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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.

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-