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Purification and Partial Characterization of a Glycoprotein Sexual Inducer from *Volvox carteri*

(alga/polyacrylamide gel electrophoresis/hormone)

GARY KOCHERT AND IDA YATES

Department of Botany, University of Georgia, Athens, Ga. 30602

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ABSTRACT **A method is described for the isolation and purification of the sexual inducer produced by male colonies of the colonial green alga *Volvox carteri*. The procedure yields a highly purified preparation of the inducer. The biologically active principal is demonstrated to be a glycoprotein with a molecular weight of 32,000.**

The colonial green alga *Volvox* provides a system for the study of cell differentiation in one its simplest manifestations. Colonies of this organism contain only two cell types (somatic and reproductive), but the two cell types are very different in both morphology and function. Moreover, the induction of sexual reproduction in *Volvox* provides an opportunity to study a developmental change. During this process male and female colonies are formed in cultures that previously produced only asexual colonies.

In all species of *Volvox* thus far studied, the production of sexual colonies is initiated by a sexual inducer released into the culture medium by male colonies (1–5). In *Volvox carteri*, the production of female colonies normally occurs only in the presence of such a sexual inducer from male colonies. Preliminary characterization of this sexual inducer has been carried out in crude preparations from two subspecies. The material was found to be heat-stable; nondialyzable; resistant to trypsin, chymotrypsin, and RNase; and sensitive to Pronase (1, 2). Only preliminary data on the other physical and chemical properties of the *V. carteri* sexual inducer have thus far been reported. In the present report we describe methods for the purification and partial characterization of the *V. carteri* sexual inducer.

MATERIALS AND METHODS

Growth of Algae. Strain KA-1 of *V. carteri* was used as the source of the sexual inducer; strain MO-1 was used as the test organism in the biological assay. Details of the morphology and maintenance culture of *V. carteri* have been published (2). For large-scale production of sexual inducer, synchronized cultures were grown in 4-liter flasks containing 3 liters of *Volvox* medium (6).

Assay for Biological Activity. The bioassay for the sexual inducer was carried out in 18 × 150-mm disposable glass test tubes containing 9 ml of *Volvox* medium. Each sample to be tested for biological activity was made up to 10 ml with volvox medium and filter-sterilized. One-milliliter serial dilutions were then made through an appropriate number of assay tubes.

After dilution, five asexual colonies from the female strain (MO-1) were added to each tube. After 5–7 days of growth at 28° in constant light of about 5000 lux, approximately 450 colonies were present in each assay tube. These were counted under a dissecting microscope and scored as vegetative or female. Results are expressed as percent female colonies.

Pipettes for use in the bioassay must be thoroughly cleaned and baked before use. A separate pipette must be used for each transfer. We use permanent glass pipettes, since the disposable pipettes we have tried all seemed to liberate some material inhibitory to growth of *Volvox*.

Column Chromatography. Chromatography of the sexual inducer was carried out on 0.9 × 8.0-cm columns of carboxymethyl (CM)–Sephadex (C-50) equilibrated with 0.01 M sodium acetate, pH 5.0. After the sample (containing 0.1–0.5 mg of protein) was adsorbed, the column was washed extensively with the acetate buffer. Elution was achieved by a 60-ml linear gradient of NaCl (0–0.5 M in the acetate buffer). A flow rate of 0.25 ml/min was maintained by an Isco Dialagrad and 1-ml fractions collected. The column fractions were assayed for protein, carbohydrate, and biological activity.

Sucrose Gradient Centrifugation. Linear sucrose gradients (12 ml, 15–30% sucrose in 10 mM Tris·HCl) were run at 40,000 rpm for 36 hr at 4° in a Spinco SW-41 rotor. Each gradient was overlaid with 100 μl of sample containing 50–100 μg of protein. Ovalbumin and gamma globulin were used as markers. After centrifugation the gradients were fractionated with an Isco density gradient fractionator equipped with UV analyzer and recorder. Fractions of approximately 0.25 ml were collected, diluted with 1 ml of H_2O, and assayed for biological activity and protein concentration.

Gel Electrophoresis. Electrophoresis in 15% polyacrylamide–urea gels (0.6 × 10 cm) was carried out by the procedure of Panyim and Chalkley (7). Twenty to 50 μl of sample (containing 10–20 μg of protein) was layered on each gel. After electrophoresis at 1 mA per gel for 4 hr at room temperature, gels were stained for 2 hr with 0.5% amido black, destained by rinsing in 7% acetic acid, and scanned at 540 nm in a Gilford 2400S spectrophotometer equipped with linear gel scanner. Parallel gels were removed from the gel tubes after electrophoresis, frozen in powdered dry ice, and sliced into 1-mm

Abbreviations: SDS, sodium dodecyl sulfate; PAS, periodic acid Schiff reagent; CM–Sephadex, carboxymethyl–Sephadex.

TABLE 1. *Purification of the sexual inducer from Volvox carteri*

Preparation	Total volume (ml)	Protein concentration (μg/ml)	Biological activity (highest dilution showing activity)
Culture medium	1500	24	10^4
1 M NaCl supernatant from CM–Sephadex	45	50	10^5
Lyophilyzed, redissolved material	2.8	800	10^6

sections with a Mickle gel slicer. Each slice was eluted overnight at 4° in 1 ml of H_2O, which was then assayed for biological activity.

Electrophoresis in 5–12.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and urea was done by the procedure of Hoober (8). Bromphenol blue was used as tracing dye and bovine-serum albumin, ovalbumin, and cytochrome *c* were used as molecular weight markers. Gels were stained overnight with 0.2% Coomassie brilliant blue and destained by washing in 7% acetic acid. Some SDS–urea gels were also stained with periodic acid Schiff stain (PAS) (9). Parallel gels were assayed for biological activity as above.

Sedimentation Equilibrium Centrifugation. Samples of the purified sexual inducer at a concentration of 0.3 mg/ml were dialyzed extensively against 0.01 M phosphate buffer, pH 7.0, and analyzed by the meniscus depletion sedimentation equilibrium method (10), with interference optics. Homogeneity of preparations was analyzed by the plot of the logarithm of the fringe displacement against the radius squared. Molecular weight calculations were made for a range of partial specific volumes.

Amino-Acid Analysis. Samples of the inducing protein were hydrolyzed in 6 N HCl at 110° for 12 hr in sealed, evacuated tubes. Amino-acid analysis was done on a Beckman model 120 C automated amino-acid analyzer (11).

Analytical Methods. Protein concentrations were determined by the method of Lowry (12) with bovine-serum albumin standards. Total carbohydrate was determined by the phenol–sulfuric-acid method (13) with mannose as standard.

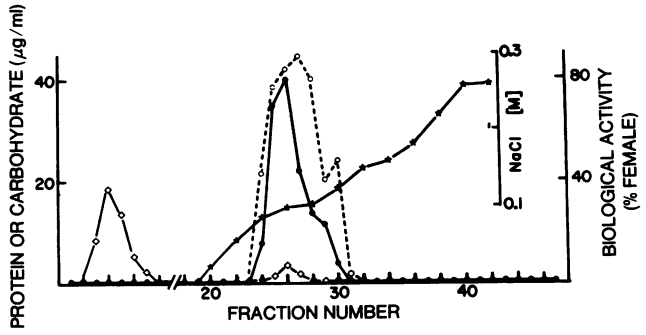

FIG. 1. Chromatography of purified sexual inducer on CM–Sephadex. Each fraction was assayed for protein, carbohydrate, and biological activity. Biological activity is shown only for the 10^4 dilution. (●——●) Protein; (◇——◇) carbohydrate; (O- - -O) biological activity; (★——★) NaCl concentration.

RESULTS

Approximately 15 liters of *Volvox* culture was used for the isolation of each batch of sexual inducer. Cultures were used 6–8 days after inoculation (about 1 day after release of male colonies and sperm bundles). Colonies were removed by filtration through Miracloth (Chicopee Mills) and discarded. The culture medium (which contains the sexual inducer) was adjusted to pH 5.0 with glacial acetic acid. Approximately 30 ml wet volume of CM–Sephadex (previously equilibrated against 0.01 M sodium acetate, pH 5.0) was added. The CM–Sephadex particles were kept in suspension on a magnetic stirrer for 2 hr at room temperature to allow adsorption of the sexual inducer. At the end of this period, CM–Sephadex particles were allowed to settle by gravity and most of the supernatant medium was removed with a siphon tube. The CM–Sephadex particles were collected from the remaining medium by low-speed centrifugation and resuspended in 20–50 ml of 0.01 M sodium acetate, pH 5.0, containing 1 M NaCl. After they were stirred on a magnetic stirrer for 1 hr at room temperature, the CM–Sephadex particles were removed by low-speed centrifugation. The supernatant containing the sexual inducer was first dialyzed extensively against deionized H_2O at 4° and then concentrated by lyophilization. The fluffy white powder that resulted was resuspended in 1–2 ml of deionized H_2O and placed on a shaker at 4° for 6–12 hr. At the end of this period, insoluble material was removed by low-speed centrifugation and the supernatant was assayed for biological activity and protein content. Batches of sexual inducer prepared in this fashion contained 0.5–2.0 mg of protein and were active in the biological assay at a dilution of 10^8 or 10^9. Protein concentrations and biological activities at various stages in the purification of a sample are given in Table 1.

When preparations of the inducing protein are rechromatographed on CM–Sephadex, essentially all the protein and biological activity is adsorbed, but considerable polysaccharide passes through the column. When a gradient of NaCl is applied to the column, the protein elutes in a single peak that parallels the biological activity. Some carbohydrate also elutes with the protein peak (Fig. 1). On 15–30% sucrose gradients the sexual inducer sediments somewhat more slowly than bovine-serum albumin, and forms a single coincident peak of protein and biological activity (Fig. 2).

To test the apparent homogeneity of the preparation, electrophoresis in polyacrylamide gels containing urea was carried out. Under these conditions a single protein band is

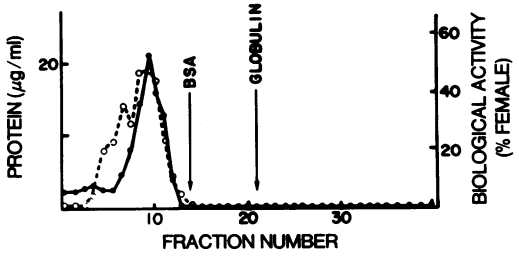

FIG. 2. Sucrose gradient centrifugation of purified sexual inducer. Bovine-serum albumin (*BSA*, molecular weight = 67,000) and gamma globulin (*globulin*, molecular weight = 120,000) were used as markers. Fractions were assayed for protein and biological activity. Biological activity is shown only for the 10^2 dilution. (●——●) Protein; (O- - -O) biological activity.

TABLE 2. *Amino-acid composition of purified sexual inducer and of colony sheath glycoprotein from Volvox carteri*

Amino acid	Percent of residues	
	Sexual inducer	Sheath glycoprotein*
Hyp	1.0	21.7
Lys	3.4	2.9
His	0.4	0.7
Arg	3.6	2.9
Asx	8.7	9.4
Thr	7.9	8.0
Ser	6.7	8.0
Glx	5.3	3.6
Pro	3.5	3.9
Gly	33.9	18.0
Ala	6.2	8.0
Val	4.5	5.8
Met	1.2	0.7
Ile	2.9	2.2
Leu	6.7	4.3
Tyr	1.7	2.2
Phe	2.4	1.5

* Lamport, personal communication.

formed (Fig. 3). Some biological activity is preserved through this procedure and elutes in a peak coincident with the protein peak.

Since the isolation procedure outlined above appeared to give a homogeneous preparation of the sexual inducer, the amino-acid composition of the material was determined. These results are presented in Table 2. Included in Table 2 for comparison is the amino-acid composition of the protein structural component of asexual colony sheath material of *V. carteri* (Lamport, personal communication).

We have determined the apparent molecular weight of the sexual inducer on SDS–polyacrylamide gels. To insure complete SDS binding, the purified sexual inducer was heated at 37° for 1 hr in 0.5% SDS and 40 mM dithiothreitol, and then

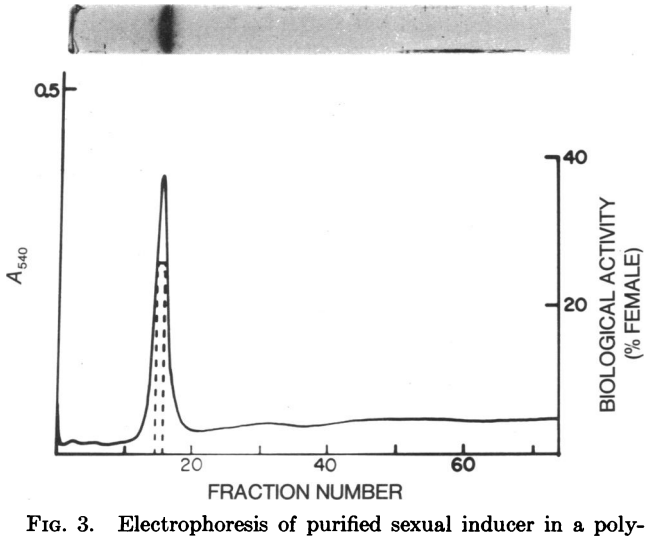

FIG. 3. Electrophoresis of purified sexual inducer in a polyacrylamide gel containing urea. The gel was stained with amido black and scanned at 540 nm. The biological activity shown was determined in a parallel gel. (——) A_{540} nm; (- - - - -) biological activity (10^2 dilution).

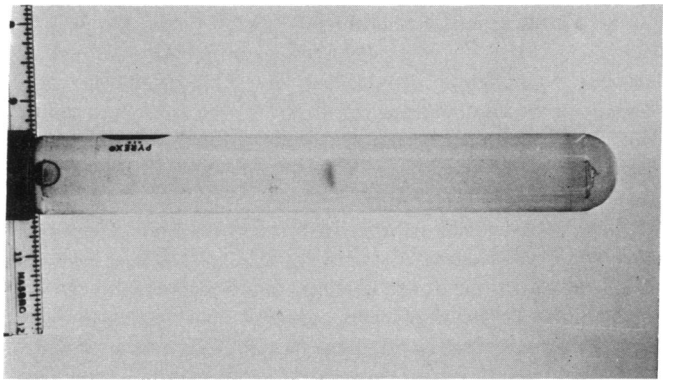

FIG. 4. SDS–polyacrylamide gel electrophoresis of the purified sexual inducer. The gel was stained with PAS after electrophoresis, and shows a single, weakly-staining band. A single protein band occurs at the same position in parallel gels (Fig. 5).

boiled for 2 min. The sexual inducer forms a single band, under these conditions in 5, 7.5, and 10 and 12% gels. The position of the band indicates an apparent molecular weight of 32,000 in all gels tested. The band stains with both protein stains and PAS (Fig. 4). Only a trace of biological activity remains after this treatment, but the remaining activity appears to be coincident with the glycoprotein band (Fig. 5).

The results of high-speed sedimentation equilibrium centrifugation experiments with purified sexual inducer indicate the material is homogeneous. Since no determinations of the partial specific volume of the sexual inducer have been made, the exact molecular weight could not be determined from the sedimentation equilibrium experiments. If calculations are made on the basis of an assumed partial specific volume of 0.70, however, the molecular weight of the inducer would be 32,700.

DISCUSSION

Our initial attempts to characterize the *V. carteri* sexual inducer were based on direct concentration of male culture medium. These efforts were complicated by the presence in the culture medium of large amounts of glycoprotein sheath material. This substance forms the matrix in which the cells

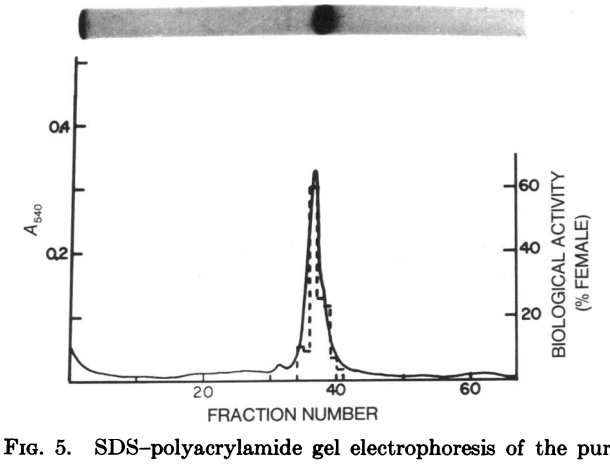

FIG. 5. SDS–polyacrylamide gel electrophoresis of the purified sexual inducer. The gel was stained with Coomassie blue and scanned at 540 nm. Biological activity was determined in a parallel gel. (——) A_{540} nm; (- - -) biological activity (10^2 dilution).

of the *Volvox* colony are embedded. After asexual reproduction, when the parental colonies disintegrate, the matrix material is released into the culture medium. Direct concentration of the medium results in a very viscous preparation that is difficult to further characterize. This problem was averted in the present study by an initial adsorption of the sexual inducer on CM-Sephadex.

The simple isolation procedure described above results in a considerable degree of purification of the sexual inducer. Most preparations form a single band when subjected to electrophoresis in polyacrylamide gels containing urea or containing both urea and SDS. When large amounts of the sexual inducer (50 μg of protein) are loaded onto a single gel, some preparations show very minor contaminants. Biological activity is always confined to the major band.

The sexual inducer appears to be a glycoprotein, based on its stainability with PAS in polyacrylamide gels and on direct analysis of the peak fractions from CM-Sephadex columns. Its apparent molecular weight, determined by electrophoresis in SDS-polyacrylamide gels, is 32,000.

Since the polysaccharide portion of glycoproteins does not bind SDS, apparent molecular weights of glycoproteins determined by SDS-polyacrylamide gel electrophoresis are usually somewhat larger than the real value. The disparity between apparent and real molecular weight increases with increasing polysaccharide content of the glycoprotein and is significant only with glycoproteins containing more than 10% polysaccharide (9). One can determine whether this is a problem for any given glycoprotein by subjecting the samples to electrophoresis in gels containing different concentrations of acrylamide. We determined the apparent molecular weight of the *Volvox* sexual inducer in 5, 7, 10, and 12.5% gels. In every case the apparent molecular weight was 32,000, and we believe this to represent the real value. We believe, however, that the material exists in an aggregated form in the culture medium. Our preliminary experiments indicate that when unconcentrated culture medium is fractionated on Sephadex G-100 gels, a portion of the biological activity elutes at a volume which would indicate a molecular weight $>$ 100,000. The high-salt elution step in our purification procedure apparently disperses these aggregates, since no high-molecular-weight material is revealed on sucrose gradient centrifugation of our purified preparations (Fig. 2).

The major difference between the amino-acid composition of the sexual inducer and that of the colony matrix glycoprotein is the hydroxyproline content (Table 2). The sheath glycoprotein contains a large amount of hydroxyproline. Our preparations of the sexual inducer contain only a small amount of hydroxyproline, indicating relatively minor contamination with sheath glycoprotein. Both the sexual inducer and the sheath glycoprotein contain a large amount of glycine, but this could represent some contamination from the glycylglycine buffer used in the culture medium.

The availability of the sexual inducer in a highly purified form should make it possible to develop an alternate assay method, based on immunological or *in vitro* labeling techniques as a substitute for the cumbersome and time-consuming biological assay. This should facilitate further studies on the mode of action of the sexual inducer.

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