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EFFECTS OF INHIBITORS ON HAEMOLYMPH PHENOLOXIDASE FROM ROSACEOUS BRANCH BORER, *OSPHERANTERIA COERULESCENS* (COLEOPTERA: CERAMBYCIDAE)

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Abstract: The rosaceous branch borer, *Ospheranteria coerulescens*, is an important pest of rosaceous trees. This insect feeds on the twigs and branches of living trees and causes their death. The characterization of the insect phenoloxidase (PO) is of interest when doing comparative investigations, and so that we may be able to understand its biochemical properties. When designing new methods of insect control such as the use of PO inhibitors, an understanding of the biochemical properties is fundamental. In this study, PO from hemolymph of the rosaceous branch borer was purified using ammonium sulfate precipitation, gel-filtration, and ion-exchange chromatography. The biochemical properties were characterized using L-dihydroxyphenylalanine (L-DOPA) as the specific substrate. The apparent molecular weights of the three isoforms of PO were determined by SDS-PAGE to be 85.23, 79.45, and 66.06 kDa. Optimal pH for PO activity was pH 8, and the optimal temperature was 45°C. Phenoloxidase lost less than 50% of its relative activity after a 60 min incubation at the optimal temperature. The effects of ions and chemical materials such as K^+, Ba^{2+}, Zn^{2+} and EDTA on PO showed that PO activity was strongly inhibited by Zn^{2+}. The Michaelis constant (K_m) and maximum velocity (V_{max}) were 88.61 mM and 0.14 µmol/min, respectively. The inhibitory effects of kojic acid, 4-hexylresorsinol, and quercetin on PO were determined, and the IC_{50s} (inhibitory concentration) were estimated as 23.31 for kojic acid, 35.75 for 4-hexylresorcinol, and 60.8 µM for quercetin. The inhibitory potency of kojic acid was 1.54 times higher than that of 4-hexylresorcinol and 2.58 times higher than that of quercetin. Phenoloxidase was effectively inhibited by 4-hexylresorcinol, and the inhibition type was competitive. The inhibition types of PO by kojic acid and quercetin were found to be mixed.

Key words: inhibitory mechanism, *Ospheranteria coerulescens*, phenoloxidase, purification

INTRODUCTION

Phenoloxidase (PO) is the key enzyme in the development and immunity of insects showing both monophenol monooxygenase activity (E.C. 1.14.18.1. tyrosine, dihydroxyphenylalanine, oxygen, oxidoreductase) and *o*-diphenoloxidase activity (E.C. 1.10.3.1. *o*-diphenol, oxygen, oxidoreductase).

Melanization is an increase in the concentration of melanin within the cuticle. Tanning is a process by which a cuticle is tanned. Sclorotization is a process by which the insect cuticle is hardened by substances other than chitin. Phenoloxidase is responsible for the biosynthesis of melanin pigment in animals and plants (Prota 1992). In addition to the melanization of the insect cuticle, this enzyme is also responsible for some important physiological processes, such as: the tanning of the cuticle, sclerotization, wound healing, encapsulation of the parasitoid, and nodule formation for defense against foreign pathogens (Sugumaran and Kanost 1993; Soderhall *et al.* 1994; Ashida and Brey 1995; Anderson *et al*. 1996; Gillespie *et al.* 1997; Sugumaran 2002).

The inhibition of PO by synthetic inhibitors could lead to the development of a novel insecticide. These inhibitors could lead to a disorder of the insect immune system against pathogens, and cause abnormal body softening. So far, the inhibitory effects of copper chelators on mushroom tyrosinase have been reported. Additionally, a natural inhibitor extracted from olive oil and saffron flower could inhibit mushroom tyrosinase (Xie *et al.* 2003). Available information on the inhibitors of insect PO is stillt the rudimentary stage. So far there is little research on the effects of these compounds on insect PO. For example, benzaldehyde, *p*-hydroxybenzaldehyde, *p*-chlorobenzaldehyde, and *p*-cyanobenzaldehyde were tested for their effects on the oxidation of L-dihydroxyphenylalanine (L-DOPA) by PO from the fifth instar of *Pieris rapae* L. The results showed that benzaldehyde, *p*-hydroxybenzaldehyde, and *p*-chlorobenzaldehyde are noncompetitive inhibitors of *P. rapae*

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PO, while *p*-cyanobenzaldehyde is a mixed-type inhibitor (Xue *et al*. 2007).

The PO of different insect species presents diverse biochemical characteristics. Due to the importance of this enzyme in insect immunity, it was characterized in some insects such as the tobacco budworm, *Musca domestica*, *Drosophila melanogaster*, *Aedes aegypti*, *Plodia interpunctella*, and *Sarcophaga bullata* (Yamaura *et al.* 1980; Ourth 1988; Fujimoto *et al*. 1993; Burks and Fuchs 1995; Chase *et al*. 2000; Wang *et al*. 2004; Hartzer *et al*. 2005). The study of this enzyme's properties is necessary not only for comparative study but also to understand the physiology of coleopteran immunity.

The rosaceous branch borer, *Ospheranteria coerulescens* Redtenbacher (Coleoptera: Cerambycidae), is the most destructive pest of rosaceous trees in Iran (Aghaali *et al*. 2012). The larvae of this insect feed on living twigs and branches of various rosaceae, and, occasionally when too abundant, they can cause damage to fruit trees. A heavy infestation results in significant crop reduction (Aghaali *et al*. 2012). There are serious problems associated with the exclusive application of pesticides against this pest, such as the intoxication of people and animals, the adverse effects of pesticides on non-target organisms, the emergence of resistant populations as well as pesticide residue and entry of the residue into the trophic network (Talebi *et al*. 2011). In the present study, PO was purified from haemolymph of the *O. coerulescens* larvae, and its biochemical properties were characterized. The inhibitory effects of kojic acid, 4-hexylresorsinol, and quercetin on PO were also surveyed.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, Tris-HCl, L-dihydroxyphenylalanine (L-DOPA), MBTH (3-methyl-2-benzothiazolinone hydrazone), and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Quercetin, kojic acid, 4-hexylresorcinol, and Sephacryl® HR-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Insects

Third instar larvae of the rosaceous branch borer were collected from the branches of amygdalus trees. Haemolymphs were collected by piercing the larvae's 2nd thorax segment with a 50 µl glass capillary tube.

Purification of PO

Haemolymph of 3rd instar *O. coerulescens* larva was centrifuged at 14 000 × g for 15 min at 4°C. The supernatant was collected and used as an enzyme source. Phenoloxidase was precipitated using an 80% saturation with crushed ammonium sulfate and then centrifuged at 13 000 × g for 30 min. The precipitate was redissolved in a minimum volume of the phosphate buffer (40 mM; pH 7.0) and then dialyzed against the 20 mM Tris-HCl cold buffer (pH 7.0). After dialysis, the sample was loaded onto a Sephacryl® HR-100 column (diameter 1.5 cm and height 47 cm), which was equilibrated with 20 mM Tris-HCl buffer (pH 7). About 18 fractions (each fraction containing 1 ml) were collected, and their absorbance levels were measured at 280 nm. The fraction with high PO activity was loaded on an ion exchange DEAE Sepharose™ fast flow column and washed with different concentrations of NaCl in the Tris-HCl buffer.

Measuring PO activity

Phenoloxidase activity was measured by monitoring the formation rate of dopachrome at 420 nm with a molar extinction coefficient of 3700 mM/cm using a microplate reader Model Stat Fax® 3200 (Awareness Technology Inc.). Enzyme activity was assayed at room temperature in 10 mM phosphate buffer (pH 7.0) containing the substrate at a final concentration of 0.1 and 30 mM MBTH and L-DOPA, respectively. In each assay, 10 µl of enzyme solution was added to each microplate well. The production of the dopachrome was monitored by measuring the absorbance at 420 nm continuously.

The effect of pH on PO activity

The optimum pH was determined by measuring the enzyme activity using sodium acetate-phosphate-borate buffer (40 mM, pH 3.0 to 12.0) as described above, at room temperature.

Optimal temperature and thermal stability of PO

The optimum temperature of PO was determined by measuring the activity as described above, at various temperatures ranging from 15 to 65°C. The thermal stability of the enzyme was monitored by incubating the purified PO at different temperatures for different times (1, 3, 5, 10, 15, 20, 30, and 60 min).

The effect of metal ions on PO activity

Phenoloxidase activity was determined in the presence of different concentrations of chloride salts of Zn^{2+}, K^{+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Hg^{2+} and EDTA (20 mM). The activity of PO was measured by incubating ions with the enzyme in phosphate buffer (20 mM; pH 7.0). The phenoloxidase activity was determined using MBTH (0.1 mM) and L-DOPA (30 mM), and monitoring the increase in absorbance. A control (no added ions) and blank (without an enzyme) containing ions were also measured.

Kinetic parameters (K_m and V_{max})

Different concentrations of L-DOPA (5, 10, 15, 30, 60, 80, and 100 mM) were mixed with 10 µl of enzyme solution. The increase in absorbance was continuously recorded at 492 nm. Enzyme activity was assayed at room temperature in 10 mM phosphate buffer (pH 7.0) containing L-DOPA and MBTH (30 mM). Then, the PO Michaelis constant (K_m) and the maximum velocities (V_{max}) were estimated by Hyper software. The experiments were performed in triplicate.

The inhibitory effects of quercetin, kojic acid, and 4-hexylresorcinol on PO

Ten µl of enzymes were incubated in different concentrations of kojic acid, 4-hexylresorcinol (0.062–2 mM), and quercetin (0.125–5 mM) for exactly 15 min at room temperature. After preincubation, the mixture of L-DOPA and MBTH was added to the reaction, and the remaining activity was determined by monitoring the increase in absorbance as previously described, at 492 nm. Probit analysis using the SPSS program (SPSS Inc. 1999) was used to estimate the I_{50} values for PO.

Determining the type of PO inhibition by quercetin, kojic acid, and 4-hexylresorcinol

To determine the inhibition type of the inhibitors, the PO assay was used as described above. Ten µl of purified PO solution was incubated with I_{25} and I_{50} concentrations of kojic acid, 4-hexylresorcinol, and quercetin for 15 min at room temperature. After preincubation, the remaining PO activity was measured with different concentrations of L-DOPA (5, 10, 15, 30, 60, 80, and 100 mM) as described above. Inhibition type was determined by analyzing the data with Lineweaver-Burk plots.

Polyacrylamide gel electrophoresis

The purity and molecular weight of the enzyme were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), based on Laemmli's (1970) method using a 4% (w/v) stacking gel and a 10% (w/v) separating gel. The molecular weight of the enzyme was estimated using the following standards: β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35.5 kDa), restriction endonuclease Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). After electrophoresis, the proteins on the gel were stained with coomassie brilliant blue R-250. Phenoloxidase activity staining was carried out using non-denaturing polyacrylamide gel electrophoresis (PAGE) based on the method of Davis (1964). The PO samples were mixed with non-denaturing sample buffer (without mercaptoethanol) and applied to 10% (w/v) polyacrylamide gel. Electrophoresis was performed at a constant voltage (100 V) in a refrigerator (at 4°C). After the electrophoresis was completed, the gel was kept in 50 mM phosphate buffer (pH 7.0) containing L-DOPA and MBTH for 5 h at 26±2°C in a dark place to develop the brown color bands corresponding with PO activity.

Protein determination

Protein concentrations were measured according to Bradford's method (1976). Bovine serum albumin was used as a standard.

Data analysis

Data were subjected to ANOVA using SAS software version 8.0 (SAS 1997).

RESULTS

Purification of PO

The phenoloxidase's haemolymph from the last instar larvae of the *O. coerulescens* was purified, and the results are presented in table 1. About one ml of the ammonium sulfate-precipitated PO was loaded onto a Sephacryl G-100 (gel filtration column). After washing with a buffer, fraction no. 9.0 showed the highest enzymatic activity using L-DOPA as the substrate (Fig. 1A). After that, this fraction was loaded onto an ion-exchange column (Fig. 1B). Fraction nos. 20, 21, and 22 showed the highest enzymatic activity levels.

Molecular weight

As shown in figure 2, under denaturing conditions on SDS-PAGE, there were three isoforms of this enzyme in the haemolymph of the *O. coerulescens*. The molecular weights of each isoform was 66.06, 79.45, and 85.23 kDa. The activity of PO was also characterized by activity staining after native PAGE, which allowed visualization of the red bands (Fig. 2B). Three isoforms from PO were detected on the gel.

PH optimum and thermal stability

The effects of different temperatures and pH values on PO activity were measured. The pH profile exhibited a typical bell-shaped curve with optimum activity at a pH of 8 (Fig. 3A). Phenoloxidase activity reached its highest level at 45°C (Fig. 3B). The stability of PO was measured at its optimal temperature, and at 10 degrees higher and lower than the optimal temperature (Fig. 3C). Results revealed that after 60 min, the enzyme lost less than 80% of its relative activity for all tested temperatures.

Table 1. Purification of PO, in which the PO comes from the haemolymph of *O. coerulescens*

Purification steps	Total activity [U]	Total protein [mg]	Recovery [%]	Specific activity [U/mg]	Purification fold
Crude extract	2.510	2.300	100.00	1.18	1.00
Ammonium sulfate 80%	1.186	1.600	47.27	1.83	1.55
Sephacryl® HR-100	0.789	0.363	30.43	2.12	1.79
DEAE Sepharose™ fast flow	0.415	0.091	16.53	4.56	3.86

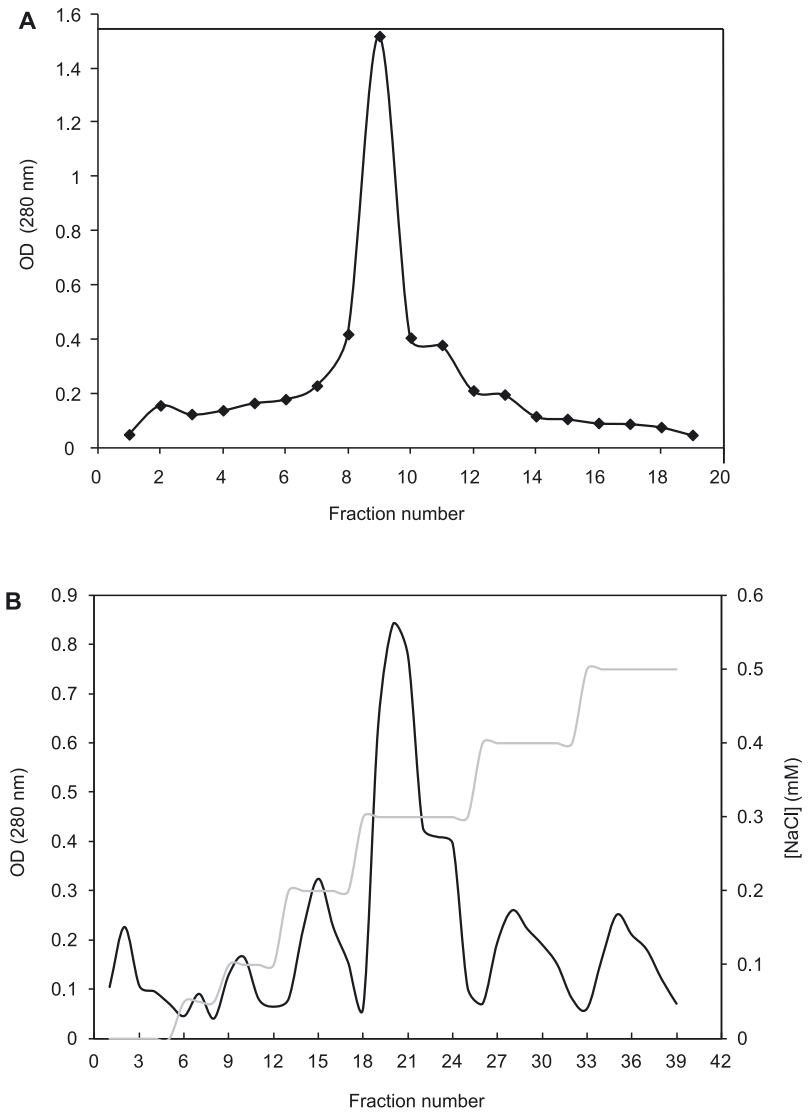

Fig. 1. Distribution pattern of protein over the fractions obtained following the Sephacryl® HR-100 gel-filtration of crude enzyme (A) (Fraction no. 9.0 contained the highest enzymatic activity using L-DOPA as the substrate) and DEAE Sepharose TM fast flow ion-exchange chromatography of fraction no. 9.0 (B) (Fraction nos. 20, 21, and 22 contained the highest PO activity). The pale line represents 0–0.5 M NaCl linear gradient

The effect of ions and chemicals

The effects of various ions and chemicals (20 mM) on PO activity were studied at a pH of 7.0, and at 30°C (Table 2). The results showed that Zn^{2+} and Co^{2+} greatly reduced the amount of enzyme activity. Enzyme activity was also somewhat reduced by Fe^{2+}, Ba^{2+}, and EDTA.

The effects of inhibitors on enzyme activity

The inhibitory effects of quercetin, kojic acid, and 4-hexylresorcinol on the L-DOPA oxidation by PO from haemolymph of the *O. coerulescens* were studied. The IC_{50s} of these inhibitors are presented in table 3. The IC_{50} of 4-hexylresorcinol, quercetin, and kojic acid on PO activity was estimated to be 35.75, 60.8, and 23.31 μM, respectively. The inhibitory potency of kojic acid on *O. coerulescens* PO was estimated at 1.54- and 2.58-fold higher than that of 4-hexylresorcinol and quercetin, respectively.

The mechanism of inhibition

The inhibition mechanisms of PO were analyzed by Lineweaver-Burk plots and there were different concentrations of inhibitors and L-DOPA. Different concentrations of 4-hexylresorcinol were represented by a group of lines with the same intercept but different slops. With an increase in the concentration of 4-hexylresorcinol, the apparent value of K_m was increased with no effect on V_{max}. Increasing L-DOPA concentrations decreased the inhibitory effect of 4-hexylresorcinol. It is likely, that 4-hexylresorcinol is similar in structure to L-DOPA, and both of them can bind to or near the active site of PO. Therefore, this inhibitor is a competitive inhibitor of *O. coerulescens* PO. Kojic acid and quercetin, however, represent different inhibition mechanisms. Changes in the concentrations of kojic acid and quercetin decreased V_{max} and K_m. Our data showed that they had mixed inhibition.

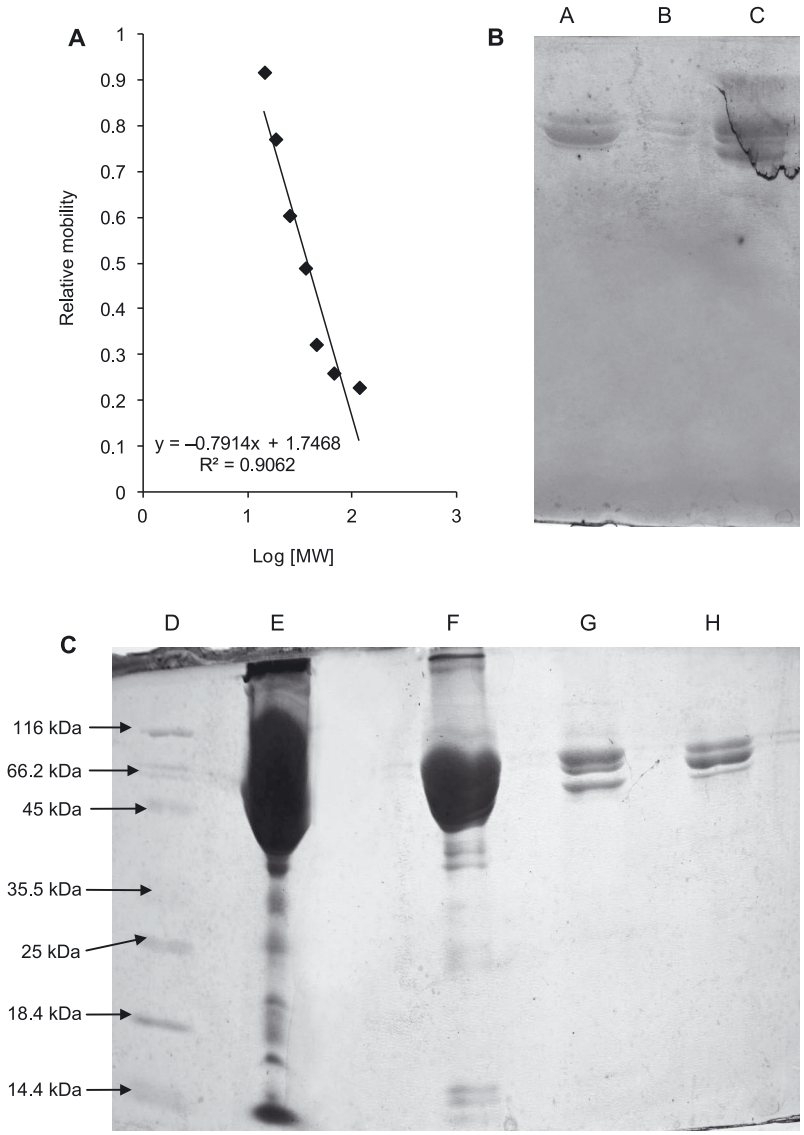

Fig. 2. The calibration curves for the estimation of the molecular weight of PO (A), zymogram of phenoloxidase and SDS-PAGE (B) of protein at different steps of purification (C). A – zymogram of PO (DEAE Sepharose™ fast flow, fraction no. 20); B – zymogram of PO (DEAE Sepharose™ fast flow, fraction no. 20); C – zymogram of PO at gel filtration step; D – Protein marker; E – Crude; F – Ammonium sulfate precipitation; G – Sephadex G-100; H – DEAE Sepharose™ fast flow

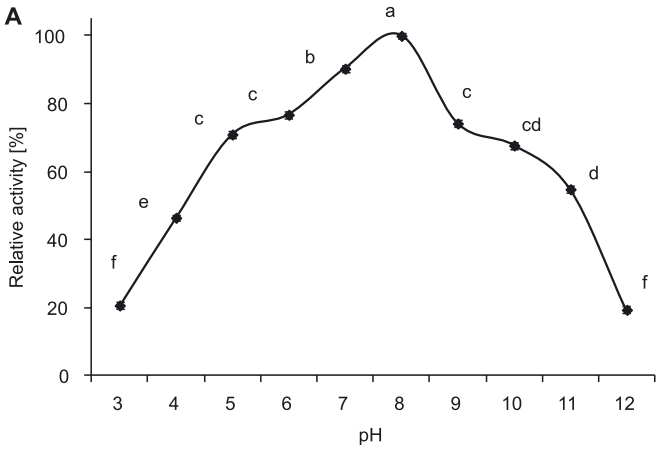

Fig. 3. Effects of different pHs (A) and temperatures (B) on purified PO (PO from *O. coerulescens*), and thermal stability of purified PO (C) Different letters (a–f) indicate that the specific activity of the enzymes is significantly different from each other, using Tukey's test ($p < 0.05$)

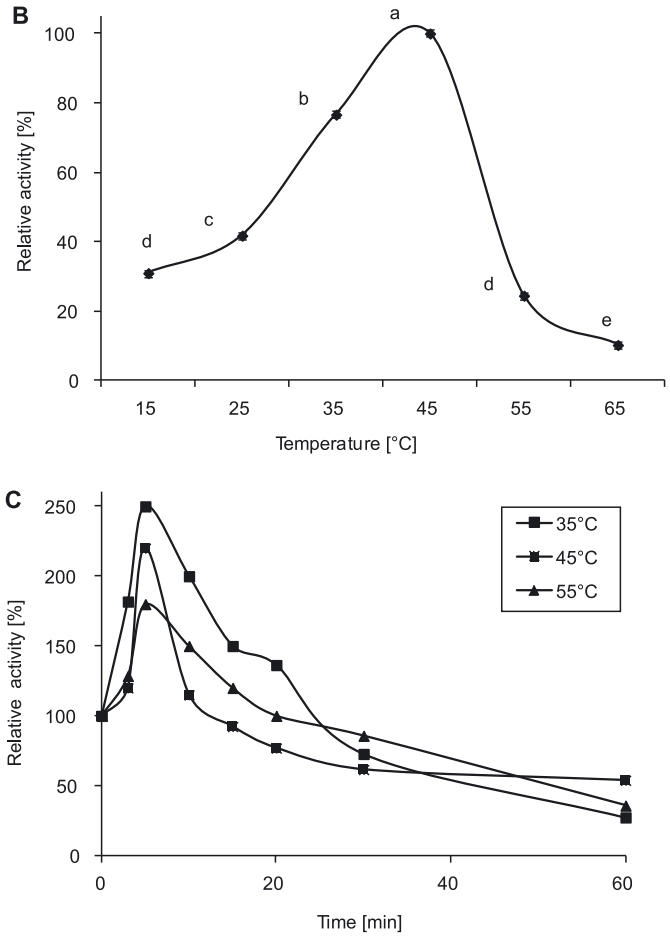

Fig. 3. Effects of different pHs (A) and temperatures (B) on purified PO (PO from *O. coerulescens*), and thermal stability of purified PO (C) – *continuation*

Table 2. Effect of different compounds on the PO activity (PO was purified from the haemolymph of *O. coerulescens*)

Compounds	Concentration [mM]	Relative activity [%]
The control	0	100.00±0.003 (a)
$FeCl_2$	20	84.24±0.004 (b)
$BaCl_2$	20	66.06±0.005 (b)
KCl	20	89.69±0.008 (ab)
EDTA	20	70.03±0.002 (b)
$HgCl_2$	20	72.72±0.006 (ab)
$CoCl_2$	20	81.81±0.008 (ab)
$ZnCl_2$	20	47.81±0.007 (c)

Different letters (a–c) indicate that the specific activity of the enzymes is significantly different from each other, using Tukey's test ($p < 0.05$)

Table 3. Inhibitions of 4-hexylresorcinol, kojic acid, and quercetin, on the PO (PO from the haemolymph of *O. coerulescens*)

Compounds	Slope ± SE	IC^a_{50}	$\chi 2$ (df)[b]	Relative potency[c] (95% confidence intervals (95% CI))
4-hexylresorsinol	1.198±0.113	35.75 (24.7–54.35)	6.15 (4)	1.54* (1.07–2.24)
Kojic acid	1.089±0.11	23.31 (18.44–22.9)	2.95 (4)	–
Quercetin	1.268±0.106	60.8 (49.15–75.3)	1.1 (4)	2.58* (1.92–3.49)

[a]the IC_{50} values are expressed as μM and their 95% confidence intervals (95% CI)
[b]values of $\chi 2$ lower than ($p \leq 0.05$) indicate a significant fit between the observed and expected regression lines
[c]relative potency = IC_{50} of compound/IC_{50} of kojic acid
*show a significant difference with IC_{50} of kojic acid

DISCUSSION

Phenoloxidase is an oxidative enzyme which represents a crucial role in insect defense via melanization (Soderhall and Cerenius 1998). This enzyme is synthesized in an inactive form called pro-phenoloxidase (pro-PO) (Liu *et al*. 2006) which is then activated by a serine protease. In this study, PO was purified from haemolymph of the *O. coerulescens* by ammonium sulfate precipitation, gel-filtration, and ion-exchange chromatography. With the ammonium sulfate precipitation and Sephacryl® HR-100 methods, the specific activity of purified PO increased almost 1.55- and 1.79-fold compared with the crude PO (Table 1). In native SDS–PAGE, three isoforms of PO were detected in the haemolymph of the *O. coerulescens*. The molecular weights of the three isoforms were 66.06, 79.45, and 85.23 kDa. The molecular weights of PO in insects and crustaceans were reported higher than 60 kDa. The molecular weight of POs depends on their isoforms, activation state, and the insect species. For example, there are two isoforms from PO in *Galleria mellonella* (Kopácek *et al*. 2001) and *Bombyx mori* (Yasuhara *et al*. 1995), six in the mosquito *Anopheles gambiae* (Müller *et al.* 1999), and three in the fruit fly *Drosophila melanogaster* (Fujimoto *et al*. 1993).

In the present study, the maximal activity of PO from *O. coerulescens* was obtained at a pH of 8.0, which is similar to that of the optimum pH for *Ostrinia furnacalis* (Guenee) (7.0), *Pieris rapae* L. (7.0), *Glyphodes pyloalis* Walker (Sharifi *et al*. 2012), and *Sarcophaga bullata* (6.5) (Wang *et al*. 2004; Xue *et al*. 2006; Feng *et al*. 2008). However, some studies have reported higher values for the optimum pH, *i.e.* pH 8.0 for *Lymantria dispar* (Dunphy 1991) and pH 9.0 for *Heliothis virescens* (Lockey and Orth 1992). Due to the buffer properties of haemolymph, it seems PO in insect haemolymph is active in neutral conditions (Sharifi *et al*. 2012). The *O. coerulescens* PO showed activity as the optimum temperature of 45°C. The optimal temperature for PO activity was reported at 35–45°C in the lepidopteran order of insects (Xue *et al.* 2006; Lockey and Orth 1992), while PO from haemolymph of bee pupae showed optimal activity at 20°C (Zufelato *et al.* 2004). As shown in figure 3c, stability of the PO activities for all three selected temperatures were similar. In the first 20 min, PO was able to retain about 85% of its activity, but enzyme activity fell to less than 40% after an hour of incubation at the optimal temperature.

The effect of some metal ions (20 mM) on PO activity was studied. The results showed that Zn^{2+} greatly inhibited PO activity. The activity of the *O. coerulescens* PO decreased somewhat with the addition of EDTA and Ba^{2+} to the reaction mixture. Zn^{2+} was the activator of PO from *P. rapae* (Xue *et al.* 2005). Similar to our results, Liu *et al*. (2006) showed that activity of PO from *Charybdis japonica* was strongly inhibited by Zn^{2+}. Also similar to our results, K^+ showed a slight inhibition in PO activity from *L. dispar*, however, K^+ had no significant influences on PO activity purified from *Gastrolina depressa*, *Sidemia depravata*, or *Holotrichia oblita*. The reason transitional metal ions have different effects on the PO from different insects is still unclear (Zhao *et al*. 2010). Some metal ions can significantly modify the structure of PO, and this modification leads to increased or decreased PO activity.

The inhibitory effect of kojic acid on PO activity was 1.54- and 2.58-fold higher than that of 4-hexylresorcinol and quercetin, respectively. The IC_{50s} of quercetin, kojic acid, and 4-hexylresorcinol on PO purified from haemolymph of the *G. pyloalis,* were 7.39, 25.98, and 4.35 μM, respectively. The inhibitory potency of 4-hexylresorcinol was 5.03- and 1.5-fold higher than kojic acid and quercetin, respectively (Sharifi *et al.* 2012). Quercetin and 4-dodecyl resorcinol showed a great inhibitory effect on PO from *L. dispar.* The IC_{50} values of quercetin and 4-dodecyl resorcinol were estimated as 0.076 mmol/l and 0.372 mmol/l, respectively, using catechol as the substrate (Yan et al. 2009). Both quercetin and 4-dodecyl resorcinol were reversible competitive inhibitors of PO from *L. dispar*. The inhibitory constants (K_i) were determined to be 1.71 mmol/l and 0.192 mmol/l, respectively. Our results showed that the IC_{50s} of quercetin and 4-hexylresworsinol on PO from the *O. coerulescens* were 60.8 and 35.75 μM, respectively. The inhibitory effects of 4-hexylresorcinol and kojic acids on the PO from *L. dispar* were also studied, using L-tyrosine or catechol as the substrate (Yan *et al*. 2009), and the IC_{50s} were 0.41 μmol/l for monophenolase and 0.35 μmol/l for diphenolase activities, respectively. The results of inhibition kinetics analyzed by Lineweaver-Burk plots on the PO of *L. dispar* indicated that 4-hexylresorcinol is a competitive inhibitor for the oxidation of catechol. The inhibition constant was determined to be 0.00015 mmol/l. Kojic acid was a competitive inhibitor for the oxidation of catechol by *P. rapae* PO. The inhibition constant was determined to be 0.51 mmol/l (Wang *et al*. 2007). Our results showed that the inhibition type of 4-hexylresorcinol on PO was competitive. Furthermore, the inhibition type of PO by kojic acid and quercetin were mixed (Table 4), but the inhibition type of PO from the loopworm *Semiothisa cinerearia* Bremer et Grea (Homoptera: Geometridae) by quercetin was proven to be a competitive inhibitor.

A number of inhibitors with inhibitory potency on insect PO have been identified. Research in this area will help us to control pests by inhibiting their PO activity. In higher plants, PO inhibitors are polyphenolic compounds and non-aromatic tyrosinase inhibitors (Casanola-Martin *et al*. 2006). A non-chemical method for controlling insect pests is the use of toxic proteins in genetically modified crops which are resistant to pests that express PO inhibitors. Quercetin and kojic acid are plant- and fungi-derived compounds, respectively, which are used in cosmetic products. However, these inhibitors may cause harmful effects on people and animals, which means further analyses need to be done.

In conclusion, PO is one of the important oxidative enzymes that are involved in insect immunity and development, and its inhibition by synthetic inhibitors is a promising approach for pest control. In this research, three PO isoforms were identified from haemolymph of *O. coerulescens.* Kojic acid, 4-hexylresorcinol, and quercetin interestingly inhibited *O. coerulescens*.

Table 4. Inhibition mechanisms of 4-hexylresorcinol, kojic acid, and quercetin, on the PO (PO from the haemolymph of *O. coerulescens*)

Inhibitors	Mechanisms of inhibition	K_m (mM) K_m (mM)	V_{max} (μmol/min) V_{max} (μmol/min)	IC_{50} (μM) IC_{25} (μM)
The control	–	88.61	0.147	–
4-hexylresorcinol	competitive	290.41	0.138	35.75
		134.50	0.141	9.75
The control	–	79.68	0.160	–
Qurcetin	mixed	22.79	0.098	60.8
		65.23	0.123	22.72
The control	–	75.68	0.161	–
Kojic acid	mixed	23.31	0.098	26.59
		5.55	0.115	39.56

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