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PURIFICATION AND CLONING OF THE SALIVARY PEROXIDASE/CATECHOL OXIDASE OF THE MOSQUITO *ANOPHELES ALBIMANUS*

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Summary

Salivary homogenates of the adult female mosquito *Anopheles albimanus* have been shown previously to contain a vasodilatory activity associated with a catechol oxidase/peroxidase activity. We have now purified the salivary peroxidase using high-performance liquid chromatography. The pure enzyme is able to relax rabbit aortic rings pre-constricted with norepinephrine. The peroxidase has a relative molecular mass of 66 907 as estimated by mass spectrometry. Amino-terminal sequencing allowed us to design oligonucleotide probes for isolation of cDNA clones derived from the salivary gland mRNA from female mosquitoes. The full sequence of the cDNA demonstrated homology between *A. albimanus* salivary peroxidase and several members of the myeloperoxidase gene family. A close comparison of *A. albimanus* salivary peroxidase with canine myeloperoxidase, for which the crystal structure is known, showed that all six disulfide bridges were conserved and demonstrated identity for all five residues associated with a Ca^{2+}-binding site. In addition, 16 of 26 residues shown to be in close proximity to the heme moiety in the canine myeloperoxidase were identical. We conclude that the salivary peroxidase of *A. albimanus* belongs to the myeloperoxidase gene family. Other possible functions for this molecule in blood feeding are discussed.

Key words: catechol oxidase, peroxidase, vasodilator, salivary gland, myeloperoxidase, mosquito, *Anopheles albimanus*.

Introduction

The saliva of blood-sucking arthropods contains a diverse array of antihemostatic chemicals (Ribeiro, 1987, 1995), including anticlotting, antiplatelet and vasodilatory substances. Interestingly, the solutions found by each arthropod species to counteract each aspect of their host hemostasis are very different, perhaps as a result of evolutionary convergence. For example, vasodilators found in the saliva or salivary homogenates of such animals include the inorganic molecule nitric oxide (NO) in the bugs *Rhodnius prolixus* and *Cimex lectularius* (Ribeiro et al., 1993; Valenzuela et al., 1995), lipids of the prostaglandin class in the tick *Boophilus microplus* (Dickinson et al., 1976; Higgs et al., 1976) and *Amblyomma americana* (Ribeiro et al., 1988, 1992) and unrelated peptides in the mosquito *Aedes aegypti* (Champagne and Ribeiro, 1994), the sand fly *Lutzomya longipalpis* (Lerner and Shoemaker, 1992) and the black fly *Simmulium vittatum* (Cupp et al., 1998). The mosquito *Anopheles albimanus* has a salivary peroxidase that displays catechol oxidase activity (i.e. it destroys catecholamines in the absence of added hydrogen peroxide). This activity has been implicated in the relaxation induced by salivary homogenates of aortic smooth muscle contracted with norepinephrine (Ribeiro and Nussenzveig, 1993).

In the present study, we report the purification of the salivary peroxidase of *A. albimanus* and demonstrate that the pure enzyme can cause vasodilation of rabbit aortic rings contracted with norepinephrine. Amino-terminal sequencing of the pure enzyme allowed cloning and sequencing of the peroxidase-encoding cDNA, which shows high homology to vertebrate and invertebrate peroxidases of the myeloperoxidase family.

Materials and methods

Anopheles albimanus (Santa Tecla strain) were reared in the Medical Entomology Section of the Laboratory of Parasitic Diseases under the expert direction of Andre Laughinghouse. Adults were offered cotton swabs containing 10 % Karo syrup (CPC International Inc., Englewood Cliffs, NJ, USA). Salivary glands from female mosquitoes at least 3 days old were dissected in groups of 20 pairs in 20 µl of phosphate-buffered saline (10 mmol l^{-1} sodium phosphate at pH 7.0 with 150 mmol l^{-1} NaCl) and kept at −75 °C until needed. These glands were used for the purification of salivary peroxidase. Homogenization of the salivary glands was achieved by sonication (Ribeiro and Nussenzveig, 1993). To construct a cDNA library, pools of salivary glands from female

mosquitoes were obtained on the day of adult emergence and 1 day following emergence, because most of the mosquito salivary gland protein is synthesized at this time (Marinotti et al., 1990; Ribeiro et al., 1984, 1985).

Rabbit aortas were purchased from Spring Valley Labs (Woodbine, MD, USA). Organs were shipped in ice-cold Hepes-buffered Tyrode's solution (see below for details). Rings could be used up to 3 days after organ removal. Rabbit aorta smooth muscle ring bioassays were performed isometrically using 10 mmol l^{-1} Hepes-buffered Tyrode's solution (Webster and Prado, 1970) at 37 °C bubbled continuously with 95 % O_2 and 5 % CO_2. Isometric transducers were obtained from Harvard Apparatus (MA, USA), and recordings were sent to a computer through an A/D card converter. Dexamethasone (0.1 μmol l^{-1}) was added to the bath to prevent induction of inducible NO synthase (iNOS), because assays were not performed under aseptic conditions and iNOS is induced by bacterial lipopolysaccharide contaminants, leading to a progressive decrease in tonus of the preparation (Moncada et al., 1991). EDTA (30 μmol l^{-1}) was also added to the bath to chelate heavy metal contaminants.

Peroxidase reaction medium contained 10 mmol l^{-1} Hepes at pH 7.4, 150 mmol l^{-1} NaCl, 0.2 mg ml^{-1} *o*-dianisidine (previously diluted to 2 mg ml^{-1} in methanol), 0.1 mmol l^{-1} H_2O_2 and the indicated amounts of salivary homogenate or protein. The progress of the reaction was followed at 450 nm in a 96-well plate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA). One unit of enzyme activity is defined at that causing a change in optical density of 1 absorbance unit min^{-1} under the conditions described.

Molecular-sieving (size-exclusion) high-performance liquid chromatography (HPLC) was performed on salivary homogenates using a TSK 2000 SW column (7.5 mm×600 mm) with 10 mmol l^{-1} Hepes, pH 7.0, and 150 mmol l^{-1} NaCl at a flow rate of 1 ml min^{-1}. A diode array detector (model SPD-M10AV; Shimadzu Corp., Columbia, MD, USA) was used to monitor the effluent light absorption in the range 250–700 nm. Fractions were collected at intervals of 0.4 min. Samples of the fractions were assayed for peroxidase activity as described above. Fractions containing peroxidase activity were concentrated in a Centricon-10 (cut-off 10 kDa) concentrator, washed with 10 mmol l^{-1} sodium phosphate, pH 6.8, and applied to a hydroxyapatite column (4.3 mm×150 mm; BioRad, USA). The hydroxyapatite column was equilibrated with 10 mmol l^{-1} sodium phosphate, pH 6.8, and 10 μmol l^{-1} $CaCl_2$. After sample injection, the sodium phosphate concentration was increased to 350 mmol l^{-1} over 20 min at a flow rate of 0.5 ml min^{-1}. Fractions were collected at intervals of 0.5 min, and samples were assayed for peroxidase activity. Active fractions were pooled and desalted by reverse-phase chromatography using a non-porous, polymer-based column (PRP-infinity; Hamilton, USA), eluted with a gradient from 10 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid in water. Eluates were monitored at 280 and 400 nm using a dual-wavelength HPLC detector (SM-4100 ThermoSeparation Products). The pumps used for HPLC were a CM-4000 and a CM-4100 from the same company.

Amino acid analysis, mass spectrometry and amino-terminal sequencing of the purified salivary peroxidase were performed using the Harvard Microchemistry facility under the direction of Dr William Lane. Laser desorption time-of-flight mass spectrometry was performed using a Finnigan LaserMat apparatus. Amino-terminal sequencing of the salivary peroxidase was performed using a Hewlett Packard G1000A protein sequencer with online 1090 high-performance liquid chromatograph.

A. albimanus salivary gland mRNA was isolated from 260 gland pairs using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) yielding a total of 250 ng of poly(A)+ mRNA. The polymerase chain reaction (PCR)-based cDNA library was constructed following the instructions for the SMART cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The mRNA was reverse-transcribed to cDNA using SuperscriptII RNase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and the CDS/3′ primer (Clontech) for 1 h at 42 °C. Second-strand synthesis was performed using a PCR-based protocol with the SMART primer (Clontech) as the sense primer and the CDS/3′ primer as the anti-sense primer in a Perkin Elmer 2400 thermal cycler and using Klen-Taq DNA polymerase (Clontech). Fractions of cDNA longer than 400 base pairs (bp) were pooled and ligated into Lambda-ZAP II vector (Stratagene). The unamplified library obtained had a complexity of 5.7×10^6 recombinants.

For PCR partial cloning of *A. albimanus* peroxidase, mRNA from 60 pairs of *A. albimanus* salivary glands was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen). The mRNA was then reverse-transcribed to cDNA using SuperscriptII RNase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and the CDS/3′ primer (Clontech) for 1 h at 42 °C. Second-strand synthesis was performed as described above. To obtain the DNA sequence of the 5′ region of *A. albimanus* peroxidase, cDNA obtained by PCR amplification containing the SMART sequence was used as a template for the PCR reaction. The primers used in this reaction were the 5′ primer that recognizes the SMART sequence (Clontech) and a primer designed from the N-terminal sequence of *A. albimanus* peroxidase [Per3R; 5′-TT(ATCG) GG(AG) TT(TC) TG(ATCG) AI(AG) TT(AG) TT-3′].

PCR products were separated on a 1.0 % agarose gel, excised and purified using the Sephaglas Bandprep Kit (Pharmacia), and cloned into a PCRscript vector (Stratagene) using the PCRscript cloning system (Stratagene). Competent cells were transformed following the manufacturer's protocol, and white colonies were isolated and grown overnight in Luria broth medium containing ampicillin (100 μg ml^{-1}) at 37 °C. Plasmids from two independent clones were isolated using the Wizard Miniprep kit (Promega, Madison, WI, USA). The clones were sequenced using dye-terminator reactions according to the manufacturer's instructions (DNA sequencing kit, part number 402079, Perkin Elmer Applied Biosystems,

Foster City, CA, USA) and analyzed using an automated ABI sequencer (ABI prism, 377 DNA sequencer; Perkin Elmer).

After confirming that the sequence of the PCR product contained the predicted partial sequence of the amino-terminal region of the *A. albimanus* peroxidase, the PCR insert was digested with *Eco*RI from the plasmid, gel-purified and cleaned as described above. The PCR insert was then labeled with dUTP–digoxigenin using now-specific forward and reverse primers. The 200 bp PCR clone labeled with dUTP–digoxigenin was used to screen an *A. albimanus* salivary gland cDNA library. Phage plaques were lifted onto a Hybond-N nylon membrane (Amersham, Arlington Heights, IL, USA) and hybridized with the digoxigenin-labeled PCR probe using the plaque hybridization protocol of the Genius system (Boehringer Mannheim). Positive plaques were selected and plated again for a secondary screening. Well-isolated positive plaques were selected, and the phagemid carrying the peroxidase clone was isolated from the phage using the *in vivo* excision protocol from the UNI-ZAP vector manual (Stratagene).

Colonies that originated from the phagemid excision protocol were isolated and grown overnight in Luria broth plus ampicillin (100 μg ml^{-1}) at 37 °C. Plasmid isolation was performed using the Wizard Miniprep kit (Promega). The insert of the isolated plasmid was sequenced as described above using the M13 and M13 reverse primers and then by custom primers constructed from the internal sequence of the peroxidase clone.

DNA sequence analysis was performed using the Blast tool for comparison of two sequences (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/blast).

Results and discussion

Initially, we tested the ability of size-exclusion, strong-cation, strong-anion and hydroxyapatite columns to resolve the salivary peroxidase of *Anopheles albimanus* chromatographically using 20 pairs of glands (approximately 20 μg of total protein) per chromatographic run. Both size-exclusion and hydroxyapatite columns gave good yields and good resolution (not shown). Accordingly, we used 1000 pairs of homogenized salivary glands to purify the salivary peroxidase after size-exclusion (Fig. 1), hydroxyapatite (Fig. 2) and reverse-phase fractionation (Fig. 3). The reverse-phase chromatographic step, using acetonitrile and 0.1 % trifluoroacetic acid, denatured the enzyme activity. However, this last step served both to concentrate the protein and to remove the salts from the previous chromatographic procedure, thus preparing the sample for mass spectrometry and Edman

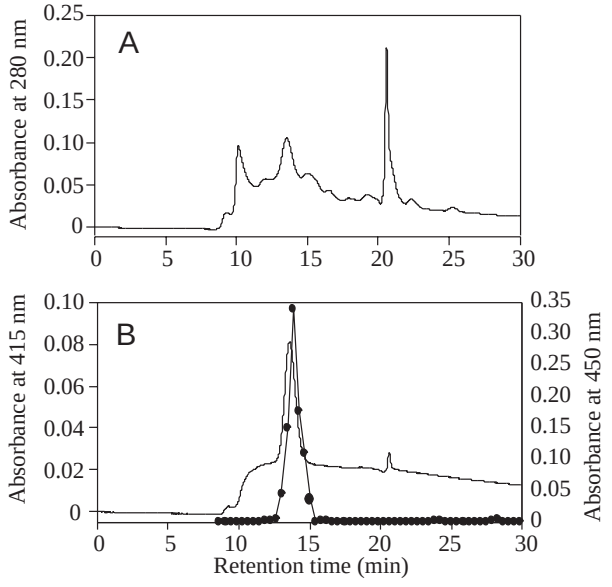

Fig. 1. Size-exclusion chromatography of homogenized salivary glands from the mosquito *Anopheles albimanus*. Salivary homogenates from 1000 pairs of salivary glands were homogenized by sonication and centrifuged at 10 000 ***g*** for 5 min, and the supernatant was applied to a TSK SW 2000 column (7.5 mm×600 mm) perfused at 1 ml min^{-1} with 10 mmol l^{-1} Hepes buffer, pH 7.0, and 150 mmol l^{-1} NaCl. (A) Absorbance profile at 280 nm. (B) Absorbance profile at 415 nm (continuous tracing). Closed circles represent peroxidase readings at 450 nm for samples collected at 0.4 min intervals and assayed with *o*-dianisidine and hydrogen peroxide.

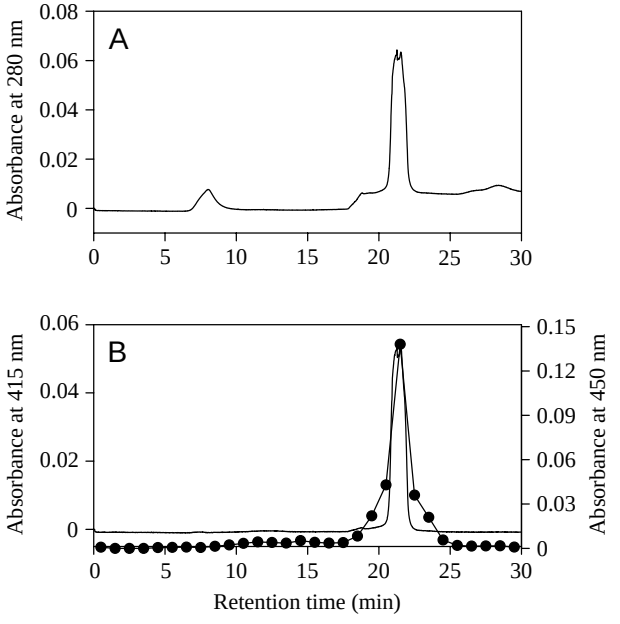

Fig. 2. Hydroxyapatite chromatography of peroxidase-active fractions from the size-exclusion chromatography shown in Fig. 1. The three most active fractions from size-exclusion chromatography were concentrated by ultrafiltration/centrifugation and applied to the hydroxyapatite column, which was equilibrated with 10 mmol l^{-1} sodium phosphate, pH 6.8, and 10 μmol l^{-1} $CaCl_2$. After sample injection, the sodium phosphate concentration was increased to 350 mmol l^{-1} over 20 min at a flow rate of 0.5 ml min^{-1}. (A) Absorbance profile at 280 nm. (B) Absorbance profile at 415 nm (continuous tracing). Closed circles represent peroxidase readings for samples assayed with *o*-dianisidine and hydrogen peroxide and read at 450 nm.

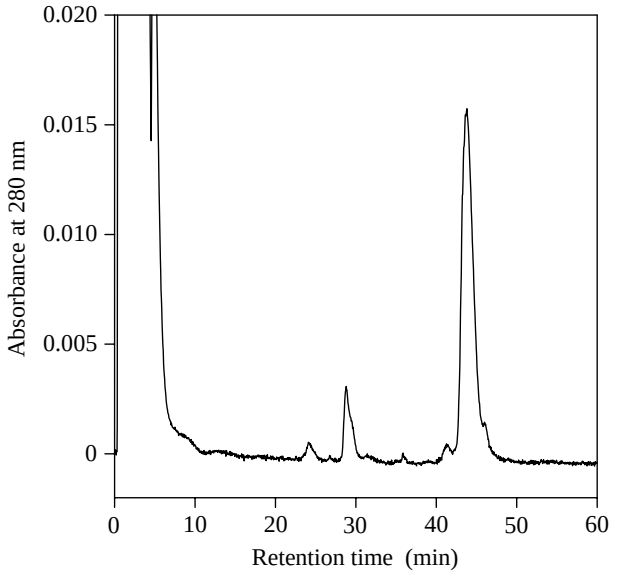

Fig. 3. Reverse-phase chromatography of peroxidase-rich fractions from the hydroxyapatite chromatography shown in Fig. 2. A PRP-infinity column was eluted at 0.5 ml min^{-1} with a gradient from 10 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid in water. The absorbance at 280 nm is shown.

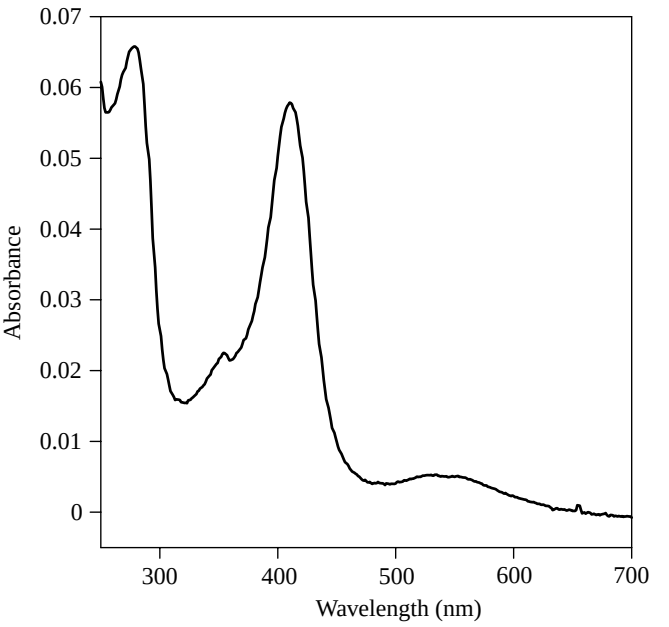

Fig. 4. Spectra of salivary peroxidase from *Anopheles albimanus* obtained after the hydroxyapatite fractionation shown in Fig. 2.

amino-terminal sequencing, both procedures requiring salt-free samples.

Size-exclusion fractionation of *A. albimanus* salivary glands showed, as reported previously (Ribeiro and Nussenzveig, 1993), co-elution of peroxidase activity with fractions also absorbing at 415 nm. Indeed, the spectra of the peroxidase containing peaks derived from the size-exclusion and hydroxyapatite columns (Fig. 4) are consistent with the presence of a heme protein with a low-spin Fe(III) (Walker et al., 1976). Reverse-phase chromatography of the peroxidase-containing fractions from the hydroxyapatite column indicated the presence of one main peak absorbing at 280 nm and eluting at 43.8 min and two other smaller peaks absorbing much (approximately 20 times) more strongly at 400 nm (results not shown), which we interpret to be non-protein, possibly hemin or hemin derivatives, eluting at 24 and 28.8 min respectively (Fig. 3). Mass spectrometry of the main product indicated a relative molecular mass of 66 907 (Fig. 5). Amino acid analysis, combined with the estimated molecular mass, indicated an overall yield of 133 pmol of the enzyme. Edman degradation of the peptide yielded nearly 45 amino acid residues of useful sequence: SN–TSPYRTLDGT–NNLQNPN–GAANTAYGRLIAADYGPQVK–P, where – indicates the absence of a signal, which could be due to the presence of cysteine, tryptophan or a modified amino acid residue. The sequence above, when submitted to the NCBI Blast 2.0 program (Altschul et al., 1990), indicated high homology to peroxidases such as the peroxidase precursor of *Drosophila melanogaster* (Swiss Prot accession number Q01603), peroxinectin from the crayfish *Pacifastacus lemiusculus* (Gene Bank accession number 1150532) and bovine lactoperoxidase precursor (Swiss Prot number P80025).

Purified *A. albimanus* salivary peroxidase following the hydroxyapatite step had a specific activity of 118 munits μg^{-1} protein, compared with 21.6 munits μg^{-1} protein for the crude homogenate, indicating that the peroxidase makes up 18 % of the total salivary gland protein. This calculation is consistent with the profile of the size-

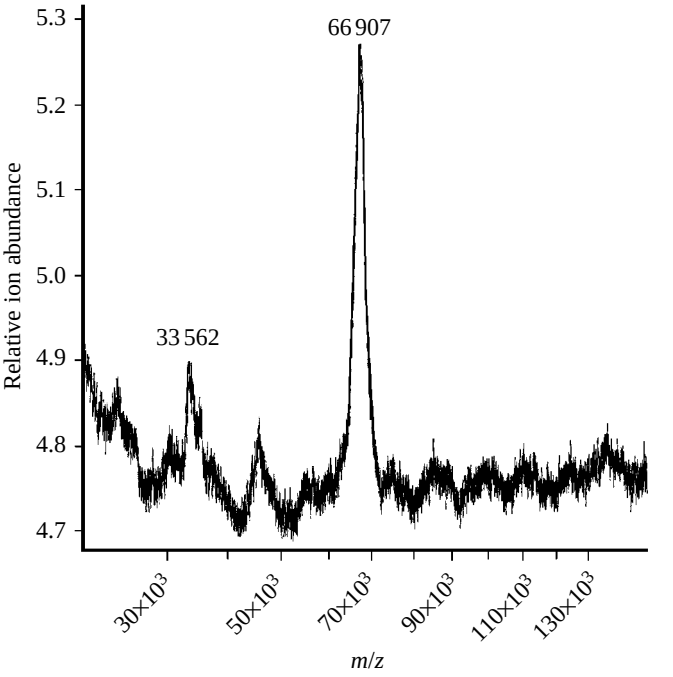

Fig. 5. Laser desorption time-of-flight mass spectrum of the peak eluting at 44 min from the reverse-phase chromatography column shown in Fig. 3. The numbers indicate the molecular mass of the monovalent and divalent positive ions. *m*, mass; *z*, valence.

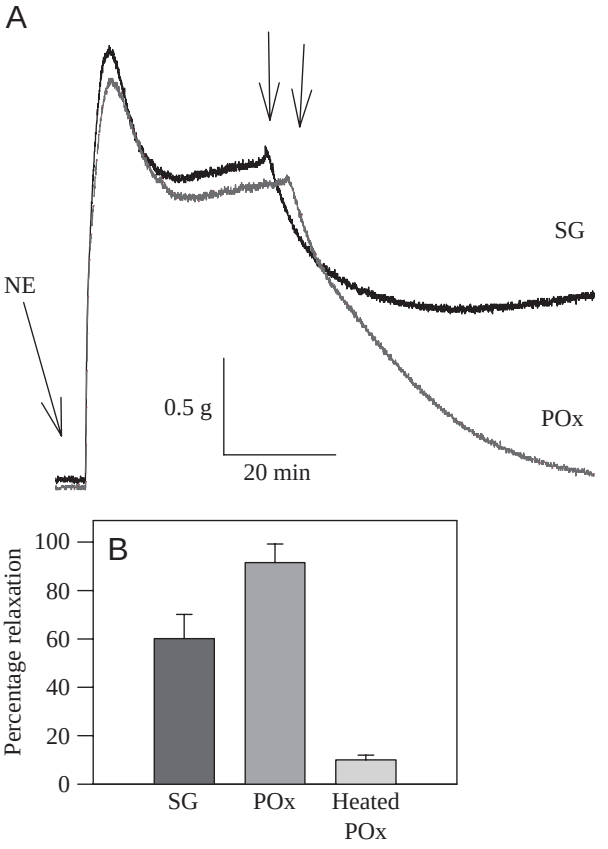

Fig. 6. Vasodilatory effects of purified salivary peroxidase and salivary gland homogenates from *Anopheles albimanus* adult female mosquitoes. (A) Aortic rings were preconstricted with 1 μmol l^{-1} norepinephrine (NE) and, following stabilization of the contractures, salivary gland homogenate (SG) representing one pair of glands (containing 21.5 munits of peroxidase) or pure peroxidase (POx, 15 munits) was added at the time indicated by the arrows. (B) Aortic relaxations measured as in A with salivary gland homogenate (SG), pure peroxidase (POx) and heat-inactivated peroxidase (HPOx). Values are means + S.E.M., *N*=4.

exclusion chromatography (Fig. 1): the peak absorbing at 280 nm and corresponding to peroxidase activity (and the peak absorbing at 415 nm) is calculated to be 19.4 % of the total area representing the 280 nm absorption for the whole chromatogram.

When 15 munits of salivary peroxidase (following the hydroxyapatite fractionation step) was added (in 20 μl) to rabbit aortic rings constricted by norepinephrine, it induced 91±8 % relaxation of the aortic ring after 30 min (Fig. 6A). In comparison, salivary gland homogenate containing 21.5 munits of peroxidase induced 60±10 % relaxation of the rings in parallel assays (means ± S.E.M. of four aortic ring preparations). The smaller vasodilatory effect observed with salivary gland homogenates may result from vasoconstrictory substances present in the homogenate. Heat treatment (100 °C, 5 min) of the purified peroxidase almost abolished the vasodilatory activity (Fig. 6B) and the peroxidase activity (results not shown), indicating that the salivary peroxidase of

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GAGTACGGCTGCAAGACGACAGAAGGGGATCAATCGATTGGTCTATTTGCATCAAGTGTA      60

CCAAGATGTGGATGTTTCTAAAGCTGCTCCTGTTCGTGTGCTCTAGTTGGTGGAGCTGTG     120
     M  W  M  F  L  K  L  L  L  F  V  C  S  S  W  W  S  C  A

CCCAAGCCTCTAATTGCAACGCGACGTCTCCGTACCGAACATTGGATGGCACCTGTAACA     180
  Q  A  S  N  C  N  A  T  S  P  Y  R  T  L  D  G  T  C  N  N

ATCTGCAGAATCCGAACTGGGGTGCGGCCAATACCGCTTACGGGCGCTTGATCGTCGCGG     240
  L  Q  N  P  N  W  G  A  A  N  T  A  Y  G  R  L  I  V  A  D

ACTACGGCGATGGCGTGAAGAGCCCGCGGAAGGCTGCGAGTGGTGCCGATCTACCCAGTG     300
  Y  G  D  G  V  K  S  P  R  K  A  A  S  G  A  D  L  P  S  A

CGAGGCTTCTTTCGATGAAACTATTCGGGGATGAGCATGTTCTGGAGCCAGCGTTCACTC     360
  R  L  L  S  M  K  L  F  G  D  E  H  V  L  E  P  A  F  T  L

TGCTTAGCATGCAGTTCGGTCAACTGGTAGCCCACGATATGGGCTTCACGAGCGGAAGTA     420
  L  S  M  Q  F  G  Q  L  V  A  H  D  M  G  F  T  S  G  S  T

CGGACATACTGCCGTGCTGTAGTGAAGGAAAGCCTGTATCAAACCCTGTCCCTCGATGCT     480
  D  I  L  P  C  C  S  E  G  K  P  V  S  N  P  V  P  R  C  Y

ACCCTATACCGGTAGCCTCAGATGATCCCGTGATGGGGTCAGCCGGTGTTCAGTGCCTAG     540
  P  I  P  V  A  S  D  D  P  V  M  G  S  A  G  V  Q  C  L  D

ACTTCCTGCGAACCATTACCGATTGCGATGCTGATCCGTCCAGCTGTTCAAATAGCAAGA     600
  F  L  R  T  I  T  D  C  D  A  D  P  S  S  C  S  N  S  K  K

AGGCGGAACAGCTCAATATTGCGACATCATTTATTGACTTGTCGGTCGTGTATGGGAACA     660
  A  E  Q  L  N  I  A  T  S  F  I  D  L  S  V  V  Y  G  N  S

GCGTTGAGGAAAACACTCCCATACGCGAGTTTACCGGTGGGCTGATGAAGGTGGAAACTC     720
  V  E  E  N  T  P  I  R  E  F  T  G  G  L  M  K  V  E  T  R

GAGATGGATCCGATTGGCCTCCGCGTAACCCGAATGCCAGCACCGCGTGTGTACAGCGTA     780
  D  G  S  D  W  P  P  R  N  P  N  A  S  T  A  C  V  Q  R  S

GCCCCGAGGATGCCTGCTACCTGACGGGTGATGCACGTGCCAACATTAGCCCACAAATGG     840
  P  E  D  A  C  Y  L  T  G  D  A  R  A  N  I  S  P  Q  M  A

CCATCCTACACATCCTGTTCCTGCGGGAGCACAACCGGATTGCCAAGCACTTGGCAGCTC     900
  I  L  H  I  L  F  L  R  E  H  N  R  I  A  K  H  L  A  A  L

TGCACCCAGAGTGGAACGACGAAAAGCTGTTCCAGGAAGCCCGGCGTATCAACAACGCTC     960
  H  P  E  W  N  D  E  K  L  F  Q  E  A  R  R  I  N  N  A  Q

AGTACCAGATCGTGTTCTACGAGTGGCTACCGAACTTCCTTCCACTACCGGATAATGGAG    1020
  Y  Q  I  V  F  Y  E  W  L  P  N  F  L  P  L  P  D  N  G  G

GTAAGCGCAGTCTGTCGAGCGTTCTCGATCACCAGTATCGTGCCGATGTGAACCCAACAA    1080
  K  R  S  L  S  S  V  L  D  H  Q  Y  R  A  D  V  N  P  T  T

CGCTAAACTCTAACGCCCATGCGGCGTTCCGATACTTCCATAGTGCCATCCTTGGTCATC    1140
  L  N  S  N  A  H  A  A  F  R  Y  F  H  S  A  I  L  G  H  L

TGCATTTGGATTACGAGAATCGAACCAAGGCTGGTGAGATCAGCTTTACCGATCACACGT    1200
  H  L  D  Y  E  N  R  T  K  A  G  E  I  S  F  T  D  H  T  L

TGAATCCCGCCATTCTCGAGGCTCCCTGCAAGTACGCTCAGCTGTCGCGTGGTATGGCCA    1260
  N  P  A  I  L  E  A  P  C  K  Y  A  Q  L  S  R  G  M  A  T

CGCAGTCGATGGGCCGAATCGATCTCAATATAGACCACGAGTTGAAGCACAATCTCTTCA    1320
  Q  S  M  G  R  I  D  L  N  I  D  H  E  L  K  H  N  L  F  K

AATTTAATGCTCCTTTCGGCAACGACCTGCGAGCGATCGACATACAGCGGGCGCGTGATC    1380
  F  N  A  P  F  G  N  D  L  R  A  I  D  I  Q  R  A  R  D  H

ACGGCCTACCTAGTTACAACAGCTTCCGCGAAAAGTGTGGTTTACCGAAGGCCGCCAGCT    1440
  G  L  P  S  Y  N  S  F  R  E  K  C  G  L  P  K  A  A  S  F

TCGATGATTTTACATCCCTGTTACACTCACCACAGGATGCTGCTCGATTAGCCTCTGTCT    1500
  D  D  F  T  S  L  L  H  S  P  Q  D  A  A  R  L  A  S  V  Y

ACGCTTCGGTGGACGATGTGGAACTGACCGTGGCCGGTTTGTTCGAAAAGCATATCCCCG    1560
  A  S  V  D  D  V  E  L  T  V  A  G  L  F  E  K  H  I  P  G

GCACACAGGTCGGTGCAACGTTCCGTTGCATCCTGCTGGAACAGTTCCATCGGACTCGGG    1620
  T  Q  V  G  A  T  F  R  C  I  L  L  E  Q  F  H  R  T  R  V

TAGGTGATCGGTTCTTCTTCGAAACATCCGATCCCATCGTTGGCTTCTCCAGAGAGCAAT    1680
  G  D  R  F  F  F  E  T  S  D  P  I  V  G  F  S  R  E  Q  F

TCAAGCAGCTGAGAAAAGCAAACATAGCCCGGCTGCTGTGCGATAATACGCCCAAGCTGG    1740
  K  Q  L  R  K  A  N  I  A  R  L  L  C  D  N  T  P  K  L  E

AGGGTATGCAAAGTAAGGCTTTTGCAGCGATCGATGCGGGATCGAACAAGGTATTGCCTT    1800
  G  M  Q  S  K  A  F  A  A  I  D  A  G  S  N  K  V  L  P  C

GTTCCAGCTTGCCGGCGGTCGATCTGGATCCATGGAAATAAACAAGAGGCTCTTATTCAT    1860
  S  S  L  P  A  V  D  L  D  P  W  K  *

GCAACGAGTCATGAGCTTACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA             1911
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Fig. 7. Sequence of *Anopheles albimanus* salivary peroxidase cDNA and its translation product. The underlined fragments represent sequence information confirmed by amino-terminal degradation. The asterisk indicates the stop codon.

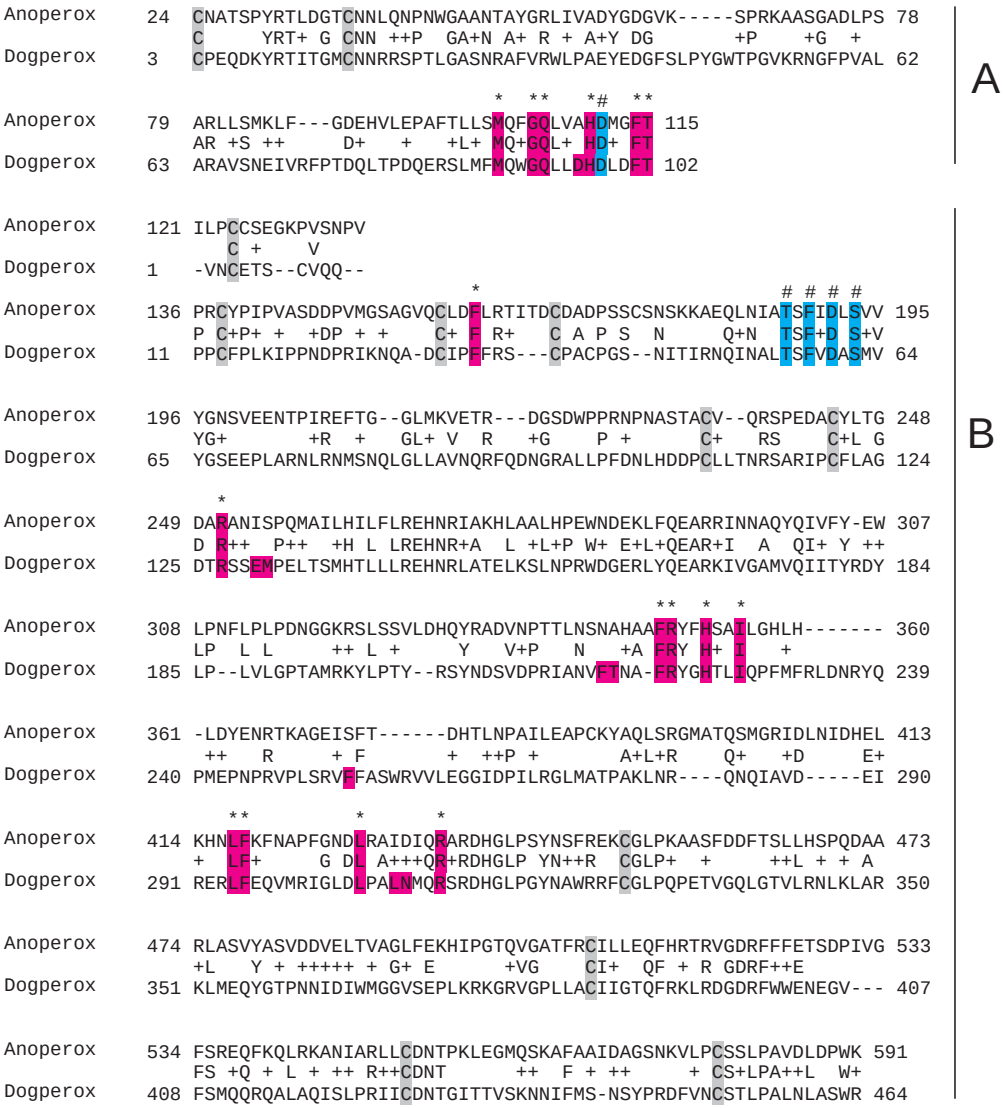

Fig. 8. Comparison of protein sequences from *Anopheles albimanus* salivary peroxidase (Anoperox) and canine myeloperoxidase (Dogperox) aligned using the Blast tool. Identical amino acid residues are shown for the two sequences. Conserved amino acid residues are marked with a + sign between the sequences. Highlighted amino acid residues indicate the 12 conserved cysteines, the five identical amino acid residues involved in Ca^{2+} binding (identified by the symbol # above the sequences) and the 16 identical amino acid residues within 0.45 nm of the heme moiety (identified by an asterisk above the two sequences). The canine sequence is also highlighted at 8 additional sites shown by Zeng and Fenna (1992) to be close the heme moiety. (A) Canine myeloperoxidase chain A (GenBank 494394); (B) chain D (GenBank 494397).

A. albimanus is responsible for most, if not all, of the vasodilatory activity observed in the salivary homogenate.

To obtain full sequence information for the salivary peroxidase of *A. albimanus*, the N-terminal amino acid sequence of the purified enzyme was used to design 14 different oligonucleotide primers (see Materials and methods) for PCR reactions, using as a template cDNA of *A. albimanus* salivary glands. Using the eighth primer prepared in this way (described in detail in the Materials and methods section), a single PCR product of 200 bp was obtained, and its sequence was found to correspond to the N-terminal region of the native *A. albimanus* peroxidase. This PCR product was then labeled with digoxigenin and used to screen an *A. albimanus* salivary gland cDNA library. One positive clone of approximately 2.1 kbp in length was obtained.

We sequenced 1911 bp of the isolated peroxidase clone, which contained an open reading frame coding for 591 amino acid residues (Fig. 7). The N-terminal sequence of the native protein was present in the deduced sequence of the peroxidase clone (Fig. 7, underlined). The first 21 residues of the predicted protein were thus considered to form the signal peptide and the remaining 570 residues to constitute the mature protein. Analysis of the predicted processed protein indicated a molecular mass of 62 919.7 Da and an isoelectric point of 6.32, with a charge of –6.50 at pH 7.0.

A search of the non-redundant protein database at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) using the gapped BLASTP program (Altschul et al., 1997) found highly homologous sequences with the myeloperoxidase gene family, the closest matches being the peroxidase precursor of *Drosophila melanogaster* (Gene Bank no. 283685), with an E value of e-144, and the sequence of peroxinectin (no. 1150532) from the crayfish, with an E value of 3e-99. A search for protein motifs at http://www.motif.genome.ad.jp/ revealed several phosphorylation and myristoylation sites and two asparagine-linked glycosylation sites at residues 203–208 and 340–343 (Marshall, 1972). Glycosylation of *A. albimanus* salivary

peroxidase could be responsible for the discrepancy between the molecular mass predicted by the clone and the observed mass obtained by mass spectroscopy of the pure enzyme. A leucine zipper motif was found at residues 61–82 (Hsi et al., 1993). This motif represents a series of at least four leucine residues separated by six amino acid residues and is implicated in a variety of proteins in the formation of dimers or other adhesion events (Zhang et al., 1996).

The X-ray crystal structure of the canine myeloperoxidase has previously been obtained at 0.3 nm resolution (Zeng and Fenna, 1992), and the information was used to map the landmarks of the myeloperoxidase family. Alignment of the *A. albimanus* salivary peroxidase with canine myeloperoxidase (using the Blast comparison tool) indicates that all 12 cysteine residues involved in disulfide bridges are conserved, as are all five residues associated with the Ca^{2+}-binding domain. Of the 26 residues indicated by Zeng and Fenna (1992) to be in close proximity to the heme moiety (24 shown in their Table 8, plus His95 and Asn421 indicated on page 203 of the same paper), 16 are identical (Fig. 8). Of the 10 non-identities, two are on non-conserved regions when several vertebrate peroxidases are compared, including met243, which is substituted in the mosquito enzyme by a serine, and leu420 of Zeng and Fenna (1992), which is substituted by isoleucine in *A. albimanus* peroxidase, an amino acid residue also found in the same location in four other vertebrate peroxidases. The leucine zipper motif is also conserved in both canine and mosquito peroxidases (not shown). These results indicate that the *A. albimanus* salivary peroxidase is similar in structure to the myeloperoxidase family of proteins.

Although the salivary peroxidase of *A. albimanus* was discovered using a smooth muscle bioassay aimed at identifying vasodilators (Ribeiro and Nussenzveig, 1993), and it has now been purified and its cDNA cloned, its full significance in the feeding process of the mosquito is still not completely understood. In addition to destroying cutaneous catecholamines that induce vasoconstriction, it may play other roles associated with the metabolism of prostaglandins and leukotrienes, as proposed previously for eosinophil and neutrophil myeloperoxidases (Henderson et al., 1982; Henderson and Klebanoff, 1983). Interestingly, the enzyme cyclo-oxygenase, which is involved in the transformation of arachidonic acid to prostaglandin H_2, is another peroxidase sharing homology to the carboxy-terminal region of the myeloperoxidase gene family (Zeng and Fenna, 1992). Nitrophorins, Fe(III) hemeproteins found in the salivary glands of some hematophagous bugs, store and carry the unstable gas nitric oxide, which has vasodilatory and antiplatelet activity. *Rhodnius prolixus* nitrophorin is completely different from *Cimex lectularius* nitrophorin (Champagne et al., 1995; Valenzuela and Ribeiro, 1998). It is possible that *A. albimanus* peroxidase could have a nitrophorin role. Other functions unrelated to the peroxidase function may also be intrinsic to the myeloperoxidase gene family. Peroxinectin, a crayfish protein from the myeloperoxidase family, is involved in cell-adhesion phenomena (Johansson et al., 1995). It was discovered and cloned for its adhesive properties. The peroxidase function of peroxinectin was discovered after its striking homology to myeloperoxidases. Johansson et al. (1997) recently discovered that vertebrate leukocyte myeloperoxidase mediates cell adhesion *via* the alpha M beta 2 integrin. It is therefore possible that soluble myeloperoxidases may act as a blocker of cell adhesion phenomena mediated by integrin receptors, such as leukocyte adhesion and platelet aggregation. These ideas remain to be tested.

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