Purification, Visualization and Characterization of the Sexual Agglutinins of the Green Alga *Chlamydomonas moewusii yapensis*

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Purification of the sexual agglutinins of both mating types of *Chlamydomonas moewusii yapensis* (= *C. moewusii* syngen II) was achieved by a three-step procedure. Electron microscopy showed both agglutinins to be long, linear molecules. The mt^+ agglutinin was a rigid molecule with one bulbous end and an average length of 251 nm. The mt^- agglutinin was longer (average length 349 nm), had a more flexible conformation and lacked a bulbous end. The M_r was estimated to be 1.0×10^6 for the mt^+ and 1.2×10^6 for the mt^- agglutinin. The mt^- agglutinin had a very high content of hydroxyproline (41%) and serine (14%); the mt^+ agglutinin had a high content of glycine, serine and hydroxyproline (18, 16 and 12%, respectively). The main sugars in the agglutinins of both mating types were arabinose and galactose. The carbohydrate portion represented about 40% of the M_r in both agglutinins. The biological activity of both agglutinins could be destroyed by periodate treatment (although their sensitivities differed), indicating the involvement of carbohydrate residues. A differential susceptibility for exoglycosidases was observed: enzymic removal of terminal glucose residues abolished the biological activity of the mt^+ agglutinin only, while removal of mannose residues selectively inactivated the mt^- agglutinin. The similarities in form and composition of the agglutinins of several *Chlamydomonas* species suggest that these recognition molecules have a common ancestry, and that the species barriers are at least partially dependent on differences in the carbohydrate side-chains.

INTRODUCTION

Many recognition reactions in plants involve highly specific macromolecules at the surface of the cells. The sexual agglutination process of *Chlamydomonas* represents an excellent model of such a cellular recognition process (Van den Ende, 1985). *Chlamydomonas* is a biflagellate, unicellular green alga. Under nutrient-limited conditions, the vegetatively growing cells differentiate into gametes. Gametes of opposite mating type, referred to as mt^+ and mt^- , recognize and adhere to each other via their flagella, forming large, twitching clumps. This flagellar adhesion triggers a number of subsequent reactions that lead to the sorting out of pairs, the fusion of two cells of opposite mating type and eventually to the formation of a zygote. The agglutination reaction itself is mediated by high- M_r glycoproteins at the flagellar surface, the so-called agglutinins, that are recognized by the partner cell (Van den Ende, 1985). The high specificity of the sexual recognition reaction, both for mating type and species, may be attributed to the specific features of the agglutinins themselves; this makes a comparison of the agglutinins of different mating type and species of great interest.

The isolation and partial characterization of the mt^+ and mt^- agglutinin of *Chlamydomonas reinhardtii* have been reported by Adair *et al.* (1982), Collin-Osdoby & Adair (1985) and Saito &

Abbreviations: HPGF, high-performance gel filtration; mt, mating type.

Matsuda (1984). Both agglutinins of *C. eugametos*, a species that cannot sexually interact with *C. reinhardtii*, have also been purified, and their composition and other properties have been determined (Samson *et al.*, 1987). Both the *C. reinhardtii* and the *C. eugametos* agglutinins appear to be long, linear glycoproteins of high molecular mass and have a high content of hydroxyproline and serine (Collin-Osdoby & Adair, 1985; Samson *et al.*, 1987).

Here we report the purification, amino acid and sugar composition, morphology and several chemical and physical properties of the sexual agglutinins of *Chlamydomonas moewusii yapensis* (= *C. moewusii* syngen II). This species is closely related to *C. eugametos* and *C. moewusii* syngen I, but cannot sexually interact with them (Wiese & Jones, 1963) or with *C. reinhardtii*. We show that the agglutinins of *C. moewusii yapensis* are also long, linear glycoproteins and that, as with those of *C. reinhardtii* and *C. eugametos* (Collin-Osdoby & Adair, 1985; Samson *et al.*, 1987) the carbohydrate part of the molecule appears to be important for their biological activity.

METHODS

Cell cultures. *Chlamydomonas moewusii yapensis* strains UTEX 792 (mt⁺) and UTEX 793 (mt⁻) from the Algal Collection of the University of Texas at Austin, USA, were cultivated in Petri dishes on agar-containing medium under a 12 h light/12 h dark regimen. Gamete suspensions were obtained by flooding 3- to 4-week-old cultures with distilled water just before the dark period and harvesting the cells at the beginning of the next light period.

Extraction and purification of the agglutinins. Extractions with 0.1% (w/v) Triton X-100 were performed as described by Klis *et al.* (1985). Biological activity in solution was assayed by the semi-quantitative method described by Musgrave *et al.* (1981). The agglutinins were purified essentially as described by Samson *et al.* (1987). Biologically active solutions were fractionated by gel filtration over Sepharose 4B-Cl (Pharmacia, 70 × 2.5 cm). The column was eluted with 10 mM-Tris/HCl, pH 7.5, at a flow rate of 15 ml h⁻¹, and fractions of 16 ml were collected. The biological activity was found in the void volume. Appropriate fractions were applied to a 9.2 × 2.5 cm column packed with DEAE-Trisacryl M (anion exchanger, LKB), equilibrated in 10 mM-Tris/HCl, pH 7.5. The mt⁻ and mt⁺ agglutinins were eluted with, respectively, a 0–150 mM and a 100–250 mM linear gradient of NaCl in buffer. The flow rate was 12 ml h⁻¹ and fractions of 5.5 ml were collected. The mt⁻ agglutinin eluted at 50 mM-NaCl and the mt⁺ agglutinin at 120 mM-NaCl. Appropriate fractions were desalted by gel filtration and concentrated using a Centricon PD30 (Amicon). Final purification was achieved by high-performance gel filtration (HPGF) of agglutinin-containing fractions, using an LKB apparatus with a 600 × 7.5 mm G6000-PW gel filtration column and a 75 × 7.5 mm TSK-PWH guard column (Toyo Soda Manufacturing Co.). Samples of 200 µl were injected and eluted with 0.1 M-Na₂SO₄ in 10 mM-sodium phosphate, pH 7.4, at a flow rate of 30 µl min⁻¹. The temperature was 18–20 °C. Absorbance at 206 nm was measured and 10-min fractions were collected. The void volume was determined with aggregated proteins and the total volume with histidine, [³²P]phosphate and water (negative absorbance peak: at 206 nm water has a lower absorbance than the eluent).

Calculation of *M_r*. The *M_r* values of the agglutinins were determined by the equation given by Siegel & Monty (1966). This states that the *M_r* of a molecule is proportional to its Stokes' radius and sedimentation coefficient and also depends on the partial specific volume. From their behaviour in HPGF the Stokes' radii of the sexual agglutinins were estimated as described by Samson *et al.* (1987). The sedimentation coefficients were determined using sucrose gradient centrifugation as described by Klis *et al.* (1985). The partial specific volume of both agglutinins was estimated from their composition according to Perkins *et al.* (1981) and Cohn & Edsall (1943).

Electron microscopy. Purified sexual agglutinins were visualized by negative staining as described by Smith & Seegan (1984) with minor modifications (Crabbendam *et al.*, 1987).

Analyses. Purified agglutinins were hydrolysed for 24 h at 110 °C under N₂ in constant-boiling HCl. The hydrolysates were used for amino acid analysis on an LKB 4151 Alpha-plus amino acid analyser. They were also used for determination of hydroxyproline according to Drozd *et al.* (1976), using the modification of Samson *et al.* (1987).

Methanolysis of the glycoprotein was carried out under N₂ in 1 M-methanolic HCl for 24 h at 85 °C. Sugars were determined by gas chromatography of the trimethylsilyl derivatives of the methanolysed sugars (Clamp, 1977). Mannitol was used as the internal standard.

Enzyme incubations. Incubation conditions were as follows: α-galactosidase (EC 3.2.1.22), 0.1 U for 3 h at 22 °C and pH 5; α-glucosidase (EC 3.2.1.20), 1 U for 22 h at 22 °C and pH 6; α-mannosidase (EC 3.2.1.24), 0.2 U for 22 h at 22 °C and pH 5; glucose oxidase (EC 1.1.3.4), 2 U for 3 h at 22 °C and pH 7; sulphatase, from *Helix pomatia*, 1 mg ml⁻¹ for 2 h at 25 °C and pH 5; phosphatase, 0.4 mg ml⁻¹ for 2 h at 25 °C and pH 5; trypsin, 0.05% (w/v) for 3 h at 22 °C and pH 7; pronase, 0.02 U for 24 h at 22 °C and pH 4.5 and 7.5. Phosphatase was obtained from Boehringer Mannheim, and pronase from Calbiochem; all other enzymes were from Sigma.

RESULTS

Purification of the agglutinins

A three-step procedure was used. The first step consisted of gel filtration of detergent-free gamete extracts over Sepharose 4B-Cl (exclusion limit for globular proteins M_r 2×10^7), which resulted in the elution of biologically active material in the void volume. Since the bulk of protein eluted later, this fractionation gave a 5- to 10-fold purification of the agglutinins. To analyse the biologically active fractions we used HPGF; the column used separated molecules with an apparent M_r from less than 2×10^4 to about 10^8 . HPGF revealed several protein components (Fig. 1). Most protein in both the mt^- and the mt^+ preparation eluted at about 19.5 ml and several minor peaks and shoulders were detected. When these fractions were analysed by SDS-PAGE (results not shown), about five carbohydrate-containing bands (as judged by staining with periodic acid-Schiff reagent) could be detected in the high- M_r region of a 4% (w/v) polyacrylamide gel.

The components present in the void volume of the Sepharose 4B-Cl column were further fractionated by anion-exchange chromatography on DEAE-Trisacryl M at pH 7.5. The gel was first eluted with 10 mM-Tris/HCl (pH 7.5) followed by a salt gradient in this buffer. For the mt^+ preparation a 100–250 mM-NaCl gradient was used and the biological activity eluted at about 120 mM-NaCl. Using a 0–150 mM-NaCl gradient for the mt^- preparations, elution of the mt^- biological activity occurred at about 50 mM-NaCl. Analysis of the biologically active fractions by HPGF gave a relatively simple profile (Fig. 2). The mt^+ preparation showed a major protein peak at 18.2 ml; only the fractions corresponding with this peak showed biological activity. A minor peak could be detected at 21.0 ml and a minute shoulder at about 19.5 ml. The mt^- preparation showed a major protein peak at 17.9 ml, which comigrated with the biological activity. The peak was well separated from the other, inactive peaks at 20.2 and 21.0 ml.

The fractions containing most biological activity were collected and run again through the same column. This resulted in a single, symmetrical protein peak; the mt^+ agglutinin eluted at 18.2 ml and the mt^- agglutinin at 17.9 ml. This material showed only one carbohydrate-stainable band on PAGE (results not shown). Examination by electron microscopy showed a homogeneous population of long, linear molecules (see below).

Electron microscopy

Examination of the purified agglutinins by electron microscopy, using the negative staining, 'pleated sheet' method of Smith & Seegan (1984), showed long, linear molecules (Fig. 3). The mt^+ agglutinins were visualized as rather rigid molecules with a length of 251 ± 12 nm ($n = 49$), and slightly bulbous at one end. The mt^- agglutinin appeared to be a considerably longer, more flexible molecule with a length of 349 ± 31 nm ($n = 24$). It showed a somewhat corkscrew-like appearance, without a bulbous end. Both agglutinins seemed to have a uniform width.

Calculation of the M_r of the agglutinins

Since most conventional methods for M_r determination (SDS-PAGE and gel filtration) are not suited for such highly asymmetrical glycoproteins as the *Chlamydomonas* agglutinins, we used the fact that the M_r is proportional to the Stokes' radius and the sedimentation coefficient and depends on the partial specific volume of a molecule (Siegel & Monty, 1966). We have shown (Samson *et al.*, 1987) that the theoretically predicted relation between behaviour in gel filtration and Stokes' radius could be confirmed for high- M_r compounds of different composition and form in our HPGF system. From the elution position of the agglutinins from the HPGF column we estimated the Stokes' radius to be 36 nm and 37 nm for the mt^+ and the mt^- agglutinin, respectively. The sedimentation coefficients of the agglutinins were determined by sucrose gradient centrifugation: 7.9S for the mt^+ , and 9.6S for the mt^- agglutinin. The partial specific volumes were calculated using the amino acid and sugar composition of the agglutinins (Table 1) according to Perkins *et al.* (1981) and Cohn & Edsall (1943); the value was 0.68 ml g^{-1} for both agglutinins. Using these data we calculated the M_r for the mt^+ agglutinin to be 1.0×10^6 , and that for the mt^- agglutinin to be 1.2×10^6 .

Fig. 1. Separation by HPGF (G6000-PW column) of the high- M_r compounds in mt^+ and mt^- extracts. The void volume fractions eluting from a Sepharose 4B-CI column were analysed. Full scale represents 0.05 absorbance units. Elution volumes (ml) are given next to the corresponding peaks.

Fig. 2. HPGF profile (G6000-PW column) of biologically active fractions eluting from the DEAE-Trisacryl M column. Full scale represents 0.05 absorbance units. Elution volumes (ml) are given next to the corresponding peaks.

Amino acid and carbohydrate composition of the agglutinins

Table 1 shows the amino acid and sugar composition of the sexual agglutinins. In both agglutinins hydroxyproline and serine were abundant amino acids. Both these amino acids can be points of attachment of *O*-glycosidically linked carbohydrate chains. In the mt^- agglutinin an extremely high content of hydroxyproline was found using two independent methods: amino acid analysis and a colorimetric hydroxyproline assay.

Arabinose represented about half the total sugar residues of both agglutinins. A high galactose content was also observed. The sugar composition of the agglutinins of the two mating types was remarkably similar. The absence of detectable *N*-acetylglucosamine in both agglutinins may indicate that *N*-linked sugar chains are not present in these glycoproteins and that only *O*-glycosidic linkages occur.

Comparison of some properties of the mt^+ and mt^- agglutinins

In Table 2 we present some of the properties of the mt^+ and mt^- agglutinins that might be relevant for our understanding of the agglutinin-mediated recognition reaction. From the electron micrographs it can be concluded that both the agglutinins are long, linear molecules,

Fig. 3. Electron micrographs of purified mt^+ and mt^- agglutinins after negative staining using the 'pleated sheet' technique. The large photograph shows a field containing numerous mt^+ agglutinin molecules. Selected mt^+ and mt^- agglutinin molecules are shown on the right. The average lengths of the mt^+ and mt^- agglutinins were 251 nm and 349 nm, respectively. Bars, 100 nm.

differing, however, in length and form. Their asymmetric shape explains the discrepancy between their behaviour on gel filtration columns as apparently extremely large molecules and their relatively low sedimentation coefficients. A clear difference between the mt^+ and mt^- agglutinin was their elution from the anion exchange column: the mt^- agglutinin was only loosely bound to DEAE-Trisacryl at pH 7.5, while the mt^+ agglutinin showed a firmer interaction.

The agglutinins differed in the susceptibility of their biological activity to various chemical treatments and enzyme incubations. Denaturing agents such as guanidine thiocyanate and SDS, and also incubation in 10 mM-sodium acetate at pH 4, inactivated the mt^+ , but not the mt^-

Table 1. *Amino acid and carbohydrate compositions of the sexual agglutinins of C. moewusii yapensis*

	Amino acids (residues per 100 residues)			Sugars (residues per 100 residues)	
	mt ⁺	mt ⁻		mt ⁺	mt ⁻
Ser(P)	2	1	Ara	46	59
Asx	8	3	Rha	4	3
Hyp	12	41	Xyl	9	6
Thr	5	5	Man	5	3
Ser	16	14	Gal	29	26
Glx	9	4	Glc	7	3
Gly	18	8	GlcNAc	ND	ND
Ala	8	5			
Val	3	1			
Ile	tr.	tr.			
Leu	6	10			
Tyr	2	1			
Phe	2	1			
Lys	5	3			
His	1	1			
Arg	3	2			

tr., trace; ND, not detectable.

Table 2. *Comparison of some properties of the mt⁺ and mt⁻ agglutinins of C. moewusii yapensis*

Property	mt ⁺	mt ⁻
Elution from Sepharose 4B-Cl*	Void volume	Void volume
Elution from DEAE-Trisacryl (pH 7.5)	120 mM-NaCl	50 mM-NaCl
HPGF†	$K_{av} = 0.54$	$K_{av} = 0.52$
Stokes' radius	36 nm	37 nm
Sedimentation coefficient	7.9S	9.6S
Form	Rod shaped, one bulbous end, rigid	Rod shaped, no bulbous end, flexible
Length	251 nm	349 nm
M_r	1.0×10^6	1.2×10^6
Sugar:protein (w/w)	40:60	35:65
M_r of protein part	6×10^5	7.5×10^5
Major amino acids	Gly, Ser, Hyp	Hyp, Ser
Major sugars	Ara, Gal	Ara, Gal
GlcNAc detectable	No	No
Concanavalin A binding	Yes	Yes
Reduction of biological activity by:		
α -Galactosidase	No	No
α -Glucosidase	Yes	No
Glucose oxidase	No	No
α -Mannosidase	No	Yes
Periodate (10 mM)	Yes	No
Phosphatase	ND	No
Sulphatase	ND	No
Phosphatase + periodate (10 mM)‡	ND	Yes
Sulphatase + periodate (10 mM)‡	ND	Yes
Trypsin	No	No
Pronase	Yes	Yes
Guanidine thiocyanate (2 M)§	Yes	No
SDS (1%, w/v)§	Yes	No
Sodium acetate (10 mM, pH 4)§	Yes	No
Sodium carbonate (20 mM, pH 10)§	Yes	Yes

ND, Not determined.

* The exclusion limit of Sepharose 4B-Cl is for globular macromolecules M_r 2×10^7 .† The exclusion limit of the HPGF is for globular macromolecules M_r 10^8 .

‡ The enzyme incubation was followed by incubation with periodate for 1 h at pH 4 and 22 °C.

§ Incubation for 3 h at room temperature.

agglutinin. Incubation at pH 10 abolished both biological activities, possibly by β -elimination of *O*-glycosidically linked sugar chains, suggesting that these sugar residues are responsible for the biological activity. Treatment with periodate showed remarkable differences: a very mild treatment (10 mM at room temperature for 60 min at pH 6) abolished only the activity of the mt^+ agglutinin, but the activity of the mt^- agglutinin seemed to be insensitive to periodate, even in harsher conditions (20 mM at room temperature for 30 h at pH 4). However, after treatment with sulphatase and phosphatase, which in itself did not affect the biological activity, periodate treatment did destroy the mt^- activity. This might indicate that sugar residues are involved in the biological activity of both agglutinins, but that sulphate and phosphate substitution inhibits direct periodate attack of carbohydrate moieties in the mt^- agglutinin. The susceptibility of the agglutinins to exoglycosidases also differed: α -glucosidase eliminated the biological activity of the mt^+ , but not of the mt^- agglutinin, while α -mannosidase reduced the biological activity of the mt^- , but not of the mt^+ agglutinin.

DISCUSSION

In this article we report the purification of the sexual agglutinins of *C. moewusii yapensis*. The purity of the agglutinins was established by their elution as single protein peaks in HPGF, by the presence of only one sugar-stainable band on SDS-PAGE, and by the fact that electron microscopy showed a homogeneous population of macromolecules. The purification steps were essentially the same as adopted for the purification of the agglutinins of *C. eugametos* (Samson *et al.*, 1987) and we presume that this procedure can also be used for purifying agglutinins of other *Chlamydomonas* species.

Electron microscopy showed that both agglutinins are long, linear molecules. This structure has also been reported for other *Chlamydomonas* agglutinins (Goodenough *et al.*, 1985; Klis *et al.*, 1985). The agglutinins of *C. reinhardtii* are both rather rigid, rod-shaped molecules with a total length of about 260 nm, and possess determinate domains such as a bulbous end, a shaft and a hook region (Goodenough *et al.*, 1985). The agglutinins of *C. eugametos* are also rod-shaped but they are somewhat longer (328 nm for the mt^+ , 345 nm for the mt^- agglutinin), and no bulbous end was seen in the mt^- agglutinin, which instead displayed a number of flexible regions at fixed positions (Crabbendam *et al.*, 1987). The agglutinins of *C. moewusii yapensis* show even larger differences between the mating types, the mt^+ agglutinin having one bulbous and one tapered end and a length of about 250 nm, and the mt^- agglutinin having two tapered ends, a length of about 350 nm and a much less rigid conformation. However, unlike the mt^- agglutinin of *C. eugametos*, the points of flexion in the molecule did not appear to occur at set positions. The agglutinins of *C. moewusii yapensis* can be considered as hydroxyproline-rich glycoproteins: the mt^- agglutinin has an exceptionally high content of hydroxyproline, as only found until now in extensins, glycoproteins which are structural compounds in the primary wall of higher plants (Lamport, 1980). One might presume that the agglutinins also possess a stable polyproline II helix conformation, by analogy with cell wall glycoproteins from higher plants (Van Holst & Varner, 1984) and from *C. reinhardtii* (Homer & Roberts, 1979); this has already been suggested for other agglutinins, which have hydroxyproline contents comparable with that of the mt^+ agglutinin of *C. moewusii yapensis* (Goodenough *et al.*, 1985; Samson *et al.*, 1987). The bulbous end of the mt^+ agglutinin is possibly its only globular domain. Goodenough *et al.* (1985) reported that the biologically important region of the *C. reinhardtii* agglutinins is probably located in the globular head region. The biological activity of the mt^+ (but not the mt^-) agglutinin of *C. moewusii yapensis* was susceptible to agents that disrupt the conformation of globular proteins, which makes it reasonable to suggest that in this mt^+ agglutinin also, the biologically active site is positioned in the globular end of the molecule.

The overall sugar and amino acid compositions of the mt^+ and mt^- agglutinins show, apart from the very high hydroxyproline content of the mt^- agglutinin, a certain similarity, and they also resemble the compositions of the *C. reinhardtii* (amino acid) (Collin-Osdoby & Adair, 1985) and *C. eugametos* (amino acid and sugar) (Samson *et al.*, 1987) agglutinins. These similarities in form and composition suggest a common ancestry for recognition molecules of this kind in different *Chlamydomonas* species.

N-Acetylglucosamine could not be detected in either of the *C. moewusii yapensis* agglutinins, suggesting the absence of *N*-linked sugar chains in these glycoproteins. This contradicts the results of Wiese *et al.* (1984), who reported that the sexual responsiveness of the *mt*⁺ gametes of *C. moewusii yapensis* could be impaired by tunicamycin, a drug which inhibits the biosynthesis of *N*-linked sugar chains. To explain this discrepancy we probably have to understand more about the molecular basis of the sexual recognition reaction in *Chlamydomonas*, especially about the incorporation of the agglutinins in the flagellar membrane and about the precise interactions of the agglutinins with their receptors on the flagellar membrane of their sexual partner.

Since the sugar residues seem to be important for the biological activity of the agglutinins (as evidenced by their sensitivity to periodate) these probably also determine the mating-type- and species-specificity of the molecules. A remarkable property of the *mt*⁺ agglutinin of *C. moewusii yapensis* was its resistance to direct periodate attack: only after treatment with phosphatase or sulphatase was its biological activity destroyed by periodate, suggesting phosphorylation and sulphation of important sugar residues, a phenomenon which has not been observed in the *mt*⁺ agglutinin or agglutinins of other species. A differential effect was apparent in incubations with exoglycosidases, the *mt*⁺ agglutinin of *C. moewusii yapensis* being susceptible to α -glucosidase and the *mt*⁺ agglutinin to α -mannosidase. Using live cells, Wiese & Wiese (1975) found that *mt*⁺, but not *mt*⁺ gametes of *C. moewusii yapensis* failed to recognize gametes of the opposite sex after exposure to α -mannosidase. This suggests that different carbohydrate groups in terminal positions are important for sexual recognition: only more knowledge about the process can explain apparent contradictions regarding mating-type specificity. A differential susceptibility has also been reported for the *C. eugametos* agglutinins, in which terminal galactose groups seem to be involved in the *mt*⁺ and terminal mannose residues in the *mt*⁺ biological activity (Samson *et al.*, 1987). Inactivation of biological activity by the same exoglycosidases could, however, not be found for the *C. reinhardtii* agglutinins (Collin-Osdoby & Adair, 1985). So the barrier between these sexually incompatible species may depend also on subtle differences in carbohydrate side-chains.

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