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Presence of hemocyanin with diphenoloxidase activity in deepwater pink shrimp (*Parapenaeus longirostris*) post mortem

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Abstract

Polyphenoloxidase and hemocyanin are two proteins which although very similar perform different physiological functions in crustaceans. This paper reports the presence of hemocyanin with diphenoloxidase activity in deepwater pink shrimp (*Parapenaeus longirostris*) post mortem. Polyacrylamide gels and specific inhibitors and substrates of mono- and diphenoloxidases were used for purposes of recognition, and MALDI-TOF mass spectrometry for identification. Presumptive polyphenoloxidase was found in an inactive form in cephalothorax following capture, subsequently becoming active during storage. Also in the course of storage, hemocyanin acquired the ability to oxidize diphenols. Ascorbic acid, sodium metabisulphite and tropolone inhibited the prooxidant activity of both presumptive polyphenoloxidase and hemocyanin in the gels. 4-Hexylresorcinol did not avoid the appearance of activity bands in the gel corresponding with hemocyanin, maybe because 4-hexylresorcinol is described as slow-binding inhibitor. The acquired prooxidant activity of hemocyanin following capture is especially important because of the rapid development of melanosis in deepwater pink shrimp during storage.
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Keywords: Polyphenoloxidase; Hemocyanin; Melanosis; Deepwater pink shrimp; *Parapenaeus longirostris*

1. Introduction

All crustaceans of the class malacostraca (which includes most commercial species) contain two proteins, polyphenoloxidase and hemocyanin, which closely resemble one another in their sequence and in their active site, which is a binuclear copper active site coordinated by six histidines (Decker & Jaenicke, 2004). However, the two perform different physiological functions.

Polyphenoloxidase (PPO) is an enzyme that is found in hemolymph and in cuticle (Nellaiappan, Vinayagan, & Kalyani, 1989). Its physiological role is important, as it is involved in cuticle sclerotization and the immune response (Aspan, Huang, Cerenius, & Söderhäll, 1995). It is normally found in an inactive state (proPPO), but if required it is activated *in vivo* after limited proteolysis with serine proteases by means of the cleavage of an N-terminal part (Decker & Tuczek, 2000). Once activated, PPO acquires both monophenoloxidase and *o*-diphenoloxidase activity; this enables it to hydroxylate monophenols to *o*-diphenols, and *o*-diphenols to quinones, which are implicated in sclerotization, wound healing and encapsulation of foreign materials. Polyphenoloxidases from arthropods are reported to associate to form structures anywhere between monomers and hexamers, the molecular weight of the monomeric forms ranging from 40 to 75 kDa (Decker & Jaenicke, 2004; Decker & Tuczek, 2000).

Also, hemocyanin (Hc) is found in hemolymph, and some authors suggest in cuticle as well (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005; Adachi, Hirata, Fujio, Nishioka, & Sakaguchi, 2003a). Its function is to carry oxygen, but Decker and Jaenicke (2004) note that it may also be implicated in the immune response. Despite the strong resemblance between hemocyanins and polyphenoloxidases, Hcs lack the ability to oxidize mono- or diphenols since a Phe residue acts as a "placeholder" for potential substrates. In vitro, diphenoloxidase activity can

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be induced in Hc with denaturing agents such as SDS, which cause the molecule to unfold, thus allowing the substrates to reach the active centre (Decker & Jaenicke, 2004). In vivo, endogenous biodefence molecules such as clotting factors and antimicrobial peptides can cause conformational changes in Hc enabling it to acquire PPO activity, as has been observed in chelicerates. This process is necessary in chelicerates because Hc has to take on the functions performed in crustaceans by PPO, which chelicerates lack (Nagai & Kawabata, 2000). Hc can also acquire PPO activity through proteolytic cleavage in its terminal amino end, in a process similar to activation of PPO by serin proteases (Adachi, Hirata, Nishioka, & Sakaguchi, 2003b; Decker & Jaenicke, 2004; Lee, Lee, & Söderhäll, 2004). As to their polymeric disposition, hemocyanins of arthropods form hexamers (Adachi et al., 2005; Decker & Jaenicke, 2004; Jaenicke & Decker, 2003) in which each subunit, of around 75 kDa, can bind an oxygen molecule. These subunits need not necessarily be the same; they appear to join by means of non-covalent bonds, although several subunits may assemble with a disulphide bond (Adachi et al., 2003b). Even hexamers may associate to form higher order quaternary structures (multimers) with up to 8×6 subunits (Markl & Decker, 1992).

Melanosis occurs during storage following the capture and death of the crustacean, caused by the oxidation of phenols to quinones followed by polymerization, which produces coloured compounds. This process, which is normally attributed to PPO activity, is especially intense in some species such as kuruma prawn. In that species PPO is a highly unstable enzyme and becomes inactive in a matter of days, while Hc is the agent chiefly responsible for blackening (Adachi, Hirata, Nagai, & Sakaguchi, 2001). Something similar may occur in deepwater pink shrimp, as melanosis develops very rapidly in this species. The fact that Hc acquires the ability to oxidize phenols could be of considerable economic importance given the large amount of this protein found in crustaceans, about 1000 times more than PPO (Adachi et al., 2005); for this could accelerate the onset of melanosis and cause severe loss of quality. The recommended amount of legally authorized additives may in some cases be insufficient to significantly inhibit the onset of melanosis in these species, which means that new anti-browning agents may be needed to enhance or replace sulphites.

The main object of this paper is to report the presence of hemocyanin with diphenoloxidase activity in deepwater pink shrimp (*Parapenaeus longirostris*) post mortem. Polyacrylamide gels and specific inhibitors and substrates of mono- and diphenoloxidases were used for purposes of recognition, and MALDI-TOF mass spectrometry for identification.

2. Materials and methods

Deepwater pink shrimp (*Parapenaeus 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 ± 1.5 g and 10.8 ± 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 and 48 h after capture were stored at −80 °C until further analysis.

2.1. Preparation of crude enzyme

Crude extract was derived from whole cephalothorax (Wang, Taylor, & Yan, 1992), in order to include all oxidizing agents naturally present in hemolymph and/or cuticle. In this way, other factors present in the hemolymph which can act as modifiers of PPO activity, such as magnesium and calcium (Williams, Davidson, & Mamo, 2003), were also taken into account. Approx 30–40 g of cephalotorax was added to 1.5 parts of 0.1 M sodium phosphate buffer pH 6.4 and homogenized in an Omnimixer–Homogenizer (model 17106, OMNI International, Waterbury, USA) for 2 min. The homogenate was centrifuged at 50,000*g*, 30 min, 4 °C (Sorvall Combiplus, Dupont, Wilmington, DE, USA). The supernatant was used as the crude preparation, and immediately frozen to −80 °C to prevent alterations prior to determination. Phenilmethanesulfonyl fluoride (PMSF), as inhibitor of serine and some cystein proteases, was added to the crude preparation (1 mg/ml) to prevent proteolysis by native proteases.

2.2. Native and SDS-PAGE (polyacrylamide gel electrophoresis)

Enzymatic extracts (35 μl) were subjected to native PAGE (6% acrylamide) using 1.5 mm thick mini slab gels. The samples were obtained from shrimps frozen 0 h and 48 h after death. 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 high molecular weight standard (Amersham Pharmacia Biotech) consisted of: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

The remaining portion of the gels was stained for PPO activity using different specific substrates. For monophenoloxidase activity, 20 mM L-tyrosine (Merck, Darmstadt, Germany) was used. For diphenoloxidase activity, 10 mM catechol (Sigma Chemical, St. Louis, USA), 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma Chemical, St. Louis, USA) and 15 mM caffeic acid (Fluka Chemie) were used. The gels were pre-equilibrated in 0.05 M sodium phosphate buffer (pH 6.5) for 20 min prior to incubation at 30 °C in the different substrates, all dissolved using the same buffer (tyrosine was previously dis-

solved in HCl 0.1 mM). Four-amino-*N*,*N*-diethylaniline sulphate (ADA, Sigma–Aldrich, St. Louis, USA) and subsequently 4-*tert*-butyl-catechol (*t*BC, Fluka Chemie, Buchs, Switzerland) were also used to detect diphenoloxidase activity, according to Rescigno et al. (1997), with slight modifications. The gels were pre-equilibrated in 0.05 M sodium phosphate buffer pH 6.5. Then, ten ml of 25 mM ADA in 10 mM HCl were added to the gel. Pink spots appeared after 5–10 min, corresponding to laccase activity. The excess solution was poured off and the gel was washed twice with the above buffer. The gel was then immersed in 25 ml of 25 mM *t*BC dissolved in 10 mM acetic acid. The deep blue spots appeared after subsequent addition of *t*BC corresponded to diphenoloxidase activity.

A second electrophoretic run in native conditions was carried out to detect the effect of one or more specific inhibitors on PPO activity, using specific substrates. As specific inhibitors, 2–200 μg/ml 4-hexylresorcinol (Sigma Chemical, St. Louis, USA), and 9 mM tropolone (Fluka) were used. Antioxidants as sodium metabisulphite (5–100 μg/ml, Sigma Chemical, St. Louis, USA), and ascorbic acid (2–175 μg/ml, Sigma chemical) were also used. Tropolone, 4-hexylresorcinol (4-HR), sodium metabisulphite or ascorbic acid were added together with ADA + *t*BC. Tropolone was also added together with L-DOPA as substrate.

Finally, crude extracts were subjected to non-reducing SDS PAGE (6% 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 high molecular weight markers (Amersham Pharmacia Biotech) consisted of: α2 macroglobulin (170 kDa), β-galactosidase (116 kDa), transferring (76 kDa), and glutamic dehydrogenase (53 kDa). They were stained with Coomassie Brilliant Blue R-250. The remaining portion of the gels was stained with ADA – *t*BC or L-tyrosine as above.

An enzymatically inactive protein (i.e. BSA) was also subjected to an electrophoretic run (native or non-reducing SDS PAGE), and no reaction was observed when the gels were stained with the specific substrates above mentioned.

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

2.3. Mass spectrometry analysis of protein spots

The gel spots of interest were manually excised from micro preparative gels using biopsy punches. Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin according to Sechi and Chait (1998). Briefly, spots were washed twice with water, shrunk 15 min with 100% acetonitrile and dried in a Savant Speed-Vac for 30 min. Then the samples were 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, samples were 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

3.1. Native PAGE

Staining of the gel with specific substrates for mono- (L-tyrosine) or diphenoloxidases (catechol, L-DOPA, ADA – *t*BC, caffeic acid) showed that the extract contained two groups of high-molecular-weight proteins with different enzymatic activity (Fig. 1).

One group of proteins (526 and 450 kDa) exhibited monophenoloxidase and diphenoloxidase activity (Fig. 1g–i), as revealed by their positive reaction to the corresponding

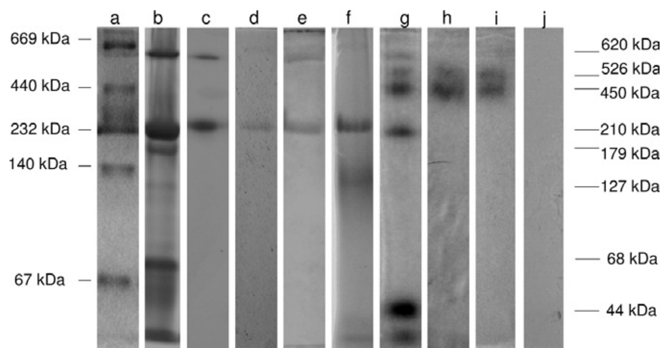

Fig. 1. Polyacrylamide gel electrophoresis (6%) under native conditions. Staining with specific substrates for PPO activity and with Coomassie Brilliant Blue R-250. (a) HMW Standard; (b, d, e, g–i) crude extract 48 h post mortem; (c, f, j) crude extract 0 h post mortem. The staining solutions were (a–c) Coomassie Brilliant Blue R-250; (d) caffeic acid; (e) Catechol; (f, g) ADA and subsequently with *t*BC; (h) L-DOPA; (i, j) tyrosine.

substrates. Diphenoloxidase activity was only observed with L-DOPA and *t*BC, and not with catechol or caffeic acid (Fig. 1d, e). These proteins were not stained with Coomassie Brilliant Blue R-250 (Fig. 1b), which suggests a low concentration in the crude extract. Both proteins did not present oxidase activity in the shrimp extract frozen on board immediately after capture (Fig. 1f, j). The other group of proteins (620 and 210 kDa) was very intensely stained by Coomassie Brilliant Blue R-250 (Fig. 1b, c) and exhibited diphenoloxidase activity (Fig. 1d, e, g), except for L-DOPA was used as substrate (Fig. 1h). Both proteins were found in the crude extract from shrimp frozen on board immediately after capture (Fig. 1c), but only the one with the lower molecular weight (210 kDa) presented diphenoloxidase activity (Fig. 1f).

Also, a protein band of about 44 kDa (Fig. 1g) appeared after staining with ADA alone and did not use either the monophenols or the diphenols mentioned above as substrates. Finally, in the electrophoretic profile stained with Coomasie there is a band corresponding to a protein of about 68 kDa lacking any phenoloxidase activity (Fig. 1b).

3.2. Non-reducing SDS-PAGE

Staining with specific substrates, in this case tyrosine for monophenoloxidases, or ADA + *t*BC for enzymes with diphenoloxidase activity, again showed up two groups of proteins with different enzymatic activity (Fig. 2).

One was a group of proteins (204 and 175 kDa) exhibiting both monophenoloxidase and diphenoloxidase activity (Fig. 2d, f). The proportion in the extract was not large enough to stain with Coomassie Blue (Fig. 2b). No such bands were observed in the crude extract from shrimp frozen on board after treatment with SDS (Fig. 2e, g).

Staining with ADA + *t*BC (Fig. 2d) and with Coomassie Brilliant Blue (Fig. 2b) revealed a second group of proteins with diphenoloxidase activity which were strongly present in the extract. Their molecular weights were approximately 218 and 126 kDa. Both protein bands also appeared in the extract frozen on board after capture (Fig. 2c).

Also, following addition of ADA a protein band of about 44 kDa appeared (Fig. 2d), as occurred in native conditions (Fig. 1g). Finally, staining with Coomassie Brilliant Blue clearly revealed an activity band corresponding to a protein of about 100 kDa (Fig. 2b) with no phenoloxidase activity.

3.3. Effect of specific inhibitors on phenoloxidase activity

Resorcinol derivatives are compounds which can inhibit browning reactions because of their structural resemblance to phenolic substrates. Fig. 3 shows the effect of 4-HR on crude extract taken 48 h post-capture, using ADA – *t*BC as substrate. The prooxidant activity of the 526 and 450 kDa proteins was inhibited by concentrations of 50 μg/ml (Fig. 3e). No such inhibiting effect was observed in the case of the 620 and 210 kDa proteins, even using 200 μg/ml of 4-HR. The activity of the 44 kDa protein band was likewise not inhibited. On the other hand 9 mM tropolone totally inhibited the oxidizing activity of the 526 and 450 kDa, as well as 620 and 210 kDa proteins when L-DOPA or ADA – *t*BC was used as substrate (Fig. 4).

Finally, two non-specific antioxidants, ascorbic acid (Fig. 5a1–3) and sodium metabisulphite (Fig. 5b1–3), were evaluated for inhibitory capacity. Concentrations of 7 μg/ml of ascorbic acid were enough to completely inhibit mono- and diphenoloxidase activity (Fig. 5a2). Regarding sulphites, 20 μg/ml of sodium metabisulphite totally inhibited the oxidizing activity of all the proteins in the gel (Fig. 5b3).

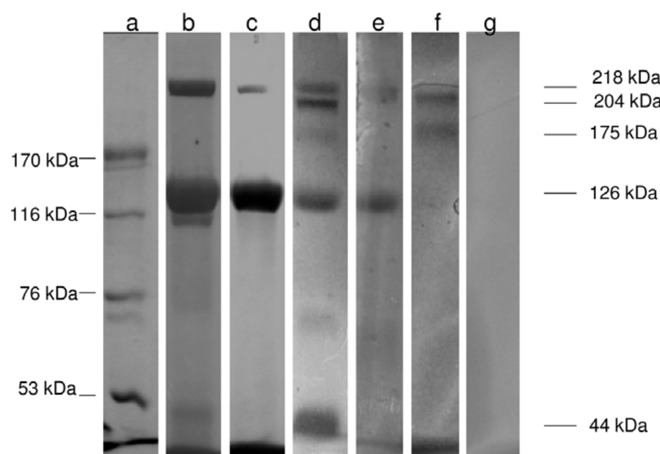

Fig. 2. Polyacrylamide gel electrophoresis (6%) with SDS in non-reducing conditions. The following extracts were used: (a) HMW pattern; (b, d, f) crude extract 48 h post mortem; (c, e, g) crude extract 0 h post mortem. The following staining solutions were used: (a–c) Coomassie Brilliant Blue R-250; (d, e) ADA + *t*BC; (f, g) tyrosine.

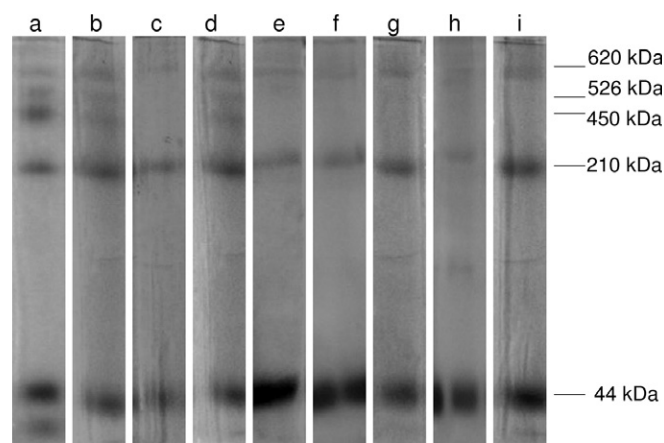

Fig. 3. Effect of 4-Hexylresorcinol (4-HR) on PPO activity in native PAGE (6% acrylamide). (a) ADA + *t*BC; (b) 2 μg/ml 4-HR + ADA + *t*BC; (c) 5 μg/ml 4-HR + ADA + *t*BC; (d) 30 μg/ml 4-HR + ADA + *t*BC; (e) 50 μg/ml 4-HR + ADA + *t*BC; (f) 60 μg/ml 4-HR + ADA + *t*BC; (g) 100 μg/ml 4-HR + ADA + *t*BC; (h) 150 μg/ml 4-HR + ADA + *t*BC; (i) 200 μg/ml 4-HR + ADA + *t*BC.

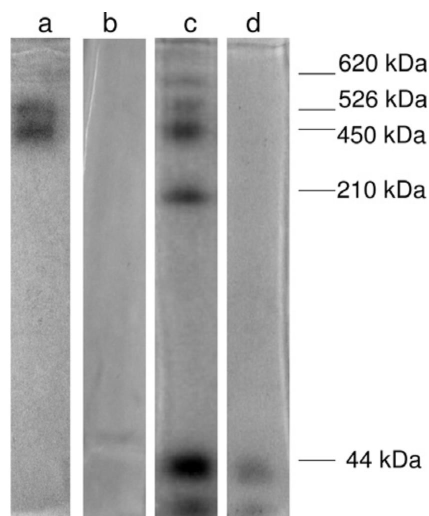

Fig. 4. Effect of tropolone on PPO activity in native PAGE (6% acrylamide). (a) L-DOPA; (b) L-DOPA + tropolone 9 mM; (c) ADA + *t*BC; (d) ADA + *t*BC + tropolone 9 mM.

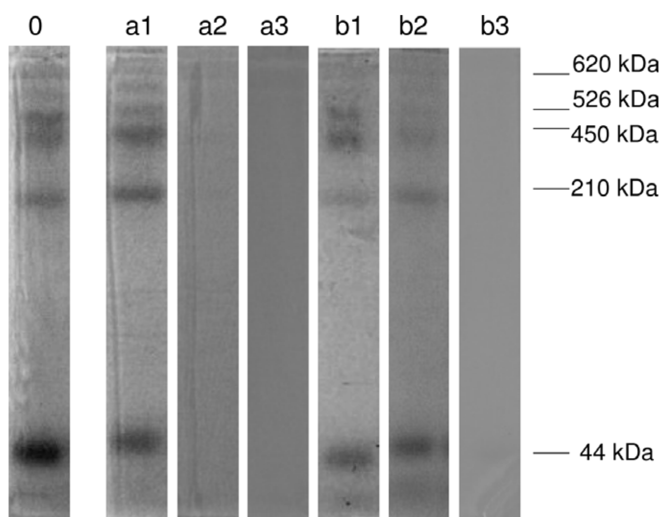

Fig. 5. Inhibitory effect of (a) ascorbic acid and (b) sodium metabisulphite on PPO activity in native PAGE (6% acrylamide). (0) ADA + *t*BC; (a1) 2 μg/ml ascorbic acid + ADA + *t*BC; (a2) 7 μg/ml ascorbic acid + ADA + *t*BC; (a3) 10 μg/ml ascorbic acid + ADA + *t*BC; (b1) 5 μg/ml sodium metabisulphite + ADA + *t*BC; (b2) 10 μg/ml sodium metabisulphite + ADA + *t*BC; (b3) 20 μg/ml sodium metabisulphite + ADA + *t*BC.

3.4. Mass spectrometry

The proteins reacting positively to ADA − *t*BC in both native and SDS-PAGE, tentatively identified as diphenoloxidase-like enzymes (620 and 210 kDa) and their oligomers (218 and 126 kDa), respectively, were cut out from the gel and, after tryptic digestion, subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The MALDI-TOF mass spectra are shown in Figs. 6 and 7. A primary database search using the peptide mass fingerprint from the four spots failed to reveal significant similarities with any known protein. For this reason, six peptides from the digested proteins, which appeared at the same time in the four spectra, were chosen for analysis by tandem mass spectrometry. The amino acid sequences of these peptides were obtained from the MALDI-TOF-TOF mass spectra after ion peptide fragmentation (Table 1).

When a database search was run on the tandem MS data, the peptides at 1203.6, 1769.8 and 1897.9 *m*/*z* showed significant homology with different hemocyanins. The peptide at 1203.6 *m*/*z* showed significant ($p < 0.05$) homology with hemocyanin of pacific white shrimp (*Litopenaeus vannamei*) (gi/7414468, total score: 32). The amino acid sequence fits the 88–96 amino acid residues identified in this protein, showing 1% sequence coverage. The peptides at 1769.8 and 1897.9 *m*/*z* exhibited a similar sequence (Table 1), with an additional arginine residue in the latter, while both showed extensive homology ($p < 0.05$) with a hemocyanin subunit three identified in Dungeness crab (*Cancer magister*) (Q5G2A6_CANMG, total score: 81). The sequence coverage was 2%, residues matching from 235 to 248, and 234 to 248, respectively. Another peptide at 1320.7 *m*/*z* also showed some homology with a hemocyanin B chain from narrow-fingered crayfish (*Astacus leptodactylus*), with a 75% sequence matching a similar length fragment of this protein (Schneider et al., 1986). The peptides at 1512.7 and 1698.8 *m*/*z* were sequenced *de novo*. The peptide at 1512 *m*/*z*, occurring as a high intensity ion peak in the MALDI-TOF spectra of all digested proteins (Figs. 6 and 7), was revealed by a SwissProt search to be a fragment of hemocyanin, presenting more than 72% identity with fragment sequences from hemocyanin subunits (score 60–62) in Green crab (*Carcinus aestuarii*), European spiny lobster (*Palinurus vulgaris*) and California spiny lobster (*Panulirus interruptus*). Similarly, the 1698.8 *m*/*z* peptide showed some homology with a phenoloxidase subunit two precursor from a silk moth, and also to a hexamerin precursor from a tropical cockroach, although scoring lower values (55 and 54, respectively).

On the other hand, the protein band with no phenoloxidase activity (Fig. 2b) of 100 kDa presented in the SDS-PAGE gel was also cut out and subjected to MALDI-TOF MS analysis after trypsin digestion. The peptide mass fingerprint yielded peptides in an *m*/*z* range from 883.34 to 3604.32 (Fig. 8). However, among peptide ions having high intensity ion peaks, there were no matches with peptides found in the above referred spectra. Five ion peptides were further fragmented; the amino acid sequences are presented in Table 2. A database search on peptides at 1125.5, 1499.8 and 1885.9 *m*/*z* showed significant homology with hemocyanin I from Norway lobster (*Nephrops norvegicus*) (gi/6118551, total scores: 29, 61 and 78), with 3%, 4% and 5% sequence coverage, respectively. The peptide at 1648.8 *m*/*z* presented high homology with a hemocyanin alpha-subunit from American lobster (*Homarus americanus*) (gi/7105883, total score: 78 and 1% sequence coverage). *De novo* sequencing was performed on the 1371.77

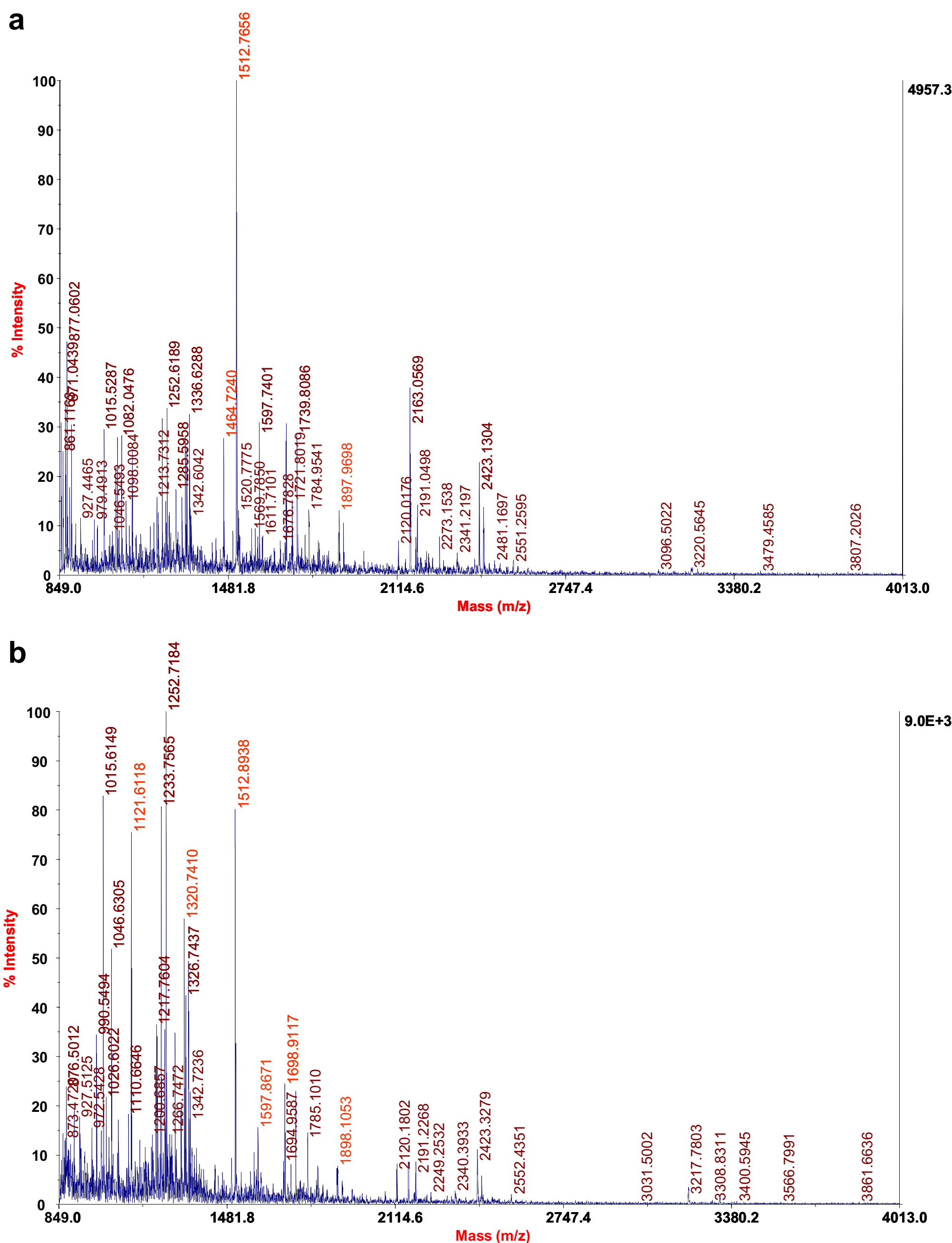

Fig. 6. MALDI-TOF MS peptide mass fingerprint (PMF) spectra of in gel tryptic digest (Native PAGE) of the protein bands of 620 kDa (a) and 210 kDa (b).

m/*z* peptide. The new fragment sequence again was strongly homologous with several hemocyanin subunits as described above, scoring values between 82% and 63%.

4. Discussion

4.1. Presence of enzymes with phenolase activity in the extract

Various methods have been reported for assessing the presence of enzymes with PPO activity in aqueous solution. One of these is detection on PAGE slab gels following incubation with specific substrates (Nellaiappan & Banu, 1991; Nellaiappan & Vinayagam, 1986; Rescigno et al., 1997). This method has the advantage that, using specific substrates, the presence of PPO can be detected with a high degree of sensitivity in both crude and purified extracts, while in addition the effect of endogenous inhibitors is avoided by separation of the components of the extract. Various different substrates have been successfully used, e.g. catechol, tyrosine or DOPA. Rescigno et al. (1997) reported consecutive use of ADA and *t*BC as substrate. *t*BC is a good substrate for PPO, whereas ADA can react with the *o*-quinones produced by the reaction between

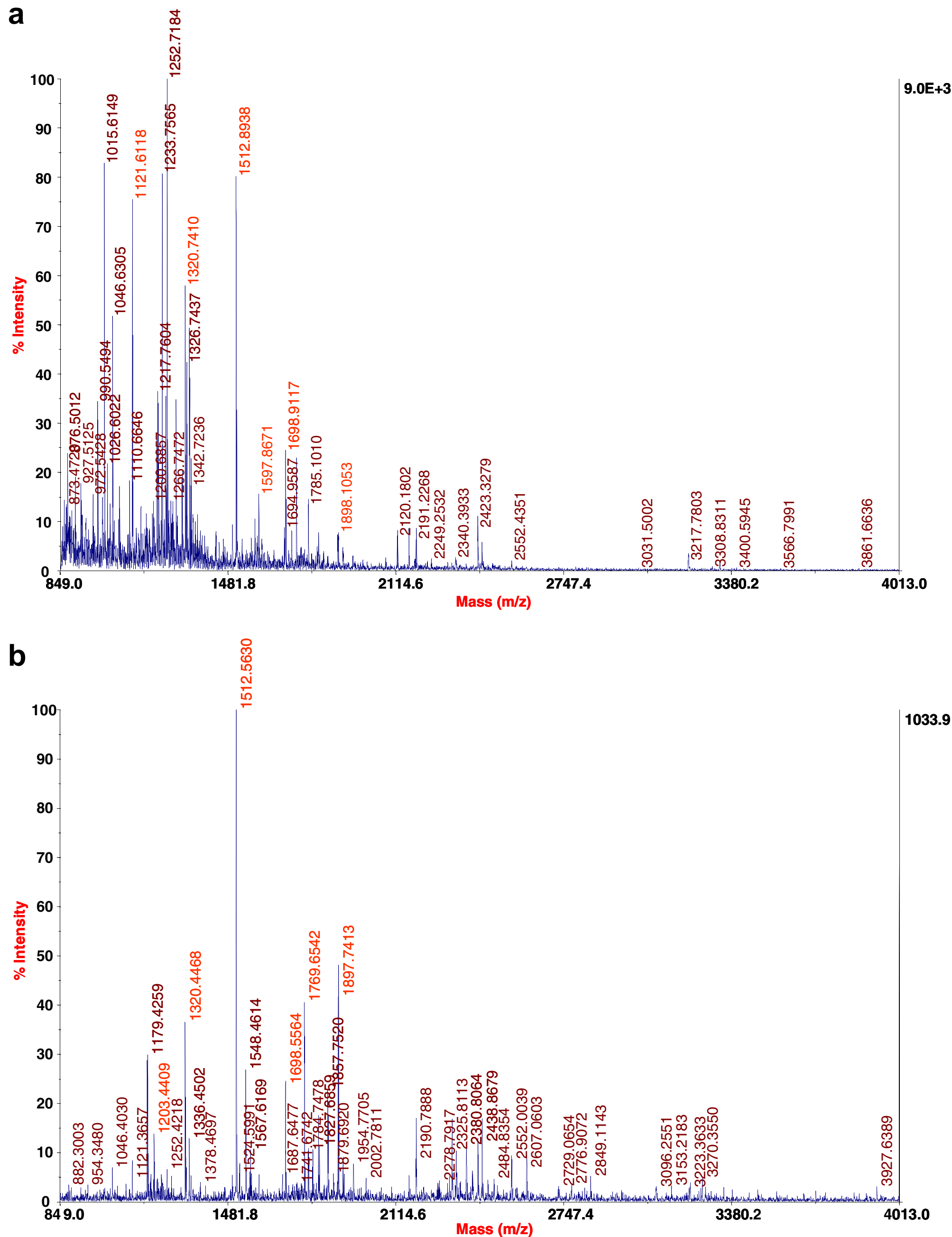

Fig. 7. MALDI-TOF MS peptide mass fingerprint (PMF) spectra of in gel tryptic digest (SDS-PAGE) of the protein bands of 218 kDa (a) and 126 kDa (b).

Table 1
Peptide sequences obtained by tandem mass spectrometry (MALDI-TOF-TOF) from protein bands appearing in both native and SDS-PAGE gels (620, 210, 218 and 126 kDa)

Monoisotropic mass (*m*/*z*)	Peptide sequence	Database
1203.6	RHWFSLFNPRQ	NCBI
1320.7	YGKGMPARVIGGV	SWISSPROT
1512.7	<u>LFTNSEVLEAAYR</u>	SWISSPROT
1698.8	<u>DFSYNLEVNNAGLSR</u>	SWISSPROT
1769.8	KGENFFWVHHQLTVRF	MSDB
1897.9	RKGENFFWVHHQLTVRF	MSDB

Underlined sequences were sequenced *de novo*.

PPO and *t*BC, creating a stable blue adduct in the area of the gel where the PPO is located.

In the crude extract of shrimp frozen 48 h post-capture, staining with different specific substrates revealed two groups of proteins with different enzymatic activities. In all cases, the high molecular weight of the protein was suggestive of an oligomeric nature.

The first group corresponded to proteins with mono- and diphenoloxidase activity, present in the extract at very low concentrations. The diphenoloxidase activity was only found with *t*BC or L-DOPA as substrate. These proteins could not be detected in the extract of shrimp frozen on

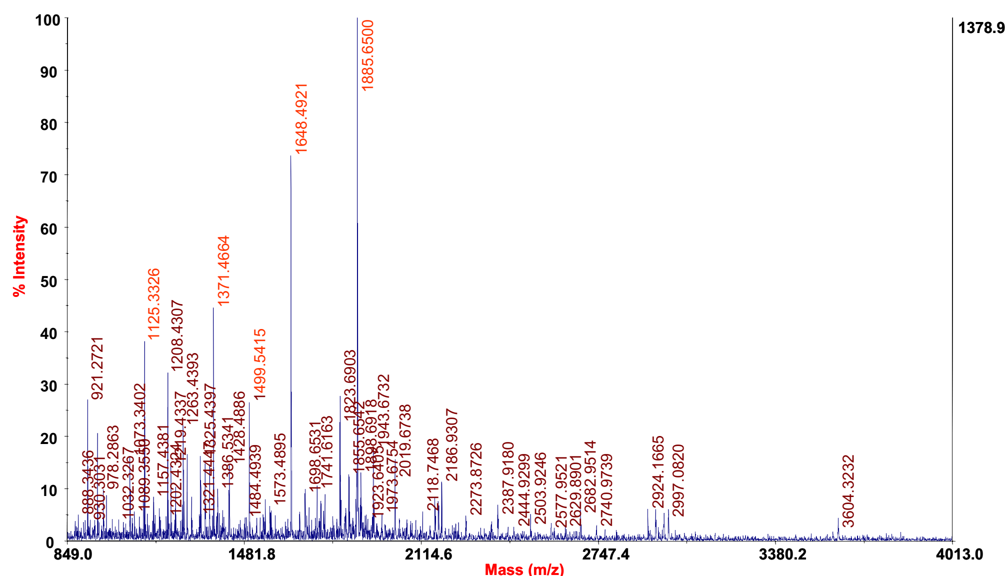

Fig. 8. MALDI-TOF MS peptide mass fingerprint (PMF) spectrum of in gel tryptic digest (SDS-PAGE) of the protein band of 100 kDa (a).

Table 2
Peptide sequences obtained by tandem mass spectrometry (MALDI-TOF-TOF) from the 120 kDa protein appearing in the SDS-PAGE gel

Monoisotropic mass (*m*/*z*)	Peptide sequence	Database
1125.5	KDSYGYHLDRK	NCBI
1371.7	IIHEGFAPHTSY	SWISSPROT
1499.8	RIIHEGFAPHTSYKY	NCBI
1648.8	KSWECFVDNAAFFRE	NCBI
1885.9	RLSNYLDPVDELHWERI	NCBI

Underlined sequence was sequenced *de novo*.

board post-capture (Fig. 1c, f), and no activation occurred following treatment with SDS (Fig. 2e, g). Presumably, another process, e.g. protease action, was responsible for their activation post-capture (Söderhäll & Smith, 1986). The monophenol tyrosine is the main substract of PPO in crustaceans (Rolle et al., 1991).The hydroxylation of tyrosine by PPO leads to the formation of dihydroxyphenylalanine (DOPA), which is also oxidised by PPO forming quinones. The presence of PPOs with the ability to oxidize mono- and diphenols was also reported by Kim, Marshall, and Wei (2000), and their presence in hemolymph and cuticle is slight compared with other proteins. These protein bands were therefore tentatively identified as PPOs. Their molecular weights were 526 and 450 kDa in native conditions (Fig. 1g, h, i), and 204 and 175 kDa following treatment with SDS (Fig. 2d and f). Given their high molecular weight, these protein bands could correspond to aggregates of PPO that survived treatment with SDS. This is possibly because the PPOs from several arthropods have "sticky" properties due to the low number of charged groups on their surface (Aspan & Söderhäll, 1991). The active phenoloxidase is therefore prone to aggregate, and these aggregates are resistant to SDS treatment. This aggregating effect even enables the subunits to bind with other proteins present in the medium. No band that could correspond to a simple subunit of lower molecular weight was clearly observed in the gels. These oligomers may therefore correspond to dimers, tetramers, pentamers, or hexamers, as has been reported in arthropods (Decker & Jaenicke, 2004). In European spiny lobster (*Palinurus elephas*) and freshwater crayfish (*Astacus leptodactylus*), Jaenicke and Decker (2003) reported an organization in hexamers made up of two stacked subunit trimers, in which the polyphenoloxidase subunit had a molecular weight of 71 kDa, giving a PPO of 526 kDa. The PPO of deepwater pink shrimp could have a similar structure, in which the 204 kDa oligomer is a trimer and the 526 kDa oligomer a hexamer. At the same time, the 175 kDa and 450 kDa protein bands could correspond to aggregates with different numbers of subunits, although they might correspond to another form of PPO. In this connection, two different forms of PPO, in hemolymph and in cuticle, have been found in other crustacean species (Kim et al., 2000; Nellaiappan et al., 1989). Other molecular weights, possibly corresponding to simple subunits, have also been reported in crustaceans, for example 40 kDa (pink shrimp, *Penaeus duodarum*, Simpson, Marshall, & Otwell, 1988); 97, 88, and 82 kDa (Florida spiny lobster, *Panulirus argus*, Chen, Rolle, Marshall, & Wei, 1991); 92 and 87 kDa (Western Australian lobster, Chen et al., 1991).

The second group of proteins present in the extract corresponded to two proteins of 620 and 210 kDa in native conditions, in high concentrations to judge by the intensity of staining with Coomassie Brilliant Blue R-250 (Fig. 1b), and exhibiting diphenoloxidase activity (Fig. 1d, e, g). However, they were unable to oxidise the diphenol L-DOPA (Fig. 1h). Both oligomers were present in the crude extract of shrimp frozen on board post-capture (Fig. 1c), although only the one with the lowest molecular weight presented diphenoloxidase activity (Fig. 1f). The crude extract was obtained from heads, including gill and

hepatopancreas, which would contain much of hemolymph (Sellos, Lemoine, & Van Wormhoudt, 1997; Yang, Perng, & Marshall, 1993). Given that Hc makes up over 90% of the constituent proteins (Adachi et al., 2005; Markl & Decker, 1992) of hemolymph, it seems likely that the two bands corresponded to two oligomers of Hc (Jaenicke & Decker, 2004). This fact was demonstrated by mass spectrometry, as it was previously described. However, Hc does not normally exhibit the ability to oxidize phenols, as a residue of Phe acts as a "placeholder" for potential substrates. The ability to oxidize phenols, and in particular diphenols, can be acquired in vitro following treatment with proteases or denaturing agents according to Jaenicke and Decker (2004). The only reported instance of in vivo acquisition of diphenoloxidase activity by Hc was in kuruma prawn (Adachi et al., 2005), although in that case the Hc also presented monophenoloxidase activity. It is possible that a similar process occurs in deepwater pink shrimp, which suggests that there may be another function of Hc in addition to oxygen transportation. Hc could acquire such activity because it has a role in the immune response (Adachi et al., 2003b; Decker & Jaenicke, 2004), or in moulting.

Furthermore, during storage the 620 kDa oligomer acquired diphenoloxidase activity (Fig. 1f, g) by means of one unidentified mechanism. This conversion did not seem to be the result of major proteolysis, given the absence of differences in electrophoretic mobility of the 620 kDa protein band immediately and 48 h after capture, as observed upon staining with Coomassie Blue (Fig. 1b, c). Nevertheless, this does not rule out the possibility of a functional conversion of Hc to PPO through minimal action of serine/cysteine proteases (Adachi et al., 2003b) or conversion by a mechanisms entailing no proteolytic cleavage. In this connection Nagai and Kawabata (2000) reported that a clotting enzyme and an unknown factor likewise implicated in the coagulation cascade may induce functional conversion of Hc to PPO in a chelicerate, the horseshoe crab *Tachypleus tridentatus*. In crustaceans, Adachi et al. (2003a) also reported that a cuticle protein of 160 kDa was a key factor in promoting melanosis in kuruma prawn by a cooperative reaction with hemocyanin-derived phenoloxidase.

In terms of quantity of protein, Fig. 1 showed the hemocyanin content in extract from shrimp frozen after mortem was apparently lower than that from a sample frozen after 48 h (Fig. 1b, c). It suggests a certain quantity of hemocyanin could be tightly bounded to carapace, but not be completely liberated during the extraction process. In this sense, Adachi et al. (2005) described the presence of hemocyanin associated with a complex of anti-bacterial peptides and chitin in kuruma prawn. Forty eight hours after capture, the endogenous enzymes could have weakened the links between hemocyanin and carapace, making the extraction process more effective.

In terms of composition, Hcs are made up of six subunits which may differ from one another, so that the protein bands of 620 and 210 kDa observed in deepwater pink shrimp in native conditions could correspond to two hexameric forms of Hc, or else to a hexamer and a dimer with diphenoloxidase activity. Following treatment with SDS, the forms of Hc with only diphenoloxidase activity presented molecular weights of approximately 218 and 126 kDa. The two protein bands also appeared in the extract frozen post-capture (Fig. 2c) and did not appear to correspond to simple subunits of Hc in view of their high molecular weight. Although they do not have "sticky" properties, subunits of Hc can on occasions be joined with a disulphide bond (Adachi et al., 2003b), which would account for their possibly being in the form of oligomers in the presence of SDS. Nellaiappan et al. (1989) also found diphenoloxidases in other crustacean species, possibly the result of aggregation following unreduced SDS PAGE. In that case, however, the molecular weights were lower (140, 250 and 350 kDa). Hc dimers and trimers of between 220 and 165 kDa have also been reported in kuruma prawn following unreduced SDS PAGE (Adachi et al., 2003b), although these presented no PPO activity. The molecular weight(s) of the Hc subunit(s) may be in the region of 100 kDa, assuming that the 620 kDa could correspond to a hexamer. In this respect, Fig. 2b clearly shows the presence, after treatment with SDS, of a band corresponding to a protein of around 100 kDa, which could be a subunit with no diphenoloxidase activity. However, the presence of this protein on the crude extract could also be the result of a process of hydrolysis during storage, as it was not observed in the extract of shrimp frozen on board post-capture (Fig. 2c). The protein band of around 68 kDa, which was stained with Coomassie Blue (Fig. 1b) in native conditions and lacked phenoloxidase activity, could also correspond to an inactive subunit, while the protein band of about 179 kDa (Fig. 1b) in the same gel could correspond to an inactive aggregate, or to another protein present in the extract. Hc subunits have been reported with somewhat lower molecular weights: 67 and 77 kDa, in *Penaeus japonicus* (Adachi et al., 2001), with weights of 66–75 kDa in crayfish *Pacifastacus leniusculus* (Lee et al., 2004), and of 70 kDa in the chelicerate horsheshoe crab (Nagai & Kawabata, 2000).

Finally, staining with ADA prior to addition of *t*BC revealed an activity band corresponding to an enzyme of about 44 kDa composed of a single subunit (Figs. 1 and 2 g, d). Since ADA is a magnificent substrate for laccases (Rescigno et al., 1997), it is possible that this activity band corresponds to a laccase, although it might actually correspond to a subunit of PPO or Hc with laccase activity.

As regards the effect of specific inhibitors of mono- or diphenoloxidase activity, tropolone and 4-hexylresorcinol (4-HR) behaved in different ways. Resorcinol derivatives are compounds which can inhibit browning reactions because of their structural resemblance to phenolic substrates. The effectiveness of 4-HR as a melanosis-inhibiting agent has been demonstrated in deepwater pink shrimp by Montero, Martínez-Álvarez, Zamorano, Alique, and Gómez-Guillén (2006). 4-HR acts as an enzyme-competitive inhibitor, specifically inhibiting PPO, and in that

respect concentrations of 50 μg/ml were sufficient to inhibit the activity of the proteins tentatively identified as PPOs (526 and 450 kDa, Fig. 3e). However, no inhibitory effect was observed on presumptive Hc (620 and 210 kDa) even although this presented similar activity to PPO. 4-HR has been described as slow-binding inhibitor (Jiménez & García-Carmona, 1997), and for that reason its inhibitory effect could have been showed after production of stained band by oxidation of *t*BC. An inhibitory effect of 4-HR on Hc has also been reported by Adachi et al. (2001, 2005) in kuruma prawn. On the other hand tropolone, as a specific inhibitor of PPO by copper chelation and/or competition with substrates (Rescigno, Sollai, Pisu, Rinaldi, & Sanjust, 2002), did exhibit inhibitory effects both on presumptive PPO and on Hc.

Finally, the two non-specific antioxidants assessed here, ascorbic acid and sodium metabisulphite, inhibited mono- and diphenoloxidase activities in the extract at very low concentrations, 7 and 20 μg/ml, respectively (Fig. 5a2 and b3). The basis of the inhibitory mechanism of ascorbic acid is reduction of the *o*-quinones that form to their diphenol precursors, thus preventing pigment formation. As for sulphites, these irreversibly inhibit PPO (Ricquebourg, Robert-Da Silva, Rouch, & Cadet, 1996); they may interact with the quinones formed, and they may also reduce the coloured *ortho*-quinones back to colourless and less reactive diphenols. According to Kim et al. (2000), when only monophenols are present, only a very small amount of sulphites is required. However, inhibition of diphenoloxidases requires larger amounts of sulphites. In the present case, the same concentration of sodium metabisulphite (20 μg/ml) totally inhibited both phenoloxidases (Fig. 5b).

Mass spectrometry confirmed that the different bands exhibiting only diphenoloxidase activity in both native and SDS-PAGE gels (620, 210, 218 and 126 kDa) corresponded to hemocyanin. These activity bands corresponded to oligomeric forms presumably generated by the same type of subunits but in different numbers. The similarity of the common peptides to different forms of hemocyanin described in the literature further suggests that these subunits may not be identical to one another, a fact already reported by other authors, such as Jekel, Neuteboom, and Beintema (1996) in other species. In the case of white shrimp (*Litopenaeus vannamei*) Hc, which presents points of similarity with *Parapenaeus longirostris* Hc aggregates, this kind of heterogeneity has also been reported along with an absence of disulphide bridges in the subunits (Sellos et al., 1997). This produces some flexibility in the molecule that is essential for cooperative oxygen binding, and it is not impossible that the structure possesses similar flexibility in deepwater pink shrimp, allowing the substrates to access the active centre. However, the fact that the 620 kDa complex does not present activity post-capture but does so after 48 h suggests that, in addition to a conformational change induced by the bonding of the subunits, there is another factor causing the acquisition of diphenoloxidase activity, presumably the action of some endogenous protease.

At the same time, among the principal proteins present in the extract which lack oxidase activity, there was one with a molecular weight of approximately 100 kDa. This protein was presumably a hemocyanin to judge by its homology with various different forms of hemocyanin described in the literature, but it was not the same as the forms of hemocyanin with diphenoloxidase activity. This indicates once again that in deepwater pink shrimp various subunits of Hc exist, but these possibly perform different physiological functions. In this connection, the composition of hemocyanins is reported to be highly variable. Some units may be expressed only in certain developmental stages or under specific physiological conditions (Markl & Decker, 1992). This fact has been described in other crustacean species. For instance they have been observed to function as buffers and osmolytes, carriers of moulting hormones, and probably as parts of the cuticle (Decker & Jaenicke, 2004). In deepwater pink shrimp (*Parapenaeus longirostris*), then, hemocyanin may also perform different functions, which are determined by the types of subunits comprising the hemocyanin.

5. Conclusion

This article shows that in freshly-caught deepwater pink shrimp there is an active form of Hc which presents diphenoloxidase activity. This activity appeared to be present in the live shrimp, and in vitro activation was not necessary. During storage, a form of Hc with a higher molecular weight also acquires such activity through a mechanism which is unidentified, although we have two hypotheses relating to mechanisms reported in the literature. The two oligomeric forms of Hc with diphenoloxidase activity seem likely to be chiefly responsible, more than PPO, for the intensity of the melanosis observed in this species. There is also at least one form of hemocyanin, different form the ones mentioned above, which lacks diphenoloxidase activity and is composed of different subunits. Two presumed oligomeric forms of PPO have also been found in the cephalothorax, in very small proportions but exhibiting intense activity. Given the difficulties involved in inhibiting melanosis in deepwater pink shrimp by means of authorized concentrations of sulphites, there is a need to find effective alternatives to inhibit the onset of melanosis in this species and prolong storage life without the risk of health problems for consumers. Future research should concentrate on characterizing Hc, examining the kinetics of the effect of PPO inhibitors on Hc, and searching for new anti-browning agents to add to commercial formulae.

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