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cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation

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ABSTRACT **Prophenoloxidase (proPO), an enzyme that is the terminal component of the so-called proPO activating system, a defense and recognition system in crustaceans and insects, has been purified and cloned from a crayfish blood cell cDNA library. The deduced amino acid sequence codes for a polypeptide with a mass of 80,732 Da, which is close to 76 kDa, the apparent mass of the purified enzyme. proPO contains two copper atoms, and two putative copper-binding sites were found in the deduced amino acid sequence. Sequence comparisons show that these putative copper-binding sites are similar to the corresponding sites in arthropod hemocyanins and also, although the sequence similarities are less extensive, similar to tyrosinases from vertebrates and microorganisms. The purified enzyme is a typical tyrosinase because it hydroxylates monophenols and oxidizes *o*-diphenols but does not oxidize *p*-diphenols. If a homogeneous preparation of crayfish proPO were incubated with a homogeneous sample of the proPO activating enzyme, a serine proteinase, the cleavage of proPO by this trypsin-like enzyme was found to occur between Arg-176 and Thr-177.**

Invertebrates lack antibodies, lymphocytes, or other features of the vertebrate adaptive immune system but have innate defense reactions (1). The invertebrates have an open circulatory system and therefore depend on immediate and constitutive mechanisms for recognition and immobilization of microorganisms and parasites for their survival. In addition, the invertebrates need a clotting system to prevent blood loss upon wounding. One candidate for an immediate noninducible system in invertebrates is the prophenoloxidase (proPO) activating system (proPO-system), and evidence is accumulating for a role of this system in recognition and defense (2–4). It has long been recognized that defense reactions in many invertebrates are often accompanied by melanization. The enzyme involved in melanin formation is phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1). PO activity has been detected in the hemolymph (blood) or coelom of many invertebrate groups, both protostomes and deuterostomes. This enzyme catalyzes the oxidation of phenols to quinones, which then polymerize nonenzymatically to melanin. The invertebrate enzyme has, wherever carefully studied, been found to exist in the blood in an inactive form, proPO, which is activated in a stepwise process involving serine proteinases activated by microbial cell wall constituents (5). This activation of the so-called proPO-system by certain polysaccharides—e.g., by 1,3-β-glucans and by bacterial polysaccharides (2–7)—shows that this system recognizes surface molecules from microorganisms and thus functions as a recognition system. The ability of a host to recognize certain conserved structures on microorganisms has been termed pattern recognition (8), so accordingly the proPO-system may be considered a pattern-recognition system.

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Several proteins that are components of, or associated with, the proPO-system have been shown to be involved in the cellular defense reactions in crustaceans (5)—for example, a 76-kDa factor (9) and a 1,3-β-glucan-binding protein (10–12). Consistent with the idea of the proPO-system as a defense system, there are also reports that certain successful parasites avoid eliciting the system or inhibit its activation or activities. Thus, some entomopathogenic fungi produce wall-less cells (protoplasts) without the 1,3-β-glucan elicitor when entering the host blood (2). The protoplasts are not encapsulated and do not degranulate host blood cells (13). Other fungi synthesize toxins that can inhibit 1,3-β-glucan-stimulated blood cell motility (14). Some parasitic wasps, at the time of oviposition, inject viruses into the host that inhibit the activation of proPO or suppress PO activity (15, 16). Of particular interest is perhaps the observation that in *Anopheles gambiae*, a vector for malaria, resistant strains of the insect can encapsulate the ookinetes of the malaria parasite with melanin. This melanization will halt infection in the mosquito larvae (17, 18). Thus, melanization and the proPO-system also appear to play an important role in defense in those insects that carry important pathogens to humans and other higher animals.

The proPO-system is activated in the presence of certain elicitors, such as the microbial polysaccharides. This system has been characterized in most detail in the freshwater crayfish, *Pacifastacus leniusculus* (5). proPO, a 76-kDa molecule, is turned into its active form, PO, through a limited proteolysis by a proPO activating enzyme (ppA), a serine proteinase (19). *In vivo* ppA gains its biological activity in the presence of microbial polysaccharides, such as lipopolysaccharides, peptidoglycans, and 1,3-β-glucans, which by binding specific pattern-recognition receptors (11, 20) will induce activation of the ppA and, as a consequence, active ppA will convert proPO to active PO. proPOs have been isolated and partially characterized from a number of arthropods, but it is only in crayfish that the endogenous activator ppA has been purified and shown to be able to convert proPO to active PO, a 62-kDa protein (19, 21). Thus, it appears as if PO is a key enzyme in the defense of arthropods against parasites, and here we report the cloning and the complete cDNA sequence of this protein‡ and the cleavage site on the zymogen proPO for the ppA.

MATERIALS AND METHODS

Protein Purification and Antibody Production. proPO and ppA were purified as described in refs. 19 and 21, respectively. The purified proPO was used to produce a rabbit antiserum that was rendered monospecific by adsorption onto proPO

Abbreviations: ppA, prophenoloxidase activating enzyme; proPO, prophenoloxidase; PO, phenoloxidase; proPO-system, proPO activating system; RACE, rapid analysis of cDNA end procedure.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X83494).

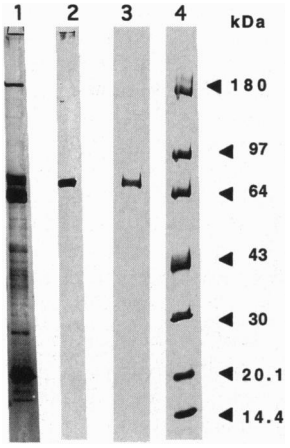

FIG. 1. Electrophoretic purity of the proPO preparation used and the specificity of the anti-proPO antibodies used for library screening. Lanes: 1, SDS/PAGE of total protein from crayfish hemocyte lysate; 2, immunoblot of the sample as in lane 1, incubated with affinity-purified antibodies toward proPO, prepared as described; 3, SDS/PAGE of pure proPO, prepared as in ref. 19; 4, molecular mass markers (180 kDa, human α_2-macroglobulin; together with low-molecular-mass protein markers from Pharmacia electrophoresis kit).

immobilized on a nitrocellulose filter according to the method of Smith and Fisher (22).

cDNA Cloning of proPO. The monospecific antiserum was used to screen a crayfish hemocyte cDNA library (23). An initial immunoscreening of 100,000 recombinants of a hemocyte cDNA library resulted in three partially overlapping clones. These clones were subcloned in pBluescript II KS(+) (Stratagene), and nucleotide sequence analysis was performed by the dideoxynucleotide chain-termination method by using Sequenase 2.0 from United States Biochemical. Nested deletions in both directions were done by using the Pharmacia nested deletion kit according to the manufacturer's instructions. Further overlapping cDNA clones were isolated by plaque hybridization of the hemocyte library using the isolated and sequenced clones as probes. The nucleotides 1–43 were determined from hemocyte mRNA using the 5′-Amplifinder RACE (for rapid analysis of cDNA end procedure) kit (Clontech) according to the manufacturer's instructions. The clone-specific primers used in the PCR reactions are indicated in Fig. 2. The sequence was established by two independent RACE experiments.

The cDNA sequence was analyzed with the MACVECTOR 4.1.1 software (Kodak). The deduced protein sequence was compared with the Entrez data base (National Center for Biotechnology, release 10.0).

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TGAATCGTGACTCAGTGCTCCGGGAGAATCCTTTTTATGCAGGTGACCCAGAAGTTGCTGAGGAGAGACACAGAGATGGCTGATGCTCAA   90
                                  M  Q  V  T  Q  K  L  L  R  R  D  T  E  M  A  D  A  Q          18

AAACAGCTGCTTTACCTGTTCGAGCGGCCGTATGATCCCATAAACGCGCCGCGAGCCGATGGCTCCTTCCTCTATGCAGTCGCCGGAGCT   180
 K  Q  L  L  Y  L  F  E  R  P  Y  D  P  I  N  A  P  R  A  D  G  S  F  L  Y  A  V  A  G  A     48

GATACAGTGGCGACCAGGTTCGGTGTGGCCCCGACCAGCACGGTGACGGTGCCGGCGCGTCCAGACGCTGACAGGAGACTCCTGGGAAGA   270
 D  T  V  A  T  R  F  G  V  A  P  T  S  T  V  T  V  P  A  R  P  D  A  D  R  R  L  L  G  R     78

GCGCCCTCCGTCCCACGGGGCGCCGTCTTCTCCTTCTTCATTAGGTCACACCGCGAGGCCGCCAGGGATCTCTGTGACGTCTTAATGAAA   360
 A  P  S  V  P  R  G  A  V  F  S  F  F  I  R  S  H  R  E  A  A  R  D  L  C  D  V  L  M  K     108

ACTCAAAATTCCACCGACCTGATGCAGTTGGCGGCCAGTGTGAGGAGACATGTCAACGAAAACCTCTTCATTTACGCACTCTCCTTCACC   450
 T  Q  N  S  T  D  L  M  Q  L  A  A  S  V  R  R  H  V  N  E  N  L  F  I  Y  A  L  S  F  T     138
          *

ATTCTTCGCAAGCAGGAATTGCGAGGTGTTCGTCTGCCGCCGATCTTGGAGGTCTTTCCCCACAAATTTATACCAATGGAAGACCTGACA   540
 I  L  R  K  Q  E  L  R  G  V  R  L  P  P  I  L  E  V  F  P  H  K  F  I  P  M  E  D  L  T     168

AGCATGCAGGTTGAAGTGAACAGGACTCCACCTACTGCTACAACACCATTGGTGATCGAATATGGACCCGAATTTGCGAACACCAACCAG   630
 S  M  Q  V  E  V  N  R  T  P  P  T  A  T  T  P  L  V  I  E  Y  G  P  E  F  A  N  T  N  Q     198
             *  ⬆

AAGGCGGAGCACCGAGTGTCATACTGGAGGGAGGACTTTGGCATCAACTCTCACCACTGGCACTGGCATCTCGTTTACCCCATTGAGATG   720
 K  A  E  H  R  V  S  Y  W  R  E  D  F  G  I  N  S  H  H  W  H  W  H  L  V  Y  P  I  E  M     228
                                    •        •

AATGTCAACCGGGATCGAAAAGGAGAACTTTTTTATTATATGCATCAGCAGATGGTCGCTAGGTACGACTGGGAGCGACTCAGCGTCAAC   810
 N  V  N  R  D  R  K  G  E  L  F  Y  Y  M  H  Q  Q  M  V  A  R  Y  D  W  E  R  L  S  V  N     258
                                       •

CTTAACCGTGTGGAGAAGCTGGAGAACTGGCGCGTGCCAATCCCAGATGGCTACTTCTCCAAGCTCACCGCTAACAACTCCGGCCGTCCC   900
 L  N  R  V  E  K  L  E  N  W  R  V  P  I  P  D  G  Y  F  S  K  L  T  A  N  N  S  G  R  P     288
                                                                   *

TGGGGCACCCGCCAGGACAACACCTTCATCAAGGATTTCAGACGTAATGATGCCGGGCTGGATTTCATAGACATCAGTGATATGGAGATC   990
 W  G  T  R  Q  D  N  T  F  I  K  D  F  R  R  N  D  A  G  L  D  F  I  D  I  S  D  M  E  I     318

TGGCGGTCCCGACTTATGGACGCCATCCACCAAGGATATATGCTAAACCGAAATGGTGAACGCGTCCCTCTCTCTGATAACGTCACAACA  1080
 W  R  S  R  L  M  D  A  I  H  Q  G  Y  M  L  N  R  N  G  E  R  V  P  L  S  D  N  V  T  T     348
                                                                   *

GGAAAGCGAGGGATTGACATCTTAGGAGACGCGTTTGAGGCAGATGCCCAGCTTAGCCCAAATTACTTATTTTATGGCGACCTTCACAAC  1170
 G  K  R  G  I  D  I  L  G  D  A  F  E  A  D  A  Q  L  S  P  N  Y  L  F  Y  G  D  L  H  N     378
                                                                            •

ACGGGCCATGTGCTGCTGGCATTCTGTCACGACAACGACAATTCACATAGGGAGGAGATAGGAGTTATGGGAGACTCTGCTACTGCTTTG  1260
 T  G  H  V  L  L  A  F  C  H  D  N  D  N  S  H  R  E  E  I  G  V  M  G  D  S  A  T  A  L     408
    •

AGAGACCCAGTCTTCTACCGCTGGCACAAGTTTGTTGACGACATCTTCCAGGAGTACAAGCTGACACAGCCGCCCTACACCATGGAGGAT  1350
 R  D  P  V  F  Y  R  W  H  K  F  V  D  D  I  F  Q  E  Y  K  L  T  Q  P  P  Y  T  M  E  D     438
                   •

CTGTCTCTGCCGGGTGTGGTGCTTGACAAGGTGGGTGTTGTGAGGAACGATCAGCTCAACACACTCACAACTGGCTGGAGCGTACGTGAG  1440
 L  S  L  P  G  V  V  L  D  K  V  G  V  V  R  N  D  Q  L  N  T  L  T  T  G  W  S  V  R  E     468

TTTGAGGCTTCCCGGGGCTTGGACTTCAACTCCCCCAACCCCGTCACTGCGCATTACCCATCTCGACCATGCACCCTTCACTTACCATCT  1530
 F  E  A  S  R  G  L  D  F  N  S  P  N  P  V  T  A  H  Y  P  S  R  P  C  T  L  H  L  P  S     498

CCAGATAACAAACAGCACAGGAAGCCGAAGTCAGTGACAGTGAGGATCTACATGGCTCCCAAACACAACGAACGGGGGCTGGAGATGGGC  1620
 P  D  N  K  Q  H  R  K  P  K  S  V  T  V  R  I  Y  M  A  P  K  H  N  E  R  G  L  E  M  G     528

TTCATGGAGCAGCGTCTCCTGTGGGCCGAGATGGATAAGTTCACCCAAGACTTGAAGCCTGGACAGAACCAGATCGTGCGTGCTTCCAAC  1710
 F  M  E  Q  R  L  L  W  A  E  M  D  K  F  T  Q  D  L  K  P  G  Q  N  Q  I  V  R  A  S  N     558
                                                                            *

CTGTCCTCCATCACCAACCCCAGCGGGTACACCTTCAGGAGCCTGGAGGCTGTCAACCCCGCTAACCCAGGACCACCAGCAAATGCTGAA  1800
 L  S  S  I  T  N  P  S  G  Y  T  F  R  S  L  E  A  V  N  P  A  N  P  G  P  P  A  N  A  E     588
                *

ACTAACTTTTGTGGGTGTGGTTGGCCCGAGCACCTGCTGCTGCCCCGGGGAAAACCAGAGGGGATGACTTACCAGCTCTTCTTCATGCTC  1890
 T  N  F  C  G  C  G  W  P  E  H  L  L  L  P  R  G  K  P  E  G  M  T  Y  Q  L  F  F  M  L     618

ACCGACCTGGAGAAGGACCAGGTGGATCAGCCAGCAGGACCCAGGAGATGTGCCAACGCTGTGTCCTTCTGCGGGATCCTGGACTCCAAG  1980
 T  D  L  E  K  D  Q  V  D  Q  P  A  G  P  R  R  C  A  N  A  V  S  F  C  G  I  L  D  S  K     648

TTCCCTGACAAACGACCTATGGGCTTCCCCTTCGACCGCCGCCCTCCGCCTCGCCTGCAAGATGCTGAAGTCACCTCAGTTGCTGACTAC  2070
 F  P  D  K  R  P  M  G  F  P  P  F  D  R  R  P  P  P  R  L  Q  D  A  E  V  T  S  V  A  D  Y  678

GCTAGACTTAGTAACATGACTGTGCAAGATATTACTATCACTTTCTTAACAACAGCCTCCAGAAGTAGACATGATGGTCCAATCTAATCA  2160
 A  R  L  S  N  M  T  V  Q  D  I  T  I  T  F  L  T  T  A  S  R  S  R  H  D  G  P  I  stop    706
          *

CTGGTAATATTAACCTTAAACAAATTCGAGTAAAGTATATGCAAACAGAATCGTGGCCCTCCATATATAATGTTCATGTTTACCTCAATA  2250
TTAAAAAGTTATTTGTTATTTTGTATGTTCTGGAAATTGGTATTTGGGTGAGGATTTATCCCAAGTAAATATTTGTGATGTCATGATGGG  2340
TGCTATGTTTAGGAAAGTATATACGTTT                                                                  2368
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FIG. 2. Nucleotide and deduced amino acid sequence of *Pac. leniusculus* proPO. The nucleotide sequence is numbered from the first base at the 5′ end; the deduced amino acid sequence is numbered from the initiating methionine. The histidine residues of Cu(A), His-219, His-221, and His-243, and of Cu(B), His-377, His-381, and His-417 are indicated by a •. Amino acid sequences confirmed by amino acid sequencing of tryptic fragments of the purified enzyme or determination of the N terminus of PO are underlined. The arrow denotes the site of cleavage of proPO by ppA. Underlined nucleotide sequences denote the sequences used to construct primers for the RACE experiments. Putative N-linked glycosylation sites are shown with asterisks. A polyadenylation signal is shown in boldface type.

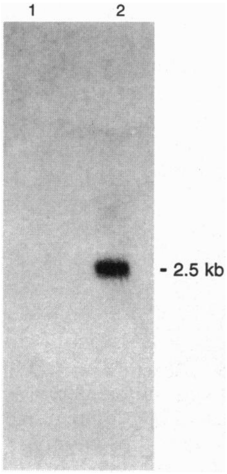

FIG. 3. Tissue-specific expression of proPO mRNA in the blood cells. Lanes: 1, 20 μg of total RNA from hepatopancreas; 2, 20 μg of blood cell RNA. The marker indicates the 2.5-kb transcript present in the blood cell RNA, whereas no proPO-specific transcript was detected in the hepatopancreas RNA.

Northern Blot Analysis. The gels were blotted onto nylon filters and hybridized with a ^{32}P-labeled 669-bp proPO clone encompassing nt 484–1152. Total RNA was isolated from hemocytes and hepatopancreas, respectively, by the acid-guanidinium method (24). The RNA was run on 1% agarose gels in the presence of formaldehyde (25) and transferred to nylon filters (Hybond-N, Amersham) by capillary blotting overnight according to the instructions supplied by the manufacturer. For hybridization 10 μCi (1 Ci = 37 GBq) of ^{32}P-labeled proPO cDNA was used in a hybridization solution containing 5× SSPE (20× SSPE = 3.6 M NaCl/0.2 M sodium phosphate/0.02 M EDTA, pH 7.7), 0.1% (wt/vol) bovine serum albumin, 0.1% (wt/vol) Ficoll (Pharmacia), 0.1% (wt/vol) SDS, and salmon sperm DNA at 100 μg/ml. The samples were hybridized overnight at 65°C and were then washed three times for 20 min with 0.1× SSPE/0.1% SDS at 65°C. After drying, the filters were subjected to autoradiography.

Proteolytic Activation of proPO by ppA and Structural Characterization of PO. Pure proPO (19) was activated by the endogenous activator, a 36-kDa hemocyte serine proteinase (ppA), and then separated on SDS/PAGE (in gradient gels; ref. 26). The separated proteins were transferred in a sandwich with one poly(vinylidene difluoride) membrane and one nitrocellulose filter, as described by LeGendre and Matsudaira (27). The poly(vinylidene difluoride) membrane was stained with Coomassie brilliant blue and then extensively destained; next bands of interest were excised. Bound proPO and PO were subjected to N-terminal amino acid determination by automated degradation on an Applied Biosystems model 470A gas phase sequencer with an online phenylthiohydantoin derivative analyzer. The N terminal of proPO, in contrast to PO, was found to be blocked. The nitrocellulose filter was stained with India ink for total protein. To obtain partial amino acid sequences for identification of cDNA clones the proPO band was excised after SDS/PAGE and digested with trypsin. Copper was determined by using the graphite furnace absorption method. For analysis, 0.1 mg of pure PO was dissolved in 1 ml of HNO_3/H_2SO_4, 1:0.15 (vol/vol).

Substrate Specificity of Purified PO. Purified proPO was used to test the ability of the enzyme to oxidize different substrates. The activity toward different phenols was assayed by the ascorbic acid method (28). In our hands elevated concentrations of ascorbic acid inhibited the enzyme activity. Thus, at higher substrate concentrations the ascorbic acid became a limiting factor. Therefore, only the relative rates of oxidation of the different substrates at a final concentration of 1 mM was determined. The reaction mixture was 1 μg of pure enzyme in 0.2 M sodium phosphate buffer, pH 7.5, activated by the addition of 10 μl of 10% SDS together with 50 μl of ascorbic acid (1 mM stock solution) and 1 mM of substrate (final concentration). The reaction was recorded as a decrease in absorbance at 265 nm. The reaction rates for the different *o*-diphenols were also determined with a continuous colorimetric assay to calculate kinetic parameters, and the two different methods gave corresponding results.

RESULTS AND DISCUSSION

proPO purified to homogeneity (19) from *Pac. leniusculus* blood cells was used to raise a polyclonal antiserum (Fig. 1). This antiserum was adsorbed onto immobilized proPO to produce monospecific antibodies for library screening (Fig. 1). For screening, a library constructed from hemocyte RNA was used because proPO is stored in its latent form in the blood cells and, thus, proPO is likely to be synthesized by this tissue. This immunoscreening resulted in the isolation of three partially overlapping clones. Further, clones were isolated by screening the hemocyte library by plaque hybridization using two of these clones as a probe. In total, seven partially overlapping clones were sequenced and found to give rise to a consensus sequence encompassing 2325 bp. To obtain the sequence of the complete open reading frame a PCR method (see Fig. 2) was used. The complete cDNA, in total 2368 bp, as constructed from these partially overlapping sequences, contains an open reading frame of 2118 bp, and the deduced

A The Cu(A) site of phenoloxidases and hemocyanins

```
                            *  *                                                *
Crayfish proPO       198  FGINSHHWHWHLVYPIEMNVN-----RDR------------------KGELFYYMHQQMVAR
Limulus HC II        167  VGINAHHWHWHLVYPSTWNPKYFGKKKDR------------------KGELFYYMHQQMCAR
Lobster HC b         188  IGMNIHHVTWHMDFPTWWQDSY-GYHLDR------------------KGELFFWVHHQLTAR
Human tyr            201  AHEAPAFLPWHRLFLLRWEQEIGKLTGDENFTIPYWDWRDAEKCDICTDEYMGGQHPTNPNL
Mouse tyr            201  AHEAPGFLPWHRLFLLLWEQEIRELTGDENFTVPYWDWRDAENCDICTDEYLGGRHPENPNL
Frog tyr             205  AHEAPGFLPWHRFYLLLWEREIQKVTGDDNFTIPFWDWRDAQQCELCTDEFFGGTHPTSNNL
Neurospora tyr        96  THSSILFITWHRPYLALYEQALYASVQAVAQKFPVEGGLRAKYVAAAKDFRAPYFDWASQPP
Streptomyces tyr      53  GHRSPSFLPWHRRFLLEFERALQSVDASVALPYWDWSADRSTRSSLWAPDFLGGTGRSRDGQ
```

B The Cu(B) site of phenoloxidases and hemocyanins

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                           *   *                                   *
Crayfish proPO       364  LHNTGHVLLAFCHDNDNSHREEIGVMGDSATALRDPVFYRWHKFVDDIFQEYK
Limulus HC II        323  LHNWGHVTMARIHDPDGRFHEEPGVMSDTSTSLRDPIFYNWHRFIDNIFHEYK
Lobster HC b         343  LHNTAHAMLGRQGDPHGKFNLPPGVMEHFETATRDPSFFRLHKYMDNIFKKHT
Human tyr            362  MHNALH-------------IYMNGTMSQVQGSANDPIFLLHHAFVDSIFEQWL
Mouse tyr            362  MHNALH-------------IFMNGTMSQVQGSANDPIFLLHHAFVDSIFEQWL
Frog tyr             366  MHNSLH-------------VFLNGSMSSVQGSANDPIFVLHHAFVDSLFEQWL
Neurospora tyr       277  VHNEIH-----------DRTGGNGHMSSLEVSAFDPLFWLHHVNVDRLWSIWQ
Streptomyces tyr     189  LHNRVH-------------VWVGGQM-ATGVSPNDPVFWLHHAYIDKLWAEWQ
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FIG. 4. Alignments of *Pac. leniusculus* proPO with other copper-containing proteins. The Copper (A) (*A*) and (B) (*B*) binding sites are each aligned. Sequences, from top to bottom, are crayfish proPO, hemocyanin II from the horseshoe crab *Limulus polyphemus* (29), hemocyanin b from the spiny lobster *Panulirus interruptus* (30), and tyrosinases from human (31), mouse (32), the frog *Rana nigromaculata* (33), the fungus *Neurospora crassa* (34), and the bacterium *Streptomyces antibioticus* (35). Amino acids found in identical positions in proPO and the other proteins are boxed. Positions of the six histidines shown to ligate the two oxygen-binding copper atoms in *Pan. interruptus* hemocyanin are indicated with asterisks above the sequences. Sequences were aligned manually, and gaps were introduced to maximize similarities.

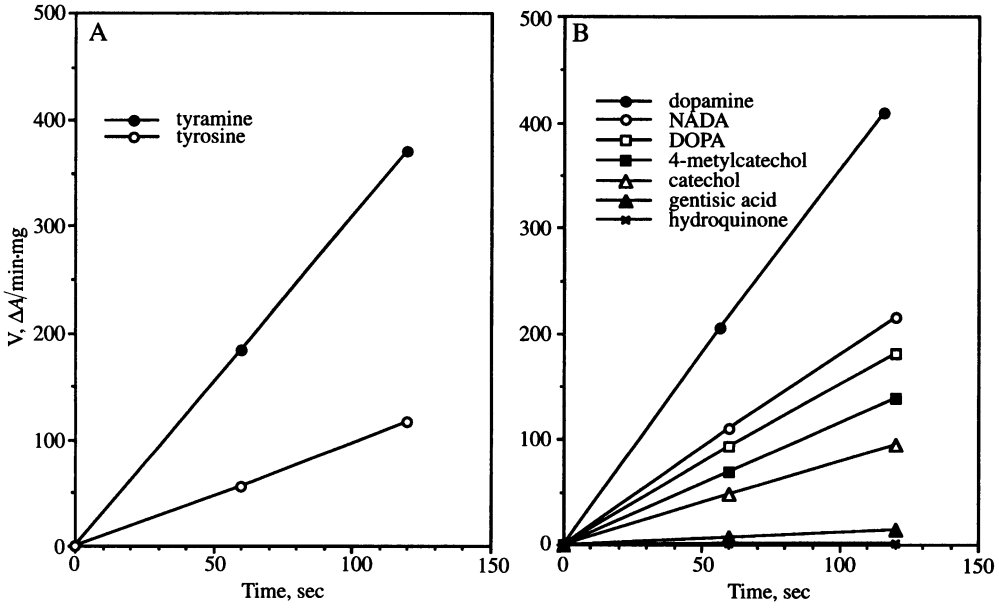

FIG. 5. Reaction rates of crayfish PO with different tyrosinase substrates. Two different monophenols, tyramine and tyrosine (*A*); five *o*-diphenols, dopamine, *N*-acetyldopamine, L-dihydroxyphenylalanine, 4-methylcatechol, and catechol (*B*), and two *p*-diphenols, gentisic acid and hydroquinone (*B*), were tested.

amino acid sequence corresponds to a polypeptide of 80,732 Da (Fig. 2). This value is close to the mass of the purified protein as judged from SDS/PAGE—i.e., 76 kDa (19). The size of the cDNA also corresponds well with the result of the Northern analysis of blood cell mRNA, which produced an ≈2.5-kb transcript (Fig. 3).

The clone was identified as coding for proPO on the basis of sequences obtained by amino acid sequencing of proteolytic digests of purified proPO (Fig. 2). Two different peptide fragments were isolated and sequenced, and the sequences obtained from these could be aligned to the deduced amino acid sequence of the cDNA clone as Phe-55–Arg-73 and as Leu-667–Arg-680 of the complete clone, respectively, thus suggesting that the clone is authentic.

Comparisons of the deduced amino acid sequence of the proPO cDNA with other copper-containing proteins such as vertebrate and microbial tyrosinases or arthropod hemocyanins show that two putative copper-binding sites are present in the proPO (Fig. 4). The amount of copper in purified PO was determined by atomic absorption to be 0.21%, which corresponds to two atoms of copper per molecule. The positions of the histidine residues are well preserved in copper-binding proteins. The six histidine residues assigned to ligation of the two oxygen-binding copper atoms in *Pan. interruptus* hemocyanin (36) appear to be present in a similar position in crayfish proPO (Fig. 4). It has been noted earlier that tyrosinases from different sources possess one putative copper-binding site, designated Cu(B), which seems to be present in all tyrosinases sequenced so far. The tyrosinases also show extensive sequence similarity with the corresponding site in hemocyanin. In contrast, the other site, Cu(A), is either absent as in *Streptomyces* tyrosinase or exhibits much less sequence similarity with the Cu(A) site of arthropod hemocyanin, as is the case with the vertebrate tyrosinases. The crayfish proPO contains putative Cu(A) and Cu(B) sites where extensive sequence similarity with arthropod hemocyanins can be found (Fig. 4). Thus, in this respect crayfish PO is more similar to hemocyanins than the tyrosinases hitherto analyzed. However, with the exception of the copper-binding sites, there is very little sequence similarity between proPO and the hemocyanins or other copper-containing proteins. Also, crustacean hemocyanin is synthesized by the hepatopancreas (midgut gland) (37), whereas proPO is synthesized by the blood cells, and no proPO expression was detectable in the hepatopancreas (Fig. 3). The deduced amino acid sequence contains seven putative N-glycosylation sites (Fig. 2), and it is therefore likely that proPO is a glycoprotein. This conclusion is corroborated by our demonstration that proPO stains for sugar with Schiff's reagent (19).

The substrate specificity of the pure protein was assessed by using different mono- and diphenols. The enzyme readily hydroxylates the monophenols tyramine and tyrosine (Fig. 5*A*). The enzyme oxidizes *o*-diphenols, such as dopamine, *N*-acetyldopamine, L-dihydroxyphenylalanine, 4-methylcatechol, and catechol but did not oxidize the two *p*-diphenols tested, gentisic acid and hydroquinone (Fig. 5*B*). Thus, crayfish proPO has a substrate specificity that is similar to other tyrosinases. The substrate specificity of the crustacean phenoloxidase is very similar to insect tyrosinases, whereas vertebrate tyrosinases have a more limited substrate specificity (38–40). In addition, inhibitors that typically inhibit tyrosinases, such as diethyldithiocarbamate, phenylthiourea, and 4-nitrocatechol, were effective in inhibiting the L-dihydroxyphenylalanine-oxidizing activity of the crayfish proPO (Table 1).

During melanin biosynthesis toxic and highly reactive intermediates are produced (41) and, therefore, the formation of catalytically active PO ought to be strictly controlled in the animal. In Fig. 6 the endogenous activation mechanism is demonstrated. As a result of partial proteolysis carried out by ppA, inactive proPO is converted into active PO with an apparent mass of 62 kDa on SDS/PAGE. The active PO was blotted onto a poly(vinylidene difluoride) membrane, and an

Table 1. Inhibition of crayfish PO activity

Inhibitor	Inhibitor conc., mM	Activity, % control
None		100
Diethyldithiocarbamate	0.001	90
	0.01	73
	0.1	16
Phenylthiourea	0.001	70
	0.01	68
	0.1	13
4-Nitrocatechol	0.1	50
	1	0

Five microliters (1 μg) of pure proPO (Fig. 1) was incubated with 750 μl of 0.2 M sodium phosphate buffer, pH 7.5, and the inhibitor (100 μl) was added. After incubation for 12 min at 20°C, the proenzyme was activated by addition of 10 μl of 10% (wt/vol) SDS. To determine enzyme activity 67 μl of L-dihydroxyphenylalanine was added to give a final substrate concentration of 1 mM. The reaction was run for 2 min before absorbance at 475 nm was monitored. conc., Concentration.

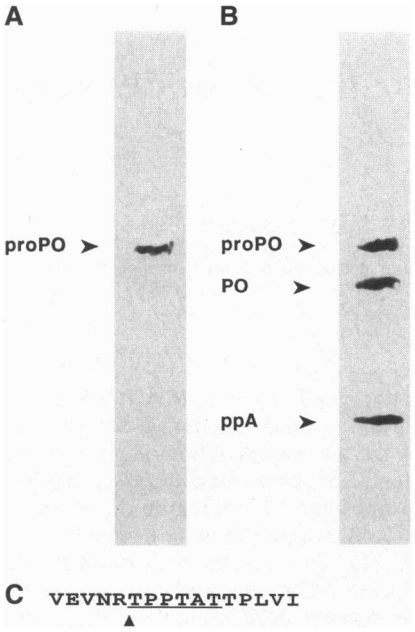

FIG. 6. Nitrocellulose blot of purified proPO (*A*) and of active PO (*B*). (*C*) Activation of proPO by the endogenous activating enzyme, ppA. The sequence obtained from the N-terminus determination of PO is underlined. The arrowhead indicates the site for cleavage of proPO by ppA.

N-terminal amino acid sequence was determined. The sequence obtained corresponds to Thr-177–Thr-182 in the deduced amino acid sequence of proPO cDNA. The amino acid residue immediately preceding Thr-177 in this sequence is an arginine (Arg-176), which shows that the cleavage site is specific for trypsin and is in accordance with the previous biochemical characterization of ppA as a trypsin-like proteinase (21). This conclusion is corroborated by earlier findings that pure ppA is inhibited by different trypsin inhibitors and also by the fact that this enzyme can use synthetic peptide substrates for trypsin-like enzymes (21). The cleaved proPO still contains both the Cu(A) and Cu(B) sites that constitute part of the active site of the PO. The deduced mass of the polypeptide sequence Thr-177–Ile-706 is 60,805 Da, which corresponds well to the mass of active PO, 62 kDa. The mass of the smaller polypeptide that would result from a cleavage between Arg-176 and Thr-177 is 19,945 Da when the whole peptide from the initiation methionine residue (Met-1) is included.

The data presented here show the primary structure of an invertebrate proPO, that a PO is produced as a zymogen, and a detailed model for how the zymogen proPO is activated. This activation is the final step in a complex activation chain which, in addition to melanin, produces several factors that in their active form exert functions of crucial importance for an adequate immune response. There are also regulatory factors present in the blood, such as proteinase inhibitors, with the capacity to modulate this response and to prevent excessive activation (42). The molecular mechanisms behind these defense reactions are now in the process of being unraveled. Such knowledge is urgently required if we are to understand how the immune system of arthropod vectors for important mammalian pathogens may be manipulated.

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