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Phenoloxidase Activity of Hemocyanin in Whiteleg Shrimp *Penaeus vannamei*: Conversion, Characterization of Catalytic Properties, and Role in Postmortem Melanosis

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Latent phenoloxidase activity of hemocyanin (Hc) in whiteleg shrimp *Penaeus vannamei* was assayed to determine its potential involvement in postmortem melanosis. Conversion of pure 12-mer, but not 6-mer, hemocyanin to phenoloxidase by endogenous (serine proteinases) and exogenous (SDS) effectors demonstrated the need of complex aggregation for displaying enzyme activity. Because Hc was converted to Hc-phenoloxidase (HcPO) by hemocytes extracts, the mechanism of conversion seems to be the same for polyphenoloxidases. HcPO has similar biochemical and kinetic properties as real polyphenoloxidases and uses mono- and diphenols as substrates. The kinetics of hydroxygenation of monophenols has a lag phase, typical for tyrosinases, contrary to oxidation of diphenols. Regardless of the structure of the substrate, melanin is finally formed. Because of the abundance, distribution, and resistance of Hc to freezing−thawing, involvement of Hc in black spot formation postmortem is suggested. This has important implications for commercialization of shrimp and related seafood.

KEYWORDS: Hemocyanin; melanosis; phenoloxidase activity; decapod crustaceans; *Penaeus vannamei*

INTRODUCTION

The copper-binding protein, hemocyanin (Hc), is present in the hemolymph of mollusks and arthropods as high-molecular weight oligomers (*1*). In crustaceans, this dioxygen-carrier protein is synthesized in the midgut gland and transported at high concentrations to hemolymph, comprising 90−95% of the total plasma proteins. The basic structure of all arthropod Hc oligomers is hexamer. In decapods, however, the hexamer (6-mer; 1 × 6) and the dodecamer (12-mer; 2 × 6) forms are common. Crustacean Hc 6-mer proteins can cluster to form aggregates up to 48 (8 × 6) subunits, resulting in molecular masses ranging from 450 to 3900 kDa.

Although this blue protein is mostly known as a dioxygen transporter, Hc is a multicopper protein that can display a remarkable range of functions, including transporting ecdysone, functioning as an osmolyte and storing protein, and acting as a precursor of antimicrobial peptides. Furthermore, Hc can display phenoloxidase (PO) activity when exposed to chemical reagents, such as percholate and SDS (*2, 4*), and endogenous defense molecules, such as clotting factors and antibacterial peptides (*5, 6*), and proteolytic enzymes, most commonly serine proteinases (*7, 8*). This has economic consequences because many crustaceans are consumed by humans, and postmortem melanosis is associated with spoilage, which makes the food product unacceptable to consumers and reduces the value (*9*) of important commercial shrimp, lobster, and crab fisheries, although the discoloration is not a health hazard.

Tyrosinase and cathecoloxidase are commonly called phenoloxidases (*10*). Tyrosinase (PO; monophenol, L-DOPA: oxygen oxidoreductase, EC 1.14.18.1), commonly called polyphenoloxidase, is a copper protein that catalyzes oxygenation of monophenols to *o*-diphenols, which oxidizes to the corresponding *o*-quinones (*11*). This enzyme is responsible for catalyzing two basic reactions, using monophenols, along with O_2, as substrates. The enzyme catalyzes hydroxylation to the *o*-position adjacent to an existing hydroxyl group, the monophenoloxidase activity. The second reaction, by diphenoloxidase activity, is the oxidation of the diphenol to *o*-benzoquinone, products that are further oxidized to melanin (brown or black products), usually by nonenzymatic reactions (*9*). Cathecoloxidase (EC 1.10.3.1), by itself, is capable of carrying out only diphenoloxidase activity. In crustaceans, PO activity is achieved by a carefully regulated series of events and brought about by the proPO-activating system (proPO system) that consists of proteins capable of binding to polysaccharides and other compounds typically associated with microorganisms, proteinases that

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become active in the presence of microbial products, and factors with the capacity to regulate the system, such as proteinase inhibitors.

PO was accepted as the enzyme involved in the oxidation of the diphenols to *o*-benzoquinones in crustaceans until the discovery that Hc acquired PO activity after treatment with several agents, including SDS and serine-proteinases (*8*). The proPO from hemocytes has been studied in detail (*12*), while Hc, acting as a PO, is of recent discovery (*2, 7*). Biochemical studies of the shrimp proPO system have been carried out in *Penaeus monodon* (*13*), *Penaeus brasiliensis* (*14*), *Penaeus californiensis* (*15*), *Penaeus paulensis* (*16*), and *Penaeus stylirostris* (*17*). Recently, proPO has been purified from hemocytes of whiteleg shrimp, and the sequence analysis with the BLAST algorithm showed that its deduced amino acid sequence exhibited the highest similarity to penaeids, such as *Penaeus monodon* (88%), *Penaeus semisulcatus* (88%), and *Marsupenaeus japonicus* (77%), but had lowest similarity (28%) to whiteleg shrimp hemocyanin (*18*). In spite of differences in amino acid sequences between tyrosinase and Hc, there is evidence indicating that the active sites of arthropod tyrosinase and Hc are quite similar and contain two copper atoms, CuA and CuB, that are coordinated by three histidines for each one (*10*).

The production of whiteleg shrimp *Penaeus vannamei* (Boone 1931), grew from 8000 t in 1980 to 1 386 000 t in 2004, involving countries in Asia, Indonesia, and the Americas. The major importer is the United States with 477 000 t in 2005. The recent finding of unconventional enzyme activity in Hc, along with the lack of characterization, encouraged us to isolate Hc from whiteleg shrimp, analyze the mechanism of conversion to HcPO, characterize the catalytic activity of HcPO, and address its likely role in postmortem melanosis.

MATERIALS AND METHODS

Chemicals. Tyrosine, tyramine hydrochloride, L-dihydroxyphenylalanine (L-DOPA), bovine trypsin, bovine chymotrypsin, Trizma base, SDS, isopropanol, acetone, methanol, urea, KCl, EDTA, HEPES, glycine, NaOH, sodium citrate, $MgCl_2$, and $CaCl_2$. (All were obtained from Sigma, St. Louis, MO.) SDS-PAGE protein standards were used (Amersham Biosciences, Little Chalfont, U.K.).

Whiteleg Shrimp and Experimental Conditions. This study was carried out at Centro de Investigaciones Biológicas del Noroeste. Experiments were performed in compliance with Mexican laws and institutional guidelines, as approved by the Committee of the Program of "Ecología Pesquera". Whiteleg shrimp were acclimated for 10 days in indoor 200-L aquariums containing filtered, running seawater at 26 ± 2 °C and 5−6 mg oxygen L^{-1} to reduce variability in results. The shrimp were fed a commercial feed ad libitum twice daily. Uneaten particles and feces were removed daily. At the end of the acclimation period, hemolymph from shrimp weighing 20−25 g was collected.

Purification of Hemocyanin. Purification was achieved by following a standard procedure (*4*) with some modifications. About 0.5 mL of hemolymph from whiteleg shrimp was extracted from the base of the pleopod of the first abdominal segment near the genital pore with a 3-mL syringe containing 0.5 mL of chilled anticoagulant solution containing 10 mM HEPES at pH 7.3 and 450 mM NaCl, 10 mM KCl, and 10 mM EDTA. The sample hemolymph was immediately centrifuged for 3 min at 4 °C at 550*g* to obtain hemocytes. The supernatant was ultracentrifuged for 4 h at 4 °C at 200000*g*. The pellet containing Hc was recovered. Finally, Hc was separated by gel filtration chromatography in Sepharose 6B (Sigma) in a 1.6 cm × 80 cm column equilibrated with 15 mM Tris·HCl buffer at pH 7.8 containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The Hc was eluted with the same buffer and then used for characterization. The Hc fraction was monitored by recording absorbance from the fractions at 340 nm, which is specific to Hc. The protein concentration was determined by the dye-binding method with bovine serum albumin as the standard.

SDS-PAGE and Native PAGE Analysis. The pure Hc obtained by chromatography was loaded in a native PAGE (4.5% acrylamide) (*4*). Electrophoresis was done at 15 mA per gel in a refrigerated device. Afterward, the electrophoresis fractions were identified by staining with Coomassie blue solution (40% methanol, 7% acetic acid). In twin lanes, aggregates were cut into ∼0.7 cm^2 pieces and electroeluted for 5 h at 10 mA using 15 mM Tris·HCl buffer at pH 7.8 that contained 5 mM $CaCl_2$ and 5 mM $MgCl_2$.

Assay for HcPO Activity. An established spectrophotometric method (*4*), with some modifications, was used to measure PO activity of Hc. HcPO activity was evaluated with a reaction mixture containing 50 μL HcPO solution (containing 300 μg protein), 50 μL substrate solution (10 mM L-DOPA in distilled water), and 200 μL 15 mM Tris·HCl at pH 7.8 that contained 5 mM $CaCl_2$, and 5 mM $MgCl_2$. The mixture was incubated for 10 min at 29 °C and absorbance measured at 490 nm. Results were continuously recorded after addition of substrate for measuring the concentration of dopachrome formed by PO activity of Hc. One unit of enzyme activity was defined as the change in absorbance of 0.001/min.

Effect of the Activators on Hc. The assay was performed as described previously for HcPO activity, where 300 μg of Hc in 250 μL of 15 mM Tris·HCl buffer at pH 7.8 was mixed separately with activators: 100 μg of bovine trypsin or chymotrypsin, 3.33 M isopropanol, 3.44 M acetone, 6.24 M methanol, or 2.77 mM SDS. The effect of the extract from hemocytes (HsE) (*8*) and digestive gland (DGE) in Hc conversion was evaluated, as described earlier. The digestive gland was recovered from the shrimp, homogenized in 15 mM Tris·HCl buffer at pH 7.8, and centrifuged for 10 min at 4 °C at 10 000 × *g*. Then, 350 μg of HC was mixed with 400 μg of DGE or 200 μg of HsE.

Effect of Temperature. HcPO activity was evaluated at 18−70 °C for 10 min using 10 mM L-DOPA as the substrate, following the standard procedure described earlier.

Effect of pH. HcPO activity was evaluated at various pH values: for pH 2.5 and 3.2, 10 mM Gly·HCl containing 0.1% SDS; for pH 3.5, 4.3, 4.9, and 5.7, 10 mM citrate buffer containing 0.1% SDS; for pH 7.0 and 7.6, 10 mM HEPES containing 0.1% SDS; and for pH 8.6, 10 mM Tris·HCl and 0.1% SDS.

Effect of Freezing on Hc. Pure Hc was frozen and maintained at −20 °C, and thawed each month, and the HcPO activity was measured by following the standard procedure.

Kinetic Parameters. K_m and V_{max} were measured with a mixture of 4 mg of Hc (200 μL) incubated in 600 μL of 15 mM Tris·HCl buffer at pH 7.8 containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% SDS for 10 min at 29 °C. Then, increasing concentrations of L-DOPA (0.1−10 mM) were added to individual test tubes, and absorbance at 490 nm was recorded for 2 min. K_m (mM) and V_{max} (U/mg) were then derived, using the Lineweaver−Burk plot.

RESULTS AND DISCUSSION

Protein Purification. The purity of the Hc was evaluated by the absorbance ratio of A340/280 = 0.214 and by SDS-PAGE (**Figure 1A**). The absorbance ratio agrees with values reported for Hc from kuruma shrimp *Penaeus japonicus* (*4*), common shore crab *Carcinus maenas*, and American lobster *Homarus americanus* (*2*). Hc from whiteleg shrimp is formed by two subunits with molecular masses of 75 and 82 kDa. **Figure 1B** shows the subunits. In this figure, some products of possible hydrolysis of the fractions, under reducing and denaturating conditions, were observed, which seems to be common for Hc in several species. With the procedure for purification, 2 mg of Hc from each shrimp was obtained, and aggregates of 6 (1 × 6) and 12 (2 × 6) subunits were purified using preparative PAGE (**Figure 2**). Though 6-mer and 12-mer aggregates maintained the same subunit pattern, we found that only the 12-mer aggregate, after conversion with SDS, can

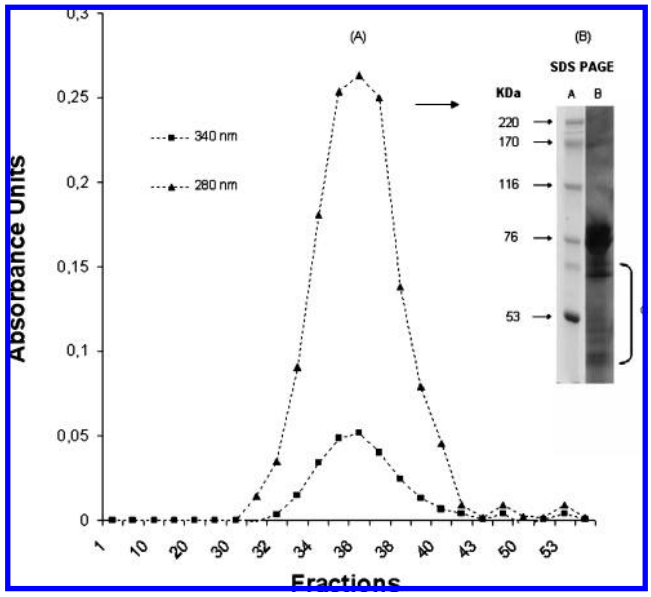

Figure 1. Purification of native *Penaeus vannamei* hemocyanin (Hc). (**A**) Sepharose 6B was loaded with ultracentrifuged plasma (10 mg mL^{-1}) with a linear flow of 14 cm h^{-1}. Fractions of 3 mL were obtained. (**B**) SDS PAGE analysis of pure Hc. A = high molecular weight markers, B = major peak, and C = products of Hc degradation.

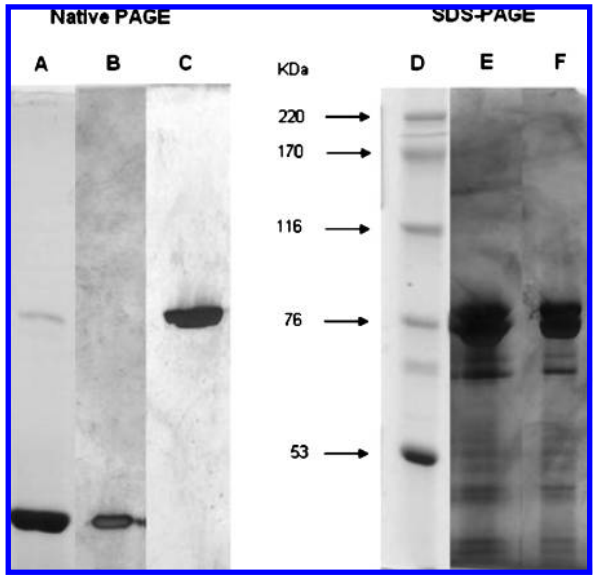

Figure 2. Native PAGE and SDS PAGE analysis of Hc and its aggregates. Native Hc (lane A: 10 μg), 6-mer (lane B: 2 μg), and 12-mer (lane C: 5 μg) were applied to native PAGE, using 4.5% acrylamide gel and stained with coomassie brilliant blue R-250 (lane A) and silver staining (lanes B and C). 6-Mer (lane E: 5 μg) and 12-mer (lane F: 5 μg) were applied to SDS-PAGE using 7.5% acrylamide gel and stained with silver. Lane D is represented by high range SDS-PAGE protein standards.

oxidize phenols, the HcPO activity. No dopachrome formation occurred when the pure 6-mer aggregate was incubated with L-DOPA and SDS. This finding agrees with those for Hc from kuruma shrimp *Penaeus japonicus* (*12*). For that species, only the 12-mer aggregate, after conversion with SDS, showed PO activity in the zymograms. Our results suggest that when subunits are associated in 12-mer aggregates, PO activity can occur; however, after conversion, the cause of the exclusive PO activity of 12-mer aggregates remains unclear. In whiteleg shrimp, HcPO activity does not depend on the native quaternary structure because even higher aggregates oxidize phenols. The

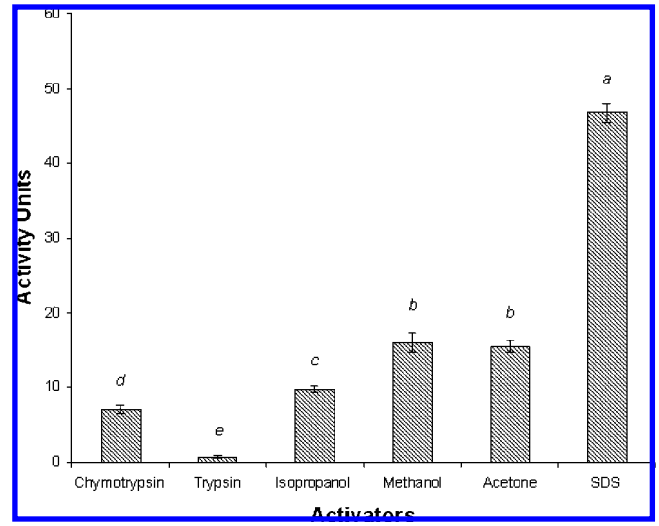

Figure 3. Conversion of hemocyanin into phenoloxidase by different activators. PO activity was evaluated using L-DOPA (10 mM) as the substrate. Experiments were performed in triplicate.

explanation seems to be based on a rearrangement of the quaternary structure, which provides free access for substrates to the active site. In 6-mer aggregates, the active sites are probably restricted.

Effect of Activators on Conversion of Hc to HcPO. HcPO activity is achieved by proteolysis or by partial unfolding of the protein structure in response to treatment with chaotropic agents, such as salts or SDS (*19*). Native Hc in whiteleg shrimp does not have PO activity; therefore, it is incapable of oxidizing *o*-diphenols. However, when incubated with SDS, Hc is converted into HcPO (**Figure 3**); these changes allow the active site to accommodate larger substrates than molecular dioxygen (*19*). We tested the effect of the concentration of SDS on conversion of Hc into HcPO. HcPO activity increased with increasing concentrations of SDS, up to 2.22 mM (data not shown). At this concentration or higher, all the possible changes had occurred; hence, no increase of phenoloxidase activity was found. For whiteleg shrimp, SDS and chymotrypsin were the major activators (*18*); however, in our study, trypsin had only a weak effect on Hc conversion to HcPO, compared to SDS. This indicates that factors involved in Hc conversion are not identical to those of the proPO activation (*8*). A recent report (*20*) showed that Hc from nine species of crustaceans displayed a weak PO activity after trypsin treatment. In our study, chymotrypsin showed a better conversion capacity, compared to trypsin (**Figure 3**). We think that, for the Hc of whiteleg shrimp, as was reported for Hc in the North American tarantula *Eurypelma californicum* (*7*), a large opening enables phenolic substrates, such as L-DOPA, to reach the active site, which depends on the elimination of the N-terminal domain along with the removal of Phe 49. This occurrence is supported by the release of some polypetides as Hc is hydrolyzed. In demonstrating that chymotrypsin is more effective in converting Hc to HcPo than is trypsin (**Figure 4**), we found that it hydrolyzes Hc subunit molecules producing peptides with molecular masses around 58 kDa. Although trypsin has a weak effect, two minor polypeptides of 52 and 56 kDa were also observed. These findings imply that the enzyme treatment breaks down Hc subunits at domain I, which allows dioxygen and L-DOPA to penetrate the active site and proceed with a chemical reaction. By comparison, SDS converts Hc by a factor of 6.5, higher than that achieved by chymotrypsin (**Figure 3**). In crustaceans, the low PO activity, compared with that found in Hc in

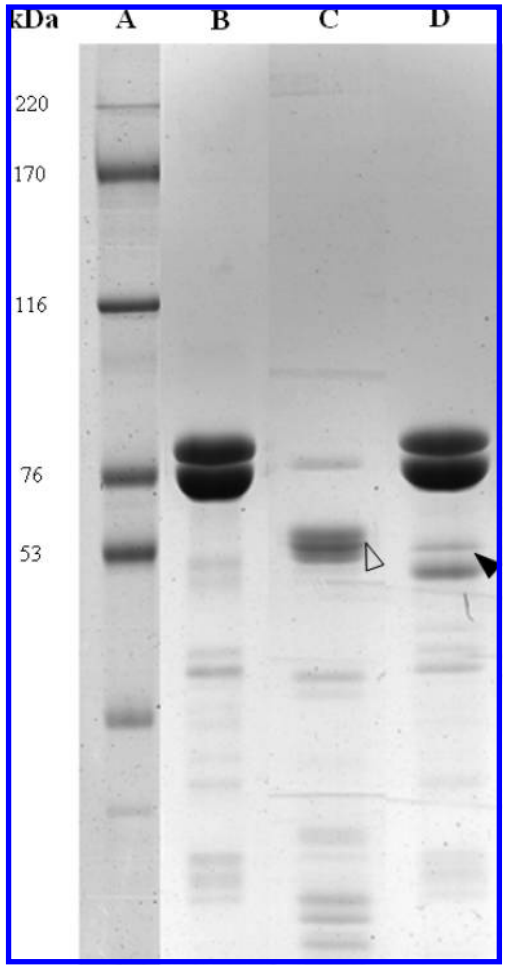

Figure 4. Analysis of proteolytic cleavage of Hc. Partially purified Hc was incubated with chymotrypsin/trypsin and then subjected to 7.5% SDS-PAGE acrylamide gel under reducing and denaturing conditions. Lane A = size marker; lane B = 10 μg Hc without proteolytic enzyme as a control; lane C = 300 μg Hc incubated with chymotrypsin 100 μg (10 μg loaded into gel); and lane D = 300 μg Hc incubated with trypsin 100 μg (10 μg loaded into gel). The open and closed arrows indicate the protein bands for chymotrypsin- and trypsin-treated Hc. Incubations lasted at least 30 min at room temperature.

horseshoe crabs after enzyme treatment, may be explained on the basis of X-ray analysis of their molecular structure (*21, 22*). A large amino acid residue in crustacean Hc sterically obstructs the entrance of any substrate larger than dioxygen from coming close to the active site, even after removing the N-terminal fragment (*7*). In one report, chymotrypsin and trypsin fail to convert Hc in *P. japonicus* (*4*). Besides SDS and serine proteinases, polar solvents were able to slightly convert whiteleg shrimp Hc to HcPO. Isopropanol showed a faint conversion, compared with chymotrypsin, while methanol and acetone had a stronger effect, but lower than SDS. These results suggest that polar solvents only produce slight conformational changes on the entire molecule.

Effect of Substrates on HcPO Activity. The Hc in whiteleg shrimp that was converted to HcPO is capable of oxidizing monophenols, such as L-tyrosine and *o*-diphenols, such as L-DOPA. Three substrates were assayed to determine specificity and mechanism of oxidation. The monophenoloxidase activity of HcPO, when using tyrosine or tyramine as the substrate, had a lag phase (**Figure 5**); this phenomenon was observed in HcPO in the North American tarantula after treatment with trypsin (*7*), as well as in most tyrosinases (*23*). The lag phase varies,

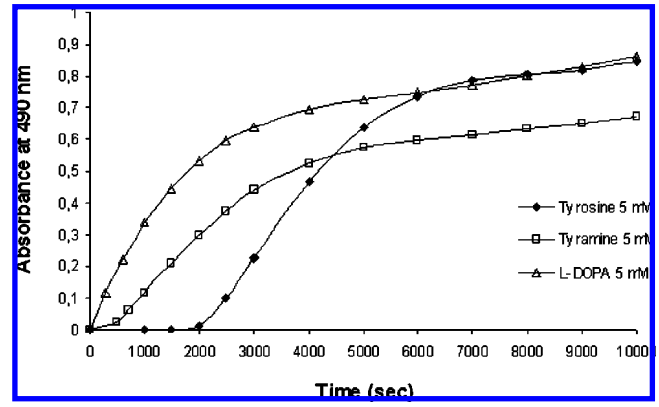

Figure 5. Mono- and *o*-diphenoloxidase activity of HcPO. Pure Hc (450 μg) was mixed with each substrate (50 μL), and the phenoloxidase activity was monitored. The activation buffer (200 μL) was composed of 15 mM Tris-HCl at pH 7.8, including $CaCl_2$ 5 mM, $MgCl_2$ 5 mM, and 0.1% SDS.

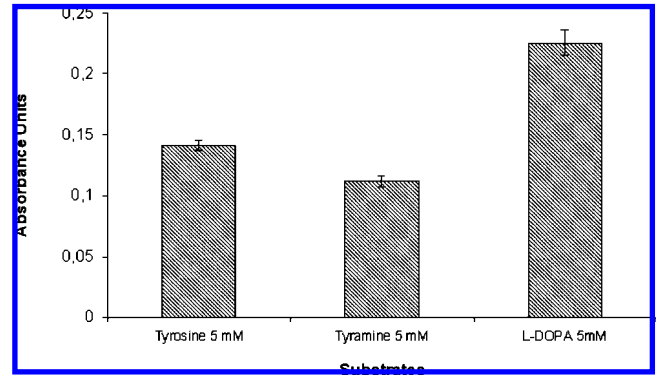

Figure 6. Dopachrome was measured against each substrate. Pure Hc (450 μg) was mixed with 200 μL of buffer (15 mM Tris-HCl at pH 7.8 and 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% SDS). Absorbance was measured after 10 min by addition of 50 μL of each substrate.

depending on the structure of the substrate and the origin of the HcPO; it can take as long as 2 h in some crustacean HcPOs (*13*). The lag phase is typical for tyrosinases and is a consequence of the slow transformation of the met-state active site, which predominates in the inactive tyrosinase, into the oxy-state, which exhibits monophenolase activity (*24*). There are some exceptions, such as monophenoloxidase of the horseshoe crab *Limulus polyphemus* and the Dungeness crab *Cancer Magister* HcPOs; they behave as cathecoloxidases when activated with SDS (*3*). On the other hand, Hc *o*-diphenoloxidase activity in whiteleg shrimp has no lag phase and is capable of oxidizing L-DOPA to the corresponding *o*-quinones, where the active site is the same as for monophenoloxidase activity (**Figure 5**). For diphenoloxidase activity, conversion of the met-state is not necessary because the met- and oxy-forms of the active site possess this specific catalytic activity (*24*).

To assess the ability of HcPO to generate dopachrome, we measured the absorbance at 490 nm 10 min after the product appeared. **Figure 6** shows that HcPO of whiteleg shrimp oxidizes L-DOPA more rapidly than monophenolic substrates. In spite of this, melanin is generated, regardless of the substrate (**Figure 5**). Tyrosine was recognized as the natural substrate for melanosis because it is present as a single amino acid in shrimp (*14*). Additionally, tyramine has not been reported as a naturally occurring phenolic constituent of shrimp; however, it is produced by bacterial activity on free L-tyrosine in some fish after death (*25, 26*).

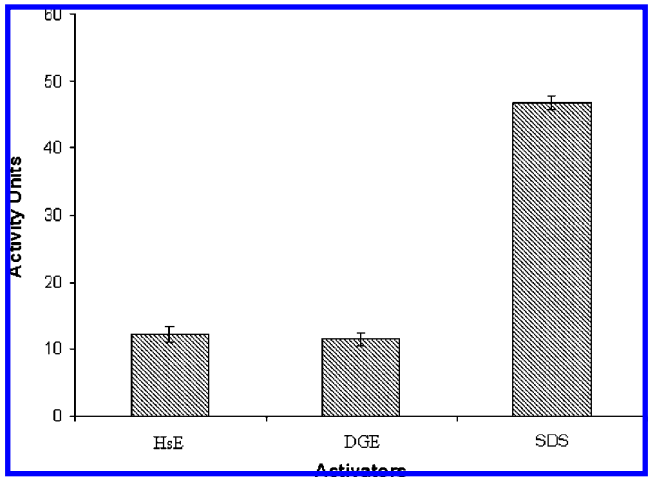

Figure 7. Effect of hemocytes extract and digestive gland extract on conversion of Hc. Both assays were carried out in similar ways: 50 μL of protein was incubated in 200 μL of 15 mM Tris-HCl buffer at pH 7.8 with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. For the first assay, 200 μg of HsE was tested in the reaction mixture. In the second assay, 400 μg of DGE was used. To start the reaction, 50 μL of substrate (10 mM L-DOPA) was added. A comparison with SDS (0.1% in buffer), as the activator, is shown.

Biochemical Properties. Despite the similarities of tyrosinases and Hc's on tertiary and quaternary structures and the fact that both proteins belong to the same protein superfamily, differences in their physicochemical properties have been revealed (*27*). Because Hc's are highly negatively charged, they are highly soluble in the hemolymph. POs, on the contrary, are sticky enzymes from the small number of charged groups on their surface (*21*).

Not long ago PO was recognized as the only enzyme catalyzing the oxidation of diphenol to dopachrome. However, PO is an unstable protein which easily aggregates and is inactivated in a few days, even under refrigeration below 4 °C (*28*). Because the development of black spots is more evident after thawing of frozen shrimp and Hc from whiteleg shrimp is stable and converted even after 3 months at −20 °C (data not shown), Hc is the scapegoat responsible for postmortem melanosis during shelf storage.

Because tissue and cell integrity is disrupted after death, a mixture of molecules coming from different tissues can converge by diffusion. Serine proteases located in the hemocytes and digestive gland can activate Hc (*12, 28*). Chymotrypsin and trypsin are abundant proteases stored in the digestive gland. If they are in contact with Hc and tyrosine, the only missing ingredient for initiating melanosis is dioxygen, which is abundant in the gills; here, melanosis starts within a few hours after death under refrigeration. Hemocytes and the content of the digestive gland were evaluated as activators of Hc. The results are shown in **Figure 7**. Endogenous activation of Hc to HcPO was demonstrated in horseshoe crabs (*5, 6*) and in *P. japonicus* (*8*). However, conclusive evidence of in situ activation is still missing (*29*). Melanosis starts in the head around the gills and spreads to the tail (*30*). For whiteleg shrimp, enzymes present in the digestive gland can initiate conversion of Hc into HcPO with chymotrypsin as one of the most active proteases in the digestive gland (*31*) (see **Figure 3**). Hence, postmortem transformation of Hc into HcPO probably occurs via this serine proteinase.

The optimal temperature of HcPO in whiteleg shrimp is ∼45 °C (data not shown). The optimal temperature is comparable to that in *P. japonicus* (*4*), *P. monodon* PO (*13*), *H. americanus* isoenzymes II and III PO (*32*), *P. duodarum*, and *P. setiferus* PO (*14*). In a similar way, optimal pH is 7.6 (data not shown), similar to that for PO in *P. duodarum*, *P. setiferus* (*14*), and *Panulirus cygnus* (*33*). There are few reports describing kinetic parameters of HcPO; our attempt to reveal these properties is one of the first for crustaceans. However, much knowledge has been accumulated about kinetic parameters of PO in crustaceans. In **Table 1**, a comparison of K_m between HcPO in whiteleg shrimp and some other crustaceans is given. These results suggest that HcPO and PO have similar substrate affinities. Catalytic properties of HcPO in whiteleg shrimp are $K_m = 1.47$ and $V_{max} = 0.122$. In contrast, catalytic properties for Hc aggregates in the green crab *Carcinus maenas* and American lobster *H. americanus* are $K_m = 193.33$ and 218 and $V_{max} = 0.222$ and 0.1176, respectively (*2*). It is notable that HcPO in whiteleg shrimp possesses a high catalytic efficiency and is distinguishable from HcPO in other crustaceans because of its higher substrate affinity.

Table 1. Catalytic Properties of PO in Several Sources and HcPO in *P. vannamei*

species	K_m (mM)	ref
L. setiferus	2.8	*12*
F. duodarum	1.6	*12*
P. monodon[1]	4.4	*12*
Hamarus americanus PPO I	1.7	*24*
H. americanus PPO II	1.3	*24*
H. americanus PPO III	1.1	*24*
L. vannamei HcPO	1.5	this study

Does Hc Participate in Postmortem Melanosis? Experiments were carried out to determine the involvement of Hc in melanosis, mostly under postmortem conditions. Results from this study indicate that Hc can attain PO activity. There are several activators in the organism that affect tertiary and quaternary structure of Hc and make it capable of shifting from adsorbing dioxygen to using phenols as substrates for oxidation. Among activators are proteinases, like those synthesized in hemocytes and the digestive gland, the latter being the most important in whiteleg shrimp, especially chymotrypsin. HcPO has monophenoloxidase activity, which is noticeable because L-tyrosine is a potential substrate in shrimp. HcPO shares some biochemical and kinetic properties with crustacean POs, indicating a similarity in the mechanism to oxidize the substrate because the active site for HcPO and PO is quite similar (*23, 34*). The Hc in whiteleg shrimp is a stable protein that retains its potential for conversion to HcPo for several months under freezing conditions. In spite of the amount of HcPO activated by serine proteinase, there is enough Hc in hemolymph, perhaps 3 orders of magnitude higher than in PO (*4*). With Hc activated and after a few days with exposure to air, HcPO is suitable for achieving the task of melanosis. Further structural information would help to clarify the PO activity mechanism of Hc, as well as biochemical characterization of whiteleg shrimp hemocyte PO for comparison, to provide conclusive evidence about the main role of hemocyanin in postmortem melanosis.

Whiteleg shrimp is heavily cultivated in northwestern Mexico, which allows production of less variable organisms than those harvested in the wild. Even so, specimens were acclimated to reduce variability produced during pond harvesting. Hemolymph was sampled and Hc converted to the active form HcPO, which is a monophenoloxidase. Because Hc is stable at freezing, it is not surprising that it became active after thawing. Freezing−thawing also disrupts cells, allowing previously compartmental-

ized contents to mix. On the other hand, proPO is unstable until freezing. Hence, we conclude that monophenoloxidase activity of Hc may be responsible for postmortem black spot formation.

ABBREVIATIONS USED

Hc, hemocyanin; HcPO, Hc converted to PO; PO, phenoloxidase; HsE, hemocytes extract; DGE, digestive gland extract.

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