Time and mode of synthesis of the sexual inducer glycoprotein of *Volvox carteri**

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The synthesis of the sex-inducing glycoprotein of *Volvox carteri* was investigated. A highly specific antiserum directed against pheromone deglycosylated with trifluoromethane sulphonic acid was used to detect inducer protein in maturing cultures of the male strain IPS-22. Immunoblot analysis showed that inducer synthesis is restricted to a very late phase of sperm development, a few hours before the sperm cells die. The pheromone is co-translationally glycosylated to yield a well-defined pattern of isoinducers.

Glycoprotein synthesis; Immunological determination; *Volvox carteri*

1. INTRODUCTION

The dioecious colonial flagellated green alga *Volvox carteri* f. *nagariensis* switches from vegetative growth cycles to the sexual reproductive cycle by means of an extremely sensitive, signal-triggered synchronization mechanism. A glycoprotein pheromone is initially formed spontaneously by a mutational switch of one single male spheroid out of about 10000 [1]. This male spheroid forms androgonidia (sperm mother cells) instead of the vegetative gonidia in the male reproductive cycle. The androgonidia divide into a tightly packed bundle of spermatozoids. On disintegration of the sperm packets the inducer appears in the medium. Only a small number of molecules of this pheromone is required to initiate the sexual cycles in both male and female strains [2]. The inducing principle consists of several isoinducers, differing in the length of their *O*-glycosylating carbohydrate side chains; the core protein of 22.5 kDa and the *N*-glycosylated oligosaccharides, which account for 50% of the glycosidic sugars and are necessary for biological activity, are identical in the native pheromonal glycoprotein [3]. The differently glycosylated isoinducers occur in variable amounts. This may be due either to inhomogeneous sugar transferring and glycosylating steps during secretory processing or to secondary modifications and deglycosylations after secretion by lytic enzymes liberated on disintegration of the sperm packets. Here it will be shown that the first possibility holds true for the formation of isoinducers in *Volvox carteri*.

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* Dedicated to Professor Johannes Willenbrink (60) with thanks and good wishes

2. EXPERIMENTAL

The culture conditions of the algae (strain IPS-22, crossbreed of HK-10, female, and 69-1b, male, which were provided by the Algal Culture Collection at the University of Texas by courtesy of Professor R.C. Starr), the biological assay of inducer activity, the determination of protein, and the electrophoretic procedures were performed as described [4–6]. Doubly quartz-distilled water was used throughout.

Immunoblotting of male culture homogenates was done as follows. Aliquots of 20 ml from induced IPS-22 cultures were homogenized by 2 times sonication at 300 W for 30 s, lyophilized to about 1 ml and dialysed against 1% urea solution. The dialysed samples were concentrated in a Speed Vac, solubilized in sample buffer by heating to 95°C for 5 min, and separated from insoluble material at 12000 × *g* in a microfuge prior to electrophoresis. After SDS-PAGE (12% polyacrylamide) proteins were electroblotted onto nitrocellulose sheets in an LKB 2005 Transphor unit at 0.5 Å for 4 h. The sheets were treated with 1% BSA (bovine serum albumin) solution in PBS (phosphate-buffered saline, pH 7.2) for 30 min to block free binding sites, then incubated with a dilution of the antiserum 1:1000 in 1% BSA at 4°C overnight and washed 4 times for 5 min with the blocking solution, containing 0.2% Tween 20. Incubation with an anti-rabbit IgG/peroxidase conjugate (1:1000 in PBS/BSA) was performed at room temperature for 4 h, followed by washing again 4 times with PBS/BSA/0.2% Tween 20, and finally incubating in substrate solution until the bands became well visible. The substrate solution contained 50 μl 3% H_2O_2 and 200 μl of 1% methanolic 4-chloro-1-naphthol in 20 ml buffer, pH 5.0 (130 mg ammonium acetate and 60 mg citric acid in 100 ml H_2O) [7].

The *ELISAs* were performed in 96-well microtiter plates with the following steps: (1) coat with 50 μl antigen, adjusted to 2 μg/ml PBS, overnight at 4°C; (2) saturate with 200 μl 1% BSA in PBS at room temperature for 1 h; (3) wash 4 times with tap water; (4) incubate with 50 μl of a dilution series of the antiserum to be assayed at 4°C overnight; (5) wash 4 times with tap water; (6) incubate with 50 μl peroxidase conjugated goat anti-rabbit antibodies, 1:1000 in PBS/BSA at room temperature for 5 h; (7) wash 4 times with tap water; (8) incubate with 100 μl substrate solution (0.9 g Na_2HPO_4,

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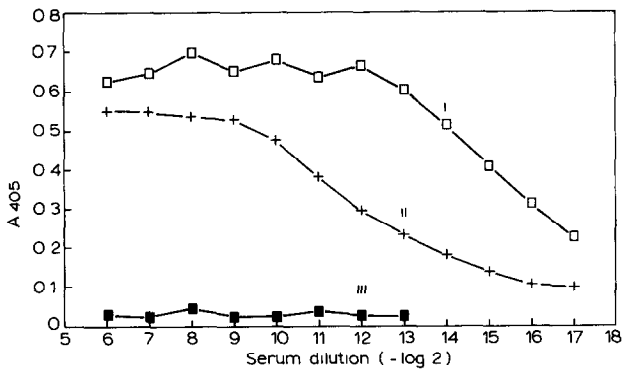


Fig. 1. Determination of the titer of the anti-inducer antiserum. The serum was diluted serially 1:2. (I) Coating with trifluoromethane sulphonic acid deglycosylated inducer (2 μg/ml). (II) Coating with native inducer (2 μg/ml). (III) Control for reaction with pre-immunisation serum, coating with native inducer.

0.5 g citric acid, 40 mg *o*-phenylene diamine, 100 μl 30% H_2O_2 in a total volume of 100 ml H_2O) [8] at room temperature for 30 min. Read absorbance at 405 nm in an automatic ELISA-reader.

3. RESULTS AND DISCUSSION

Earlier studies on the synthesis of the inducer glycoprotein indicated the expression of a pre-inducer protein which is glycosylated later [9]. However, the use of enzyme inhibitors, such as tunicamycin, is beset with uncertainties, and the previous antisera [9] were of little specificity, acting on the sugar epitopes of the inducer glycoprotein. Therefore a rabbit antiserum was raised against the trifluoromethane sulphonic acid deglycosylated core protein. As shown in Fig. 1 its titer, as determined by ELISA, is 8 times higher with the deglycosylated protein than with the native inducer. The reduced accessibility of the antigenic determinants in the native glycoprotein as compared with its protein moiety is in accordance with its great resistance against proteases [10] and indicates that the sugars are coating the protein effectively.

The specificity of the serum was determined on blots of media of disintegrated male cultures. Fig. 2 compares immunostaining with the antiserum against inducer core protein with that against the glycosylated inducer. In the former (lane 1) only the bands of the isoinducers are seen, whereas the latter (lane 2) shows a multi-banded pattern up to high molecular masses, difficult to analyse.

Inducer action can be inhibited completely and stoichiometrically, if increasing amounts of the antiserum are added to the standard bioassay (Fig. 3). Addition of pre-immunisation serum does not have any effect on sexual induction. Unreacted excess antiserum at higher titers, however, damages the algae.

The kinetics of inducer synthesis was followed in induced cultures of *Volvox carteri* strain IPS-22 beginning at the time of cleavage of androgonidia to complete disintegration of sperm packets. Aliquots of the unfiltered cultures were counted for free sperm packets, assayed for biological activity, and directly electrophoresed. The overall pattern of Coomassie blue-stained proteins remains almost unchanged, and the amount of inducer is hardly detectable. Synthesis of inducer begins surprisingly late. Even at the time of maximum concentration of free sperm packets (Fig. 4A, lane 6) very little of the protein is synthesized, and only on disintegration of the packets (Fig. 4A, lane 7) synthesis begins very quickly. The increase in biological activity in the supernatant of disintegrating male cultures runs parallel to the intensity of the inducer bands on the nitrocellulose blots. No additional activity can be released by sonication of the samples. These facts indicate that the fully processed inducer is directly released into the medium. If unglycosylated pre-pheromone

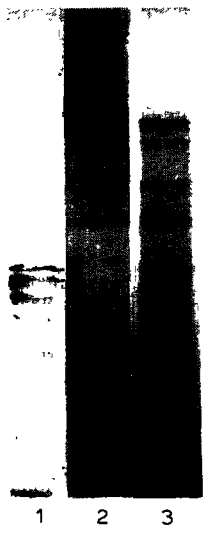


Fig. 2. Specificity of antisera, tested with crude medium. Parallel samples of 20 ml each from a disintegrated male culture were separated in a 12% SDS-gel. (1, 2) Immunostaining after transfer to nitrocellulose with antiserum against inducer core protein (1) and antiserum against native α-inducer (2). (3) Coomassie blue staining.

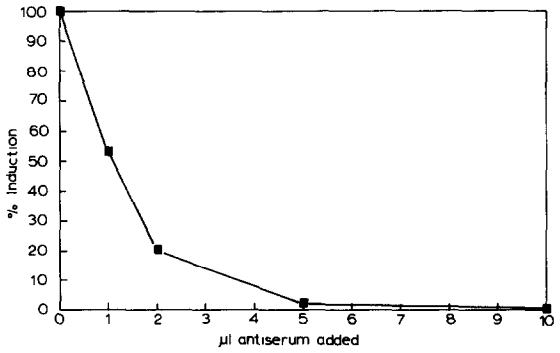
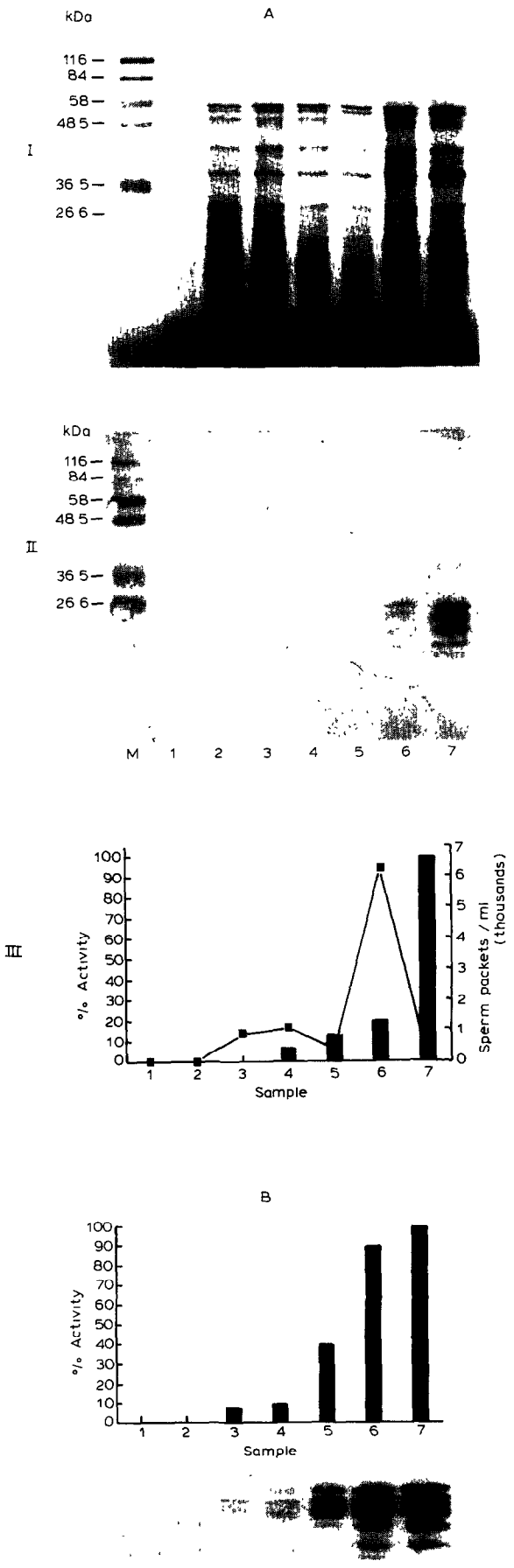


Fig. 3. Inhibition of sexual induction by anti-core protein serum. Indicated volumes of antiserum were mixed with 10 ml of *Volvox*-medium, containing just the amount of inducer needed for complete induction of test-algae.



were stored first, bands of lower molecular mass should prevail at the beginning of translation. This is definitely not the case, as can be seen from Fig. 4B. The pattern of bands with their relative intensities is unchanged during increase of the total amount of inducer, indicating that glycosylation of the core protein occurs as soon as the protein comes off the ribosomes to yield the heterogeneous inducer forms.

The late translation of the inducer during development of sperms is remarkable. It requires stored inducer-mRNA as well as stocks of energy since the finally differentiated sperm packets are unable to photosynthesise. The occurrence of isoinducers may also be explained by this late timing of production, when sperm cells may have already degraded part of their anabolic and processing enzymes shortly before their programmed function and death. The restriction of inducer synthesis to such a late phase of male development indicates a strict regulation of the process. It remains to be investigated whether this is achieved on the level of transcription or RNA-processing and -translation.

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Fig. 4. Time course of inducer synthesis and secretion. (A) Panel I: Coomassie blue staining of samples after SDS-PAGE. Panel II: Immune staining of blots (see section 2). Panel III: Concentration of sperm packets (line) and inducing activity (columns). Titer at complete disintegration of sperm packets = 30% induction at 10^8 dilution. Samples: (1) 0 time, all androgonidia uncleaved; (2) 16 h, 50% androgonidia divided; (3) 24 h, all androgonidia divided, 50% spheroids hatched; (4) 32 h, 80% spheroids hatched; (5) 42 h, all spheroids hatched; (6) 47 h, mostly free swimming sperm packets; (7) all sperm packets disintegrated; (M) protein standards. (B) Immune staining and activity from a less synchronous, higher titer culture than in (A). Maximum activity after disintegration: 100% induction at 10^8 dilution. Samples were taken parallel to (A).