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# Tyrosinase localization in mollusc shells

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**Abstract**

In molluscan shellfish, pigmentation is frequently observed in the calcified shell, but the molecular basis of this process is not understood. Here, we report two tyrosinase proteins (Pfty1 and Pfty2) found in the prismatic shell layer of the pearl oyster *Pinctada fucata*; this layer is recognized as the pigmented region in *P. fucata.* The protein sequences were deduced from the corresponding cDNAs and confirmed by MALDI-TOF/TOF analysis. The sequences suggest that both tyrosinases have two copper-binding sites in similar N-terminal domains that are homologous to tyrosinases of cephalopods and hemocyanins of gastropods. In turn, this suggests that bivalve tyrosinases are evolved from a common ancestral copper-binding protein in the mollusc. Pfty1 and Pfty2 were specifically expressed in the mantle, and their expression in the mantle is different from each other, suggesting that these tyrosinases have distinctive roles in melanogenesis in shells.


*Keywords:* Mollusc; Mantle; Pearl oyster; Shell; Pigmentation; Tyrosinase; Prismatic layer; Calcification

## 1. Introduction

In the last decade, several copper oxygen-binding enzymes have been characterized and their two main classes have been identified: hemocyanins as oxygen-transport proteins and phenoloxidases as enzymes (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) that catalyze hydroxylation of monophenols and oxidation of *o*-diphenols. Tyrosinase, which is found in many organisms, including vertebrates (Kwon et al., 1987, 1988; Mochii et al., 1992; Miura et al., 1995), invertebrates (Naraoka et al., 2003) and plants (van Gelder et al., 1997), has an important enzymatic function as one of the phenoloxidases in melanogenesis, which starts with transformation of tyrosine to L-DOPA. The series of reactions in melanin biosynthesis regulated by tyrosinase and related phenoloxidases are thought to be responsible for sclerotization of cuticles in insects, and for protection against ultraviolet radiation and other insults (Hearing and Jimenez, 1987; Marmaras et al., 1996; Sugumaran, 2002) in many organisms.

Both hemocyanin and tyrosinase are found in molluscs, and the nucleotide sequence of hemocyanin has been reported for a few cephalopods (Miller et al., 1998) and gastropods (Lieb et al., 1999, 2000, 2001, 2004; Altenhein et al., 2002), and the sequence and biochemical properties of tyrosinase and the mechanism of melanin biosynthesis in ink sacs have been investigated in some cephalopod species (Palumbo et al., 1994; Naraoka et al., 2003; Palumbo, 2003). In contrast, little is known about tyrosinase and its intrinsic role in melanin biosynthesis in mollusc shellfish. The shells of pearl oyster have two distinct calcareous layers: the pigmented prismatic layer and the nacreous layer (Wilt et al., 2003). Formation of both layers is regulated by proteins secreted from the mantle, which surrounds the internal organ and is divided into three parts: the mantle center, mantle pallial, and mantle edge. The mantle pallial is thought to contribute to formation of the nacreous layer, and the mantle edge to formation of the prismatic layer.

We speculated that the pigmented layer of shells might contain key regulators involved in melanin production in

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molluscan bivalves. Focusing our attention on proteins in the pigmented prismatic layer, here we show that two types of tyrosinase can be found in the prismatic layer in the pearl oyster *Pinctada fucata*.

## 2. Materials and methods

### 2.1. Isolation of proteins from the prismatic layer of shells

The prismatic layer of shells of *P. fucata* was rinsed in dilute sodium hypochlorite and powdered. The powdered prismatic shell material (about 20 g) was incubated in 20 vol of 8 M urea, 1% CHAPS and 50 μg/mL *p*-amidinophenylmethanesulfonyl fluoride (APMSF) for 3 days at 37 °C, and then centrifuged to remove insoluble shell material. The supernatant was dialyzed in $H_2O$ overnight and then centrifuged. The water-insoluble proteins precipitated in this process were then separated by SDS-PAGE, followed by visualization with Coomassie Brilliant Blue R-250 (CBB R-250).

### 2.2. MALDI-TOF/TOF analysis

After visualization of proteins with CBB R-250, an intensely stained portion of the gel corresponding to bands of 43 to 49 kDa was excised and subjected to MALDI-TOF MS analysis. Picking of gel spots, destaining of the gel pieces, in-gel trypsin digestion, and sample loading onto MALDI plates were performed automatically using an Xcise robotic protein processing system (Shimadzu Biotech). The gel spots were cut into pieces and transferred into 96-well microtiter plates. The pieces were washed with 100 μL of 50% acetonitrile in 50 mM ammonium bicarbonate twice for 20 min and then with 100 μL of 100% acetonitrile for 20 min. Following trypsin (Promega) digestion of proteins in the gel pieces in 30 μL of 25 mM ammonium bicarbonate at 30 °C overnight, the resulting peptides were purified and concentrated using ZipTip™ μC18 (Millipore), and subsequently spotted onto the MALDI sample plate with a 2.5 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. MS or tandem MS spectrometric analysis of the tryptic digests was performed using a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). MS spectra were measured in the reflector mode with a mass range from 700 to 3500 Da. External calibration was performed using five standard peptides. MS/MS spectra were measured in CID-off mode. Prediction of amino acid sequence from MS/MS data were carried out using denovo explorer ver. 1.22 (Applied Biosystems) with MS/MS tolerance of 0.2 Da.

### 2.3. Sequence analysis of cDNAs

The sequences of 3214 cDNAs derived from the mantle were searched for peptide sequences that were determined by MALDI-TOF/TOF MS analysis using GENETYX-PDB database software (Genetyx). This resulted in identification of two cDNA clusters. The longest cDNAs in the two clusters were sequenced using a Big-Dye terminator kit and an ABI 3730 DNA sequencer (Applied Biosystems).

### 2.4. Northern blot analysis

Tissues (whole mantle, gill, mid gut, edge and pallial of the mantle) from adult specimens of the pearl oyster *P. fucata* were isolated in artificial seawater (ASW) and homogenized in 10 vol of Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions (Invitrogen). RNA samples (10 μg) were separated by electrophoresis in a 1% agarose gel containing formaldehyde, and were then transferred to a Hybond-N filter (GE Healthcare Bio-Sciences). Hybridization was performed using $^{32}$P-labeled Pfty1 and Pfty2 cDNA probes in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 4 mM EDTA and 100 μg/mL of salmon sperm DNA at 65 °C. The filter was washed in 0.5× SSC at 65 °C. Signals were detected using a BAS2500 Image analyzer (Fuji film).

## 3. Results

### 3.1. Isolation of insoluble proteins from the pigmented prismatic layer in the pearl oyster

First, we attempted to solubilize the proteins found in the pigmented prismatic layer. After incubation of the powdered prismatic layer in the lysis buffer containing 8 M urea, the solubilized protein fraction was separated by SDS-PAGE, and this resulted in identification of three discrete bands (43, 47 and 49 kDa) in the polyacrylamide gel (Fig. 1). To investigate the partial sequences of the proteins corresponding to these bands, the bands were isolated and subjected to MALDI-TOF/TOF analysis following trypsin digestion. Since the 47 and 49 kDa proteins showed similar peaks in the MS spectrum, the 49 kDa protein was taken to be representative of the two bands (Fig. 2A).

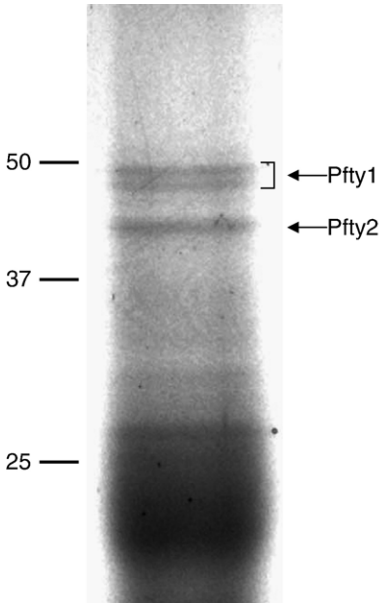


Fig. 1. SDS-PAGE of water-insoluble proteins isolated from the prismatic layer of the shell. Water-insoluble proteins isolated from the prismatic layer were subjected to SDS-PAGE and stained with CBB R-250. Three discrete bands (43, 47 and 49 kDa) corresponding to Pfty1 and Pfty2 are indicated by arrows. Molecular mass standards (kDa) are shown on the left side of the gel.