RADIOIMMUNOASSAY FOR THE SEX INDUCER OF *VOLVOX CARTERI* F, NAGARIENSIS

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1. Introduction

The Volvocales are colony-forming green algae consisting of two types of cells: up to several thousand somatic cells form the sheath of a spheroid, enclosing within a gel matrix the gonidia which are capable of sexual or asexual reproduction. Sexual development is induced by a hormone [1]. This was first described in a strain of *Volvox aureus* [2], and later in many other species of *Volvox* [3–6]. In *Volvox carteri* f. nagariensis the sex inducer is secreted into the medium by the mature male strain. The hormone was shown to be a glycoprotein of 28 000 mol. wt composed of one polypeptide chain [7,8]. When the sex inducer is added to vegetative female colonies their gonidia develop into eggs in the next generation. This induction phenomenon can be used to quantitate the hormone [9] by adding vegetative female colonies to a decadic dilution series of a solution containing the sex inducer; after two successive lifecycles only the tube with the threshold dilution will contain both sexual and asexual offspring indicating that the hormone was present in limiting concentration. Up to now this bioassay is the sole method to determine the concentration of the sex inducer in a fluid. Although being very sensitive (10^{-10} g/l stimulates 100% formation of sexual embryos) it is not satisfying for the following reasons:

(i) The high sensitivity of the bioassay requires that test samples have to be diluted in many successive steps (e.g., $1{:}10^8$ for an expected hormone concentration of 1 μg/ml which is an average concentration of inducer in the media) and therefore dilution errors potentiate;
(ii) The bioassay is time consuming; and
(iii) Strongly dependent on exactly reproducible conditions of the vegetative female colonies;
(iv) The test is easily disturbed even by traces of contaminating inducer in buffers or media.

Here we establish an alternative rapid and quantitative determination of the sex inducer that avoids the drawbacks of the bioassay. The test is based on the principle of a radioimmunoassay (RIA) employing radioiodinated rabbit antibodies. The RIA detects 30 ng sex inducer/ml and is highly hormone specific. In the course of this work [10] we made the surprising observation that on introducing a radioactive label into the sex inducer, the latter, although not degraded by iodination, loses its immunological and biological activity almost entirely.

2. Materials and methods

2.1. *Preparation of sex inducer*

The female strain HK-10 and the male strain 69-1b of *Volvox carteri* f. nagariensis Iyengar were generously provided from the Algal Culture Collection at the University of Texas by R. C. Starr. The algae were grown axenically in *Volvox* medium [11] at 28°C in a light/dark rhythm of 16:8 h (10 000 lux). Methods for large scale culture of the male strain and purification procedures for the sex inducer were essentially as in [8]. Details of the bioassay have been published [9].

2.2. *Preparation of antiserum*

A 6 month old rabbit was obtained from a local breeder. The rabbit was sensitized subcutaneously

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with 100 μg purified hormone in 'complete Freund's adjuvant' (Difco Lab., Detroit, MI). Booster injections were given subcutaneously on day 35 (100 μg in 'incomplete Freund's adjuvant' (Difco Lab., Detroit, MI)) and intravenously on day 56 (50 μg in 0.01 M phosphate-buffered saline (pH 7.2) (PBS)). The rabbit was bled on day 68. The antiserum was obtained by centrifugation of the clotted blood at 2 000 × *g* and stored at −20°C. The immunoglobulin (Ig) fraction containing the specific antibodies was prepared by dialysis of the serum against 1.75 M ammonium sulphate; the precipitated gamma globulin was dissolved in PBS. These steps were repeated once and the Ig fraction was dialysed against PBS for 16 h at 4°C.

2.3. *Iodination procedures*

Radioiodinations were carried out according to the chloramine T method [12] using 35–50 μg protein and 1 mCi carrier-free $Na^{125}I$ (Amersham Buchler, Braunschweig). In the case of the sex inducer, separation from unbound iodide was achieved by gel filtration through a column of Sephadex G-25 (Pharmacia, Uppsala) 0.6 × 25 cm, equilibrated with PBS and presaturated with 1 ml 1% bovine serum albumin (BSA) in PBS. For elution with PBS, fractions of 400 μl were collected, and a 5 μl sample of each fraction was counted in a γ scintillation counter (Prias, Packard Instr., Frankfurt). The radioactive protein fractions were pooled and stored at 4°C. For the separation of antibodies and *Staphylococcus* protein A the reaction mixture was passed through a syringe filled with 0.5 ml Dowex 1 × 8 (Serva, Heidelberg). In each case the amount of the trichloroacetic acid-precipitable radioactivity (antibodies, 96%, protein A, 90%) or the ammonium sulphate-precipitable radioactivity (sex inducer, 80%) was determined.

2.4. *Radioimmunoassays*

Plastic plate assays [13,14] were carried out using plastic microtiter plates (Cooke Lab. Prod., Alexandria, VI). For coating each well was filled with 50 μl protein solution and incubated. The fluid was removed and the wells were washed 3 times with 100 μl PBS. They were then filled with 100 μl 1% BSA in PBS, and incubated for 1 h at 37°C to saturate free adsorption sites. For layer S 2 and 3, these coating and washing steps were repeated, omitting the saturation step. The details of incubation and of the conditions for the coating steps are given in the figure legends. During the whole procedure the plates were kept in a moist chamber. Finally, the plates were dried, and the wells were sliced off by a hot wire. Each well was counted in a γ scintillation counter.

2.5. *Other analytical procedures*

Double diffusion in 0.8% agarose containing 1.5% polyethylene glycol was done as in [15]. Sodium dodecylsulphate–polyacrylamide slab gel electrophoresis (SDS–PAGE) was done as in [16]. Autoradiography of double diffusion slides and SDS–PAGE gels was performed on X-ray film (3M, Düsseldorf). Protein concentrations were determined by a modified Lowry method [17]. All chemicals were of analytical grade.

3. Results

In a first series of experiments the rabbit antiserum was tested by double diffusion for specific antibodies. A single, sharp precipitation line was formed with the purified sex inducer (10 μg/ml) at antiserum dilutions down to 1:32. Controls with normal rabbit serum gave no detectable precipitates.

To exclude interferences by contaminating material in the sex inducer preparation used for eliciting the antiserum, the hormone was iodinated by the chloramine T method to a spec. act. 1.2 μ Ci/μg protein. On SDS–PAGE this material showed a single sharp band at a position corresponding to mol. w 28 000 which is the same as for the unlabelled hormone [7]. Double diffusion analysis with radioactive sex inducer revealed, however, that only a fraction of this radioactivity is precipitated (fig.1). As will be demonstrated below the bulk of the hormone has lost reactivity towards our antibodies upon iodination and therefore a direct radioimmunoassay could not be established.

To circumvent this difficulty we traced the antigen–antibody complex with *Staphylococcus aureus* protein A which binds specifically to the constant domain of certain immunoglobulin heavy chains [18]. The protein A was labelled by the chloramine T method, and plastic plates were coated in three successive steps in the following order: (i) sex inducer; (ii) antiserum dilution; (iii) ^{125}I-labelled protein A (fig.2). In step (ii) a 1:30 dilution of the antiserum was simultaneously incubated with free hormone.

Fig.1. Double diffusion analysis of radioiodinated sex inducer. Each hole was filled with 5 μl. Central hole: ^{125}I-labelled hormone (50 000 cpm); outer holes, counter clockwise: antiserum dilutions: 1:3; 1:10; 1:30; 1:100; 1:300; 1:1000; 1:3000; 1:10 000. Diffusion overnight at 4°C; gel was washed with PBS and water, dried and exposed to X-ray film for 30 days.

However, inhibitory effects of this treatment as judged by protein A binding were observed only at inducer concentrations >1 μg/ml which makes this method unsuitable for practical application. The lack of sensitivity may in part be due to the fact that protein A does not bind to all classes of immunoglobulin [19].

The final approach was to set up a sandwich-radioimmunoassay (RIA) by the principle in [20]. For this purpose, the plastic plates were coated with: (i) the Ig-fraction of the antiserum; (ii) dilutions of the hormone; (iii) ^{125}I-labelled Ig-fraction (chloramine T method, 1 μ Ci/μg). The resulting standard curve is presented in fig.3 which shows that under our experimental conditions up to 2.5% of the input radioactivity could be bound. Using this sandwich-assay, unknown concentrations of the sex inducer were

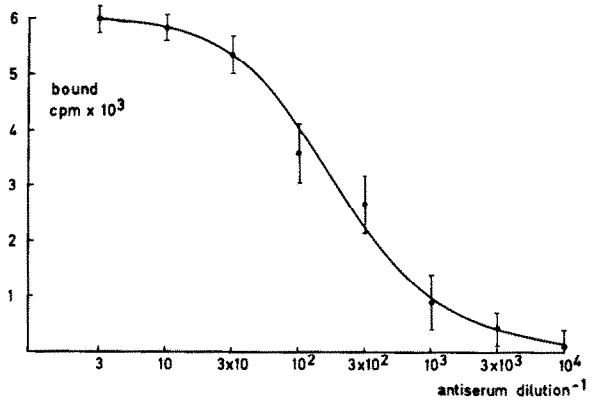

Fig.2. Antiserum dilution curve for protein A assay. Dilution of the antiserum (reciprocal) vs specifically bound cpm. Each well was incubated with: 50 μl sex inducer 10 μg/ml, overnight at 4°C (1st coating); 50 μl antiserum dilution, 2 h at 37°C (2nd coating); 50 μl ^{125}I-labelled protein A, 1 h at 37°C (3rd coating). Input, 90 000 cpm; high control, 6240 cpm; low control, 350 cpm (2nd coating with normal rabbit serum).

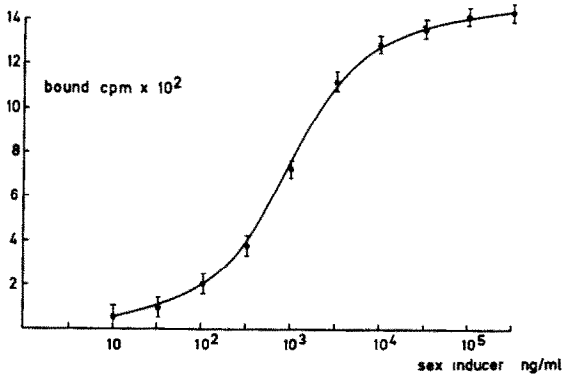

Fig.3. Standard curve for the sandwich-assay. Log concentration of the hormone vs specifically bound cpm. Each well was incubated with: 50 μl Ig-fraction 100 μg/ml, overnight at 4°C (1st coating); 50 μl sex inducer dilution or sample, 2 h at 37°C (2nd coating); 50 μl ^{125}I-labelled Ig-fraction, overnight at 4°C (3rd coating). Input, 70 000 cpm; high control, 1730 cpm; low control, 280 cpm (1st coating with the Ig-fraction of normal rabbit serum).

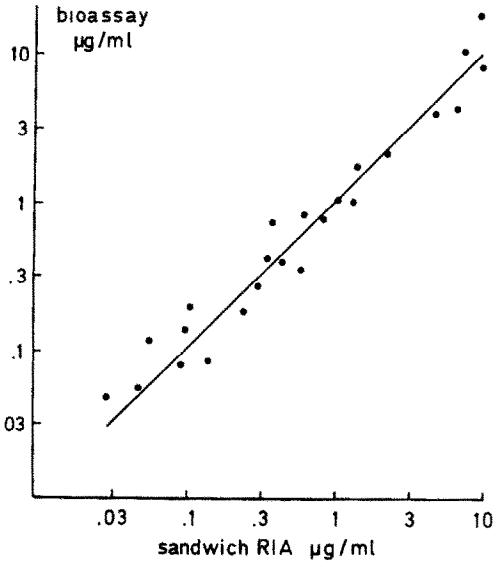

Fig.4. Comparison of radioimmunoassay and bioassay. The hormone concentration of the samples was determined: (i) by the sandwich-RIA using the standard curve (fig.3); (ii) by the bioassay, (the standard curve is not shown; 1 mg/ml results in 80% sexual embryos at a dilution of 1:10^{11}).

successfully measured between 30 μg/ml and 10 μg/ml.

In a detailed study it was shown that determinations of sex inducer concentrations by the bioassay and by the sandwich-RIA yield concordant results over a wide range of concentrations. This is demonstrated in fig.4. Most important, this holds also for culture media containing unpurified inducer. Thus, the sandwich-RIA is unaffected by other proteins found in the culture media, confirming that the antibodies are hormone specific. Erratic results will only arise if the samples for the sandwich-RIA are not dialysed against or diluted with PBS (>1:10).

This RIA avoids the iodination of the sex inducer. We were, nevertheless, interested in labelling the hormone; for the ^{125}I-labelled inducer would be a valuable tool to investigate the (hypothetical) hormone receptor. With the RIA at hand we determined the hormone concentration before and after iodination by the chloramine T method, using non-radioactive iodide. We also analysed the effect of each reagent in the iodination mixture separately. The results of these experiments as judged by both, the sandwich-RIA and the bioassay, appear in table 1. As seen from column D the iodination of the hormone resulted in an almost complete loss of reactivity against our antibodies and of biological activity.

4. Discussion

Somewhat unexpectedly, the iodination of the sex inducer of *Volvox carteri* yields a molecule of unchanged molecular weight but with little biological and immunological activity. Consistent with this result:

(i) A modified Farr assay [21,22] with radioiodinated inducer glycoprotein under varying conditions; and

(ii) An indirect precipitation assay [22] yielded maximally 10% of the total counts in the precipitate;

(iii) In a direct binding assay on plastic plates [23] ~3% of the added label were bound (50% inhibition by 5 μg/ml unlabelled sex inducer).

Since the iodination of the hormone by the lactoperoxidase/glucose oxidase method [24,25] as well as with the Bolton-Hunter reagent [26] (yielding an incorporation of 0.01 and 0.3 μCi/μg inducer protein, respectively) gave identical low immunological and biological responses we conclude that the introduction of the iodine into tyrosine or lysine residues of the sex inducer (and not the iodination conditions as such) change the structure or conformation of the molecule to an extent incompatible with the binding to receptor and/or antigenic sites.

The described sandwich-RIA based on antibodies contained in the radioiodinated Ig-fraction of a rabbit antiserum elicited against a highly purified sex inducer preparation gave a linear correlation to the bioassay with great specificity and satisfactory sensitivity and reproducibility. It is thus amenable to quick and exact quantitative analysis of inducer preparations in biological material.

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Table 1
Effect of chloramine T, iodine and sodium metabisulfite on the immunological and biological activity of the sex inducer

	Exp. A	Exp. B	Exp. C	Exp. D
Chloramine T	–[a]	–	+	+
NaI	–	+	–	+
$Na_2S_2O_5$	–	+	+	+
Remaining activity (sandwich-RIA)	100	99, 96[b]	25, 35, 28	1, 3, 5
Remaining activity (bioassay)	100	95, 100	10, 5, 20	0.5, 4, 1

[a] +, reagent present in the reaction mixture; –, reagent replaced by buffer
[b] Results of independent experiments; data are given in % of the activity found in exp. A

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