The sexual agglutinins in *Chlamydomonas eugametos* are sulphated glycoproteins

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Sexual adhesion in the green alga *Chlamydomonas eugametos* is mediated by hydroxyproline-containing glycoproteins (agglutinins). The agglutinins were metabolically labelled with [³⁵S]sulphate. The sulphur-containing amino acids methionine and cysteine were not detectably labelled; little or no sulphated tyrosine was detected. This indicated that the radioactive label had been largely or exclusively incorporated into sulphated sugar residues. Acid hydrolysis released essentially all radioactivity as free sulphate. Short periods of hydrolysis released sulphated oligosaccharides. β -Elimination released about 20–25% of the sulphated saccharides, indicating that these are O-linked to serine or threonine or both. The remaining sulphated sugar chains are presumably O-linked to hydroxyproline. It is proposed that the sulphated sugars of the agglutinins play a role in sexual adhesion.

Introduction

In the biflagellate, unicellular green alga *Chlamydomonas eugametos*, cell fusion is initiated by adhesion of the flagella between gametes of opposite mating type. This process represents an excellent model system for studying cell–cell recognition (van den Ende *et al.*, 1990). In both mating types, designated *mt*⁺ and *mt*[−], hydroxyproline-containing glycoproteins, termed agglutinins, are involved in flagellar adhesion (Musgrave *et al.*, 1981; Klis *et al.*, 1985; Samson *et al.*, 1987; Versluis *et al.*, 1992). Both *mt*⁺ and *mt*[−] agglutinins are long, fibrous molecules (Crabbendam *et al.*, 1986) with estimated molecular masses of 1200 and 1300 kDa, respectively (Samson *et al.*, 1987), and they are responsible for the specificity of sexual adhesion (van den Ende *et al.*, 1990).

In many biological processes, including sexual interaction and adhesion, sulphated polysaccharides play an important role. For example, bindin, an adhesive protein from sea urchin sperm, specifically binds sulphated fucan polysaccharides on the surface of the eggs (DeAngelis & Glabe, 1990). It has also been

suggested that adhesive proteins from ram and boar sperm interact with sulphated polysaccharides of the egg (Williams & Jones, 1990; Jonáková *et al.*, 1991). Finally, fertilization is blocked by the sulphated heteropolysaccharide fucoidan in widely diverging species, suggesting that sulphated polysaccharides mediate this process (Jonáková *et al.*, 1991). Host plant specificity in the *Rhizobium*–legume symbiosis is determined by 6-O-sulphation of lipo-oligosaccharide signals (Nod factors) (Roche *et al.*, 1991). Sulphated signal molecules of *Rhizobium meliloti* elicit root hair curling and nodulation on alfalfa but not on common vetch, while on the other hand nonsulphated factors of mutants of this soil bacterium elicit these biological responses on vetch but not on alfalfa. Other adhesive proteins, including fibronectin (Yamada *et al.*, 1980), laminin, thrombospondin and von Willebrand factor (Roberts *et al.*, 1986), selectively bind sulphated polysaccharides, the binding of which is essential for their biological function.

The following observations suggested that sulphated sugars might also play a role in sexual adhesion of *C. eugametos* gametes. Firstly, the agglutination between gametes of opposite mating type is sensitive to relatively low salt concentrations (Köhle *et al.*, 1980; Goodenough, 1986) and, secondly, sugars appear to be essential for the biological activity of both agglutinins (Samson *et al.*, 1987). Finally, sulphated hydroxyproline-containing glycoproteins are known in green algae. They have been found in the cell wall of *Chlamydomonas reinhardtii*

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; *mt*[−]/*mt*⁺, mating type minus/plus; HP gel filtration, high-performance gel filtration.

(Roberts *et al.*, 1980) as well as in the extracellular matrix of the related genus *Volvox* (Ertl *et al.*, 1989). Studies were accordingly undertaken to determine whether or not the sexual agglutinins of *C. eugametos* possess sulphated sugars.

Methods

Materials. ^{35}S as carrier-free sulphate in aqueous solution (sp. act. 370 MBq mmol $^{-1}$; 185 MBq) was purchased from Amersham. Glycopeptidase F (PNGase F, EC 3.2.2.18) was obtained from Boehringer.

Cell cultures and in vivo ^{35}S -labelling of the sexual agglutinins. *Chlamydomonas eugametos* strains 5.39.4 (mt^{-}) and 17.17.2 (mt^{+}) were cultivated in Petri dishes on agar-containing medium in a 12 h light/12 h dark regimen (Schuring *et al.*, 1987). Cells were scraped from 2–3-week-old cultures, which had been cooled for 1 h at 5 °C, and suspended in 5 mM-HEPES/NaOH buffer, pH 7.2, containing 1 mM- CaCl_2 and 1 mM- MgCl_2 . [^{35}S]Sulphate (37 MBq) was added to 20 ml cell suspension (3.5×10^8 cells ml $^{-1}$) and the cells were gently shaken at room temperature in a 12 h light/12 h dark regimen. When the cells had incorporated 30–50% of the added radioactivity (after 1 or 2 d), they were harvested. ^{35}S -labelled agglutinins were isolated and purified as described below.

Extraction and purification of agglutinins. The mt^{-} and mt^{+} agglutinins were extracted as described previously (Musgrave *et al.*, 1981; Klis *et al.*, 1985). The extracts were fractionated on a ZetaPrep disk (DEAE-anion exchanger; CUNO Inc.). Elution was carried out stepwise with increasing NaCl concentrations (50, 150, 250, and 500 mM, and 1 M) in 10 mM-Tris/HCl, pH 7.4. Agglutinin-containing fractions, as detected by a semi-quantitative assay (Musgrave *et al.*, 1981), were collected and concentrated with a Centricon-30 microconcentrator (Amicon). The concentrate was applied onto a Bio-Gel TSK-60XL (7.5 mm $\times$ 300 mm; Bio-Rad) gel filtration column. Agglutinin-containing fractions were collected and run again. The column was eluted with 10 mM-sodium phosphate, 100 mM-sodium sulphate at pH 7.4 (Samson *et al.*, 1987). Protein concentrations were calculated from the absorption at 206 nm (Scopes, 1982).

High-performance gel filtration of hydrolysed ^{35}S -labelled agglutinins. Desulphation of metabolically labelled agglutinin was performed essentially according to Freeze & Wolgast (1986). Labelled agglutinin was hydrolysed in 0.25 M-HCl at 100 °C for different time periods. Samples were cooled and neutralized with NaOH or NaHCO_3 . Acid hydrolysates were analysed by HP gel filtration using the following columns connected in series: a Bio-Gel TSK guard column (7.5 mm $\times$ 75 mm), a Bio-Gel TSK-10 column (7.5 mm $\times$ 300 mm; Bio-Rad), and a self-packed Bio-Gel P-2 column (7.5 mm $\times$ 600 mm), which separates salts from amino acids and monosaccharides. The columns were eluted with 10 mM-sodium phosphate, 100 mM-sodium sulphate at pH 7.4, at a flow rate of 100 $\mu\text{l min}^{-1}$, and 0.4 ml fractions were collected. The ^{35}S activity in each fraction was determined. The column was calibrated with a partial dextran hydrolysate (Yamashita *et al.*, 1982). Carbohydrates were measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956).

β -Elimination of ^{35}S -labelled agglutinins. Purified, ^{35}S -labelled agglutinin was incubated in 0.05 M-NaOH and 1 M- NaBH_4 for 6 h at 45 °C (Lens *et al.*, 1983). The reaction mixture was cooled, titrated with 4 M-acetic acid to pH 4.5, and then neutralized with NaOH and concentrated in a SpeedVac concentrator. The samples were analysed by

gel filtration using the HPLC system as described above for the acid hydrolysates or fractionated into high and low molecular mass material by gel filtration on a Sephadex G-25 column (15 mm $\times$ 50 mm).

Sugar and enzyme analysis. Agglutinins were hydrolysed in 0.25 M-HCl for 15 min at 100 °C. The hydrolysates were applied onto a Bio-Gel P-6 desalting column (cut-off 6000 Da). High and low molecular mass materials were separately analysed for sugars by gas-liquid chromatography (Gerwig *et al.*, 1984). Glycopeptidase-F (PNG-ase F) incubations were performed in 20 mM-sodium phosphate, 10 mM-EDTA, 10 mM- β -mercaptoethanol, 0.5% (w/v) CHAPS, pH 7.0, at 37 °C for 24 h. Samples were heated for 2–3 min at 100 °C prior to this treatment.

Barium hydroxide hydrolysis of ^{35}S -labelled agglutinins. To determine whether tyrosine-*O*-sulphate was present, ^{35}S -labelled agglutinin (1000–1500 d.p.m.) was hydrolysed with barium hydroxide (Huttner, 1984). After neutralization with sulphuric acid and sedimentation of the precipitate by centrifugation at 10000 g for 10 min, tyrosine-*O*-sulphate, if present, was in the supernatant fraction. The agglutinins were assayed for the presence of tyrosine-*O*-sulphate by measuring the radioactivity in the neutralized supernatant.

Results

The presence of sulphate groups in the agglutinins was established by metabolically labelling gametes with [^{35}S]sulphate, and isolating and purifying the mt^{+} and mt^{-} agglutinins. The standard procedure for purifying the sexual agglutinins consisted of anion-exchange chromatography followed by gel filtration. When the agglutinin-containing fractions were subsequently applied to a 2.2%–20% (w/v) SDS-PAGE gradient gel, a single high-molecular-mass glycoprotein was observed (Samson *et al.*, 1987). For the present set of experiments, the agglutinin-containing fractions of the first gel filtration run were isolated and applied again to the same column. A single peak of ^{35}S activity was observed which coincided with a single absorption peak at 206 nm. The specific activities were 73 ± 10 Bq μg^{-1} (mean $\pm$ SEM; $n = 4$) for the mt^{+} agglutinin and 38 ± 3 Bq μg^{-1} ($n = 3$) for the mt^{-} agglutinin, corresponding to at least 238 and 130 sulphur atoms, respectively, per molecule of agglutinin.

There are three known possibilities by which sulphur can occur in glycoproteins. Firstly, it can be incorporated into the sulphur-containing amino acids methionine and cysteine. Secondly, sulphation of sugar residues can occur. Thirdly, sulphate can be *O*-esterified to tyrosine (Huttner, 1984). If present, tyrosine-*O*-sulphate can be detected in the supernatant fraction after $\text{Ba}(\text{OH})_2$ hydrolysis and subsequent neutralization with sulphuric acid to co-precipitate released sulphate as its barium salt (Huttner, 1984). However, this treatment released only 4–6% of the ^{35}S label of both agglutinins into the supernatant fraction (not shown). It was thus concluded that the agglutinins possessed little or no tyrosine-*O*-sulphate and that about 95% of the ^{35}S label had been incorporated otherwise.

Fig. 1. HP gel filtration analysis of ^{35}S -labelled mt^+ agglutinin after mild acid hydrolysis. Incubations were performed in 0.25 M-HCl at 100 °C for different periods of time. The percentages of the total ^{35}S activity eluting in fractions 40–89 were as follows: 22 % (hydrolysis for 10 min), 43 % (25 min), 56 % (50 min), and 22 % (150 min). Arrows indicate the following elution positions: V_0 , (void volume) – the cut-off was about 2300 Da; V_t , total volume; 1, glucose; 2, sucrose; 3, raffinose; 4 to 7, dextran hydrolysate, presumably Glc_4 to Glc_7 ; M, ^{35}S -methionine; and S, ^{35}S -sulphate. The fraction volume was 0.4 ml. Similar HP gel filtration profiles were obtained for the mt^- agglutinin.

In order to discriminate between the other two possibilities, ^{35}S -labelled agglutinins were subjected to mild acid hydrolysis for different periods of time. The hydrolysates were analysed by HP gel filtration. Increasing hydrolysis periods resulted in a gradual shift of ^{35}S activity from void-volume to total-volume fractions, where free sulphate elutes after monosaccharides and single amino acids (Fig. 1). After acid hydrolysis for 150 min, all the ^{35}S activity eluted as free sulphate in the

Table 1. Composition of the saccharides released from the sexual agglutinins as a percentage of the total amount of each monosaccharide (Klis *et al.*, 1989) after acid hydrolysis for 15 min in 0.25 M-HCl at 100 °C.

The means of two separate determinations are presented. The variations in the sugar determinations were less than 10 %.

Sugar	Composition (%) of saccharides released from	
	mt^- agglutinin	mt^+ agglutinin
Ara	91	94
Rha	60	100
Xyl	81	96
Man	75	98
Gal	89	86
Glc	94	96
GlcNAc	73	< 1

Fig. 2. Determination of half-lives of the release of free ^{35}S -sulphate from labelled agglutinins in 0.25 M-HCl at 100 °C. ●, mt^+ agglutinin; ○, mt^- agglutinin.

total volume. No radioactivity was found in cysteine or methionine. Thus, the ^{35}S label in both agglutinins was predominantly or exclusively present as sugar-linked sulphate groups. Depending on the duration of hydrolysis, intermediate compounds (≤ 2300 Da) were also found. The percentage of radioactivity in the fractions between the void and total volume (viz. fractions 40–89 in Fig. 1) increased from 22 % after a 10 min hydrolysis to 56 % after a 50 min hydrolysis. The results for the mt^- agglutinin were 19 % and 59 %, respectively (not shown). This suggested that both agglutinins had been partially deglycosylated and had released sulphated oligosaccharides. This was supported by gas chromatographic analysis of the released material, which showed that after 15 min all major sugars were, to a large extent, released (Table 1). The $t_{1/2}$ for sulphate release appeared to be 74 min for the mt^+ agglutinin and 53 min for the mt^- agglutinin (Fig. 2). These half-lives indicate an axial position of the sulphate residues in the sugar rings (Rees, 1963).

Fig. 3. HP gel filtration analysis of ^{35}S -labelled mt^+ agglutinin after β -elimination. The β -elimination products eluted after the void volume (V_0) and contained 21% of the total ^{35}S activity (fractions 40–45). Arrows indicate the following elution positions: V_0 , void volume – the cut-off was about 2300 Da; V_1 , total volume; 1, glucose; 2, sucrose; 3, raffinose; 4 to 7, dextran hydrolysate, presumably Glc_4 to Glc_7 . An HP gel filtration profile representative of four individual experiments is presented. Similar results were obtained for the mt^- agglutinin.

The *O*-glycosidic bond between sugars and the β -hydroxyamino acids, serine and threonine, is relatively sensitive to alkaline cleavage by a β -elimination type reaction (Sharon, 1975). In the presence of the strong reducing agent NaBH_4 , the released oligosaccharide side-chains can be recovered to determine whether or not they contain ^{35}S label. β -Elimination of both agglutinins released 30% of the sulphated sugars as low-molecular-mass material recovered after Sephadex G-25 gel filtration (not shown). After HP gel filtration of the β -elimination products of the mt^+ agglutinin, approximately 20–25% of the ^{35}S -label eluted directly after the void volume peak (viz. fractions 40–45 in Fig. 3). These oligosaccharides were at least nine residues long as determined with a partial dextran hydrolysate for standards. The results of Sephadex G-25 gel filtration and HP gel filtration of the β -elimination products showed that a considerable proportion of the sulphate groups were attached to sugars *O*-glycosidically linked to serine or threonine or both. The alkali-insensitive part of the ^{35}S label is presumably exclusively attached to hydroxyproline-linked saccharides, since treatment with glycopeptidase-F, which hydrolyses *N*-glycosidic bonds, did not release any labelled material.

Discussion

In this report both the mt^+ and mt^- sexual agglutinins of *Chlamydomonas eugametos* are shown to be sulphated glycoproteins. Sulphation is a post-translational modification which results in sulphate being linked as an ester to either tyrosine or sugar residues. Our results indicate

that in these agglutinins, sulphate is largely or exclusively bound to sugars *O*-linked to either serine and threonine or to hydroxyproline. By β -elimination it was shown that about a quarter of the ^{35}S label was present in *O*-linked sugars attached to serine or threonine. The remainder is probably attached to glycosides linked to hydroxyproline.

After mild acid hydrolysis, a number of labelled products were obtained which probably represent sulphated oligosaccharides and free sulphate. The high sensitivity to acid hydrolysis of the sugar side-chains in the sexual agglutinins is in agreement with observations on other hydroxyproline-containing glycoproteins. Firstly, because arabinose is one of the predominant sugars in the sexual agglutinins (Samson *et al.*, 1987; Klis *et al.*, 1989), it seems likely that the sexual agglutinins of *C. eugametos* will contain arabinofuranosyl hydroxyproline linkages as similar hydroxyproline-containing glycoproteins do (Lamport, 1980). Such linkages are very acid-labile: they can be hydrolysed within 1 h in 0.1 M-HCl at 100 °C (Lamport, 1980). Secondly, Miller *et al.* (1972) found that hydroxyproline glycosides isolated from *Chlamydomonas* cell wall protein could be partially degraded by treatment with 0.1 M-trifluoroacetic acid at 90 °C for 30 min.

The half-lives for release of free sulphate of both agglutinins differ from those found for sulphated cell wall glycoproteins in *C. reinhardtii* (Roberts *et al.*, 1980), implying that sugars in *C. eugametos* agglutinins are esterified at different positions. The occurrence of sulphate groups in both agglutinins probably also explains the low isoelectric points of both the mt^+ agglutinin (pI 4.3) and the mt^- agglutinin (pI 3) (Samson *et al.*, 1987).

The fact that sulphated carbohydrate side-chains are often involved in biological recognition and in the binding and interaction of macromolecules raises the question whether or not the sulphated oligosaccharide side-chains in the sexual agglutinins play a role in sexual agglutination. The following observations indicate that they might indeed be involved in this process. Relatively low concentrations of salts can completely and reversibly inhibit sexual adhesion in *Chlamydomonas* (Köhle *et al.*, 1980; Goodenough, 1986), suggesting that the adhesion reaction is due to ionic binding of positively and negatively charged groups at the recognition site. On the other hand, there is strong evidence that in both sexual agglutinins sugar residues are essential for the biological activity of the agglutinins. For example, both molecules lose their activity after a short treatment with periodate, which specifically attacks sugars (Woodward *et al.*, 1985). It is, therefore tempting to speculate that the sulphated sugars in both agglutinins play a role in sexual adhesion.

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