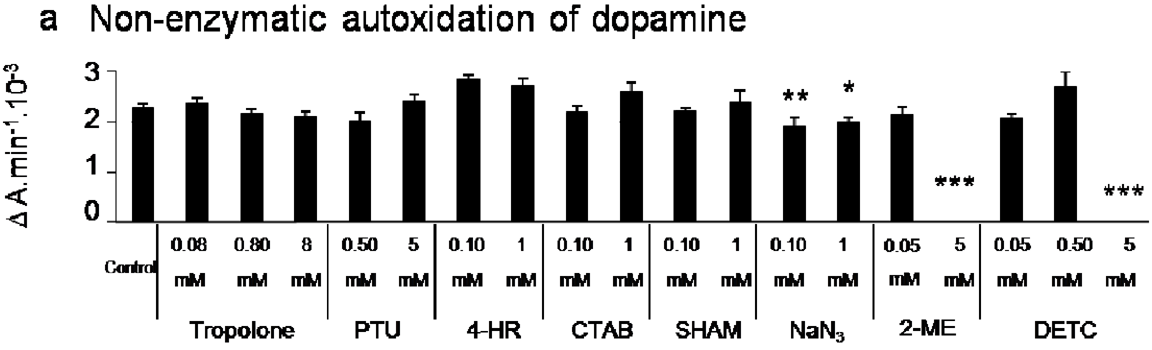


Fig. 3 Effect of inhibitors on autoxidation and enzymatic oxidation of dopamine. (a) Non-enzymatic autoxidation (without plasma). (b) Enzymatic oxidation (with plasma). 'Control' corresponds to the condition without inhibitor. PO inhibitor concentrations correspond to final concentrations in the assay.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


a Non-enzymatic autoxidation of dopamine

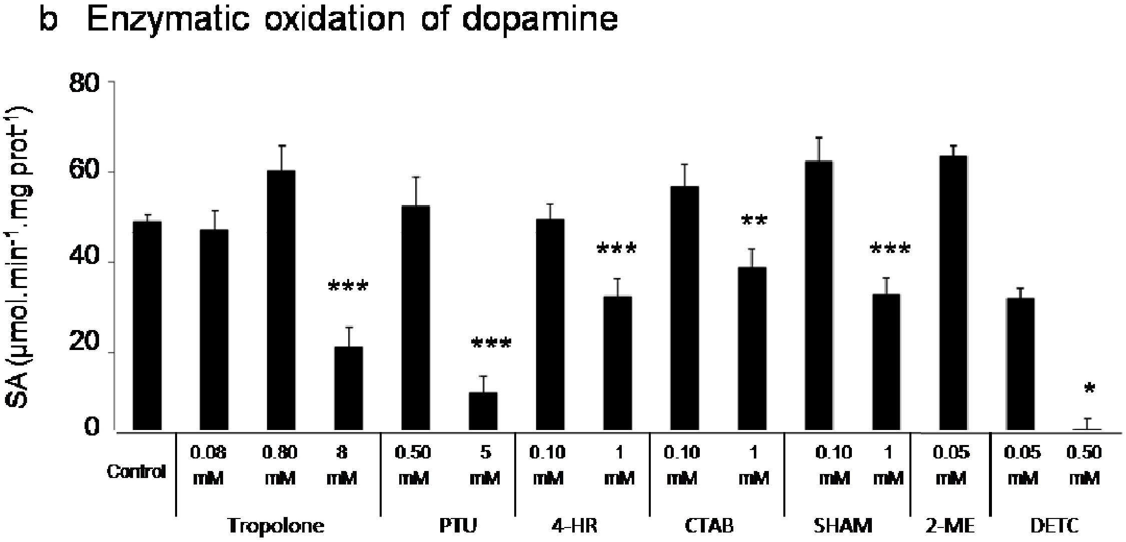
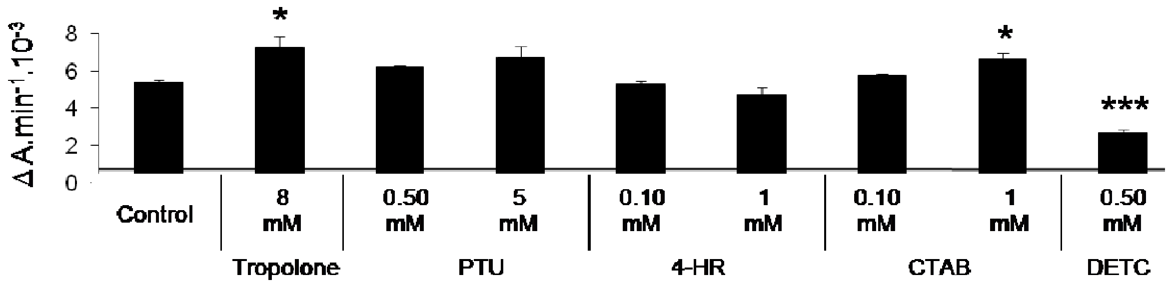

b Enzymatic oxidation of dopamine

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

a Non-enzymatic autoxidation of PPD



b Enzymatic oxidation of PPD

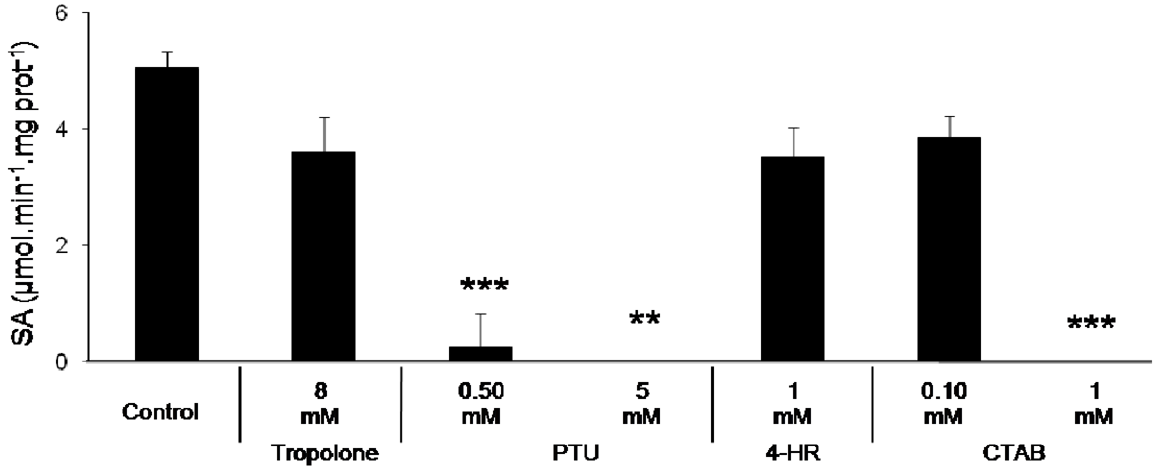


Fig. 5 Inhibition of phenoloxidase-like activity in precipitated protein fractions from plasma and hemocyte lysate supernatant (HLS). Both dopamine (a, b) and PPD (c, d) were used as substrates. ‘Control’ corresponds to the condition without inhibitor. PO inhibitor concentrations correspond to final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

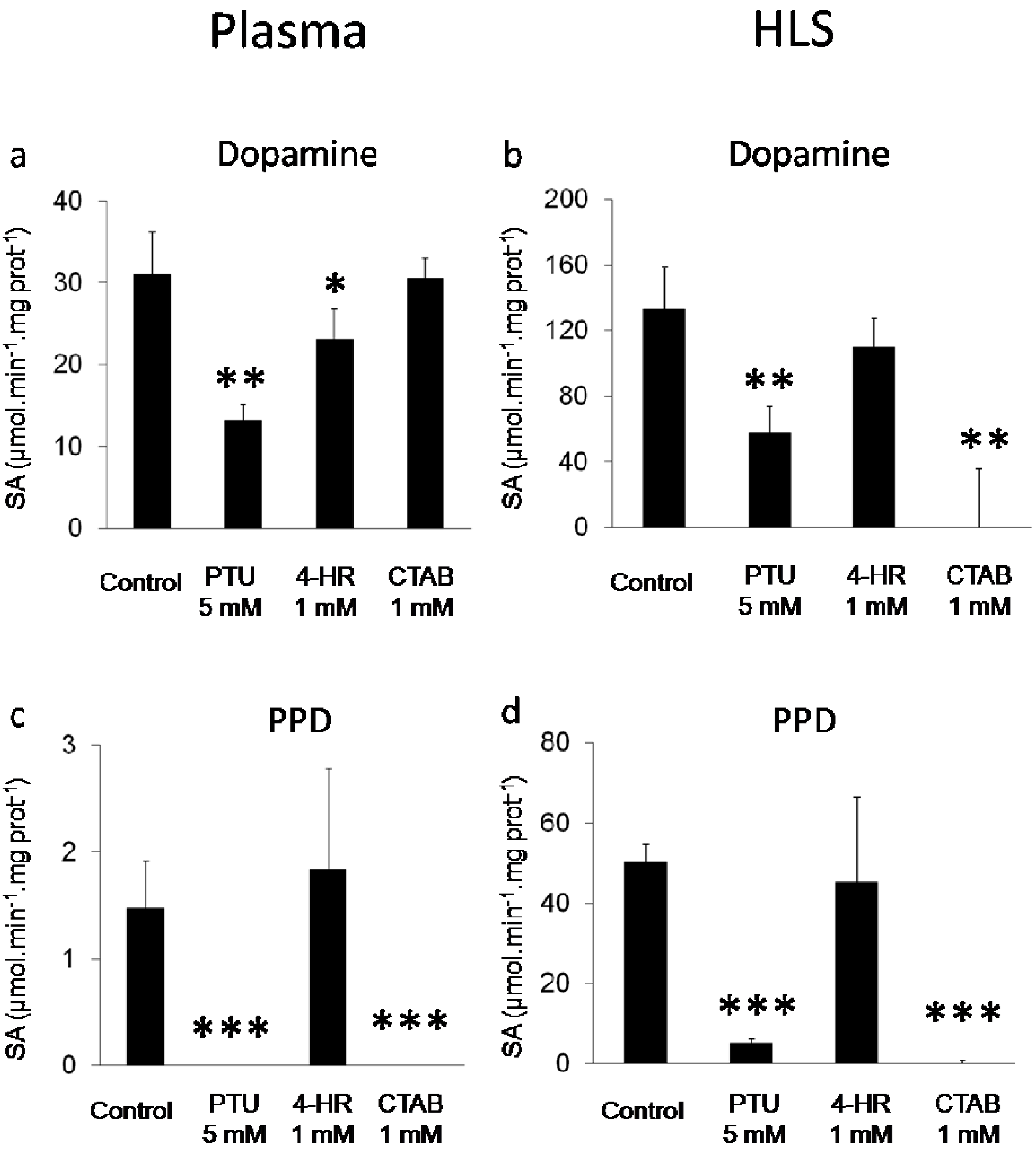


Table 1

Phenoloxidase-like inhibitors and modes of action. DETC: diethyldithiocarbamate; PTU: 1-phenyl-2-thiourea; 2-ME: 2-mercaptoethanol; NaN_3: sodium azide; 4-HR: 4-Hexylresorcinol; SHAM: salicylhydroxamic acid; CTAB: cetyl trimethyl ammonium bromide.

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]

[1] Tyrosinase inhibitor

[2] Catecholase inhibitor

[3] Laccase inhibitor

Table 2

Identification of phenoloxidase-like activity in plasma of *Crassostrea gigas* by using a panel of substrates. ø, no PO-like activity detected.

Type of substrate	**Substrate**	**λ (nm)[4]**	**Final substrate concentrations tested (mM)**	**Substrate saturating concentration (mM)**	***Km*app (mM)**	***Vm*app (ΔA min^{-1}.10^{-3})**
Monophenol[1]	L-tyrosine	490	4, 6, 8, 10, 20	ø	ø	ø
	4-HA	490	4, 6, 8, 10, 20	ø	ø	ø
	PHPPA	490	4, 6, 8, 10, 20	ø	ø	ø
o-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	ø	ø	ø
Metoxi phenol[3]	Syringaldazine	525	0.01, 0.1, 1	ø	ø	ø
Non-phenolic substrates[3]	ABTS	420	1, 2, 3, 4, 5	ø	ø	ø
	PPD	420	5, 10, 25, 50, 100	50	45	0.59

[1] Tyrosinase substrate, in Tris buffer

[2] Catecholase substrate, in Tris buffer

[3] Laccase substrate, in methanol

[4] Wavelengths used to measure by spectrophotometry the formation of each *o*-quinone derivative