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# 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 activities 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 and 60°C, however thermal stability was greatest at temperatures below 35°C. The enzyme was most active at pH 5 and 8, but most stable at basic pH. Pressurization of the enzymatic extract was assayed within a range of 0.1–400 MPa, for 10 min at $<10$°C. Pressure-induced inactivation was evident, particularly at 300–400 MPa. Total inhibition of the extract was achieved only with ascorbic acid and citric acid at pH 3.0. 80 μg/ml sulphite, 150 μg/ml of kojic acid, 1 g/l of 4-hexylresorcinol or 0.1 g/l of sodium benzoate was required for 80% inhibition. 

*Keywords:* Polyphenoloxidase; Prawns; Melanosis; Inhibitors; High pressure

## 1. 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 has received 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, Perdigao, Santiago, & Kozima, 1984). Moreover, there are some indications that it also occurs in the surface membrane covering the muscle and in the haemolymph (Nakagawa & 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, Marshall, & Otwell, 1987). In fact, in the few reports of partial characterization of polyphenoloxidase (PPO), such as in pink shrimp (*Penaeus duorarum)* (Simpson, Marshall, &

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Otwell, 1988), white shrimp (*Penaeus setiferus*; Madero & Finne, 1982; Simpson et al., 1987), black tiger shrimp (*Penaeus monodan*; Rolle, Guizani, Chen, Marshall, Yang, & Wei, 1991) and Florida prawn (*Panulirus argus*; Ali, Gleeson, Wei, & Marshall, 1994; Ali, Marshall, Wei, & Gleeson, 1994), 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 & 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 susceptibilities 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, Otwell, & Marshall, 1989); however, these compounds are known to produce allergic reactions and serious disturbances in asthmatic subjects (Dewitt, 1988; Taylor & Bush, 1986). 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, Rolle, Marshall, & Wei, 1991). 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, Wei, Rolle, Otwell, Balaban, & Marshall, 1991; Chen, Wei, & Marshall, 1991; Taoukis, Labuza, Lillemo, & Lin, 1990), chitosan or phytic acid in shrimp (*Trachypenaeus curvirostris*; Yu, Wang, Liu, & Tian, 1996), or derivatives of resorcinol in shrimp and prawn (McEvily, Iyengar, & Otwell, 1991; Otwell, Iyengar, & McEvily, 1992; Slattery, Williams, & Cusack, 1995).

There are also several processes that strongly inhibit PPO. In this connection, particularly interesting are atmospheres that reduce the oxygen in the medium. Also, high hydrostatic pressure has been tried as a PPO inhibitor in various vegetable products. Some studies show that pressurization, not only inhibits the enzyme, but could actually cause slight enzymatic activation (Asaka & Hayashi, 1991; Estiaghi & Knorr, 1993); on the other hand, Seydarhelm, Boguslawski, Michaelis, and Knorr (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, Ludikhuyze, Van den Broecic, & Hendrickx, 1998). In the present study, many of these possibilities were restricted; the level and temperature of pressurization were low enough to achieve 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.

## 2. Materials and methods

### 2.1. General

The specie 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 $13.0 \pm 0.9$ cm and $21.5 \pm 4.5$ g, respectively. Forty kilograms 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 $-80^{\circ}C$.

### 2.2. Enzyme extraction

PPO was extracted according to Chen, Rolle et al. (1991) 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 for 3 h at $4^{\circ}C$ under $N_2$ atmosphere and the suspension was centrifuged (8000 *g*, 30 min, $4^{\circ}C$). The supernatant was collected and precipitated with ammonium sulphate at 0, 0–40 and 40–70% saturation, depending on the assay, at $4^{\circ}C$ under $N_2$ atmosphere. After subsequent centrifugation (12 500 *g*, 30 min, $4^{\circ}C$), 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 $4^{\circ}C$ 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 $-80^{\circ}C$ in order to prevent alterations, as far as possible, prior to determination.

### 2.3. Protein quantification

Protein was estimated according to Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard.

### 2.4. 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, St. 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 sodium phosphate buffer. Unless otherwise stated, experiments were repeated, at least in duplicate, and the results are expressed as absorbance increment/min. *Relative activity* describes enzymatic activity as the percentage of enzymatic activity expressed as $(A/A_{max})\times 100$, where $A$ indicates the increase in optical density per minute.

### 2.5. Isoelectric focusing

Ten microliters of extract were loaded onto 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 carbonic anhydrase B, horse myoglobin, lectin and trypsinogen, supplied by Pharmacia LKB. Isoelectric focusing was performed on a Pharmacia apparatus (FBE-3000) at 10°C, 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 M sodium hydroxide and 1 M orthophosphoric acid, respectively. The gels were then fixed in 10% trichloroacetic acid and 5% sulphosalicylic acid 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.

### 2.6. 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, St. 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 temperatures (from 25 to 60°C) for 30 min. After cooling, residual activities were determined at 25°C by addition of the substrate.

### 2.7. 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.

### 2.8. 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 thermocouple. 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.

### 2.9. 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 Co., St. Louis, MO, USA), kojic acid (Sigma), 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 $[(A–A^*)/A]\times 100$, where $A$ and $A^*$ indicate the variation of absorbance in absence and presence, respectively, of the inhibitor.

## 3. Results and discussion

### 3.1. 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 (Fig. 1). The activity in the carapace was the greatest; next the caudal zone (uropods and telson), and last, the muscle and cuticle. This could be due to the fact that PPO activity appears to be involved in the sclerotization process. The PPO in the muscle would come from the haemolymph and 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 were not considered.

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 a desalting column. Dialysis causes considerable loss of enzyme activity because it takes at least 8 h: so a desalting column is considered more suitable, because the salt can be removed much more quickly (5–10 min). 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 with respect to the activity of the 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 355 (Δ OD/min/ml) and specific activity 80.5 (total activity/mg protein). Close values of specific activity were reported by Savagaon and Sreenivasan (1978) in lobster: 109 OD increment/mg protein/5 min at 25°C.

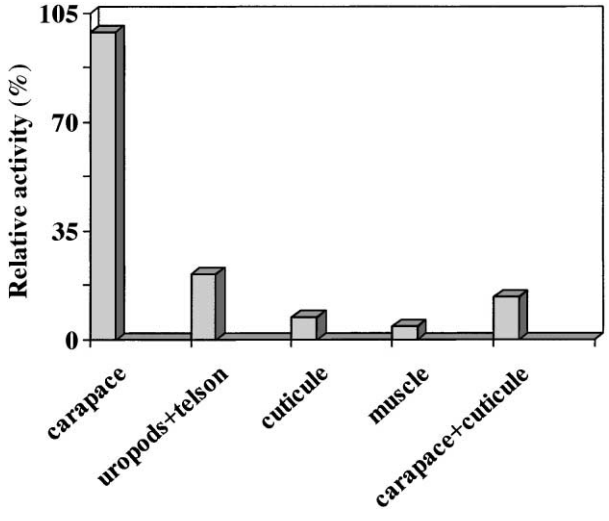


Fig. 1. Polyphenoloxidase activity of different anatomical locations.

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 pH characteristics. In other 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 values were not reported. In the case of the prawn, *Penaeus setiferus*, on the other hand, the best precipitation for purification was between 40 and 75% ammonium sulphate saturation, yielding a smaller purified enzyme (30 kDa; Simpson et al., 1987). This confirms the variations in PPO characteristics among species. Also, in Florida lobster (*Panulirus argus)*, isomorphic forms of varying molecular weight and one single pI have been found (Ali, Marshall et al., 1994). There are few reports 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 & Janave, 1986).

### 3.2. Effect of temperature

Fig. 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°C. The activity increased by around 20% for every 10°C of temperature to reach both plateaus; this could be 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, was possibly due to thermal denaturation of the enzyme.

These results relate to other studies (Chen, Rolle 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, Marshall et al., 1994) 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 (Rolle et al., 1991; Simpson et al., 1988); in *Penaeus setiferus*, the upper limit was 50°C (Simpson et al., 1987), and in lobster (*Panulirus argus*) it was 40°C (Ali, Marshall et al., 1994). Moreover, even within the same species there are differences depending on the state of activation of the enzyme itself (Ferrer, Koburger, Otwell, Gleeson, Simpson, & Marshall, 1989). What the foregoing suggests is that some enzymes can be active within a wide temperature range but further unstable.

### 3.3. Effect of pH

Fig. 3 shows the enzymatic activity profile of the extract from the carapace of the cephalothorax at 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 maxima of acid and alkaline activities.

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 $7.16 \pm 0.07$, while, in the abdominal cuticle, it was $8.76 \pm 0.04$. Therefore, the enzyme would probably present different optimum pH characteristics, depending on the locus of extraction, although, in our case, both pH activity peaks were located in the carapace. The pH profile for PPO isolated from other crustaceans varies according to species; for example, Chen, Rolle et al. (1991) 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 pH range 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*) 6–6.5 (Ali, Marshall et al., 1994).

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 was used in the following assays.

As with temperature, the activity and stability differed according to enzyme state of activation (Ferrer, Koburger et al., 1989). In this connection, almost all studies seem to agree that PPO is not stable at acid pH. Both in lobster (*Panulirus argus*; Ferrer, Koburger et al., 1989; Ali, Marshall et al., 1994) 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 $pH \leqslant 7.5$, 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

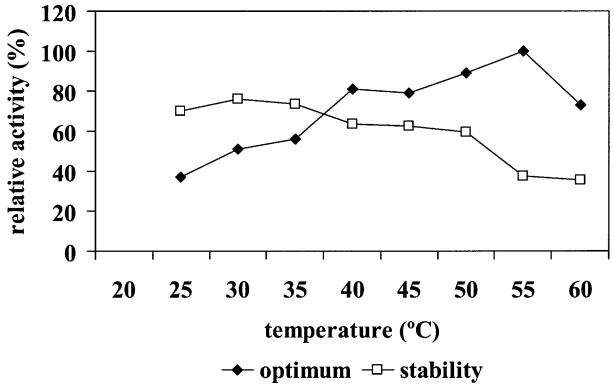


Fig. 2. Optimum temperature and stability curves of PPO of carapace extract of prawn.

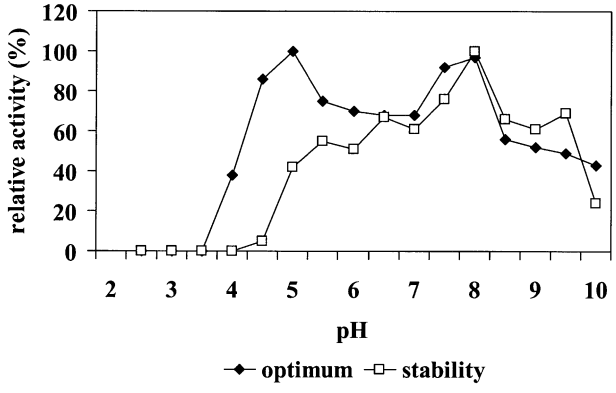


Fig. 3. Optimum pH and stability curves of PPO of carapace extract of prawn.

spiny lobster (*Panulirus argus*) was not stable at acid pH values, and the pattern was similar in Western Australian lobster (*Panulirus Cygnus*; Chen, Rolle et al., 1991). It seems that the species most resembling this in temperature profile were the imperial tiger prawn and, in pH, the white shrimp (*Penaeus setiferus)* from the Florida coast.

### 3.4. Effect of high pressure

In basic conditions (pH 8), activity was greater 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 from 100 to 200 MPa and more pronounced from 300 to 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, Nicoli, Dall'Aglio, & Lerici, 1995; Seyderhelm et al., 1996; Gomes & Ledward, 1996) or on products such as juices and purees (Cano, Hernandez, & de Ancos 1997; Hernández and Cano, 1998; Palou, López-Malo, Barbosa-Cánova, Welti-Chanes, & Swanson 1999; Quaglia, Gravina, Paperi, Paoletti, 1996). PPO enzymes from different origins display 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, 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).

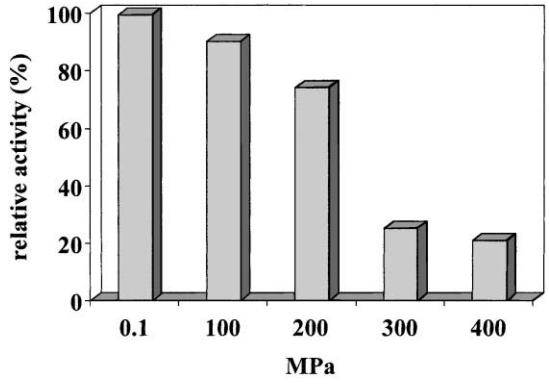


Fig. 4. Effect of high hydrostatic pressure on relative activity of PPO, 7°C 10 min.

### 3.5. Inhibiting compounds

The behaviour of the inhibitors in the model system cannot 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 from 80 to 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 & Embs, 1966); it modifies the protein structure (Sayavedra-Soto & Montgomery, 1986), or it reduces the quinones (Ferrar & 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, Wei, Rolle et al. (1991) 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 these temperatures the PPO in this species would be highly unstable, with little relative activity. Kahn, Shalom, & Zakin (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 in which it is used.

Hexylresorcinol (Fig. 5) produced 40% inhibition with concentrations of 0.15 g/l, and 80% inhibition with 1 g/l under the given conditions of addition (0°C, 5 min). The studies published on hexylresorcinol were conducted on the prawn itself and showed that it was a good inhibitor since, under the given conditions, melanosis was inhibited to a greater extent by 50 ppm of hexylresorcinol than by the usual concentrations of sulphite (12 500 ppm; Otwel et al., 1992).

Sodium benzoate (Fig. 5) was more effective than hexylresorcinol, producing around 70% inhibition with

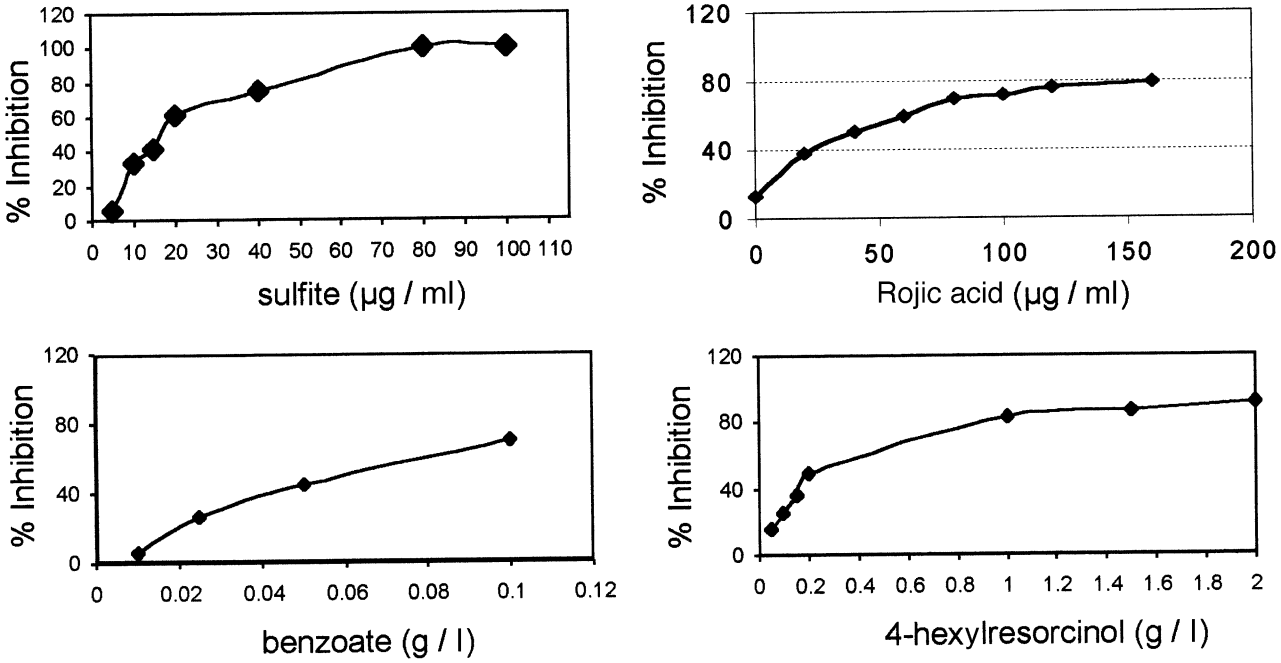


Fig. 5. Concentration, as related to the inhibitory effects of bisulphite, kojic acid, 4-hexylresorcinol and benzoic acid, on the relative activity of PPO.

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; benzoic acid is one such compound. Additionally the seeds of *Cuminum cyminum*, commonly known as cumin, contain cumic acid, a phenol derivative with some inhibition capacity (Kubo & 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 acidities.

## 4. 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 similarities to some shrimps, but there was no great resemblance to any one in particular. High pressures (300–400 MPa, 10 min, $<10^{\circ}$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.

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## References

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

Ali, M. T., Marshall, M. R., Wei, C. I., & Gleeson, R. A. (1994). Monophenol oxidase activity from the cuticle of Florida spiny lobster (*Panulirus argus*). *Journal of Agricultural and Food Chemistry*, *42*, 53–58.

Anese, M., Nicoli, M. C., Dall'Aglio, G., & Lerici, C. R. (1995). Effect of high pressure treatment on peroxidase and polyphenoloxidase activities. *Journal of Food Biochemistry*, *18*, 285–293.

Asaka, M., & Hayashi, R. (1991). Activation of polyphenoloxidase in pear fruits by high pressure treatment. *Agricultural and Biological Chemistry*, *55*(9), 2439–2440.

Cano, M. P., Hernandez, A., & de Ancos, B. (1997). High pressure and temperature effects on enzyme inactivation in strawberry and orange products. *Journal of Food Science*, *62*, 985–988.

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

Chen, J. S., Wei, C. I., Rolle, R. S., Otwell, W. S., Balaban, M. O., & Marshall, M. R. (1991). Inhibitory effect of kojic acid on some plant and crustacean polyphenoloxidases. *Journal of Agricultural and Food Chemistry*, *39*, 1396–1401.

Chen, J. S., Wei, C. I., & Marshall, M. R. (1991). Inhibitory mechanism of kojic acid on polyphenoloxidase. *Journal of Agricultural and Food Chemistry*, *39*(11), 1897–1901.

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

Estiaghi, M. N., & Knorr, D. (1993). Potato cubes response to water blanching and high hydrostatic pressure. *Journal of Food Science*, *58*(6), 1371–1374.

Ferrar, P. H., & Walker, R. L. (1999). Microorganisms as potential sources of novel diphenol oxidase inhibitors. *Journal of Food Biochemistry*, *23*, 1–15.

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

Ferrer, O. J., Otwell, W. S., & Marshall, M. R. (1989). Effect of bisulfite on lobster shell phenoloxidase. *Journal of Food Science*, *54*(2), 478–480.

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

Hernández, A., & Cano, M. P. (1998). High pressure and temperature effects on enzyme inactivation in tomato puree. *Journal of Agricultural and Food Chemistry*, *46*, 266–270.

Kahn, V., Shalom, N. B., & Zakin, V. (1998). Effect of kojic acid on the hydroxylation of L-tyrosine and tyramine by mushroom tyrosinase. *Journal of Food Biochemistry*, *22*, 455–474.

Kubo, I., & Kinst-Hori, I. (1998). Tyrosinase inhibitors from cumin. *Journal of Agricultural and Food Chemistry*, *46*, 5338–5341.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265.

Madero, C. F., & Finne, G. (1982). Properties of phenoloxidase from Gulf shrimp. In *Proceedings of Annual Tropical & Subtropical Fisheries Technology Conferences of Americas* (pp. 328–339).

Markakis, P., & Embs, R. J. (1966). Effect of sulfite and ascorbic on mushroom phenol oxidase. *Journal of Food Science*, *31*, 807–811.

McEvily, A. J., Iyengar, R., & Otwell, S. (1991). Sulfite alternative prevents shrimp melanosis. *Food Technology*, *September*, 80–86.

Nakagawa, T., & Nagayama, F. (1981). Distribution of catechol oxidase in crustaceans. *Bulletin of the Japanese Society of Scientific Fisheries*, *47*(12), 1645.

Ogawa, M., Perdigao, N. B., Santiago, M. E., & Kozima, T. T. (1984). On physiological aspects of black spot appearance in shrimp. *Bulletin of the Japanese Society of Scientific Fisheries*, *50*(10), 1763–1769.

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

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

Quaglia, G. B., Gravina, R., Paperi, R., & Paoletti, F. (1996). Effect of high pressure treatments on peroxidase activity, ascorbic acid content and texture in green peas. *Food Science and Technology—Lebensmittel-Wissenschaft & Technologie*, *29*, 552–555.

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

Savagaon, K. A., & Sreenivasan, A. (1978). Activation mechanism of pre-phenoloxidase in lobster and shrimp. *Fish Technology*, *15*, 49–55.

Sayavedra-Soto, L. A., & Montgomery, M. W. (1986). Inhibition of polyphenoloxidase by sulfite. *Journal of Food Science*, *51*(6), 1531–1536.

Seyderhelm, I., Boguslawski, S., Michaelis, G., & Knorr, D. (1996). Pressure induced inactivation of selected food enzymes. *Journal of Food Science*, *61*(2), 308–310.

Simpson, B. K., Marshall, M. R., & Otwell, W. S. (1987). Phenoloxidase from shrimp (*Penaeus setiferus*): purification and some properties. *Journal of Agricultural and Food Chemistry*, *35*, 918–921.

Simpson, B. K., Marshall, M. R., & Otwell, W. S. (1988). Phenoloxidase from pink and white shrimp: kinetic and other properties. *Journal of Food Biochemistry*, *12*, 205–217.

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

Taylor, S. L., & Bush, R. K. (1986). Sulphite as food ingredients. *Food Technology*, *40*, 47–52.

Taoukis, P. S., Labuza, T. P., Lillemo, J. H., & Lin, S. W. (1990). Inhibition of shrimp melanosis (black spot) by ficin. *Lebensmittel-Wissenschaft & Technologie*, *23*, 52–54.

Thomas, P., & Janave, M. T. (1986). Isoelectric focusing evidence for banana isoenzymes with mono- and diphenolase activity. *Journal of Food Science*, *51*(2), 384–387.

Yu Guang-li, Wang Yuan-hong, Liu Shu-qing, Tian Xue-lin. (1996). Effects of low molecular weight chitosan (LMC-1) on shrimp preservation. *Chinese Journal of Oceanology and Limnology*, *14*(2), 189–192.

Weemaes, C., Ludikhuyze, L., Van den Broeck, I., & Hendrickx, M. (1998). High pressure inactivation of polyphenoloxidase. *Journal of Food Science*, *63*(5), 873–877.