Identification of a Steroidogenic Neurohormone in Female Mosquitoes*

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In the female mosquito, Aedes aegypti, neurohormones are released from the brain in response to a blood meal and stimulate the ovaries to secrete ecdysteroid hormones, which modulate yolk protein synthesis in the fat body. Peptides with this bioactivity were isolated from head extracts, and partial sequences from these peptides when aligned gave a 31-residue sequence at the amino terminus. Oligonucleotide primers for this sequence were used to amplify with the polymerase chain reaction a genomic DNA product that hybridized to a clone from a head cDNA library. The cDNA encodes a 149-residue preprohormone that is processed into an 86-residue peptide, as indicated by the mass value obtained from the native peptide, with the expected amino-terminal sequence. After modification, the cDNA for the putative neurohormone was expressed in a bacterial system, and the purified peptide had high specific activity in bioassays, thus confirming that it is a steroidogenic gonadotropin, the first to be identified for invertebrates.

Among vertebrates, the gonadotropic neurohormones, follicle-stimulating hormone and luteinizing hormone, regulate steroidogenesis during reproductive cycles; for invertebrates, the only characterized steroidogenic neurohormones are the prothoracicotropic hormones, which initiate molting in insect larvae (1). The first insect gonadotropin was discovered by Lea (2, 3), who described a neurohormone controlling reproduction in mosquitoes. For the yellow fever mosquito, Aedes aegypti (Diptera: Culicidae), each reproductive cycle in a female begins with the ingestion of a blood meal and ends with egg deposition. The blood meal, in turn, stimulates release of gonadotropic neurohormones from medial neurosecretory cells in the brain for up to 12 h post ingestion (2, 3). These neurohormones stimulate the ovaries to secrete ecdysteroids and thus are referred to as “ovary ecdysteroidogenic hormones” (OEHs) (4). The ecdysteroids modulate fat body secretion of yolk proteins (5, 6), which are stored selectively in the oocyte for embryonic development.

Despite many attempts to purify gonadotropins from mosquitoes (4, 5, 7), only their peptide nature and apparent heterogeneity (3,500–24,000-Da range) are known. This report describes the purification of OEH I from an extract of six million heads and its structural characterization by a combination of biochemical and molecular techniques. To verify the biological activity of the putative neurohormone, a recombinant OEH I was expressed and purified for testing in vivo and in vitro, and with an OEH I antisera, the source of the neurohormone was identified.

EXPERIMENTAL PROCEDURES

Bioassays—To test chromatographic fractions and bacterial-expressed protein for bioactivity, samples (0.5 μl of saline solution/female) were injected into 3–5-day-old, sugar-fed females (nonoogenic), decapitated within 1 h after a blood meal. After 16–24 h at 30 °C, ovaries were dissected from the females to measure yolk deposition in oocytes. Bioactive peptides induced >100 μm of yolk deposition, and no yolk was evident in females treated as controls.

Samples with in vivo activity were tested for ecdysteroidogenesis by incubation with ovaries from nonoogenic females (four or five ovary pairs in 60 μl of saline solution, three replicates/dose). After 6 h at 30 °C, incubation media (50 μl) were assayed for ecdysteroid content by radioimmunoassay (4), using an antiserum characterized by Kingan (9). Typically, bioactive peptides stimulated secretion of ecdysteroids 2–3-fold over that of controls, and where indicated in the text (*), ecdysteroidogenesis stimulated by expressed OEH I was significantly greater than that of controls (Student’s t test, p < 0.05).

Preparation and Extraction of Mosquito Heads—Heads from sugar-fed females, 5–7 days post eclosion (most males had died by this time), were separated from bodies (4) and extracted in batches of one million heads (50 g), for a total of six million heads. Each batch of heads was homogenized three times in 200 ml of ice-cold 0.2 M acetic acid solution with phenylmethylsulfonyl fluoride (40 mg/ml), EDTA (128 mg/ml), and thiodiglycol (0.1% v/v), after which the solution was sonicated and centrifuged. The supernatant solutions were pooled, partially lyophilized, rehydrated with 100 ml of 0.02 M ammonium acetate buffer (pH 4.5, containing 0.1% thiodiglycol), and centrifuged. The resultant supernatant solution was stripped of lipids by hexane partitioning prior to chromatography.

Purification of Native OEHs—Prior to the extraction and purification of the OEH described in this report, bioactive peptides had been isolated from extracts of one or two million heads, as described below. An amino-terminal sequence from one of these peptides (PTNVLEIR- WKLYSGK-ASGLM) was obtained by M.R.B. at the Molecular Genetics Core-stimulating hormone and luteinizing hormone, regulate steroidogenesis stimulated by expressed OEH I was significantly greater than that of controls (Student’s t test, p < 0.05).

Each extract of one million heads was fractionated first with cation exchange chromatography (CM-Sepharose CL-6B (Pharmacia Biotech Inc.), 30 × 2.6 cm inside diameter column; gradient of 0.02–0.5 M ammonium acetate buffer with 0.1% thiodiglycol; 30–40 ml/h, 6–8 ml/fraction; 280 nm). Bioactive fractions were divided into two groups, and each was subjected to ultrafiltration (10 kDa filter/150 ml Omegacell, Filtron Corp.); 40–55 p.s.i.). Afterward the bioactive retentate solutions (>10 kDa) were recombined for two semipreparative HPLC
steps (Alltech, Macropore C18 column, 3 μm, 300 Å, 1 cm × 35 cm) and eluted with a gradient of solvent B: 20–100% over 90 min (2 mM/formin/fraction, 280 nm). In the first step, solvent B was 60% 1-propanol, 20% CH3CN, 20% water, 0.1% heptfluorobutyric acid (HFBA; solvent A, water with 0.1% HFBA) and, in the second step, 0.1% trifluoroacetic acid was used for HFBA in the solvents. After the second step, bioactive fractions from all six batches were divided into three groups: A, fractions eluting in the earliest time range; B, the intermediate time range; and C, the latest time range.

For the next chromatography step, each group of fractions was subdivided into three portions, and each portion fractionated with cation-exchange HPLC (Bio-Rad TSK SP60W, 7.5 × 250 mm) using a gradient of 0.02–0.5 mM ammonium acetate buffer (pH 4.5), 10% 1-propanol over 50 min (0.8 mL/min/fraction; 280 nm). Bioactive fractions were obtained from the previous HPLC step for each group were divided into early and late eluting portions and separately fractionated with reversed-phase HPLC on a C4 column (Alltech Microspheric, 5 μm, 300 Å, 250 × 4.6-mm inside diameter) with a gradient of solvent B (30% CH3CN, 30% 1-propanol, 40% water, 0.1% HFBA; solvent A, water with 0.1% HFBA; 20–30% for 5 min, 30–60% for 60 min, and 60–100% for 20 min (1 mL/min/fraction, 206 nm). Material from the A group of fractions was not purified further because the bioactivity was lost.

For groups B and C, bioactive fractions eluting early (B1 and C1) and late (B2 and C2) in the last HPLC step were combined and separately fractionated with the same reversed-phase conditions, using 0.1% trifluoroacetic acid for HFBA. Bioactive fractions from the chromatography of the early and late fractions step were combined for another fractionation on the same column but with a different gradient of solvent B: 20–30% for 5 min, 30–60% for 60 min, and 50–100% for 15 min (peaks were manually collected). Bioactive material eluted with this step was resolved further by narrow bore HPLC (two Brownlee Aquapak C2 columns in series; 300 Å, 250 × 2.1-mm inside diameter) with a gradient of solvent B (60% CH3CN, 20% 1-propanol, 20% water, 0.1% trifluoroacetic acid; solvent A, water with 0.1% trifluoroacetic acid): 20–60% over 60 min (200 μL/min; peaks were manually collected; 214 nm). The bioactive material eluted as two peaks, the first and major peak was subjected to another purification step, and the second minor peak was not purified further.

For the last step, the material from the first peak was eluted from a microcapillary HPLC system (Fig. 1A) (10, 11) using a C18 column (Vydac C18, 300 Å, 3 μm, 0.53-mm inside diameter × 250 mm) with a gradient of solvent B (90% CH3CN, 10% water, 0.07% trifluoroacetic acid; solvent A, water with 0.1% trifluoroacetic acid): 20–60% over 60 min (20 μL/min; peaks were manually collected; 214 nm). The bioactive material eluted as a single peak and was used for mass spectrometry and sequencing in the laboratory of K. M. Swiderek.

**Sequence Analyses—** For in situ reduction and alkylation of the peptide prior to endoproteolytic digestion, 22.5 μL of 45 mM dithiothreitol were added to the peptide aliquot (~5 μL), and after a 15-min incubation at 50 °C, 2.5 μL of 100 mM iodoacetamide acid were added for an additional 15-min incubation at room temperature, pH 8.0. Alkylation of the peptide was performed with iodoacetamide (1:25 w/w, enzyme:peptide in 70 μL/w) with the addition of 50 mM sodium phosphate buffer (pH 7.4), resuspended, and lysed in 1.0 mL of 25 mM Tris/HCl buffer (pH 8.0; 10 mM EDTA and 50 mM dextrose) with lysozyme (1 mg/mL). Five milliliter of 30 mM Tris/His buffer (pH 7.5; 1 mM EDTA, 30 mM NaCl, 0.3 mM Pefablock, and 1 mM dithiothreitol) were added to the suspension, which was then sonicated and centrifuged (12,000 × g, 4 °C, 15 min). The pellet was resuspended with the same buffer as above containing 1% Triton X-100, centrifuged as above, and dissolved in 5.0 mL of 30 mM Tris/His buffer (pH 7.0) containing 6 M guanidine HCl, 1 mM EDTA, and 5 mM dithiothreitol. The solubilized material was transferred to washed dialysis tubing (3,500-Da cut-off) and dialyzed against 100 mM Tris/HCl buffer (pH 8.0; 10 mM EDTA and 50 mM dextrose) with lysozyme (1 mg/mL). Five milliliter of 30 mM Tris/His buffer (pH 7.5; 1 mM EDTA, 30 mM NaCl, 0.3 mM Pefablock, and 1 mM dithiothreitol) were added to the suspension, which was then sonicated and centrifuged (12,000 × g, 4 °C, 15 min). The pellet was resuspended with the same buffer as above containing 1% Triton X-100, centrifuged as above, and dissolved in 5.0 mL of 30 mM Tris/His buffer (pH 7.0) containing 6 M guanidine HCl, 1 mM EDTA, and 5 mM dithiothreitol. The solubilized material was transferred to washed dialysis tubing (3,500-Da cut-off) for equilibration with distilled water, centrifuged as above, and lyophilized with a small portion aliquoted for bioassay and Western blotting.

To purify the expressed OEH I peptide from 100 mL of culture broth, the pellet and lyophilized supematant solution were subjected to two steps of semipreparative HPLC on a C18 column (Waters DeltaPak, 15 μm, 300 Å matrix; 25 × 100 cm with a guard cartridge). For the first step, the expressed protein was eluted with a gradient of solvent B (CH3CN with 0.1% HFBA; solvent A, water with 0.1% HFBA): 20–40% for 20 min, 40–80% for 40 min, and 80–100% for 10 min (6 mL/min/fraction, 280 nm). Fractions active in vivo were pooled and subjected to a second HPLC step with a gradient of solvent B (CH3CN...
with 0.1% trifluoroacetic acid; solvent A, water with 5% CH₃CN and 0.1% trifluoroacetic acid: 10–20% for 10 min, 20–50% for 40 min, and 50–100% for 10 min (6 ml/min/fraction; 206 nm). Bioactive peptide represented by a single peak in the last HPLC step was subjected to amino acid analysis and mass spectrometry, and its specific activity was determined with bioassays.

**Electrophoresis and Western blotting**—The expressed protein was solubilized (0.5 m Tris/HCl buffer (pH 6.8) with 12% glycerol, 1% SDS, and 2% 2-mercaptoethanol; boiled for 10 min), separated in 16.5% Tris-Tricine gels (Bio-Rad) at 100 V in 0.1 m Tris, 0.1 m Tricine buffer (pH 8.25) with 0.1% SDS, and stained with Coomassie Blue R-250. Polyamide standards (Bio-Rad) were used to evaluate the molecular weights of samples.

After electrophoresis, peptides from unstained gels were transferred onto polyvinylidene difluoride membranes (Bio-Rad, 0.2 µm; 100V at 4 °C in a 25 mm Tris, 192 mm glycine buffer, 20% methanol). The membranes were dried, washed in a mixture of 50% methanol and 20% Tris buffer (pH 7.5) containing 150 mM NaCl (TBS) and 0.05% Tween-20 (TBS-T), blocked for 1 h in TBS-T with 5% non-fat dry milk, and washed three times in TBS-T. After incubation at room temperature for 1 h with the OEH I antiserum (see “Immunocytochemistry,” 1:4000 in TBS-T), the membranes were washed in TBS-T and incubated with anti-rabbit IgG antibodies conjugated to peroxidase (Sigma, 1:4000 in TBS-T) for 1 h at room temperature. Following washes in TBS-T and another wash in TBS, membranes were immersed in a staining solution (3.3′-diaminobenzidine tetrahydrochloride (Sigma), 10 mg in 10 ml of TBS with 1.0 mg of NiCl₂ and 0.08% H₂O₂).

**Amino Acid Composition**—Prior to analysis, the partially purified OEH I from the bacterial expression was dried in a 63 mm glass tube. The sample was hydrolyzed with 6 N HCl at 110 °C for 24 h. Analysis was accomplished by separating amino acids with HPLC and by postcolumn derivatization with ninhydrin on a Beckman 6300 high performance analyzer running a low pH sodium citrate gradient (Na-A, 50–100% for 10 min (6 ml/min/fraction; 206 nm). Bioactive peptide was synthesized, conjugated to thyroglobulin with glutaraldehyde, and DNA/PCR product (PTNVLEMRCKLYSGPAVQNTGECVHGAELN) resolved residue ambiguities, especially where a Cys was encoded, and differed from that of the peptide sequence at residues seven, Met for Ile, and nine, Cys for Gly/Trp. The PCR product then was used to probe a head cDNA library, and a clone was identified and sequenced. The cDNA clone is 973 nucleotides long and has an open reading frame encoding a 149-residue peptide that begins with a putative signal peptide followed by the PCR product sequence (Fig. 2). Residue 30 of the encoded peptide matches the corresponding residue seven, Ile, in the composite amino-terminal sequence of the bioactive peptide sequence and not the Met of the PCR product. Also, the sequence from residue 64 to 86 of the encoded peptide matches that of a tryptic fragment obtained from a bioactive peptide (FVGDK-GESTAGIIMSGK-ASGLM, see “Purification of Native OEHs,” under “Experimental Procedures”). A search of protein data bases revealed that the encoded peptide sequence from residue 23 to 109 had the highest sequence similarity (29%) to that of a locust neurohormone, neuroparsin A (Fig. 3) (17). No other protein sequences were significantly similar, even when searches were limited to the sequence, KGVGDKCG (residue 62–68), that is seemingly conserved between OEH I and neuroparsin A.

A bacterial system was chosen for expression of the OEH I cDNA to determine whether the encoded peptide from residue 24–108 was bioactive. A peptide of ~9 kDa was the predominant form in a Western blot of solubilized protein from BL21/OEH I cells (Fig. 4); a dimer and trimer of the peptide also were evident. No immunoreactive peptides were evident in the protein from BL21/pET cells. Solubilized BL21/OEH I protein stimulated yolk deposition in all females injected over a range of 0.06 to 1.8 µg/female, whereas BL21/pET protein showed no activity over the same range. Ovary ecysteoidogenesis was stimulated approximately three fold with 1.0 µg of the BL21/OEH I protein (306 ± 47 pg* (X ± S.E.) ecysteoids/50 µl of incubation media) above that with the same amount of BL21/pET protein (83 ± 30 pg). Greater amounts of the BL21/OEH I protein stimulated a similar level of ecysteoidogenesis (4.0 µg of protein, 394 ± 25 pg* ecysteoids; 2.0 µg, 339 ± 22 pg* above that of BL21/pET protein (4.0 µg, 192 ± 30 pg, 2.0 µg, 192 ± 30 pg, 206 nm). The solubilized BL21/OEH I protein was purified further by semipreparative HPLC to determine its specific activity in the bioassays. In addition, mass spectrometry of a portion of the purified BL21/OEH I peptide revealed a predominant peak of M⁺—8691. The amount of purified peptide was calculated from the results of the amino acid analysis. This analysis also indicated that the amino acid composition of the expressed peptide...
was similar to that of OEH I (15 of the 16 amino acids quantified were $\pm 1.0$ mol % of the value known for OEH I, excluding Cys residues). For the in vivo bioassay, the minimum amount of peptide found to stimulate yolk deposition in more than half of the injected females was 0.15 ng (0.017 pmol)/female (11/13 females injected); 0.58 and 0.29 ng/female had the same effect. Bioactivity was evident at 0.07 ng/female (4/13 females injected) and not at lower amounts. Ovary ecdysteroidogenesis in vitro was stimulated 2-fold (87 $\pm 13$ pg ecdysteroids/50 ml of incubation medium) with 57 ng (6.6 pmol) of the purified peptide above that of ovaries incubated in medium alone (39 $\pm 10$ pg). With greater quantities of peptide, ecdysteroid production increased 3-fold (114 ng of peptide, 120 $\pm 6$ pg ecdysteroids; 228 ng, 115 $\pm 10$ pg) and decreased with a smaller quantity (29 ng, 58 $\pm 9$ pg).

To identify the source of OEH I in the mosquito head, the antiserum specific for the genomic DNA PCR product was used for immunocytochemistry. Immunoreactivity was observed in two or three pairs of medial neurosecretory cells in the female brain (Fig. 5) and in their axons extending to the corpus cardiacum, a neurohemal organ associated with the aorta. Preabsorption of the antiserum with the synthetic peptide blocked immunoreactivity.

**DISCUSSION**

Neuropeptides with gonadotropic and ecdysteroidogenic bioactivity in female mosquitoes were isolated from different extracts of several million heads and subjected to sequencing either before or after enzyme digests. Of those partial sequences, all but one could be aligned within a 31-residue sequence at the amino-terminal region. With this sequence information and molecular techniques, the preprohormone sequence was determined. The signal peptide sequence is indicated in **bold** letters. The peptide sequence for OEH I is **underlined** with its termini indicated, and the sequence of the peptide cleaved from the carboxyl terminus is in **italics**. Polyadenylation signals at the 3' end are **underlined**.

**Fig. 2.** Nucleotide sequence and translated open-reading frame of the OEH I cDNA (GenBank™ accession no. U69542). The signal peptide sequence is indicated in **bold** letters. The peptide sequence for OEH I is **underlined** with its termini indicated, and the sequence of the peptide cleaved from the carboxyl terminus is in **italics**. Polyadenylation signals at the 3' end are **underlined**.

**Fig. 3.** Amino acid sequences for OEH I and neuroparsin A have 29% identity. Cys residues are indicated in bold.
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for the first OEH (designated as OEH I) identified in mosquitoes was determined from a cDNA sequence (Fig. 2). Partial peptide sequences for the amino-terminal region and for a tryptic fragment of bioactive peptides are found within the prohormone sequence. In addition, the original studies of Lea (2, 3) are supported by the immunocytochemical localization of OEH I in the medial neurosecretory cells of female mosquitoes.

 Structural and molecular characterization, taken together, suggest a putative sequence for the native OEH I. The value obtained by mass spectrometry of a bioactive peptide, M_r-8803, is the same as that predicted for an 86 residue peptide extending from a pGlu at residue 23, a converted Gln, to the carboxyl terminus, an Arg at residue 108 (an endoproteinase site), assuming four internal disulfide bonds (Fig. 2). Based on sequencing results from native peptides, the amino terminus may either be the Pro at residue 24 or the Gln at residue 23. The sequence beginning with the Pro at residue 24 and extending to residue 108 would have an estimated mass of 8692, which is less than the mass spectrometry value, thus indicating that pGlu is the more likely amino terminus. Unsuccessful attempts to sequence the amino terminus of the peptide subjected to mass spectrometry and other peptides previously isolated (4) adds further support for a pGlu, which in this position prevents Edman sequencing. With 12 Cys residues in the peptide sequence, up to six internal disulfide bonds can be formed; only with additional studies will the specific Cys residues forming the bonds be identified. The above sequence has a predicted pI of 7.8, consistent with its basic nature observed during chromatography, and there are no glycosylation sites. The fate and role of the putative peptide spanning residues 109–149 that is part of the OEH I prohormone are unknown.

Bacterial expression of the OEH I cDNA sequence from residues 24 to 108, without the putative pGlu at the amino terminus, yielded a peptide that was recognized by the OEH I antiserum in a Western blot. The purified peptide had high specific activity in both the in vivo and in vitro bioassays, thus confirming that OEH I is an ecdysteroidogenic gonadotropin. Yolk deposition in a blood-fed decapitated female was stimulated by as little as 11 nM expressed OEH I (0.017 pmol/female with m_r-8754, 6.6 pmol/60 l of medium) was needed to stimulate ecdysteroidogenesis by isolated ovaries. The high specific bioactivity of the expressed peptide suggests that the pGlu amino terminus is not an absolute requirement for bioactivity and that the peptide is folded in the same way as the native peptide. The mass value of 8692 obtained for the expressed peptide is the same as that estimated for this sequence with four disulfide bonds.

 The amino acid sequence of OEH I has limited similarity with that of neuroparsin A (Fig. 3), a neurohormone in locusts; both peptides have twelve Cys residues, eight of which are positioned similarly. Neuroparsin A (M_r-8754) was isolated from the corpora cardiaca of adult Locusta migratoria, based on its antidiuretic activity, and sequenced (17). It is synthesized in the medial neurosecretory cells of the brain and stored in the corpora cardiaca, where it is processed into several truncated forms at the amino terminus, including neuroparsin B, with 78 residues (M_r-8185). Additionally, a cDNA of 683 base pairs encoding neuroparsin A was cloned from an adult brain cDNA library. Neuroparsins are best characterized for their stimulation of fluid uptake in the rectum of locusts through the inositol phosphatase cascade (18). It is not known whether neuroparsins are steroidogenic in locusts.

Heterogeneity of OEHs was observed in this purification effort and in other reports (4, 7, 8). The different bioactive peptides may be due to the processing of a larger peptide into truncated forms during secretion, as with neuroparsin A, to peptide degradation during extraction and purification, to multiple genes encoding related peptides, or to the existence of structurally unrelated but functionally similar neuropeptides. Preliminary results from Southern analysis indicates the existence of a single copy of the OEH I gene with at least four alleles represented in the population of mosquitoes in our laboratory. These polymorphisms may account for the sequence differences between the PCR product and the OEH I cDNA. An insulin-like neuropeptide also may have a role in ovary ecdysteroidogenesis in mosquitoes, as suggested by characterization of an insulin receptor-like protein in mosquito ovaries and stimulation of this process by vertebrate insulin (15). Neuropeptides with very different molecular weights are known to stimulate ecdysteroidogenesis by the prothoracic glands of several insect species (19–21). The ovary maturing parsin (M_r-6927) of locusts (22, 23) is the only other structurally characterized gonadotropin known for adult insects. It is secreted from neurosecretory cells and stimulates ovarian development and vitellogenesis over several days; a direct effect on ovarian ecdysteroidogenesis has not been reported.

With the recombinant OEH I, direct effects of this neurohormone on vitellogenesis in the female mosquito can be studied, since it has been shown that 20-hydroxyecdysone indirectly stimulates yolk protein synthesis by the fat body (24) and that “head factors” affect yolk protein uptake by oocytes (25). In addition, the structural characterization of OEH I provides clues for the identification of related peptides that may be evolutionarily conserved for regulation of gonad steroidogenesis in other insects and invertebrates.

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