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Characterisation and tissue distribution of polyphenol oxidase of deepwater pink shrimp (*Parapenaeus longirostris*)

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ABSTRACT

Characterisation and tissue distribution of polyphenol oxidase (PPO) was studied in deepwater pink shrimp (*Parapenaeus longirostris*) post mortem. PPO activity was the highest in the carapace, followed by that in the abdomen exoskeleton, cephalotorax, pleopods and telson. No PPO activity was found in the abdomen muscle and in the pereopods and maxillipeds using the enzymatic assay. Storage of whole shrimps and of the different organs showed that melanosis (blackening) required the presence of the cephalotorax to be initiated, indicating that its development depends on other factors in addition to the PPO levels. Further characterisation was carried out in extracts partly purified using 40–70% ammonium sulfate fractionation. The enzyme had the highest activity at pH 4.5 and was most stable at pH 4.5 and 9.0. No clear maximum was observed in the 15–60 °C range but the higher stability was achieved at 30–35 °C. Apparent kinetic constants in the partly purified PPO from carapace were K_M = 1.85 mM and V_{max} = 38.5 U/mg of protein, pointing to a high affinity and reactivity of the enzyme when assayed with DOPA. Electrophoretic mobility was studied in native PAGE and non-reducing SDS–PAGE followed by staining with DOPA. Approximate MW of 500 kDa and 200 kDa were observed, respectively. These two forms could correspond to aggregates of minor PPO subunits that could not be resolved in these electrophoretic systems. The peptide mass fingerprinting obtained by MALDI-TOF analysis showed some peptides whose homology with hemocyanins and different PPO subunit precursors has already been demonstrated in the same species.

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1. Introduction

Discoloration in crustaceans, called melanosis or blackspot is a natural post-mortem process originated by the polymerization of phenols into insoluble black pigments, the melanins. The presence of melanins strongly reduces the marketability of the products (Kim, Marshall, & Wei, 2000). Phenol polymerization is catalyzed by polyphenoloxidase (also called phenoloxidase), an enzymatic complex found in almost all organisms. The term polyphenoloxidase (PPO) is generally used to refer to two similar enzymes involved in phenol oxidation: tyrosinases (EC 1.14.18.1), which catalyze the *o*-hydroxilation of monohydroxyphenols (i.e. monophenolase or cresolase activity) and the oxidation of *o*-dihydroxyphenols to *o*-quinones (i.e. catecholoxidase or diphenolase activity), and catecholoxidases (EC 1.10.3.1) which only catalyze the oxidation of *o*-dihydroxyphenols. The *o*-quinones may react nonenzymatically with a variety of compounds in the presence of O_2 and form melanins. Monophenol oxidases generally also act as *o*-diphenoloxidases, as reported in Kim et al. (2000) and Ramírez, Whitaker, and Virador (2003), often at a faster rate.

In invertebrates, PPO is synthesised as a proPPO, a zymogen that is activated by a proteinase cascade triggered by microbial compounds (carbohydrates and lipopolysaccharides) and which involves a series of other proteins (Wang, Chang, & Chen, 2006). This system has a key role in the primary immune response, cuticle sclerotization and healing of injuries in crustaceans.

During post-mortem storage of crustaceans, proPPO can be also activated into PPO by the action of proteolytic enzymes leaching from the digestive tract (Ali, Gleeson, Wei, & Marshall, 1994). Moreover, protein hydrolysis by these proteases originates substrates for active PPO (Ali et al., 1994).

PPO has been studied in different tissues from various crustacean species, but in most cases only a tissue has been selected as the source to extract the enzyme. Furthermore, due to the different names used, initial studies were somewhat confusing as regards the tissue being sampled. On the other hand, despite deepwater pink shrimp (*Parapenaeus longirostris*) is a species of great economic relevance in Mediterranean countries and it shows high susceptibility to melanosis, there are no published kinetic studies of

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PPO from this species. Since in this and other shrimps, as well as in prawns, melanosis is firstly detected in the head and then down spreads to the other tissues during chilled storage, we were interested in assessing the PPO activity in different tissues. Moreover, chemical treatments used to prevent melanosis in other crustaceans have been proven ineffective when applied to deepwater pink shrimp (Montero, Martínez-Alvarez, Zamorano, Alique, & Gómez-Guillén, 2006) and therefore characterisation of its PPO could be useful to explain these findings and to design more appropriate formula for controlling melanosis.

2. Materials and methods

2.1. Sampling of shrimp tissues

Deepwater pink shrimp (*P. longirostris*) were caught by a commercial shrimp vessel fishing off the port of El Puerto de Santa María, Cádiz (in the Atlantic coast of Spain), placed in polystyrene boxes, covered with ice and kept in a cold room. Packages were stored at −80 °C immediately after arrival of the vessel at the port (about 7 h after catch), later shipped by truck to the Madrid laboratory and stored at −80 °C until further analysis. Shrimps were then thawed and washed under cold tap water, and the carapace (exoskeleton of the cephalothorax), cephalothorax including the first three thoracic segments, exoskeleton of the abdomen, the remaining thorax segments and the abdomen (hereinafter named as muscle), pereopods and maxillipeds, 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. Storage of whole shrimp and individual tissues

Whole shrimps and samples of the above tissues (carapace, cephalothorax, abdomen exoskeleton, muscle, pereopods and maxillipeds, pleopods and telson), as well as of heads (i.e. carapace + cephalothorax + pereopods and maxillipeds), heads with the carapace removed (i.e. cephalothorax + pereopods and maxillipeds), whole abdomen (including the exoskeleton, muscle, pleopods and telson), whole abdomen with the muscle removed (i.e. abdomen exoskeleton + pleopods + telson) and whole shrimps with only the carapace removed, were stored at 4 °C under a saturating relative humidity. Development of melanosis in those samples was evaluated periodically. At least three samples from each tissue or combination of tissues were stored and the whole experiment was repeated twice using shrimps from different dates in the same season.

2.3. Partial purification of PPO

PPO was extracted according to Simpson, Marshall, and Otwell (1988) 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% Brij 35 and 2% polyvinylpolypirrolidone (PVPP). The suspension was stirred for 3 h at 4 °C and then centrifuged at 20,000*g* for 30 min at the same temperature. The supernatant was filtered through eight layers of muslin, an aliquot frozen in liquid nitrogen and stored at −80 °C (crude extract) and the remaining extract was fractionated with ammonium sulfate. After centrifugation of the corresponding supernatants at 20,000*g* for 30 min at 4 °C, the pellets precipitating between 0–40% and 40–70% saturation were resuspended in 0.1 M sodium phosphate buffer (pH 7.2) containing 40 or 70% ammonium sulfate, respectively, stirred for 1 h at 4 °C and centrifuged as described previously. This step was repeated for the 0–40% ammonium sulfate fraction to facilitate removal of the pigments and lipid materials (Rolle et al., 1991). The three fractions (0–40, 40–70, and 70–100%) were finally dissolved in 0.1 M sodium phosphate buffer (pH 7.2), desalted using PD-10 columns (Amersham Biosciences) and the corresponding eluates (designed as partly purified PPO extracts) frozen in liquid nitrogen and stored at −80 °C.

2.4. Enzyme activity

PPO activity was determined spectrophotometrically using a Shimadzu UV-1601 (Kyoto, Japan) equipped with a CPS-240 thermostatic controller. The reaction mixture containing 50 μL of either crude or partly purified PPO extract and 700 μL of 10 mM DL-3,4-dihydroxyphenylalanine (DL-DOPA) in 0.05 M sodium phosphate buffer (pH 6.5) was incubated at 35 °C and the absorbance at 475 nm measured every 15 s during 10 min. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per minute (Bartolo & Birk, 1998; Cong et al., 2005). Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein.

2.5. Protein determination

The protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

2.6. pH optimum and stability

The influence of pH on PPO activity was assayed by mixing 50 μL of the partly purified PPO extract from the carapace with 350 μL of 20 mM DL-DOPA dissolved in distilled water and 350 μL of different buffers in a pH range from 1.5 to 9.5. The following buffer solutions at a final concentration of 0.1 M were used: glycine–HCl (pH range: 1.5–3.5), sodium acetate (pH range: 3.0–6.0), sodium phosphate (pH range: 5.5–9.5), and Tris–HCl (pH range: 6.5–9.5). The influence of pH on PPO stability was determined by incubating 50 μL of the same extract with 100 μL of buffer (pH range and buffer composition was as above) for 1 h at room temperature. This mixture was incubated at 35 °C for 5 min prior to the addition of 2.1 mL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) at 35 °C and the residual enzymatic activity was determined as previously described. Experiments were performed in triplicate, and the results expressed as the percentage of maximum PPO activity.

2.7. Temperature optimum and thermostability

The influence of temperature on PPO activity was assayed by mixing 50 μL of the partly purified PPO extract from the carapace with 700 μL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5), pre-equilibrated at different temperatures (15–60 °C) and measuring the enzymatic activity at the same temperature. Thermostability was determined incubating the partly purified carapace extract at different temperatures (25–70 °C) for 30 min, cooling in ice for 3 min and mixing 50 μL of the incubated extract with 350 μL of 0.05 M sodium phosphate buffer (pH 6.5) at 35 °C. After 3 min, 350 μL of 20 mM DL-DOPA in the same buffer were added to this mixture and the residual activity measured as above. To account for any possible evaporation of sample during incubation, protein quantification was performed in aliquots of the incubated extract and calculations were done on the basis of residual specific activity. Experiments were performed in triplicate, and the results expressed as the percentage of maximum PPO activity.

2.8. Determination of kinetic parameters

The apparent kinetic constants (K_M and V_{max}) were estimated in the partly purified PPO extract from carapace under assay conditions similar to those previously described, but the reaction temperature was set at 25 °C to facilitate the comparison with most published results. The concentration of DL-DOPA ranged from 0.5 to 20 mM. The kinetic parameters were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph.

2.9. PPO electrophoretic mobility

Partly purified enzymatic extracts from the different tissues were subjected to native PAGE and non-reducing SDS–PAGE (6% acrylamide in both cases) using 1.5 mm thick mini slab gels. Crude extracts of carapace and cephalothorax were also included in both electrophoretic analyses. High Molecular Weight and High Molecular Weight-SDS calibration kits of Amersham were used for native PAGE and non-reducing SDS–PAGE, respectively. The samples to be separated by SDS–PAGE were previously incubated with an equal volume of loading buffer (final SDS concentration was 2% w/v) for 1 h at room temperature. 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 remaining portion of the gels were stained for PPO activity by pre-equilibration in 0.05 M sodium phosphate buffer (pH 6.5) for 20 min prior to incubation at 30 °C in 10 mM DL-DOPA dissolved in the same buffer. The lanes containing the carapace and cephalothorax extracts showed a brownish band corresponding to PPO after approximately 45 min of incubation, but enzymatic staining of other samples required longer periods of incubation. Therefore gels were routinely incubated overnight and the substrate solution was changed several times.

2.10. Influence of trypsin, cold storage and repeated freezing and thawing on PPO electrophoretic mobility

The influence of trypsin treatment was assayed in PPO extracts partly purified from all tissues by incubation with one volume of 1% (w/v) trypsin (Sigma, 16,000 U per mg) for 1 h on ice (Ali et al., 1994; Ferrer et al., 1989). The effects of cold storage (1 d at 4 °C) and repeated freezing at −20 °C and thawing at room temperature (3 cycles) on the electrophoretic mobility of PPO were determined in crude and partly purified extracts of the carapace. All samples were subjected to native PAGE and the activity of PPO detected in the electrophoretic gel as above.

2.11. Mass spectrometry analysis of protein

The partly purified extract from cephalotorax subjected to native PAGE was used to perform the mass spectrometry analysis. The (non-stained) area in the lane stained with Coomassie Brilliant Blue R-250 corresponding to the same position than the band in the parallel lane stained with DL-DOPA was manually excised from the native gel using biopsy punches. Protein was in-gel reduced, alkylated and digested with trypsin according to Sechi and Chait (1998). Briefly, the spot was washed twice with water, shrunk 15 min with 100% acetonitrile and dried in a Savant SpeedVac for 30 min. Then the sample was reduced with 10 mM dithioerytritol in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, sample was digested with 12,5 ng/μl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a MALDI-tandem time-of flight mass spectrometer 4700 Proteomics Analyzer (PerSeptives Biosystems, Framingham, MA). The instrument was operated in positive reflector mode, with an accelerating voltage of 20,000 V. Peptides from the auto digestion of the trypsin were used for the internal calibration. MALDI-TOF MS analysis produces peptide mass fingerprints and the peptides observed can be collected and represented as a list of monoisotopic molecular weights. The suitable precursors for MS/MS sequencing analyses were selected and fragmentation was carried out using the CID. *Denovo* sequencing from fragmentation spectra of peptides was performed using DeNovo tool software (Applied Biosystems) and homology search of the sequences was obtained by Blast (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results and discussion

3.1. PPO partial purification and distribution

Preliminary experiments were carried out to study the effect of extraction time on PPO recovery from carapace of deepwater pink shrimp, since most researchers have used a 3 h stirring time to extract PPO from powder preparations of different crustaceans, but others (Chen, Charest, Marshall, & Wei, 1997; Rolle et al., 1991) have suggested that stirring for longer than 0.5 and 1 h, respectively, may decrease total and specific PPO activity. We found that stirring of shrimp powder in extraction buffer from 1 to 3 h enhanced both total and specific PPO activities in the crude extracts (data not shown). It is generally assumed that the reaction of PPO with phenolic substrates during enzyme extraction and the subsequent polymerization of the intermediate products can lead to enzyme precipitation and loss of enzymatic activity (Chen et al., 1997). Most researchers, including Chen et al. (1997) and Rolle et al. (1991) did not include any protective agents in the extraction buffer; therefore extended extraction periods could facilitate the interaction of the enzyme with the substrates, producing a decrease in extractable PPO activity. This negative effect was likely prevented by the addition of PVPP (a phenol scavenger) in the buffer used in our extraction protocol.

Apparently, the crude extract from the muscle had the highest activity (Fig. 1a). However this higher activity was likely due to interference in the assay caused by turbidity of the extract, as there was no browning of the corresponding reaction mixture after 3 days at room temperature whereas reaction mixtures containing extracts from the other organs precipitated producing black precipitates after 1 day. This interfering effect was confirmed by the absence of PPO activity in the corresponding partly purified extract (see below). After applying these confirming criteria, the highest PPO activity was found in the carapace extract, followed by the activities shown by the extracts from the cephalothorax, abdomen exoskeleton and telson. Very low activity was shown by the extract from the pleopods, whereas the pereopods extract had no measurable PPO activity.

Further purification of the PPO was achieved by ammonium sulfate fractionation. This fractionation has been used by several authors to purify PPO from crustaceans (Adachi et al., 1999; Chen et al., 1997; Cong et al., 2005; Liu et al., 2006; Montero, Avalos, & Pérez-Mateos, 2001), and the best range of ammonium sulfate saturation to precipitate the enzyme appears to depend on the species considered, reflecting the variations in biochemical characteristics of PPO enzymes from different sources. We found that the

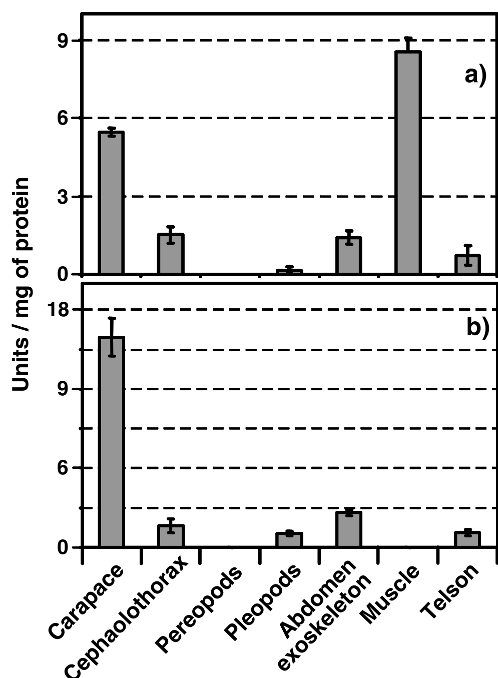

Fig. 1. PPO activity in crude (a) and partly purified using 40–70% ammonium sulfate (b) extract from different organs of deepwater pink shrimp. Columns represent the average of three replicated experiments (with three determinations per experiment) and error bars represent the standard deviation.

majority of PPO activity from deepwater pink shrimp was recovered in the 40–70% ammonium sulfate fraction for all tissues.

Considering these partly purified extracts, the greatest PPO activity was found in the carapace (Fig. 1b), followed by the abdomen exoskeleton, cephalothorax and pleopods and telson (which showed practically the same activity). The muscle and the pereopods and maxillipeds showed no detectable PPO activity. This profile of PPO distribution was similar in shrimps caught in two different seasons (data not shown).

To the best of our knowledge this is the first report of the distribution of PPO activity in different organs for a crustacean species using a partly purified enzyme. Crude extracts were used by Nakagawa and Nagayama (1981) to study PPO distribution in tissues and organs of several crustaceans (tiger shrimp, crayfish, taishoebi shrimp, two species of deep sea crab, snow crab and swimming crab). They found that monophenol oxidase activity of PPO was preferentially found in the gill, whereas diphenol oxidase activity of the enzyme was shown only in the hemolymph and gill or in the hemolymph of two species (crayfish *Cambarus clarkii* and tiger shrimp *Penaeus japonicus*, respectively). Montero et al. (2001) determined PPO activity in crude extracts of different tissues from imperial tiger prawn and found the highest activity in the carapace, next in the telson and last in the abdomen and in its exoskeleton. They performed further studies using ammonium sulfate-fractionated PPO only from the carapace.

We evaluated the potential physiological relevance of our findings on the distribution of PPO by assessing the contribution of the different tissues in the initiation of melanosis during chilled storage of whole shrimps as well as of individualised tissues and combinations thereof (Fig. 2). Marked melanosis developed in the head (both as separated organ and attached to the shrimp) and cephalothorax on day 1, but the other individual organs showed no evidence of black spots even after 7 days of storage. In the whole shrimp, melanosis first appeared in the ventral area of the head that contains the gills (day 1). It then extended towards the dorsal area, the most proximal articles (sometimes misleadingly called segments) of the pereopods and maxillipeds, and affected slightly the first segment of the exoskeleton of the abdomen (day 4). On day 7 blackening had spread to the exoskeleton of the first three abdominal segments, the first pair of pleopods and the proximal articles of the following two pairs, with minor black patches also found in the telson and distal abdominal segment. Removal of the carapace resulted in a faster melanosis development in the excised heads and the whole shrimp, probably due to higher accessibility of oxygen to the enzyme and the substrates. Neither the whole shrimps nor any individual tissue or combination of tissues showed marked melanosis when the cephalothorax was not present. Slight black discoloration developed in some beheaded shrimps only when residual portions of the cephalothorax were attached to the muscle, a result similar to that reported by Ali et al. (1994).

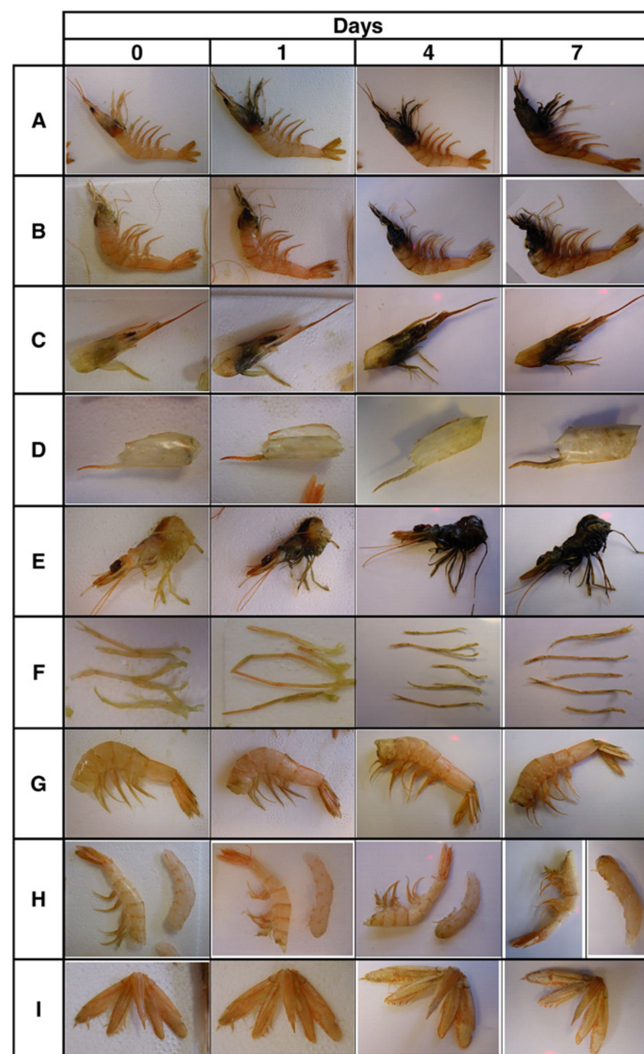

Fig. 2. Melanosis appearance in different anatomical parts of deepwater pink shrimp during 0, 1, 4 and 7 days of storage at 4 °C. (A) whole shrimp, (B) whole shrimp with the carapace removed, (C) head (carapace + cephalothorax + pereopods and maxillipeds), (D) carapace, (E) head with the carapace removed, (F) pereopods + maxillipeds, (G) whole abdomen (including the exoskeleton, muscle, pleopods and telson), (H) abdomen with the muscle removed (left) and corresponding muscle (right), and (I) telson.

Absence of noticeable melanosis in the carapace, in which we found the highest PPO activity, confirms that the development of melanosis in the different tissues of the shrimp depends on several factors in addition to the PPO levels. Since all known arthropods

PPO are synthesised as zymogens (proPPOs) that are activated by limited proteolysis (Burmester, 2002), the accessibility of the proteolytic enzymes to proPPOs is likely to be an important factor controlling melanosis in the different tissues. It has been suggested that in the living animal the activation of proPPO is regulated by a serine protease and that both proteins are found in the hemolymph (Decker & Jaenicke, 2004; Gollas-Galván, Hernández-López, & Vargas-Albores, 1999). Therefore a faster rate of proPPO activation is expectable in the well-irrigated cephalothorax, particularly in the gills, where melanosis first appeared in our experiments. On the other hand, several evidences suggest that proteolytic enzymes leaching from the digestive tract may contribute to proPPO activation during storage of crustaceans. Thus, Ali et al. (1994) showed that, during storage of cross-sections of lobster tail, darkening was preferentially developed around the gut, but the same area did not show black discoloration when this organ had been previously removed. Furthermore, Ali et al. (1994) found that an extract of hepatopancreas (an organ with digestion and nutrient adsorption/storage functions) can activate cuticle proPPO. These results are in accordance with our findings of the faster development of melanosis in shrimp cephalothorax and head (cephalothorax + carapace), both as excised organs and as part of the complete animal, since the hepatopancreas and the bigger portion of the gut (the foregut) are located inside the cephalothorax.

Other factors that should be considered in determining the extent of melanosis during cold storage of crustaceans are the presence of activators of the proteases converting proPPO, the availability and concentration of substrates and cofactors for PPO activity, the molt stage, and nonenzymatic melanin formation. Finally, it must be noted that there is increasing evidence for a potential contribution of hemocyanins to the PPO activity found in some crustacean species (Adachi, Hirata, Nagai, & Sakaguchi, 2001; Martínez-Alvarez, Gómez-Guillén, & Montero, 2008a). Hemocyanins are oxygen carrier proteins present in the hemolymph of various arthropods and mollusks and are also involved in the immune defense. They may be converted into the PPO enzyme by a yet unknown mechanism, though limited proteolysis, changes in pH and conformational changes induced by some hemolymphs factors have been hypothesised as activating processes (Adachi et al., 2001; Decker & Jaenicke, 2004). Moreover, it has been suggested that hemocyanin-derived PPO is the key enzyme in the development of melanosis during chilled storage, since it is highly stable under low temperatures while PPO originated from proPPO easily aggregates and is inactivated at chilling and freezing temperatures (Adachi et al., 2001).

3.2. pH and temperature optimum and stability

Partly purified carapace PPO showed a maximum activity at pH 4.5 (Fig. 3a). At higher pH, PPO activity was in the range of 50–25% of that maximum, whereas it was completely inhibited at pH below 3.0. The optimal pH was similar to that reported for PPO extracted from tiger shrimp hemocytes (Adachi et al., 2001, pH 4.9) and carapace (Montero et al., 2001; pH 5.0). Though both research groups also found a second maximum at pH 8.0 and pH 8.3, respectively, most crustaceans PPO show only one pH maximum, generally within the pH 6–8 range (Ali et al., 1994; Chen, Rolle, Marshall, & Wei, 1991a; Fan & Wang, 2002; Ferrer et al., 1989; Gollas-Galván et al., 1999; Liu et al., 2006; Opoku-Gyamfua, Simpson, & Squires, 1992; Rolle et al., 1991; Simpson, Marshall, & Otwell, 1987; Simpson et al., 1988). We carried out similar experiments using PPO partly purified from the cephalothorax, abdomen exoskeleton and the pleopods, and found single pH maxima in the 4.5–6.0 range (data not shown).

The stability of the partly purified enzyme showed a maximum also at the optimal pH (pH 4.5), with another maximum at pH 9.0 (Fig. 3b). The activity loss after 1 h of incubation in the different buffers at pH between 3.0 and 9.5 was always lower than 40% (i.e. remaining activity was at least 60% of the maximum activity observed at pH 4.5), whereas at pH below 3.0 the loss was more marked (remaining activity was less than 37% of the maximum). Most authors have found that maximum stability for PPO from different species of shrimp and prawn was in the pH 6–8 range (Ali et al., 1994; Chen et al., 1991a; Ferrer et al., 1989; Gollas-Galván et al., 1999; Montero et al., 2001; Opoku-Gyamfua et al., 1992; Rolle et al., 1991; Simpson et al., 1987, 1988).

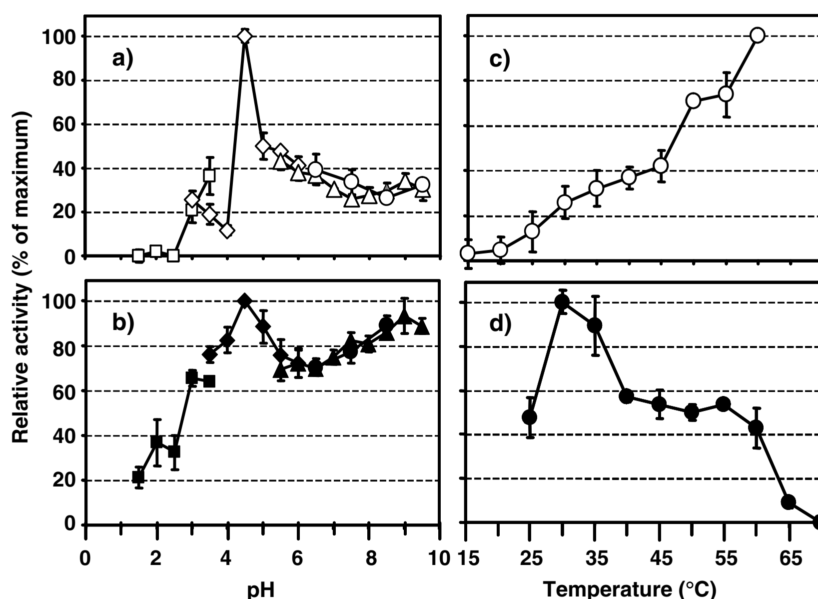

Fig. 3. Optimum pH (a), pH stability (b), optimum temperature (c) and stability at different temperatures (d) of partly purified (using 40–70% ammonium sulfate) PPO from carapace. Each point represents the average of three replicated experiments (with three determinations per experiment) and error bars represent the standard deviation. In figures (a) and (b) square symbols (■,□) correspond to results obtained using glycine–HCl (pH range: 1.5–3.5), diamonds (◇,♦) correspond to results obtained using sodium acetate (pH range: 3.0–6.0), triangles (Δ,▲) correspond to results obtained using sodium phosphate (pH range: 5.5–9.5), and circles (○,●) correspond to results obtained using Tris–HCl (pH range: 6.5–9.5).

The high stability of PPO from *Parapenaeus longirostris* at lower pH combined with its maximum activity at pH 4.5–6.0 may explain why dipping shrimps of this species in different combinations of acids results in faster melanosis development as compared to dipping in non-acid solutions (Montero et al., 2006). Likewise, we have observed that when deepwater pink shrimp is dipped in acid solutions (0.1, 0.5 and 1 M of acetic or citric acids) melanosis progresses faster at the higher concentrations.

The activity of the PPO from carapace gradually increased with temperature in the range of 15–60 °C and thus no maximum was shown in this range of temperature (Fig. 3c). Temperatures higher than 60 °C were avoided due to enhanced DL-DOPA oxidation without concur of PPO. Chen et al. (1991a) also reported a continuously increasing activity of PPO from Western Australian and Florida spiny lobsters in the range of 20–60 °C and Williams, Davidson, and Mamo (2003) found that PPO from the hemolymph of Western Rock lobster (*Panulirus cygnus*) was activated between 60 and 80 °C. However, in different crustaceans a number of authors found maximum activities of PPO in the range of 40–45 °C (Adachi et al., 1999; Ali et al., 1994; Fan & Wang, 2002; Liu et al., 2006; Rolle et al., 1991; Simpson et al., 1987, 1988) while Opoku-Gyamfua et al. (1992) and Montero et al. (2001) reported maxima at 30 °C and 55 °C for lobster *Homarus americanus* and imperial tiger prawn, respectively.

The higher stability of PPO from deepwater pink shrimp was shown in the 30–35 °C range and no remaining activity could be found after incubation of the enzyme at 70 °C for 30 min (Fig. 3d). A number of PPO purified from different organs in different shrimp and prawn species are stable in the 20–40 °C range (Adachi et al., 1999; Ali et al., 1994; Chen et al., 1991a; Ferrer et al., 1989; Montero et al., 2001; Opoku-Gyamfua et al., 1992; Rolle et al., 1991; Simpson et al., 1987, 1988).

3.3. Enzyme kinetics

The Lineweaver-Burk plot showed a correlation coefficient of $R^2 = 0.954$. The apparent K_M value of carapace PPO for DOPA was calculated as 1.85 ± 0.29 mM. The values closer to that found in our study are those shown for PPO from the cephalothorax of pink shrimp *Penaeus duorarum* (1.6 ± 0.1 mM, Simpson et al., 1988) and from the abdomen cuticle of *H. americanus* lobster (2.13 ± 0.6 mM, Opoku-Gyamfua et al., 1992). Except for PPO of the cuticle of Florida spiny lobster, in which the apparent K_M for DOPA was 0.81 ± 0.04 mM (Ferrer et al., 1989) most crustaceans PPO characterised so far have higher apparent K_M for this substrate, depending on the species and the source organ: 2.8 ± 0.2 and 3.48 mM for PPO from the head of white shrimp *Penaeus setiferus* (Chen, Wei, & Marshall, 1991b; Simpson et al., 1988, respectively); 4.45 mM for PPO of the head of black tiger shrimp (Rolle et al., 1991); 9.85 and 3.27 mM for PPO of the head and of the cuticle of beheaded Florida spiny lobster, respectively (Chen et al., 1991a, 1991b, respectively) and 3.41 mM for PPO of hemolymph from crab *Charybdis japonica* (Liu et al., 2006).

Besides a relatively high affinity towards its substrate as compared to other crustaceans PPO, the PPO characterised in this study showed an apparent V_{max} = 38.5 ± 5.9 U/mg of protein. Comparable studies carried out in PPO from the cephalothorax of pink and white shrimp by Simpson et al. (1988) showed a much lower V_{max} of 3.1 ± 0.2 and 5.6 ± 0.1 U/mg of protein, respectively.

The relatively high affinity of PPO from the carapace of deepwater pink shrimp towards its substrate is indicated by the low apparent K_M value, as well as a high reaction rate once the substrate is bound by the enzyme (as indicated by the apparent V_{max}). This is a possibility in explaining why higher concentrations of either 4-hexylresorcinol (a PPO inhibitor) or commercial formulations (based on this compound), are required to prevent melanosis in pink shrimp, as compared to the concentration preventing melanosis in other crustacean species (McEvily, Iyengar, & Otwell, 1991; Montero et al., 2006). Nonetheless, it may also be affected by the presence of a laccase-like enzyme and also of hemocyanin with the capacity to oxidise phenols in pink shrimp, as reported in Martínez-Alvarez, Montero, and Gómez-Guillén (2008b) and Martínez-Alvarez et al. (2008a).

3.4. Electrophoretic mobility

The native PAGE obtained from partly purified extracts of carapace, cephalothorax and muscle following staining with DOPA is shown in Fig. 4. Extracts from carapace and cephalothorax showed a band of approximately 500 kDa, whereas no PPO activity could be detected in the extract from muscle even after prolonged incubation of the gel with the substrate, confirming our results on the absence of PPO in this tissue. This protein band may correspond to the presumptive PPO oligomer of about 526 kDa described by Martínez-Alvarez et al. (2008a) in the same species, which also oxidised tyrosine. Therefore, it behaves as a typical PPO, as it conveys mono- and dihydroxyphenoloxidase activity. A similar band was found after staining for PPO activity, native PAGE gels loaded with partly purified extracts from the tissues that had shown PPO activity in the enzymatic assays (Fig. 5) and when crude extracts were used (Fig. 5, lane 1 and data not shown). Trypsin treatment, repeated freezing and thawing, and 1-day storage at 4 °C of crude or partly purified extracts did not have a noticeable effect on electrophoretic mobility of PPO (Fig. 5 and data not shown). Ferrer et al. (1989) and Ali et al. (1994) reported changes in the electrophoretic profile when trypsin was used to treat the samples and thus a band showing PPO activity could only be resolved in low percentage PAGE following this treatment. In brown shrimp hemocytes, high MW protein aggregates showing PPO activity could not be resolved in SDS–PAGE if the partly purified extract was not treated with trypsin (Gollas-Galván et al., 1999). Nonetheless it should be noted that native PAGE may not allow resolving some proteins with similar MW differences particularly when low acrylamide percentage is used (6% in our study), specially when these proteins have high MW. Therefore we cannot exclude that some undetectable change might occur in the PPO extracts when subjected to the different treatments.

The bands showing PPO activity in extracts from carapace and cephalothorax were not stained by Coomassie blue (compare lanes A and B with C and D, respectively in Fig. 4). Coomassie blue staining is considered to detect even 0.1 µg of protein in polyacrilamide gels. Since electrophoretically-separated crude and partly purified

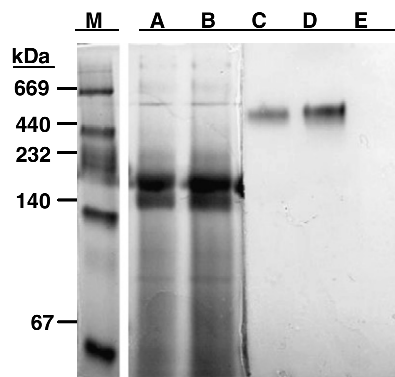

Fig. 4. Polyacrylamide gel electrophoresis (6%) in native conditions of partly purified extracts from different organs. M, High Molecular Weight calibration kit; A and C, carapace; B and D, cephalothorax; E, muscle (abdomen). Lanes M, A and B were stained with Coomassie Brilliant Blue R-250 and lanes C, D and E were stained for PPO activity using DL-DOPA.

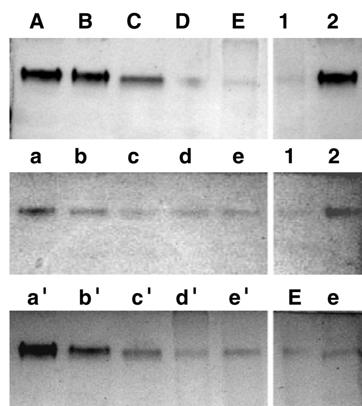

Fig. 5. Polyacrylamide gel electrophoresis (6%) in native conditions followed by staining for PPO activity with DL-DOPA. Lanes A–E, partly purified extracts; lanes a–e, partly purified extracts treated with trypsin; lanes a'–e', partly purified extracts stored for 1 day at 4 °C; lane 1, crude extract from carapace; and lane 2, partly purified extract after 3 cycles of freezing/thawing. A, a, a′, carapace; B, b, b′, cephalothorax; C, c, c′, abdomen exoskeleton; D, d, d′, pleopods; and E, e, e′, telson.

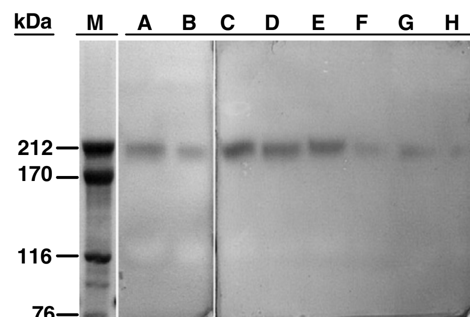

Fig. 6. SDS–PAGE electrophoresis (6%) in non-reducing conditions. M, High Molecular Weight-SDS calibration kit; A and B, crude extracts of carapace and cephalothorax, respectively; C–H, partly purified extracts from C, carapace; D, cephalothorax; E, abdomen exoskeleton; F, pleopods; G, telson; and H, pereopods. Lane M was stained with Coomassie Brilliant Blue R-250, Lanes A–H were stained for PPO activity using DL-DOPA.

extracts from cephalothorax and carapace developed clear brownish bands within 45 min of incubation of the gel with DOPA, this would indicate a high PPO activity towards DOPA which is in accordance with the kinetic parameters results discussed above.

As shown in Fig. 6, a protein band of about 200 kDa was resolved by non-reducing SDS–PAGE followed by incubation with DOPA in extracts of tissues that had enzymatic PPO activity. PPOs with a MW around 200 kDa have been reported in other crustacean species, such as gulf brown shrimp (210 kDa; Madero & Finne, 1982), Florida spiny lobster *Panulirus argus*, white shrimp *Penaeus setiferus* and brown shrimp *Penaeus aztecus* (200, 190 and 190 kDa, respectively, Chen et al., 1992). Gollas-Galván et al. (1999) found a PPO in brown shrimp hemocytes of 272 kDa and Ali et al. (1994) reported a MW of 156 kDa for a PPO from spiny lobster cuticle. However, simple subunits of PPO have been reported to have lower molecular weight, about 71 (Jaenicke & Decker, 2003), 40 kDa (Simpson et al., 1988), 97, 88 or 82 (Chen et al., 1991a), so its possible that the protein band of 212 kDa corresponds to aggregates of PPO that survived treatment with SDS. However, no band that could correspond to a simple subunit of higher electrophoretic mobility (i.e. lower apparent MW) was clearly observed in the gels. It should be noted that PPOs from several arthropods have "sticky" properties due to the low number of charged groups on their surface. Therefore active phenoloxidase is prone to form aggregates, and these are resistant to SDS treatment (Aspan & Söderhäll, 1991).

3.5. Mass spectrometry

The MALDI-TOF peptide mass fingerprinting yielded peptides in an m/z range from 873.46 to 3699.92 (Fig. 7). A primary database search using this peptide mass fingerprint failed to reveal significant similarities with any known protein. The mass spectrum was very similar to those described by Martínez-Alvarez et al. (2008a) for different hemocyanin oligomers of deepwater pink

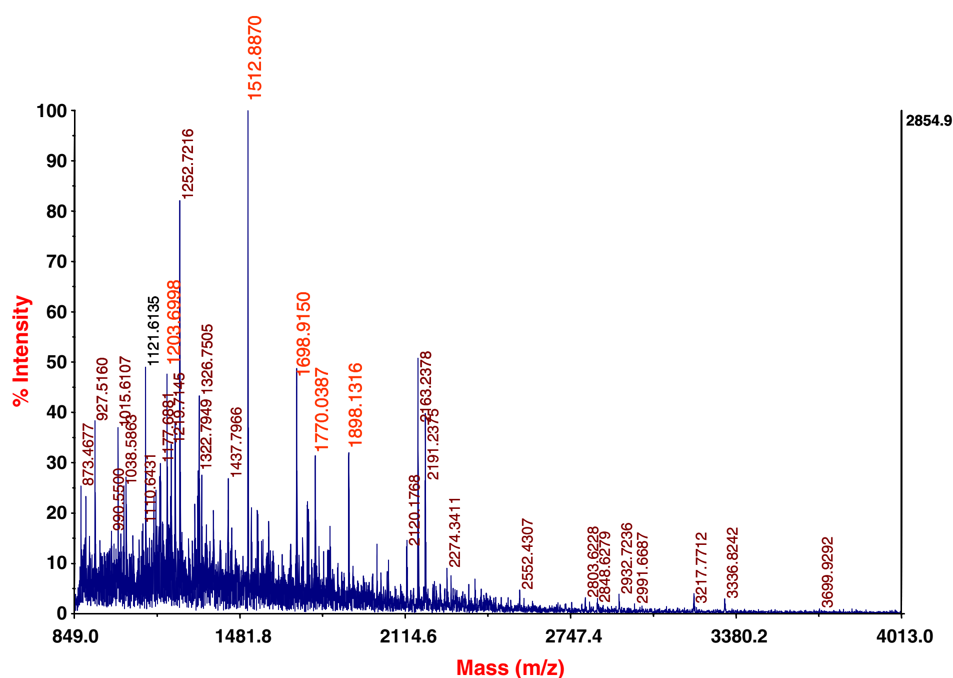

Fig. 7. MALDI-TOF MS peptide mass fingerprint spectra of in gel tryptic digest (Native PAGE) of the PPO band stained with DL-DOPA.

shrimp. In that study, several peptides from the digested proteins were then chosen for analysis by tandem mass spectrometry, and their amino acid sequences were obtained from the MALDI-TOF-TOF mass spectra after ion peptide fragmentation. Those authors described significant homology of peptides at 1203 m/z (RHWFSLFNPRQ), 1512 m/z (LFTNSEVLEAAYR), 1698 m/z (DFSYN-LEVNNAGLSR), 1770 m/z (KGENFFWVHHQLTVRF) and 1898 m/z (RKGENFFWVHHQLTVRF), with different hemocyanins. As shown in Fig. 7, all these peptides could be also found in the peptide mass fingerprint of the presumptive PPO studied in the present work, suggesting therefore a high resemblance with hemocyanin. However, the hemocyanin oligomers described by those authors had different molecular weight than that of presumptive PPO and showed only dihydroxyphenoloxidase activity. The strong resemblances between PPO and hemocyanins could be explained by the strict sequence similarity between the dicupric clusters of both proteins in crustaceans (Burmester, 2002; Kim et al., 2000). The absence of higher homology of our presumptive PPO with another polyphenoloxidases may be attributed to crustaceans PPO differences in sequence as well as in domain structure from vertebrate, plant and fungal PPOs (Hughes, 1999). Moreover, scarcely information about crustacean PPO is found in the data bases (Burmester, 2002; Hughes, 1999), unlike hemocyanin, a similar protein much more abundant and easier to purify. Nonetheless, it is worth mentioning that peptide at 1698.9 did show significant homology with different phenoloxidase subunit precursors, as subunit 2 precursor of silkmoth, *Bombix mori* (PRP2_BOMMO, identities = 64%, positives = 71%), or hexamerin precursor of tropical cockroach, *Blaberus discoidalis* (HEXA_BLADI, identities = 75%, positives = 87%).

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