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Inhibitory effect of cysteine and glutathione on phenoloxidase from kuruma prawn (*Penaeus japonicus*)

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Abstract

The inhibitory effect of cysteine and glutathione on phenoloxidase (PO) from kuruma prawn were investigated. Cysteine and glutathione inhibited the oxidation of 3-(3,4-dihydroxylphenyl)-L-alanine (L-DOPA) catalyzed by kuruma prawn PO. Those thiol compounds showed competitive inhibition with K_i values of 0.45–0.46 mM. The inactivated PO could be partially recovered by the addition of copper acetate (0.01–0.2 mM). Almost complete restoration of PO activity was achieved with the addition of *N*-ethylmaleimide (NEM) in the range 0.05–0.2 mM, suggesting the importance of sulfhydryl groups of cysteine and glutathione for their inhibitory activity. Additionally, both cysteine and glutathione prevented the colour development by trapping the colour intermediates or reducing *o*-quinone to colourless compounds.

Keywords: Phenoloxidase; Cysteine; Glutathione; Prawn; Melanosis; Inhibitor

1. Introduction

Melanosis or blackening is a problem occurring in crustaceans during post-mortem storage. This phenomenon causes loss in nutrients, market value and consumer acceptability (Bartolo & Birk, 1998; Ogawa, Meneses, Perdigão, & Kozima, 1983; Ogawa, Perdigão, Santiago, & Kozima, 1984). This browning process has been known to be induced by phenol oxidase (PO; EC 1.14.18.1), also known as tyrosinase, polyphenol oxidase (PPO) and phenolase. PO catalyzes the hydroxylation of tyrosine to *o*-dihydroxyphenylalanine (DOPA) and oxidation of DOPA and other *o*-phenols to *o*-quinones (Friedman, 1996). The *o*-quinone can undergo further oxidation to brown melanin pigments or participate in polymerization reactions with protein functional groups to form cross-linked polymers (Friedman, 1996). PO is distributed differently in many parts of shrimp and lobster (Montero, Ávalos, & Pérez-Mateos, 2001; Ogawa et al., 1984). Generally, the melanosis in crustaceans starts in the head portions and spreads to the tails. The intensity of occurrence depends on species (Montero et al., 2001; Simpson, Marshall, & Otwell, 1987).

Melanosis in crustaceans can be controlled by some compounds, especially sulfiting agents (Ferrer, Otwell, & Marshall, 1989). Due to the allergy problem of sulfite compounds (Taylor & Bush, 1986), alternative compounds have been intensively studied. 4-Hexylresorcinol was used successfully in preventing melanosis in post-mortem shrimp and frozen crab (McEvily, Iyengar, & Otwell, 1991). Kojic acid could inhibit PPO from shrimp and spiny lobster (Chen et al., 1991). Melanosis in the

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pink shrimp was also inhibited by ficin (Taoukis, Labuza, Lillemo, & Lin, 1990). Montero et al. (2001) reported that kojic acid, benzoate and 4-hexylresorcinol could potentially be used as sulfite substitutes to prevent melanosis in prawn.

Sulfhydryl compounds, such as cysteine and glutathione, have been reported to inhibit the enzymatic browning reaction, especially in fruits and vegetables (Arslan, Erzengin, Sinan, & Ozensoy, 2004; Jiang, Fu, Zauberman, & Fuchs, 1999). Sulfur amino acid and sulfur-rich protein prevent the browning reaction by trapping the intermediates with the subsequent prevention of transformation to the brown pigment (Friedman, 1996). Furthermore, the compounds could inhibit PO by changing the copper-containing active site of the enzyme (Friedman, 1996) or showed direct irreversible inhibition (Robert, Richard-Forget, Rouch, Pabion, & Cadet, 1996). Therefore, sulfhydryl compounds should be alternatives for preventing melanosis in crustaceans. So far, no information of the use of sulfhydryl compounds in crustacean has been reported. Our investigation aimed to study the inhibitory activity of cysteine and glutathione toward PO from kuruma prawn.

2. Materials and methods

2.1. Chemicals

L-cysteine, glutathione (reduced form), 3-(3,4-dihydroxylphenyl)-L-alanine (L-DOPA), copper acetate and *N*-ethylmaleimide were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Preparation of PO from kuruma prawn

PO was extracted and 19-fold purified from the chephalothorax of kuruma prawn (*Penaeus japonicus*), as described by Benjakul, Visessanguan, and Tanaka (2005). PO activity was 1.5 U/ml. The PO solution was kept under nitrogen atmosphere at −20 °C until used.

2.3. PO activity assay

PO activity was assayed using L-DOPA as a substrate according to the method of Simpson et al. (1987), as modified by Benjakul et al. (2005). The assay system consisted of 100 μl of the enzyme solution, 500 μl of 15 mM L-DOPA in water, 1200 μl of assay buffers [McIlvaine buffer (0.2 M sodium phosphate, 0.1 M sodium citrate, pH 6.5)] and 200 μl of deionized water. The PO activity was determined for 3 min at 35 °C by monitoring the formation of dopachrome formation at 475 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PO activity was defined as an increase in the absorbance at 475 nm/min/ml.

2.4. Effect of cysteine and glutathione on PO activity

Cysteine or glutathione solutions (100 μl) were mixed with the enzyme (100 μl) to obtain a final concentration of 2 mM. Then, the assay buffer (1200 μl) and deionized water (100 μ) were added. To initiate the reaction, 500 μl of 15 mM L-DOPA were added. The reaction was conducted at 25 °C and the absorbance at 475 nm was monitored up to 5 min.

2.5. Kinetic analysis

The enzyme solution (100 μl) was mixed with cysteine or glutathione (100 μl) to obtain final concentrations of 0.25, 0.5 and 0.75 mM. The mixture was incubated for 5 min at room temperature (25 °C). To initiate the reaction, 1800 μl of L-DOPA in 50 mM sodium phosphate buffer, pH 6.5 were added. At each concentration of cysteine or glutathione, seven different concentrations of L-DOPA (0.11–4.5 mM) were used as the substrate. The reaction was assayed for 3 min at 35 °C. Absorbance at 475 nm was measured and the velocity was calculated as A_{475}/min. Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of PO were determined by Lineweaver–Burk plots (Lineweaver & Burk, 1934).

2.6. Effect of copper acetate and NEM on the inhibitory activity of cysteine and glutathione

Cysteine or glutathione at a final concentration of 0.2 mM was used. Copper acetate or NEM solution (100 μl) was mixed with cysteine or glutathione (100 μl) and enzyme (100 μl) to obtain the final concentration of 0–0.2 mM. The mixture was incubated for 5 min at room temperature. The assay buffer (1200 μl) was added and the reaction was then started by addition of 500 μl of 15 mM L-DOPA. The reaction was conducted for 3 min at 35 °C. The PO activity was calculated. The effect of copper acetate or NEM on PO activity was determined in the same manner in the absence of cysteine or glutathione.

2.7. Effects of cysteine and glutathione on the intermediate browning products

The reaction mixture containing 100 μl of enzyme solution, 100 μl of deionized water, 1200 μl assay buffer and 500 μl of L-DOPA was incubated at 25 °C for 3 min. To the reaction mixture, 100 μl of 4 mM cysteine or 4 mM glutathione were added immediately and mixed thoroughly. The change in red colour developed was monitored by measuring the absorbance at 475 nm for up to 3 min at 25 °C. For the control, deionized water (100 μl) was added instead of cysteine or glutathione. Decrease in absorbance at 475 nm indicates the forma-

tion of quinone-sulfhydryl compound or the reduction of *o*-quinone to phenols.

3. Results and discussion

3.1. Effect of cysteine and glutathione on PO activity

A lowered A_{475} in the sample treated with cysteine or glutathione indicated their inhibitory activity against kuruma prawn PO (Fig. 1). Cysteine exhibited a slightly greater inhibition, toward kuruma prawn PO, than did glutathione. For the control, the increase in A_{475} was observed as the reaction time increased. PO is the enzyme, which uses molecular oxygen to catalyze the *o*-hydroxylation of monophenols to *o*-diphenol and their further oxidation to coloured and highly reactive *o*-quinones (Arslan et al., 2004). From the result, it was noticeable that a lag phase was found in the sample containing cysteine or glutathione. In general, the lag phase is known to be extended by monophenolase inhibitor (Kubo & Kinst-Hori, 1998; Sánchez-Ferrer, Rodríguez-López, Garcia-Cánovas, & García-Carmona, 1995). Cysteine can act primarily with *o*-quinones and form colourless addition compounds (Peñalver, Rodríguez-López, García-Molina, García-Cánovas, & Tudela, 2002; Richard-Forget, Goupy, & Nicolas, 1992). Cysteine also reduces *o*-quinones to their phenol precursors (Cilliers & Singleton, 1990; Negishi & Ozawa, 2000). Cysteine was also reported to inactivate palmito PPO directly (Robert et al., 1996). Reduced glutathione was shown to effectively inhibit litchi PPO (Jiang et al., 1999). From the result, PO from kuruma prawn might be inhibited by both thiol agents, cysteine and glutathione, with multiple mechanisms, which need to be further investigated.

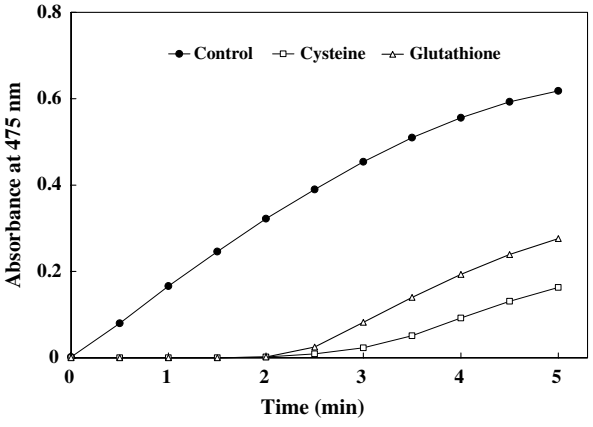

Fig. 1. Inhibitory effect of cysteine and glutathione on dopachrome formation induced by kuruma prawn PO. The formation of dopachrome was monitored by measuring the absorbance at 475 nm, at 25 °C, for up to 5 min.

3.2. Mode of PO inhibition by cysteine and glutathione

From Lineweaver–Burk plots, K_m and V_{max} of L-DOPA, induced by kuruma prawn PO under the experimental conditions were 0.26 mM and 0.20 $\Delta A_{475}\text{min}^{-1}$, respectively. The K_m observed for kuruma prawn PO was lower than those found in PO from white shrimp (2.8 mM) and pink shrimp (1.6 mM) (Simpson, Marshall, & Otwell, 1988). The result suggested the greater affinity between kuruma prawn PO and L-DOPA, compared with PO from those two species.

Inhibition kinetics for cysteine and glutathione toward kurama prawn PO were determined by Lineweaver–Burk plots as shown in Fig. 2. No lag phase was observed with cysteine and glutathione at any concentrations used (0.25, 0.5 and 0.75 mM) (data not shown). Since K_m values increased with cysteine and glutathione concentrations, whereas V_{max} values remained unchanged, the inhibitory mode of both compounds was shown to be of the competitive type. Competitive inhibition takes place when a molecule that is structurally similar to the substrate for a particular reaction competes for a position at the active site on the enzyme.

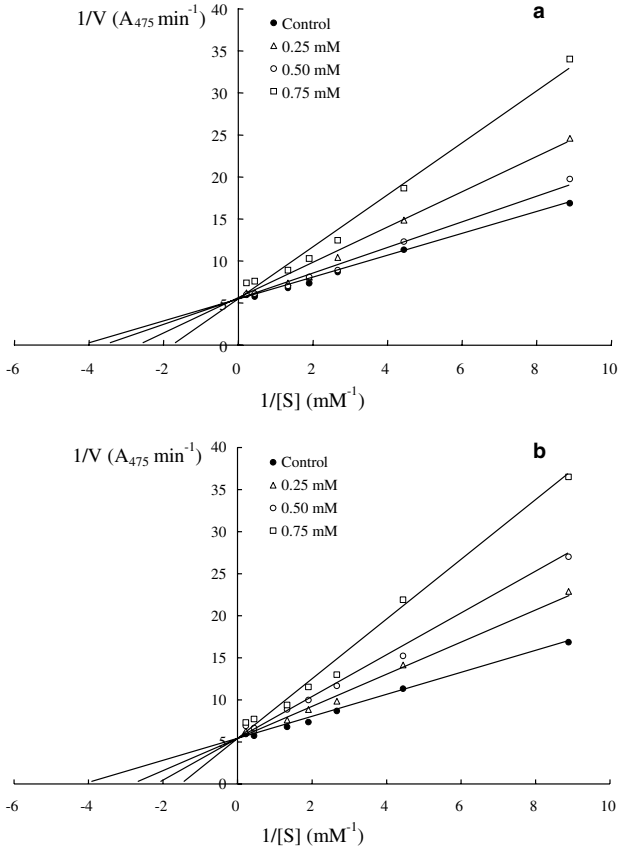

Fig. 2. Lineweaver–Burk plots of kuruma prawn PO and L-DOPA without and with cysteine (A) and glutathione (B) at different concentrations.

The result was in agreement with Richard-Forget et al. (1992) who reported that cysteine exhibited competitive inhibition on apple PPO. Arslan et al. (2004) also found competitive behaviour of cysteine and glutathione, when tested against mulberry PPO. K_i values of cysteine and glutathione, calculated from the double reciprocal plots, were 0.46 and 0.45 mM, respectively. Therefore, the two thiol compounds showed similar effectivities in inhibiting PO from kuruma prawn under the experimental conditions. Nevertheless, cysteine had a higher K_i value than glutathione, when tested with mulberry PPO (Arslan et al., 2004). This might be due to differences in enzyme conformation in which the inhibitors competed with substrate to bind enzyme differently. Inhibition of thiol compounds is attributed either to the stable colourless products formed by an addition reaction with *o*-quinone or binding to the active centre of PPO (Arslan et al., 2004).

3.3. Effects of copper acetate and NEM on the inhibitory activity of cysteine and glutathione

Copper acetate, at 0.01–0.2 mM, had no influence on kuruma prawn PO activity (Fig. 3). On the addition of copper acetate to the reaction mixture containing cysteine or glutathione, a restoration of PO activity was observed, to different degrees. In general, PO activity in the presence of cysteine was effectively recovered by the addition of copper acetate up to 0.05 mM. Concentrations above 0.05 mM did not show increased efficacy in restoration of PO activity. For glutathione, PO activity was more recovered by increasing copper acetate concentration up to 0.1 mM. No differences in PO activity were found by increasing copper acetate above 0.1 mM. At the same concentration tested, copper acetate addition resulted in less restoration of PO activity in the presence of glutathione, than in the presence of cysteine. This result suggested that copper ion might have different mechanisms for the restoration of PO inactivated by cysteine and glutathione. Son, Moon, and Lee (2000) reported that inactivated mushroom PPO partially recovered when cupric ion was added. Copper in the active site of PO is primarily involved in the browning reaction. Met-PPO [Cu(II)Cu(II)] is first reduced by reductants to deoxy-PPO [Cu(I)Cu(I)], which then interacts with oxygen, forming oxy-PPO [Cu(II)Cu(II)O_2], capable of catalyzing mono- or diphenol oxidation (Winkler, Lerch, & Solomon, 1981). Thiol reagents might interact with copper at the active site of PO, leading to loss of the activity. Therefore, added free copper ion might bind with thiol reagents more effectively than copper at the active site, resulting in the recovered activity.

Restoration of PO from kuruma prawn was observed with addition of NEM, a sulfyhydryl blocking agent (Fig. 4). The activity was recovered in the presence of NEM up to 0.05 mM. No changes in activity were observed with the addition of NEM at 0.05–0.2 mM and almost complete recovery was obtained within this concentration range. NEM had no adverse effect on the activity of PO from kuruma prawn. This result suggested that PO from kuruma prawn had no cysteine or sulfhydryl groups in the active site. Therefore, the restoration of PO by NEM was most likely due to its effect on both inhibitors, cysteine and glutathione. Cysteine can react with dopaquinone, forming 5-*S*-cysteinyldopa. Furthermore, it can reduce dopaquinone to dopa (Friedman, 1996). Therefore, the blocking of sulfhydryl groups by NEM prevented those phenomena. Additionally, the blocked sulfhydryl groups could not bind with the copper in the active site.

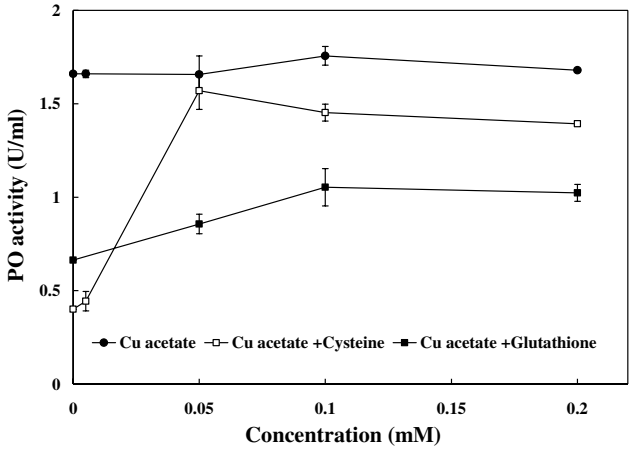

Fig. 3. Effect of copper acetate at different concentrations on the restoration of kuruma prawn PO in the presence or absence of cysteine or glutathione. Bars represent the standard deviation from triplicate determinations.

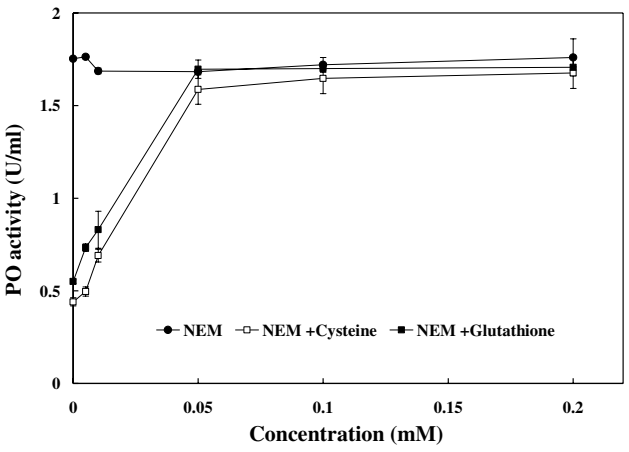

Fig. 4. Effect of NEM at different concentrations on the restoration of kuruma prawn PO in the presence or absence of cysteine or glutathione. Bars represent the standard deviation from triplicate determinations.

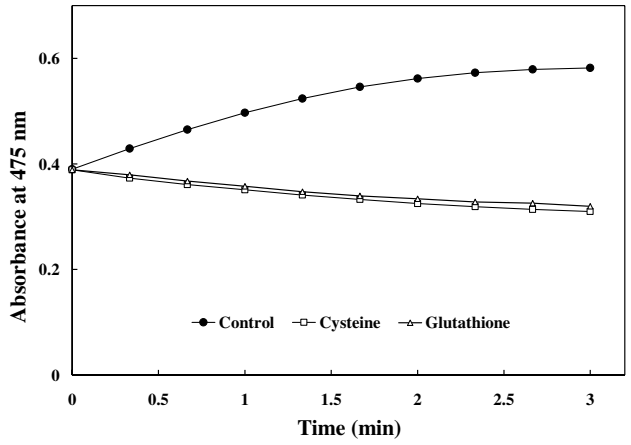

Fig. 5. Absorbance of dopachrome after the addition of cysteine or glutathione at different times. The cysteine or glutathione was added to the assay mixture after incubation at 25 °C for 3 min. The absorbance at 475 nm was monitored for another 3 min at 25 °C.

3.4. Effect of cysteine and glutathione on the intermediate browning products

A gradual decrease in A_{475} of the reaction mixture containing produced dopachrome was observed after the addition of cysteine or glutathione (Fig. 5). Conversely, $A_{475}$of the control increased continuously with increasing reaction time. The decrease in A_{475} indicated transformation of the dopaquinone, a red colour pigment, to other products. Cysteine was reported to trap *o*-quinone by forming cysteinyl adducts, the colourless compounds (Richard-Forget et al., 1992). Also, thiol-containing compounds can reduce *o*-quinone to *o*-diphenol (Friedman, 1996). Kojic acid was capable of reducing *o*-quinones to diphenols to prevent the blackening (Chen, Wei, & Marshall, 1991). From this result, the formation of colour compounds through the action of PO was affected in the presence of cysteine or glutathione, probably by the reduction of quinone or formation of the cysteinyl adducts.

4. Conclusion

Cysteine and glutathione exhibited competitive inhibition toward PO from kuruma prawn. The inactivated PO recovered upon copper acetate and NEM addition. The cysteine and glutathione could also reduce *o*-quinone or interact with the intermediate, leading to colourless compounds. Thus, inhibition of PO by both compounds might occur directly or indirectly. To prevent the melanosis in prawn or other crustaceans, both thiol compounds can be used.

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