CHARACTERIZATION OF POLYPHENOLOXIDASE OF PRAWNS (*Penaeus japonicus*). ALTERNATIVES TO INHIBITION: ADDITIVES AND HIGH-PRESSURE TREATMENT

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ABSTRACT

Polyphenoloxidase (PPO) presented different specific activity at different locations in the imperial tiger prawn (*Penaeus japonicus)*, with the highest values in the carapace. The procedure achieved a degree of purification, close to 70 times, increasing relative activity by means of ammonium sulphate 0-40 % saturation. Isoelectric focusing showed two bands around pI 5.0. The optimum temperature for PPO reaction with DOPA was between 40-60 ºC, however thermal stability was greatest at temperatures lower 35ºC. The enzyme was most active at pH 5 and 8, but it was most stable at pH basic. Pressurization of the enzymatic extract was assayed within a range of 0.1 to 400 MPa, for 10 min at <10 °C. Pressure-induced inactivation was evident, particularly at 300 – 400 MPa. Regarding Inhibiting compounds, total inhibition of the extract was only achieved with ascorbic acid and citric acid at pH 3.0. With 80 μg / mL sulphite, 150 μg / mL of kojic acid, 1 g / L of 4-hexilresorcinol or 0.1 g / L of sodium benzoate was required for 80 % inhibition.

Key words: polyphenoloxidase prawns, melanosis, inhibitors, high pressure

INTRODUCTION

Melanosis is a process that is triggered by a biochemical mechanism consisting of oxidation of phenols to quinones by means of an enzymatic complex known as polyphenoloxidase (PPO). This is followed by non-enzymatic polymerization of the quinones, giving rise to pigments of high molecular weight and very dark or black colouring. In prawns and other crustaceans, this degenerative reaction occurs post-mortem. Although the pigmentation seems to be harmless to consumers, it drastically reduces the product's market value, hence occasioning considerable financial loss. This process has been and continues to be widely studied in vegetable products, but less attention in the case of crustaceans. A better understanding of the properties and mechanics of these enzymes is needed in order to be able to control and ultimately inhibit their action.

In live prawns and other crustaceans, the enzyme appears to be involved in sclerotization. Bearing this in mind, post-mortem melanosis occurs in several zones along the exoskeleton. It is located principally in the carapace of the cephalothorax, in the caudal zone - telson and uropods-, and in the cuticle of the abdomen, mainly in the zones where the cuticle segments are joined and where the cuticle is joined to the pleopods (Ogawa *et al*. 1984). Moreover, there are some references indicating that it also occurs in the surface membrane covering the muscle and in the haemolymph (Nagagawa and Nagayama 1981; Ogawa *et al*. 1984).

In chilled prawns and shrimps, the melanotic reaction begins at the head and then spreads to the tail, the rate of spread of melanosis differs among the various species. This could be related to differences in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson et al. 1987). In fact, in the few references of partial characterization of polyphenoloxidase (PPO) such as in pink shrimp (*Penaeus duorarum)* (Simpson et al. 1988), white

shrimp (*Penaeus setiferus)* (Madero and Finne, 1982; Simpson et al. 1987), black tiger shrimp (*Penaeus monodan)* (Rolle et al. 1991) and Florida prawn (*Panulirus argus)* (Ali et al. 1994a,b), the enzyme presented differences in molecular weight, isoelectric point, optimum pH, thermal stability and kinetic parameters. Moreover, the PPO enzyme was much more active in some species than in others; thus, in the pink shrimp (*Penaeus duorarum)* (Simpson et al. 1988) PPO oxidized faster than in the white shrimp (*Penaeus setiferus)* (Madero and Finne, 1982; Simpson et al. 1987); however the spread of melanosis was much slower in the black tiger shrimp (*Penaeus monodan)* (Rolle et al. 1991). This variety in behaviour may be influenced by differences in habitat. A comparison of the PPO enzymes in different prawn and shrimp species exhibiting different susceptibility to melanosis could lead to a better understanding of the biochemical basis determining high melanotic activity in certain species.

Melanosis in crustaceans is normally controlled by means of certain sulphite derivatives (Ferrer et al. 1989a); however, these compounds are known to produce allergic reactions and serious disturbances in asthmatic subjects (Taylor and Bush, 1986; Dewitt, 1988). It is therefore necessary to find other compounds or alternative processes. The mechanics of melanosis production are complex and consist of several phases, so that there is a wide range of inhibitory procedures: inhibition of the PPO enzyme by reduction of o-quinones to diphenols; interaction with formation of o-quinones; reduction of the reaction oxygen or Cu^{+2} to Cu^{+} (Chen et al. 1991a). Many of these inhibitors have been studied in PPO extracted from vegetables, and only few have been studied in PPO from crustaceans. Examples include kojic acid in PPO from shrimp (*Penaeus duorarum)* (Chen et al. 1991b,c; Taoukis *et al*., 1990), chitosan or phytic acid in shrimp (*Trachypenaeus curvirostris)* (Yu et al., 1996), or derivatives of resorcinol in shrimp and prawn (McEvily *et al*., 1991; Otwell *et al*., 1992; Slattery *et al*., 1995).

There are also a number of processes that strongly inhibit PPO. In this connection, particularly interesting are atmospheres that reduce the oxygen in the medium. Also, high pressure hydrostatic has been tried as a PPO inhibitor in various vegetable products. Some studies show that pressurization did not only inhibit the enzyme but could actually cause slight enzymatic activation (Asaka and Hayashi, 1991; Estiaghi and Knorr, 1993); on the other hand, Seydarhelm et al (1996) reported total inactivation of PPO at very high pressure (900 MPa). Noting that pressure-induced inactivation depends on the immersion medium, pH, temperature and time; and moreover, there are differences depending on the species of origin (Weemaes et al. 1998). In the present study, many of these options were restricted, the level and temperature of pressurization was not too high in order to get sensory characteristics like those of fresh prawns

This study describes the partial purification and characterization of PPO extracted from imperial tiger prawn (*Penaeus japonicus)* and the possible inhibiting effect of some chemical substances or high pressure on PPO activity.

MATERIALS AND METHODS

The species utilized was live farmed imperial tiger prawn (*Penaus japonicus*) (Acuinova Andalucia, S.A., Ayamonte, Spain) harvested in December. Average sizes and weights of individuals were 13.0 ± 0.9 cm and 21.5 ± 4.5 g, respectively. 40 kilos of prawns were headed following anaesthesia by low-temperature shock. The carapace was separated from the cephalothorax, the cuticle from the abdomen, telson and uropods, and the muscle from the tail. Samples were washed in cold water, frozen in liquid nitrogen and ground into a fine powder in an Osterix blender (Osterizer, Par Sunbeam, Mod. 867 50 E, USA). The various powdered extracts were vacuum-packed and stored at –80 °C.

Enzyme extraction. PPO was extracted according to Chen *et al*. (1991a) with a slight modification. One part of the powder (10 g) was added to three parts (w/v) of 0.05 M sodium phosphate buffer pH 7.2 containing 1 M NaCl and 0.2 % Brij 35 (Sigma Diagnostics, St. Louis, USA). The extract was stirred 3 hr at 4 °C under N_2 atmosphere and the suspension was centrifuged (8000*g*, 30 min, 4 °C). The supernatant was collected and precipitated with ammonium sulphate at 0, 0-40 and 40-70 % saturation depending on the assay, at 4 °C under N_2 atmosphere. After subsequent centrifugation (12500*g*, 30 min, 4 °C), the pellet was dissolved in 2.5 mL of 0.05 M phosphate buffer pH 6.5. Finally, molecular exclusion filtration (PD – 10 column, Sephadex G – 25 M, Amersham Pharmacia Biotech) at 4 ºC was used to remove salts. The final volume (3.5 mL) was mixed with 0.05 M phosphate buffer, pH 6.5, and immediately frozen to - 80 °C in order to prevent alterations as far as possible prior to determination.

Protein quantification. Protein was estimated according to Lowry et al (1951) using bovine serum albumin as standard.

Measurement of PPO activity. The enzyme activity was measured continuously at 475 nm 25 °C for 5 min in a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) with a CPS-240 thermostatic controller. The substrate was DL-3,4-dihydroxyphenylalanine (DL-DOPA) (Sigma Chemical, Sto. Louis, USA). Measurements were made in a mixture of 40 μL of enzyme extract with 280 μL of 20 mM DL - DOPA, in water and 280 μL of 0.05 M buffer sodium phosphate. Unless otherwise stated, experiments were repeated at least in duplicate and the results are expressed as absorbance increment / min. *Relative activity* described enzymatic activity as the percentage of enzymatic activity expressed as $(A / A_{máx}).100$, where A indicates the increase in optic density per minute.

Isoelectric Focusing. 10μL of extract was loaded on to the gel (Ampholine, PAGplate 3-9.5, Pharmacia Biotech, Pharmacia LKB, VS). The isoelectric focusing patterns used to run parallel to the sample in the same gel covered a wide pH range (4-9) and contained amyloglucosidase (a soy trypsin inhibitor), β-lactoglobulin A, bovine carbon anhydrase B, horse myoglobin, lectin and trypsinogen, supplied by Pharmacia LKB. Isoelectric focusing was performed on a Pharmacia apparatus (FBE-3000) at 10 ºC, conditions 1500 V, 30 mA and 30 W, up to 2500 v. The samples were applied 1 cm from the cathode in the gel. The cathode and anode electrolytes were 1 mol sodium hydroxide and 1mol orthophosphoric acid, respectively. The gels were then fixed in 10 % trichloroacetic acid and 5 % sulphosalicylic solution. After fixing, the gels were stained by immersion in 0.04 % coomassie blue R-250 in ethanol/acetic acid / water (25:10:65) overnight. Excess tincture was removed by repeated washing of the same solution without coomassie blue. Analyses were carried out in duplicate.

Optimum temperature and thermostability. PPO activity was measured continuously at 475 nm for 5 min at different temperatures (25-60 °C). The assay was performed by mixing 40 μL of enzyme extract with 280 μL of 20 mM DL-DOPA (Sigma Chemical, Sto. Louis, USA) in water and 280 μL of 0.05 M phosphate buffer pH 7. To estimate thermostability, enzyme solution was preincubated in 0.05 mM phosphate buffer pH 7 at different temperature (from 25 °C to 60 °C) for 30 min. After cooling, residual activities were determined at 25 °C by addition of the substrate.

Optimum pH and Stability. The optimal pH of the PPO extract was determined by mixing 40 μL of enzyme extract with 280 μL of 20 mM DL-DOPA (Sigma Chemical, Sto. Louis, USA) in water and 280 μL of 0.05 M phosphate buffer with the desired pH values (2.5-9.5). To determine the influence of pH on the stability of the enzyme, 40 μL enzyme extract was incubated with 280 μL of 0.05 M phosphate buffer at the desired pH values (2.5-9.5) for 30 min at ambient temperature.

Residual enzyme activity was measured at 25 °C by additon of 280 μL of 20 mM DL-DOPA in water.

Effect of high pressure. High - pressure treatments were performed in a high pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France) where the temperature of the immersion medium was controlled via a thermo-couple. Pressure was increased at 2.5 MPa/s. One part of the enzyme extract was added to seven parts 0.05 M phosphate buffer at pH 8 (v/v) and was pressurized in totaly full Eppendorf tubes of this solution at 100-400 MPa for 10 min at <10 °C. Residual enzyme activity was measured at 25 °C by additon of 280 μL of 20 mM DL-DOPA in water to 320 μL of the pressurized solution.

Influence of chemical reagents. To determine the influence of specific inhibitors of the enzyme, 40 μL enzyme extract was incubated with 140 μL of 0.10 M phosphate buffer at pH 8 and 140 μL of different concentrations of inhibitor solution for 5 min at 0 °C. Residual enzyme activity was measured at 25 °C by additon of 280 μL of 20 mM DL-DOPA in water. The inhibitors used were: sodium bisulphite (Sigma Chemical, St. Louis, USA), kojic acid (Sigma, Chemical Co., St. Louis, MO, USA), 4-hexylresorcinol (Sigma), sodium benzoate (Panreac Química, Montcada i Rexac, Barcelona, Spain), citric acid (Panreac) and L (+)ascorbic acid (Panreac). The percentage of inhibition was expressed as [(A - A*)/ A].100, where A and A* indicate the variation of absorbance in absence and presence, respectively, of the inhibitor.

RESULTS AND DISCUSSION

Extraction of polyphenoloxidase

First, a study was carried out on enzyme extracted from different parts of the prawn (carapace of cephalotorax, uropods/telson, abdominal muscle and cuticle of abdomen) in order to determine in what zones most of the enzyme activity took place in this specie (Fig. 1). The activity in the carapace was the greatest; next the caudal zone (uropods and telson), and last, the muscle and cuticle. It could be due to the fact that PPO activity appear to be involved in sclerotization process. The PPO in the muscle would come from the haemolymph, this could explain the low activity. According with Ogawa et al. (1984), the PPO in the cuticle is located largely in a small ventral area in the pleuron, close to the pleopod joints. This means that when extraction is performed on the carapace and the cuticle together, there is much lower PPO activity than when only the carapace is used. Therefore only the carapace of the cephalothorax was used for partial purification of PPO.

In a study of the distribution of catecholoxidase in the tissues and organs of several crustaceans, Nakagawa and Nagayama (1981) observed no activity at all in muscle of tiger shrimp (*Penaeus japonicus)* and in other species, however some activity was detected in the case of crabs. These authors also found that the values were highest in gill and cuticle, but different locations in the cuticle was not consider.

With the addition of ammonium sulphate, protein solubility can be reduced, thus the enzyme is partially purified. Ammonium sulphate interferes with the determination of protein concentration; therefore, it must be eliminated by dialysis or desalting column. Dialysis caused considerable loss of enzyme activity because it takes at least 8 hours: so a desalting column was considered more suitable, because the salt can be removed much more quickly (5-10 minutes). It was also necessary to assay different degrees of precipitation with ammonium sulphate, ranging from 0 % to 70 %, since the best percentage depends on the characteristics of the species. In our case, the greatest activity was found within the range 0-40 %; relative activity was seven times higher than

without precipitation, that is, in the crude extract. The precipitation fraction with 40 - 70 % ammonium sulphate represented only 5-10 % of relative activity respect to the activity of 0-40 % fraction, suggesting that recovery of PPO was not efficient in this fraction. The total activity in the carapace, partially purified by precipitation with 40 % ammonium sulphate was 354.8 (Δ DO/min/mL) and specific activity 80.5 (total activity/mg protein). Closed values of specific activity were reported by Savagaon and Sreenivasan (1978) in lobster: 109.0 DO increment /mg protein/ 5 min at 25 °C.

Isoelectric focusing of the PPO extract precipitated with 0-40 % ammonium sulphate showed two bands, very close together, at around pH 5.2, which suggests the presence of two isoenzymes with slightly different characteristics versus pH. In another species, such as the black tiger prawn *(Penaeus monodon)*, a larger amount of enzyme has also been reported with this proportion of ammonium sulphate, coinciding with two isomorphic forms of 80 and 63 kDa respectively (Rolle et al. 1991), although their pI was not reported. In the case of the prawn *Penaeus setiferus*, on the other hand, the best precipitation for purification was between 40-75 % ammonium sulphate saturation, getting a smaller purified enzyme (30 kDa) (Simpson et al. 1987). This confirms the variation in PPO characteristics for every species. Also in Florida lobster (*Panulirus argus)*, isomorphic forms of varying molecular weight and one single pI have been found (Ali et al. 1994a). No much more information about isomorphic forms in crustaceans, however, at least 14 isoenzymes have been found in some vegetable species, with pI ranging from 4 to 5.5, and from 10 to 13 isoenzymes with pI ranging from 4 to 5.8 in pulp of several varieties of banana (Thomas and Janave, 1986).

Effect of temperature

Figure 2 shows the evolution of the temperature of enzyme extract activation and the thermal

stability. The activation temperature profile shows two plateaus between 30-35 and 40-45 º C. The activity increased by around 20 % for every 10 °C of temperature to reach both plateaus; it could be explained due to the fact than the increase of temperature produces more activity, as the reaction is accelerated by the enhanced kinetic energy; however, a loss of activity over 55 °C, possibly due to thermal denaturation of the enzyme.

These results relate to other studies (Chen *et al*., 1991a) carried out with PPO isolated from lobster (*Panulirus argus* or *Panulirus cygnus*), in which enzyme activity increased with increasing temperature up to 60 °C. In other cases, maximum activity occurs at 40 - 45 °C, for example: in PPO extracted from lobster *(Panulirus argus)* (Ali *et al*, 1994a) or divers shrimps (*Penaeus duorarum)* (Simpson *et al*., 1988), *Penaeus monodon* (Rolle *et al*., 1991) and *Penaeus setiferus* (Simpson *et al*., 1987).

Thermal stability was considerably reduced when the enzyme extract was subjected to temperatures up to 35 °C. The optimum temperature for determination of enzymatic activity was below 30 °C, even although activity values were lower than at higher temperatures. The results from other genera and/or species have consistently shown a considerable decrease with increased temperature, pointing to species-dependent differences in thermal stability, which may in turn be related to the specific habitat of each one. In shrimps *(Penaeus duorarum* and *Penaeus monodon)*, the enzyme has been found to be unstable at temperatures over 30 - 35 °C (Simpson *et al*., 1988; Rolle *et al*., 1991); in *Penaeus setiferus*, the upper limit was 50 °C (Simpson *et al*., 1987), and in lobster *(Panulirus argus)* it was 40 °C (Ali *et al*., 1994a). Moreover, even within the same species there are differences depending on the state of activation of the enzyme itself (Ferrer *et al*., 1989b). What the foregoing suggests is that some enzymes can be active within a wide temperature range but further unstable.

Effect of pH

Figure 3 shows the enzymatic activity profile of the extract from the carapace of the cephalothorax versus different pH values. There are two pronounced peaks of high activity, one in the acid zone (pH 5) and the other in the basic zone (pH 8). In this connection, the two isoenzymes shown by isoelectric focusing could correspond respectively to maxim of acid and alkaline activity.

Moreover, the optimum pH depends to a large extent on the physiological pH in which the enzyme activity occurs in nature. For example, the pH of the carapace of the cephalothorax was 7.16 ± 0.07, while in the abdominal cuticle it was 8.76 ± 0.04. Therefore, the enzyme would probably present different optimum pH characteristics depending on the locus of extraction, although in our case both activity peaks versus pH were located in the carapace. The pH profile for PPO isolated from other crustaceans varies according to species, for example, Chen *et al*. (1991a) reported that the optimum pH varied from pH 6 - 8 in Western Australian lobster (*Panulirus cygnus)* and pH 6.5 in Florida Spiny Lobster (*Panulirus argus)*. In the case of shrimp (*Penaeus setiferus*), PPO was active in the range of pH 6.5 - 9 (Simpson *et al*., 1988). In *Penaeus monodon* the maximum activity has been reported at pH 6.0 (Rolle *et al*., 1991), and in lobster *(Panulirus argus)* in the range of pH 6 – 6.5 (Ali *et al*., 1994a).

There was a total decrease of stability versus pH (Fig. 3) at acid levels (pH < 5), indicating that the enzyme is highly unstable within that range. Therefore, despite the occurrence of maximum activity at pH 5, it is not advisable to determine enzyme activity at that level since it is too close to the critical point. The high instability at acid pH seems suggest that treatment of prawns with acid solutions would inhibit melanosis to a large extent; on the other hand, at pH closer to neutral (pH 7.5) or slightly basic (pH 8), the enzyme was more stable, for this reason, pH 8 will be used in the following assays.

As with temperature, the activity and stability differed according to enzyme state of activation (Ferrer *et al*., 1989b). In this connection, severalalmost all studies seem to agree that PPO was not stable at acid pH. Both in lobster (*Panulirus argus)* (Ferrer *et al*., 1989 b; Ali *et al*., 1994a) and in white shrimp (*Penaeus setiferus*) (Simpson *et al*. 1987), the pH profile for PPO activity was unstable at pH below 5. Thus, the PPO of black tiger shrimp (*Penaeus monodan)* (Rolle et al.1991) became unstable at pH $\leq$ 7.5, possibly due in part to its slow melanotic activity. The pink shrimp (*Penaeus duorarum)* presented very high stability between pH 6 and 12, with maximum activity at pH 8-9 (Simpson et al. 1988). The PPO Florida spiny lobster (*Panulirus argus*) was not stable at acid pH values, and the pattern was similar in Western Australian lobster (*Panulirus cygnus*) (Chen *et al*. 1991a). It seems that the species most resembling in temperature profile was the imperial tiger prawn and in pH was the white shrimp (*Penaeus setiferus)* from the Florida coast.

Effect of high pressure

In basic conditions (pH 8), activity was greatest at atmospheric pressure (0.1 MPa) than in pressurized samples. There was a decrease in activity as the pressure was gradually increased (Fig. 4); the decrease was slight between 100 - 200 MPa and more pronounced between 300 – 400 MPa. The enzyme extract was therefore inactivated by pressure.

No studies were found in the literature on the effect of high pressure on PPO in crustaceans, however, this effect has been studied in vegetable products, where the response to pressurization varied. For example, Asaka and Hayashi (1991) found that pressurization caused activation of PPO from pear in slightly acid conditions (pH 5 - 6), the optimum for activity being pH 6.5. On the other hand, there are examples of PPO inhibition when pressurization was used on vegetable

extracts (Anese *et al*., 1995; Seyderhelm *et al*., 1996; Gomes and Ledward, 1996) or on products such as juices and purees (Quaglia *et al*., 1996; Cano *et al*., 1997; Hernández and Cano, 1998; Palou *et al*., 1999). PPO from different origins displays different pressure stabilities; pressure inactivation behaviour of PPO is dependent on the enzyme source (Weemaes et al., 1998). Generally, enzyme inactivation was greater with high pressures (700 - 900 MPa) than when lower pressures were applied; but, the results vary much more according to the treatment conditions. Inhibition can be favoured if the pH of the pressurizing medium is one at which the enzyme is unstable (Seydarhelm et al., 1996).

Inhibiting compounds

The behaviour of the inhibitors in the model system can not be extrapolated to a real system, that is to the actual prawn. However, such behaviour is useful as an approach to the mechanics of inhibitor action and as a means of comparing the effectiveness of the various additives with a view to selecting those that are capable of inhibiting melanosis.

The presence of sodium metabisulphite in the enzyme extract at pH 8 (Fig. 5) indicates that the sulphite produced 30 % inhibition at concentrations of 10 μg / mL, and total inhibition at concentrations between 80 –100 μg / mL. Sulphite inhibition appears to operate through various different mechanisms: the sulphite combines irreversibly with the quinones, preventing their polymerization to pigmented compounds (Markakis and Embs, 1966); it modifies the protein structure (Sayavedra - Soto and Montgomery, 1986); or it reduces the quinones (Ferrar and Walker, 1999).

Higher concentrations of kojic acid than of sulphite were required to achieve similar degrees of enzyme inhibition; thus, it took 150 μg / mL of kojic acid to achieve 80 % inhibition as compared to

40 μg / mL of sulphite (Fig.5). On the other hand, kojic acid is free of the toxicity problems presented by sulphite derivatives. Chen *et al*. (1991b) reported similar results, they found that the addition of 20 μg / mL of kojic acid produced around 20 % inhibition of DL - DOPA oxidation by PPO of black tiger shrimp (*Penaeus monodon*) in buffered solution pH 6.5, and around 80 % inhibition with 100 μg / mL. However, in our case, kojic acid appears to have been more effective; because, inhibition took place at lower temperatures and over shorter times (0 °C versus 37 °C, 5 min versus 15 min); at the these temperatures the PPO in this species would be highly unstable, with little relative activity. Kahn et al. (1998) reported that kojic acid produces inhibition on the one hand by preventing the oxidation of o-dihydroxyphenol to quinones, and on the other hand by preventing the polymerization of the quinones and forming a stable yellow product. For this reason it may not be desirable in certain products, but that would depend on the concentration of the inhibitor and the type of food on which it is used.

Hexylresorcinol (Fig. 5) produced 40 % inhibition with concentrations of 0.15 g / L, and 80 % inhibition with 1 g / L in the given conditions of addition (0 ºC, 5 min). The studies published on hexylresorcinol were conducted on the prawn itself and concluded that it was a good inhibitor since in the given conditions, melanosis was inhibited to a greater extent by 50 ppm of hexylresorcinol than by the usual concentrations of sulphite (12500 ppm) (Otwel *et al*., 1992).

Sodium benzoate (Fig. 5) was more effective than hexylresorcinol, producing around 70 % inhibition with concentrations of 0.1 g / L. According to Kubo and Kinst - Hori (1998), any soluble compound derived from phenolic acid or mixes may provide good protection against tyrosin oxidation, thus preventing melanosis in shrimps; being benzoic acid one of this compounds. Additonal, in the same work, it was reported that the seeds of *Cuminum cyminum*, commonly known as cumin, contain cumic acid, a phenol derivative with capacity of inhibition (Kubo and Kinst-Hori, 1998). Therefore cumin, a natural compound, could be used to help inhibit melanosis.

When enzyme activity was measured at the pH given by addition of the acids (ascorbic acid pH 3.1, citric acid pH 2.8), inhibition was total; however, when the measurement was performed at pH 8 as indicated above, no inhibition was detected. These results suggest that ascorbic acid and citric acid caused inhibition by destabilizing the enzyme at the pH resulting from their own acidity.

CONCLUSIONS

There is little information available regarding this activation process in the literature for seafood, the characterization of the various enzymes from divers species/genera indicates different structural and functional properties. In the present case, the PPO of imperial tiger prawn (*Penaeus japonicus*) exhibited some similarities with some shrimps, but there was no great resemblance to any one in particular. High pressures (300-400 MPa, 10 min, <10 °C) inhibited 80 % of enzyme activity in the extract. It is necessary to study inhibitors in model systems in order to get standard forms suitable for each species. Briefly, our results suggest that kojic acid, 4-hexylresorcinol, sodium benzoate, citric acid and L (+) ascorbic acid could potentially be used as sulphite substitutes to prevent melanosis in seafood products, and that their use, singly or in combination, could have useful effects in terms of quality and health. However, this is not always possible or effective in fresh crustaceans, and further research is required in these areas.

Acknowledgements

This research was financed by the Comisión Interministerial de Ciencia y Tecnología (CICyT) under project ALI-97-0759 and the Comunidad Autónoma de Madrid (CAM) under project

06G/053 / 96. The authors wish to Consejo Superior de Investigaciones Científicas (CSIC) for the postdoctoral fellowship awarded to MPM and to thank HASENOSA S.A. for cofinancing the research and providing the samples.

LITERATURE CITED

Ali, M.T.; Marshall, M.R.; Wei, C.I.; Gleeson, R.A. Monophenol oxidase activity from the cuticle of Florida spiny lobster (*Panulirus argus*). *J. Agric. Food Chem.* **1994**a, 42:53-58.

Ali, M.T.; Gleeson, R.A.; Wei, C.I.; Marshall, M.R. Activation mechanisms of pro-phenoloxidase on melanosis development in Florida spiny lobster (*Panulirus argus*) cuticle. *J. Food Sci.* **1994**b, 59 (5):1024-1030.

Anese, M.; Nicoli, M.C.; Dall'Aglio, G.; Lerici C.R. Effect of high pressure treatment on peroxidase and polyphenoloxidase activities. *J. Food Biochem*. **1995,** 18:285-293.

Asaka, M.; Hayashi, R. Activation of polyphenoloxidase in pear fruits by high pressure treatment. *Agric. Biol. Chem*. **1991**, 55(9) 2439-2440.

Cano, M. P.; Hernandez, A.; de Ancos, B. High pressure and temperature effects on enzyme inactivation in strawberry and orange products. *J. Food Sci.* **1997**, (62)985-988.

Chen, J.S.; Rolle, R.S.; Marshall, M.R.; Wei, C.I. Comparison of phenoloxidase activity from Florida Spiny Lobster and Western Australian Lobster. *J. Food Sci.* **1991**a, 56(1)154-160.

Chen, J.S.; Wei, C.I.; Rolle, R.S.; Otwell, W.S.; Balaban, M.O.; Marshall, M.R. Inhibitory effect of kojic acid on some plant and crustacean polyphenoloxidases. *J. Agric. Food Che*m. **1991**b, 39:1396-1401.

Chen, J.S.; Wei, C.I.; Marshall, M.R. Inhibitory mechanism of kojic acid on polyphenoloxidase. *J. Agric. Food Chem.* **1991**c, 39(11)1897-1901.

DeWitt, B. J. An improved methodology for the estimation of sulfur dioxide in shrimp. Resumen Internacional B, **1988**, 48(12)3463-3464.

Estiaghi, M.N.; Knorr, D. Potato cubes response to water blanching and high hydrostatic pressure. J. Food Sci. **1993**, 58(6):1371-1374.

Ferrar, P.H.; Walker, R.L. Microorganisms as potential sources of novel diphenol oxidase inhibitors. *J. Food Biochem*. **1999**, 23:1-15.

Ferrer, O.J.; Otwell, W.S.; Marshall, M.R. Effect of bisulfite on lobster shell phenoloxidase. *J. Food Sci.* **1989a**, 54(2)478-480.

Ferrer, O.J.; Koburger, J.A.; Otwell, W.S.; Gleeson, R.A.; Simpson, B.K.; Marshall, M.R. Phenoloxidase from the cuticle of Florida Spiny lobster (*Panulirus argus*): mode activation and characterization. *J. Food Sci*. **1989b**, 54(1):63-67, 176.

Gomes, M.R.A.; Ledward, D.A. Effect of high-pressure treatment on the activity of some polyphenoloxidases. *Food Chem*. **1996**, 56(1)1-5.

Hernández, A.; Cano, M.P. High pressure and temperature effects on enzyme inactivation in tomato puree. *J. Agric. Food Chem.* **1998**, 46:266-270.

Kahn, V.; Shalom, N.B.; Zakin, V. Effect of kojic acid on the hydroxylation of L-tyrosine and tyramine by mushroom tyrosinase. *J. Food Biochem*. **1998**, 22:455-474.

Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitors from cumin. *J. Agric. Food Chem*. **1998**, 46, 5338-5341.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. **1951**, 193, 265.

Madero, C.F.; Finne, G. Properties of phenoloxidase from Gulf shrimp. In Proc. 7th Ann. Trop. and Subtrop. Fish. Tech. Conf. Americas; **1982**, 328-339

Markakis, P.; Embs, R.J. Effect of sulfite and ascorbic on mushroom phenol oxidase. *J. Food Sci*. **1966**, 31:807-811.

McEvily, A.J.; Iyengar, R.; Otwell, S. Sulfite alternative prevents shrimp melanosis. *Food Technol*. **1991**, sept: 80-86.

Nakagawa, T.; Nagayama, F. Distribution of catechol oxidase in crustaceans. *Bull. Jap. Soc. Sci. Fish*. **1981**, 47(12) 1645.

Ogawa, M.; Perdigao, N.B.; Santiago, M.E.; Kozima, T.T. On physiological aspects of black spot appearance in shrimp. *Bull. Jap. Soc. Sci. Fish*. **1984**, 50(10)1763-1769.

Otwell, W.S.; Iyengar, R.; McEvily, A.J. Inhibition of shrimp melanosis by 4-hexylresorcinol. *J. Aquatic Food Product Technol*. **1992**, 1(1):53-65.

Palou, E.; López-Malo, A.; Barbosa-Cánova, G.V.; Welti-Chanes, J.; Swanson, B.G. Polyphenoloxidase activity and color of blanched and high hydrostatic pressure treated banana puree. *J. Food Sci.* **1999**, 64(1)42-45.

Quaglia, G.B.; Gravina, R.; Paperi, R.; Paoletti, F. Effect of high pressure treatments on peroxidase activity, ascorbic acid content and texture in green peas. *Lebensm. -Wiss. U.- Technol*. **1996**, 29:552-555.

Rolle, R.S.; Guizani, N.; Chen, J.S.; Marshall, M.R.; Yang, J.S.; Wei, C.I. Purification and characterization of phenoloxidase isoforms from Taiwanese black tiger shrimp (*Penaeus monodon*). *J. Food Biochem*. **1991**,15:17-32.

Savagaon, K.A.; Sreenivasan, A. Activation mechanism of pre-phenoloxidase in lobster and shrimp. *Fish. Technol.* **1978**, 15:49-55.

Sayavedra-Soto, L.A.; Montgomery, M.W. Inhibition of polyphenoloxidase by sulfite. *J. Food Sci*. **1986,** 51(6):1531-1536.

Seyderhelm, I.; Boguslawski, S.; Michaelis, G.; Knorr, D. Pressure induced inactivation of selected food enzymes. *J. Food Sci.* **1996**, 61(2)308-310.

Simpson, B.K.; Marshall, M.R.; Otwell, W.S. Phenoloxidase from shrimp (*Penaeus setiferus*): purification and some properties. *J. Agric. Food Chem*. **1987,** 35:918-921.

Simpson, B.K.; Marshall, M.R.; Otwell, W.S. Phenoloxidase from pink and white shrimp: kinetic and other properties. *J. Food Biochem*. **1988,** 12:205-217.

Slattery, S.L.; Williams, D.J.; Cusack, A. A sulphite-free treatment inhibits blackspot formation in prawns. *Food Australia* **1995**, 47(11)509-514.

Taylor, S.L.; Bush, R.K. Sulphite as food ingredients. Food Technol. **1986,** 40:47-52.

Taoukis, P.S.; Labuza, T.P.; Lillemo, J.H.; Lin, S.W. Inhibition of shrimp melanosis (black spot) by ficin. *Lebensm.-Wiss.u.- Technol*. **1990**, 23:52-54.

Thomas, P.; Janave, M. T. Isoelectric focusing evidence for banana isoenzymes with mono- and diphenolase activity. J. Food Sci. **1986**, 51(2):384-387.

Yu Guang-li; Wang Yuan-hong; Liu Shu-qing; Tian Xue-lin. Effects of low molecular weight chitosan (LMC-1) on shrimp preservation. *Chin. J. Oceanol. Limnol*. **1996,** 14(2):189-192.

Weemaes, C.; Ludikhuyze, L.; Van den Broeck, I.; Hendrickx, M. High pressure inactivation of polyphenoloxidase. J. Food Sci. **1998**, 63(5):873-877.

Figure 1. Poliphenoloxidase activity of different anatomical locations

Figure 2. Optimum temperature and stability curves of PPO of carapace extract of prawn

Figure 3. Optimum pH and stability curves of PPO of carapace extract of prawn

Figure 4. Effect of high hydrostatic pressure on relative activity of PPO, 7 ºC 10 min

Figure 5. Concentration as related to the inhibitory effect of bisulphite, kojic acid, 4-hexylresorcinol and benzoate acid on relative activity of PPO