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# Purification and characterisation of phenoloxidase from amphioxus *Branchiostoma belcheri tsingtauense*

Qiuxiang Pang[a], Shicui Zhang[a,*], Xiaodong Shi[b], Feng Su[a], Di Wu[a]

[a]*Department of Marine Biology, Ocean University of China, Qingdao 266003, PR China*
[b]*Institute of Biophysics of Chinese Academy of Sciences, Beijing 100101, PR China*

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**Abstract**

Phenoloxidase (PO) from the humoral fluid of amphioxus *B. belcheri tsingtauense* was purified using a sequential combination of ammonium sulphate precipitation, Sephadex G-200 chromatography and DEAE Sepharose™ Fast Flow chromatography. In PAGE, the purified enzyme exhibited a single band of 150 kDa under non-reducing conditions, and was resolved to three bands with molecular masses of 72, 46 and 44 kDa, respectively, under reducing conditions, suggesting that the PO in amphioxus humoral fluid seems to be a heterotrimer of three polypeptides held together by disulphide bonds. The substrate specificity and inhibition characteristics both indicate that the PO isolated from amphioxus humoral fluid is a tyrosinase-type enzyme. In addition, mouse antisera against the purified PO were prepared, and their specificity was confirmed by Western blotting, facilitating the future determination of the origin of PO in the humoral fluid and the distribution of PO-synthesising tissues in amphioxus.


*Keywords:* Amphioxus; *Branchiostoma belcheri tsingtauense*; Phenoloxidase; Prophenoloxidase; Humoral fluid; Purification; Chromatography

## 1. Introduction

Phenoloxidase (PO), is a bifunctional copper-dependent enzyme possessing both tyrosinase activity (monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) and laccase activity (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) [1,2]. PO catalyses the oxidation of dopa rapidly to dopaquinone, which in

* Corresponding author. Tel./fax: +86 532 2032787.
*E-mail address:* sczhang@ouc.edu.cn (S. Zhang).

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turn polymerises non-enzymatically into insoluble melanin [3]. PO has been found in a variety of animals including both vertebrates and invertebrates [4,5]. It is not only present intracellularly in blood cells of vertebrates and invertebrates, but also present extracellularly in cell-free plasma and in cuticle of some insects [6–10]. In haemocytes and plasma, PO usually exists as an inactive precursor, prophenoloxidase (proPO) [6,10]. ProPO can readily be activated to PO by both an endogenous activating system and exogenous activators such as trypsin, lipopolysaccharide and zymosan (β-1,3-glucan) [1,11–14]. It has been shown that the proPO system, comprising proPO together with its activating enzyme, plays a role in defence reactions, including melanisation [15], sclerotisation [16], encapsulation [17–19] and wound healing [20], in both arthropods and molluscs [21,22]. In addition, the proPO system is also involved in several physiological processes such as neurotransmitter synthesis, eye pigmentation and production of inks [23–26].

Given such important functions, numerous investigations have been carried out on the characterisation of the enzyme PO. However, quite a few problems such as its instability, "stickiness" and loss of activity during purification have prevented the detailed characterisation of PO, and it has thus far been purified only from arthropods [11,13,14,27–31], molluscs [21] and ascidians [23]. Consequently, knowledge of the biochemical properties of this enzyme is very limited.

Amphioxus or lancelet, an intermediary species from invertebrates and vertebrates, has long been regarded as a basal lineage of chordates. Our previous study has shown that PO activity is present in the epidermal cells of gill and intestine and in the humoral fluid and epidermal mucus of amphioxus *Branchiostoma belcheri tsingtauense*, and PO in the mucus exhibits an antibacterial activity [32–35]. However, purification and characterisation of PO in this evolutionarily important animal remains open. The purpose of this study was thus to purify PO from the humoral fluid of amphioxus *B. belcheri tsingtauense*, and to examine its biochemical properties.

## 2. Materials and methods

### 2.1. Animals and chemicals

Amphioxus *B. belcheri tsingtauense* with an average body length of about 4 cm, were collected from the "amphioxus ground" near Shazikou in the vicinity of Qingdao, and reared in the laboratory on a diet of single-cell algae fed daily. The seawater was aerated continuously and changed once a day.

L-3,4-Dihydroxyphenyl-alanine(L-dopa), 4-methylcatechol, 3-methyl-2-benzothiazolinon hydrazonehydrochloride (MBTH), dopamine, hydroquinone, methylhydroquinone, phenylthiourea (PTU), sodium cacodylate (CAC), sodium azide and sodium dodecyl sulphate (SDS) were all purchased from Sigma (St Louis, MO). Sephadex G-200 was procured from Pharmacia Fine Chemicals (Nutley, NJ), and DEAE Sepharose™ Fast Flow was from Amersham Biosciences (Uppsala, Sweden). All other chemicals were analytical reagents.

### 2.2. Preparation of humoral fluid

The humoral fluid was prepared by the method of Wang et al. [36]. Briefly, about 1000 amphioxus were rinsed with distilled water, wiped out thoroughly with sterilised gauze, and then cut into about 2 $mm^3$ pieces on ice to bleed. After centrifugation at 12,000×$g$ for 30 min at 4 °C, the supernatant was collected and stored at −70 °C until used.

### 2.3. Enzyme purification

All operations were performed at 0–5 °C unless stated otherwise. PO from the humoral fluid was initially enriched by trial and sequential fractionation using $(NH_4)_2SO_4$ at 0–80% saturation levels following Scopes [37]. Since the trial fractionation yielded PO activity from the humoral fluid within 0–60% $(NH_4)_2SO_4$ saturation, the sequential fractionation of PO was, therefore, performed subsequently within the saturation limit. The pellet obtained with 30–40% $(NH_4)_2SO_4$ saturation in the sequential fractionation was found to contain the highest amount of PO and hence this $(NH_4)_2SO_4$ fraction was routinely used as the starting material for isolation of PO by gel permeation column chromatography. The pellet was dissolved in a minimum amount of 50 mM sodium phosphate buffer (SPB), pH 7.2, and applied to a Sephadex G-200 column (1.8×75 cm) equilibrated with the same buffer. The elution was performed with 50 mM SPB (pH 7.2) at a flow rate of 15 ml/h. Eluted fractions (3 ml each) were collected and their PO activity was measured by spectrophotometric assay (see below). Fractions containing PO activity were pooled. They were concentrated with sucrose, dialysed against 50 mM Tris–HCl (pH 7.2) and chromatographed on a DEAE Sepharose™ Fast Flow column (1.6×20 cm) equilibrated with 50 mM Tris–HCl (pH 7.2). Bound proteins were eluted with 50 mM Tris–HCl (pH 7.2) containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl (50 ml each), respectively, at a constant flow rate of 240 ml/h. Collected fractions were assayed for PO activity, and those with PO activity were pooled, concentrated with sucrose and used directly for biochemical property assay.

### 2.4. PO activity assay

PO activity in the humoral fluid and eluted fractions were assayed by the method of Horowitz and Shen [38] with slight modifications. An aliquot of 100 μl of the humoral fluid and eluted fractions was mixed with 360 μl of 10 mM sodium cacodylate (CAC) buffer (pH 7.0), 25 μl of 200 mM $CaCl_2$ and 15 μl of 1% SDS. They were pre-incubated at 30 °C for 2 min before addition of 950 μl of distilled water and 50 μl of 20 mM L-dopa [39]. The absorbance at 490 nm was measured every 1 min. The control was processed similarly except that the humoral fluid or eluted fractions were substituted with distilled water. The enzyme activity was expressed as units, where one unit is defined as 0.001 OD per milligram protein.

### 2.5. Electrophoresis and enzyme staining

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Davis [40], and SDS-PAGE according to Laemmli [41]. For native PAGE, the gel was run at 100 V for 2.5 h, and the molecular mass markers used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa) (Pharmacia). The reducing SDS-PAGE was run at 120 V for 2 h, and the molecular mass markers were myosin (200 kDa), calmodulin binding protein (130 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa) and rabbit actin (43 kDa). PO activity was detected on polyacrylamide gels by the method of Nellaiappan and Vinayagem [42] slightly modified. The native gels were washed with 50 mM SPB (pH 6.5) and pre-incubated in 50 mM SPB (pH 6.5) with 0.1% SDS for 15 min. The gels were then washed with distilled water twice and incubated in 50 mM SPB (pH 6.5) containing 10 mM 4-methylcatechol and 0.3% MBTH dissolved in 25% ethanol, which was prepared freshly. For SDS-PAGE, the gels were washed twice in ice-cold distilled water, and incubated in 50 mM SPB (pH 6.5) containing 10 mM 4-methylcatechol and 0.3% MBTH dissolved in 25% ethanol. Both native PAGE and reducing SDS-PAGE gels were also stained with 0.1% Coomassie brilliant blue R-250 to reveal the total proteins in the humoral fluid and isolated enzyme.

### 2.6. Inhibition assay of PO activity

The isolated enzyme was assayed for PO activity in the presence of PO inhibitors PTU and sodium azide [22,43]. A total of 100 μl of the isolated enzyme was added to 1230 μl of 1 mM PTU or 1 mM sodium azide in 10 mM CAC, and pre-incubated at 30 °C for 20 min. It was then mixed with 75 μl of 200 mM $CaCl_2$ and 45 μl of 1% SDS. The mixtures were incubated at 30 °C for 2 min. After addition of 50 μl of 20 mM L-dopa, the absorbance at 490 nm was measured. The control was processed similarly except that PTU and sodium azide were withdrawn from the reaction mixture.

### 2.7. Substrate specificity assay of PO

The substrate specificity assay was carried out according to the method of Andersen [44]. A reaction mixture containing 100 μl of the isolated enzyme, 360 μl of 50 mM SPB (pH 7.0), 25 μl of 200 mM $CaCl_2$ and 15 μl of 1% SDS was incubated at 30 °C for 2 min, and then 1 ml of 50 mM SPB (pH 7.0) with 1.5 mM of different substrates was added. The absorbance at 490 nm was monitored every 1 min against the control consisting of SPB, substrate and SDS in the presence of $Ca^{2+}$. Substrates used were 4-methylcatechol, dopamine, L-dopa, hydroquinone and methylhydroquinone.

### 2.8. Polyclonal antibody production

Specific polyclonal antisera against the isolated PO were raised in mice. In brief, approximately 80 μg of the purified PO was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites of the mice. Two booster injections of the same amount of antigen mixed with Freund's incomplete adjuvant were administered subcutaneously at intervals of 2 weeks. Eight days after the final booster, blood was collected and serum prepared. The antisera were aliquoted and stored at −70 °C. Antibody titre in the sera was determined by dot blot assay [45].

### 2.9. Western blotting analysis

The humoral fluid and isolated enzyme were run on native PAGE gel and reducing SDS-PAGE gel. The gels were washed for 15 min in the transfer buffer of 100 mM SPB containing 0.1% Tween-20, and proteins on the gels were blotted on nitrocellulose membrane (Hybond, Amersham Pharmacia). Blotted membranes were incubated at 30 °C for 1.5 h in 100 mM SPB containing 3% defatted milk powder, and then for 2 h in mouse anti-PO sera, diluted 1:100 with 100 mM SPB containing 3% BSA and 0.1% Tween-20. After washing in 100 mM SPB, membranes were incubated at 30 °C for 2 h in peroxidase-conjugated anti-mouse antibody, diluted 1:300. Bands were visualised using DAB and 0.03% $H_2O_2$.

### 2.10. Protein determination

The protein concentrations were determined by the method of Bradford [46] with bovine serum albumin as standard.

### 2.11. Statistical analysis

All experiments were repeated at least three times. The values are the means ($\pm$SD) of three assays performed. Significance was determined with Student's $t$ test and differences between results were considered significant at $p < 0.05$.

Table 1
Summary of the isolation of PO from the humoral fluid of amphioxus *B. belcheri tsingtauense*

| Step | Total volume (ml) | Total protein (mg) | Total activity (U $min^{-1}$) | Specific activity (U $min^{-1}$ $mg^{-1}$ protein) | Purification (-fold) |
|---|---|---|---|---|---|
| Humoral fluid | 20 | 276 | 1982 | 7.18 | 1 |
| $(NH_4)_2SO_4$ | 2.4 | 12.84 | 750 | 58 | 8.1 |
| Sephadex G-200 | 3 | 1.31 | 120 | 92 | 12.8 |
| DEAE- FF | 3 | 0.189 | 80 | 423 | 60 |

## 3. Results

PO was purified from the humoral fluid of amphioxus *B. belcheri tsingtauense* using a sequential combination of ammonium sulphate precipitation, Sephadex G-200 chromatography and DEAE Sepharose™ Fast Flow chromatography. The purification scheme and results are summarised in Table 1.

Fig. 1A is the typical elution profile of PO isolated from 2 ml of amphioxus humoral fluid after passing $(NH_4)_2SO_4$ fraction through Sephadex G-200. PO was detectable in fraction numbers 27–35, which

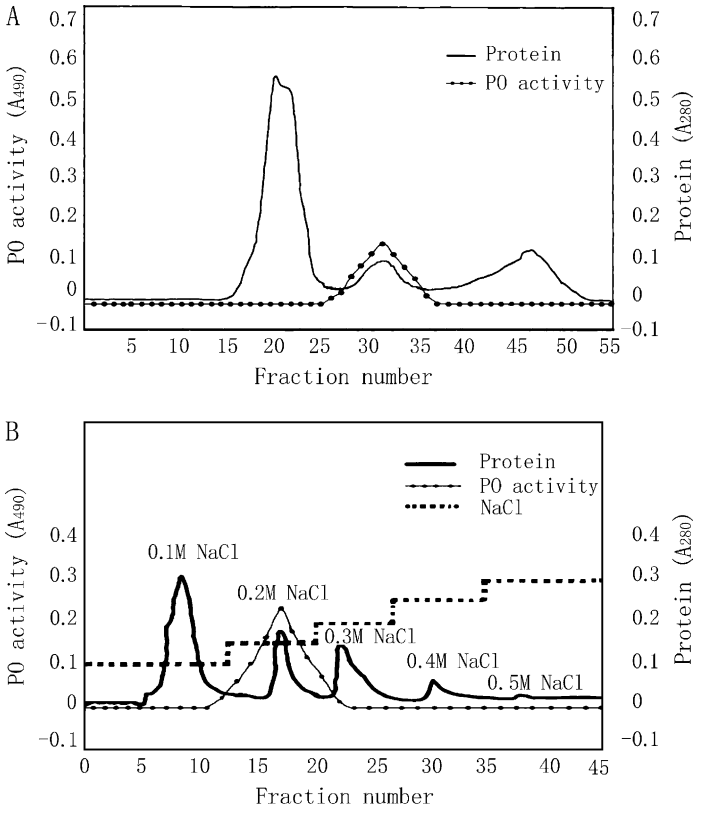


Fig. 1. Purification of PO. (A) Sephadex G-200 chromatography of the proteins obtained with 30–40% $(NH_4)_2SO_4$ saturation. Column (1.8×75 cm) equilibrated with 50 mM SPB (pH 7.2) buffer; flow rate 15 ml/h. (B) DEAE Sepharose™ Fast Flow ion exchange chromatography of PO-containing fractions eluted from Sephadex G-200 column. Column (1.6×20 cm) equilibrated with 50 mM Tris–HCl (pH 7.2); flow rate 240 ml/h.

coincided with the second prominent peak of proteins from the column. Among these fractions, fraction numbers 29–33, that were found to contain the highest amount of PO, were pooled and concentrated. The concentrated sample was subjected to separation by DEAE Sepharose™ Fast Flow ion-exchange chromatography, and resulted in the elution profile shown in Fig. 1B. From this profile, it is clear that most of the high molecular weight non-PO proteins were eluted early from the column, and were well separated from the PO elution peak. The PO activity in these isolated fractions was highly enriched, amounting to a 60-fold purification from the humoral fluid and a specific activity of 423 U $min^{-1}$ $mg^{-1}$ protein (Table 1).

Native PAGE of the purified protein revealed a single band with an estimated molecular mass of approximately 150 kDa with the specific enzyme activity (Fig. 2A). When subjected to reducing SDS-PAGE followed by Coomassie brilliant blue staining, it was resolved to three bands with molecular masses of 44, 46 and 72 kDa, respectively (Fig. 2B). All three bands were also stained positively for PO activity (Fig. 2B).

Mouse antisera against the purified PO with a titre of 1:1600 were obtained. They were capable of recognising one band of approximately 150 kDa on native PAGE gels corresponding to the PO band in the zymogram (Fig. 3A). The antisera also cross-reacted with three bands of 72, 46 and 44 kDa on reducing SDS-PAGE gels (Fig. 3B).

As shown in Fig. 4, the PO activity was poorly inhibited by sodium azide, but strongly inhibited by PTU. Table 2 shows the substrate specificity of activated PO. Dopamine, L-dopa and 4-methylcatechol were all suitable substrates, while hydroquinone and methylhydroquinone were poor ones.

## 4. Discussion

Previously, we have demonstrated the presence of PO activity in the humoral fluid of amphioxus *B. belcheri tsingtauense* [32]. Here we describe for the first time the purification of PO from the humoral fluid. The purified enzyme exhibited a single band of 150 kDa under non-reducing conditions, and was resolved to

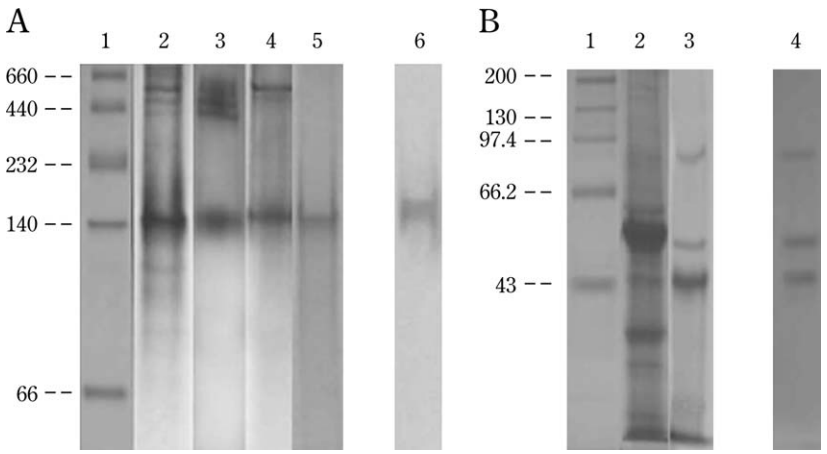


Fig. 2. Electrophoresis and enzyme staining. (A) Native PAGE. Lane 1, marker proteins; lane 2, the humoral fluid; lane 3, the pellet obtained by 30–40% $(NH_4)_2SO_4$ saturation; lane 4, PO-containing fractions eluted from Sephadex G-200 column; lanes 5 and 6, PO-containing fractions eluted from DEAE Sepharose™ Fast Flow column. Lanes 1–5, stained with Coomassie brilliant blue R-250; lane 6, stained with 4-methylcatechol and MBTH. (B) SDS-PAGE under reducing conditions. Lane 1, marker proteins; lane 2, the humoral fluid; lanes 3 and 4, PO-containing fractions eluted from DEAE Sepharose™ Fast Flow column. Lanes 1–3, stained with Coomassie brilliant blue R-250; lane 4, stained with 4-methylcatechol and MBTH.

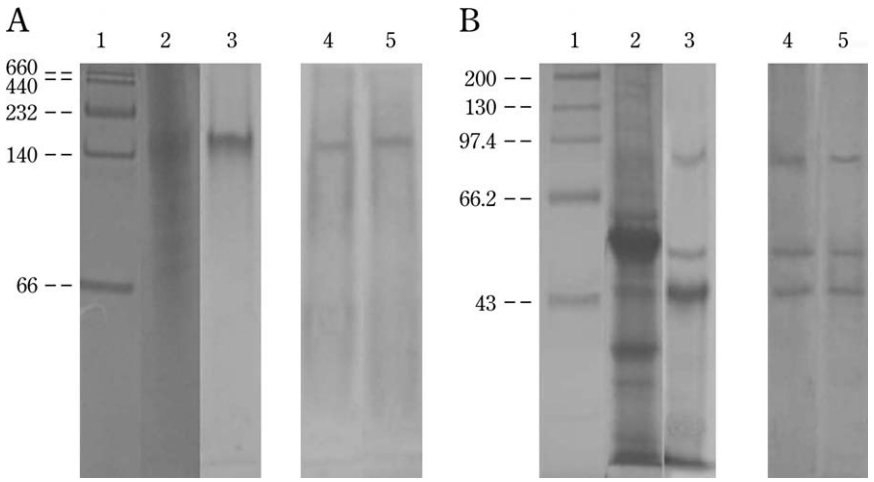


Fig. 3. Electrophoresis and immunoblotting. (A) PAGE and immunoblotting of the humoral fluid and purified PO. Lane 1, marker proteins; lanes 2 and 5, the humoral fluid; lanes 3 and 4, the purified PO; lanes 1–3, stained with Coomassie brilliant blue R-250; lanes 4 and 5, stained with mouse anti-PO sera. (B) SDS-PAGE under reducing conditions and immunoblotting of the humoral fluid and purified PO. Lane 1, marker proteins; lanes 2 and 5, the humoral fluid; lanes 3 and 4, the purified PO. Lanes 1–3, stained with Coomassie brilliant blue R-250; lanes 4 and 5, stained with mouse anti-PO sera.

three bands with molecular masses of 72, 46 and 44 kDa, respectively, under reducing conditions, that were all cross-reactive with mouse anti-PO sera. It has been shown that PO and proPO generally exist as a homodimer [23,47] or heterodimer [28,48]. However, our results here suggest that the PO in amphioxus humoral fluid seems to be a heterotrimer of three polypeptides held together by disulphide bonds. Interestingly, the yellow mosquito proPO also contains three subunits of 76, 62 and 58 kDa [25].

The substrate specificity and inhibition characteristics both indicate that the PO isolated from amphioxus humoral fluid is a tyrosinase-type enzyme, not a laccase-type enzyme. This is similar to the enzymes from the haemocytes of *Penaeus californiensis* and *Pacifastacus leniusculus*, and from the egg masses of *Biomphalaria glabrata* [22,47,49].

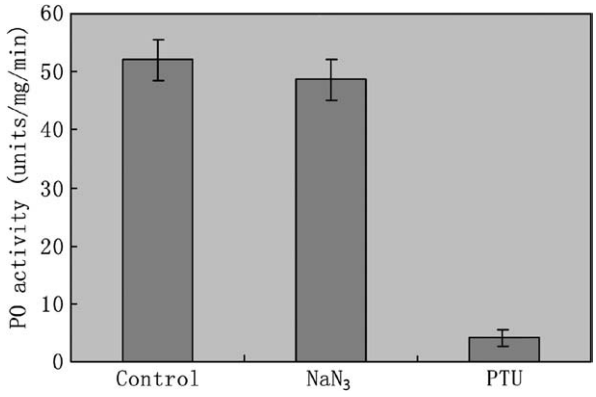


Fig. 4. Effects of PTU and sodium azide on activity of the purified PO. The purified enzyme was assayed for PO activity in the presence of 1 mM PTU or 1 mM sodium azide. The control was processed similarly except that PTU and sodium azide were withdrawn from the reaction mixture.

Table 2
Activity of the purified PO in the humoral fluid of *B. belcheri tsingtauense* towards various substrates

| Substrate | PO activity |
|---|---|
| 4-Methylcatechol | 9960.2±138.5 |
| L-dopa | 9073.4±424.6 |
| Dopamine | 16296±698.5 |
| Hydroquinone | 269.6±33.4 |
| Methylhydroquinone | 253.5±54.6 |

All experiments were performed at least three times. The values are the means (±SD) of the three assays done.

PO enzyme in the body surface mucus of the amphioxus has been suggested to originate from the secretion of the epidermal cells [34]. The source of PO in the humoral fluid remains unknown at present. Mouse antisera against the purified PO have been prepared, and their cross-reactivity has been confirmed by Western blotting. This will contribute to the future determination of the origin of PO in the humoral fluid and the distribution of PO-synthesising tissues in amphioxus.

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## References

[1] Barrett FM. Phenoloxidase from larval cuticle of the sheep blowfly, *Lucilia cuprina*: characterization, developmental changes, and inhibition by antiphenoloxidase antibodies. Arch Insect Biochem Physiol 1987;5:99–118.
[2] Sugumaran M. Molecular mechanisms for mammalian melanogenesis. Comparison with insect cuticular sclerotization. FEBS Lett 1991;295:233–9.
[3] Lerch K. Protein and active-site structure of tyrosinase. Prog Clin Biol Res 1988;256:85–98.
[4] Cicero R, Sciuto S, Chillemi R, Sichel G. Melanosynthesis in the Kupffer cells of amphibia. Comp Biochem Physiol A 1982;73:477–9.
[5] Johansson MW, Söderhäll K. The prophenoloxidase activating system and associated proteins in invertebrates. Prog Mol Subcell Biol 1996;15:46–66.
[6] Asokan R, Arumugam M, Mullainadhan P. Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel *Perna viridis* Linnaeus. Dev Comp Immunol 1997;21:1–12.
[7] Binnington KC, Barrett FM. Ultrastructural localization of phenoloxidases in cuticle and haemopoietic tissue of the blowfly *Lucilia cuprina*. Tissue Cell 1988;20:405–19.
[8] Coles JA, Pipe RK. Phenoloxidase activity in the haemolymph and haemocytes of the marine mussel *Mytilus edulis*. Fish Shellfish Immunol 1994;4:337–52.
[9] Johansson MW, Söderhäll K. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J Comp Physiol B 1985;156:175–81.
[10] Smith VJ, Söderhäll K. A comparison of phenoloxidase activity in the blood of marine invertebrates. Dev Comp Immunol 1991;15:251–61.
[11] Hall M, Scott T, Sugumaran M, Söderhäll K, Law JH. Proenzyme of *Manduca sexta* phenoloxidase: purification, activation, substrate specificity of the active enzyme, and molecular cloning. Proc Natl Acad Sci U S A 1995;92:7764–8.
[12] Jiang H, Kanost MR. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. J Biol Chem 1997;272:1082–7.
[13] Kopacek P, Weise C, Gotz P. The prophenoloxidase from the wax moth *Galleria mellonella*: purification and characterization of the proenzyme. Insect Biochem Mol Biol 1995;25:1081–91.

[14] Yasuhara Y, Koizumi Y, Katagiri C, Ashida M. Reexamination of properties of prophenoloxidase isolated from larval hemolymph of the silkworm, *Bombyx mori*. Arch Biochem Biophys 1995;320:14–23.
[15] Waite JH. The phylogeny and chemical diversity of quinoned-tanned glues and varnishes. Comp Biochem Physiol B 1995;156: 491–6.
[16] Lee MJ, Anstee JH. Phenoloxidase and its zymogen from the haemolymph of larvae of the lepidopteran *Spodoptera littoralis* (Lepidoptera: Noctuidae). Comp Biochem Physiol B 1995;110:379–84.
[17] Christensen BM, Forton KF. Hemocyte-mediated melanization of microfilariae in *Aedes aegypti*. J. Parasitol 1986;72:220–5.
[18] Christensen BM, Tracy JW. Arthropod-transmitted parasites: mechanisms of immune interaction. Am Zool 1989;29:387–98.
[19] Vass E, Nappi AJ, Carton Y. Alteration in the activities of tyrosinase, N-acetyltransferase and tyrosine aminotransferase in immune reactive larvae of *Drosophila melanogaster*. Dev Comp Immunol 1993;17:109–18.
[20] Ratcliffe NA, Rowley AF, Fitzgerald SW, Rhodes CP. Invertebrate immunity: basic concepts and recent advances. Int Rev Cytol 1985;97:183–350.
[21] Naraoka T, Uchisawa H, Mori H, Matsue H, Chiba S, Kimura A. Purification, characterization and molecular cloning of tyrosinase from the cephalopod mollusk, *Illex argentinus*. Eur J Biochem 2003;270:4026–38.
[22] Bai G, Brown JF, Watson C, Yoshino TP. Isolation and characterization of phenoloxidase from egg masses of the gastropod mollusc, *Biomphalaria glabrata*. Comp Biochem Physiol B 1997;118:463–9.
[23] Frizzo A, Guidolin L, Ballarin L, Sabbadin A. Purification and partial characterisation of phenoloxidase from the colonial ascidian *Botryllus schlosseri*. Mar Biol 1999;135:483–8.
[24] Waite JH. The phylogeny and chemical diversity of quinone-tanned glues and varnishes. Comp Biochem Physiol B 1990;97: 19–29.
[25] Waite JH. The DOPA ephemera: a recurrent motif in invertebrates. Biol Bull Mar Biol Lab Woods Hole 1992;183:178–84.
[26] Rzepecki LM, Hansen KM, Waite JH. Characterization of a cystine-rich polyphenolic protein family from the blue mussel *Mytilus edulis* L. Biol Bull Mar Biol Lab Woods Hole 1992;183:123–37.
[27] Ashida M. Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm *Bombyx mori*. Arch Biochem Biophys 1971;144:749–62.
[28] Burks CS, Fuchs MS. Partial purification of plasma phenoloxidase of the yellow fever mosquito *Aedes aegypti* L. (Diptera: Culicidae). Comp Biochem Physiol B 1995;110:641–7.
[29] Chase MR, Raina K, Bruno J, Sugumaran M. Purification, characterization and molecular cloning of prophenoloxidase from *Sarcophaga bullata*. Insect Biochem Mol Biol 2000;30:953–67.
[30] Durrant HJ, Ratcliffe NA, Hipkin CR, Aspan A, Söderhäll K. Purification of the pro-phenol oxidase enzyme from haemocytes of the cockroach *Blaberus discoidalis*. Biochem J 1993;289:87–91.
[31] Kwon TH, Lee SY, Lee JH, Choi JS, Kawabata S, Iwanaga S, et al. Purification and characterization of prophenoloxidase from the hemolymph of coleopteran insect, *Holotrichia diomphalia* larvae. Mol Cell 1997;7:90–7.
[32] Pang QX, Zhang SC, Wang CF, Shi XD, Sun YN. Presence of prophenoloxidase in the humoral fluid of amphioxus *Branchiostoma belcheri tsingtauense*. Fish Shellfish Immunol 2004;17:477–87.
[33] Li GR, Zhang SC, Xiang JH. Phenoloxidase, a marker enzyme for differentiation of the neural ectoderm and the epidermal ectoderm during embryonic development of amphioxus *Branchiostoma belcheri tsingtauense*. Mech Dev 2000;96:107–9.
[34] Zhang SC, Li GR. Presence of phenoloxidase and prophenoloxidase in the epidermal cells and the epidermis mucus of the lancelet *Branchiostoma belcheri tsingtauense*. Ophelia 2000;52:207–12.
[35] Li GR, Zhang SC, Li HY. Ultrastructural localization and antibacterial activity of phenoloxidase in amphioxus *Branchiostoma belcheri tsingtauense*. J Mar Biol Assoc UK 2001;81:705–6.
[36] Wang CF, Zhang SC, Luo Y, Xu TT. Presence and induction by bacteria, of D-galactoside-specific lectins in the humoral fluids of amphioxus *Branchiostoma belcheri tsingtauense*. Inflammopharmacology 2002;9:241–8.
[37] Scopes RK. Protein purification: principles and practice. New York: Springer-Verlag; 1982.
[38] Horowitz NH, Shen SC. Neurospora tyrosinase. J Biol Chem 1952;197:513–20.
[39] Ashida M, Söderhäll K. The prophenoloxidase activating system in crayfish. Comp Biochem Physiol B 1984;77:21–6.
[40] Davis BJ. Disk electrophoresis. II. Method and application to human serum proteins. Ann N Y Acad Sci 1964;121:404–27.
[41] Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage $T_4$. Nature 1970;227:680–5.
[42] Nellaiappan K, Vinayagam A. A rapid method for detection of tyrosinase activity in electrophoresis. Stain Technol 1986;61: 269–72.
[43] Seed JL, Bennett JL. *Schistosoma mansoni*: phenol oxidase's role in eggshell formation. Exp Parasitol 1980;49:430–41.
[44] Andersen SO. Cuticular sclerotization. In: Miller TA, editor. Cuticle techniques in arthropods. New York: Springer; 1980. p. 185–215.
[45] Shibata S, Yoneda S, Yanagishita M, Yamashita Y. Isolation of proteoglycan (versican) aggregate from rat dental pulp. Arch Oral Biol 2000;45:563–8.
[46] Brandford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[47] Gollas-Galvan T, Hernandez-Lopez J, Vargas-Albores F. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. Comp Biochem Physiol B 1999;122:77–82.
[48] Ashida M, Yamazaki H. Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In: Ohnishi E, Ishizaki H, editors. Molting and metamorphosis. Berlin: Springer-Verlag; 1990. p. 239–65.
[49] Asano T, Ashida M. Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*. J Biol Chem 2001;276:11100–12.