Original Article

Purification and characterization of phenoloxidase from brine shrimp *Artemia sinica*

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Phenoloxidase from *Artemia sinica* (AsPO) was purified by Superdex 200 gel-filtration and Q Sepharose fast flow ion-exchange chromatography, and its properties were characterized biochemically and enzymatically by using *L*-dihydroxyphenylalanine (*L*-DOPA) as the specific substrate. Results showed that AsPO was isolated as a monomeric protein of 125.5 kDa in molecular mass. The optimal pH value and temperature are 7.0 and 50°C, respectively, for its PO activity. The AsPO had an apparent $K_m$ value of 4.2 mM on *L*-DOPA, and 10.9 mM on catechol, respectively. Oxidase inhibitor on PO activity showed that the AsPO was extremely sensitive to ascorbic acid, sodium sulfite, and citric acid; and was very sensitive to cysteine, benzoic acid, dihydroxyphenylalanine; and was very sensitive to ascorbic acid, sodium sulfite, and dihydroxyphenylalanine (L-DOPA) as the specific substrate. Results showed that AsPO was isolated as a monomeric protein of 125.5 kDa in molecular mass. The optimal pH value and temperature are 7.0 and 50°C, respectively, for its PO activity. The AsPO had an apparent $K_m$ value of 4.2 mM on *L*-DOPA, and 10.9 mM on catechol, respectively. Oxidase inhibitor on PO activity showed that the AsPO was extremely sensitive to ascorbic acid, sodium sulfite, and citric acid; and was very sensitive to cysteine, benzoic acid, and 1-phenyl-2-thiourea. Combined with its specific enzyme activity on *L*-DOPA and catechol, it can be concluded that AsPO is most probably a typical catechol-type *O*-diphenoloxidase. Its PO activity was also sensitive to metal ions and chelators, and 20 mM DETC-inhibited PO activity was obviously recovered by 15 mM Cu$^{2+}$, indicating that AsPO is most probably a copper-containing metalloenzyme. All these data about specific substrate, sensitivity to oxidase inhibitor metal ions and chelators indicate that the AsPO has the properties of a catechol-type copper-containing *O*-diphenoloxidase that functions as a vital humoral factor in host defense via melaninization as in other Crustaceans.

Keywords: phenoloxidase; *Artemia sinica; L*-dihydroxyphenylalanine; *O*-phenoloxidase; metalloenzyme

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Introduction

It has been well proved that phenoloxidase (PO), an active form of prophenoloxidase (proPO), is a copper-containing key enzyme in the innate defense system of invertebrates [1–11]. PO, under stimulation of invading pathogens, can be activated by the vital cascade reaction of ‘proPO activating system’ in a stepwise process, which results in pathogen encapsulation by formation of melamins that is a vital melanogenic pathway involved in immune reactions of invertebrate hemocytes [1,4,5,8,12]. For example, PO binds to infected cells via a thiol-ester-like motif, leading to the production of toxic intermediates of phenol, which might prevent viruses from entering into the body cavity in taura syndrome virus-infected shrimp [13].

Crustacean and Mollusca proPOs, synthesized in blood cells, have been isolated and characterized from several species [3–5,7,9–11,14]. It has been found that the purified Crustacean and Mollusca proPOs are monomers with a molecular mass of 70–87 kDa, while their activated forms were 60–77 kDa [7,9,10,14–16]. However, the proPO from *Octopus ocellatus* has been identified as a heterodimeric protein with a molecular mass of 153.8 kDa and presents two subunits with molecular mass of 75.6 and 73.0 kDa after sodium dodecyl sulfate (SDS) activation [11]. Moreover, Crustacean and Mollusca POs have properties of either tyrosinase-type of metalloenzymes in brown shrimp *Penaeus californiensis* [17], shrimp *Penaeus chinensis* [7], and clam *Ruditapes philippinarum* [9], or *O*-diphenoloxidase-type of metalloenzymes in *Charybdis japonica*, [10] and *O. ocellatus* [11].

The brine shrimp *Artemia sinica*, a typical inhabitant of hypersaline environments in the central parts of China, is an intensive Crustacean model animal for various kinds of researches in Developmental Biology, Cell Biology, Immunology, and Molecular Biology [18]. Although POs of various Crustacean species have been characterized, there is no report on the properties of PO from *A. sinica* by now. To clarify the properties of AsPO and establish an animal model for non-specific immunity studies of Crustaceans, this study was intended to purify AsPO and characterize its biochemical and enzymatic properties.

Materials and Methods

Materials

Fresh adult *A. sinica*, with an average length of ~8 mm, was purchased from Chengyang Brine Shrimp Breeding Company (Qingdao, China) and kept at −80°C until use.
Isolation of AsPO

About 40 g adult *A. sinica* were homogenized on ice in 15 ml of 20 mM Tris-HCl buffer (pH 7.1) with an XHF-D tissue grinders (Shenhua Biotech, Guangzhou, China). The homogenate was then centrifuged for 1 h at 9000 g (4°C) and the supernatant was precipitated overnight with 67% saturated (NH₄)₂SO₄ solution (67P-SAS) at 4°C. The precipitate was collected by centrifugation for 1 h at 9000 g (4°C), and dissolved in 4 ml of 20 mM Tris-HCl buffer (pH 7.1), and then dialyzed against 2000 ml distilled water. The dialyzate was lyophilized and kept at 4°C.

Enzyme assay

The AsPO activity was measured according to the method of Ashida and Dohke [19] with minor modifications. Briefly, AsPO sample (20 μl) was mixed with 20 μl of 15 mM L-dihydroxyphenylalanine (L-DOPA) dissolved in 20 mM Tris-HCl buffer (pH 7.1). In some experiments, L-DOPA was replaced by catechol, tyrosine hydroquinone, or phenol. After 40 min of incubation at 28°C, 260 μl of ice-cold distilled water was added to each sample to stop reaction. Then the reaction mixture was measured with an UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan) at wavelength of 490 nm. The AsPO activity was estimated as the increment in the rate of absorbance. An increase of 0.001 per minute was taken to be 1 unit (U): activity = A₄₉₀ × 10⁻³/min.

Purification of AsPO

Column chromatography and purification was performed as described previously [7] with modifications. The lyophilized crude AsPO sample (15 mg) was dissolved in 0.3 ml of 20 mM Tris-HCl buffer (pH 7.1), and centrifuged at 10,000 g (4°C) for 1 h. The collected supernatant was loaded onto a Superdex 200 gel-filtration column (1.5 cm × 40 cm) (GE Healthcare, Wauwatosa, USA), pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.1) and eluted with the same buffer. A flow rate of 0.5 ml/min was maintained throughout elution and fractions of 1.0 ml were collected. Ultraviolet absorption was monitored at 280 nm and the AsPO activity of each collected fraction was measured with 15 mM L-DOPA as described above. The fractions with high AsPO activities were collected and pooled together as Superdex 200 purified AsPO sample. The gel-filtration purified AsPO sample was then applied to a Q Sepharose fast flow (FF) ion-exchange column (1 cm × 30 cm; GE Healthcare) and eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl buffer (pH 7.1). A flow rate of 1.0 ml/min was maintained throughout elution and fractions of 1.0 ml were collected, respectively. The 280 nm absorbance and PO activity of each fraction was measured as described above. The fractions with high AsPO activities were pooled together, and desalted and concentrated with Amicon Ultra-4 centrifugal filter units (Millipore-Amicon, Billerica, USA). The Q Sepharose FF-purified AsPO sample was applied to a Superdex 200 gel-filtration column (1.5 cm × 40 cm; GE Healthcare) again, and eluted under the same conditions as described above. After AsPO activity measurement, the fractions with high AsPO activities were pooled together, desalted and concentrated with Amicon Ultra-4 centrifugal filter units. This desalted and concentrated sample was used as purified AsPO. The above chromatographic steps were all performed at 4°C. The protein concentrations of different AsPO preparations were measured as described by Bradford [20], using BSA (Sigma-Aldrich, St. Louis, USA) as the protein standard.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli [21] on 12.5% gels, using low-molecular-weight protein markers [Escherichia coli β-galactosidase, 116.0 kDa; bovine serum albumin (BSA), 66.2 kDa; chicken egg ovalbumin, 45.0 kDa; porcine muscle lactate dehydrogenase, 35.0 kDa; *E. coli* REase Bsp981, 25.0 kDa; chicken egg lysozyme, 14.4 kDa] (0.15 mg/ml) (Fermentas Life Sciences, St. Leon-Rot, Germany). The samples (10 μl; 0.15 mg/ml) and the protein markers (10 μl) were electrophoresized. After electrophoresis, proteins were stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

Optimal pH value and temperature determination

To determine the optimal temperature of AsPO activity, the reaction mixture containing 20 μl of the purified AsPO and 15 mM L-DOPA was incubated for 40 min at 20, 30, 40, 50, 60°C, respectively. The reaction was stopped and each reaction mixture was measured spectrophotometrically under the same conditions as described above, respectively.

To determine the optimal pH value of AsPO activity, the reaction mixture containing 20 μl of 15 mM L-DOPA and 20 μl of the purified AsPO at pH 5.0, 6.0, 7.0, 8.0, and 9.0, respectively, was incubated for 40 min at 28°C as described previously [11]. The reaction was stopped and each reaction mixture was measured spectrophotometrically under the same conditions as described above, respectively.

Kinetic studies

Kinetic parameters were determined following Michaelis–Menten equation according to the method described by Eisenthal and Cornish-Bowden [22]. For 20 μl of purified AsPO solution, 20 μl of different concentrations of L-DOPA, catechol, hydroquinone, tyrosine, or phenol dissolved in 20 mM Tris-HCl buffer (pH 7.1) were added, respectively. The concentration of L-DOPA was 5, 10, 15, 20, and 25 mM, respectively, and the concentrations of...
catechol, hydroquinone, tyrosine, and phenol was 10, 20, 30, 40, 50, and 60 mM, respectively. The enzymatic activities of AsPO were measured spectrophotometrically under the same conditions as described above.

Effects of inhibitors and metal ions
For 20 μl of purified AsPO, 20 μl of inhibitors (benzoic acid, sodium sulfite, citric acid, thiourea, 1-phenyl-2-thiourea, cysteine, and ascorbic acid), ions (Cu^{2+}, Ca^{2+}, Zn^{2+}, and Mg^{2+}), or chelators (EDTA, and DETC) dissolved in 20 mM Tris-HCl buffer (pH 7.1) at different concentrations of 2.5, 5, 10, and 20 mM, was added, respectively. After a pre-incubation for 20 min at the optimal temperature, 20 μl of 15 mM L-DOPA was added, respectively, and incubated for 40 min. Then 240 μl of ice-cold DW was added, and the reaction mixture was measured spectrophotometrically under the same conditions as described above.

The recovery effect of some metal ions on DETC-pretreated AsPO activity was investigated as described previously [23]. For 20 μl of purified AsPO, 20 μl of 20 mM DETC dissolved in 20 mM Tris-HCl buffer (pH 7.1) was added and incubated at 0°C for 20 min. Then 20 μl of 5, 10, 15, and 20 mM metal ions was added, respectively, and incubated at 0°C for 3 h. After 20 μl of 15 mM L-DOPA was added and incubated for 40 min at the optimal temperature, 220 μl of ice-cold DW was added and measured spectrophotometrically under the same conditions as described above.

Statistical analysis
Data are expressed as the mean ± SD in triplicates and are analyzed for statistical significance with ANOVA single factor. P value <0.05 is considered to be statistically significant.

Results
Purification of AsPO
AsPO was isolated from adult brine shrimp homogenate using a sequential combination of 67P-SAS precipitation, Superdex 200 gel-filtration (I), Q Sepharose FF ion-exchange, and Superdex 200 gel-filtration (II) column chromatography. The purification scheme and results are summarized in Table 1. It shows that the specific activity of the purified AsPO is 1.04, 3.94, 18.73, and 53.87 folds higher than that of crude homogenate sample.

The crude homogenate from adult brine shrimp, with a protein content of ~4.65 mg, has a total L-DOPA oxidizing activity of 93.08 U (Table 1). After 67P-SAS precipitated, desalted, and concentrated, ~4.2 mg of lyophilized AsPO sample was obtained. The lyophilized crude AsPO was dissolved in 0.3 ml of 20 mM Tris-HCl buffer (pH 7.1) and it has a total L-DOPA oxidizing activity of 87.63 U (Table 1).

About 0.25 ml of the 67P-SAS precipitated AsPO was loaded onto a Superdex 200 gel-filtration column [Fig. 1(A)]. Peak fractions 44–46, with highest AsPO activities, were collected and pooled together as Superdex 200 (I) purified AsPO sample. The 2.97 ml Superdex 200 (I) purified AsPO had a protein concentration of 0.32 mg/ml and a total L-DOPA oxidizing activity of 75.00 U (Table 1). On SDS-PAGE, the Superdex 200 (I) purified AsPO contained a 125.5 kDa AsPO molecule in absolute majority (Fig. 2).

The pooled fractions were then loaded on a Q Sepharose FF ion-exchange column [Fig. 1(B)]. Peak fractions 50–53, at a NaCl concentration of 0.283 M, with highest AsPO activities, were collected and pooled together. The 3.96 ml pooled fraction had a protein concentration of 0.04 mg/ml and a total enzymatic activity of 60.00 U (Table 1). Then the pooled fractions were desalted and concentrated as Q Sepharose FF-purified AsPO. On SDS-PAGE, the Q Sepharose FF-purified AsPO mainly contained a 125.5 kDa AsPO molecule (Fig. 2).

The Q Sepharose FF-purified AsPO sample was loaded onto the Superdex 200 gel-filtration column again [Fig. 1(C)]. Peak fractions 46–48, with highest AsPO activities, were collected and pooled together. The 2.97 ml pooled fraction had a protein concentration of 0.013 mg/ml and a total enzymatic activity of 43.13 U (Table 1). The pooled Superdex 200 (II) fraction contained only a 125.5 kDa AsPO molecule on both reducing and non-reducing SDS-PAGE, the Superdex 200 (II) purified AsPO contained a 125.5 kDa AsPO molecule on both reducing and non-reducing SDS-PAGE.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Enzymatic activity (U)</th>
<th>Yield (%)</th>
<th>Purification (folds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (U)</td>
<td>Specific (U/mg)</td>
<td></td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>4.65</td>
<td>93.08</td>
<td>20.02</td>
<td>100.00</td>
</tr>
<tr>
<td>67P-SAS precipitate</td>
<td>4.20</td>
<td>87.63</td>
<td>20.86</td>
<td>94.14</td>
</tr>
<tr>
<td>Superdex 200 I</td>
<td>0.95</td>
<td>75.00</td>
<td>78.95</td>
<td>80.58</td>
</tr>
<tr>
<td>Q Sepharose FF</td>
<td>0.16</td>
<td>60.00</td>
<td>375.00</td>
<td>64.46</td>
</tr>
<tr>
<td>Superdex 200 II</td>
<td>0.04</td>
<td>43.13</td>
<td>1078.25</td>
<td>46.34</td>
</tr>
</tbody>
</table>

*Derived from 40 g adult *A. sinica*.

The PO activities were measured by using 15 mM L-DOPA as a substrate.
reducing SDS-PAGE (Fig. 2), and was used as purified AsPO for property characterization.

**Optimal temperature and pH**

The effect of different temperatures and pH values on the enzymatic activity of the purified AsPO on L-DOPA is shown in Fig. 3. The optimal temperature of the purified AsPO was $\sim 50^\circ C$ [Fig. 3(A)], and its optimal pH value was $\sim 7.0$ [Fig. 3(B)].

**Enzyme kinetics**

Kinetic assay showed that the purified AsPO could oxidize L-DOPA and catechol, and its $K_m$ value on L-DOPA and catechol was calculated to be 4.2 and 10.9 mM, respectively, (Fig. 4). However, no AsPO activity was detected against tyrosine, hydroquinone, or phenol. These results indicated that the AsPO was probably a kind of $O$-diphenoloxidase.

**Inhibition studies**

The effects of different oxidase inhibitors, several metal ions, and chelators were examined with respect to the oxidizing activity of the purified AsPO on L-DOPA. The AsPO activity was completely inhibited by ascorbic acid.
and sodium sulfite, greatly inhibited by citric acid, cysteine, benzoic acid, 1-phenyl-2-thiourea, and less inhibited by thiourea. Besides these, AsPO was sensitive to chelators and metal ions, and its enzymatic activity was greatly inhibited by DETC and EDTA in a dose-dependent manner, obviously inhibited by Zn$^{2+}$ and Mg$^{2+}$, but activated by Ca$^{2+}$ and Cu$^{2+}$ (Table 2).

The recovery effect of metal ions on the activity of DETC-pretreated AsPO was also evaluated in this study. It was found that the enzymatic activity of the purified AsPO, greatly inhibited by 20 mM DETC, was restored to 71% of its original level by 15 mM Cu$^{2+}$ (Table 3). These results, combined with its sensitivity to metal ions and chelators, indicated that the AsPO was probably a copper-containing metalloenzyme.

**Table 2** Effects of inhibitors, chelators, and metal ions on purified PO from *A. sinica* (AsPO)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Maximum inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>20</td>
<td>100 ± 3.00*</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>2.5</td>
<td>100 ± 3.00*</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2.5</td>
<td>92.0 ± 2.76*</td>
</tr>
<tr>
<td>Cysteine</td>
<td>20</td>
<td>84.1 ± 2.52*</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2.5</td>
<td>73.5 ± 2.21*</td>
</tr>
<tr>
<td>1-phenyl-2-thiourea</td>
<td>10</td>
<td>60.5 ± 1.80*</td>
</tr>
<tr>
<td>Thiourea</td>
<td>20</td>
<td>31.0 ± 0.93*</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>20</td>
<td>39.5 ± 1.19*</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>20</td>
<td>34.2 ± 1.03*</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>10</td>
<td>−13.2 ± 0.40*</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>10</td>
<td>−44.7 ± 1.34*</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.5</td>
<td>31.6 ± 0.95*</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>36.8 ± 1.1*</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>39.5 ± 1.1*</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>50.0 ± 0.63*</td>
</tr>
<tr>
<td>DETC</td>
<td>2.5</td>
<td>47.4 ± 1.42*</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>55.3 ± 1.7*</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>60.5 ± 1.82*</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>65.8 ± 2.0*</td>
</tr>
</tbody>
</table>

Concentrations of inhibitors indicated those exhibiting highest inhibition so far tested. The enzymatic activities were measured by using 15 mM L-DOPA as a specific substrate. Benzoic acid was dissolved in anhydrous ethanol. ‘+’ means activity had been enhanced. All the values are shown as the average of three pooled samples in triplicate ± SD.

**Table 3** Recovery effects of Cu$^{2+}$ on the activity of DETC-pretreated PO from *A. sinica* (AsPO)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzymatic activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO only</td>
<td>100 ± 3.00</td>
</tr>
<tr>
<td>PO + 20 mM DETC</td>
<td>34.2 ± 1.03*</td>
</tr>
<tr>
<td>PO + 20 mM DETC + 5 mM Cu$^{2+}$</td>
<td>58.1 ± 1.70**</td>
</tr>
<tr>
<td>PO + 20 mM DETC + 10 mM Cu$^{2+}$</td>
<td>61.3 ± 1.80**</td>
</tr>
<tr>
<td>PO + 20 mM DETC + 15 mM Cu$^{2+}$</td>
<td>71.0 ± 2.13**</td>
</tr>
<tr>
<td>PO + 20 mM DETC + 20 mM Cu$^{2+}$</td>
<td>64.5 ± 1.94**</td>
</tr>
</tbody>
</table>

The enzymatic activity of AsPO was measured by using 15 mM L-DOPA as a specific substrate. All the values are the average of three pooled samples in triplicate ± SD. *P < 0.01 vs. PO only; **P < 0.01 vs. DETC-inhibited PO.

**Discussion**

PO, one of the most important components in the invertebrate-specific immune response, is the final component of a complex serine protease cascade terminating with the activation of proPO, and facilitates the synthesis of melanin by oxidation of phenols to quinones [4,13,17,24]. There are many reports of the properties of POs in insects and arthropods [2–11,14–17], but no information is available on AsPO. The present study was intended to purify AsPO and characterize it biochemically and enzymatically.

PO, purified by gel-filtration and ion-exchange chromatography from *A. sinica* homogenates, was characterized by using L-DOPA as a substrate in this study. Since L-DOPA is a specific substrate only for PO oxidizing activity, combined with the results that only a single band was visualized in SDS-PAGE, it can be concluded that the
protein purified and characterized in this study is AsPO and contamination with other proteins has been avoided.

With the purification procedures used, the specific activity of purified AsPO increased almost 53.87-folds compared with the crude AsPO, with an overall recovery of 46.34% of the initial activity. The estimated molecular mass of the purified AsPO was determined to be 125.5 kDa in both reducing and non-reducing SDS-PAGE, which indicated that only one type of active PO in brine shrimp *A. sinica*. The molecular mass of AsPO is much higher than that of the other Crustacean POs (60–77 kDa) [7,10,11,13–15,17], and lower than that of heterodimeric proPOs from *O. ocellatus* (153.8 kDa) [11] and squid *Illex argentinus* tyrosinase (140.2 kDa) [25]. These results imply that AsPO is different from the other Crustacean POs both in molecular forms and molecular masses.

The purified AsPO had an optimal pH of 7.0 against *L*-DOPA, identical to that of other Crustacean POs such as *O. ocellatus* [11] and *Nephrops norvegicus* [26], higher than that of *P. chinensis* (pH 6.0) [7] and *C. japonica* (pH 6.0) [10], and lower than that of *Penaeus setiferus* (pH 7.5) [27], *I. argentinus* (pH 8.0) [25], and *P. californiensis* (pH 8.0) [17]. The optimal temperature of purified AsPO was 50°C that was higher than that of *Penaeus japonicus* (40°C) [28], *P. chinensis* (40°C) [7], *C. japonica* (40°C) [10], and *P. setiferus* (45°C) [27]. The differences of optimal pH and temperature may be correlated with the different species and the different survival conditions such as temperatures of their living environment and invading pathogens, and so on. The differences of species and survival conditions might affect their properties during long-term evolution.

The purified AsPO could oxidize both *L*-DOPA and catechol effectively, but failed to oxidize tyrosine, hydroquinone, or phenol. The *K_m* value of AsPO on *L*-DOPA and catechol is 4.2 mM and 10.9 mM, respectively, indicating that AsPO has a lower affinity for catechol than that for *L*-DOPA. The *K_m* value of AsPO on *L*-DOPA was higher than that of POs from *P. chinensis* (1.99 mM) [7], *P. californiensis* (2.5 mM) [17], *O. ocellatus* (3.1 mM) [11], *C. japonica* (3.41 mM) [10], and *P. japonicus* (3.45 mM) [28]. The *K_m* value of AsPO on catechol was higher than that of *P. californiensis* (7.97 mM) [17] and *O. ocellatus* (6.3 mM) [11]. All these results indicated that the affinity of AsPO for *L*-DOPA and catechol is lower than that of the other Crustacean POs, implying that oxidizing activity of AsPO might be weaker than that of POs from other Crustaceans animals. As is well known, POs from insects have been classified into three types: laccase (E.C.1.10.3.2; *p*-diphenol), catechol oxidase (E.C.1.10.3.1; *p*-diphenol), and tyrosinase (E.C.1.14.18.1; monophenol, *L*-DOPA) [29]. These results of substrate specificity showed that AsPO was probably a kind of *O*-diphenoloxidase, not a laccase or a tyrosinase.

Results of oxidase inhibitors showed that the purified AsPO was extremely sensitive to ascorbic acid, sodium sulfite and citric acid, very sensitive to cysteine, benzoic acid, and 1-phenyl-2-thiourea. Combined with the specific oxidizing activity on *L*-DOPA and catechol, it can be concluded that AsPO is most probably a typical catechol-type *O*-diphenoloxidase. The *O*-diphenoloxidase property of AsPO is the same as that of POs from *O. ocellatus* [11] and *C. japonica* PO [10], and different from the tyrosinase property of POs from some other crustaceans such as crayfish *Pacifastacus leniusculus* [30], shrimp *P. chinensis* [7], and *P. californiensis* [17].

The activity of the purified AsPO was very sensitive to metal ions (inhibited by Zn²⁺ and Mg²⁺, activated by Ca²⁺ and Cu²⁺) and chelators (inhibited by DETC and EDTA in a dose-dependent manner), indicating that AsPO was probably a kind of metalloenzyme. Besides, the DETC-inhibited AsPO activity could be greatly restored by Cu²⁺, and was restored to 71% of its original level by 15 mM Cu²⁺. All these results indicate that AsPO is most probably a kind of copper-containing metalloenzyme, which is similar to that of POs from the other crustaceans, such as *I. argentinus* [25], *P. chinensis* [7], *C. japonica* [10], and *O. ocellatus* [11]. The copper-containing metalloenzyme property of AsPO is consistent with crayfish proPO that contains two Cu atoms and its Cu binding sites are similar to those in crustacean hemocyanin [30].

From above, it can be concluded that the AsPO is most probably a catechol-type copper-containing *O*-diphenoloxidase, which functions as one of the most important enzymes in host defense via melanization as in other crustaceans [4,31]. Further studies on the gene expression pattern of AsPO and its regulation mechanism are required.

**Acknowledgements**

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**References**

Phenoloxidase from *Artemia sinica*


4. Song YL, Yu CI, Lien TW, Huang CC and Lin MN. Haemolymph par-  

5. Sugumaran M, Duggaraju P, Jayachandran E and Kirk KL. Formation of a  

6. Asano T and Ashida M. Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori* purification and demonstration of its transport from hemo-  


16. Kwon TH, Lee SY, Lee JH, Choi JS, Kawabata S, Iwanaga S and Lee BL. Purification and characterization of prophenoloxidase from the haemo-  


