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Phenoloxidase in the scallop *Chlamys farreri*: Purification and antibacterial activity of its reaction products generated *in vitro*

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ABSTRACT

Phenoloxidase (PO) was purified from hemocytes of the scallop *Chlamys farreri* using native-PAGE and gel permeation column chromatography, and then substrate specificity and antibacterial activity generated from reaction products of purified PO were analyzed. The results showed purified PO had a molecular mass of 576 kDa in native-PAGE and 53 kDa in denatured PAGE, and could catalyze the substrates L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, catechol and hydroquinone suggesting it is a type of *p*-diphenoloxidase. Using dopamine as a substrate, PO reaction products significantly inhibited the growth of *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Aeromonas salmonicida*. No significant inhibition was found in *Streptococcus dysgalactiae*, *Streptococcus iniae*, *Micrococcus lysodeikticus* and *Edwardsiella tarda*. When L-DOPA was used as a substrate, significant inhibition occurred in *A. salmonicida* only.

1. Introduction

Phenoloxidase (PO) is a critical component of the immune system of molluscs [1,2]. As the product of a complex cascade of reactions, PO is generated from proPO through a limited proteolysis by a proPO activating enzyme (ppA) [3]. *In vivo* ppA gains its biological activity in the presence of microbial polysaccharides, such as lipopolysaccharides, peptidoglycans and 1,3-β-glucans, which by binding specific pattern-recognition receptors induce the activation of the ppA, and as a consequence, active ppA converts proPO to PO [4,5], finally PO is involved in melanization, encapsulation, wound healing, phagocytosis, and pathogen extermination [6–8]. In molluscs, PO is mainly found in hemolymph [2,8], and exists as soluble or cellular form, the soluble PO is always involved in humoral immunity, while the cellular PO which binds to the surface of hemocytes is more associated with cell-mediated immunity [9,10], although there are other molecules like hemocyanin or hemoglobin showing PO-like activity [11,12]. The PO is reported to use phenols as substrates, and the substrate phenols are various and mainly include monophenols (typically tyrosine), *o*-diphenols (typically catechol), *p*-diphenols (typically hydroquinone) etc. [13].

PO activity always had close relationship to the challenge of pathogens or alien materials. Bacterial infection can cause a significant increase in PO activity in whole hemolymph (PAH) of the bivalves *Crassostrea madrasensis* and *Chlamys farreri* [14,15]. Furthermore, bacterial lipopolysaccharides strongly enhance PAH in scallops (*Argopecten ventricosus*, *Nodipecten subnodosus* and *C. farreri*), oysters (*Crassostrea gigas* and *Saccostrea glomerata*), and pen shell *Atrina maura* [16,17]. Bacterial DNA incubated with hemolymph also causes an increase of PAH in the mussel *Hyriopsis cumingii* [18]. In most studies, characterization of bivalve POs was performed using whole hemolymph in which other molecules were involved in defending against bacteria [19]. Therefore, we believe that it is necessary to investigate the role of purified PO in bacterial resistance. In this study, *Vibrio alginolyticus, Vibrio parahaemolyticus, Edwardsiella tarda, Aeromonas salmonicida, Streptococcus dysgalactiae, Streptococcus iniae and Micrococcus lysodeikticus*, which were reported to be the pathogenic species in marine animals [20–26] were selected in our tests.

This paper reports on the purification of PO from the scallop *C. farreri*, and determines the PO molecular mass, substrate specificity, and antibacterial activities of PO reaction products generated *in vitro* against seven species of bacteria. The aim of the study was to provide primary data on PO as a potential immune index in this scallop.

Abbreviations: PO, phenoloxidase; PAH, PO activity in whole haemolymph; PBS, phosphate-buffered saline; HLS, hemocyte lysate supernatant; SDS, sodium dodecyl sulfate; L-DOPA, L-3,4-dihydroxyphenylalanine; BSA, bovine serum albumin; CBB, Coomassie brilliant blue R-250.

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2. Materials and methods

2.1. Experimental animals

One-year old *Chlamys farreri* (5.8 ± 0.44 cm shell length) were purchased from Qingdao in China and acclimated in a re-circulating seawater system at room temperature for 24 h before use. Totally, about 400 scallops were used for hemolymph collection.

2.2. Bacteria strains

V. alginolyticus, *V. parahaemolyticus*, *E. tarda*, *A. salmonicida*, *S. dysgalactiae*, *S. iniae* and *M. lysodeikticus*, were used in the experiment. Bacteria sources, strains, isolates, culture media, conditions, and CFU/ml at A_{600} of 1.0 are given in Table 1. For preparation of bacterial suspensions, culture media were centrifuged at 5000 × *g* for 15 min, the cell pellets were resuspended in PBS to a final A_{600} of 1.0 for the antibacterial activity test.

2.3. Hemocyte lysate supernatant preparation

Hemolymph, withdrawn from the adductor muscle sinus using sterilized syringes, was centrifuged at 700 × *g* for 10 min at 4 °C. The pellet was collected and suspended in phosphate-buffered saline (PBS, 2.7 mM KCl, 0.137 M NaCl, 1.47 mM KH_2PO_4, 8.09 mM $Na_2HPO_4{\cdot}12H_2O$, pH 7.6). The samples were then sonicated and centrifuged at 15,000 × *g* for 30 min at 4 °C. The resulting supernatant, hereinafter referred to as the hemocyte lysate supernatant (HLS), was collected and stored at −80 °C until use.

2.4. PO purification

One hundred microliters of HLS was subjected to electrophoresis with a 5% stacking gel and 6–20% linear-gradient separating gel (gel size is 180 × 160 × 1.0 mm) in Tris-Glycine buffer (0.025 M Tris, 0.2 M Glycine, pH 8.0) for 12 h at 3 W using high molecular weight markers ranging from 67 to 669 kDa (GE Healthcare). One lane of the gel was cut and stained using 1% (W/V) catechol dissolved in PBS to label the PO-containing bands. Based on catechol staining, the unstained lanes were excised for PO-containing bands, which were sonicated in PBS and centrifuged at 17,000 × *g* for 30 min. The resulting supernatant was condensed using centrifugal concentrators (Millipore) and successively applied to a Sephacryl S-300 gel-filtration column (1.6 × 60 cm, GE Healthcare) equilibrated with PBS. The elution was performed with PBS at a flow rate of 0.5 ml/min and fractions of 1.5 ml were collected, then PO activities in each fraction were measured by spectrophotometric assay (see Section 2.5). The fractions with high PO activities were collected and pooled, desalted and concentrated using centrifugal concentrators (Millipore), and the resulting solution used as purified PO solution. All of the procedures except staining were performed at 4 °C, and none of the buffers that were used in the PO purification contained sodium dodecyl sulfate (SDS) or reducing agents.

Purified PO was applied to native-PAGE and SDS–PAGE respectively to determine the molecular mass. SDS–PAGE was carried out on a 5% stacking gel and a 12% separating gel (gel size is 140 × 120 × 0.75 mm) according to the method of Laemmli [27] using standard proteins ranging from 14.4 to 116 kDa as molecular marker (Fermentas). The electrophoresis was performed at constant electricity of 30 mA in stacking gel and 60 mA in separating gel. After electrophoresis, gels were stained with Coomassie brilliant blue R-250.

2.5. PO activity assay and protein assay

PO activity was determined by L-3,4-dihydroxyphenylalanine (L-DOPA) transformation to dopachromes method [28] with some modifications. Briefly, 100 μl of the sample was added to 2.0 ml of 15 mM L-DOPA that was dissolved in PBS, and control was performed by replacement of the sample with same volume of PBS. The dopachrome formed was measured spectrophotometrically at 490 nm every 3 min for 30 min using a U-2001 spectrophotometer (Hitachi), at the same time, the spontaneous oxidation of L-DOPA was measured. The PO activity was estimated based on the increment in the rate of absorbance. An increase of 0.001 per min was considered one unit (A_{490} 10^{-3} min^{-1}).

The protein concentrations were determined by the Bradford method [29], using bovine serum albumin (BSA) (Sigma) as the protein standard.

2.6. Substrate specificity

Substrates of catechol, L-DOPA, dopamine, hydroquinone and tyrosine were each dissolved in PBS at a concentration of 15 mM, and 2.0 ml of each substrate solution was added to 100 μl of purified PO, and controls were performed by replacement of purified PO with same volume of PBS, then melanochrome formation was measured spectrophotometrically at 490 nm every 3 min for 30 min.

2.7. Antibacterial activity test

Catechol, L-DOPA, dopamine and hydroquinone were used as the substrates in this test. Twenty microliters of purified PO solution, 160 μl substrate solution and 20 μl of each bacterial suspension were mixed and incubated at 30 °C for 1.5 h, then the mixture was centrifuged at 5000 × *g* for 15 min, and the cell pellet was resuspended with 200 μl PBS. Afterwards, 20 μl of the resuspensions were sampled for microscopic observation. Eighty microliters of the resuspensions were inoculated in 2.0 ml of fresh medium and incubated at 28 °C or 37 °C for 9 h and shaken, during incubation, 100 μl of bacterial incubation suspensions were sampled every 1 h and read in a microplate reader at 600 nm. As

Table 1
Bacteria information.

Bacteria species	Source/strain	Medium	Temperature (°C)	CFU/ml at A_{600} of 1.0
V. alginolyticus	LMG 4408T/283[a]	2216E Broth	28	$1.93 \pm 0.03 \times 10^9$
V. parahaemolyticus	LMG 12094/HW458[a]	2216E Broth	28	$1.92 \pm 0.07 \times 10^9$
E. tarda	Japanese flounder/N17[a]	Nutrient agar	28	$2.25 \pm 0.06 \times 10^9$
M. lysodeikticus	ATCC 4698/L11[a]	Nutrient agar	28	$2.13 \pm 0.04 \times 10^9$
S. dysgalactiae	Japanese flounder/SD[a]	Brain heart infusion broth	37	$6.77 \pm 0.05 \times 10^8$
S. iniae	Japanese flounder/SI7[a]	Brain heart infusion broth	37	$6.93 \pm 0.03 \times 10^8$
A. salmonicida	Sea cucumber/H1[a]	Luria-Bertani	28	$1.74 \pm 0.03 \times 10^9$

[a] Bacteria strain code used in our laboratory.