RAPID HETEROLOGOUS HAPTENE RADIOIMMUNOASSAY FOR INSECT MOULTING HORMONE

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1. Introduction

Several recent studies suggest that moulting hormone (MH) is involved in regulating both moulting and ecdysis in insects. Although this hormone has been shown to effect dramatic changes in the metabolism of macromolecules, little is known about the mechanisms by which these changes are elicited. In addition, nothing is known about the controls involved in the synthesis of the hormones themselves.

The hormones, ecdysone and ecdysterone, have been shown to have specific and different effects on different tissues, but their precise roles in metamorphosis are still not fully understood. It is possible that a detailed study of their titers during developmental stages in which specific effects caused by them are known, will help to elucidate the roles played by the two hormones.

Until recent years, bioassay was the only method available for determining ecdysone titers. Micromethods for determining ecdysone titers have been recently reviewed [1]. The radioimmunoassay (RIA) proved to be the most suitable technique for the examination of the physiological MH levels of insects [2–6]. The specificity of the RIA technique, coupled with TLC, provides perhaps the most elegant means for determining the presence of molecules of a specific structure.

All moulting hormone RIA methods described to date are homologous RIA systems, i.e., they utilize the same steroid as antigen and as tracer. The system we describe uses polypodine B for the immunization and ecdysone as the tracer and is thus a heterologous system. The antigen used is the conjugate of RSA and the 6-oxime derivative of the steroid, the side chain acting as antigenic determinant. The conditions of assay are such that the time of assay is 50 min as compared to previous systems which generally required a day.

This paper is the first part in a series on moulting hormone levels of insects

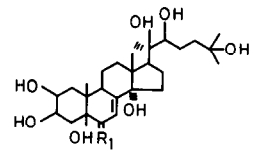
2. Materials and methods

2.1. *Chemicals and solvents*

Polypodine B (I) was isolated from *Polypodium vulgare* according to Jizba [7]. Ecdysterone (VI) and inokosterone (VII) were purchased from Rohto Co., ecdysone (V) from Schering A.G., Berlin. Ponasterone A (VIII) was a generous gift of D. H. S. Horn, 2β,3β-dihydroxy-5βcholest-6-one (IX), 2β,3β dihydroxy-5αcholest-6-one (X) were synthesized by F. Hodosan, and 3β,5β,6β tryhydroxycoprostane (XI) was synthesized by I. Vincze. Carboxymethoxilamine hemihydrochloride (CMA, Aldrich), dicyclohexylcarbodiimide (DCC, Fluka), rabbit serum albumin (RSA, Serva) were used without further purification. [23,24-^{3}H]Ecdysone (spec. act. 68 Ci/mmol), kindly provided by J. D. O'Connor, was used as tracer. All solvents used were of analytical grade and obtained from Reanal, Hungary.

2.2. *Antigen synthesis*

Polypodine B-oxime–RSA (IV) antigen was synthesized for the immunization.

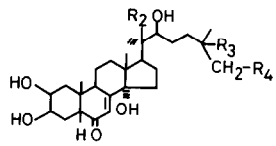


I. R_1: O

II. R_1: =N-O-CH_2-COOH

III. R_1: =N-O-CH_2-COO-N

IV. R_1 =N-O-CH_2-CONH-RSA

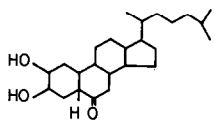


V. R_2=R_4=H; R_3=OH

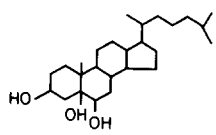
VI. R_2=R_3=OH; R_4=H

VII. R_2=R_4=OH; R_3=H

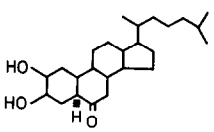
VIII. R_2=OH; R_3=R_4=H



IX.



XI.



X.

Scheme 1

2.2.1. Polypodine B-carboxymethoxime (II)

Polypodine B, 10 mg ((I) 20 μmol) and 23 mg CMA (105 μmol) were dissolved in 670 μl absolute pyridine and the reaction mixture was incubated at 60°C overnight. Pyridine was removed by evaporation under reduced pressure. The residue, dissolved in the water phase of a butanol/cyclohexane/water (9:1:10) biphasic system, was chromatographed on a reverse phase hydrophobe celite column [8]. Fractions, 7.5 ml, were collected, monitored by ultraviolet light and analysed by thin-layer chromatography (TLC, silica gel; chloroform/methanol/water, 60 : 30 : 5). The polypodine B-oxime (II) fractions were pooled, lyophilized and characterized by ultraviolet and infrared spectra. The conversion of polypodine B to the oxime form was measured colorimetrically using 2 ml 50% sulphuric acid with 0.5% vanillin in ethanol as a reagent and 0.1 ml sample. At 630 nm polipodine B gives an intense green colour, whereas the oxime (II) does not.

2.2.2. Polypodine B-oxime–RSA coupling

Polypodine B-oxime, 4 μmol, were dissolved in 40 ml absolute dioxane containing 10% dimethyl-sulphoxide. 0.48 mg *N*-OH–succinimide (4.16 μmol) and 0.84 mg DCC (4.06 μmol) were then added. After two days of continous stirring at room temperature the solvent was removed under reduced pressure and 10 mg RSA (0.147 μmol), dissolved in 2 ml 0.2 M phosphate buffer, pH 7.0, were added in order to couple the oxime to the RSA. After 4 h stirring at room temperature the small molecules were removed by one hour's dialysis in a Bio-Fiber 50 Beaker (Bio Rad Lab). The protein was precipitated at 4°C by the addition of 1.5 vol. acetone, concentrated by centrifugation, and lyophilized. The ultraviolet spectrum of the antigen was used to determine the steroid–protein ratio. Protein measurements were made according to standard Lowry procedure [9].

2.3. *Immunization procedure*

Three white New Zealand rabbits (1.5 kg) were immunized intramuscularly and subcutaneously with a suspension of 1 mg antigen in 0.5 ml physiological saline and 0.5 ml complete Freund's adjuvante. Three and five weeks later booster injections, each containing 0.5 mg antigen in the above mixture, were given. Ten days after the second booster injection the rabbits were bled and the serum prepared.

Immunoglobulin (Ig) was purified with serial ammonium sulphate precipitation according to Kabat [10]. The Ig was stored either lyophilized or dissolved in RIA buffer in 2 ml aliquots at −20°C.

2.4. *Standard RIA procedure*

Tracer, 20 μl, (4000 cpm) and 20 μl test steroid solution in ethanol were introduced into small plastic tubes (Eppendorf reaction vial) and evaporated under nitrogen stream. Diluted serum 100 μl or Ig solution 100 μl in RIA buffer (0.9% NaCl, 6% foetal bovine serum, 0.1% merthiolate in 0.05 M borate buffer, pH 8.4) were added, stirred and incubated for 25 min at 38°C, followed by 5 min at 0°C to stop the reaction. Ice-cold saturated ammonium sulphate, 100 μl, was added, stirred vigorously, and after 10 min incubation at 0°C the tubes were centrifuged at 1500 × *g* for 5 min. The precipitate was washed with 50% saturated ammonium sulphate in 0.05 M borate buffer (pH 8.4). The radioactivity of the washed precipitate was determined in a Tritone X-100 toluene scintillation cocktail (counting efficiency 26%) in a Packard Tricarb 3200 liquid scintillation counter.

3. Results and discussion

We found that the polypodine B-carboxymethyloxime production is optimal at 1:5 ratio of polypodine B and CMA, when conversion to the oxime reaches 73%.

The reverse phase chromatography (fig.1) was found to be effective in separating the oxime from unreacted polypodine B and CMA. The polypodine B-carboxymethyloxime has 0.25 R_F value and an ultraviolet absorption maximum at 255 nm (ϵ 10 000) in ethanol. In the infrared spectrum it has characteristic signals at 1600, 1324 and 1080 cm^{-1}.

The oxime derivative coupled to RSA by the hydroxysuccinimide reaction [11] was found to have a haptene density of 8 steroid molecules/RSA molecule, which is sufficient for successful immunization [12].

The purified immunoglobulin at 1.8 mg/ml concentration or the serum at 98-fold dilution binds 50% of the tracer. This is comparable to that obtained for an homologuos system [2].

Figure 2 shows a typical calibration curve. The sensitivity of the assay calculated according to Midgley [13] is 80 pg (1.74×10^{-13} M). The dissociation constant is 9.56×10^{-8} M, and the number of binding sites is 7.73×10^{-9} M as determined from a Scatchard plot. The calibration curve was linearized for practical use by the help of the logit-log transformation.

The cross reactivity of the serum is shown in table 1. As expected, polypodine B proved to be a more effective competitor than ecdysone and ecdysterone. The hydroxyl function at position 25 apparently plays an important role in the interaction between the Ig and hapten, since either the absence of the 25 hydroxyl function (ponasterone A (VIII)) or the presence of a hydroxyl group at the position 26 instead of 25 (inokosterone (VII)) significantly alters the competition of the compound with the radioactive ecdysone. The absence of the 20 hydroxyl function (ecdysone (V)) does not show this effect. The molecules which have only the 2β,3β-diol, 6-one or 3β,5β-diol system are not effective competitors.

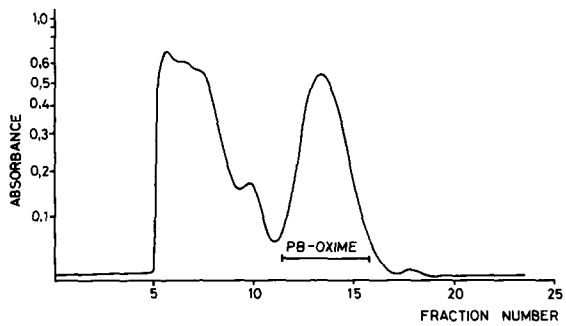


Fig.1. Purification of polypodine B-carboxymethoxime by chromatography on a hydrophobe celite column (1.5 × 20 cm). Fraction vol. 7.5 ml.

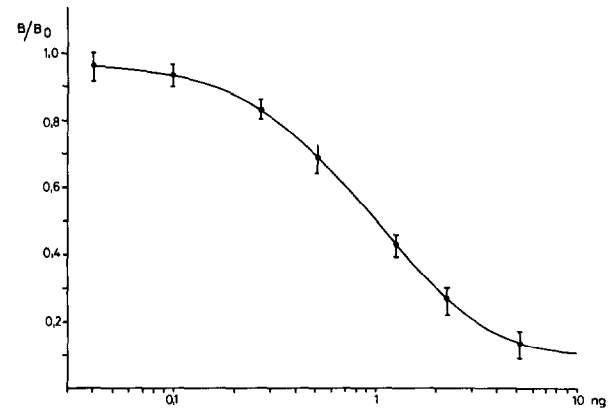


Fig.2. Inhibition of labeled ecdysone binding to antiecdysone immunoglobuline (2 mg/ml) in the presence of increasing amount of unlabeled ecdysone. (B_0) Bound radioactivity in the absence of the competitor. (B) Bound radioactivity in the presence of the competitor.

Table 1

		Nanograms required 50% inhibition of [^{3}H]ecdysone binding
Polypodine B	(I)	0.109
Ecdysone	(V)	1.004
Ecdysterone	(VI)	1.200
Inokosterone	(VII)	6.17
Ponasterone A	(VIII)	72.50
(IX)		a
(X)		a
(XI)		a

[a] 50% Inhibition was not reached at a concentration up to 1000 ng

Thus the antiserum is more specific to the hydroxylated side chain than to the tetracyclic nucleus of the ecdysones, and in this respect is similar to other 6-oxime RIA systems [2,5].

At 38°C the equilibrium of the RIA system is achieved within 10 min: 25 min incubation was chosen for the routine assay. The total length of the assay, including the separation of the bound and free reactants, is 50 min. By making 32 parallel assays, it is possible to perform as many as 200–300 assays a day.

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