Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2964–2968, March 1995
Biochemistry

Primary structure of a potent endogenous dopa-containing inhibitor of phenol oxidase from *Musca domestica*

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Communicated by Klaus Biemann, Massachusetts Institute of Technology, Cambridge, MA, December 28, 1994 (received for review September 26, 1994)

ABSTRACT **The complete amino acid sequence of a low molecular weight peptide from the hemolymph of the housefly *Musca domestica* L., which had been determined to competitively inhibit phenol oxidase (PO; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) in the nM range, was unambiguously established by employing both automatic Edman degradation and mass spectrometry. The physiologically active peptide, which was designated phenol oxidase inhibitor (POI), has an observed molecular weight of 4213.1 ± 0.2 by electrospray ionization mass spectrometry. The relatively short and structurally dense peptide contained 38 amino acid residues rich in cysteine and lysine. Comparison of the observed and calculated molecular mass indicates that apparently all six cysteine residues form disulfide bridges. Interestingly, sequence analyses of both the intact and protease-digested *S*-pyridylethylated POI showed that one of the two tyrosine residues (Tyr-32) is hydroxylated to a 3,4-dihydroxyphenylalanine (dopa) residue. This agreed with the increase of 16 mass units observed in mass spectrometric measurements. This was further verified by submission of free L-dopa to the sequencer, which gave a retention time consistent with the atypical peak observed at the Edman cycle of the peptide containing dopa. This study demonstrates the existence of a biologically active, dopa-containing peptide among the insects. Since the POI activity was most prominent in aged pupae, especially pharate adults, the POI may play an important role in smoothing the way of adult emergence through hindering excessive melanization, as well as hardening, of cuticular proteins under the epicuticle.**

Phenol oxidase (PO; monophenol, dihydroxyphenylalanine: oxygen oxidoreductase; EC 1.14.18.1) , a widely distributed copper-containing enzyme in nature, which is also known as tyrosinase, catalyzes two successive reactions: hydroxylation of monophenol to *o*-diphenol and the corresponding oxidation of *o*-diphenol to *o*-quinone (1, 2). This enzyme is responsible for a cascade of reactions in which the end product of the spontaneous nonenzymatic reactions that follow is melanin, a brownish or yellowish pigment.

Various studies suggest that PO-mediated reactions play important physiological roles during insect development, such as in cuticular tanning and sclerotization, as well as in wound healing and defense against foreign pathogens (3–6). PO is found in the hemolymph of most insects as an inactive precursor called pro-PO or latent PO. Thus, most extensive studies have been carried out on the assumption that the level of PO activity is controlled by pro-PO activation systems (7–9).

Although PO in insects has been well monitored, no detailed biochemical study on the regulation of the fate of the active form of this enzyme has ever been reported. Recently, we reported the presence of an endogenous PO inhibitor (POI) in the housefly *Musca domestica* (10). The activity of POI increased throughout pupariation, attaining its highest level during the pharate adult stage, but disappeared in the newly emerged adults. In this study, we present the complete structure of POI and the identification of a modified tyrosine residue (dopa) by using both Edman and mass spectrometric analyses.

MATERIALS AND METHODS

Insects. Pupae of the housefly *Musca domestica* L. were raised and collected according to our previous report (10).

Materials. L-3,4-Dihydroxyphenylalanine (L-dopa) was obtained from Sigma. Reversed-phase HPLC (RP-HPLC) columns (YMC-Pack Protein-RP and Cosmosil $5C_{18}$-AR-300) were from YMC (Kyoto) and Nacalai Tesque (Kyoto), respectively. The Wakosil $5C_4$-200 column, *Achromobacter lyticus* I protease (lysyl endopeptidase; LEP) and 4-vinylpyridine were purchased from Wako Pure Chemical (Osaka). Endoproteinase Asp-N (EAN) was from Boehringer Mannheim and $H_2{}^{18}O$ was a product of Commissariat a L'Energie Atomique (France). All other chemicals used were of analytical grade or of the highest quality commercially available.

PO and POI Assay. PO activity was assayed according to the method of Horowitz and Shen (11) but modified such that an appropriate amount of POI was added to the reaction medium. A typical reaction mixture (3.0 ml) containing 0.3 ml each of PO and POI solution in 50 mM phosphate buffer, pH 6.0, was incubated at 25°C for 10 min, after which dopa was added to a final concentration of 2 mM. The changes in absorbance were monitored at 470 nm, and the residual PO activity was measured by using a molar extinction coefficient of 3715 for dopachrome (1). One unit of POI activity was defined as the amount required to inhibit one unit of PO. One unit of PO was taken as the amount of enzyme that produced 1 μmol of dopachrome per min. The PO used in the assay was purified from housefly final instar larvae as an electrophoretically homogeneous protein by using a previously described method (12).

Purification of POI. Crude POI solution was prepared and purified from housefly pupae shortly before adult emergence (pharate adult) by a combination of ion-exchange chromatography and gel filtration as described (10). However, the succeeding RP-HPLC purification steps were performed as described in this report. Fractions exhibiting POI activity from the gel filtration step were pooled and further purified through a series of RP-HPLC separations using a Tosoh (Tokyo) HPLC

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Abbreviations: PO, phenol oxidase; POI, phenol oxidase inhibitor; PE, *S*-pyridylethyl; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase HPLC; ESI, electrospray ionization; FAB, fast atom bombardment; LEP, lysyl endopeptidase; EAN, endoproteinase Asp-N; PTH, phenylthiohydantoin.

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system equipped with two UV detectors and an SC-8020 HPLC system controller. Initially, the partially purified POI was loaded onto a YMC-Pack Protein-RP column (150 × 4.6 mm i.d.). After washing the column with water containing 0.1% trifluoroacetic acid (TFA), elution was performed by employing a linear gradient of 5–55% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min over 45 min. Absorbances of the eluate were monitored at 280 and 214 nm. Fractions were lyophilized and redissolved into 100 μl of Milli-Q (Millipore) water, and an aliquot of each fraction was taken for the assay. Active fractions were rechromatographed twice on a Wakosil $5C_4$-200 column (250 × 4.6 mm i.d.) under the same elution conditions as above until POI was isolated to apparent homogeneity.

Reduction and *S*-Pyridylethylation of POI. Purified POI (≈5–10 μg) in 50 μl of 6 M guanidine·HCl containing 1.0 mM EDTA buffered with 0.25 M Tris·HCl, pH 8.0, was mixed with 2.5 μl of 10% (vol/vol) 2-mercaptoethanol and overlaid with N_2 gas. The mixture was incubated at room temperature in the dark for 2 h. Alkylation of the reduced POI was achieved by adding 2 μl of 4-vinylpyridine to the mixture (13). Incubation was continued as above for 2 h under N_2 gas, after which the mixture was immediately desalted through a Wakosil $5C_4$-200 column. The *S*-pyridylethyl POI (PE-POI) fraction was collected and rechromatographed on a Cosmosil $5C_{18}$-AR-300 column to ensure that the modified inhibitor was free from contamination by reagents or other modified products.

Amino Acid Analysis. Samples were hydrolyzed in 6.0 N HCl containing 1% phenol at 110°C for 21 h by using a Waters Pico·Tag work station. The hydrolysates were concentrated under reduced pressure, derivatized with phenylisothiocyanate, and analyzed in a Waters amino acid analyzer equipped with a Pico·Tag column for phenylthiocarbamoyl-amino acid analysis.

Amino Acid Sequence Determination. Automatic Edman degradation analysis was carried out by using either an Applied Biosystems 477A with an online 120A phenylthiohydantoin (PTH)-amino acid analyzer or a Shimadzu PPQS-10 coupled to an LC-AS10 equipped with a WS-PTH column for PTH-amino acid analysis.

Enzymatic Digestion of POI. Reduced and PE-POI was incubated with either LEP or EAN (10:1; wt/wt; substrate to enzyme) in 50 μl of the appropriate buffer containing 40 atom % $H_2{}^{18}O$ at 35°C for 2 h. The buffer was either 100 mM ammonium carbonate, pH 7.8, for LEP digestion or 100 mM sodium phosphate, pH 7.85, for EAN digestion. Products of enzymatic cleavage were separated by RP-HPLC by using a Cosmosil $5C_{18}$-AR-300 column (150 × 4.6 mm i.d.). Fragments were eluted with a 5–55% linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min over 45 min. The absorbances of eluates were monitored simultaneously at 254 and 214 nm. Peptides were lyophilized and subjected to either automatic Edman or fast atom bombardment (FAB) mass spectrometry analysis.

Proteolytic digestion in the presence of $H_2{}^{18}O$ allows partial incorporation of ^{18}O during hydrolysis of the peptide bond into the carbonyl group of constituent fragments originating from the N terminus of peptides or proteins, while leaving the original C-terminal fragment unlabeled (14). On FAB mass spectrometry analysis, the masses of N-terminal fragments were shifted to 2 units over that of the C-terminal fragment. Thus, unambiguous assignment of the C-terminal fragment is achieved.

Mass Spectrometry. A JMS-HX/HX110A double-focusing mass spectrometer (JEOL) equipped with an electrospray ionization (ESI) ion source (Analytica, Branford, CT) and an FAB ion source was operated at an accelerating voltage of 7 and 10 kV, respectively. The mass spectra were acquired with a mass resolution of 1000 for ESI and 3000 for FAB. The mass calibration was performed by using a mixture of KI and CsI (1:2; wt/wt). About 200 pmol of purified POI and its pyridylethylated derivative was dissolved into 20 μl of acetonitrile/water (1:1, vol/vol) containing 2% acetic acid, and the solution was infused into the ESI ion source at a flow rate of 1 μl/min by a Harvard syringe pump (Harvard Apparatus). Heated N_2 (150°C) was used as a drying gas for ESI and introduced into the capillary region at a flow rate of 25 liters/min. The proteolytic peptides were dissolved in 50% acetonitrile solution and mixed with the matrix, dithiothreitol/dithioerythritol (5:1; wt/wt) on the tip of the stainless target for FAB mass measurements. All mass spectra were processed in a complement data acquisition system (JEOL).

RESULTS

Purified POI was obtained from housefly pupae crude extract after a combination of ammonium sulfate fractionations, ion-exchange chromatography, and gel filtration, as described earlier (10), and by a series of RP-HPLC steps, as employed in this study (Fig. 1). The Lineweaver–Burk plot of dopa oxidation by larval PO in the presence of POI (Fig. 2) indicated that POI competitively inhibited PO-catalyzed oxidation of dopa with a K_i of 40 nM, a value comparable to our previous report (10).

Amino acid analysis showed that POI is rich in cysteine and basic amino acid residues but does not contain phenylalanine, methionine, or tryptophan (Table 1). The observed molecular mass of intact, native POI on an ESI mass spectrometer was

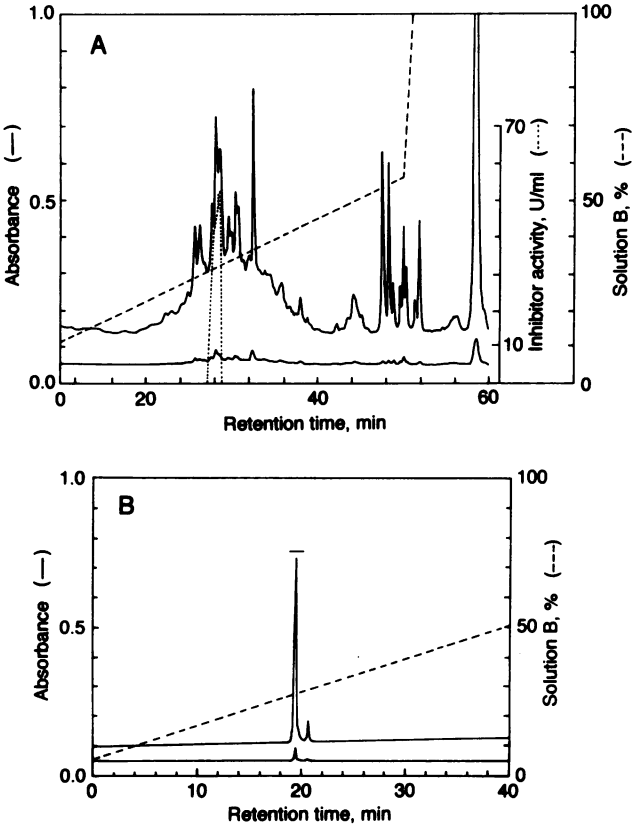

FIG. 1. RP-HPLC elution patterns of POI. (*A*) Purification of POI on a YMC-Pack Protein-RP column. Fractions exhibiting PO inhibition were collected, concentrated, and further separated through a series of RP-HPLC steps. (*B*) Typical elution pattern of purified POI (bar) that had been subjected twice to chromatography on a Wakosil $5C_4$-200 column. All elution procedures were performed as described in *Materials and Methods*. Absorbances were simultaneously monitored at 214 nm (upper tracing) and 280 nm (lower tracing). Solution A, 0.1% TFA in Milli-Q water; solution B, 90% acetonitrile in Milli-Q water containing 0.1% TFA.

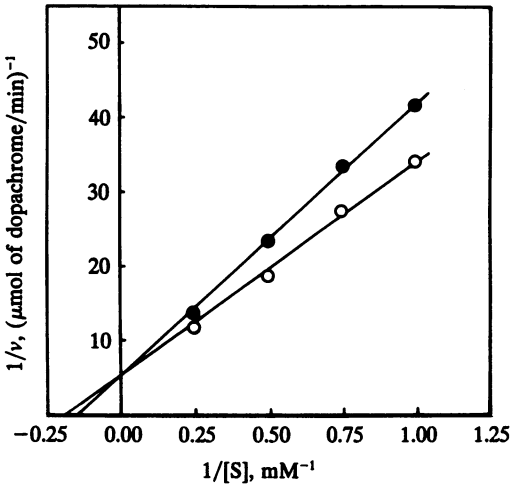

FIG. 2. Lineweaver–Burk plot of dopa oxidation by larval PO in the presence of POI under the initial steady-state velocity at 25°C. Reaction conditions: concentration range of substrate (dopa), 0–4.0 mM in 50 mM phosphate buffer, pH 6.0; POI concentration as determined by amino acid analysis, 10 nM. In this particular assay, the reaction was initiated by addition of enzyme to a mixture of substrate and inhibitor. ○, Control, no POI; ●, with POI.

4213.1 ± 0.2 (Fig. 3), whereas the pyridylethylated derivative gave a molecular weight of 4850.1 (data not shown). The increase in mass by 637.0 units implies the presence of six cysteine residues in the molecule. Direct amino acid sequencing of intact, native POI by automated Edman degradation provided a sequence up to the C-terminal amino acid that complements the amino acid composition except for the six undetectable amino acid residues at positions 11, 18, 24, 25, 29, and 36. These were later identified as cysteine residues after subjecting reduced and PE-POI to the sequencer.

To further establish the amino acid sequence, confirmation of the C terminus of POI was performed by digesting PE-POI with LEP in a buffer prepared with 40 atom % ^{18}O-substituted water and then analyzing the FAB mass spectral pattern of the resulting proteolytic fragment. By this treatment, ^{18}O should be incorporated into the newly generated carboxyl groups but

Table 1. Amino acid composition of POI

Amino acid	Method of analysis	
	Acid hydrolysis*	Edman degradation
Asp	5.47	2
Asn		3
Glu	2.17	1
Gln		1
Ser	1.91	2
Gly	1.14	1
His	2.10	2
Arg	1.20	1
Thr	3.12	3
Ala	3.00	3
Pro	1.61	1
Dopa	—†	1
Tyr	1.13	1
Val	3.33	4
1/2Cys	—†	6
Ile	0.73	1
Leu	1.13	1
Lys	3.16	4
Total		38

*Values are shown as mol of the indicated amino acid per mol of Ala, which was calculated to be 303 pmol in the 21-h hydrolysate.
†Not determined.

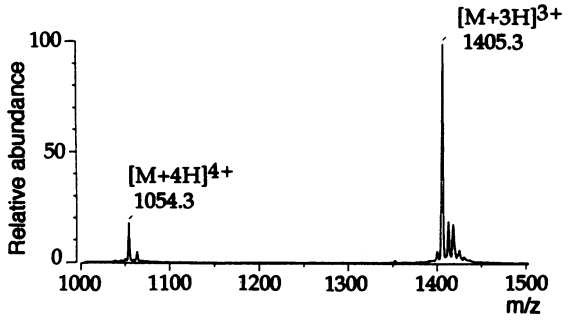

FIG. 3. ESI mass spectrum of intact native POI (15). From each m/z value of the multiply charged ion signals ($[M+nH]^{n+}$), the molecular mass of POI was calculated to be 4212.9 (n = 3) or 4213.2 (n = 4). The average molecular mass obtained was 4213.1 ± 0.2.

not into the original C-terminal carboxyl group of PE-POI. The FAB mass spectrum of the C-terminal sequence PE-Cys-Val-Thr showed a distinct molecular ion signal at m/z = 427.2 (Fig. 4), with the natural isotopic distributions clearly indicating the fragment to be from the C terminus of POI.

The EAN digestion of PE-POI gave two main fragments, with molecular ion signals at m/z = 2460.2 and m/z = 2409.8 (data not shown). Sequence analyses and FAB mass isotopic distribution patterns showed that the fragments coincided with the N terminus (position 1–21) and C terminus (position 22–38) of POI, respectively. Furthermore, the sequence elution pattern at cycle 11 (position 32) of the C-terminal EAN fragment yielded an atypical PTH-amino acid peak that eluted close to PTH-Gly, at a retention time of 5.23 min (Fig. 5*A*). The same peak was also detected at cycle 32 during the Edman degradation of intact POI (data not shown). The molecular mass of POI calculated from the amino acid sequence results, excluding an unidentified amino acid at position 32, was 4033.9. The difference in the molecular mass (179.2) from the observed mass (ESI mass spectrometer) should account for the mass of the amino acid residue at position 32. The amino acid residues having the closest mass were tyrosine and tryptophan, but tryptophan was excluded since POI had no atypical absorption maximum at around 280 nm. Although there is the possibility of other amino acids which were modified at position 32, the possible modification of tyrosine was considered first because the oxidation of tyrosine to dopa at position 32 was a high probability due to the action of PO. In addition, the mass of a dopa residue is exactly 179.2. We then identified the product at cycle 11 on Edman degradation

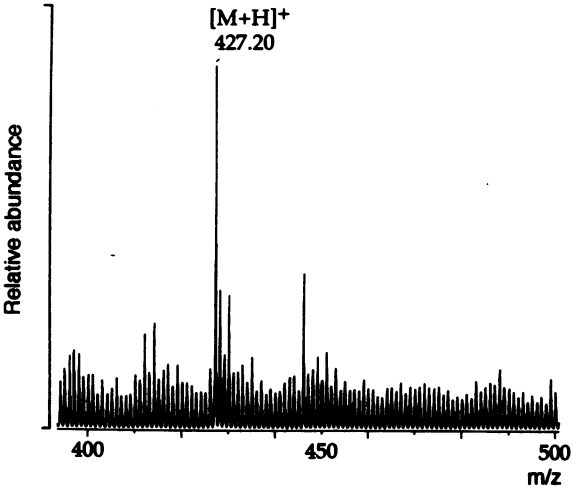

FIG. 4. FAB mass spectrum of the C-terminal fragment of POI derived from LEP digestion of PE-POI in $H_2{}^{18}O$.

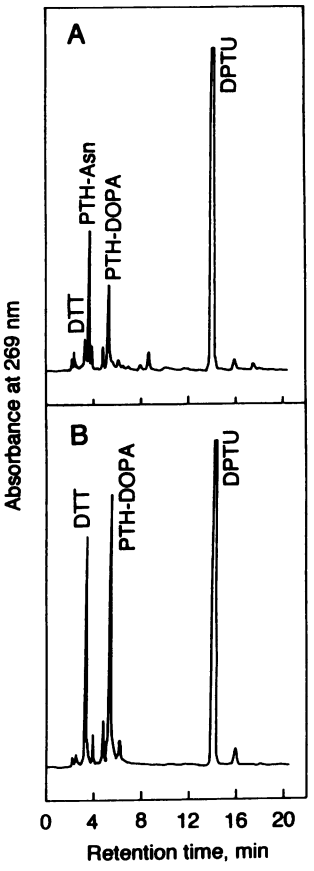

FIG. 5. Identification of dopa residue by Edman analysis. (*A*) The 11th cycle of dopa-containing EAN fragment showing an atypical PTH-amino acid peak. The PTH-Asn peak was a carryover from the previous cycle. (*B*) Standard L-dopa after submission to the sequencer (Shimadzu PPQS-10).

of the C-terminal EAN fragment as that obtained for L-dopa (Fig. 5*B*). The atypical peak at 5.23 min in the elution profile was completely identical to PTH-L-dopa. As a result, POI consisted of 38 amino acid residues with six half cystines in disulfide linkages and one tyrosine residue (C-terminal Tyr at position 32) modified to a dopa residue (Fig. 6), the theoretical mass (4213.8) of which agreed well with the observed value (4213.1) for intact, native POI (Fig. 3).

DISCUSSION

The complete amino acid sequence of a low molecular weight peptide that is a potent reversible endogenous inhibitor of PO

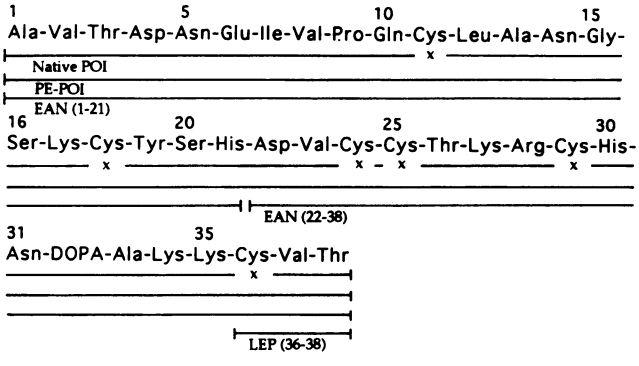

FIG. 6. The primary structure of housefly POI. The complete amino acid sequence was deduced from Edman and mass spectrometric analyses of native POI, intact PE-POI, and proteolytic fragments of PE-POI. x, Unidentified amino acid residue.

found in pupae of the housefly *Musca domestica* (10) was unambiguously established by employing a combination of Edman degradation and mass spectrometric analyses (Fig. 6). A homology search (IDEAS; Kyoto University) revealed no sequence similarity to other known peptides. The POI is apparently characterized by a highly rigid structure containing relatively large amounts of lysine and cysteine residues that had been deduced by ESI mass analysis to form three disulfide bonds. Besides the POI isolated and characterized in this report, a variant peptide with a truncated N terminus was also obtained in minute quantities during purification (data not shown).

In most POI purification procedures, a POI whose Tyr-32 was modified to dopa was isolated. However, it should be noted that in some preparations unoxidized POI was present as a minor product. It is likely that the mature POI is folded into a rigid structure which renders one particular tyrosine residue susceptible to oxidation. Thus, when POI is released into the hemolymph, the exposed intrachain tyrosine undergoes modification by hydroxylation of its phenolic ring during POI–PO complex formation. The enzymatic oxidation of tyrosine residues by tyrosinase in some peptides and proteins has been well described (16, 17). Similar to other insect POs, housefly PO has two catalytic activities: monophenol monooxygenase and *o*-diphenol oxidase. However, it should be clarified whether the peptidyltyrosine is converted to peptidyl-dopa (POI) by monophenol monooxygenase activity of housefly PO or other enzymes such as tyrosine hydroxylase or peroxidase, although the hydroxylation of peptidyltyrosine by tyrosine hydroxylase or peroxidase appeared not to have been demonstrated. The binding of substrates with this enzyme is believed to involve coordination to the binuclear copper center, the active site of catalysis (18). Since POI inhibits the enzyme in a competitive manner with respect to dopa, the position at or around the dopa-32 residue of POI may have an important role in inhibitor binding. The incubation of POI with PO for 10 min at 25°C did not form detectable oxidative products such as dopa-quinone-containing peptide as a result of RP-HPLC separation combined with Edman analysis of the reaction mixture (data not shown). However, it also seems possible that, through PO action, some of the dopa-32 residue of POI is converted to dopa-quinone, which rapidly binds to PO, because we have not done an extensive search for dopa-quinone containing peptide in PO after its incubation with POI.

The complex process associated with cuticular tanning and sclerotization in insects involves cross-linking of the chitin–protein matrix with catechols and quinones derived from the oxidation of tyrosine (3, 19). Although an important physiological process in the metamorphosis of insects, excessive melanization and hardening of cuticular proteins under the epicuticle would most likely hinder rupturing of the cuticle during adult emergence. Moreover, some stable quinones exhibit considerable resistance against reactions that eventually form melanin pigments. Thus, if not properly quenched, stable quinones could be toxic and damage the insect itself (20). Therefore, an effective control mechanism for the regulation of active PO is essential. Indeed, the high PO activity observed in the homogenates of aged pupae suddenly drops in the homogenates of the pharate adult of the housefly (unpublished data). However, by a Western blot detection method using polyclonal antibodies raised against housefly PO, no significant change in PO protein content was observed in the homogenates of the pharate adult compared with that of the homogenates of other stages of aged pupae (data not shown). As a matter of fact, large amounts of PO could be purified from the pharate adult of housefly according to the method described earlier (12), where most of the POI contained in the homogenates was removed (or dissociated) by 60% ammonium sulfate fractionation. Thus, we suggest that the POI functions as a regulator of the necessary levels of PO, espe-

cially during postactivation of pro-POs. This may introduce a new dimension in our understanding of the complex process of insect metamorphosis, particularly prior to adult emergence, when the physiologically active POI exhibits its highest activity (10). Housefly POI also inhibits PO from insects other than housefly (gadfly, *Ptecticus tenebrifer*) and a noninsect PO (mushroom tyrosinase), both with high affinity (data not shown). Thus, POI may prove to be a powerful tool in elucidating the structures and functions not only of insect POs but also of POs (or tyrosinases) from other sources.

Although dopa has been considered to be universally distributed as a free or peptide-bound amino acid in tissues and fluids of invertebrates (21), we have not found any reports concerning dopa-containing peptides isolated from insects. Therefore, it is reasonable to consider that housefly POI is the only dopa-containing peptide which has been demonstrated to possess physiological activity at least among the insects. High molecular weight proteins containing dopa as a covalent part of the polypeptide backbones were suggested as precursors of polymerized structures in some invertebrates (22, 23). Protein containing high amounts of dopa is thought to contribute to the byssal adhesion of the marine mussel, *Mytilus edulis* (22) and in eggshell formation by the liver fluke, *Fasciola hepatica* (23). A dopa-rich iron-binding glycoprotein called ferreascidin from the ascidian *Pyura stolonifera*, is the basis of the ascidian's test (24). Although not oligopeptides, dopa-containing tetrapeptide-like substances, halocyamines, from *Halocynthia roretzi*, show apparent antimicrobial activities with minimum inhibition concentrations at the μM level (25). However, the details of the antimicrobial activities have not as yet been well characterized. Our study supports the conclusion that POI has a single dopa-containing sequence motif that is different from the above proteins and tetrapeptides. It possesses potent inhibitory activity in the nM range. We propose that POI represents a class of biologically active dopa-containing peptides.

We are grateful to Drs. M. Funatsu (Kumamoto Institute of Technology), T. Nagahama (Kagoshima University), K. Watanabe (Saga University), and M. Nakamura (Osaka University) for many helpful discussions and Ms. L. F. Tsukamoto (Saga University) and Dr. M. B. Yatvin (Oregon Health Sciences University) for carefully reading the manuscript. We thank Dr. K. Yoshino (Osaka University) for his assistance in the mass spectrometric analyses and Mr. T. Kondo (Saga Medical School) and Drs. Y. Ito and T. Imoto (Kyushu University) for the use of the amino acid sequencer. We also thank Mr. I. Konoike and Mr. K. Kimura (Milbon Corporation) for their continued encouragement. This work was supported by grants from the Milbon Corporation of Japan.

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