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The host-seeking inhibitory peptide, Aea-HP-1, is made in the male accessory gland and transferred to the female during copulation

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ABSTRACT

Male accessory glands (MAGs) of insects are responsible for the production of many of the seminal fluid proteins and peptides that elicit physiological and behavioral responses in the post-mated female. In the yellow fever mosquito, *Aedes aegypti*, seminal fluid components are responsible for stimulating egg production, changing female behavior away from host-seeking toward egg-laying and mating refractoriness, but hitherto no behavior-modifying molecule from the MAGs has been structurally characterized. We now show using mass spectrometry and HPLC/ELISA that the MAG is a major site of synthesis of the biologically active decapeptide, Aea-HP-1 (pERPhPSLKTRFamide) that was first characterized by Matsumoto and colleagues in 1989 from mosquito head extracts and shown to have host-seeking inhibitory properties. The peptide is localized to the anterior portion of the MAG, occurs at high concentrations in the gland and is transferred to the female reproductive tract on copulation. Aea-HP-1 has a pyroglutamic acid at the N-terminus, an amidated carboxyl at the C-terminus and an unusual 4-hydroxyproline in position 4 of the peptide. The structure of the peptide with its blocked N- and C-termini confers resistance to metabolic inactivation by MAG peptidases; however the peptide persists for less than 2 h in the female reproductive tract after copulation. Aea-HP-1 is not a ligand for the mosquito sex peptide/myoinhibitory peptide receptor. *A. aegypti* often mate close to the host and therefore it is possible that male-derived Aea-HP-1 induces short-term changes to female host-seeking behavior to reduce potentially lethal encounters with hosts soon after insemination.

1. Introduction

The mosquito, *Aedes aegypti*, is the main insect vector of yellow fever, chikungunya fever and dengue fever viruses in tropical and sub-tropical regions of the world [25]. The close association of *A. aegypti* with urban populations and its changing geographic distribution are contributing to the spread and increased incidence of dengue fever and the life-threatening dengue hemorrhagic fever [40]. Accordingly, there is interest in understanding the factors and mechanisms that determine reproductive success and influence behavior of the biting females, to aid the development of new vector control strategies.

It has been known for a long time that components of seminal fluid made by the male accessory glands (MAGs) and donated to the female during copulation are important for the reproductive success of *A. aegypti*, not only by facilitating the safe transfer of sperm, but also by directly influencing reproductive physiology and diverse behaviors of the post-mated female, including a lifetime refractoriness to mating [5–7,20,29]. Mature females couple repeatedly with males, but are in fact monogamous because they become refractory to a second insemination [8]. This refractoriness can be induced by either transplanting intact MAGs from mature males into the thorax of virgin females or by injecting females with a MAG homogenate [14,35]. Other behavioral responses attributed to MAG components in blood-fed female *A. aegypti* include activation of egg development [22], stimulation of oviposition [28] and pre-oviposition behavior [43] and reduction in host-seeking and biting behavior [18]. Surprisingly, the molecules responsible for eliciting these behavioral responses have not been chemically characterized, hindering our understanding the molecular basis of how MAGs modulate the behavior of female mosquitoes. Historically, the attempts at purification of active MAG constituents of mosquitoes have been limited to primitive fractionation techniques

Abbreviations: Aea-HP, *Aedes aegypti* head peptide; SP, sex peptide; SPR, sex peptide receptor; MIP, myoinhibitory peptide; SV, seminal vesicles; MALDI/TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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and have resulted in confusion about the number and nature of the molecules responsible (for review see [5]). Only recently have advanced analytical techniques been applied to the chemical analysis of *A. aegypti* MAG secretions, but this work has only focused on proteins and not peptides that might be involved in changing the behavior of the female [36,37].

We now report that the MAGs of *A. aegypti* are a source of the head peptide Aea-HP-1 and that the peptide is transferred during copulation to the female reproductive tract. Aea-HP-1 was first isolated from heads and, subsequently, bodies of adult *A. aegypti* and is known to inhibit host-seeking behavior in adult females [4,30,39]. A recent peptidomics study notably failed to identify the source of Aea-HP-1 in endocrine and neuroendocrine cells of adult insects suggesting that the MAG is possibly the principal source of Aea-HP-1 in adults [34].

2. Materials and methods

2.1. Rearing of insects

A. aegypti mosquitoes, originating from the Liverpool School of Tropical Medicine, were reared at a temperature of 26–27 °C and 80–85% relative humidity. Newly emerged adult males and females were maintained together in netted population cages (30 cm^3) and provided with sterile glucose solution (0.5% w/v) as continual food source. Females at four days old were additionally provided with a meal of murine blood. Eggs were collected from blood-fed females on damp filter paper and kept at 26–27 °C and 82.5% relative humidity. Established procedures were used for culturing larvae [32]. Virgin males and females were collected after placing pupae in individual tubes and were grouped in separate cages with access to glucose until required for either dissection or for mating. *Drosophila melanogaster* were maintained on oatmeal/molasses/agar medium at 25 °C.

2.2. Tissue extraction with acidified methanol

Tissues were dissected from adult mosquitoes in phosphate buffered saline (PBS, MP Biomedicals, Cambridge, UK) and collected into acidified methanol (86%, v/v, aqueous methanol and 5% v/v glacial acetic acid). MAGs and male seminal vesicles (SVs) (5 pairs per 100 μl) were typically prepared for analysis by infusing whole tissues in acidified methanol for 30 min, then centrifuging for 10 min at 13,000 rpm in a bench-top microcentrifuge, retaining the supernatant. Homogenization was avoided to provide a cleaner sample for analysis. Reproductive tracts from individual females (virgin or mated females as required) were collected in 25 μl of the acidified methanol and stored at −20 °C until required. The samples were centrifuged as above to provide a clear supernatant for chemical analysis.

2.3. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS)

Mosquito tissues were analyzed for Aea-HP-1 by subjecting either acidified methanol extracts or intact tissues to MALDI/TOF-MS analysis. For the methanolic extracts, an aliquot (1 μl) of MassPREP™ MALDI CHCA matrix (Waters Ltd., Manchester, UK) solution (2 mg/ml α-cyano-4-hydroxycinnamic acid in 25% v/v acetonitrile/25% v/v methanol/0.1% v/v trifluoroacetic acid (TFA)) was mixed with 1 μl of peptide sample and applied to a MALDI sample plate. After allowing samples to dry naturally in the air, the dried MALDI plate was transferred to a M@LDI L/R MALDI/TOF mass spectrometer (Waters Ltd.). The instrument used a N_2 laser at 337 nm; source voltage was set at 15,000 V, pulse voltage was set at 2450 V, reflectron voltage was set at 2000 V, microchannel plate detector voltage was set at 1950 V. Laser energy was set to medium with fine adjustment to optimize signal for each sample. A minimum of 100 laser shots were accumulated and combined to produce a raw spectrum of positive ion monoisotopic peptide masses ($[M+H]^+$) within the mass range m/z 800–4000. Spectra were processed (background subtraction, smoothing and peak centroiding) using MassLynx 4.0 software (Waters Ltd.) and calibrated externally using a datafile obtained for a tryptic digest of yeast alcohol dehydrogenase.

For whole tissue analysis, intact tissues were applied directly to a MALDI sample plate where they were rinsed with water to minimize salt contamination before the application of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile/0.05% aqueous TFA). Dried samples were then analyzed using a Voyager DE STR MALDI/TOF mass spectrometer (Applied Biosystems, Warrington) as described previously [2]. Spectra represent the resolved monoisotopic $[M+H]^+$ masses in positive reflector mode within the mass range m/z 500–2500. The MALDI laser was directed to areas close to, but not within, the tissue samples to avoid interference with energy transfer during ionization.

Peptide sequence information was obtained by MALDI Post-Source Decay (PSD) analysis of an acidified methanol extract of MAGs and SVs, performed using the Voyager instrument and angiotensin I as the standard for calibration. A PSD spectrum was produced from 7 to 8 spectral segments and stitched together using the Voyager software. Sequences were interpreted manually.

MALDI/TOF-MS of HPLC fractions was performed by drying each fraction and re-dissolving in 10 μl of 70% (v/v) acetonitrile. A 0.5 μl aliquot of the fraction was then added to 0.5 μl matrix and mixed before transfer to a MALDI sample plate. After drying at room temperature, mass spectra were acquired on a Voyager DE STR MALDI/TOF instrument [2].

2.4. HPLC (high-performance liquid chromatography) separation of peptides

Samples were diluted 10-fold in 0.1% (v/v) TFA for fractionation by reversed phase high-performance liquid chromatography (RP-HPLC) performed using a System Gold liquid chromatography system (Beckman Coulter UK Ltd., High Wycombe, UK), utilizing a dual pump programmable solvent module 126 and a UV detector module 166 [2]. Samples were loaded via a Rheodyne loop injector onto a Jupiter C_{18} 5 μm 300 Å column (250 mm × 2.1 mm internal diameter) fitted with a 30 mm × 2.1 mm guard column (Phenomenex, Macclesfield, UK). The column was eluted with a linear gradient of 10–60% acetonitrile/0.1% TFA, over 50 min at a flow rate of 0.2 ml/min, and elution monitored at 215 nm. Fractions (0.2 ml) were collected and dried by centrifugal evaporation for immunoassay or mass analysis.

2.5. Immunoassay

Peptides were quantified using an indirect enzyme-linked immunosorbent assay (ELISA) for peptides with a C-terminal RFamide, as described previously [1]. Briefly, either HPLC fractions or synthetic Aea-HP-1 (pERPhPSLKTRFamide; pE, pyro-glutamic acid, hP, 4-hydroxyproline; amide, amidated C-terminus) custom synthesized by Biomatik, Cambridge, Canada) were dried onto multiwell plates (Sigma–Aldrich Co., Dorset, UK) at 37 °C, then incubated overnight at 4 °C with 100 μl of 0.1 M bicarbonate (coating) buffer (pH 9.6). Plates were washed three times with 150 μl of 10 mM phosphate–buffered saline 0.1% (w/v) Tween-20 (PBS-T), blocking solution (150 μl; 2% w/v non-fat milk in PBS-T) was added, and the plates incubated for 90 min at 37 °C. After a further PBS-T wash, 100 μl of primary anti-FMRFamide antiserum (Bachem UK Ltd., St. Helens, UK; diluted 1:3000 in PBS-T) was added to each well and the plates incubated for another 90 min at 37 °C. After