Plant Physiol. (1985) 79, 740–745
0032-0889/85/79/0740/06/$01.00/0

Sexual Agglutination in the Unicellular Green Alga *Chlamydomonas eugametos*

IDENTIFICATION AND PROPERTIES OF THE MATING TYPE PLUS AGGLUTINATION FACTOR

Received for publication April 26, 1985

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ABSTRACT

Gametes of the unicellular green alga *Chlamydomonas eugametos* agglutinate via their flagella. The mating type plus agglutination factor was solubilized by relatively mild treatments such as a short pH shock or an osmotic shock indicating that it is an extrinsic membrane component. It was also extracted in the nonionic detergent Triton X-100. A simple two-step procedure consisting of gel filtration over Sepharose 4B-cross-linked followed by anion exchange chromatography of the void volume yielded an electrophoretically pure preparation of a single high molecular weight glycoprotein. The agglutination factor sedimented as a 9.3 S particle (assuming a density of 1.50) in sucrose gradients. This low value, compared with the high apparent molecular weight seen during gel filtration and electrophoresis, suggests that the agglutination factor is a rod-like molecule. This was confirmed by viewing rotary-shadowed preparations in the electron microscope. A population of long slender molecules was revealed (328 ± 20 nanometers), many of which had a knob at one end and a flexible region about one fourth of the length from the other end.

Cell-cell recognition in plants plays a role in a remarkable range of phenomena including sexual reproduction, parasitism, grafting, and many others (15). The sexual agglutination process shown by gametes of *Chlamydomonas* represents a spectacular example of such a recognition process (11, 27, 28). *Chlamydomonas* is a unicellular green alga propelled by two flagella at the anterior end of the cell body. Under normal conditions, the population grows vegetatively but when certain nutrients become limiting (23), vegetative cells are transformed into gametes. Gametes are morphologically similar to vegetative cells but their flagella are agglutinable. When gametes of opposite mt[1] (mt^+ and mt^-) are mixed, they adhere to each other via their flagella and form large vibrating clumps. Agglutination triggers a sequence of physiological and morphological reactions that lead to cell fusion and zygote formation (11, 27, 28). The first event known to occur after agglutination is a temporary rise in the cAMP level of the cells, which is detectable within 5 s (20). Other early reactions are an accelerated efflux of calcium ions (3) and a morphological transformation of the flagellar tip (8, 18). How these and other subsequent events are interrelated is not yet known. The agglutination reaction itself is mediated by high mol wt glycoproteins at the flagellar surface. In *C. reinhardtii*, the mt^+ agglutination factor has been identified and partially characterized by Adair *et al.* (1, 2; see also 7). More recently, the isolation of the mt^- agglutination factor was also reported (6, 24). In *C. eugametos*, a species that cannot sexually agglutinate with *C. reinhardtii*, the mt^- agglutination factor has been purified and analyzed by Musgrave *et al.* (19) and by Homan (12). In this paper we report the purification of the mt^+ agglutination factor of this species, and show that it is a long linear glycoprotein.

[1] Abbreviations: mt, mating type; PAS, periodic acid-Schiff.

MATERIALS AND METHODS

Cell Cultures. *Chlamydomonas eugametos* strains 11-5/9 (mt^+) and 11-5/10 (mt^-) from the Sammlung von Algenkulturen, Göttingen, Federal Republic of Germany, were cultivated in Petri dishes on agar-containing medium in a 12-h light/12-h dark regimen as described by Mesland (17). Gamete suspensions were obtained by flooding 2- to 3-week-old cultures with distilled H_2O just before the start of the dark period. They were harvested the following light period. A Petri dish culture contained on the average 2.5×10^8 cells. Suspensions of vegetative cells were obtained by flooding with 0.5% (w/v) NH_4Cl (23).

Detergent Extraction of mt^+ Agglutination Factor. Gamete suspensions were centrifuged at 5°C at 1500*g* for 15 min. The cells were resuspended in ice-cold 0.1% (w/v) Triton X-100 (1 ml/culture) and gently shaken at 5°C for 1 h. The suspension was then centrifuged at 48,000*g* for 30 min. The supernatant was collected and mixed with extensively washed Amberlite XAD-2 (20 g wet weight/100 ml) to remove Triton X-100 (4). The Amberlite suspension was shaken at 5°C for 15 min and filtered to remove the Amberlite. Fresh Amberlite was added to the filtrate and the procedure was repeated. The final filtrate, which was slightly cloudy, was free from Triton X-100. It was stored at −70°C; at this temperature the biological activity was stable for at least 6 months. After thawing, samples were routinely centrifuged at 10,000*g* for 15 min. This step resulted in a relatively large pellet that was discarded. The biological activity was entirely in the supernatant.

pH Shock Extraction of mt^+ Agglutination Factor. Gamete suspensions were concentrated by centrifuging (1,000*g*, 15 min) at room temperature. The cells were resuspended in distilled H_2O (0.5 ml/culture) and illuminated for 30 min. The suspension was quickly cooled with ice-cold water (1.5 ml/culture) and the pH was lowered to 4.3 with 0.5 N acetic acid. After 1 min the solution was neutralized with 0.5 N KOH. During this treatment the flagella were released and some flagellar membrane proteins including the mt^+ agglutination factor were solubilized. The cell bodies were spun off (1,000*g*, 15 min) and the supernatant was further clarified by centrifuging at 48,000*g* for 30 min. The final supernatant contained the biological activity and was stored at −70°C. After thawing, the solution was centrifuged (10,000*g*, 10

min) to remove water-insoluble components.

Assay of Biological Activity. The biological activity in solution was assayed by adsorbing it onto charcoal particles (19). Gametes of the opposite mating type avidly adhere to such activated particles and show the same twitching behavior as observed with agglutinating gametes. An initial sample of 100 μl was diluted with 10 mM sodium phosphate (pH 7.4) in binary steps. To each solution 10 μl of 1% (w/v) charcoal suspension was added. The charcoal particles were resuspended and allowed to settle again. Two μl were pipetted from the bottom and placed on a microscopic slide; 20 μl of test gametes were added and thoroughly mixed with the charcoal particles. Biological activities were expressed as titers (2^0, 2^1, etc). The most dilute solution in which cell adhesion to the charcoal particles was observed was arbitrarily defined to contain one unit of biologically active material per ml (1 unit/ml). Specific activity was defined as units/mg protein. The charcoal assay has the same specificity as the sexual agglutination reaction and is sex specific, gamete specific, and species specific. One unit of biological activity (mt^+) corresponds to 5 to 10 ng agglutination factor (see "Results").

Gel Filtration. Biologically active solutions were fractionated by gel filtration over Sepharose 4B-Cl, 38 × 2.6 cm, or Fractogel TSK HW-75 (20–40 μm grade; Merck), 90 × 2.6 cm. The gels were either eluted with 10 mM sodium phosphate (pH 7.4) at a flow rate of 10 ml/h or, when gel filtration was followed by anion exchange chromatography, with 10 mM histidine-HCl (pH 6.0) to avoid changing buffers. Fractions of 5 ml were collected. Void volume and total volume were determined with 0.5% (w/v) dextran (Sigma; industrial grade; mol wt = 45 × 10^6) and 0.5% (w/v) glucose, respectively. Carbohydrates were measured with phenol-sulfuric acid as described in (13). K_{av} values were calculated according to (elution volume − void volume)/(total volume − void volume).

Ion Exchange Chromatography. Ion exchange chromatography was carried out in a 20 × 1.5 cm column packed with QAE-Sephadex A25 equilibrated in 10 mM histidine-HCl (pH 6.0). The column was eluted with a linear gradient (0.10–0.35 M NaCl) in buffer at 4 ml/h. Seventy fractions of 2.5 ml were collected. Fractions were desalted by gel filtration over Sephadex G-25.

Polyacrylamide Electrophoresis. SDS-PAGE was carried out according to Laemmli (14) in 4% acrylamide gels. Since we were only interested in the separation of high mol wt compounds, stacking gels were omitted, because they had no effect on either sharpness or separation of the bands in the high mol wt region of the gels. To improve the separation in this region of the gels, the front marker was routinely allowed to run off the gels. Separation was optimal using long electrophoresis times at low amperage (1 mamp/gel for 24 h); at higher amperages the bands broadened. The gels were stained with PAS reagent (30).

Protein Concentration. Protein concentrations of crude extracts were measured with Serva Blue G (21) except in chromatography experiments when protein content was measured fluorometrically (9). The excitation wave length was set at 285 nm (band width 15 nm) and the emission was recorded at 338 nm (band width 15 nm). The lowest concentration of BSA that could be accurately measured was 0.1 μg/ml. The protein concentration of the purified preparation of mt^+ agglutination factor was also measured at 206 nm (25).

Sucrose Gradient Centrifugation. Sedimentation coefficients were estimated according to McEwen (16). A Triton X-100 extract in 4% (w/w) sucrose (300 μl) was layered onto a 7 to 30% linear sucrose gradient and centrifuged at 38,000*g* for 22 h (Beckman SW 40 rotor; 5°C); 0.5-ml fractions were collected from the bottom of the tube. The refraction index was determined and the fractions were filtered over Sephadex G-25 before further analysis.

Electron Microscopy. A solution of the purified mt^+ agglutination factor was suspended in 70% glycerol to give a final concentration of approximately 50% glycerol. Using a nebulizer, the solution was sprayed onto carbon-coated grids to give a dense covering of droplets, which were dried in an evaporator for 2 h and then rotary shadowed with platinum/palladium at an angle of 5°. The preparations were viewed in a Jeol/1200-EX at 80 kV.

RESULTS

Extraction. Biologically active material of mt^+ gametes could be solubilized by extraction in nonionic detergents such as Triton X-100, by lowering the pH to 4.3 for 1 min, or by an osmotic shock (Table I). Various concentrations of Triton X-100 were tried, from 0.01 to 2% (w/v); 0.1% appeared to be the optimal concentration. At higher concentrations, slightly higher yields were obtained, but the extracts were green, because the cell contents were partially solubilized. It was further determined that the biologically active material was quantitatively recovered in the water phase after removal of Triton X-100 with Amberlite XAD-2. In most bulk extraction experiments 0.1% (w/v) Triton X-100 was therefore used. About 40 units of biologically active material per culture were released. The pH shock procedure brought about the same amount of mt^+ agglutination factor into solution, but considerably less contaminating protein (Table I). Unfortunately, this procedure was only suitable for small volumes up to 25 ml, for at higher volumes the results became variable since the amount of contaminating glycoprotein tended to increase. Table I also shows that a hypertonic shock followed by a hypotonic shock was relatively effective in solubilizing the biologically active material from mt^+ gametes. In contrast to the mt^- agglutination factor (19), the mt^+ factor irreversibly lost its activity when extracted in SDS or guanidine isothiocyanate.

Gel Filtration. Gel filtration of gamete extracts over Sepharose 4B-C1, which has an exclusion limit for globular proteins of 2.10^7, resulted in the biological activity eluting in the void volume (Fig. 1A). Since the bulk of the protein eluted later, this produced a 5- to 10-fold purification of the agglutination factor depending on whether the extracts were obtained by a pH shock or by detergent extraction. When the void volume fractions were subjected to SDS-gel electrophoresis they were seen to contain five

Table I. *Solubilization of the mt^+ Agglutination Factor*

Treatment	Biological Activity Extracted per Culture[a]	Protein Extracted per Culture[a]	Specific Activity
	units	*mg*	10^{-2} × *units/mg*
0.1% (w/v) Triton X-100	40	0.15	2.7
pH 4.3 for 1 min	46	0.09	5.1
Hypertonic shock[b]	1		
Hypotonic shock[b]	20		

[a] A culture contains on the average 2.5 × 10^8 cells. [b] The cells were incubated in 7% (w/w) sucrose at 5°C for 1 h (hypertonic shock); the cells were then sedimented (10,000g, 5 min) and resuspended in water (hypotonic shock).

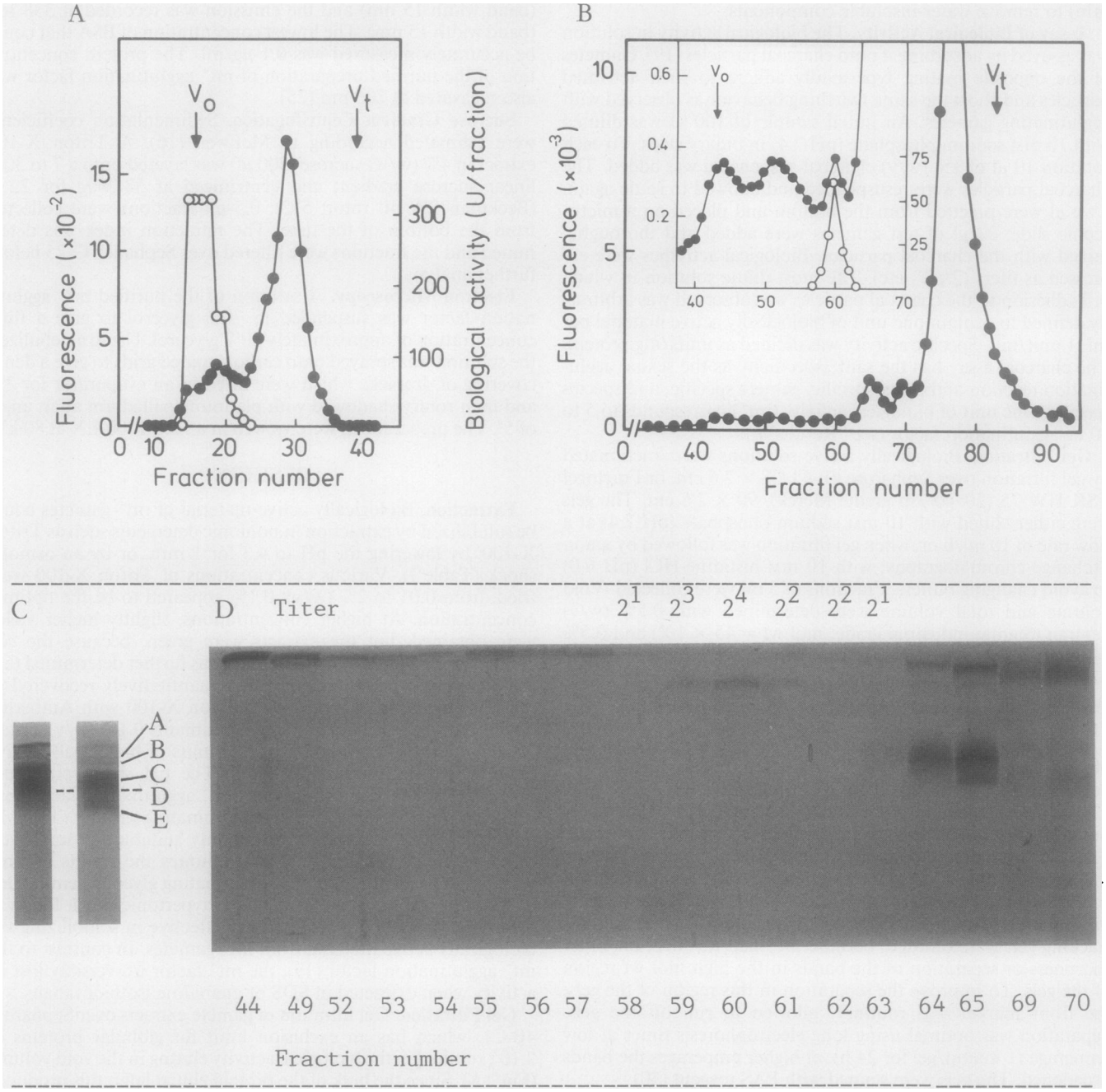

FIG. 1. Gel filtration of mt$^+$ extracts followed by SDS-PAGE of the eluate. A, Gel filtration over Sepharose 4B-C1. The extract was obtained by the pH shock procedure. Protein was determined by measuring its intrinsic fluorescence (●); 1,000 units corresponded to 1.4 μg/ml BSA. The biological activity was measured via the charcoal assay (○). The void volume (V_0) and total volume (V_t) were at fractions 16 and 39, respectively. B, Gel filtration over Fractogel TSK HW-75 (20–40 μm grade). The extract was obtained by the pH shock procedure. The void volume and total volume were at fractions 43 and 83, respectively. The high mol wt region of the elution profile is shown enlarged in the inset; left y-axis: fluorescence (× 10^{-3}; ●); right y-axis: biological activity (units/fraction; ○). C, SDS-PAGE of the voiding fractions from Sepharose 4B-C1 followed by staining with PAS reagent. The fractions were freeze dried, taken up in sample buffer, and electrophoresed in 4% gels. Since bands were detected only in the upper part of the gels, the front marker was routinely allowed to run off the gels to improve separation. The gel on the left contains 5 bands (PAS-A, -B, -C, -D, and -E), which partially overlap. PAS-D could be removed from the mixture by hydroxyapatite chromatography because it was the only band that was not retained. In this way, the existence of three separate bands (PAS-C, -D, and -E) was made clear (compare gel at the right to gel at the left). When a detergent extract was analyzed, an identical pattern was obtained. D, SDS-PAGE of the high mol wt region of the eluate followed by staining with PAS reagent. Since the lower halves of the gels were empty, the front marker was routinely allowed to run off the gels.

dominant PAS-staining bands, called PAS-A, -B, -C, -D, and -E (Fig. 1C, left lane). Staining with Coomassie brilliant blue did not reveal more bands and was less sensitive. PAS-A and PAS-B were minor bands and were not always separated from each other. To demonstrate that the subsequent region indeed consisted of three bands (PAS-C, -D, and -E), the entire mixture was first chromatographed over hydroxyapatite. All the bands were retained except for PAS-D. The retained fraction was eluted with 0.1 M sodium phosphate (pH 7.0), and subjected to electrophoresis (Fig. 1C, right lane). Extracts from vegetative cells were

likewise fractionated by filtration over Sepharose 4B-C1; in this case, the voiding fractions contained only three bands (PAS-A, PAS-B, and PAS-D) indicating that PAS-C and PAS-E are gamete-specific and possibly responsible for the agglutination activity.

When extracts were fractionated by filtration over Fractogel-75 (exclusion limit about 1.10^8), the biological activity eluted as a slightly tailing peak at $K_{av} = 0.42$ which corresponded to a minor peak in the protein elution profile (Fig. 1B). Protein was measured on the basis of its intrinsic fluorescence in the UV region because other methods (A_{280}, staining with Serva Blue G, and Lowry) were not sensitive enough in the high mol wt region. Fractions 44, 49, 52–65, 69, and 70 were analyzed by SDS-PAGE followed by PAS-staining (Fig. 1D). Only the distribution of the PAS-E band was correlated with that of the biological activity. PAS-A and -B were present in some biologically active fractions but in general eluted earlier. The distribution of PAS-C also overlapped the biologically active fractions, but its maximal staining intensity was found in fraction 65, beyond the biologically active region. PAS-D eluted even later (fractions 69 and 70). The lower halves of the gels illustrated in Figure 1D did not contain any PAS- or Coomassie-staining bands. Purification of the agglutination factor varied from 30- to 60-fold.

Ion Exchange Chromatography. The material in the void volume from the Sepharose 4B-C1 column was further fractionated by ion exchange chromatography on QAE Sephadex G-25 (Fig. 2) at pH 6. This pH was chosen because preliminary experiments indicated that the mt+ agglutination factor had a low isoelectric point. The gel was first eluted with 10 mM histidine-HCl (pH 6.0), followed by a 0.10 to 0.35 M NaCl gradient in the same buffer. The biological activity eluted at about 0.18 M NaCl (Fig. 2, inset) and coincided with a peak of fluorescing material in the same region. Gel electrophoresis of this peak resulted in a single band running at the position of PAS-E (Fig. 2, inset). PAS-D was detected in the nonretarded material, and PAS-C, together with PAS-A and -B, was found in the large peak eluting at 0.28 M NaCl. The gels of the remaining peaks were empty; the compounds in these peaks were not identified.

Sucrose Gradient Centrifugation. Assuming that the mt+ agglutination factor is a flexible coiled molecule when in solution under nondenaturing conditions, one might expect it to behave as a globular protein during gel filtration and during centrifugation in a sucrose gradient. Its exclusion from Sepharose 4B-C1 and retention ($K_{av} = 0.42$) in Fractogel 75 would then correspond to a mol wt of at least 2.10^7 (Fig. 1). This, in its turn, would correspond to a sedimentation coefficient of at least 200 S (22). In practice, the mt+ agglutination factor sedimented as a narrow peak (Fig. 3) with a sedimentation coefficient of 9.3 to 10.2, depending on the density assigned to it (1.50 and 1.30, respectively). This huge discrepancy between theory and practice can best be explained by assuming that the mt+ agglutination factor is not a globular protein, but a stiff, rod-like molecule (26). This assumption was tested by visualizing the agglutination factor in the electron microscope.

Electron Microscopy. The biologically active material eluting at 0.18 M NaCl from the ion exchange column was further analyzed by EM. The rotary-shadowed preparation consisted entirely of long, linear molecules having an average length of 328 nm (SD = 20 nm; 18 measurements) and a width of about 9 nm (Fig. 4). Many molecules were slightly bulbous at one end (Fig. 4, left-hand side) and some molecules seemed to possess a flexible region or kink at about 70 to 80 nm from the other end of the molecule.

DISCUSSION

In this paper, the mt+ agglutination factor from *Chlamydomonas eugametos* is identified as a long, linear glycoprotein that can easily be extracted from mt+ gametes. The pH shock procedure used in this paper was originally designed to isolate biologically active flagella (29) and is normally carried out in the presence of sucrose. However, when the sucrose was omitted, the agglutination factor was released into the medium (this paper) and the flagella became inactive (5, 10). The fact that the mt+ agglutination factor can be solubilized by lowering the pH to 4.3 for 1 min, or by an osmotic shock (Table I), suggests that the plus factor is only loosely bound to the cell surface and is probably an extrinsic membrane protein. This is also in accordance with the observation, that freeze-dried, detergent-free preparations of the agglutination factor rapidly dissolve in water. From the fact that the agglutination factor was stainable with periodic acid-Schiff reagent on acrylamide gels it was inferred that the agglutination factor is glycosylated. This was also supported by the observation that the sexual agglutination factor was tightly adsorbed by Con A-Sepharose indicating the presence of external mannose and/or glucose residues (data not shown). The sedimentation coefficient was calculated according to McEwen (16); one of the variables in this calculation is the specific density of the mt+ agglutination factor depending on the degree of glycosylation assigned to it. Assuming that the degree of glycosylation is comparable to that of the mt− agglutination

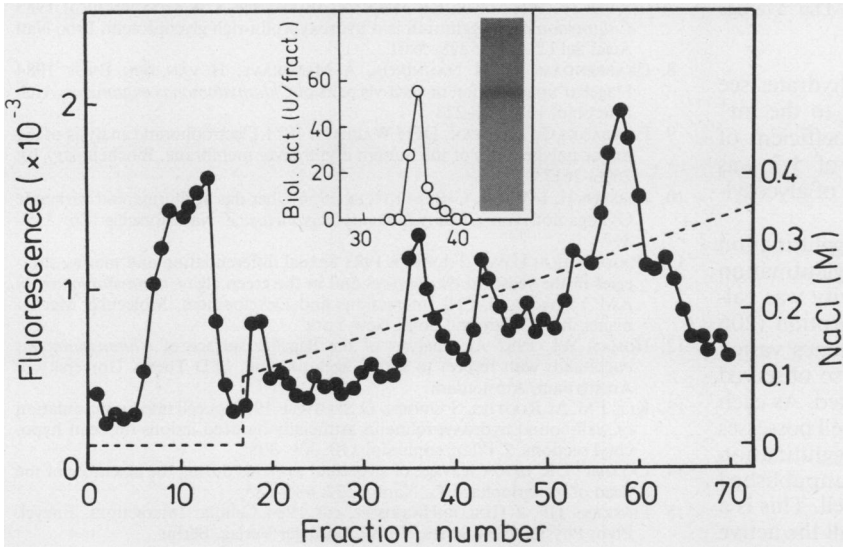

FIG. 2. Ion exchange chromatography on QAE Sephadex A-25 of a mt+ extract after gel filtration over Sepharose 4B-C1. The column (20 × 1.5 cm) was equilibrated with 10 mM histidine-HCl (pH 6.0). After applying the sample and eluting first with buffer, a linear gradient of NaCl (0.10–0.35 M) in buffer was applied. Fractions of 2.5 ml were collected. Protein concentrations were measured fluorometrically. A thousand units corresponded to 1.4 μg/ml BSA. The left part of the inset shows the biological activity in fractions 33–41; these fractions were pooled, desalted, and freeze dried before analysis by SDS-PAGE in combination with PAS-staining (right part of the inset).

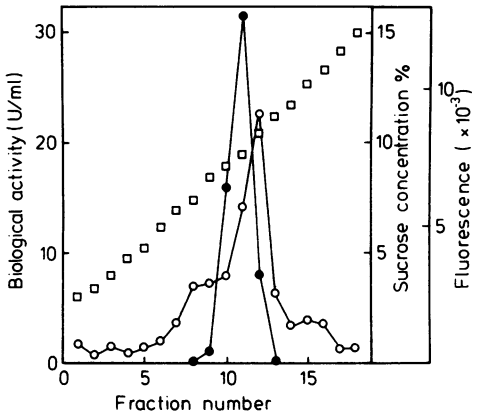

FIG. 3. Sucrose gradient centrifugation of the mt⁺ agglutination factor. A Triton X-100 extract in 4% (w/w) sucrose was layered onto a 7 to 30% (w/w) linear sucrose gradient and centrifuged at 38,000*g* for 22 h. Fractions of 0.5 ml were collected from the bottom of the tube; □, sucrose concentration (%, w/w); ○, protein concentration measured as intrinsic fluorescence (1,000 units correspond with 1.4 μg BSA); ●, biological activity.

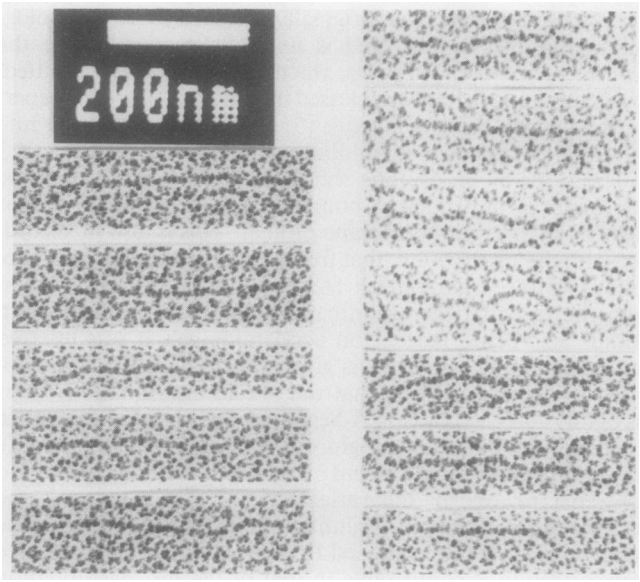

FIG. 4. Electron micrograph pictures of the purified mt⁺ agglutination factor after rotary shadowing with platinum/palladium. The average length of the agglutination factor is 328 nm.

factor of *Chlamydomonas eugametos* (45% carbohydrate; see reference 12) a specific density of 1.5 was assigned to the mt⁺ agglutination factor resulting in a sedimentation coefficient of 9.3 S. For comparison, when a specific density of 1.3 was assumed corresponding to a low or negligible degree of glycosylation, a value of 10.2 S was obtained.

It is possible to estimate how many copies of the agglutination factor are present in each cell. First, the amount of agglutination factor corresponding to one unit of biological activity was calculated. From the fluorescence (338 nm) and absorption (206 nm) of the pure glycoprotein in solution, the estimates varied from 5 to 10 ng protein per unit of activity. We also observed that on average 40 units per culture could be extracted. As each culture contains approximately 2.5×10^8 cells, each cell possesses $40 \times 7.5 \times 10^{-9}/2.5 \times 10^8$ g = 1.2×10^{-15} g of agglutination factor. Assuming a mol wt of 1.5×10^6 (F. M. Klis, unpublished data), this corresponds to about 500 molecules per cell. This is a preliminary estimate based on the assumption that all the active molecules were extracted and should therefore be considered to be a minimal value.

The mt⁻ agglutination factor of *C. eugametos* has been previously identified and purified (12, 19). It also has a low sedimentation coefficient (8.65; [12]) while behaving in gel filtration media as if it was a much larger molecule. When applied onto Fractogel 75, it eluted at the same position as the mt⁺ agglutination factor (data not shown). It is therefore highly likely that the mt⁻ agglutination factor will also prove to be a large linear glycoprotein. This has already been shown to be the case for the mt⁺ agglutination factor from *C. reinhardtii* (1). Although it is shorter, 220 nm compared with 328 nm, it bears a strong resemblance to the equivalent *C. eugametos* factor, for it also possesses a knob or swelling at one end with a flexible region close to the other end of the molecule (1). On the whole, the sexual agglutinins of both species have much in common despite the fact that their gametes do not agglutinate with each other. In both species, they are identified as long, linear glycoproteins, apparently bound to the flagellar membrane in an extrinsic way, and present in low copy numbers (1, 2, 6, 19, this paper). Indeed, the similarity is such that one automatically feels that these characteristics may prove typical for agglutination factors generally within this group of algae. This raises the question of how the species specificity of the sexual agglutination reaction within this group is controlled. There is strong evidence that the biological activity of the sexual agglutinins depends on specific carbohydrate side-chains (28). One might speculate that differences in composition and sequence of these side-chains are responsible for the specificity of the sexual agglutination reaction.

Acknowledgments.—We thank Piet Van Egmond for general assistance. We also thank the cell biology group at the John Innes Instiute in Norwich for their help in preparing the electron micrographs.

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