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IDENTIFICATION AND ISOLATION OF ENDOGENOUS INSECT PHENOLOXIDASE INHIBITORS

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SUMMARY Inhibitors of phenoloxidase were identified in pupae of the housefly, Musca domestica L. The phenoloxidase inhibitors were purified from final instar pupae of the housefly by a combination of ammonium sulfate fractionation, ion-exchange chromatography, gel filtration and reverse-phase high performance liquid chromatography. The potent phenoloxidase inhibitors were heat-stable low molecular weight peptides with an inhibition constant of nM range. To the best of our knowledge, this is the first time that endogenous phenoloxidase inhibitors have been identified among the insects, and probably also among the invertebrates. It is likely that the inhibitors play a central role in regulating the action of active phenoloxidases and will also serve as important tools for understanding the structures and functions of phenoloxidases, as well as their role in insect metamorphosis.

Phenoloxidase (monophenol, dihydroxyphenylalanine:oxidoreductase, EC 1.14.18.1 in part), also called tyrosinase, is known to catalyze two successive reactions: cresolase (or monophenol monooxygenase) hydroxylation of monophenols to o-diphenol, and catecholase (or o-diphenoloxidase) oxidation of o-diphenol to o-quinone (1,2).

Insect phenoloxidase is a widely distributed enzyme which is considered to play a key role in cuticular tanning and sclerotization, as well as in wound healing and defense against foreign pathogens (3-6). Many of the biochemical studies recently carried out on this enzyme

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have been focussed on the characterization and activation mechanisms of the proenzymes, prophenoloxidases (7-9) in insects. However, there are very few reports on the regulation of the active form of insect phenoloxidase. Here we report the identification, purification and some biochemical characterizations of phenoloxidase inhibitors which were isolated from pupae of the housefly, Musca domestica L.

MATERIALS AND METHODS

Materials: Larvae of the housefly, Musca domestica L., were reared on a moist mixture of wheat bran and yeast powder. The temperature and humidity of the insect room were kept at 25°C and 50-60%, respectively. Six to seven days after hatching, the larvae metamorphosed to white pupae (pupariation), and adults emerged five to six days after the day of pupariation.

3,4-Dihydroxyphenylalanine (DOPA) was obtained from Sigma Chemical, St. Louis. High performance liquid chromatography (HPLC) columns of YMC C_8 and Wakopak WS-PTC were obtained from JASCO, Toyko, and Wako Pure Chemical, Osaka, respectively. SP-Toyopearl 550 C and Sephadex G-25 were obtained from TOSOH, Tokyo, and Pharmacia, Uppsala, respectively. Other chemicals were of the highest grade commercially available.

Preparation of crude extract: Houseflies were homogenized at 0-4°C with a precooled mortar and pestle in 4 fold (w/v) 0.1 M acetate buffer, pH 4.0. Whole homogenates were filtered through gauze and the filtrate was heated at 70°C for 30 min, afterwhich all the proteins coagulated by the heat were removed by centrifugation. The clear supernatant, which contained no phenoloxidase activity, was collected as crude extract.

Preparation of crude phenoloxidase inhibitor: 1.5 kg of final instar pupae were subjected to the extraction and heat treatment procedures described above. The obtained clear supernatant (crude extract) was brought to 60% saturation with solid ammonium sulfate. The resulting precipitate was collected by centrifugation and the supernatant was further brought to 80% saturation with solid ammonium sulfate. The resulting precipitate was collected by centrifugation and then dialyzed against water. After dialysis, centrifugation was performed and the clear supernatant was obtained as the crude phenoloxidase inhibitor solution.

Phenoloxidase and phenoloxidase inhibitor assays: Phenoloxidase activity was determined essentially according to the method of Horowitz and Shen (10). A typical incubation medium (3.0 ml) contained 50 mM phosphate buffer (pH 6.0), purified phenoloxidase (0.014 U/ml) and 2 mM DOPA. The activity was measured at 25°C by monitoring the changes in absorbance at 470 nm using a molar extinction of 3715 for dopachrome (1). Phenoloxidase inhibitor activity was assayed under the above assay conditions by measuring the ability of inhibitor to suppress the phenoloxidase activity. One unit (U) of phenoloxidase inhibitor activity was defined as the amount required to inhibit one unit of phenoloxidase; one unit of phenoloxidase was taken as the amount of enzyme that produced 1 μmol of dopachrome per minute. The phenoloxidase used in this study was purified from either final instar larvae or aged pupae, three days after pupariation, as an electrophoretically homogeneous protein using a previously described method (11).

Protein chemical analyses: Amino acid compositions of phenoloxidase inhibitors were determined by a PTC-prelabelling method (12) using a Wakopak WS-PTC column, after hydrolysis with constant-boiling 6N HCL containing 0.05% 2-mercaptoethanol in an evacuated sealed glass tube

at 110°C for 24 h, 48 h and 72 h. Cysteine was determined as cysteic acid after performic acid oxidation (13).

N-terminal amino acid sequences of phenoloxidase inhibitors were determined by the 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate/phenylisothiocyanate (DABITC/PITC) double coupling method (14).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli (15) using a 12.5% acrylamide running gel. The proteins in the gels were visualized by silver staining (16). Amounts of protein were determined by the method of Lowry (17), except the protein content of the purified phenoloxidase inhibitors, which was estimated by amino acid analysis.

RESULTS AND DISCUSSION

The phenoloxidase inhibitor activity increased throughout pupation, was most prominent in aged pupae, especially final instar pupae, and disappeared in newly emerged adults (Fig. 1). To better define the inhibitor activities present in aged pupae of the housefly, a crude soluble extract of whole aged pupae just prior to adult emergence was prepared and subjected to heat-treatment and subsequently to ammonium sulfate fractionation, as described in Materials and Methods. The obtained crude phenoloxidase inhibitor solution, having a specific activity of 0.5 U/mg, was purified by a combination (Fig. 2) of

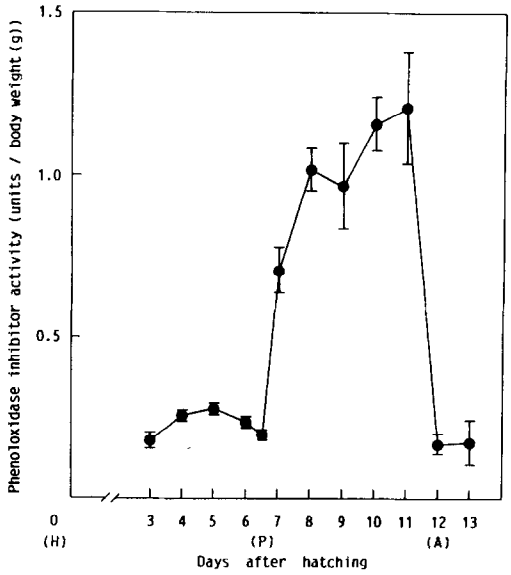

Fig. 1. Fluctuation of phenoloxidase inhibitor activity during the process of metamorphosis of the housefly. Crude extract prepared from 10 g of houseflies at each stage of metamorphosis was assayed for phenoloxidase inhibitor. H, P and A indicate hatching, pupariation and adult emergence, respectively. Values are the means ± SE of replicate determinations from six experiments.

ion-exchange chromatography, gel filtration, and repeated reverse-phase HPLC. It was then resolved into three fractions having molecular weights of 2,850 (housefly phenoloxidase inhibitor A, POI A), 3,100 (POI B) and 3,350 (POI C), by SDS-PAGE, as shown in Fig. 2c. The purified inhibitors had specific activities of 195 U/mg (POI A), 1767 U/mg (POI B) and 399 U/mg (POI C), and overall yields of 0.24% (POI A), 0.67% (POI B) and 0.29% (POI C). We estimate that 5 to 10 μg of each purified inhibitor could be obtained from 86,000 housefly pupae

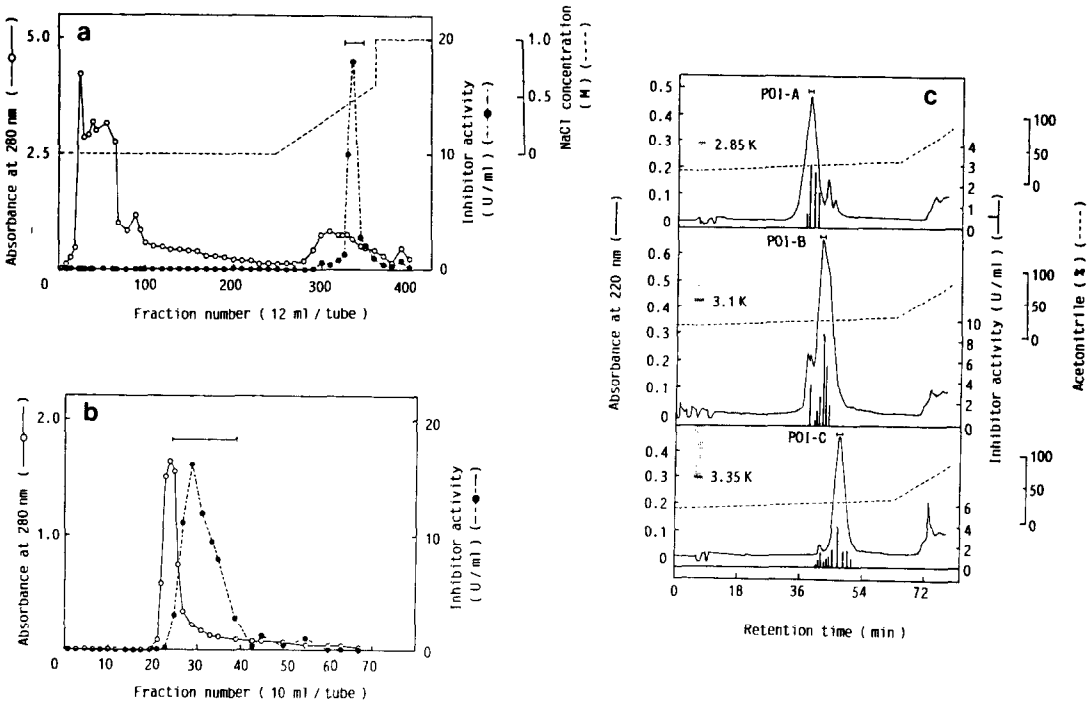

Fig. 2. Purification of housefly phenoloxidase inhibitors. Crude phenoloxidase inhibitor solution (2.56 g of protein) prepared from 1.5 kg of final instar pupae of the housefly was applied to a 440 ml column of SP-Toyopearl 550 C equilibrated with 50 mM phosphate buffer (pH 6.0). Bound proteins were eluted with a gradient (0 to 0.6 M) of NaCl (a). The eluate containing phenoloxidase inhibitor activity (bracketed) was lyophilized and dissolved in water and applied to a column (2.8 x 110 cm) of Sephadex G-25 equilibrated in 50 mM phosphate buffer, pH 6.0 (b). The collected fractions containing phenoloxidase inhibitor activity (bracketed) were applied to a 70 ml column of SP-Toyopearl 550 C and eluted, in the same manner as described above (data not shown). The material that inhibited phenoloxidase was dialyzed, lyophilized, and dissolved in water and applied to an HPLC column of YMC C_8 (4.6 x 250 mm). A preliminary run on the HPLC with a gradient of acetonitrile (0 to 50%) in 0.1% trifluoroacetic acid (TFA) implied the existence of several phenoloxidase inhibitors which were closely eluted. Therefore, a very mild gradient (12 to 24%) of acetonitrile in 0.1% TFA with a flow rate of 1 ml/min in the above HPLC was employed as the elution conditions. The first HPLC run dissolved the applied materials into three phenoloxidase inhibitors (not shown). The three active fractions were rechromatographed under the same conditions (c) and the active regions (bracketed) were subjected to SDS-PAGE (c).

(1.5 Kg). Each of the inhibitors apparently exists as a monomer, since they were all eluted through a Sephadex G-25 gel at a molecular weight range of approximately 3,000 to 3,500. The purified inhibitors were fairly heat-stable. They retained greater than 60% of their activity after 1 h heating at 80°C (not shown). The inhibitors were stable over a wide range (4 to 10) of pH (not shown). The amino acid compositions and partial N-terminal amino acid sequence analyses revealed that the three phenoloxidase inhibitors are structurally similar peptides which are rich in cysteine and glycine and also possess the same N-terminus, Glu (Table 1). The kinetic studies revealed that all three inhibitors were potent and reversible inhibitors of phenoloxidase purified from housefly larvae. The inhibitory properties of the three inhibitors, when characterized by both Lineweaver-Burk and Dixon plots (18), appeared to occur in a competitive manner (Table 2). Although the affinity (Km = 3.93 mM) of housefly larval phenoloxidase for DOPA appeared to be fairly similar to that of pupal phenoloxidase (Km=4.3 mM), phenoloxidase inhibitor (POI C) indicated a rather high affinity towards larval phenoloxidase (Table 2). Similar to other insect

TABLE 1. Amino acid compositions and partial N-terminal amino acid sequences of phenoloxidase inhibitors

Amino acid compositions	POI A (%)	POI B (%)	POI C (%)
Asp/Asn	4.1	3.0	4.2
Glu/Gln	3.9	3.5	4.5
Ser	3.0	7.1	3.1
Gly	22.3	17.9	19.4
His	3.0	4.1	1.2
Arg	6.2	5.0	3.8
Thr	4.9	5.7	4.1
Ala	8.1	7.6	6.5
Pro	2.3	1.6	3.7
Tyr	3.0	3.3	3.4
Val	6.8	9.2	8.8
Met	2.3	---	---
Cys*	11.9	15.3	16.1
Ile	3.3	3.9	3.4
Leu	6.3	6.8	7.3
Phe	1.9	---	1.4
Lys	6.1	5.9	7.6
Trp	ND	ND	ND
N-terminal sequences	E-I/L-V-P-X	E-I/L-V-P-X	E-I/L-X

*, determined as cysteic acid after performic acid oxidation. ND, not determined. I/L indicates isoleucine or leucine and "X" represents undetermined sequences.

TABLE 2. Kinetic analyses of housefly phenoloxidase inhibitors

	Type of inhibition	K_i^* (nM) (a)	(b)
POI A	Competitive	6.9	5.3
POI B	Competitive	88.9	66.3
POI C	Competitive	15.4	18.0
POI C	(Competitive)	(60.0)	(54.5)

*Inhibition constants obtained through Lineweaver-Burk (a) and Dixon (b) plot analyses under the initial steady-state velocity at 25°C. Data in parentheses indicate the mode and K_i for the interaction between POI C and phenoloxidase isolated from aged housefly pupae, three days after pupariation. All other experiments were performed using phenoloxidase isolated from final instar larvae of the housefly. The concentration range of substrate (DOPA) used was 1.25 to 5.0 mM. The concentration ranges of phenoloxidase inhibitors used were 1.5 to 6.0 nM for POI A, 20.0 to 80.0 nM for POI B, and 6.0 to 24.0 nM for POI C. The reactions were initiated by adding enzyme to the reaction media containing substrate and inhibitor.

phenoloxidases (3), housefly phenoloxidases have two catalytic activities: monophenol monooxygenase and o-diphenoloxidase. Apparently the three phenoloxidase inhibitors isolated from the housefly could inhibit only the o-diphenoloxidase activity of the enzymes (not shown). Studies to clarify whether all three phenoloxidase inhibitors are genetically coded or derived from one inhibitor as a result of post-translational modification or degradation due to protease attack, are currently underway in our laboratory. The role of phenoloxidase in the normal development of housefly pupae after pupariation is clearly a significant one. However, as excessive melanization and sclerotization of cuticular proteins under the epicuticle would most likely hinder rupturing of the cuticle during adult emergence, an effective control mechanism for phenoloxidase activity is essential. Until the present, it has been assumed that the level of phenoloxidase activity was controlled exclusively by a specific activator system (7-9). The identification of these endogenous phenoloxidase inhibitors, however, suggests that endogenous phenoloxidase inhibitors also play a critical role in the regulation of phenoloxidase activity, particularly at the time of adult emergence. Further, they should prove to be powerful tools in understanding the structures and functions of insect phenoloxidases.

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