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# Evidence of an active laccase-like enzyme in deepwater pink shrimp (*Parapenaeus longirostris*)

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**Abstract**

This paper demonstrates the presence of an active laccase-like enzyme from deepwater pink shrimp (*Parapenaeus longirostris*) using polyacrylamide gel electrophoresis. This enzyme was found in all anatomical parts of the deepwater pink shrimp, but particularly in the cephalothorax, and became active during the course of storage. Gel staining with laccase-specific substrates such as ADA, DMP and DAB was used to characterize a protein of around 44 kDa as containing laccase activity. The enzyme was inhibited by a specific inhibitor, CTAB. 4-Hexylresorcinol, a specific inhibitor of polyphenoloxidase (PPO), did not inhibit the laccase-like enzyme. Low concentrations of antioxidants ascorbic acid or sodium metabisulphite were sufficient to inhibit the laccase-like enzyme. ABTS and DMP were subsequently used to characterize the enzyme. Given the evidence of this enzyme in deepwater pink shrimp, new melanosis-inhibiting compounds that are suitable for consumption need to be found to complement specific inhibitors of PPO activity.


*Keywords:* Crustacean; Laccase; Melanosis; Pink shrimp; Polyphenoloxidase

## 1. Introduction

Polyphenoloxidase is an enzyme found in many plants, some fungi, and animals such as insects, crustaceans, man and others. All polyphenoloxidases (PPOs) are copper-containing proteins which can act on two types of substrate: monohydroxyphenols and dihydroxyphenols. Monophenoloxidases (EC 1.14.18.1) hydroxylate *o*-monohydroxyphenols in *o*-position with respect to the original hydroxyl group, forming dihydroxyphenols. Diphenoloxidases (EC 1.10.3.1) eliminate the hydrogen of the hydroxyl groups from *o*-dihydroxyphenols by oxidation, forming *o*-benzoquinones. The *o*-benzoquinones are highly reactive products and can polymerize spontaneously to form brown pigments (melanins), or react with amino acids and proteins that enhance the brown colour produced. Monophenoloxidases generally also act as *o*-diphenol oxidases, but not all *o*-diphenoloxidases can act as monophenoloxidases (Rivas & Whitaker, 1973).

Laccases, which may be considered a third type of polyphenoloxidase (EC 1.10.3.2), mainly oxidize *p*-isomers (Rescigno et al., 1997). They can also oxidize a wide range of organic and inorganic substances (Flurkey, 2003; Lertsiri, Phontree, Thepsingha, & Bhumiratana, 2003), including *o*-dihydroxyphenols (Barrett, 1991; Cárdenas & Dankert, 2000), but not *o*-monohydroxyphenols (Andersen, 1985). Laccases have been found in fungi, plants, some bacteria (Claus, 2004) and also in arthropods, particularly insects (Ashida & Yamazaki, 1990; Barrett, 1987; Charalambidis, Bournazos, Zervas, Katsoris, & Marmaras, 1994). In another arthropod group, the crustaceans, their presence has only been reported in red swamp crayfish (*Procambarus clarkii*, Cárdenas & Dankert, 2000). However, their presence there could be important given that oxidation of *o*- and *p*-dihydroxyphenols by laccases (Flurkey, 2003; Lertsiri et al., 2003) may complement the oxidizing action of the PPO, causing browning, decreasing consumer product appeal, nutritional value, and marketability. These

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substrates include *p*-diphenols and hydroquinone, which can form in appreciable amounts during storage, through a non-enzymatic reaction based on phenylalanine and tyrosine (Dennell, 1958a, 1958b). Specific PPO inhibitors such as 4-hexylresorcinol do not inhibit laccases (Flurkey, Ratcliff, Lopez, Kuglin, & Dawley, 1995), and therefore, if the latter are present in crustaceans, it may be necessary to add food-grade laccase inhibitors to melanosis-inhibiting formulations.

The main object of this research was to determine the presence of a laccase-like enzyme in deepwater pink shrimp (*Parapenaeus longirostris*), using polyacrylamide gels and laccase-specific inhibitors and substrates for identification. Specific PPO inhibitors and substrates were used to distinguish the two enzymes. A second objective was subsequent characterization of the laccase-like enzyme with two specific substrates, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP).

## 2. Materials and methods

### 2.1. General

Deepwater pink shrimp (*P. longirostris*) were caught off the South coast of Spain (Cádiz) by trawl in November. Mean shrimp weights and average lengths at the time of capture were $6.00 \pm 1.5$ g and $10.8 \pm 0.5$ cm. On board they were separated from the by-catch, washed with seawater, placed in perforated polystyrene boxes (approximately 2 kg per box) and covered with flake ice. One batch was immediately frozen on board using liquid nitrogen. Packages were shipped by refrigerated truck to the laboratory in Madrid (Spain) and 48 h after capture the carapace (exoskeleton of the cephalothorax), cephalothorax (which comprises the visceral content and the first three thoracic segments), exoskeleton of the abdomen, pereopods, pleopods, and telson (including the uropods) were individually sampled and frozen in liquid nitrogen. The different tissues were mixed with dry ice, ground to a fine powder using a coffee mill and stored at –80 °C for enzyme purification.

### 2.2. Preparation of partially purified enzyme

PPO was extracted according to Chen, Rolle, Wei, and Marshall (1991), with slight modifications. One part of ground powder was added to three parts (w/v) of 0.1 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl, 0.2% (w/v) Brij 35 (Panreac química, Barcelona, Spain) and 2% (w/v) poly(vinylpolypirrolidone) (Sigma–Aldrich, St. Louis, USA). The suspension was stirred for 3 h at 2 °C and then centrifuged (Beckman J2-MC Centrifuge, JA-20 Rotor, Beckman Instruments Inc., California, USA) at 27,000*g* for 30 min at 4 °C. The supernatant was filtered through eight layers of muslin, and was fractionated with ammonium sulphate as follows: after centrifugation of the corresponding supernatants at 27,000*g* for 30 min at 4 °C, the pellets precipitating between 0–40% and 40–70% saturation were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) and desalted using PD-10 columns (Sephadex G-25 M, Amersham Pharmacia Biotech, Uppsala, Sweden) at 2 °C. Samples were eluted with 3.5 ml of 0.05 M phosphate buffer solution pH 6.5. The corresponding eluates (designed as semipurified laccase extracts) were mixed with phenylmethyl sulfonyl fluoride (PMSF), as inhibitor of serine and some cysteine proteases (final concentration 18 mg/ml), frozen in liquid nitrogen and stored at –80 °C.

### 2.3. Native and SDS–PAGE (polyacrylamide gel electrophoresis)

The enzymatic extracts (35 μl) from the different anatomical parts were subjected to native PAGE (6% acrylamide) using 1.5 mm thick mini slab gels. Electrophoresis was run at constant voltage (200 V) until the bromophenol blue front begun to run out of the gel. The lanes containing the molecular weight markers were cut and stained with Coomassie Brilliant Blue R-250. The broad molecular weight standard (Amersham Pharmacia Biotech) consisted of urease (trimer, 272 kDa), bovine serum albumin (dimer, 132 kDa; monomer, 66 kDa), chicken egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

The remaining portion of the gels was stained for laccase activity, using different specific substrates. The gels were previously soaked in 20 ml of 0.1 M sodium phosphate buffer pH 7.2 at ambient temperature for 10 min. To detect laccase activity, the gels were soaked in 25 mM 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, USA), 20 mM 2,6-dimethoxyphenol (DMP, Sigma–Aldrich Chemie, Steinheim, Germany) or 25 mM 4-amino-*N*,*N*-diethylaniline sulphate (ADA, Sigma–Aldrich Chemie, St. Louis, USA) according to Rescigno et al. (1997). To detect both mono- and diphenoloxidase activity in the same polyacrylamide gel, 4-amino-*N*,*N*-diethylaniline sulphate (ADA, Sigma–Aldrich, St. Louis, USA) and subsequently 4-*tert*-butyl-catechol (*t*BC, Fluka Chemie, Buchs, Switzerland) were used, according to Rescigno et al. (1997), with slight modifications. The gels were previously soaked in 20 ml of 0.1 M sodium phosphate buffer pH 7.2 at ambient temperature for 10 min. Ten milli liters of freshly prepared ADA (25 mM) in 10 mM HCl were added to the gel. After 8 min the excess solution was poured off, and the gel was rapidly washed with the above buffer. The gel was subsequently soaked briefly in 15 ml of 25 mM *t*BC dissolved in 10 mM acetic acid. The deep blue spots appeared corresponded to mono- and diphenoloxidase activity. The gels were also soaked with the monohydroxyphenol tyrosine (20 mM, previously dissolved in HCl 0.1 M) and the dihydroxyphenols L-3,4-dihydroxyphenylalanine (L-DOPA, 15 mM), 3,4-dihydroxy-*trans*-cinnamate (caffeic acid, 10 mM) and pyrocatechol (catechol, 15 mM). All chemicals were dissolved in 0.1 M phosphate buffer pH 7.2.

Subsequently, the 40–70% fraction from semi-purified extracts of cephalothorax was subjected to non-reducing

SDS–PAGE (12.5% acrylamide). The samples were previously incubated with an equal volume of loading buffer (final SDS concentration was 2% w/v) for 1 h at room temperature. The low molecular weight markers (Amersham Pharmacia Biotech) consisted of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). It was stained with Coomassie Brilliant Blue R-250. The remaining portion of the gels was stained with 25 mM ADA and 15 mM L-DOPA.

Finally, a second electrophoretic run in native conditions was carried out to detect the effect of one or more specific inhibitors on laccase activity, using specific substrates. Semi-purified extract of cephalothorax (40–70%) from shrimps frozen 48 h after capture was used. As specific inhibitors on polyphenoloxidase activity, 20–200 μg/ml 4-hexylresorcinol (Sigma Chemical, St. Louis, USA), and 9 mM tropolone (Fluka Chemie) were used. As specific inhibitors on both polyphenoloxidase and laccase activity, 5–70 μg/ml sodium metabisulphite (Sigma Chemical, St. Louis, USA), and 50–150 μg/ml ascorbic acid (Sigma Chemical) were used. Cetyltrimethyl-ammonium bromide (CTAB, Sigma Chemical, St. Louis, USA) (200–3000 μg/ml) was used as specific inhibitor on laccase activity. Tropolone, sodium metabisulphite or ascorbic acid were added using ADA as substrate. CTAB and 4-hexylresorcinol were added using either ADA or DAB as substrate.

The hypothesis that the appearance of staining in the gels was due to non-specific reactions of proteins to the assayed substrates was ruled out after no reaction was observed to staining of an enzymatically inactive protein – i.e. BSA – subjected to electrophoresis with any of these reagents.

The molecular weight of each band was determined by the 1-D Manager (version 2.0) image analysis and quantification tool (Tecnología para diagnóstico e Investigación, S.A., Spain).

### 2.4. Measurement of laccase activity

To estimate kinetic parameters, soluble fraction in 40–70% ammonium sulphate from shrimps 48 h after mortem was used. Laccase activity was assayed at 30 °C using (a) 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Fluka Chemie, Buchs, Switzerland) and (b) 2,6-dimethoxyphenol (DMP, Sigma Chemical, St. Louis, USA) as substrates as follows.

(a) The assay mixture contained 0.2 mM ABTS and 0.1 M sodium phosphate buffer, pH 7.5. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) for 4 min. (b) The assay mixture contained 0.2 mM DMP and 0.1 M sodium phosphate buffer, pH 6.5. Oxidation of DMP was followed by absorbance increase at 477 nm ($\varepsilon = 14{,}800\ M^{-1}\ cm^{-1}$) for 4 min. As blank, DMP or ABTS dissolved in buffer solution was used. The absorbance was monitored in a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) with a CPS-240 thermostatic controller. Kinetic properties were determined by the Lineweaver-Burk plot using different concentrations of ABTS (0.1–70 μM) or DMP (1–100 μM). All kinetic studies were performed at least three times. All enzyme activity was expressed as units/ml of semipurified extract, considering the unity as μmol of substrate oxidized per minute.

### 2.5. Optimum temperature and thermostability

To estimate optimum temperature, laccase activity was measured at different temperatures between 10 and 60 °C, using either ABTS or DMP as substrates. To estimate thermostability, the semi purified extract was preincubated in sodium phosphate buffer at temperatures between 10–80 °C for 30 min. As blank, phosphate buffer was also incubated at the same temperatures. After cooling in an ice bath, residual activity was determined at 25 °C by addition of the substrate.

Results were expressed as relative activity, who describes enzymatic activity as the percentage of enzymatic activity expressed as $(A/A_{max}) \times 100$, where $A$ indicates the increase in optical density per minute.

### 2.6. Optimum pH and stability

The optimal pH of the enzyme extract was determined at 30 °C by incubating 30 μl of semi purified extract with 970 μl of either 0.2 mM ABTS or DMP as substrates. ABTS and DMP were previously dissolved in 0.1 M buffer solutions at the desired pH values (2.5–9.5). Glycine (pH 2–3), Mc Ilvaine's citrate–phosphate (3.5–6) and sodium phosphate (6.5–9) were used as buffer solutions. As blank, DMP or ABTS dissolved in buffer solution was used.

To determine the influence of pH on the stability of the enzyme, 30 μl of semi purified extract were incubated with 900 μl of buffers solutions at the desired pH values (2–9.5) at 25 °C for 30 min. As blanks, 30 μl of 0.1 M sodium phosphate buffer were also incubated at the same pHs. Residual enzyme activity was measured at 25 °C by addition of 0.2 mM DMP or ABTS dissolved in sodium phosphate buffer. Results were also expressed as relative activity.

## 3. Results

### 3.1. Effect of laccase-specific substrates. Native PAGE

Post-gel electrophoresis detection of laccase has the advantage that with specific substrates it is possible to detect the presence of laccase in both crude and purified extracts with a high degree of sensitivity, moreover obviating the effect of endogenous enzyme inhibitors. This technique has been described by Rescigno et al. (1997), who used ADA as substrate, by Cárdenas and Dankert (2000), Flurkey et al. (1995), and Binnington and Barrett (1988), who used DAB as substrate. Similar techniques have been used to determine the presence of enzymes with PPO activity in gels. PPOs, which can use many of the laccase substrates, were

also present in the partially-purified extract and therefore specific substrates had to be used to differentiate between the two. Staining with ADA and then with *t*BC has the advantage that the two enzymes can be distinguished in the same gel (Rescigno et al., 1997). When ADA was used as substrate in semipurified extract of cephalothorax frozen 48 h post mortem, pink spots denoting laccase activity only appeared in the 40–70% fraction. This activity corresponded to an enzyme having a molecular weight of around 44 kDa (Fig. 1b). Subsequent addition of *t*BC, following the technique described by Rescigno et al. (1997) produced various bands possibly corresponding to high molecular weight oligomers with PPO activity (Fig. 1b). Other PPO substrates such as L-tyrosine (monohydroxyphenol), L-DOPA, caffeic acid or catechol (dihydroxyphenols) were not oxidized by the laccase-like enzyme (data not shown), but the dihydroxyphenols could also be considered laccase substrates. When another two laccase-specific substrates were used – 3,3′-diaminobenzidine (DAB) (Binnington & Barrett, 1988) and 2,6-dimethoxyphenol (DMP) (Flurkey et al., 1995) – a band appeared which was similar to the one observed with ADA (Fig. 1d and e). DAB is also a substrate for peroxidases, but the presence of this enzyme in the gel was ruled out in view of the lack of a reaction to addition of ADA + $H_2O_2$, as reported by Rescigno et al. (1997) (data not shown). Fig. 1f highlights ADA–*t*BC staining of the 40–70% fraction corresponding to deepwater pink shrimp frozen on board immediately upon capture, where there was no sign of laccase activity.

ADA staining also revealed the presence of a laccase-like enzyme in all examined anatomical parts of shrimp frozen 48 h post-capture (Fig. 2). Here again, activity was only found in the 40–70% fraction. Most activity was concentrated essentially in the cephalothorax (Fig. 2b), although there was also evident activity in the cuticle (Figs. 2a and c). There were only slight traces in parapods and pleopods and practically nothing in the telson.

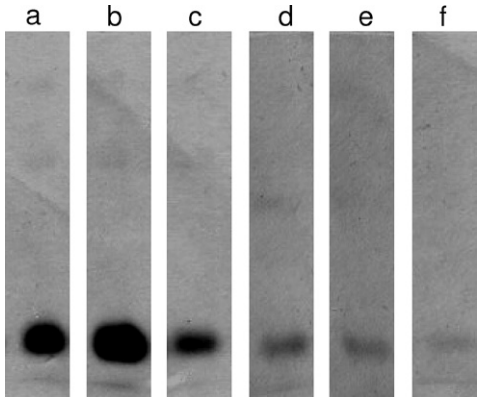


Fig. 2. Polyacrylamide gel electrophoresis (6%) showing the presence of a laccase-like enzyme in various anatomical parts of deepwater pink shrimp. ADA was used as a staining solution. (a) Cephalothorax cuticle; (b) cephalothorax; (c) abdominal cuticle; (d) parapods; (e) pleopods and (f) telson.

### 3.2. Effect of laccase-specific substrates. Non-reducing SDS–PAGE

#### 3.2.1. General

ADA staining after non-reducing SDS–PAGE showed that the laccase enzyme was active in the gel. The denaturing effect of SDS, then, was insufficient to inhibit its activity. Denaturation with SDS also induced oxidation of L-DOPA by the laccase-like enzyme (Fig. 3c). Its appar-

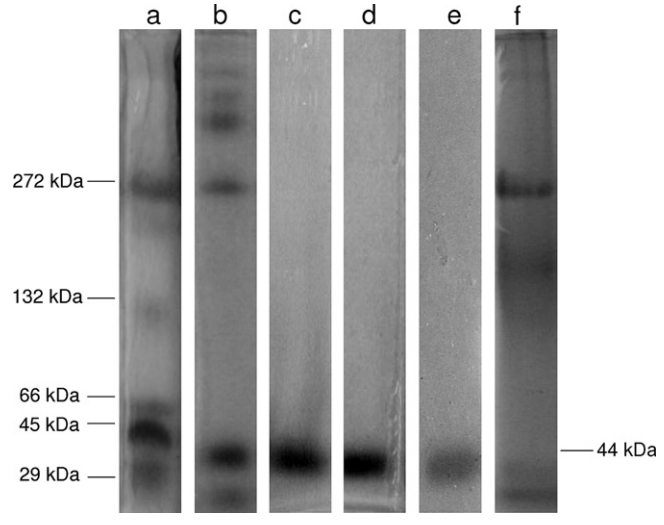


Fig. 1. Polyacrylamide gel electrophoresis (6%) in native conditions of the 40–70% fraction of deepwater pink shrimp from cephalothorax frozen 0 and 48 h post-capture. Staining with laccase-specific substrates, PPO and Coomassie Brilliant Blue R-250. (a) Standard LMW; (b–e) semipurified extract 48 h post mortem; (f) semipurified extract 0 h post mortem; (b, f) ADA + *t*BC; (c) ADA; (d) DMP and (e) DAB.

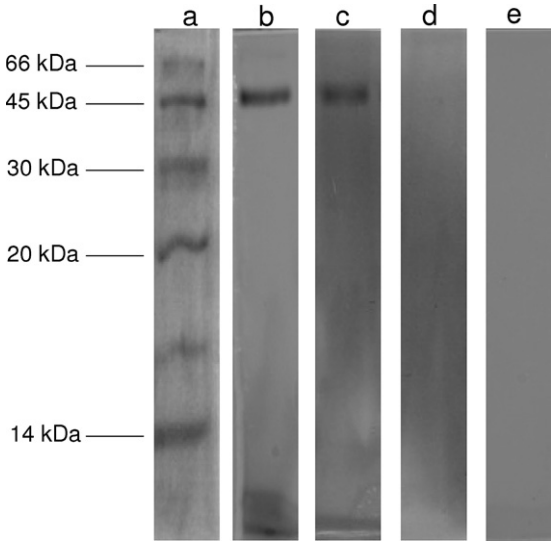


Fig. 3. Polyacrylamide gel electrophoresis (12.5%) with SDS in non-reducing conditions. Gels were stained with Coomassie Brilliant Blue R-250 (a), ADA (b, d) and L-DOPA (c, e). (a) LMW Standard; (b, c) semipurified extract of cephalothorax from shrimps frozen 48 h post mortem and (d, e) semipurified extract of cephalothorax from shrimps frozen 0 h post mortem.

ent molecular weight was similar to that observed in native conditions, suggesting that this protein consisted of a single enzyme of approximately 44 kDa (Fig. 3b and c). Incubation with SDS did not trigger activation of the laccase-like enzyme in pink shrimp frozen immediately upon capture (Fig. 3d and e).

### 3.2.2. Effect of specific inhibitors on laccase activity

Specific inhibitors have been used to distinguish laccase from PPO (Flurkey et al., 1995). A specific laccase inhibitor, cetyl trimethyl ammonium bromide (CTAB), inhibited laccase activity at a concentration of 40 μg/ml using DAB as substrate (Fig. 4a and b). However, when the substrate was ADA, concentrations of up to 3 mg/ml were insufficient to produce an inhibitory effect (Fig. 4c and d). The inhibitory effect of CTAB therefore appears to depend on the type of substrate used.

Another way to differentiate PPO-like activity from laccase-like activity is to use specific PPO inhibitors which do not affect laccase activity (Flurkey et al., 1995). Two specific PPO inhibitors were used for this purpose: tropolone (Rescigno, Sollai, Pisu, Rinaldi, & Sanjust, 2002) and 4-hexylresorcinol (4-HR) (Flurkey et al., 1995). The combination of tropolone and ADA produced some inhibition, although not total, of laccase (Fig. 4).

However, 4-HR failed to inhibit activity of the laccase-like enzyme when added along with ADA or DAB (Fig. 5). Concentrations of up to 200 μg/ml were assayed without achieving inhibition. At this last concentration the 4-HR was added to the gel without any other substrate; there was no staining and therefore 4-HR was ruled out for use as a substrate for the laccase-like enzyme.

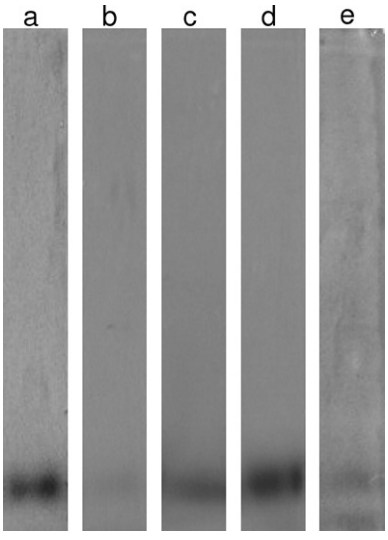


Fig. 4. Native polyacrylamide gel electrophoresis (6%) of semipurified extract of cephalothorax of deepwater pink shrimp frozen 48 h post capture. Effect of CTAB or tropolone using DAB or ADA as substrates. (a) DAB 5 mM; (b) DAB + CTAB 40 μg/ml; (c) ADA 5 mM; (d) ADA + CTAB 3 mg/ml and (e) ADA + tropolone 9 mM.

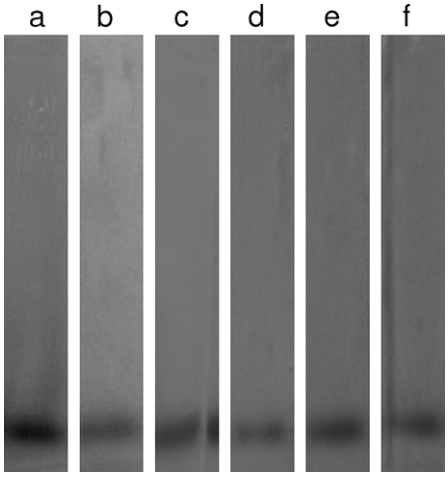


Fig. 5. Polyacrylamide gel electrophoresis (6%) under native conditions of the 40–70% fraction of deepwater pink shrimp from cephalothorax 48 h post-capture. Effect of 4-hexylresorcinol (4-HR) on laccase using ADA or DAB as substrate. (a) ADA 5 mM; (b) ADA + 4-HR 20 μg/ml; (c) ADA + 4-HR 200 μg/ml; (d) DAB 5 mM; (e) DAB + 4-HR 20 μg/ml and (f) DAB + 4-HR 200 μg/ml.

### 3.2.3. Effect of non-specific inhibitors on laccase activity

Finally, two non-specific antioxidants, ascorbic acid and sodium metabisulphite, were evaluated for inhibitory capacity. The basis of the inhibitory mechanism of ascorbic acid is reduction of the quinones to their diphenol precursors, thus preventing pigment formation (Iyengar & McEvily, 1992). Immersion of the gels in 150 μg/ml ascorbic acid was sufficient to achieve total inhibition of laccase (Fig. 6a) using ADA as substrate. In the case of sulphites, 70 μg/ml was enough to cause total inhibition of the lacasse-like enzyme

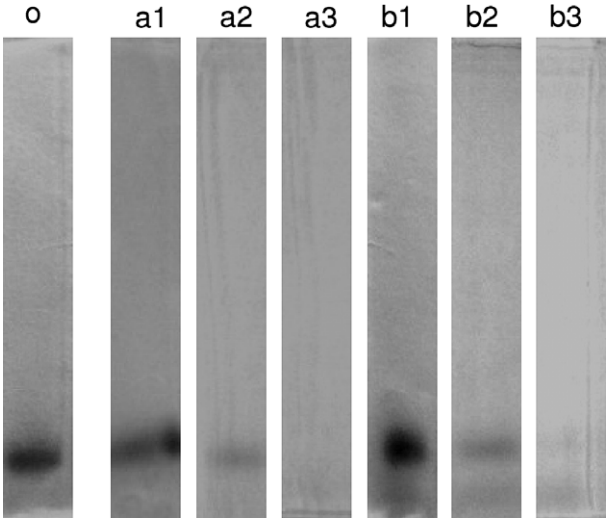


Fig. 6. Polyacrylamide gel electrophoresis (6%) under native conditions of the 40–70% fraction of deepwater pink shrimp from cephalothorax 48 h post-capture. (0) ADA 5 mM; (a) inhibitory effect of ascorbic acid on the laccase-like enzyme, (a1) ADA + 50 μg/ml ascorbic acid, (a2) ADA + 125 μg/ml ascorbic acid, (a3) ADA + 150 μg/ml ascorbic acid; (b) inhibitory effect of sodium metabisulfite on the laccase-like enzyme, (b1) ADA + 5 μg/ml metabisulfite; (b2) ADA + 40 μg/ml metabisulfite, (b3) ADA + 70 μg/ml metabisulfite.

(Fig. 6b), possibly through the reduction of quinones to phenols by reacting with quinones to give colourless compounds, or by acting as an irreversible PPO inhibitor (Ricquebourg, Robert-Da Silva, Rouch, & Cadet, 1996).

### 3.3. Characterization of laccase-like activity

Two specific substrates, ABTS and DMP, were used to characterize the laccase-like activity. Fig. 7a and b show the enzyme activity at different temperatures and the thermal stability, in the presence of each of the substrates. The activity increased with temperature in both substrates (Fig. 7a). When the substrate was ABTS, the enzyme activity remained relatively close to 40% and more or less constant between 15 and 45 °C; however, at higher temperatures the relative activity increased sharply and was practically trebled at 60 °C, the point at which 100% relative activity was reached. When the substrate was DMP, the profile was very similar, with very similar values up to 40 °C; thereafter there was a considerable increase up to 50 °C, at which temperature relative activity reached 100%. Above 50 °C the activity declined, eventually reaching relative activity values of around 60%.

Thermal stability was very high at 10 and 30 °C irrespective of the substrate used (Fig. 7b). The relative activity gradually decreased thereafter as the incubation temperature rose, although values in excess of 40% were registered with DMP or ABTS at temperatures up to 55 °C. At that point, in the assay with DMP relative activity was constant at close to 60% following incubation at 50–70 °C. After incubation at 75 °C, activity was minimal with either substrate, and at 80 °C the enzyme was inactivated.

In view of the high stability and acceptable level of activity observed at 30 °C, this was the temperature chosen to determine the stability of the enzyme versus pH and the optimum pH (Fig. 7c and d).

The optimum pH profiles identified with ABTS or DMP as substrate were very similar. Fig. 7c shows a very pronounced peak at pH 4.5–5 in both cases. Much lower peaks of activity, in the region of 20–40% relative activity, were observed with both substrates at pH 6.5 and with ABTS at pH 7.5. At pH values of 8 and upwards, the relative activity was very low. As regards the effect of the pH of the medium, the enzyme presented high stability at pH 4 with both substrates (Fig. 7d). At pH 6.5–8.5, the stability of relative activity reached 60% with DMP, while values were much lower with ABTS.

As regards the kinetic constants ($K_m$ and $V_{max}$) recorded for DMP and ABTS, the latter presented the lowest $K_m$ value (0.81 μM). The $K_m$ for DMP was about eight times higher (6.27 μM). Although the enzyme exhibited a considerably greater affinity for ABTS, the maximum rate of oxidation of the substrate was similar in both cases, 0.002 Units/ml.

## 4. Discussion

This paper reports the presence of a laccase-like enzyme active in deepwater pink shrimp 48 h post mortem, with an affinity for oxidation of laccase-specific substrates like ADA, DAB and DMP. A similar enzyme has only been reported in one crustacean species, the red swamp crayfish (*P. clarkii*) (Cárdenas & Dankert, 2000). Its characterization and the effect of specific inhibitors remain to be determined. This is the first time that has been done in

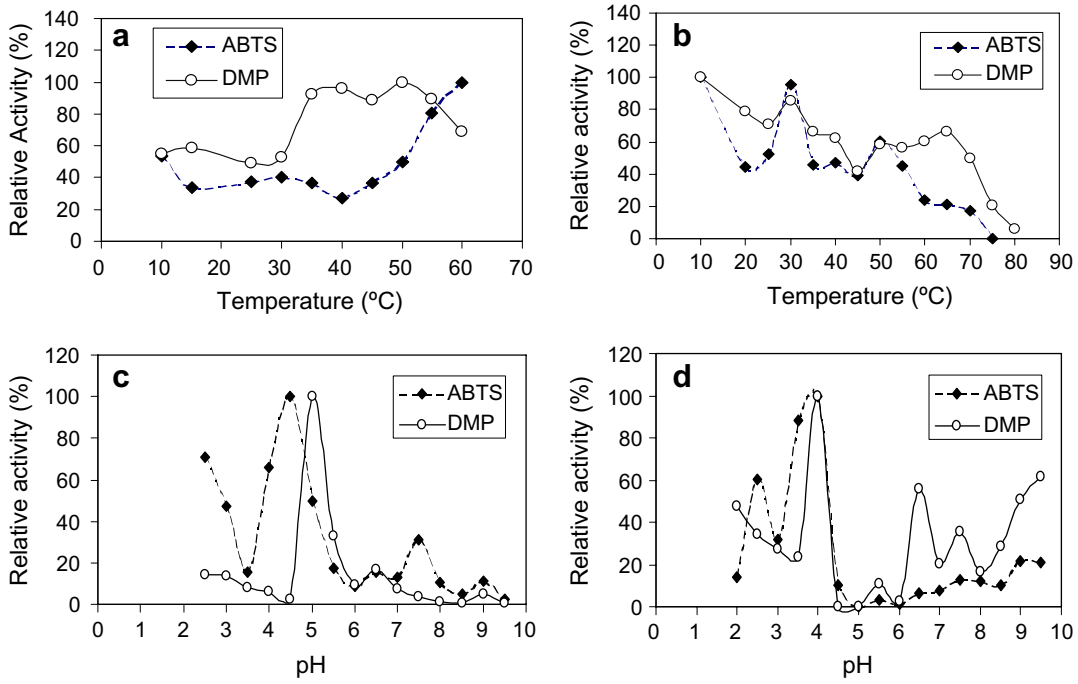


Fig. 7. Optimum temperature (a), thermal stability (b), optimum pH (c) and stability versus pH of the medium (d) of the laccase-like enzyme in the presence of ABTS or DMP. The 40–70% fraction of deepwater pink shrimp from cephalothorax 48 h post-capture was used as enzymic extract.

crustaceans. The discovery of laccase-like enzyme in seafood would be of importance, as specific inhibitors of PPO (as 4-hexylresorcinol) are unable to prevent the oxidative effect of laccases. In a previous work (Montero, Martínez-Alvarez, & Gómez-Guillén, 2004), we reported formulations including 4-hexylresorcinol was effective as melanosis-inhibiting agent on deepwater pink shrimp. However, we also reported 4-HR was unable to avoid the appearance of yellow-greenish coloration in cephalothorax during storage. Apparently, this coloration could be the consequence of the activity of endogenous proteases. Nevertheless, the fact of this coloration was the result of oxidant processes mediated by the laccase-like enzyme should not be dismissed.

Like laccases, this enzyme was unable to oxidize *o*-monohydroxyphenols like L-tyrosine. However, it was likewise incapable of oxidizing dihydroxyphenols susceptible of being oxidized by laccases, such as caffeic acid and catechol. The laccase-like enzyme appears to be a simple protein with a molecular weight in the region of 44 kDa. Its molecular weight differs from that observed in crayfish by Cárdenas and Dankert (2000), who recorded molecular weights of 80 and 72 kDa, and from insect laccases, between 65 and 140 kDa (Barrett, 1991). Also, this enzyme is found in practically all anatomical parts of the deepwater pink shrimp (Fig. 2), although chiefly in the cephalothorax, where it may be located preferentially in the haemolymph. Cárdenas and Dankert (2000) also reported the presence of laccase in the haemolymph of crayfish, particularly inside haemocytes. According to these authors, laccase could be implicated in the immune system, as has also been proposed in some insect species (Barrett, 1991; Nappi, Vass, Carton, & Frey, 1992). The laccase-like enzyme has also been found in cuticle, the same as in insects (Ashida & Yamazaki, 1990; Barrett, 1987; Charalambidis et al., 1994), where it possibly plays a role in sclerotization. In this connection, it has been reported that in various insect species the enzyme is only found in cuticles destined to become sclerotized (Andersen, 1985; Barrett, 1987). The reason why the laccase-like enzyme remained inactive post-capture may be that, as in insects, it was present in the cuticle as an inert proenzyme. Given that in insects the enzyme is activated at the time of use by limited proteolysis (Ashida & Yamazaki, 1990; Yamazaki & Kambara, 1984), it is possible that its activation in deepwater pink shrimp during the first 48 h post-capture was triggered by similar mechanisms and not by conformational changes, given the absence of activity following treatment with SDS (Fig. 3d and e). However, conformational changes can trigger oxidation of L-DOPA, which is utilized for melanin biosynthesis, 48 h post-capture. Therefore, it would be possible the laccase-like enzyme had a role in the immune system, and proteolysis and conformational changes were induced in living pink shrimp if necessary. Regarding this subject, Cárdenas and Dankert (2000) reported a role of laccase from red swamp crayfish in the hardening of humoral or cellular capsules around invading parasites.

Also, the enzyme was inhibited by a laccase-specific inhibitor, CTAB, but only with DAB as substrate (Fig. 4). CTAB is a cationic detergent, and it seems to inhibit laccases in a competitive or non competitive manner (Walter & McCallion, 1980). Our results suggest that CTAB may competitively inhibit laccase, but the affinity of the enzyme for it is likely to be far weaker than its affinity for ADA. This would account for the absence of inhibition with ADA as substrate, at least at the concentrations assayed in this research. In this connection Flurkey et al. (1995) suggested that CTAB may not be a preferential laccase inhibitor or that CTAB inhibition may be dependent on the type of substrate employed. Another inhibitor, in this case tropolone, which is specific to PPOs, produced mild inhibition of activity (Fig. 4). Tropolone could react with products during the oxidation process and interfere with laccase estimations, according to Flurkey (2003). It would not be dismissed that tropolone inhibition could be dependent on the type of substrate employed. Another PPO inhibitor, 4-HR (McEvily, Iyengar, & Otwell, 1992), failed to inhibit the activity of the laccase-like enzyme (Fig. 5). The effectiveness of 4-HR as a melanosis-inhibiting agent has been demonstrated both in laboratory tests and on board simulating commercial caught with fresh crustacean (Guandalini, Ioppolo, Mantovani, Stacchini, & Giovannini, 1998; Montero et al., 2004), and it is authorized for use as such in numerous countries. However, given the lack of an inhibitory effect on laccases, commercial formulations based on 4-HR would need to have specific inhibitors added, at least for treatment of pink shrimp post mortem, where melanosis progresses rapidly. Antioxidant agents like ascorbic acid and sodium metabisulphite (Iyengar & McEvily, 1992) inhibited the laccase-like enzyme at very small concentrations (Fig. 6), demonstrating again the oxidant activity of this enzyme.

At the same time the enzyme is capable of achieving augmented activity with rising temperature in contact with a substrate of ABTS or DMP (Fig. 7a and b). This could be due to the fact that the reaction is accelerated by the enhanced kinetic energy. We would note that the enzyme attains relative activity levels of 40% or more at temperatures where it is generally very stable, which coincide with ambient temperatures.

Then again, the enzyme exhibits good stability at temperatures between 50 and 60 °C; its activity in that range is relatively very high, indicating considerable resistance to thermal denaturation. Cárdenas and Dankert (2000) reported high thermal stability of laccases in crayfish, although the times of incubation used were much shorter. High thermostability has also been reported in insects, in *Calliphora vicina* (Barrett & Andersen, 1981), and *Sarcophaga bullata* (Barrett, 1987). As regards the effect of pH, the enzyme exhibits high activity at pH 4–5 with both DMP and ABTS (Fig. 7c and d). Similar pH optima (between 4.5 and 5.5) have also been reported for various insect species (Barrett, 1987, 1991; Barrett & Andersen, 1981). However, the highest levels of stability are attained at slightly lower pHs. According to Flurkey (2003), the pH stability

of laccases varies considerably; some are stable to a high pH range (pH 3–7), while others are far more pH sensitive. It is very possible that the high activity observed at pH 4–5 is due to unfolding of the molecule caused by the acidic pH in the early moments, which would help the substrate to reach the active centre. Subsequently, over time the pH of the medium would produce such denaturation as to block the link between substrate and active centre. In the pH range 6.5–8, relative activity is low, but stability – at least with DMP – is relatively high. The pH of pink shrimp muscle is within this range during chilled storage, according to Huidobro, López-Caballero, and Mendes (2002). The fact that the enzyme is stable at these pH values is of particular interest in that it would presumably remain stable enough post mortem to oxidize *p*-dihydroxyphenols and produce melanins. This is all the more important in the cephalothorax, where the enzyme appears to concentrate, and where the pH would presumably be in the range 6.5–8. Lastly, we would note the low level of activity at pH 8–9, at which Cárdenas and Dankert (2000) observed a high level of laccase activity in crayfish.

Finally, the $K_m$ and $V_{max}$ values show that the enzyme has considerably more affinity for ABTS, but despite that, the maximum rate of oxidation of the substrate is similar with ABTS and DMP alike. A weaker affinity for different substrates ($K_m$ 0.1–10 mM) has been reported in various insect, fungus and plant laccases, but both the grade of purification of the enzyme and the pH of the medium used were different (Barrett & Andersen, 1981; Solomon, Sundram, & Machonkin, 1996; Yarapolov, Skorobogatko, Vartanov, & Varfolomeyev, 1997).

To summarize, our research has shown the existence of a laccase-like enzyme in pink shrimp (*P. longirostris*) post mortem, especially in the cephalothorax. Its characterization reveals similar behavior to that of various insect laccases versus temperature and pH of the medium. This enzyme exhibits very intense activity, and during storage melanosis may continue to occur as a result of oxidation of *p*-dihydroxyphenols produced mainly by non-specific hydroxylation of aromatic amino acids. Its presence in pink shrimp prompts a need to seek new melanosis-inhibiting formulations that incorporate laccase inhibitors along with PPO-exclusive inhibitors like 4-hexylresorcinol. In this sense, several formulations with laccase inhibitors have recently been patented (Montero, Martínez-Alvarez, & Gómez-Guillén, 2007). Further studies are needed to consider the effect of specific inhibitors, their concrete location in the animal, their sequencing, their physiological role, and lastly their relationship with other forms of polyphenoloxidase present in crustaceans or with insect laccases.

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