1   **PRESENCE OF HEMOCYANIN WITH DIPHENOLOXIDASE ACTIVITY**
2   **IN DEEPWATER PINK SHRIMP (*Parapenaeus longirostris*) POST MORTEM**

3
4   Oscar Martínez-Alvarez*, Carmen Gómez-Guillén and Pilar Montero

5   Instituto del Frío (C.S.I.C.), José Antonio Novais 10, 28040 Madrid (Spain)

6   Tel: +345492300; Fax: +345493627

7
8   Author   to   whom   correspondence   should   be   addressed   [e-mail
9   oscar_martine50@hotmail.com]

10   **Abbreviated running title: Oxidant hemocyanin in deepwater pink shrimp**

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.

Keywords: polyphenoloxidase, hemocyanin, melanosis, deepwater pink shrimp, *Parapenaeus longirostris*.

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 and 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 and Tucek, 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 and Tucek, 2000; Decker and Jaenicke, 2004).

Also, hemocyanin (Hc) is found in hemolymph, and some authors suggest in cuticle as well (Adachi, Hirata, Fujio, Nishioka, & Sakaguchi, 2003a; Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005). 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 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 and 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 (Nagay and 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 (Decker and Jaenicke, 2004; Lee, Lee, & Söderhäll, 2004; Adachi, Hirata, Nishioka, & Sakaguchi, 2003b). As to their polymeric disposition, hemocyanins of arthropods form hexamers (Adachi et al, 2005; Decker and Jaenicke, 2004; Jaenicke and 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 x 6 subunits (Marckl and 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 enzyme 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.

MATERIAL 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 hours after capture were stored at -80°C until further analysis.

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 cephalothorax 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 $50000 \times g$, 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. Phenylmethanesulfonyl fluoride (PMSF), as inhibitor of serine and some cysteine proteases, was added to the crude preparation (1 mg/ml) to prevent proteolysis by native proteases.

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 began 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 dissolved 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, Sanjust, Montanari, Sollai, Soddu, Rinaldi, Oliva, & Rinaldi (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 minutes, 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, 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), transferrin (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 1-D Manager (version 2.0) image analysis and quantification tool (Tecnología para diagnóstico e Investigación, S.A., Spain).

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 SpeedVac for 30 min. Then the samples were reduced with 10mM dithioerythritol 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 3mg/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 20000 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>).

RESULTS

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, h, i), as revealed by their positive reaction to the corresponding 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 Coomassie there is a band corresponding to a protein of about 68 kDa lacking any phenoloxidase activity (Fig. 1b).

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 and 2f). 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.

Effect of specific inhibitors on phenoloxidase activity

Resorcinol derivatives are compounds which can inhibit browning reactions because of their structural resemblance to phenolic substrates. Figure 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 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).

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 Fig. 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 to 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 3 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, Voll, Lehmann, Grisshammer, Goettgens, & Linzen, 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 (Fig. 6, 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 2 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 (Figure 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 m/z peptide. The new fragment sequence again was strongly homologous with several hemocyanin subunits as described above, scoring values between 82 and 63 %.

DISCUSSION

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 and Vinayagam, 1986; Nellaiappan and Banu, 1991; 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 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 hours 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 board post-capture (Fig. 1c, f), and no activation occurred following treatment with SDS (Fig. 2e, 2g). Presumably, another process, e.g. protease action, was responsible for their activation post-capture (Söderhäll and Smith, 1986). The monophenol tyrosine is the main substrate of PPO in crustaceans (Rolle, Guizani, Chen, Marshall, Yan & Wei, 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, & 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

306 SDS (Fig. 2d and 2f). Given their high molecular weight, these protein bands could
307 correspond to aggregates of PPO that survived treatment with SDS. This is possibly because
308 the PPOs from several arthropods have “sticky” properties due to the low number of charged
309 groups on their surface (Aspan and Söderhäll, 1991). The active phenoloxidase is therefore
310 prone to aggregate, and these aggregates are resistant to SDS treatment. This aggregating
311 effect even enables the subunits to bind with other proteins present in the medium. No band
312 that could correspond to a simple subunit of lower molecular weight was clearly observed in
313 the gels. These oligomers may therefore correspond to dimers, tetramers, pentamers, or
314 hexamers, as has been reported in arthropods (Decker and Jaenicke, 2004). In European spiny
315 lobster (*Palinurus elephas*) and freshwater crayfish (*Astacus leptodactylus*), Jaenicke and
316 Decker (2003) reported an organization in hexamers made up of two stacked subunit trimers,
317 in which the polyphenoloxidase subunit had a molecular weight of 71 kDa, giving a PPO of
318 526 kDa. The PPO of deepwater pink shrimp could have a similar structure, in which the 204
319 kDa oligomer is a trimer and the 526 kDa oligomer a hexamer. At the same time, the 175 kDa
320 and 450 kDa [protein](#) bands could correspond to aggregates with different numbers of
321 subunits, although they might correspond to another form of PPO. In this connection, two
322 different forms of PPO, in hemolymph and in cuticle, have been found in other crustacean
323 species (Kim et al., 2000; Nellaiappan et al., 1989). Other molecular weights, possibly
324 corresponding to simple subunits, have also been reported in crustaceans, for example 40 kDa
325 (pink shrimp, *Penaeus duodactylus*, Simpson, Marshall & Otwell, 1988); 97, 88, and 82 kDa
326 (Florida spiny lobster, *Panulirus argus*, Chen, Rolle, Marshall & Wei, 1991); 92 and 87 kDa
327 (Western Australian lobster, Chen et al, 1991).

328 The second group of proteins present in the extract corresponded to two proteins of 620 and
329 210 kDa in native conditions, in high concentrations to judge by the intensity of staining with
330 Coomassie Brilliant Blue R-250 (Fig. 1b), and exhibiting diphenoloxidase activity (Fig. 1d,

1e, 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 (Yang, Perng, & Marshall, 1993; Sellos, Lemoine, & Van Wormhoudt, 1997). Given that Hc makes up over 90% of the constituent proteins (Marckl and Decker, 1992; Adachi et al., 2005) of hemolymph, it seems likely that the two bands corresponded to two oligomers of Hc (Jaenicke and 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 (Decker and Jaenicke, 2004; Adachi et al., 2003b), 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 hours 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 to 75 kDa in crayfish *Pacifastacus leniusculus* (Lee et al, 2004), and of 70 kDa in the chelicerate horseshoe crab (Nagai and 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 (Fig. 1g, 2d). 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

406 compounds which can inhibit browning reactions because of their structural resemblance to
407 phenolic substrates. The effectiveness of 4-HR as a melanosis-inhibiting agent has been
408 demonstrated in deepwater pink shrimp by Montero, Martínez-Álvarez, Zamorano, Alique, &
409 Gómez-Guillén (2006). 4-HR acts as an enzyme-competitive inhibitor, specifically inhibiting
410 PPO, and in that respect concentrations of 50 µg/ml were sufficient to inhibit the activity of
411 the proteins tentatively identified as PPOs (526 and 450 kDa, Fig. 3e). However, no
412 inhibitory effect was observed on presumptive Hc (620 and 210 kDa) even although this
413 presented similar activity to PPO. 4-HR has been described as slow-binding inhibitor
414 (Jiménez and García-Carmona, 1997), and for that reason its inhibitory effect could have
415 been showed after production of stained band by oxidation of *t*BC. An inhibitory effect of 4-
416 HR on Hc has also been reported by Adachi et al. (2001, 2005) in kuruma prawn. On the
417 other hand tropolone, as a specific inhibitor of PPO by copper chelation and/or competition
418 with substrates (Rescigno, Sollai, Pisu, Rinaldi, & Sanjust, 2002), did exhibit inhibitory
419 effects both on presumptive PPO and on Hc.

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

430 concentration of sodium metabisulphite (20 µg/ml) totally inhibited both phenoloxidases
431 (Fig. 5b).

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

449 At the same time, among the principal proteins present in the extract which lack oxidase
450 activity, there was one with a molecular weight of approximately 100 kDa. This protein was
451 presumably a hemocyanin to judge by its homology with various different forms of
452 hemocyanin described in the literature, but it was not the same as the forms of hemocyanin
453 with diphenoloxidase activity. This indicates once again that in deepwater pink shrimp
454 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 (Marckl and 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 and 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.

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 from 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.

ACKNOWLEDGEMENTS

This research was financed under project between Consejería de Agricultura y Pesca (Junta de Andalucía) and Consejo Superior de Investigaciones Científicas (C.S.I.C.).

REFERENCES

1. Adachi, K., Hirata, T., Nagai, K., & Sakaguchi, M. (2001). Hemocyanin: a most likely inducer of black spots in kuruma prawn *Penaeus japonicus* during storage. *Journal of Food Science*, 66, 1130-1136.
2. Adachi, K., Hirata, T., Fujio, A., Nishioka, T., & Sakaguchi, M. (2003a). A 160-kDa is essential for hemocyanin-derived melanosis of prawn. *Journal of Food Science* 68(3), 765-769.
3. Adachi, K., Hirata, T., Nishioka, T., & Sakaguchi, M. (2003b). Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. *Comparative Biochemistry and Physiology Part B*, 134, 135-141.
4. Adachi, K., Endo, H., Watanabe, T., Nishioka, T., & Hirata, T. (2005). Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization. *Pigment Cell Research*, 18, 136-143.
5. Aspan, A., Huang, T.S., Cerenius, L., & Söderhäll, K. (1995). cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. *Proceedings of the National Academy of Sciences of USA*, 99, 939-945.
6. Aspan, A., & Söderhäll, K. (1991). Purification of prophenoloxidase from crayfish blood-cells, and its activation by an endogenous serine proteinase. *Insect Biochemistry*, 21 (4), 363-373.
7. Chen, J.S., Rolle, R.S., Marshall, M.R., & Wei, C.I. (1991). Comparison of phenoloxidase activity from Florida spiny lobster and Western Australia lobster. *Journal of Food Science*, 56, 154-160.

- 505 8. Decker, H., & Jaenicke, E. (2004). Recent findings on phenoloxidase activity and
506 antimicrobial activity of hemocyanins. *Developmental and Comparative Immunology*,
507 28, 673-687.
- 508 9. Decker, H., & Tuzek, F. (2000). Tyrosinase/catecholoxidase activity of hemocyanins:
509 structural basis and molecular mechanism. *Trends in Biochemical Sciences*, 25, 392-
510 397.
- 511 10. Jaenicke, E., & Decker, H. (2003). Tyrosinases from crustaceans form hexamers.
512 *Biochemical Journal*, 371 (Part 2), 515-523.
- 513 11. Jaenicke, E., & Decker, H. (2004). Conversion of crustacean hemocyanin to
514 catecholoxidase. *Micron*, 35, 89-90.
- 515 12. Jekel, P.A., Neuteboom, B., Beintema, J.J. (1996). Primary structure of hemocyanin
516 from *Palinurus vulgaris*. *Comparative Biochemistry and Physiology B*, 115 (2), 243-
517 246.
- 518 13. Jiménez, M., & García-Carmona, F. (1997). 4-Substituted resorcinols (sulfite
519 alternatives) as slow-binding inhibitors of tyrosinase catecholase activity. *Journal of*
520 *Agricultural and Food Chemistry*, 45, 2061-2065.
- 521 14. Kim, J., Marshall, M.R., & Wei, C. (2000). Polyphenoloxidase. In F. Norman, & K.
522 Benjamin, *Seafood enzymes. Utilization and influence on post harvest seafood quality*,
523 pp. 271-315. New York: Marcel Decker.
- 524 15. Lee, S. Y., Lee, B. L., & Söderhäll, K. (2004). Processing of crayfish hemocyanin
525 subunits into phenoloxidase. *Biochemical and Biophysical Research Communications*,
526 322, 490-496.
- 527 16. Markl, J., & Decker, H. (1992). Molecular structure of the arthropod hemocyanins. In
528 C.P. Magnum, *Advances of Environmental Biochemistry and Physiology*, pp. 325–376.
529 Berlin: Springer-Verlag.

- 530 17. Montero, P., Martínez-Álvarez, O., Zamorano, P., Alique, R. & Gómez-Guillén, M.C.
531 (2006). Melanosis inhibition and 4-hexylresorcinol residual levels in deepwater pink
532 shrimp (*Parapenaeus longirostris*) following various treatments. *European Food*
533 *Research and Technology*, 223, 16-21.
- 534 18. Nagai, T. & Kawabata, S. (2000). A link between blood coagulation and prophenol
535 oxidase activation in arthropod host defense. *Journal of Biological Chemistry*, 275,
536 29264-29267.
- 537 19. Nellaiappan, K. & Vinayagam, A. (1986). A rapid method for detection of tyrosinase
538 activity in electrophoresis. *Stain Technology*, 61, 269-272.
- 539 20. Nellaiappan, K., Vinayagan, A., & Kalyani, R. (1989). Electrophoretic pattern of blood
540 and cuticular phenoloxidase of different crustaceans. *Experimental Biology*, 48, 177-
541 179.
- 542 21. Nellaiappan, K. & Banu, M. (1991). Demonstration of monophenoloxidase activity of
543 tyrosinase after electrophoresis. *Biotechnic & Histochemistry*, 66, 125-130.
- 544 22. Rescigno, A., Sanjust, E., Montanari, L., Sollai, F., Soddu, G., Rinaldi, A.C., Oliva, S.,
545 & Rinaldi, A. (1997). Detection of laccase, peroxidase, and polyphenol oxidase on a
546 single polyacrylamide gel electrophoresis. *Analytical Letters*, 30(12), 2211-2220.
- 547 23. Rescigno, A., Sollai, F., Pisu, B., Rinaldi, A., Sanjust, E. (2002). Tyrosinase inhibition:
548 general and applied aspects. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 17,
549 207-218.
- 550 24. Ricquebourg, S.L., Robert-Da Silva, C.M.F., Rouch, C.C., & Cadet, F.R. (1996).
551 Theoretical support for a conformational change of polyphenol oxidase induced by
552 metabisulfite. *Journal of Agricultural and Food Chemistry*, 44, 3457-3460.

- 553 25. Rolle, R. S., Guizani, N., Chen, J. S., Marshall M. R., Yan, J. S., & Wei, C. I. (1991).
554 Purification and characterization of phenoloxidase isoforms from Taiwanese black tiger
555 shrimp (*Penaeus monodon*). *Journal of Food Biochemistry*, 15, 17-32.
- 556 26. Schneider, H.J., Voll, W., Lehmann, L., Grisshammer, R., Goettgens, A., & Linzen B.
557 (1986). Partial amino acid sequence of crayfish (*Astacus leptodactylus*) hemocyanin. In
558 B. Linzen, *Invertebrate Oxygen Carriers* (pp.173-176), Heidelberg: Springer-Verlag.
- 559 27. Sechi, S. & Chait, B.T. (1998). Modification of cysteine residues by alkylation. A tool
560 in peptide mapping and protein identification. *Analytical Chemistry*, 70, 5150-5158.
- 561 28. Sellos, D., Lemoine, S., & Van Wormhoudt, A. (1997). Molecular cloning of
562 hemocyanin cDNA from *Penaeus vannamei* (Crustacea, Decapoda): structure,
563 evolution and physiological aspects. *FEBS Letters*, 407 (2), 153-158.
- 564 29. Simpson, B. K., Marshall, M. R., & Otwell, W. S. (1988). Phenoloxidases from pink
565 and white shrimp: kinetic and other properties. *Journal of Food Biochemistry*, 12, 205-
566 217.
- 567 30. Söderhäll, K., & Smith, V.J. (1986). The prophenoloxidase activating system: the
568 biochemistry of its activation and role in arthropod cellular immunity, with special
569 reference to crustaceans. In M. Brehélin, *Immunity in invertebrates. Cells, molecules,*
570 *and defence reactions*, pp. 209-223. Berlin Heidelberg: Springer-Verlag.
- 571 31. Wang, Z. K. D., Taylor, A., & Yan, X. (1992). Studies on the protease activities in
572 Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation
573 process. *Food Chemistry*, 45, 111–116.
- 574 32. Williams, H.G., Davidson, G.W., & Mamo, J.C. (2003). Heat-induced activation of
575 polyphenoloxidase in western rock lobster (*Panulirus cygnus*) hemolymph: implications
576 for heat processing. *Journal of Food Science*, 68 (6), 1928-1932.

33. Yang, J.S., Perng F.S., & Marshall M. R. (1993). Immunohistochemical localization of phenoloxidase in hepatopancreas, muscle and epidermis of grass shrimp (*Penaeus monodon* f.). *Journal of Food Biochemistry*, 17, 115-124.

FIGURE CAPTIONS

- Figure 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) 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.

- Figure 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, b, c) Coomassie Brilliant Blue R-250; d, e) ADA+*t*BC; f-g) Tyrosine.

- Figure 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.

- Figure 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.

- Figure 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

601 metabisulphite+ADA+*t*BC; b2) 10 µg/ml sodium metabisulphite+ADA+*t*BC; b3) 20 µg/ml
602 sodium metabisulphite+ADA+*t*BC.

603 - Figure 6. MALDI-TOF MS peptide mass fingerprint (PMF) spectra of in gel tryptic digest
604 (Native PAGE) of the protein bands of 620 kDa (A) and 210 kDa (B).

605 - Figure 7. MALDI-TOF MS peptide mass fingerprint (PMF) spectra of in gel tryptic digest
606 (SDS-PAGE) of the protein bands of 218 kDa (A) and 126 kDa (B).

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

609

610 **TABLE CAPTIONS**

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

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

616

617

618 **TABLES**

619 Table 1

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

620 Underlined sequences were sequenced *de novo*.

621

Table 2

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

Underlined sequence was sequenced *de novo*.

FIGURES

Figure 1

649
650
651

652 Figure 2

653
654
655

656

657 Figure 3

658

659

660

661 Figure 4

662

663

664

665 Figure 5

666

667

Figure 6.

A)

B)

Figure 7.

A)

B)

751 Figure 8:
752
753
754