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Evaluation of ecdysteroid antisera for a competitive enzyme immunoassay and of extraction procedures for the measurement of mosquito ecdysteroids

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Abstract

Ecdysteroid hormones regulate several aspects of insect development and reproduction. The predominant ecdysteroids produced by insects including mosquitoes are ecdysone (E) and 20-hydroxyecdysone (20E). The ability to measure E and 20E titers is essential for many studies, but few sensitive, low cost options are currently available for doing so. To address this deficiency, we developed a new enzyme-linked immunoassay (EIA). In the first part of the study, we compared the affinity of two new antisera named EAB25 and EAB27 to other available ecdysteroid antisera. EAB25 had a 27-fold higher affinity for 20E than E, while EAB27 had a four-fold higher affinity for 20E. In the second part of the study, EIA protocols were developed for analyzing E and 20E produced by the mosquito *Aedes aegypti*. Results indicated that pelts from fourth instar larvae and ovaries from blood-fed, adult females produced E and 20E. Methanol extraction in the presence of magnesium from whole body samples altered antibody recognition of E and 20E by EIA. However, extraction with 1-butanol and two organic/water phase separations eliminated this problem and improved assay performance. We conclude the new antisera used in the EIA provide a low-cost, flexible, and sensitive method for measuring E and 20E in insects.

Keywords

steroid hormone; arthropod; insect; development; reproduction

1. Introduction

Ecdysteroid hormones regulate many aspects of insect development and reproduction (Brown et al., 2009; Nijhout and Callier, 2015). The predominant forms of ecdysteroids present in most insects are ecdysone (E) and 20-hydroxyecdysone (20E). E is usually synthesized de novo from ingested sterols by the prothoracic glands in immature stages or cells in the gonads and other tissues in adults (Brown et al., 2009; Lafont et al., 2012). Secretion of E is regulated by different neuropeptides and is typically phasic (Brown et al.,

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2009; Marchal et al., 2010; Smith and Rybczynski, 2012; Heinrich, 2012; Bonneton and Laudet, 2012). Related ecdysteroids, such as makisterone A, are produced in select insects such as the honeybee, *Apis mellifera*, *Drosophila melanogaster*, and Pentatomomorpha (Hemiptera infraorder) (Tohidi-Esfahani et al., 2011; Lafont et al., 2012; Lavrynenko et al., 2015).

Many studies of E and 20E function require the ability to measure titers in whole insects, tissue extracts, hemolymph, or medium after ex vivo assays. Bioassays and different chromatography techniques were initially used to qualitatively assess and measure titers (Borst and O'Connor, 1974; Wilson, et al., 1981; Lafont et al., 2012). Subsequent development of immunoassays provided more sensitive, quantitative measurements but require antisera that preferentially recognize E and/or 20E. For more than four decades, the radioimmunoassay (RIA) was the predominant format for measuring E and 20E, as first developed by Borst and O'Connor (1972) and modified by different laboratories (Porcheron et al., 1976; Takeda et al., 1986; Warren and Gilbert, 1988). The durability of the RIA as the primary approach for measuring E and 20E was due to several factors including: 1) a commercial source of radiolabeled E with a high specific activity and long half-life, 2) freely available specific antisera, 3) repeatability, 4) flexible protocol, and 5) low sample cost. This situation changed a few years ago when Perkin-Elmer (Waltham, MA) ended production of radiolabeled E (23,24-³H(N)] E) used in RIAs. Stocks of characterized ecdysteroid antisera also have diminished or are no longer available.

Enzyme-linked immunoassays (EIAs) provide an alternative approach for measuring ecdysteroids that do not require radiolabelled E (Kingan, 1989; Porcheron et al. 1989; Pascual et al. 1995; Shiotsuki et al., 2005; Blais et al., 2010). However, EIAs have not been used as commonly as RIAs due to enzyme tracer effects on antigen-antibody interactions and variable well binding due to evaporation and other factors (Shiotsuki et al., 2005; Skrzipczyk and Verdier, 2013). However, EIAs are now the only realistic option for measuring E and 20E titers outside of chromatographic and mass spectrometry (MS) approaches. Antisera and secondary tracers for EIAs are currently available from two commercial sources: Bertin Pharma (Montignyle-Bretonneux, France)/Cayman Chemical Company, (Ann Arbor, MI) and Cosmo Bio, (Tokyo, Japan). These reagents and kits are only offered in small amounts and single-plate formats, which limit the number of experimental samples that can be examined when combined with controls and standards.

The goal of this study was to improve the EIA for measurement of E and 20E by developing antisera and protocols that can be easily used at low cost. We first report the sensitivity and specificity of two new antisera for the predominant ecdysteroids in insects, and compare their properties to two older antisera and one commercial antiserum in a competitive EIA. Results indicated that one of the new antisera binds E, 20E, and makisterone, while the other showed strong preference for binding 20E. We then conducted studies in the yellow fever mosquito, *Aedes aegypti*, to assess the utility of these antisera in EIAs for quantifying E and 20E in larvae and adult females after consuming a blood meal. Previous studies using high performance liquid chromatography (HPLC) and RIA showed that *A. aegypti* primarily if not exclusively produce E and 20E (Hagedorn et al., 1975; Greenplate et al., 1985; Borovsky et al., 1986; Jenkins et al., 1992; Sieglaff et al., 2005; Telang et al., 2007). Inconsistencies in

the E and 20E titers generated by our EIAs with prior results generated by RIA prompted the development of a new extraction protocol for use with whole mosquitoes, particular tissues, and ex vivo assays.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), bovine beta-lactoglobulin (BLG), E, 20E, and other reagents were obtained from Sigma Aldrich. Phosphate buffered saline 1× (PBS; HyClone™, GE Life Sciences) was used to prepare samples and as a rinse solution with 0.05% Tween 20 (PBS-T). Wells were blocked with non-fat dry milk (0.08% in PBS; BioRad, blotting grade, 170-6404) instead of serum or BSA, which was the conjugate antigen for the new antisera. HPLC-grade methanol (Fisher, A452) was used for extraction and HPLC. The buffered saline was made as previously described (Dhara et al. 2013).

2.2. EIAs

2.2.1. Ecdysteroid-protein conjugate preparation—We conjugated E or 20E to BSA for use as antigens in generating new antisera, but used E- or 20E-BLG conjugates to coat plate wells. BLG was chosen for the latter because it is commercially available as a pure protein, strongly absorbs to polystyrene, and is similar to BSA in conjugate site-to-weight ratio, solubility and globular shape. As a major milk protein, BLG also provided a competitive match for non-specific antibody binding sites with the milk block used in the EIA.

E and 20E were separately conjugated to BSA or BLG using the two-step process of Kingan (1989). First, aminooxyacetic acid (AOA, 5 mg) was linked to 1 mg of each ecdysteroid at carbon 6 in the B ring by mixing with 0.4 ml of pyridine in a microfuge tube overnight at 30°C. Pyridine was evaporated with an air stream. The AOA-ecdysteroid mixture was solubilized in water (2 ml) and loaded onto a C₁₈ solid phase extraction (SPE) cartridge (3 ml, Analytichem International 606303) activated with 100% methanol. The column was then rinsed with 5% methanol (5 ml), followed by 60% methanol (2 ml) to elute the conjugant, which was dried in a vacuum centrifuge. Second, the solubilized AOA-ecdysteroid (2 ml water with 10 µl of 10% HCl to optimize pH for the reaction) was covalently linked to BSA or BLG with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (approximate ratio 1:2.5 protein) by stirring overnight at room temperature (RT). The AOA-ecdysteroid-protein mixtures were diluted with PBS to 0.4 mg/ml protein, loaded into dialysis tubing (MWCO 3500, Fisher) for three changes in PBS overnight at 4°C. Sodium azide (0.02%) was then added to the E or 20E-protein conjugant stocks for storage at -80°C.

2.2.2. Antisera production and screening—Antisera to E-BSA and 20E-BSA were produced in rabbits by Pacific Immunology (Ramona, CA). Preimmune sera were collected prior to injecting two rabbits in multiple sites with E-BSA and another two rabbits with 20E-BSA in Freund's complete adjuvant. After three antigen boosts every two weeks and four production bleeds for sera, the rabbits were exsanguinated to obtain the final sera, which were treated with 0.02% sodium azide and stored at -80°C. Antisera collected post

immunization displayed differential binding to the ecdysteroid-BLG conjugates in the EIA (see below), whereas the preimmune sera did not. Combinatorial EIAs were then used over a range of dilutions for the primary ecdysteroid-BSA antisera, secondary antibody, and E or 20E-BLG conjugates to optimize protocol conditions as described below.

2.2.3. EIA Protocol—For all steps, 96-well microplates (Corning 3590) were covered and held in closed wet chambers to minimize evaporation between solution changes. The protocol began with adsorption of either E-BLG or 20E-BLG in PBS (28 ng of protein conjugate/100 μ l per well) to wells overnight at 4°C. Plate wells were then emptied, rinsed with PBS-T (100 μ l), and emptied again before adding blocking solution (100 μ l/well of 0.1% milk powder in PBS-T; Bio-Rad 170-6404; solution filtered to remove particles prior to use) for 1 h at RT. Wells were emptied and rinsed as above with PBS-T, and then 10 μ l of PBS was added per well to keep the surface wet. Free E or 20E standards (4 to 2000 pg) and samples (50 μ l) were added to replicate wells, followed by diluted primary antisera (50 μ l/well) for all but the nonspecific binding (blank) wells and then incubated overnight at 4°C. Wells were emptied, rinsed two times, and treated with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma SAB3700831) diluted 1:15,000 in 100 μ l of blocking solution for 3 h at RT. Wells were emptied, rinsed with two changes of PBS-T, and treated with substrate (3,3',5,5'-tetramethylbenzidine, 100 μ l/well, KPL 50-76-00) for 20-30 min at 27°C. Addition of 1 M phosphoric acid (100 μ l/well) stopped the reaction so that the absorbance values could be recorded with a plate reader at 450 nm (BioTek MQX200). In addition, each EIA plate has replicate wells designated for secondary antibody alone to monitor nonspecific binding and for ecdysteroid-BLG with no free E or 20E to determine maximum binding of the primary antiserum. Quality control wells with 250 pg of E or 20E were positioned independently of the standard line wells.

The binding characteristics of the new antisera were compared in competitive EIAs to three antisera (named L2, H22 and 482202 from Cayman Chemical Company) previously developed and validated to preferentially bind E and 20E. Our laboratory had also previously used the L2 and H22 antisera to measure E and 20E titers in *A. aegypti* by RIA (Jenkins et al., 1992; Sieglaff et al., 2005; Telang et al., 2007). For the EIAs, the L2 antiserum (Aribi et al., 1997; Blais, et al., 2010; donated by J.-P. Delbecque, Université Bordeaux 1, Talence, France) was diluted 1:14,000. The H22 antiserum (Warren et al., 2009; donated by L. I. Gilbert, University of North Carolina) was diluted 1:7000. The 482202 Cayman was dissolved in 6 ml of PBS plus milk block for a final dilution 1:2 of prepared stock as recommended by the manufacturer.

2.2.4. Specificity assessment—EAB25 and EAB27 were tested for relative specificity by conducting competitive binding EIAs with free 7-dehydrocholesterol, cholesterol, beta-estradiol, corticosterone, makisterone A, ponasterone A, and testosterone (Santa Cruz Biotechnology, Sigma Aldrich). Each compound was dissolved in ethanol and then diluted with water to make 40 pg/ μ l stocks. A standard range of each free steroid or sterol (16 to 1000 pg) was then prepared for incubation with EAB25 antiserum (1:30,000) and bound 20E-BLG or EAB27 antisera (1:4,000) with E-BLG for three replicate EIAs as above.

2.3. Ex vivo ecdysteroid production by larval tissues and ovaries

A. aegypti (UGAL strain) was reared as previously described (Telang et al., 2007; 2010). Newly molted last (fourth) instar larvae were transferred from laboratory rearing pans to small plastic cups and provided with food. Previous studies determined that E and 20E are produced by the body wall of last instar larvae, and that titers peak 30 to 36 h post-ecdysis (Jenkins et al., 1992; Telang et al., 2007). In this study, 30-36 h fourth instars were opened laterally in saline using micro-scissors followed by removal of the head and gut. The remaining larval pelt was then transferred to an upside down cap with saline (60 μ l) that was cut from a 1.5 ml polypropylene microfuge tube and incubated for 6 h at 27°C. During the incubation period, individual caps were housed in wells of a 12-well culture plate (Corning) that each contained 200 μ l of water. Each culture plate was held in a covered plastic box. This setup resulted in no loss of volume from individual caps. The saline from the cap was then collected and frozen. In adult females, ovary production of E and 20E peaks 18 to 24 h post blood meal (PBM; Sieglaff et al., 2005). Ovaries with the last two abdominal segments were dissected from females (3-5 days old) at 24 h PBM and transferred to caps (2 pairs in 60 μ l of saline) cut from 0.6 ml polypropylene microfuge tubes for incubation and collection as above. Fifty μ l aliquots from collected larval pelt and ovary samples were diluted with 200 μ l of ultrapure water, partitioned against 300 μ l of chloroform, and centrifuged for 2 min at 20,000 $\times g$. The aqueous fraction was transferred to a new tube, and the chloroform was repartitioned with 200 μ l of water and centrifuged. The aqueous fractions were pooled and reduced to ~80 μ l in a vacuum centrifuge (Thermo Electron Corp., UVS400; medium setting, 40°C). Samples so prepared were promptly analyzed by EIA. Sample sizes varied between experiments and are given in the figure captions.

2.4. Sample extraction from whole larvae and adult females

Three approaches were used to extract samples from mosquitoes. First, last instar larvae or adult females 24 h PBM (up to 50 per batch) staged as above were homogenized in methanol (95 or 100%, 1 ml) in 1.5 ml polypropylene tubes using plastic pestles, followed by vortexing and centrifugation for 5 min and 13,000 $\times g$. Each supernatant was transferred to another tube, and the remaining pellet homogenized in 500 μ l of methanol and centrifuged. After which, the supernatant was pooled with the first one. The supernatant extracts were dried in a vacuum centrifuge, frozen, and later rehydrated in PBS for EIA or HPLC.

Second, cohorts of 1, 5, or 10 non-blood fed adult females (3 days old) were placed into tubes containing 500 μ g of 20E and 1 ml of methanol for extraction, processing, and drying of the pooled supernatants as described above. After rehydrating the supernatant samples in 1 ml of 5% methanol, each one was passed over an activated C₁₈ SPE cartridge (Phenomenex), rinsed once with 1 ml of 5% methanol, and retained molecules eluted using 1 ml of 100% methanol. As controls, 20E (100 ng) alone or with 30 mM MgCl₂ was dissolved in 1 ml of 95% methanol, held on ice for a few minutes, and then loaded and eluted from C₁₈ SPE cartridges as above. All eluates were dried and reconstituted in PBS for the EIAs or 30% methanol (15 μ l) for MS analysis.

Third, cohorts of 1, 5, or 10 last instar larvae or adult females 24 h PBM were homogenized in 300 μ l of PBS followed by the addition of an equal volume of 1-butanol. Samples were

vortexed until uniformly cloudy and then centrifuged at $20,000 \times g$ for 10 min. The organic phase (top) was collected into a new tube and dried in a vacuum centrifuge. The dried organic extracts were each reconstituted in 200 μ l of chloroform to which 200 μ l of water was added. After vortexing and centrifugation, the water phase was transferred to another tube, and the organic phase was again extracted with water following the same procedure. The water phases were pooled, frozen, and lyophilized to remove trace chloroform. Control samples containing 500 pg of E or 20E extracted in the same manner served as recovery controls. All samples were then reconstituted in PBS for the EIAs.

2.5. Sample analysis from mosquito larvae and adult females

To assess whether EAB25 and EAB27 bound E and 20E from mosquito samples, ex vivo and whole body extracts reconstituted in 200 μ l of PBS were diluted in an equal volume of 10% methanol. Each sample (400 μ l) was injected onto a Spherisorb ODS1 column (4.6×150 mm, Waters PSS830613) and eluted with a gradient (10 – 100% methanol, 60 min, 1 ml/min) on a Beckman Gold 125s/166 HPLC system. Absorbance of the eluted solvent was monitored at 254 nm. E, 20E, and makisterone standards were injected and eluted using the same conditions. Fractions (1 ml/min) were collected for the samples and ecdysteroid standards. Aliquots from the fractions were dried and rehydrated in PBS. Duplicate 50 μ l aliquots from each fraction were then added to plate wells for the EIAs. For a subset of the samples extracted by methanol/ $MgCl_2$ treatment, HPLC fractions containing the major immunoreactive ecdysteroids were diffused into a Bruker Esquire 3000 mass spectrophotometer with APCI while running in negative ion mode. This provided soft ionization of ecdysteroids, giving dominant M-1 values for masses of standards.

2.6. Data analysis

For the EIAs, a regression line for E or 20E was generated by calculating the percentage absorbance for the different amounts of free ecdysteroid in replicate wells of a 96 well plate as a percentage of the maximum absorbance control (StatPlus 5.8.2.0). The standard regression line was used to calculate the quantity of ecdysteroid in experimental samples based on the absorbance of replicate wells. Data from HPLC fraction detection by EIA was prepared in Excel 2011 (Microsoft, 14.7.0). Treatment differences were examined by analysis of variance (ANOVA) followed by a post-hoc Tukey-Kramer Honest Significant Difference Test at the $p < 0.05$ level.

3. Results

3.1. Comparison of antisera for recognition of E and 20E

Sera from the final bleeds of four rabbits were tested for binding to E-BLG and 20E-BLG in the EIA. EAB25 from a rabbit immunized with 20E-BSA exhibited the strongest binding to 20E-BLG, while EAB27 from a rabbit immunized with E-BSA exhibited the strongest binding to E-BLG. Binding curves generated over a range of dilutions indicated that EAB25 used at 1:30,000, EAB27 antiserum used at 1:4000, and coating wells with BLG conjugates at 28 ng/100 μ l was optimal for competitive EIAs (Table 1; Supplementary Fig. S1). As expected, EAB25 and EAB27 also bound to BSA alone but did not exhibit any binding above background to BLG alone (Supplementary Fig. S2). To compare the sensitivities of

EAB25 and EAB27 to the L2, H22, and 482202 Cayman antisera, we generated binding curves over a 4 to 2,000 pg range of free E or 20E standards with their respective BLG conjugate (Fig. 1A, B). EAB25 had the lowest sensitivity for E of the antisera tested but showed similar sensitivity for 20E as H22 and (Fig. 1 A). EAB27 had the highest sensitivity for 20E, and a similar sensitivity for E as H22 and 482202 Cayman (Fig. 1B).

3.2. New antisera preferentially bind E and 20E

We next examined the binding affinities of EAB25 and EAB27 for precursor sterols in E biosynthesis (cholesterol and 7-dehydrocholesterol), related steroids with an unmodified E chain (makisterone and ponasterone, a plant ecdysteroid), or steroids with no E chain (beta-estradiol, corticosterone, and testosterone). Binding curves were generated to each free sterol for comparison to free E and 20E over a 16 to 1,000 pg range with EAB25/20E-BLG and EAB27/E-BLG. EAB25 antiserum bound only 20E (Fig. 2A). EAB27, in contrast, exhibited parallel binding curves with nearly identical high binding to E and 20E and lower binding to makisterone and ponasterone. Neither antiserum recognized 7-dehydrocholesterol as illustrated by its respective flat lines (Fig. 2A, B). Cholesterol, beta-estradiol, corticosterone, and testosterone also were not recognized by either antiserum (data not shown).

Using data from the EAB25 and EAB27 EIAs, we calculated E/20E affinity ratios for each antiserum by dividing the E value for 250 pg by the 20E value for 250 pg (Fig. 2A, B): 0.037 for EAB25 and 0.226 for EAB27. The published affinity ratios for the established antisera are 0.498 for L2 (Pascual, et al., 1995), 0.649 for H22 (Horn, et al., 1976), and 0.307 for 482202 Cayman (Porcheron, et al., 1989). Comparison of these ratios indicated EAB25 had a 27-fold higher affinity for 20E than E, while EAB27 had a four-fold higher affinity for 20E, with L2 and H22 having a two-fold higher and 482202 Cayman a three-fold higher affinity for E than 20E. Additional information about the antisera is given in Table 1.

3.3. New antisera detect E and 20E produced by *A. aegypti* tissues in ex vivo assays

We previously used ex vivo tissue incubations and RIAs to measure titers of E and 20E in medium after culture of 30-36 h old larval pelts prepared from staged *A. aegypti* fourth instars (Jenkins et al., 1992; Telang et al., 2007), or ovaries collected from adult females at 24 h PBM (Hagedorn et al., 1975; Greenplate et al., 1985; Borovsky et al., 1986; Sieglaff et al., 2005). The antisera used in previous RIAs (e.g. L2) could not distinguish between E and 20E. We repeated these assays using the new EIAs to both compare values to prior results and assess the abundance of E versus 20E. Results from the EAB25 and EAB27 EIAs indicated that 20E was present in the medium after ex vivo incubation of both larval pelts and ovaries (Fig. 3). This conclusion was supported by HPLC, which sufficiently separated the 20E and E standards (Fig. 4A, B). With the EAB25 EIA, the content of fraction 31 from larval pelts and ovaries was determined to be 20E, as it was the same fraction that the 20E standard eluted into under the same HPLC conditions. E in fraction 36 (where the E standard eluted) was detected in both samples with the EAB27 EIA but not with the EAB25 EIA. No makisterone was detected in fraction 33 or 34 (where the makisterone standard eluted) of either larval pelt or ovary samples after HPLC. Because of the high threshold of detection for ecdysteroids in our HPLC system (about 100 ng at 254 nm), chromatograms of the

mosquito samples had no peaks in the elution range of relevant steroids, and hence are not shown.

3.4. New antisera also detect E and 20E in *A. aegypti* tissue extracts

Another method for measuring E or 20E from mosquitoes and other insects is extraction from larvae or adults. This is usually done using methanol followed by organic phase separation or SPE over C₁₈ cartridges to enrich ecdysteroid content for measurement by immunoassay. We therefore assessed whether: 1) E and 20E content of *A. aegypti* last instars and adult females 24 h PBM were similar to previously reported data, and 2) the EIAs in combination with HPLC fractionation recognized E and 20E with minimal or no interference from other extracted molecules. Extractions of staged larvae and females with methanol followed by SPE yielded higher than expected values from the EAB25 and EAB27 EIAs. We therefore extracted groups of 1, 5, and 10 non-blood fed adult females in methanol alone or spiked with 500 pg of 20E to determine the efficiency of its recovery as measured with the EIAs. Results indicated that values increased non-linearly, with samples of 5 and 10 females yielding higher than expected titers than extractions from fewer females (Fig. 5A, B). This outcome suggested that homogenization of mosquitoes in methanol altered the immunoreactivity of the extracted E and/or 20E.

A review of the literature indicated that magnesium ions (Mg²⁺) are commonly involved in redox and non-redox catalysis in biological systems, and that the combination of methanol and Mg²⁺ is often used to reduce many types of chemical functional groups, including those found on steroids (Lee et. al. 2004). In all cellular systems, Mg²⁺ is present in the millimolar range with insect hemolymph exceeding 10 mM (Clark and Craig, 1953) independent of diet (Wyatt, 1961). Thus, we questioned whether homogenizing insect bodies in methanol might result in the reduction of 20E and alter its recognition by the new antisera. To examine this, 20E (500 pg) was processed in methanol or water (500 µl) with and without 30 mM MgCl₂. Processing of 20E in water or methanol alone had little to no effect on its recovery or immunoreactivity in both EIAs (Fig. 6). In contrast, adding MgCl₂ to water resulted in both EIAs yielding 20E values that were almost two-fold higher, while adding MgCl₂ to methanol increased values three-to-fourfold. These inflated results were similar to the values for the whole body female samples extracted in methanol with a 20E spike (see Fig. 5). Collectively, these data suggested that methanol/MgCl₂ altered the structure of 20E, which in turn influenced titer estimates generated by the EIAs. This was further supported by MS analysis, which showed a 20-fold decrease in the mass intensity of 20E in methanol/MgCl₂ along with new mass species absent for 20E in methanol alone (Supplementary Fig. S3). The identity and relative immunoreactivity of the new species that formed in methanol/MgCl₂ were not determined.

Other organic solvents have been used to extract E and 20E from insects that do not affect their structure as determined by MS (Hikiba et al., 2013). We found that 1-butanol extracted E and 20E from whole larvae and adult female *A. aegypti*, but an additional step of phase separation was required to remove non-specific EIA material and readily rehydrate samples for use in the EIAs. This procedure gave reliable results and scaled E and 20E recovery for

staged last instar larvae (Fig. 7A, B) and adult females (Fig. 7C, D). More than 95% of the standards extracted in parallel were also recovered (Fig. 7E).

The E and 20E standards plus mosquito samples extracted in 1-butanol were fractionated by HPLC, and the content of fraction aliquots measured with both EIAs. E and 20E standards were recovered within 95% of expected values (600 pg for these aliquots) using the EAB27 EIA (total value for two fractions covering the elution of each standard) (Fig. 8A, B). Makisterone A in contrast was 61% of its value, which was consistent with the lower affinity of the EAB27 antiserum for this ecdysteroid. EAB25 detected two-fold more 20E than present in the fraction aliquots, but not the other standards. The discrepancy in values may be an effect of the methanol carrier or UV light through the flow cell on the 20E standard during HPLC (Canonica et al., 1985; Harmatha et al., 2002), since the EAB25 EIA reliably measured 20E content in aqueous solutions. HPLC of the mosquito extracts resolved 20E as the predominant species (Fig. 8 C, D), but only 45% of the amount estimated to be in the respective samples was eluted. Notably, similar amounts of 20E were measured with both EIAs in the fractions from larval or adult female extracts. No E was detected in the extract from larvae after HPLC (Fig. 8C), but it was present in the extract from adult females (Fig. 8D). No makisterone was detected in the mosquito extracts, and only negligible amounts of other immunoreactive factors were detected in fractions flanking those shown in Fig. 8C and D.

4. Discussion

The primary goal of this study was to develop a low cost, reliable method for measuring E and 20E titers in insects such as *A. aegypti*, because the RIAs primarily used for this purpose are no longer useable due to loss of essential reagents. EIAs offer an alternative for this need. Results of this study show that two new antisera we generated preferentially bind E and 20E or 20E. The EIAs we developed also offer several advantages. First, they are not proprietary, do not require specialized equipment, and the cost of preparing the E- and 20E-BLG conjugates is low. Second the EIAs are flexible because the two antisera can be used with E- or 20E-BLG as the bound competitor and a peroxidase-conjugated secondary antibody as the reporter. Third, the overall costs of these EIAs are approximately one hundred-fold lower than commercially available EIA kits for measuring E and 20E titers (Supplementary Table 1). All EIA protocols, including the one presented here, are similar in that they indirectly quantify the abundance of a given molecule of interest by enzymatic colorimetric reaction. We found that using E- or 20E-BLG as the well coating extends the functional range of the EAB25 and EAB27 antisera beyond the limit of the well surface area, which allows for an economical use of each. However, it also limits the upper range of detection, as only a finite amount of the BLG conjugates adsorb to wells. With a checkerboard titration, we determined this maximum to be around 400 ng for 20E-BLG with the EAB25 antiserum, or 200 ng for E-BLG with the EAB27 antiserum (Supplementary Fig. S1). On the other hand, the quantity of absorbed epitope-protein conjugate does not represent the actual quantity of potential binding sites.

Under the conditions developed for our competitive EIAs, the limit of detection (LOD; defined as 80% maximal binding, I_{80}) for 20E with the EAB25 antiserum and 20E-BLG is

84.2 pg (1.8×10^{-13} moles), and for E with the EAB27 antiserum and E-BLG, is 4.5 pg (9.7×10^{-15} moles). The working range for the EAB25 EIA is 80 to 2000 pg of 20E and for EAB27, 5 to 500 pg of E and 20E. The sensitivity at the lowest values are similar to the commercially available 20E EIA kit (A05120, Bertin Pharma/501390, Cayman Chemical Company), which reports a linear standard range of 39 to 5000 pg/ml of E and 20E, LOD of 31 pg 20E (6.5×10^{-14} moles), and cross reactivity to E (100%) and ponasterone A (43%). As used in our EIA, 482202 Cayman had an I_{80} of 57.3 pg (1.2×10^{-13} moles) for 20E in the presence of 20E-BLG, and 17.9 pg (3.9×10^{-14} moles) for E in the presence of E-BLG. The sensitivity and the working range of the EAB27 EIA for E and 20E is similar to the values reported for the H22 and L2 antisera used in RIAs (Warren et al., 1984; Pascal et al., 1995).

We used the new antisera in EIAs to quantify E and 20E in mosquito larvae and females at times of peak titer to compare with previously reported results generated by RIA. We also confirmed by HPLC the immunoreactive factors in these insect samples were predominantly E and 20E with EIA screening of the fractions. Previous RIA data for *A. aegypti* last instar larvae (30-36 post eclosion) measured 20 to 200 pg of E and 20E per individual in hemolymph, and 80 to 360 pg of ex vivo-produced E and 20E per pelt over 6 h (Jenkins et al., 1992; Telang et al., 2007). Approximately 200 pg of E and 20E per individual was also measured by EIA after batch extraction of larvae using methanol (Margam et al., 2006). The values reported in Fig. 3 for total E and 20E produced ex vivo by pelts are less than two-fold higher than these previous results, while values from extracted larvae are similar. Both E and 20E were present in the media after ex vivo pelt incubation, but 20E was the predominant form extracted from larvae, as quantified with the EAB25 EIA and confirmed by HPLC (Fig. 4A and 8C). The absence of E in the larva extract may be due to the rapid conversion of E produced by tissues 20E, so it is the only circulating form in vivo, or conversion may have occurred during homogenization of the larvae.

Hagedorn et al. (1975) was the first to analyze and quantify the ecdysteroids in *A. aegypti* adult females after blood feeding by using a multistep extraction procedure that began with 60% methanol, which was followed by RIA. These data indicated that individual females at 20 h PBM contain approximately 250 pg of E and 20E. Using HPLC and gas-chromatography, the authors further showed that ovaries from blood-fed females predominantly secreted E in ex vivo assays while extracts from whole females predominantly contained 20E, which supported the conclusion that E is converted to 20E in vivo. A subsequent study reported that similarly staged adult females after a single-step 50% methanol extraction contained on average 600 pg of E plus 20E although absolute values varied between replicates (Greenplate et al., 1985). A third study, using a different extraction method, also measured ~600 pg of E plus 20E in the body of similarly staged *A. aegypti* by RIA, while HPLC and thin layer chromatography identified only E in ovaries but two-fold more 20E than E in whole bodies (Borovsky et al., 1986). Our own previous studies have primarily focused on ex vivo production of E plus 20E by ovaries from adult female *A. aegypti*. Results generated by RIA indicate that ovaries from females 18-30 h PBM produce 100-120 pg of E plus 20E when incubated in the same saline used in this study (Sieglaff et al., 2005). With the EAB27 EIA, E plus 20E produced ex vivo by ovaries were almost six-fold higher, but the amount extracted from an adult female was very similar to previously

reported values (Fig. 3). Results from the current study also indicate that 20E is present in both the ovary incubation medium and whole body extracts (Fig. 3, Fig. 4B and 8D).

Altogether, the differences between values reported here versus previous studies likely reflect the use of different antisera and assay formats. The antisera used for RIAs in the earlier studies of mosquitoes differed in their relative recognition of E and 20E, so values were reported as total E plus 20E. The RIA we previously used to measure E plus 20E in *A. aegypti* was based on the L2 antiserum. The new EIA described here using EAB27 measures E plus 20E with similar binding affinity for each while EAB27 preferentially binds 20E. Together, data from the two EIAs can be used to generate estimates for the amount of E and 20E in a sample. The relative amount of circulating E and 20E is important because binding to the ecdysteroid receptor (EcR)-Ultraspiracle (USP) complex likely differs between these ligands (Wang et al., 2000; Heinrich, 2012). Homogenization of insects in methanol followed by its evaporation is widely used for the extraction of E, 20E and other ecdysteroids from insects including mosquitoes (Wilson et al., 1990; Margam et al., 2006). In contrast, our results indicated methanol was not the best choice for E and 20E extraction from whole *A. aegypti* due to interactions with Mg^{2+} . We did not find any reference to this interaction in the insect literature at-large, or to any prior studies that compared the efficiency of methanol for extraction of endogenous ecdysteroids from whole insect bodies alone or with known amounts of added ecdysteroids. In contrast, directed reduction of insect steroids has been used to enhance identification (Dinan and Rees, 1978; Savchenko and Odinokov, 2012) while prior studies also provide evidence for reductive cleavage of hydroxyl groups under specific conditions (Horn and Bergamasco, 1985; Harmatha, et al., 2002). Our MS data showed the presence of multiple species rather than a single identifiable reduced form after treatment of 20E with methanol and $MgCl_2$ (Supplementary Fig. S3). One possibility is that these multiple species are immunoreactive and account for the anomalous values obtained from the EIAs. Another is that extraction results in the methanol/ Mg^{2+} reduction of other endogenous sterols that are recognized by the antisera we used in the EIAs.

Regardless of why methanol extraction elevated E and 20E values, we now avoid its use with mosquitoes and recommend that extractions be done macerating samples in PBS or water, followed by 1-butanol extraction and water/chloroform phase separation to purify ecdysteroids from the other lipids. This approach did not alter the immunoreactivity of known ecdysteroid standards, and consistent values for E and 20E were obtained from one to ten last instar larvae or adult female mosquitoes. Extraction of larger numbers of mosquitoes was less efficient, because a larger pellet rests between the butanol-water phases, reducing the volume of retrievable butanol, which contains E and 20E. As a side note, butanol was the solvent originally used in preparing extracts from insects that led to the structural characterization of E (Karlson, 1996). In using this EIA, we also suggest that known amounts of E and 20E be processed with or without mosquitoes to monitor efficiency of recovery and any changes in immunoreactivity. Further studies will help to resolve whether methanol extraction of insects that have high Mg^{2+} concentrations is a significant problem for the quantification of E plus 20E by EIAs in other insects. In conclusion, the EIA protocol described here takes advantage of the high specificity of the EAB25 antiserum for 20E, which is unique among the available antisera. We further demonstrate that our EIAs in

combination with HPLC provide quantification of E and 20E in samples prepared from *A. aegypti*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A new enzyme-linked immunoassay (EIA) for two ecdysteroids was developed.
- Antisera used in the EIA preferentially recognized different ecdysteroids.
- Ecdysteroid titers in mosquito larvae and adults were measured with the EIA.
- 1-butanol was more suitable than methanol for extracting samples from mosquitoes.

Figure 1.

Comparison of EAB25, EAB27, H22, L2, and 482202 Cayman antisera recognition of free ecdysone (E) and bound E-bovine lactoglobulin (BLG) (A) or free 20-hydroxyecdysone (20E) and bound 20E-BLG (B). The standard range is 4-2000 pg free E or 20E. Antisera dilution: EAB25, 1:30,000; EAB27, 1:4,000; H22, 1:7,000, L2, 1:14,000, 482202 Cayman (Cayman) recommended concentration (actual concentration undisclosed). Each standard value on the curve represents the mean of 6 replicates $\pm$ standard error (S.E).

Figure 2.

EAB25 and EAB27 antisera binding affinity for different free steroids. (A) EAB25 (1:30,000 with bound 20E-BLG) strongly preferentially bound 20E. (B) EAB27 (1:4000 with E-BLG bound) recognized 20E and E equally while also recognized makisterone and ponasterone. Neither antiserum recognized 7-dehydrocholesterol. Non-specific binding of EAB25 and EAB27 are $5.2 \pm 0.5\%$ and $5.1 \pm 1.7\%$ of maximum binding respectively. Each value on the curve represents the mean of 3 replicates $\pm$ S.E. over a standard range for each free steroid of 4 to 1000 pg. Cholesterol, beta-estradiol, corticosterone, and testosterone were tested identically to the sterols shown but were omitted from the graphs because they produced flat lines that were identical to 7-dehydrocholesterol.

Figure 3.

E and 20E are detected in ex vivo assays and extracts of *A. aegypti*. Pelts from last instar larvae (30-36 h post ecdysis) and ovaries from females (24 h post blood meal, PBM) were incubated for 6 h in saline, afterwards the saline samples were phase separated with chloroform. Whole bodies of similarly staged larvae and females were extracted in 1-butanol followed by chloroform/water phase separation. Aqueous samples were diluted in PBS to represent a single individual and then quantified with the EAB25 (black bars) and EAB27 (white bars) EIAs, which primarily recognize 20E or E plus 20E, respectively. Each bar represents the mean value of 9 replicates $\pm$ S.E. for each treatment.

Figure 4.

Identification of immunoreactive E and 20E in HPLC fractionated samples from ex vivo cultures of *A. aegypti* (A) last instar body walls (30 to 36 h post eclosion) or (B) ovaries from females 24 h PBM. Tissues were incubated in saline for 6 h. Samples were then pooled and subjected to HPLC. 20E (*) and E (**) eluted in the designated fractions was determined by loading and elution of E and 20E standards along with makisterone (100 ng each) in a later HPLC run. The amount of E and 20E in the fractionated aliquots was determined using the EAB25 and EAB27 EIAs. Each bar represents the mean value of two replicates.

Figure 5.

Amounts of E and 20E extracted from 1, 5, or 10 non-blood fed *A. aegypti* females using 95% methanol. Extracted samples were analyzed with or without a spike of 20E (500 pg) using the (A) EAB25 or (B) EAB27 EIA, which primarily recognize 20E or E plus 20E, respectively. Samples were diluted to remain within the EIA standard range. Note that the Y axes of the graphs are presented as a Log2 scale to capture the wide range of values presented. Each bar represents the mean value of 8 or 9 replicates $\pm$ S.E. for each treatment.

Figure 6.

The amount of 20E measured with the EAB25 and EAB27 EIAs differs between samples prepared in water or 95% methanol (MeOH) with MgCl₂ (100 mM). Each sample contained a known amount of 20E (500 pg). Aqueous samples (H₂O) were directly analyzed by EIA, while the methanol samples were dried and rehydrated before analysis. Letters indicate a statistical difference between groups at $p < 0.05$. EAB25 $F_{4, 39} = 51.26$; EAB27, $F_{4, 39} = 31.14$, $P < 0.0001$. Each bar represents the mean value of 8 replicates $\pm$ S.E. for each treatment.

Figure 7.

Total E plus 20E extracted with 1-butanol and water/chloroform phase separation from 1, 5, and 10 (A,B) last instar larvae 30-36 h post eclosion, (C,D) female 24 h post blood meal, and (E) ecdysone (E) and 20-hydroxyecdysone (20E) standards (145 pg), as measured with the EAB25 and EAB27 EIAs, which primarily recognize 20E or E plus 20E, respectively. Samples were diluted to remain within the EIA standard range. Each bar represents the mean value of 9 replicates $\pm$ S.E. for each treatment.

Figure 8. HPLC fractionation of ecdysteroid standards and whole body extracts from *A. aegypti* followed by quantification using the EAB25 or EAB27 EIA. (A) Absorbance profile (254 nm) for the elution of 20E (*), makisterone (***) and E (**) standards (100 ng each). (B) Quantity of ecdysteroid detected in 600 pg equivalent aliquots of each fraction from A as measured by the EAB27 or EAB25 EIA. (C) HPLC fractionation and quantification of 20E and E in extracts of *A. aegypti* last instars (30 – 36 h post eclosion). (D) HPLC fractionation and quantification of 20E and E in extracts of *A. aegypti* adult females (24 h PBM). Larvae and adult females were extracted in butanol.

Characteristics of the antisera used for competitive EIAs with free 20-hydroxyecdysone (20E) or ecdysone (E) and 20E or E conjugated to bovine beta-lactoglobulin. EAB25 and EAB27 antisera data presented herein. L2 antisera was produced and characterized, as described in Pascual et al. 1995. H22 antisera was produced, as described in Horn et al., 1976, and characterized by Warren and Gilbert, 1986. Information about the 482202 Cayman is referenced as Porcheron et al., 1989 in the EIA manual from Cayman Chemical Co.

Table 1

Antiserum	Source	Antigen	Dilution	E/20E specificity ratio	Makisterone A cross reactivity
EAB25	Current study	20E-6-BSA	1:30,000	0.037	< 0.1%
EAB27	Current study	E-6-BSA	1:4,000	0.226	5%
L2	Delbecque	E-thyroglobulin	1:14,000	0.498	9%
H22	Gilbert	E-22-thyroglobulin	1:7,000	0.649	22%
482202 Cayman	Cayman Chemical Company	20E-6-BSA	1:2	0.307	4%