General and Comparative Endocrinology 253 (2017) 60–69

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

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

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ARTICLE INFO

Article history:
Received 30 May 2017
Revised 8 August 2017
Accepted 29 August 2017
Available online 1 September 2017

Keywords:
Steroid hormone
Arthropod
Insect
Development
Reproduction

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.

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., 2009; Marchal et al., 2010; Smith and Rybczynski, 2012; Henrich, 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-^{3}H(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

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http://dx.doi.org/10.1016/j.ygcen.2017.08.028
0016-6480/

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 (Montigny-le-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 1X (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 C_{18} 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 µl per well) to wells overnight at 4 °C. Plate wells were then emptied, rinsed with PBS-T (100 µl), and emptied again before adding blocking solution (100 µl/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 µl of PBS was added per well to keep the surface wet. Free E or 20E standards (4–2000 pg) and samples (50 µl) were added to replicate wells, followed by diluted primary antisera (50 µl/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 µl 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 µl/well, KPL 50-76-00) for 20–30 min at 27 °C. Addition of 1 M phosphoric acid (100 µl/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 non-specific binding and for ecdysteroid-BLG with no free E or 20 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