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Endogenous Phenoloxidase Purified from an Earthworm, *Lumbricus rubellus*

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An endogenous phenoloxidase (EPO) from earthworm, *Lumbricus rubellus*, has been purified and characterized. The purified EPO using ammonium sulfate fractionation, Blue-2, Phenyl-, and Q-sepharose chromatography steps was revealed in SDS-PAGE as a single protein band with Mr. of 59 kDa. A native structure of the enzyme was examined with an *in situ* staining of a nondenaturing-PAGE using DL-dopa as a substrate. The result showed that a single band due to the EPO activity was located slightly above a standard polypeptide with Mr. of 210 kDa. These facts indicate that the EPO is an oligomeric enzyme. The presence of a monophenolase activity of the purified EPO, which hydroxylates tyrosine to dopa, was confirmed by observing dopachrome accumulation at 475 nm at pH 8.0 with a typical lag phase during 60 min. of meausrement. A series of inhibition study has been performed for the enzyme with several divalent cation chelators such as phenylthiourea (PTU), 1, 10-phenanthroline, EDTA, and EGTA. Among them, only PTU inhibited the enzyme with $IC_{0.5}$ of 65 μM, which indicated that copper was critical for the catalysis of EPO. The enzyme was maximally active at 35°C and pH 8.0 when L-dopa to dopachrome conversion was spectrophotometrically monitored at 475 nm. The apparent Km values of PO for L-dopa were obtained as 1.86 mM and 13.8 mM at pH 6.5 and 8.0, respectively. The catalytic efficiencies at both pH were almost identical [$(kcat/Km)_{pH8.0}/(kcat/Km)_{pH6.5}$ = 0.92] while the Vmax at pH 8.0 was 6.6-fold higher than that at pH 6.5. This fact may indicate that pH affects the catalysis at substrate and/or enzyme-substrate complex level rather than the enzyme itself. Taken together, the EPO was an oligomeric enzyme which did not require proteolysis for its activation. These results also indicated that the enzyme can exist, at least, in part as a latent form *in vivo*, which might be distinct from the prophenoloxidase activating system. Therefore, it is pertinent to consider that there must be certain regulatory molecules or phenomena in *L. rubellus* which make the PO in a latent form *in vivo* before the foreign invasions.

KEY WORDS: Phenoloxidase, Tyrosinase, Earthworm, *Lumbricus rubellus*

Phenoloxidase [PO: monophenol, dihydroxyphenylalanine: oxygen oxidoreductase; EC 1.14.18.1], also known as tyrosinase, is a widely distributed bifunctional copper-containing enzyme

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which catalyzes two successive reactions in melanin biosynthesis (Cabanes *et al.*, 1987). One due to cresolase or monophenolase activity converts L-tyrosine to L-dopa[1] by hydroxylation as a rate-determining step. The other which rapidly oxidizes L-dopa to o-dopaquinone is catalyzed by catecholase or diphenolase activity. The resulting unstable o-dopaquinone experiences spontaneous nonenzymatic reactions, which eventually forms melanin deposit as a brownish or yellowish pigment (Daquinag *et al.*, 1995).

In insects, PO-mediated reaction plays pivotal roles in not only development such as cuticular tanning and sclerotization but also cellular defence against foreign pathogens such as microorganism and parasite (Li and Christensen, 1993; Ratcliffe *et al.*, 1984). In general, melanization has been considered as an important defence mechanism in arthropods. The toxic quinones generated as an intermediate during the melanization process could act as bacteriocidal or fungistatic agents. In addition, the reaction between melanin and proteins on foreign pathogens could physically isolate them, which are ultimately removed from a host through encapsulation and opsonization followed by phagocytosis (see Boman and Hultmark, 1987 for review).

It has been suggested that the activity of PO is regulated in several ways to discriminate self and nonself in insects. As the most well-established pathway, PO has been activated by removing a 5 kDa peptide from its zymogen, so called prophenoloxidase (pro-PO), by the action of serine protease(s) in a cascade reaction (Ashida and Dohke, 1980; Ashida, 1981; Ashida and Söderhäll, 1984). This cascade is initiated by fungal or bacterial cell wall constituents such as β-1,3-glucan and lipopolysaccharide through specific binding proteins which, in turn, activate the serine protease(s) (Söderhäll and Smith, 1984, 1986; Ratcliffe *et al.*, 1985). Besides this pro-PO activation system by a limited proteolysis, the PO is activated from its latent form with artificial agents such as lipids (Heyneman and Vercauteren, 1968), sodium dodecylsulfate (Inaba and Funatsu, 1964), 2-propanol (Asada *et al.*, 1993), nitrocellulose membrane (Brey *et al.*, 1991), and heat (Ashida and Söderhäll, 1984). It has been also shown that PO was activated by self-aggregation (Mitchell and Weber, 1965). A physiological implication of the activation by the above agents is yet to be known.

Recently, we have shown that *Lumbricus rebellus* also contains the PO activity which has been activated by both a limited proteolysis and a heat treatment (Bahk *et al.*, 1995). We have suggested that there must be more than one pathway in the earthworm, which activate PO from its latent form. In order to understand activation pathway and its involvement in defence mechanism of annelids, it is necessary to purify and characterize endogenous PO, which has been shown in this report.

Materials and Methods

Materials

Adult *L. rubellus* earthworms had been provided by Shinpoong Pharmaceutical Co. located in Ansan, Korea. After washing three times with distilled water, the fresh earthworms were divided into several batches (50 g/batch) and kept frozen at −70°C. Sodium cacodylate, L-dopa, tyrosine, Tris, MES, phenylthiourea, EDTA, EGTA, 1,10-phenanthroline, and NaCl were obtained from Sigma Chemical Co. Ammonium persulfate and TEMED were from BioRad. Other reagents for gel electrophoresis, Blue-2 sepharose, and Q-sepharose were also purchased from Sigma. Phenyl-sepharose CL-6B was obtained from Pharmacia. Ammonium sulfate, ethyl-eneglycol, acetic acid, and boric acid were purchased from Junsei Pure Chemical Co., Japan.

1 The abbreviations used are: L-dopa, 3,4-dihydroxyphenylalanine; PO, phenol-oxidase; EPO, endogenous phenoloxidase; proPO, prophenoloxidase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PTU, phenylthiourea; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid; TRIS, tris (hydroxymethyl)aminomethane; MES, 2-(N-morpholino)ethane- sulfonic acid; Cac buffer, sodium cacodylate buffer; HPLC, high performance liquid chromatography.

Other chemicals were of the highest purity available.

PO assay

PO activity was assayed with the method of Horowitz and Shen (1952) with modifications. A typical reaction was done directly in a cuvette which contained 0.4 ml of 50 mM sodium cacodylate buffer, pH 6.5 and 50 μl of each fraction from each purification step. As soon as L-dopa prepared in water was added to reach a final concentration of 5 mM in a total volume of 0.6 ml, dopachrome generation was continuously monitored at 475 nm with Uvikon spectrophotometer 930 from Kontron instruments. The initial rates were obtained from slopes of the curves within 40 sec. after the addition of substrate. One unit of PO activity is defined as the amount of enzyme which increases absorbance of 0.001 within 1 min. in a reaction.

Purification of endogenous PO

The endogenous PO was purified from a supernatant prepared by thawing 50 g of the frozen earthworm in 100 ml of sodium cacodylate buffer, pH 6.5 (Cac buffer) in a ratio of 1 : 2 (w/v). The trunks of earthworms were chopped and stirred at 4°C for 4 hrs. A centrifugation was followed with the extract at 25,000 × g for 20 min. with Centrikon T-324, Kontron instrument. PO was further extracted from the supernatant by sonication during 1 min. period with 3 min. intervals to keep the solution cold. This sonication was repeated for three times with Fisher sonic dismembrator model 300. Ultracentrifugation was done at 105,000 × g for 60 min. at 4°C with Centrikon T-2080, Kontron instrument and the resulting supernatant was collected. Ammonium sulfate fractionation was performed between 20% and 60% at 4°C. After a centrifugation at 25,000 × g for 15 min., the resulting pellet was resuspended in 50 mM Cac buffer, pH 6.5 and dialyzed with one change against 2 L of the same buffer at 4°C for 3 hrs. for each time. As the first step, PO was purified with Blue-2 sepharose chromatography (1.6 cm × 10 cm) which was equilibrated with the Cac buffer and eluted with a solution containing 1 M NaCl in the Cac buffer. The active fraction in high salt concentration was further purified with phenyl-sepharose hydrophobic interaction chromatography (1.6 cm × 10 cm) already equilibrated with the eluting buffer of the previous step. After washing the column extensively with 50 mM Cac buffer, the bound PO was eluted with a step gradient of ethylene glycol prepared in the Cac buffer from 10% to 80% with 10% increase for each step. This partially purified PO was dialyzed twice against 2 L of 50 mM Tris-Cl, pH 8.0 at 4°C for 3 hrs. for each time. Finally, the endogenous PO was recovered from Q-sepharose chromatography equilibrated with the Tris buffer as a flow-through fraction.

Temperature and pH optima

PO activities at various temperatures from 5°C to 50°C were measured at 475 nm in 50 mM Cac buffer, pH 6.5 with the thermostated spectrophotometer (Uvikon 930, Kontron instrument) after 10 min. preincubation at the temperatures. A pH optimum of the enzyme was obtained from the activities after 10 min. preincubation at various pH maintained with a complex buffer composed of 50 mM each of acetic acid, MES, Tris, and boric acid from pH 4 to 10 adjusted with 1 N HCl and NaOH.

Inhibition

Inhibition of PO was examined at 475 nm in the presence of phenylthiourea, 1,10-phenanthroline, EDTA, and EGTA at various concentrations without preincubation. The activities of PO were compared in the presence and absence of inhibitors as v/vo and plotted against the inhibitor concentrations, where v and vo represented the activities in the presence and absence of the inhibitors, respectively. From the plots, inhibitory concentrations which reduced an original activity to 50% were obtained as $IC_{0.5}$.

Monophenolase activity of PO

Tyrosine prepared in water as 2 mM was used as an initial substrate. To a reaction mixture, tyrosine was added to a final concentration of 1 mM in 250 μl of 100 mM Tris-Cl, pH 8.0 and 50 μl of PO. Dopachrome accumulation was

continuously monitored at 475 nm for 60 min.

Kinetic parameters

The apparent K_m and V_{max} of PO for L-dopa were obtained at various substrate concentrations with Lineweaver-Burk plots in two different pH buffered with 50 mM Tris-Cl, pH 8.0 and 50 mM Cac, pH 6.5. Specificity constants were obtained as k_{cat}/K_m.

Gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) and the protein was visualized with a silver staining procedure (Sammons *et al.*, 1981). Nondenaturing-PAGE was done accordingly to Davis (1961) and the PO activity was detected by soaking the gel in 10 mM DL-dopa in 50 mM Tris-Cl (pH 8.0) with the method of Nellaiappan and Vinayagam (1986).

Protein assay

Protein was quantitated with the method of Bradford (1976) using bovine serum albumin as a standard protein.

Results

Purification of endogenous PO

An endogenous PO (EPO) which did not require a limited proteolysis for activation was purified to about 900-fold with ammonium sulfate fractionation, Blue-2, phenyl, and Q-sepharose chromatography steps (Table 1). The initial extract before the sonication step contained proPO as well as EPO in a 1:1 ratio. The amount of proPO was quantitated with 150 μg of trypsin treatment at 37°C during various time intervals. At each time point, the total PO activity due to both the endogenous PO and the PO converted from proPO was measured. From the time point which gave the highest activity, the amount of proPO was calculated by substracting the endogenous activity from the total. Before the ultra-centrifugation, the sample was reextracted with sonication, which increased the endogenous PO to 3.5-fold. The sonication was turned out to be the most effective artificial way to activate PO from its latent form among other treatments such as 0.02% melittin, 0.02% Triton X-100, 0.01% SDS, and freezing-thawing, although all these agents increased the activity from 1.5 to 2.5-fold (data not shown). These facts indicate that a large amount of PO could exist in an inhibited state or as a latent form that is distinct from the proPO which must be processed by protease(s). During the purification, the total catalytic activity was increased after ammonium sulfate fractionation, which again indicated that the PO was liberated or activated from its latent form with this salt.

As the first chromatography step, Blue-2 sepharose was used (Fig. 1). While most proteins did not bind to the matrix, EPO was attached and eluted with 1 M NaCl without any gradient. In this active fraction, proPO was still present along with EPO. The next purification was to use the phenyl-sepharose hydrophobic interaction chromatography, which was proved to be very effective (Fig. 2). In fact, the proPO seemed to be completely removed with a reverse gradient and a washing

Table 1. Purification of endogenous phenoloxidase (EPO) from *Lumbricus rubellus*

Methods	Total units[a]	Total proein (mg)	Specific activity[b]	Yield (%)	Purification (fold)
Supernatant	32100	820	39.1	(100)	(1.0)
$(NH_4)_2SO_4$ (20-60%)	35400	298	118.8	110	3.04
Blue-2	34986	19.0	1841.4	109	47.1
Phenyl	25333	2.10	12063.3	79	308.5
Dialysis	21000	1.85	11351.4	65.4	290.0
Q-Sepharose	14728	0.414	35574.9	45.9	909.8

a One unit is defined as the amount of enzyme which increases 0.001 of absorbance within 1 min at 475 nm.
b Specific activity is expressed in units/mg protein.

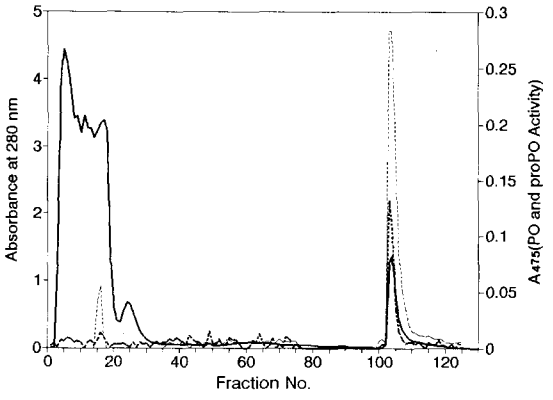

Fig. 1. An elution pattern of proteins and the PO and proPO activities from Blue-2 sepharose chromatography. The column was previously equilibrated with 50 mM sodium cacodylate, pH 6.5. After the sample was applied, the colume was extensively washed with the cacodylate buffer until absorbance of the eluent reached below 0.01 at 280 nm. The first rise in absorbance at 280 nm was due to the flow-through where proteins did not bind to the matrix. After the extensive wash, the adsorbed protein was eluted with 1 M NaCl and collected in 3.5 ml each fraction, whose elution profile was shown as the second absorbance peak at fraction number 104. It showes proteins as a solid line, PO as a broken line, and proPO as a thick-broken line.

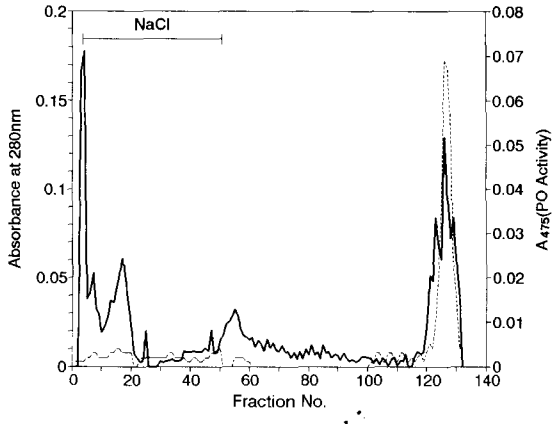

Fig. 2. PO purification with phenyl-sepharose chromatography. The active fractions from the Blue-2 chromatography step was loaded on the phenyl column already equilibrated with 1 M NaCl in the cacodylate buffer, pH 6.5. The proteins indicated as a solid line were initially eluted with a reverse gradient between 1 M NaCl and the Cac buffer without NaCl. After the column was further washed with the Cac. buffer, PO shown as a broken line was eluted with a step gradient of ethylene glycol. The fractions obtained with the reverse gradient were also indicated.

procedure before the ethylene glycol step gradient because the EPO active fractions was not further activated with trypsin treatment. After the dialysis which slightly decreased purification fold, any undesirable proteins were easily removed by passing the sample through the Q-sepharose column. The EPO was obtained in a flow-through.

The purity of EPO was examined with SDS-PAGE and a silver staining procedure, which showed that a homogeneous band with Mr. of 59 kDa was apparent in a rather diffused manner (Fig. 3A). Any reason for the diffused appearance of EPO is not clear at present even though there would be several possibilities such as presence of isoforms due to different posttranslational modifications. In order to know a native structure of the enzyme, a nondenaturing-PAGE was performed and subjected to the *in situ* staining with DL-dopa. The converted dopachrome was precipitated as a band located slightly above a 210 kDa standard protein of myosine heavy chain (Fig. 3B). Although molecular weight estimation was particularly difficult on a nondenaturing-PAGE, the EPO in *Lumbricus rubellus* could be considered as a tetrameric protein since the band was very closely located to the standard protein. In any event, it is clear that the EPO has been existed as an oligomeric form in nature. In order to obtain more solid evidence for the native structure of EPO, gel permeation chromatography was performed with a TSK G3000SW$_{XL}$ column for HPLC. Unfortunately, a dopachrome-generating activity came out at a total volume which was the same volume as free copper ions eluted (data not shown). Since phenoloxidase has been known as a copper-containing enzyme, which is the case for this EPO (see below), only explanation with which we can come up is that loosely bound copper ions have been dissociated during the chromatography for unknown reason.

Characterization of PO

The effect of temperature on EPO activity was studied by preincubating the enzyme for 10 min. at the temperatures indicated before the substrate, 5 mM L-dopa, was added (Fig. 4). The enzyme

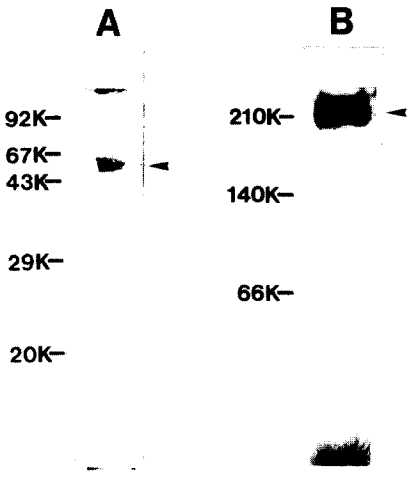

Fig. 3. Gel electrophoresis of the purified EPO. (A) 12 % SDS-PAGE of the enzyme visualized with a silver staining. The molecular weight standard proteins used were phosphorylase a (92,500), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (20,100). (B) 5% nondenaturing-PAGE of EPO. The dopachrome accumulation was detected by incubating the gel overnight in 10 mM DL-dopa in 50 mM Tris-Cl, pH 8.0. The markers used were myosin heavy chain (212,000), lactate dehydrogenase (140,000), and bovine serum albumin (66,000).

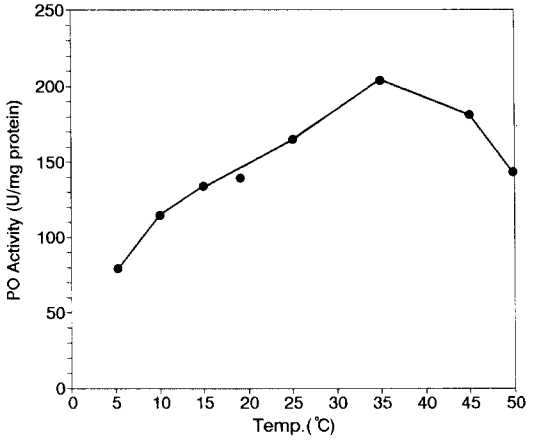

Fig. 4. EPO activities at various temperatures. The activities shown as units/mg protein were measured with a thermostated spectrophotometer after 10 min. of preincubation at the temperatures with 5 mM L-dopa.

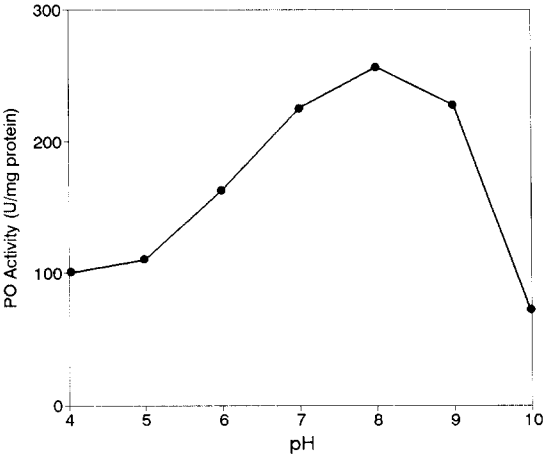

Fig. 5. pH effects on the enzyme activities. The activities were obtained by measuring initial rates in a complex buffer composed of 50 mM each of acetic acid, MES, TRIS, and boric acid. The pH was adjusted with either 1 N HCl or NaCl. The enzyme was preincubated at each pH for 10 min. before the measurements.

was maximally active at 35°C with a specific activity of 200 units/mg protein although fairly good activities were observed at rather broad temperature range. Recently, we have shown that the proPO activation system in *L. rebellus* was maximally operated at 50°C, which did not require any exogenous protease for activation (Bahk *et al.*, 1995). At this temperature, EPO was inhibited instead, which indicated that the EPO preparation did not contain any latent form of the enzyme.

An optimum pH for EPO was obtained by measuring initial rates of the activities in a complex buffer consisted in 50 mM each of acetic acid, MES, TRIS, and boric acid after pH were adjusted with 1 N HCl and NaOH (Fig. 5). After 10 min. of preincubation, L-dopa was added to 5 mM. The data were presented after substraction of a spontaneous chemical conversion of L-dopa to dopachrome at basic pH from the total observed activities. The result showed that the enzyme was maximally active at pH 8.0 with a specific activity of 250 units/mg protein.

In order to confirm the purified protein as a phenoloxidase, a series of inhibition study was carried out using several divalent cation-specific

chelators such as PTU, known as a specific inhibitor of dopamine β-hydroxylase, used for Cu^{2+}, 1,10-phenanthroline for Fe^{2+}, EGTA for Ca^{2+}, and EDTA (Fig. 6). Only PTU inhibited the enzyme with IC0.5 of 65 μM, indicating that PO

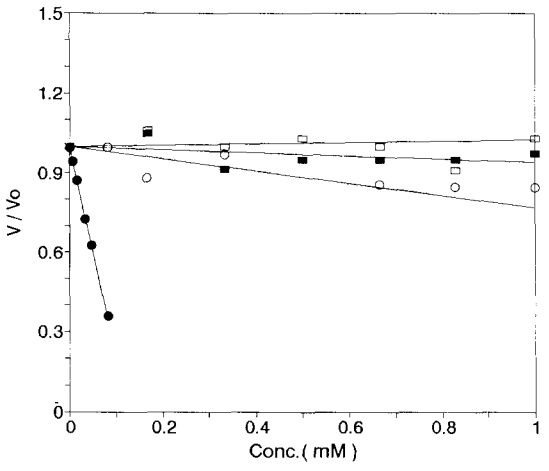

Fig. 6. Inhibition of EPO with several divalent cation chelators. The activities of the enzyme in the presence of chelators at various concentrations were measured without any preincubations. The amount of inhibition was shown in v/vo, where vo and v were the activities in the absence and presence of the chelators, respectively. Phenylthiourea (●), 1,10-phenanthroline (○), EDTA (■), EGTA (□).

from *L. rubellus* was a copper-containing protein like other tyrosinases. Interestingly, although both Ca^{2+} and EDTA did not affect EPO activity, Ca^{2+}-EDTA complex formed prior to reactions inhibited the enzyme as the same extent as 1,10-phenanthroline (data not shown). The inhibition by PTU, however, did not substantiate the enzyme as phenoloxidase because even an isolated copper ion could oxidize L-dopa to dopachrome. In order to make sure of EPO as an enzyme, a catalysis of the rate-limiting step from L-tyrosine to L-dopa must be examined. When dopachrome accumulation was measured with tyrosine as a substrate in the presence and absence of the enzyme, it was clear that the isolated EPO recognized tyrosine and converted it to dopachrome through L-dopa even though the activity was slow (Fig. 7). The activity was appeared with a typical lag which might be due to the accumulation of L-dopa. Since peroxidase and basic pH (pH 8.0) could be responsible for productions of L-dopa and dopachrome, respectively, a possible contamination of peroxidase in our enzyme preparation was examined with the method of Maehly and Chance (1954). The result showed that there had been no peroxidase activity observed (data not shown). Taken together, PO isolated from *Lumbricus rubellus* was a copper containing enzyme which

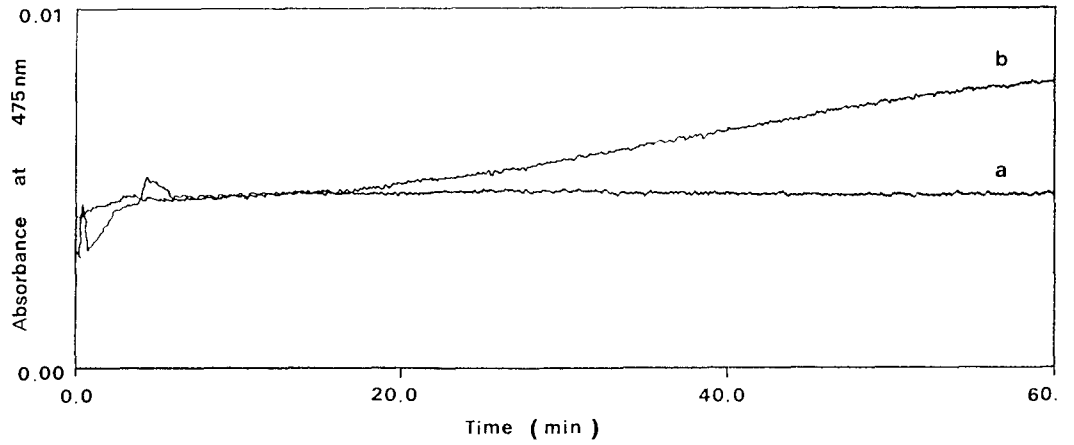

Fig. 7. Tyrosin recognition of EPO. Dopachrome accumulation from tyrosine was continuously monitored for 60 min. at 475 nm in the absence (a) and presence (b) of EPO. The reaction mixture was composed of 1 mM tyrosine in 50 mM Tris-Cl, pH 8.0.

contained both monophenolase and diphenolase activities.

Finally, steady state kinetic parameters, K_m and V_{max}, were obtained with L-dopa at both pH 6.5 and 8.0 from Lineweaver-Burk Plots (Fig. 8). At pH 6.5, EPO exhibited an apparent K_m of 1.86 mM for L-dopa with V_{max} of 0.02 expressed in absorbance change for 1 min. at 475 nm. At pH 8.0, on the other hand, the affinity was decreased to an apparent K_m of 13.8 mM while Vmax was increased 6.8-fold to 0.137. When specificity constants (k_{cat}/K_m) were compared, 2.4×10^3 M^{-1} sec^{-1} and 2.6×10^3 $M^{-1}sec^{-1}$ were obtained at pH 8.0 and 6.5, respectively, with a ratio of 0.92. The fact that a ratio of k_{cat}/K_m was 0.92 even though the Vmax was increased to 6.8-fold at the optimum pH indicated that the pH did not affect the enzyme itself but it could influence at an enzyme-substrate complex level.

Discussion

In invertebrate, phenoloxidase has been extensively studied with respect to its involvement in host defence mechanism from invading foreign substances such as bacteria, fungal spores, and parasites. There have been two well-known immunity-related factors to protect the hosts. Lectin is one of them which agglutinates invading microorganisms carrying the corresponding sugar residues as their surface components. This process results in a formation of agglutinated cells which makes them to be easily phagocytosed or encapsulated (Boman and Hultmark, 1987). The other is an enzyme called PO. In arthropods, this enzyme was generated from its precursor, proPO, by the action of serine protease(s). This proPO activating system was initially proposed to be responsible for discrimination between self and nonself molecules based on a finding that the enzme in crayfish blood was activated by fungal cell wall constituent, β-1,3-glucan (Söderhäll *et al.*, 1994). Although vast majority of research dealing with the PO in insects confirmed the activating system, our recent observations in annelids made us doubtful on the fact that the enzyme existed exclusively as a precursor. Our previous study showed that PO could be present as a form of both the precursor and a latent form which did not require proteolysis (Bahk *et al.*, 1995). We undertook, therefore, this study to know the nature of PO in *L. rubellus*, which would eventually relate the enzyme to the host defence mechanism in annelids.

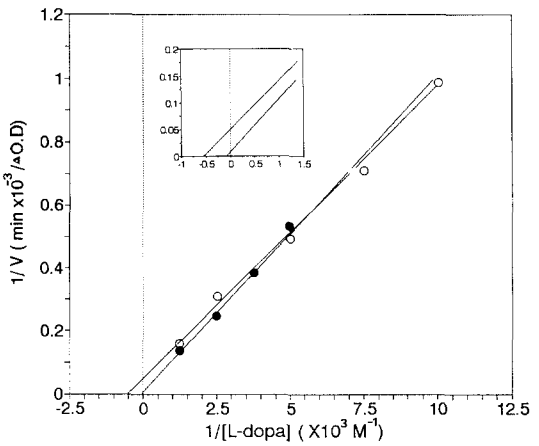

Fig. 8. Lineweaver-Burk plot of EPO activity at pH 6.5 and 8.0. The enzyme activities were obtained at the various L-dopa concentrations indicated in either 50 mM sodium cacodylate buffer, pH 6.5 (○) or 50 mM Tris-Cl, pH 8.0 (●). The inset is a magnification of the intercepts.

The EPO from the earthworm was purified with the relatively simple method by minimizing salt gradient steps. A difficulty we had to encounter during this purification was due to individual variations in *Lumbricus rubellus*. It had been shown that other species in annelids such as *Eisenia foetida* and *Lumbricus terrestris* also exhibited the variations when serine proteases were isolated from their coelomic fluids (Leipner *et al.*, 1993). This variations might be caused by different growing conditions such as prey, temperature, humidity, and other environmental factors.

The purified EPO could be possibly a tetrameric protein with Mr. of slightly above 210 kDa because SDS-PAGE of the enzyme showed a single band with Mr. of 59 kDa. The high molecular weight oligomeric phenoloxidase is not an unique feature for the enzyme. A phenoloxidase from *Heliothis virescens* larvae is 250

kDa (Lockey and Ourth, 1992). Another well-characterized enzyme from mushroom is a tetramer with Mr. of 128 kDa (Duckworth and Coleman, 1970). A tetrameric phenoloxidase is also found in the eichinoderm, Holothuria tubulosa, with Mr. of 60 kDa (Roch *et al.*, 1992). Although exact substoichiometry of the EPO is not definitive at present, the fact that the enzyme exists as an oligomeric form in nature may have significant meaning. It could indicate that the phenoloxidase might not require proteolysis to become an active form since most phenoloxidases known to experience the prophenoloxidase activating system are monomeric proteins. It is difficult to imagine that both proteolysis and oligomerization must be preceded to protect the host from foreign invasions. It can be suggested that the enzyme in *L. rubellus* could exist in part as a latent form possibly associated with an inhibitor(s). This contention is supported by the observation that EPO was stimulated with various artificial agents such as sonication, SDS, TX-100, mellitin, and heat without the use of proteases. It was also suggested that the phenoloxidase could be activated through various pathways in the earthworm (Bahk *et al.*, 1995).

The EPO was a copper-containing enzyme which contained not only diphenolase but also monophenolase activity which recognized and converted tyrosine to L-dopa. In general, prophenoloxidase and its resulting phenoloxidase have been studied with L-dopa as substrate and its conversion to dopachrome which absorbs light at 475 nm after exogenous trypsin treatment. However, this can not prove a protein to be phenoloxidase since isolated copper ion itself can convert the L-dopa to dopachrome. In other words, the L-dopa conversion to dopachrome by PO could be a necessary condition for defining the enzyme, but it can not be a sufficient one because of copper's own ability to generate dopachrome from the substrate. Therefore, many studies dealing with prophenoloxidase and phenoloxidase must be substantiated by their tyrosine recognizing abilities to define them as the real enzymes. For example, extensive trypsin digestion of any copper-containing or any transition metal-containing proteins could show the diphenolase activities due to the ions free from proteins. The purified EPO converted tyrosine to dopachrome with a lag phase which might be a period to accumulate enough L-dopa to be sensed by the enzyme. Although the apparent K_m values for L-dopa at both pH 6.5 and 8.0 are high, this does not necessarily mean that the enzyme would not be effective to protect the host because real substrates for the enzyme *in vivo* might not be free amino acids. Any tyrosine residues in peptides and proteins could interact with the enzyme more effectively because of the additional amino acids around the tyrosine or L-dopa residues in the substrates.

The EPO exhibited its maximum activity at 35° C and pH 8.0. In arthropods, proPO was maximally stimulated at 58°C (Ashida and Söderhäll, 1984). Our previous report also showed that maximal PO activity was observed at 50°C from the coelomic fluid of *L. rubellus* (Bahk *et al.*, 1995). Taken together, the purified EPO did not contain any latent form of the enzyme. This was further supported by the fact that the enzyme was not stimulated by any of calcium, LPS, or β-1,3-glucan which had been shown to activate PO in the coelomic fluid (unpublished observation).

Generally, phenoloxidases have basic pH optima. The pH optimum studies were usually performed by measuring rates of enzyme catalyzed reactions in various pH values. However, this approach does not seem to be appropriate for the reactions using substrates which are unstable at particular pH like L-dopa at basic conditions because the pH optimum is influenced by both enzyme and substrate. In order to understand the pH effect on the enzyme itself, k_{cat}/K_m must be considered. When V_{max} and k_{cat}/K_m of EPO were compared at pH 6.5 and 8.0, it was clear that the pH optimum we obtained as 8.0 was not solely due to the enzyme itself. The pH definitely affected either substrate or enzyme-substrate complex. However, since the chemical conversion of substrate at each pH was considered and substracted from the activity in the presence of the enzyme, the pH optimum could be due to the enzyme-substrate complex. As one of possible reasons, the substrate, L-dopa, could be activated

by the pH to form a different kind of enzyme-substrate complex.

In conclusion, we have isolated an oligomeric endogenous phenoloxidase from *L. rubellus* in an active form which did not require proteolysis for its activation. It is pertinent to consider that the EPO can exist, at least, in part as a latent form *in vivo* even though a possibility of proPO activating system present in the earthworm can not be totally excluded. Therefore, it is predicted that there must be certain regulatory molecules or phenomena in *L. rubellus* which make the PO in a latent form in vivo before the foreign invasions.

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붉은 지렁이(*Lumbricus rubellus*) 체내로부터 정제한 Phenoloxidase

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붉은지렁이(*Lumbricus rubellus*)로부터 체내에 존재하는 phenoloxidase(EPO)를 ammonium sulfate, Blue-2, Phenyl-, Q-sepharose chromatography등을 이용하여 정제하였다. 이 효소는 SDS-PAGE상에서 59 kDa의 분자량을 갖는 단일 단백질로 나타났으며 nondenaturing-PAGE를 이용하여 DL-dopa를 기질로 *in situ* 염색 결과, 210 kDa 보다 다소 큰 단일 band가 dopachrome 침착에의해 형성되었다. 이는 곧 이 효소가 자연상태에서 복합체의 형태로 존재하고 있음을 의미한다. 또한 이 효소는 monophenolase 활성도, 즉 tyrosine을 dopa로 전환시키는 활성도도 갖고 있음을 470 nm에서 dopachrome의 축적을 관찰함으로써 확인할 수 있었다. Phenylthiourea (PTU), 1,10-phenanthroline, EDTA, EGTA등을 사용한 효소억제실험 결과, PTU 만이 65 μM의 $IC_{0.5}$로 효소 활성도를 효과적으로 억제시켰다. 이는 EPO의 촉매기작에서 구리가 매우 중요한 역할을 하고 있음을 의미한다. 이 효소는 L-dopa를 기질로 사용하였을 때 35°C와 pH 8.0에서 최적의 활성도를 나타내었다. EPO의 L-dopa에 대한 K_m은 pH 6.5와 8.0에서 각각 1.86 mM과 13.8 mM로 나타났다. 또한, pH 8.0에서 V_{max}는 pH 6.5에서 보다 약 6.6배 높은 반면, 각 조건에서의 촉매효율성은 거의 차이가 없음 $[(k_{cat}/K_m)_{pH8.0}/(k_{cat}/K_m)_{pH6.5} = 0.92]$을 알 수 있었다. 따라서, 이 사실은 EPO 촉매기작에 미치는 pH의 효과가 효소 자체에보다는 기질 또는 효소-기질 복합체 형성과정에 영향을 줌을 의미한다. 이와같은 사실을 종합해 보면, *L. rubellus*에 존재하는 phenol-oxidase는 oligomeric form을 가지며 활성화되기 위한 제한적 단백질 가수분해를 필요로 하지 않는다. 따라서, prophenoloxidase activating system의 존재가능성을 완전히 배재할 수는 없으나 지렁이 체내의 PO는 최소한 부분적으로나마 latent form으로 존재함을 확인할 수 있었다. 이는 외부 침입시 host를 보호하기 위한 방법으로 EPO를 latent form으로 유지 시킬 수 있는 또는 활성화 시킬 수 있는 조절기작의 존재를 예측하게 한다.