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Three new antisera with high sensitivity to ecdysone, 3-dehydroecdysone and other A-ring derivatives: Production and characterization

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Abstract. Three new antisera have been produced in rabbits immunized with E-2/3-hemisuccinate conjugates. In a competitive enzyme immunoassay (EIA) with 20-ecdysone-peroxidase as a tracer, the antisera exhibit high specificity towards the side chain of the ecdysone (E) molecule. Modifications of the A-ring do result in no or small changes of sensitivity.

One antiserum (RUD-2) allows the direct quantification of total ecdysteroids in samples containing E and 3-dehydroecdysone in varying ratios, without further sophisticated sample treatment. At least one of the other antisera allows the direct quantification of mixtures of E and 25-deoxyecdysone (RUD-1). Thus, these antisera may be a useful tool in studying molting control in arthropods.

INTRODUCTION

Since the isolation of ecdysone (E) (Butenandt & Karlson, 1954) it was believed for a long time that mainly this hormone is produced in the molting glands of most arthropods. More recently it has been shown that at least in vitro molting glands of some arthropods also secrete other ecdysteroids than E (for review see Grieneisen, 1994; Lachaise et al., 1993). The molting glands of several insect and crustacean species in vitro produce a mixture of E and 3-dehydroecdysone (3DE) (Warren & Gilbert, 1988; Spaziani et al., 1989; Kiriishi et al., 1990; Blais & Lafont, 1991; Sonobe et al., 1991; Böcking et al., 1993), the relative concentrations of which may vary during the molting or reproductive cycle (Smith et al., 1993).

Exact quantification of the total ecdysteroid content is a prerequisite of investigations on molting gland regulation. The most sensitive quantification methods are the well-established immunoassays. Above all, the specificity of these methods is dependent on the kind of antisera. Except for the S3-antiserum (Kiriishi et al., 1990), no antiserum has been described that displays the same specificity for E and 3DE. The method of production and the properties of the S3-antiserum were not extensively detailed, but it was shown that its affinity to E and 3DE was the same in RIA. However, using more common antiecdysteroid antisera (see Reum & Koolman, 1989) may lead to incorrect quantification due to varying E/3DE ratios and for determination of the total amount of ecdysteroids in biological

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samples it has been necessary to measure the samples twice with two different antisera, bearing different cross-reactivity factors for E and 3DE. Alternatively, prior to immunoassay, 3DE can be reduced to E with lepidopteran hemolymph (Smith et al., 1993). Both methods are rather time-consuming when measurement of large numbers of samples is required.

In the present paper we report the production of three new polyclonal rabbit antisera termed RUD-1, -2 and -3. They have been developed with the primarily aim to gain antisera for one step ecdysteroid quantification of samples containing E and 3DE. These antisera were obtained after immunization of rabbits with E-3/2-hemisuccinate that has been coupled to thyroglobuline. The antisera were characterized in an EIA using a conjugate of 20E-2-hemisuccinate and peroxidase as tracer.

MATERIAL AND METHODS

Chemicals

Ecdysone (E), 20-hydroxyecdysone (20E) and makisterone A (MaA) were obtained from Simes (Milan, Italy). 2-deoxyecdysone (2dE) and ponasterone A (PoA) were generous gifts from Dr D. Horn (Acheron, Australia). Turkesterone was a gift from Dr A. Suksamrarn (Bangkok, Thailand). Makisterone C (MaC), poststerone, 20,26-dihydroxyecdysone (20,26E), inokosterone, 22-iso-E, triol (22,25dE) and ketodiol (2,22,25dE) were obtained from various sources (Lafont & Wilson, 1992). 3-dehydro-derivates (3DE, 3D20E and 3D2dE) were prepared from corresponding compounds by chemical oxidation (Girault et al., 1989). 5alpha-isomer was obtained from E on equilibration under alkaline conditions (Horn, 1971). 25-deoxyecdysone (25dE) was prepared according to Píš et al. (1994). 3H-ecdysone was prepared from tritium labeled 2dE, generous gift of Drs C. Hétru and J.A. Hoffmann, Strasbourg (F). All other reagents were of analytical grade.

Abbreviations of ecdysteroids are given according to Lafont et al. (1993).

Preparation of immunogen

Succinate was coupled to E (a portion of which was tritium labeled) according to Soumoff et al. (1981) producing E-3-hemisuccinate as well as E-2-hemisuccinate. Since the two products occur in an equilibrium, silicagel chromatography can only increase the concentration of E-3-hemisuccinate but does not exclude the reformation of the C-2 conjugate.

Conjugation to bovine thyroglobulin (Sigma) followed the method of Horn et al. (1976). 12 mg E-hemisuccinate and 33.7 mg bovine thyroglobulin yielded 25.3 mg of the conjugate. The coupling rate determined by measuring of radioactivity was 160 mol ecdysone per mol thyroglobulin.

Immunization

Three male New Zealand rabbits received one injection of 2 mg antigen followed by three booster injections of 1.5 mg. Antigen was emulsified in 750 µl phosphate buffer (50 mM, pH 7.4, 0.9% NaCl) and 750 µl Freund's adjuvant (complete adjuvant for the first injection, incomplete for boosters) and injected intra- and subcutaneously. Bleedings were taken from the earveins. The antisera of the three different rabbits are termed RUD-1, RUD-2 and RUD-3. Thimerosal (Merck) was added to a final concentration of 0.01%. The crude antisera were lyophilized and stored at –80°C.

Tracer

Synthesis of 20E-2-succinate coupled to peroxidase has been as described elsewhere (Delbecque et al., in prep.). A partial isomerisation to the 3-succinate cannot be excluded.

Enzyme immunoassay (EIA)

The competitive EIA followed the protocol of Porcheron et al. (1989) with minor modifications according to Delbecque et al. (in prep.). In brief: the anti-ecdysteroid antibodies (primary antibodies) are immobilized to microtiteplates (Nunc maxisorp) by goat anti rabbit IgG. During a 3 h incubation period, ecdysteroid sample and tracer compete for binding sites at the primary antibodies. After plate washing the remaining peroxidase is assayed by a 20 min color reaction using tetramethylbenzidine as substrate. Reaction is stopped with H_2SO_4 and the absorption is measured at 450 nm. After subtraction of non specific

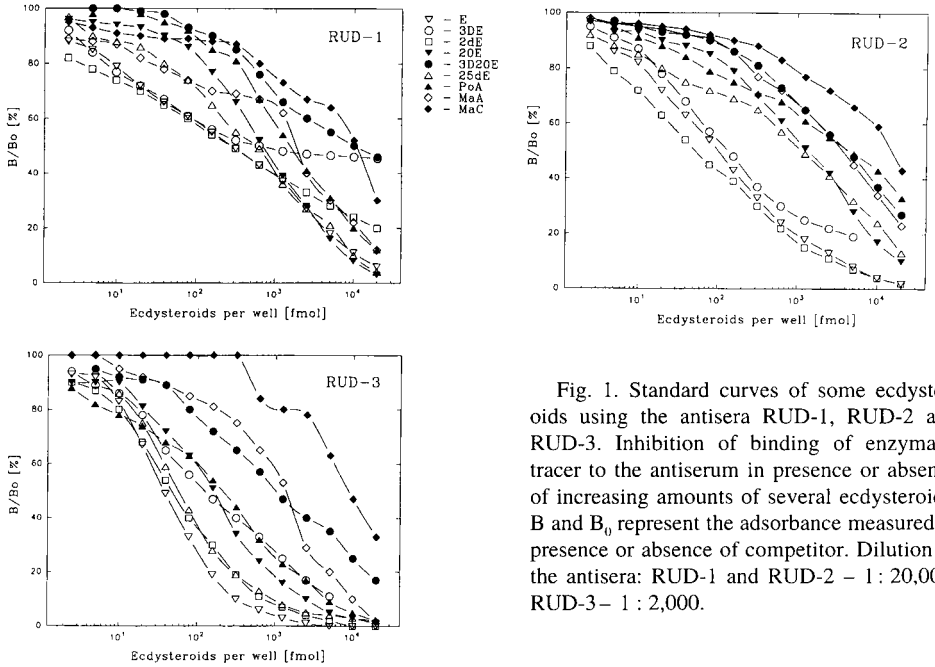

Fig. 1. Standard curves of some ecdysteroids using the antisera RUD-1, RUD-2 and RUD-3. Inhibition of binding of enzymatic tracer to the antiserum in presence or absence of increasing amounts of several ecdysteroids. B and B_0 represent the adsorbance measured in presence or absence of competitor. Dilution of the antisera: RUD-1 and RUD-2 – 1 : 20,000; RUD-3 – 1 : 2,000.

binding (NSB) results are expressed as $(B/B_0) \times 100$. B and B_0 represent the absorption values in presence or absence of ecdysteroid sample, respectively. NSB was always less than 6% of B_0.

RESULTS AND DISCUSSION

Titer of the antisera and precision of the test

Optimal dilution of the antisera was determined from titration curves. RUD-1 and RUD-2 were used at concentrations of 1 : 20,000, whereas RUD-3 was used at 1 : 2,000 dilution. The variability of B_0 is less than 5%. Thus, a reliable detection limit can be set at 80% maximal binding (I_{80}). Intra-assay variance [(standard deviation/mean) × 100] was determined with samples of 3DE (n = 10). In the range of 3.9 to 1,000 pg, covering the range of usual biological samples, it is less than 8% for RUD-1 and RUD-2 or 12% for RUD-3.

Sensitivity and specificity

Figure 1 exhibits standard curves of some zooecdysteroids for the three antisera (in the range of 2.4 to 20,000 fmol). In Table 1 the sensitivity [B/Bo = 50% (I_{50})] of the antisera to E and 3DE is given as well as the detection limit (I_{80}).

RUD-3 shows the highest sensitivity to E, RUD-1 the lowest. Compared with the sensitivity of antisera characterized in EIAs by Porcheron et al. (1989), Royer et al. (1993) and Delbecque et al. (in prep.), RUD-3 is in the same range of sensitivity. For practical use, the sensitivity of all antisera is sufficient to determine the titers in usual biological samples. Of more interest is the specificity, expressed as cross-reactivity factors (cr-factors) to E [I_{50}(test steroid)/I_{50}(ecdysone)] Table 2 presents the cr-factors of some ecdysteroids, calculated from standard curves. In addition to the zooecdysteroids shown in Fig.

1, we also tested other steroids (zoo- and phytoecdysteroids, as well as putative biosynthetic intermediates) in order to examine possible effects of specific derivations.

TABLE 1. Sensitivity (50% maximal binding; I_{50}) and detection limit (80% maximal binding; I_{80}) of the three antisera for E and 30E.

	I_{50} (fmol)		I_{80} (fmol)	
	E	3DE	E	3DE
RUD-1	300	580	8.8	7.0
RUD-2	105	135	11.5	16.5
RUD-3	38	130	11.0	17.0

As expected from the antigen used, all three antisera exhibit high specificity towards ecdysteroids having an unmodified E side chain. In contrast to that, modifications in proximity of the coupling side (C-3/C-2) result in no or small changes of sensitivity. However, there are exceptions of this general rule. For example, RUD-1 and RUD-3 display also high sensitivity to 25-deoxyecdysone (25dE).

In conclusion, we have produced one antiserum (RUD-2) optimal for the quantification of E/3DE containing samples and at least one (RUD-3) that permits quantitative measurements of E/25dE containing samples. Simultaneous secretion of E/25dE is observed for brachyuran crustaceans, and the ratio changes dramatically during molting cycle (Saïdi et al., 1994).

Thus, our antisera may provide promising tools to unravel the physiological significance of the appearance of distinct ecdysteroids under a variety of internal (and external) conditions in arthropods.

TABLE 2. Cross reactivity factors of ecdysteroids in relation to ecdysone [I_{50}(ecdysteroid)/(ecdysone)].

	RUD-1	RUD-2	RUD-3
Ecdysone (E)	1.0	1.0	1.0
3DE	2.1	1.3	3.4
2dE	1.0	0.5	1.3
20E	2.4	12.8	4.5
3D20E	33.3	38.5	26.3
25dE	1.9	10.5	1.5
PoA	5.1	43.5	5.5
MaA	5.8	34.5	38.5
MaC	37.0	13.9	250.0
3D2dE	1.7	1.1	2.5
5alphaE	12.3	3.1	6.3
20,26E	18.5	1,000.0	83.3
Inokesterone	5.8	40.5	15.6
Turkesterone	50.0	111.0	13.9
22-iso-E	18.5	37.0	111.0
Triol	300.0	1,000.0	500.0
Ketodiol	300.0	1,000.0	500.0
Poststerone	55.6	1,000.0	333.0

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