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Green algae *Chlamydomonas reinhardtii* possess endogenous sialylated N-glycans

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ABSTRACT

Green algae have a great potential as biofactories for the production of proteins. *Chlamydomonas reinhardtii*, a representative of eukaryotic microalgae, has been extensively used as a model organism to study light-induced gene expression, chloroplast biogenesis, photosynthesis, light perception, cell–cell recognition, and cell cycle control. However, little is known about the glycosylation machinery and N-linked glycan structures of green algae. In this study, we performed mass spectrometry analysis of N-linked oligosaccharides released from total extracts of *Chlamydomonas reinhardtii* and demonstrated that *C. reinhardtii* algae possess glycoproteins with mammalian-like sialylated N-linked oligosaccharides. These findings suggest that *C. reinhardtii* may be an attractive system for expression of target proteins.

1. Introduction

Glycosylation is one of the most common and important post-translational modifications of proteins, and the biological activity of many therapeutic glycoproteins may depend on their glycosylation status. Currently, the N-linked glycosylation status is well established for yeast, insects, mammals and plants. However, little is known about the N-linked glycosylation pathway and N-linked glycan structure in green algae. All available studies have shown that proteins of green microalgae contain predominantly high-mannose glycans; however, in some species hybrid and complex types of N-linked glycans are also reported [1,2]. For example, a structure analysis of N-linked glycans of the diatom microalgae *Phaeodactylum tricornutum* showed that while its proteins mostly carry the high-mannose-type N-linked glycans ranging from Man-5 to Man-9, minor glycans Man-3 and Man-4 carrying a 1,3-linked fucose are also present [1]. Furthermore, the 66-kDa glycoprotein from the cell wall of red microalgae of the *Porphyridum* sp. contains a novel glycan structure with 6-*O*-MeMan and xylose monosaccharides that differed from the glycan structures found in other algae organisms so far [2].

Chlamydomonas reinhardtii is a well-studied representative of eukaryotic microalgae that has been used as a model organism for a number of physiological, biochemical and genetic studies for more than a decade [3,4]. The recently released version 4 of *Chlamydomonas* genome revealed that *Chlamydomonas* and humans share 706 protein families [5]. Eukaryotic microalgae have been also used for the recombinant protein expression [6,7]. Features that make this algae attractive as a potential protein production platform include the low biomass cost, safety, the ease of genetic manipulation to introduce genes of interest into the nuclear, chloroplastic or mitochondrial genome, the possession of eukaryotic post-translational modification machinery, rapid growth and scalability, as well as the ability to grow phototrophically or heterotrophically utilizing acetate as a carbon source. Despite the listed advantages, there is a number of questions that need to be addressed, including glycosylation, before green algae can be utilized for commercial manufacturing.

To this point, in this study, by using both mass spectrometry (MS) and biochemical analyses, we have demonstrated that the green algae *Chlamydomonas reinhardtii* possess glycoproteins with mammalian-type sialylated N-linked oligosaccharides.

2. Materials and methods

2.1. Preparation of cell protein extract

C. reinhardtii CC-125 cells were grown photoautotrophically under ambient air at 25 °C and collected by centrifugation at 3000*g* for 10 min. The cells were washed with phosphate buffered saline

Abbreviations: 2-AB, 2-aminobenzamide; CST, CMP-sialic acid transporter; ER, endoplasmic reticulum; hEPO, human erythropoietin; HPLC-FLD, high-performance liquid chromatography using fluorescence detection; HRP, horseradish peroxidase; MS, mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; RCA, *Ricinus communis* agglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline

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(PBS), suspended and disrupted by sonication in PBS buffer, pH 7.0, containing 1 mM phenylmethanesulfonyl fluoride, and then centrifuged at 150,000*g* for 30 min. The supernatant was desalted by passing through a Sephadex G-25 column (PD-10, Amersham Pharmacia, Uppsala, Sweden) and used as a total soluble protein preparation for biochemical and MS analyses. The pellet was further incubated in PBS plus 0.1% Triton X-100 at 4 °C for 30 min followed by centrifugation at 150,000*g* for 30 min. The supernatant was desalted by passing through a PD-10 column and used for analyses as a membrane protein preparation. All experiments were carried out at 4 °C.

2.2. Sialic acid-specific lectin blotting analysis

Lectin blotting for detection of sialic acid was performed according to a method described previously [8] with some modifications. Briefly, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was washed three times with Tris-buffered saline (TBS) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) and blocked with Carbo-free blocking buffer (Cat. No. SP-5040, Vector Laboratories, Burlingame, CA) for 1 h. After blocking, the membrane was incubated in the lectin incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) containing 10 μg/ml biotinylated SNA-1 (*Sambucus nigra*, Cat. No. BA-6802-1, EY Laboratories, San Mateo, CA) and 50 μg/ml MAA (*Maackia amurensis*, Cat. No. BA-7801-5, EY Laboratories) for 2 h. After three washes with TBS, the membrane was incubated with avidin plus biotinylated horseradish peroxidase (HRP) using ABC kit (Cat. No. PK-4000, Vector Laboratories) for 30 min and washed three times with TBS. Lectin binding to sialic acid-containing proteins was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). For the lactose inhibition experiment, lectins were pre-incubated in 100 mM lactose solution, and the blocking buffer, lectin incubation buffer and wash buffer contained 100 mM lactose, as described previously [8].

2.3. Galactose-specific lectin blotting analysis

Lectin blotting specific for galactose was performed using *Ricinus communis* agglutinin RCA_{120} (Cat. No. B-1085, Vector Laboratories) according to the manufacturer's protocol. Briefly, proteins were separated by SDS–PAGE and transferred to a PVDF membrane. The membrane was washed three times with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) and blocked using Carbo-free blocking buffer (Cat. No. SP-5040, Vector Laboratories) for 30 min. After blocking, the membrane was incubated in PBS containing the biotinylated lectin at 20 μg/ml, washed three times with PBS-T (PBS containing 0.05% Tween-20), and incubated with avidin plus biotinylated HRP using VECTASTAIN-ABC (Cat. No. PK-6100, Vector Laboratories) for 30 min. Avidin plus biotinylated HRP was prepared in PBS-T according to the manufacturer's instructions. The lectin binding to galactose-containing proteins was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL).

2.4. Construction of binary construct for trans-Golgi targeting of human β1,4-galactosyltransferase

The binary vector pBI121 [9] was used for the expression of modified β1,4-GalT in *Nicotiana benthamiana*. Briefly, the N-terminal CMP-sialic acid transporter (CST) domain of human GalT was replaced with the CST from the rat α2,6-sialyltransferase (ST, GenBank accession number M187609) as described previously [10]. ST-GalT was optimized for the expression in *N. benthamiana* (for codon optimization, mRNA stability, etc.) and synthesized by GENEART AG (Regensburg, Germany) with flanking *PacI* (5′-terminus) and *XhoI* (3′-terminus, after stop codon) sites. pBI121-ST-GalT was then introduced into *Agrobacterium tumefaciens* strain GV3101. The resulting bacterial strain was grown in the BBL medium (10 g/L soy hydrolysate, 5 g/L yeast extract, 5 g/L NaCl, 50 mg/L kanamycin) overnight at 28 °C. The bacteria were introduced by manual infiltration into 6-week-old *N. benthamiana* plants grown in soil. Five, six and seven days after infiltration, leaf tissue was harvested and homogenized using a bullet blender (Zymo research). Extracts were clarified by centrifugation (13,000*g* for 30 min) and used for Western blot analysis.

2.5. Analysis of N-linked oligosaccharides by HPLC-FLD

Cleavage of N-linked carbohydrates from glycoprotein samples was performed using N-Glycosidase A (PNGase A, Roche). Once released, glycans were extracted and dried by centrifugal concentration. The recovered oligosaccharides were labeled with 2-aminobenzamide (2-AB) in the presence of sodium cyanoborohydride under acidic conditions. Subsequent to the derivatization step, excess dye and other reagents remaining in the samples were removed by means of Glycoclean® S sample filtration cartridges. The following high-performance liquid chromatography using fluorescence detection (HPLC-FLD) procedure was then applied. Mobile Phase A was 65% acetonitrile/35% water, and Mobile Phase B was 250 mM ammonium formate, pH 4.4. Chromatography mode was normal phase, and detection was performed using fluorescence at 330 nm (Ex) and 420 nm (Em). Chromatographic peaks were integrated, and based on peak retention times were compared to those from fetuin. Results were expressed as % area of each glycoform of the total peak area. Peak samples resulted from the HPLC-FLD separation were collected and dried by centrifugal concentration. Each peak sample was re-suspended in 12 μl of 0.1% formic acid in water. A 2-μl injection volume of each peak was loaded on a mass spectrometer. MS mobile phase A was water with 0.1% formic acid and MS mobile phase B was 90% acetonitrile in water with 0.1% formic acid. Chromatography was a graphitized carbon chip (Reverse Phase). The obtained molecular masses were compared with the data in the Consortium for Functional Glycomics structural databases to identify matches to known oligosaccharide structures. In addition, oligosaccharide fragmentation spectra were manually verified to match the assigned structures. Oligosaccharides are represented by singly or doubly charged $(M+2H)^{++}$ ions in electrospray ionization mass spectrometry (ESI-MS). Mass increase was 120 Da for the 2-AB derivative compared to the monoisotopic mass of the oligosaccharide.

3. Results

3.1. N-Linked oligosaccharide analysis

To release glycans, total proteins of *C. reinhardtii* were treated with PNGase A (N-Glycosidase A). PNGase A is known to cleave N-linked glycans, including molecules carrying a fucose linked by an α1,3 bond to Asn-GlcNAc [11]. The released oligosaccharides were then analyzed as described in Section 2. In brief, N-linked glycans released from total soluble and total membrane fractions of *C. reinhardtii* were separated by HPLC, shown in Fig. 1A and B as traces of chromatographic signals from 2-AB-labeled glycans in the test samples. The peak fractions were collected and analyzed using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Intact mass analysis by MS and fragmentation of the detected oligosaccharide masses by the collision-induced dissociation (CID) method allowed for the

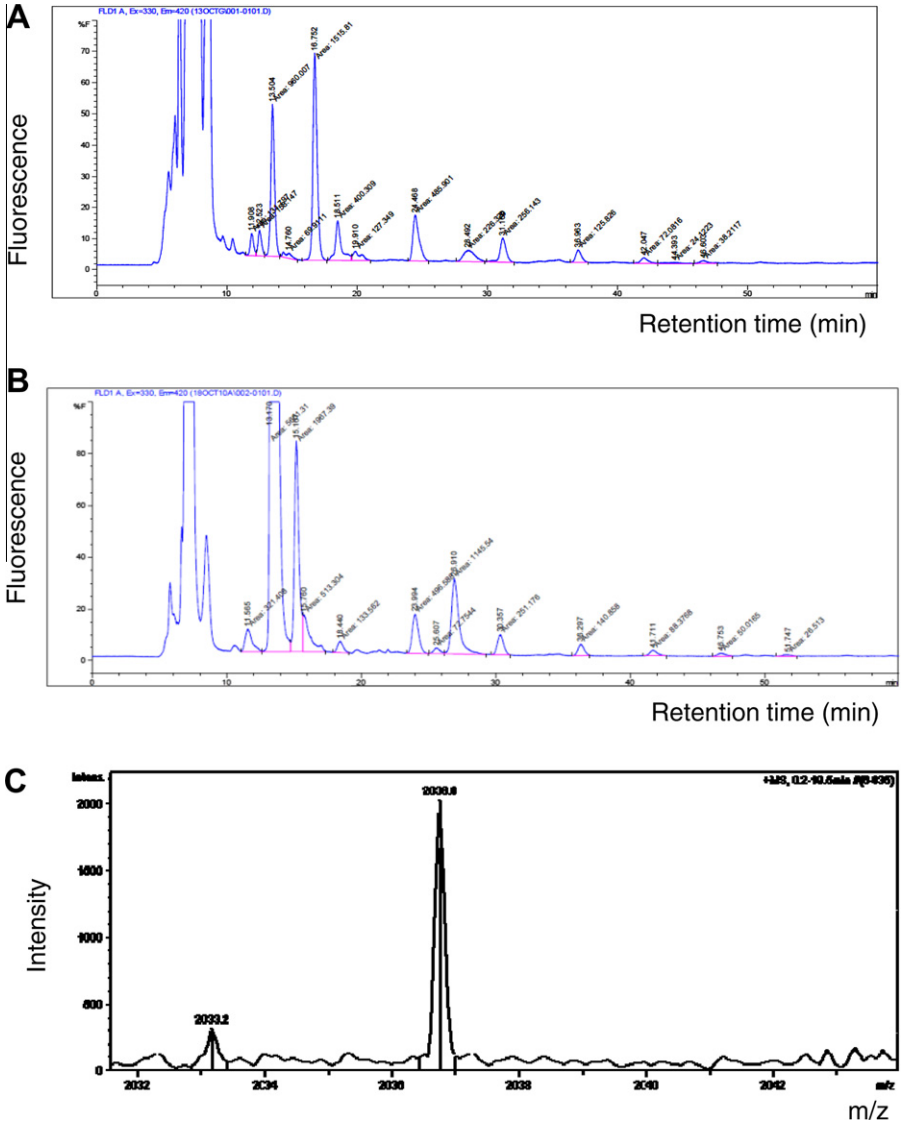


Fig. 1. Glycan profiles of extracts from *C. reinhardtii*. Cleavage of N-linked carbohydrates from glycoproteins of the total soluble fraction of *C. reinhardtii* was performed using N-glycosidase A (PNGase A, Roche). The released glycan pool was extracted and brought to dryness by centrifugal concentration. Analysis of N-linked oligosaccharides by HPLC-FLD was performed as described in Section 2. (A) Profile of glycans released from *C. reinhardtii* total soluble glycoproteins by PNGase A. (B) Profile of glycans released from *C. reinhardtii* total membrane glycoproteins by PNGase A. (C) Selected MS spectrum of ion at mass/charge (m/z) 2036.8 corresponding to Neu5Ac. The peak fraction N6 (from total membrane fraction) was analyzed using MALDI-TOF-MS. Number of sugars [4,4,1,1,0] in the structure: 4 (N-acetylhexosamines), 4 (hexose, mannose or galactose), 1 (fucose), 1 (Neu5Ac), 0 (Neu5Gc).

determination of the oligosaccharide species containing two sialic acids Neu5Gc (N-glycolylneuraminic acid) and Neu5Ac (N-acetylneuraminic acid). MS spectrum of ion at mass/charge (m/z) 2036.8 corresponding to Neu5Ac is shown in Fig. 1C. The additional glycan structures identified in *C. reinhardtii* extracts are summarized in Tables 1 and 2. Predominant oligosaccharide structures present in the samples were asialo, biantennary, with core fucosylation, and with or without galactosylation. High-mannose species corresponding to MAN5, MAN6 and MAN8 were also detected. Xylose-containing oligosaccharides in these fractions were not detected.

3.2. Galactose-specific lectin blotting analysis of *C. reinhardtii* proteins

The analysis of MS spectra confirmed the presence of galactose associated with sialic acid in N-linked glycans of *C. reinhardtii*. Because of the difficulty in distinguishing between mannose and Gal in complex glycan structures by means of MS, we further analyzed *C. reinhardtii* proteins using affinity blotting with the RCA_{120} lectin from *R. communis* that binds to the Galβ1–4GlcNAc sequence and, to a small extent, to other terminal β-linked Gal residues [12]. The results of the lectin blotting analysis showed that RCA_{120} binds to a number of *C. reinhardtii* proteins, and the binding pattern is similar to that observed in extracts from *N. benthamiana* expressing human β1,4-galactosyltransferase (Fig. 2), suggesting the presence of N-linked glycans containing 1,4-Gal residues. In contrast, in extracts from control *N. benthamiana* plants which did not express human β1,4-galactosyltransferase, RCA_{120} reacted only with high-molecular-weight proteins (Fig. 2, lane PC). These results are consistent with previously published data and suggest that the proteins that bound RCA_{120} might contain arabinogalactan [12].

3.3. Sialic acid-specific lectin blotting analysis of *C. reinhardtii* proteins

For sialic acid-specific lectin blotting, we probed *C. reinhardtii* glycoproteins with a mixture of biotinylated lectins from *S. nigra*

Table 1
Identified structures of glycans released from *C. reinhardtii* total soluble glycoproteins. Ion trap mass spectroscopy oligosaccharide nomenclature: acetylhexosamines (square ■); hexoses (circle ● or ●); fucose (triangle ▼); NeuAc (diamond ♦); NeuGc (diamond ◊).

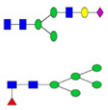
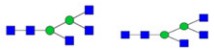
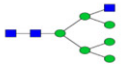
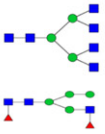
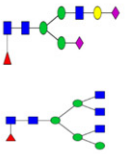
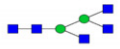
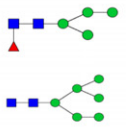
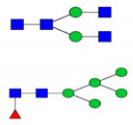
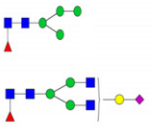
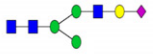
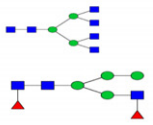
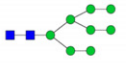
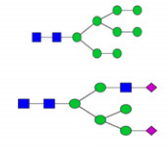
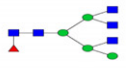
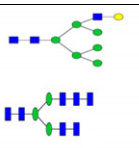
Peak No.	Mass (Da) [2AB mass subtracted] Observed	Mass (Da) [2AB mass subtracted] Theoretical	Adduct	Identified Glycans	Glycan Structures
1	1301.4	1301.2	$[M+H]^+$	[4,2,1,0,0]	
2	1302.4	1301.2	$[M+2H]^{2+}$	[4,2,1,0,0]	
3	1,382; 1567.6	1381.2, 1567.7	$[M+2H]^{2+}$, $[M+2H]^{2+}$	[2,5,1,0,0], [3,4,0,1,0]	
4	1358.2,1478.8	1358.2, 1478.8	$[M+H]^+$, $[M+2H]^{2+}$	[5,2,0,0,0], [4,4,0,0,0]	
5	1235.6, 1396.8	1235.1, 1396.8	$[M+2H]^{2+}$, $[M+2H]^{2+}$	[2,5,0,0,0] (MAN5), [2,6,0,0,0] (MAN6)	
6	1601.2	1600.4	$[M+H]^+$	[3,6,0,0,0]	
7	1567.1	1567.4	$[M+H]^+$	[3,4,0,1,0]	
8	1568.9, 1724.3	1568.4, 1723.6	$[M+H]^+$, $[M+H]^+$	[3,4,2,0,0], [6,3,0,0,0]	
9	1542.6, 1812.5	1543.4, 1812.5	$[M+2H]^{2+}$, $[M+H]^+$	[2,6,1,0,0], [5,3,2,0,0]	
					
10	1829	1828.7	$[M+2H]^{2+}$	[5,4,1,0,0]	
11	1681.7	1682.5	$[M+H]^+$	[5,4,0,0,0]	
12	1932.5	1932.7	$[M+H]^+$	[4,4,1,0,1]	
13	1828.2, 2005.0	1828.7, 2004.8	$[M+H]^+$ $[M+2H]^{2+}$	[5,4,1,0,0],[3,4,1,2,0]	
14	1990.9	1990.8	$[M+H]^+$	[5,5,1,0,0]	

Table 2
Identified structures of glycans released from *C. reinhardtii* total membrane glycoproteins. Ion trap mass spectroscopy oligosaccharide nomenclature: acetylhexosamines (square ■); hexoses (circle ● or ●); fucose (triangle ▼); NeuAc (diamond ♦); NeuGc (diamond ◊).

Peak No.	Mass (Da) [2AB mass subtracted] Observed	Mass (Da) [2AB mass subtracted] Theoretical	Adduct	Identified Glycans	Glycan Structures
1	1234.8	1235.1	$[M+2H]^{2+}$	[2,5,0,0,0](MAN5)	
2	1359	1358.3	$[M+2H]^{2+}$	[5,2,0,0,0]	
3	1218.8, 1397.2	1219.1, 1397.2	$[M+2H]^{2+}$, $[M+2H]^{2+}$	[2,4,1,0,0], [2,6,0,0,0](MAN6)	
4	1381.6	1381.6	$[M+H]^{+}$	[4,2,0,0,0], [2,5,1,0,0]	
5	1708.4	1707.6	$[M+2H]^{2+}$	[6,2,1,0,0]	
6	1916.6	1916.7	$[M+H]^{+}$	[2,4,1,0,0], [4,4,1,1,0]	
7	1765.0	1764.6	$[M+2H]^{2+}$	[5,2,0,0,0], [7,2,0,0,0]	
8	1771.8, 1951.0	1770.6, 1948.8	$[M+2H]^{2+}$, $[M+2H]^{2+}$	[4,4,0,1,0],[4,6,1,0,0]	
9	1721.8	1721.5	$[M+2H]^{2+}$	[2,8,0,0,0](MAN8)	
10	1720.8, 2021.0	1721.5, 2020.8	$[M+2H]^{2+}$, $[M+2H]^{2+}$	[2,8,0,0,0](MAN8), [3,5,0,2,0]	
11	1828.6	1828.7	$[M+2H]^{2+}$	[5,4,1,0,0]	
12	1828	1828.7	$[M+2H]^{2+}$	[5,4,1,0,0]	
13	1762.7, 1926.0	1762.6, 1926.8	$[M+H]^{+}$, $[M+2H]^{2+}$	[3,7,0,0,0], [7,3,0,0,0]	

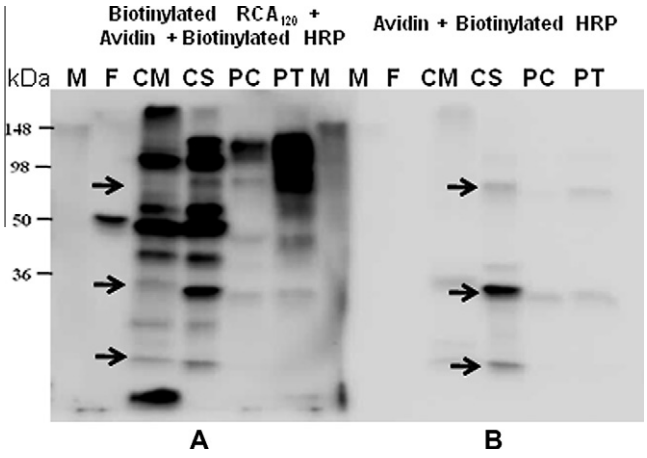


Fig. 2. Lectin blotting analysis of *C. reinhardtii* proteins using RCA_{120}. Eight microgram of *C. reinhardtii* total soluble protein (CS), 8 μg of *C. reinhardtii* total membrane protein (CM), 8 μg of total soluble proteins from *N. benthamiana* (PC), 8 μg of total soluble proteins from *N. benthamiana* infiltrated with human β1,4-galactosyltransferase (PT), and 250 ng of fibrinogen (F) were loaded onto the SDS–PAGE gel followed by a Western blot analysis. Galactosylated proteins were detected using the RCA_{120} lectin at 10 μg/ml in PBS with 0.05% Tween. Fibrinogen (Cat. No. F8630, Sigma, St. Louis, MO) was used as a positive control. (A) After blocking with Carbo-free blocking buffer, membranes were incubated with the 1,4-galactose residue-specific lectin followed by avidin plus biotinylated HRP using ABC kit (Vector Laboratories); (B) after blocking, the membrane was incubated with avidin plus biotinylated HRP using ABC kit. M – protein marker. Arrows show non-specific signals due to algae-derived biotin staining.

(SNA-I) and *Maackia amurensis* (MAA). These lectins bind to terminal SA-α2,6-Gal and SA-α2,3-Gal structures, respectively [13,14]. The specificity of lectin binding to sialylated glycoproteins of *C. reinhardtii* was confirmed by protein treatment with sialidase (Fig. 3) and inhibition of lectin binding by 100 mM lactose (data not shown). Sialidase-treated proteins showed a significantly weaker binding to lectins compared to non-treated proteins from *C. reinhardtii* as well as fibrinogen (Fig. 3), indicating the removal of α2-3,6-linked sialic acids from *C. reinhardtii* glycoproteins. Taken together, these results along with the results of the MS analysis confirm the presence of sialic acid residues in *C. reinhardtii* glycoproteins.

4. Discussion

Sialic acids are ubiquitous in animals of the deuterostome lineage, from starfish to human. On the other hand, it has been commonly accepted that in plants, protists, archaea, eubacteria and fungi sialic acids are absent [15–17]. However, some pathogenic organisms including certain bacteria, protozoa and fungi have been shown to have sialic acids [18–20]. It has been demonstrated that some strains of pathogenic bacteria synthesize sialic acids *de novo* to generate sialylated glycolipids on the cell surface [21]. The primary role of sialic acids is believed to protect these pathogenic bacteria from recognition by host immune system; however, they are also important for protein targeting, cell–cell interaction, and cell–substrate recognition and adhesion [22].

It has been reported that sialylation affects biological activity of many therapeutically important proteins [23]. In studies of the recombinant human erythropoietin (hEPO) protein, it has been demonstrated that asialylated hEPO has a very low erythropoietic activity *in vivo* compared to sialylated hEPO [24,25]. In addition, galactosylation may be critical for the pharmacokinetic activity of some therapeutic antibodies [12]. Although N-linked glycosylation and N-linked glycan structures have been well studied in mammals and other high eukaryotes, very little attention has been paid to studying of N-linked glycosylation in green algae. In a recent

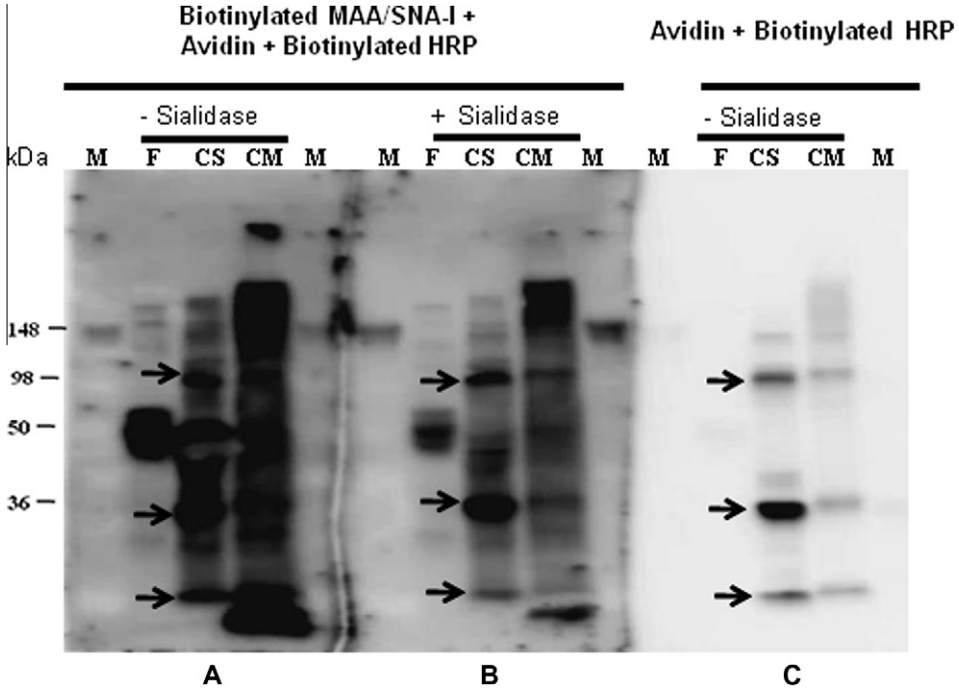


Fig. 3. Lectin blotting analysis of *C. reinhardtii* proteins using MAA and SNA-I. Twenty microgram of *C. reinhardtii* total soluble protein (CS) and 20 μg of *C. reinhardtii* total membrane protein (CM) along with 2 μg fibrinogen (F), treated with sialidase (A, as indicated) and non-treated (B and C), were loaded onto the SDS–PAGE gel followed by a Western blot analysis. Sialic acid was detected using the MAA and SNA-I lectins at 50 μg/ml and 10 μg/ml, respectively, in the reaction buffer (RB) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Fibrinogen was used as a positive control. (A and B) After blocking with Carbo-free blocking buffer, the membrane was incubated with the lectins followed by avidin plus biotinylated HRP using ABC kit; (C) after blocking, the membrane was incubated with avidin plus biotinylated HRP using ABC kit. M – protein marker. Arrows show non-specific signals due to algae-derived biotin staining.

study, an analysis of N-linked glycan structure from the diatom microalgae *P. tricornutum* has been performed. Results of this analysis have demonstrated that proteins of these algae carry mostly high-mannose-type N-linked glycans ranging from Man-5 to Man-9. In addition, minor glycans Man-3, Man-4 carrying a 1,3-linked fucose have been identified. However, the presence of 1,4-linked galactose and sialic acid in *P. tricornutum* proteins has not been confirmed using both immunodetection with glycan-specific probes and 4,4-dimethyl-2,2-bipyridine coupling [1].

In this study, we have demonstrated the presence of sialylated glycoproteins in unicellular green algae *C. reinhardii*. In previous studies, the sialyltransferase activity has been detected on the external surface of gametes of *Chlamydomonas moewusii* and suggested to be associated with mating [26]. Here, we performed an *in silico* analysis of the genomes of *C. reinhardtii* and *Ostreococcus lucimarinus* in search for genes that may encode homologs of mammalian enzymes involved in sialylation. Although we failed to find putative gene homologs for mammalian sialyltransferases in these databases, in the *C. reinhardtii* database we have identified an expressed sequence tag sequence (AV628473) that has some similarity to CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase (XP_546003.2). We also found that both *C. reinhardtii* (BP098341) and *O. lucimarinus* (XP_001422322.1, XP_001422301.1, XP_003083530.1 and XP_001416575) genomes contain putative gene homologs for CSTs. When a biochemical analysis using the lectin RCA_{120} was performed, the presence of 1,4-Gal in *C. reinhardtii* was also confirmed, however, homologs of the mammalian β1,4-galactosyltransferase in the *C. reinhardtii* genome were not found. It should be noted that the putative homologs for the mammalian β1,4 galactosyltransferase have been found in *O. lucimarinus* (XP_001416803.1). Together with our MS and lectin blotting data, this finding supports the presence of the glycan sialylation machinery in green algae. Screening of the *C. reinhardtii* genome database (Chlamydomonasreinhardtiiv4.0) also revealed the presence of a gene encoding putative α-mannosidase I, the first enzyme that modifies N-glycans transported from the endoplasmic reticulum (ER) to the Golgi by removing one to four α1,2-Man residues, thus converting $Man_9GLcNAc_2$ to $Man_5GlcNAc_2$. However, we could not identify any genes which putatively encode enzymes involved in N-glycosylation, including N-acetylglucosaminyltransferase (which initiates complex N-linked carbohydrate formation by catalyzing the transfer of GlcNAc from UDPGlcNAc to the oligomannosyl acceptor Man5GlcNAc2-Asn) and N-acetyl-glucosaminyltransferase II. The absence of these enzymes and homologs of the mammalian sialyltransferase in *C. reinhardtii* could be potentially explained by the difference between the algal and eukaryotic N-protein glycosylation pathways. In our study, using both biochemical and MS analyses, we showed that *C. reinhardtii* have mammalian-like N-linked glycans, with terminal sialylated complex glycan structures and core fucosylation. However, fucose in *C. reinhardtii* N-glycans is probably α1,3-linked, because we only found an α1,3-fucosyltransferase homolog in the *C. reinhardtii* database (accession no. XP_001695259). It is well established that in plant N-linked glycans fucose is most commonly linked by an α1,3 bond to N-acetylglucosamine, while in human N-linked glycans fucose is most commonly linked by an α1,6 bond to the reducing terminal β-N-acetylglucosamine. Human α1,3-fucosyltransferase catalyzes the transfer of the L-fucose moiety from guanosine diphosphate-β-L-fucose to acceptor sugars to form biologically important fucoglycoconjugates, including sialyl $Lewis^x$ (SLe^x) carbohydrate [27]. A homology search showed that although α1,3-fucosyltransferase of *C. reinhardtii* shares low-level identity with α1,3-fucosyltransferase of *Arabidopsis thaliana* (28.8%), it contains a SNC motif which is highly conserved across plant transferases. *C. reinhardtii* α1,3-fucosyltransferase also shares low-level identity with human α1,3-fucosyltransferase (24.4%).

PNGase A (from almonds) that cleaves all types of asparagine-bound N-glycans including high mannose, hybrid, biantennary, triantennary and tetra-antennary complex glycans [11] can also cleave N-linked glycans carrying a fucose linked by an α1,3 bond to Asn-GlcNAc [28] that is present in plant and insect glycoproteins. Thus, our data suggest that *C. reinhardtii* has mammalian-like N-linked glycans, with 1,4-Gal associated with a sialylated complex glycan structure and a plant-like core 1,3 fucosylation – perhaps, a novel N-linked glycan structure. Unlike plant N-linked glycan structures, the presence of any xylose-containing oligosaccharides in *C. reinhardtii* has not been confirmed. Thus, since green algae are capable of performing important post-translational modifications such as N-glycosylation with terminal sialylation, they are attractive for biotechnological applications for expression of biologically active therapeutic glycoproteins. Further investigations are in progress to fully elucidate the N-linked glycosylation status of *C. reinhardtii*.

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