
N-Glycosylation of the 66-kDa Cell-Wall Glycoprotein of a Red Microalga

Oshrat Levy-Ontman

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/46580>

1. Introduction

1.1. Cell-wall polysaccharide of *Porphyridium* sp.

1.1.1. Chemical studies

The cells of the red microalga *Porphyridium* sp. are encapsulated within a polysaccharide, one of the main products of this alga and that is vital for its survival [1-4]. Due to the unique properties of this polysaccharide, which has tremendous value in the biotechnology field, strenuous, interdisciplinary efforts have been devoted in the past few decades to the study of its chemical structure, rheological properties, and bioactivities [3].

The red microalga *Porphyridium* sp. cell-wall polysaccharide comprises negatively charged heteropolymers with a relatively high molecular mass, apparently in the 2×10^6 Da range [5-8]. The precise pK_a value of dissociation is very low and is somewhat difficult to determine due to the heterogeneous nature of the molecules. The polysaccharide is composed of 10 different monosugars, proteins, and sulfate groups (~7.6 %w/w) [5-6, 8-10]. The prominent sugars are glucose:galactose:xylose in a molar ratio of 1:1.9:3.2, respectively [10]. The minor sugars include rhamnose, arabinose, mannose, and methylated monosugars [5-6, 8, 10]. Moreover, it is anionic due to the presence of uronic acid groups and half-ester sulfate groups [1, 5-6, 11], the latter of which are attached to the 3 or 6 position of glucose and galactose [11].

During growth, the external part of the polysaccharide (known as the "soluble fraction") is released to the surrounding aqueous medium and accumulates in the medium, while the remainder, i.e., most of the polysaccharide (~ 50-70%, known as the "bound fraction"), remains attached to the cell [1-4, 12]. When red microalgae are grown in a liquid medium, the viscosity of the medium increases continuously as the polysaccharides are released from

the cell surface [1, 13]. As a result, during the logarithmic phase of growth polysaccharide capsules are thinnest, while during the stationary phase they are thickest. Cell-wall polysaccharide production (quantity and quality) has been found to be affected by environmental conditions and genetic modifications [4]. For example, growing *Porphyridium* sp. in a medium deprived of nitrate and sulfate enhances production and solubilization of the polysaccharide [14]. Moreover, these conditions have also been shown to change polysaccharide compositions [14].

The precise structure of the *Porphyridium* sp. cell-wall polysaccharide is not fully understood due to its complexity and the lack of known specific enzymes that degrade it [3]. Although several studies have been conducted on *Porphyridium* sp. polysaccharide structure, most have focused mainly on the soluble fraction of the polysaccharide. The soluble polysaccharide of *Porphyridium* sp. was found to have a basic building block comprising aldobiouronic acid 3-O-(α -D-glucopyranosyluronic acid)-L-galactopyranose disaccharide [15]. This building block has also found in other red microalgal cell-wall polysaccharides, i.e., *Porphyridium aeruginosum* and *Dixonella grisea* [15]. Moreover, it was also discovered to be part of a bigger structure, composed of a larger, linear building block that contains (1 \rightarrow 2 or 1 \rightarrow 4)-linked xylopyranosyl, (1 \rightarrow 3)-linked galactopyranosyl, and (1 \rightarrow 3)-linked glucopyranosyl or glucopyranosyluronic acid residues [16]. Two oligosaccharides isolated from the bound fraction were also investigated. They were shown to comprise three major neutral monosaccharides – Xylose, Glucose Galactose, and GlcA – the last of which did not contain the disaccharide building block that was found in the soluble polysaccharide fraction [17].

1.1.2. Rheological studies

Their physicochemical natures make the red microalgal polysaccharides potentially valuable candidates for various industrial applications. One of the most important properties of the polysaccharides is their capacity to yield highly viscous solutions, comparable with those of industrial polysaccharides such as xanthan and carageenan, under relatively low polymer concentrations [4]. The *Porphyridium* sp. polysaccharide is composed of an oriented single, two-fold helical structure with a pitch of 1.6 nm (i.e., a single chain helix with two chemical repeats, which are probably the aldobiouronic acid) [18-19]. Chain stiffness is in a range comparable to that of rigid helicels such as xanthan gum and DNA [18].

A heteropolyelectrolyte, the polysaccharide of *Porphyridium* sp. shows marked shear thinning (with no evidence of a Newtonian plateau, typical of a structured medium), thixotropic, and elastic behaviors [18-19]. It was suggested that the shear thinning behavior and the capability to yield highly viscous solutions under relatively low polymer concentrations can be attributed to the ability of the polysaccharide to form a weakly cross-linked, elastic, gel-like network structure (that breaks down under shear).

The similarity of the physicochemical properties of red microalgal polysaccharides to those of other polysaccharides currently used in industry as gelling agents, thickeners, stabilizers, and emulsifiers (such as xanthan) make the red microalgal polysaccharides a valuable

alternative source to these existing industrial polysaccharides. One of their unique advantages over those of other phycocolloids, mainly for commercial applications, is their stability over wide temperature, pH value, light, and salinity ranges [4, 6, 18-22].

There is evidence that upon heating the *Porphyridium* sp. cell-wall polysaccharide in solution, the weak elastic gel, transforms into a stronger elastic network that is reversible by cooling [19]. Aqueous preparations that gel with heating and melt upon cooling are rare and may have unique industrial applications. This phenomenon demonstrates again the notable stability attributed to the polysaccharides that differentiate them from other known phycocolloids, e.g., agar-agar. Another significant property of the red microalgal polysaccharides that confers on them great industrial value is their effectiveness in drag reduction [4, 23]. Indeed, they were proposed as ideal for cargo ships to lessen the drag felt by the marine transport vessels, thereby reducing the needed propulsion power and fuel costs or, alternatively, increasing ship speeds [24].

The *Porphyridium* sp. cell-wall polysaccharide was found to adsorb onto mica surfaces (negatively charged), forming ultrathin coating layers in the nanometer range [21-22]. The polysaccharide layer appeared to remain highly mobile at the surface, as flexible microfibrils (~ 10 nm in width and 1 to 2 nm in height), [21]. However, hyaluronic acid under the same conditions did not show any sign of adsorption onto the mica surfaces [22].

One of the most outstanding properties of *Porphyridium* sp. polysaccharide lubricating films is that only a subnanometric (0.5-1 nm) monolayer is needed to provide a stable, low-friction coefficient, robustness (high load carrying capacity), and good wear protection, and the friction force exhibits a weak dependence on sliding velocity [22]. Moreover, pressing/shearing was shown to affect the adhesion such that the application of pressure plays an important role in reordering the polysaccharide molecules between two surfaces, binding them together to protect them from damage and control their friction.

In comparison to the other biopolymers investigated so far, the *Porphyridium* sp. polysaccharide at once possesses most of the tribological requirements for efficient biolubrication, e.g., steady low friction, stability at high pressures, stability at high and low velocities, and wear protection and stability over large shearing distances. In addition, the polysaccharide was shown to be superior to hyaluronic acid as biolubricants in terms of stability, friction reduction, and adsorption [21-22]. Also, the polysaccharide was affected neither by hyaluronidase activity, in contrast to hyaluronic acid [22], nor by carbohydrases [25]. In summary, these properties combine to make the red microalgal polysaccharide of *Porphyridium* sp. ideal for applications in various industries, i.e., marine transport, biomedical, cosmetics, and nutrition.

1.1.3. Bioactivities

Among its bioactivities whose potential has been at least partially realized are the *Porphyridium* sp. polysaccharide's anti-inflammatory and anti-irritating activities [26]. Found to be generally well-suited for a variety of skin applications, the polysaccharide was

shown to have a marked soothing affect on the irritation associated with common skin inflammations. Moreover, using TBA and FOX methods, the polysaccharide was also shown to have potent antioxidant properties [27]. Indeed, these proven polysaccharide bioactivities led to its current application as an anti aging agent by a leading global cosmetics company and have promoted further research aimed at discovering additional uses.

In addition to bioactivities with dermal applications, the polysaccharide was also found to possess anti-viral activity against animal viruses [28-33]. Moreover, it was shown to significantly inhibit infection with retroviruses (murine leukemia virus, HIV-1, and HIV-2) and cellular transformation by murine sarcoma virus [29].

The *Porphyridium* sp. polysaccharide can also be used as a nutritional agent, as the fibers it contains constitute a viable dietary supplement. Animal feeding experiments have shown that rats whose diets were supplemented with low concentrations of polysaccharide had considerably lower levels of serum cholesterol, triglycerides, and very low-density lipoprotein (VLDL) [34-36]. This diet also resulted in an increase in feces mass (by 130%) and in bile acid excretion (5.1 fold or more). Moreover, rats fed the polysaccharide exhibited longer small intestines (by 17%) and colons (by 8.5%) [36]. An important finding is the complete absence of toxic effects following the *Porphyridium* sp. polysaccharide diet in comparison to diets based on other known sulphated polysaccharides that were found to be toxic [37]. It was thus suggested that the *Porphyridium* sp. polysaccharide could be produced and marketed commercially as a dietary fiber supplement [35-36].

It is noteworthy to add that the beneficial bioactivities and fluid dynamic behavior observed in *Porphyridium* sp. polysaccharide are probably the direct results of the their general role in their natural surroundings, isolated from the sea sand, where the environmental conditions are subject to harsh, widely fluctuating conditions, i.e., extreme light and drought during ebb tides. Most likely the unique polysaccharide structure is responsible for these special properties. Indeed, the polysaccharide form the boundary between the cell and its surroundings, functioning as a capsular defense barrier.

2. The 66-kDa glycoprotein

Almost no work has been reported on the cell-wall proteins of red microalgae. However, a number of non-covalently-bound cell-wall proteins were detected in SDS polyacrylamide gel electrophoresis (SDS-PAGE) when the cell-wall polysaccharide complex of *Porphyridium* sp. was loaded on the gel after it had been boiled in sample buffer containing SDS and β -mercaptoethanol (Figure 1). The most prominent of those proteins is named after its molecular mass: the 66-kDa cell-wall glycoprotein. The total mass of all N-glycans attached to the protein was estimated at 8 kDa [38-39].

The 66-kDa glycoprotein was found to be non-covalently, tightly bound to the polysaccharide [38-39]. Although it could not be co-eluted with the polysaccharide in size-exclusion chromatography (SEC) by increasing NaCl concentrations (0.25-1.5M), it could be partially dissociated from the polysaccharide by SEC in the presence of 2M guanidine

hydrochloride. Furthermore, the glycoprotein could not be completely separated from the polysaccharide that had been denatured by boiling, in buffer containing SDS and β -mercaptoethanol before loading it into an SEC column. Western blot analysis (using polyclonal antiserum raised against the 66-kDa glycoprotein) revealed that the glycoprotein is specific to *Porphyridium* sp. and its closely related isolates, but it is not detected in other red microalgae, blue-green algae, or plants [38-39]. Indirect immunofluorescent assay and immune-gold labeling with the antiserum showed that the 66-kDa glycoprotein is located in the Golgi and on the cell surface of *Porphyridium* sp.

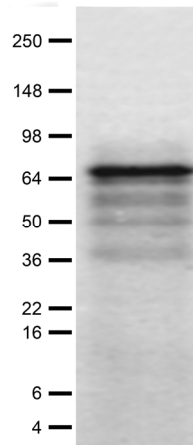


Figure 1. Cell-wall proteins of the *Porphyridium* sp. polysaccharide. The polysaccharide (36 μ g) was subjected to SDS-PAGE and stained with Coomassie blue.

The 66-kDa glycoprotein was also detected in genetically spontaneous mutants that are resistant to the cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB) or in physiologically modified cell-wall complexes of *Porphyridium* sp. (from sulfate, nitrate, calcium starved cultures) [38-39].

By means of an *in vitro* assay, it was demonstrated that the 66-kDa glycoprotein binds to the cell-wall polysaccharide of *Porphyridium* sp. Furthermore, it also binds to the cell-wall polysaccharides of two other species of red microalgae, *Dixoniella grisea* and *Porphyridium aeruginosum*, and to λ -carrageenan from a red seaweed. But it does not bind to the other polysaccharides examined, i.e., dextran, dextran sulfate, xylan, and xanthan gum [38-39].

Sequencing of a cDNA clone encoding the 66-kDa glycoprotein revealed that this is a novel protein, with four potential N-glycan sites, which does not show similarity to any protein in the public domain databases.

Although the sequencing clone revealed this glycoprotein to be a novel protein, it does show structural similarities, within the carbohydrate-binding domain (CBD), to some protein superfamilies, namely, glycosyltransferases, pectin lyase-like, sialidases, and conA-like lectins/glucanases in the SCOP and PROSITE databases, indicating a possible role of the 66-

kDa glycoprotein in cell-wall polysaccharide synthesis/modification [38-39]. In addition, two amino acid sequences of the N-terminus and several internal peptides showed some homology to endo β 1-4 xylanase [38-39]. Moreover, this protein was found in the early stages of the cell-wall cycle as an intermediate product [40-41] and in all mutants characterized by modified cell walls [39], which indicates that it may be involved in polysaccharide biosynthesis. In addition, the glycoprotein was shown to play a role in biorecognition [42]: *Porphyridium* sp. cells that were treated with antibodies to the 66-kDa glycoprotein were not recognized by the microalga's predator, the dinoflagellate *Cryptothecodinium cohnii* [42].

3. N-Glycan structures of the 66-kDa glycoprotein

The primary structures of the 66-kDa N-glycan have been investigated by various methodologies. Preliminary characterization of glycan moieties attached to the 66-kDa protein was done by lectin array analysis. The SDS-PAGE-resolved polysaccharide proteins (containing the 66-kDa glycoprotein) were blotted onto nitrocellulose membranes and probed with lectin-conjugated-biotin and streptavidin-conjugated-HRP according to Gravel [43]. The glycoprotein was detected by the lectins ConA (Concanavalin A), GNA (Galanthus nivalis lectin), and GSL I (*Griffonia* (Bandeiraea) simplicifolia lectin I (Figure 2). ConA has high affinity to α -D-mannose and lower affinity to α -D glucose [44]. GNA recognized a terminal mannose via α (1-3), α (1-6) or α (1-2) to another mannose residues [45]. The positive reactions with the lectins ConA and GNA suggest the presence of N-glycosidically-linked "high mannose" or hybrid"-type glycan chains while that with GSL I indicates the possible presence of O-linked chains comprising α -Gal/ α -GalNAc monosaccharides. In contrast, the glycoprotein could not be detected by the lectins DSA (*Datura stramonium*), AAA (*Aleuria aurantia* agglutinin), RCA I (*Ricinus communis* agglutinin I), PNA (Peanut Agglutinin), WGA (Wheat Germ Agglutinin), SNA (*Sambucus nigra*), or MAA (*Maackia amurensis* lectin, suggesting that it lacks the Gal β (1-4)GlcNAc, GlcNAc-Ser/Thr, α (1-6)-linked fucose, terminal β -D-galactose, Gal β (1-3) GalNAc, GlcNAc β (1-4)GlcNAc, sialic acid terminally linked to α (2-6)Gal, or GlcNAc and sialic acid terminally linked to α (2-3) Gal groups, respectively.

Other direct, well-known methods for N-glycan analysis have also been conducted as follows. The glycoprotein was separated using a funnel-shaped polyacrylamide gel under conditions described previously [38-39]. The 66-kDa glycoprotein was detected by Coomassie blue staining, and its N-glycans were separated using in-gel digestion with PNGase F according to Küster et al. [46]. Following several cleaning steps [47], part of the separated N-glycans were labeled with the fluorescence agent 2AB according to the method described by Bigge et al. [48], and the rest were kept for mass spectrometry analysis. An NP-HPLC analysis of the 2AB-labeled N-glycans revealed four main peaks, indicating a minimum of four different N-glycans in the sugar moieties of the 66-kDa glycoprotein (ranging in size from 7 to 8.5 GU values in terms of the glucose ladder standard) [47]. To test whether the N-glycan moieties contain other types of sugars, i.e., those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue, the 66-kDa glycoprotein was

digested with PNGase A [47]. Following labeling of the PNGase-A-released N-glycans, they were run on an NP-HPLC. The resulting NP-chromatogram was identical to that of the PNGase-F-released glycans, indicating the absence of a core α 1,3-linked Fuc.

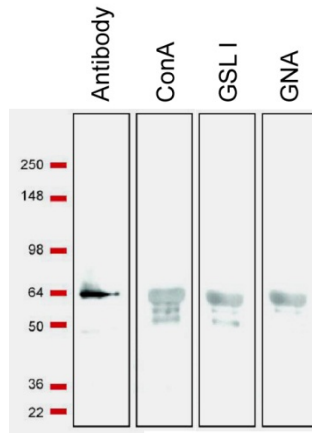


Figure 2. Lectin analysis of the 66-kDa cell-wall glycoprotein. The cell-wall polysaccharide (36 μ g) was subjected to SDS-PAGE. Following electrophoresis, the proteins were blotted onto a nitrocellulose membrane and probed with ConA, GNA, and GSL I.

To elucidate the N-glycan structures, the next step was to use an exoglycosidase array of enzymes that normally cleave the non-reducing end of typical N-glycans. Following the digestion of the 2AB- labeled PNGase-F-released N-glycans with an exoglycosidase array (ABS, BTG, SPH, BKF, XYL, JBM), the glycan NP-HPLC chromatogram did not change in comparison to that prior to digestion, indicating that the mixture of N-linked glycans obtained from the 66-kDa glycoprotein of *Porphyridium* sp. differs from glycans known to date [47]. To obtain more information about N-glycan structures, the labeled and unlabeled N-glycans were analyzed by mass spectrometry (positive-ion MALDI-TOF MS and negative-ion ESI-MS). As expected, the results did not match the typical mass values of other known, investigated N-glycans. Moreover, the 2AB-labeled N-glycan fraction released after PNGase F/A was also run on WAX-HPLC, and all the glycans were found to be neutral [47].

Traditionally, the gold standard for such studies would have been to include GC/MS and/or NMR data, which is not the case here. Since the glycoprotein is associated with the soluble polysaccharide, first it has to be separated from the polysaccharide (loading volume was 1.7 ml). Working with the polysaccharide is tedious and time consuming due to its high viscosity with excessive shear thinning. Moreover, the polysaccharide contains numerous other compounds, which dictated that we first dialyze it against double distilled water and dilute its concentration to 0.3 w/v. At the gel loading point, the polysaccharide concentration was lower since it was diluted again with Laemmli sample buffer (lowering the final concentration to about 0.2 w/v). Each gel run yielded one band (1 cm \times 0.5 mm) that contained about 30 μ g of the glycoprotein and a relatively small amount of total N-glycans

(about 500 pmol, calculated by 2AB calibration standards). To analyze the glycan structures using GC-MS, it was vital to collect enough material. Glycans from 40 gel pieces were collected, separated from the gel pieces, and cleaned. We hydrolyzed the glycans by rigorous acid conditions, methylated them, and tried to compare their GC-MS spectra to that of known methylated monosaccharide standards. The methylated monosaccharide GC-MS spectra derived from the unknown glycans contained a lot of background noise that presumably hid the monosaccharide peaks. The noise probably derived from the preparation of the polyacrylamide gel pieces together with the steps leading to the GC-MS analysis. We now understand that conventional strategies (collection of small glycan amounts from relatively large gel pieces) are insufficient in this research. As a result, it was necessary to use indirect methodologies to obtain structural information. In future research, we hope to develop a method for producing uncontaminated proteins from the polysaccharide.

To understand the N-glycan compositions suggested by the mass values, the identities of the constituent monosaccharides of the N-glycans were determined. The N-glycans were hydrolyzed and labeled with 2AB and analyzed using a combination of MS spectrometry and a comparison of monosaccharide standards to the hydrolyzed monosaccharide chromatograms of the N-glycans obtained by NP/RP-HPLC [47]. The analysis indicated that each of the N-glycans derived from the 66-kDa glycoprotein comprised the same four monosaccharides: GlcNAc, mannose, probably 6-O-MeMan and xylose. Integrating monosaccharide identity data with the MS analysis, a sugar composition can be determined for each N-glycan feature (Table 1):

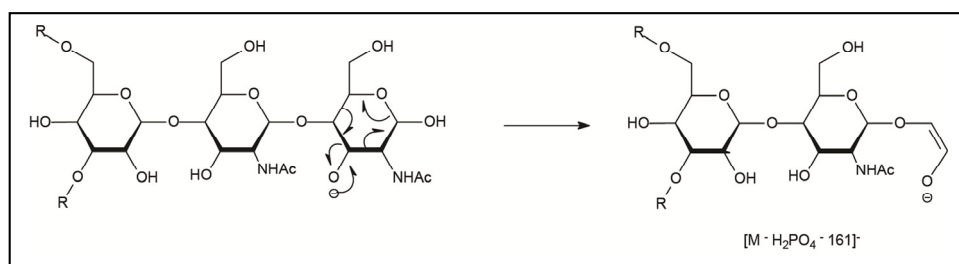
N-Glycan calculated mass (Da)	Composition			
	Mannose	MeMan	GlcNAc	Xylose
1894.70	5	3	2	1
2026.75	5	3	2	2
2056.76	6	3	2	1
2188.80	6	3	2	2

Table 1. Compositions of the N-glycans from the 66-kDa glycoprotein [47]

The N-glycans were also released by Endo-H and then analyzed by NP-HPLC. To estimate the difference in glucose unit values between the PNGase-F- and Endo-H-released N-glycans from the 66-kDa glycoprotein, the NP-HPLC chromatograms of the two preparations were compared to the NP-HPLC chromatogram of a known standard of PNGase-F- and Endo-H-released N-glycans derived from RNase B. The differences in the elution times (in the NP-chromatogram) of the 66-kDa glycoprotein fractions compared to those of the RNase B fractions showed the same pattern. The NP-HPLC chromatogram of the N-glycans released from the RNase B glycoprotein (data not shown) indicated that the size difference between 2AB-labeled glycans released by the action of PNGase F and Endo H is very small (0.16 GU or less). For example, the size of the oligomannose structure with five mannose residues, which was separated from RNase B by PNGase F action, exceeded that

obtained by Endo H digestion by a mere 0.16 GU. It was also shown that the size difference between the PNGase-F- and Endo-H-released glycans decreased as glycan size increased: Oligomannose structures with eight or nine mannose residues exhibited no size difference between PNGase-F- and Endo-H-released materials. The small difference in glycan size (in GU values) between the smallest glycan, released by PNGase F and yielding the smallest GU value (m/z 1895), and the major peak obtained in the NP-HPLC chromatogram after Endo H digestion, indicates that the glycan features are the same as those associated with N-glycans from RNase B. A comparison of the sizes of the minor peaks for the derived glycans released by Endo H compared to those released by PNGase F digestion (corresponding to 2027, 2057, 2189 Da) was in good agreement with the differences found in the measurements of the RNase B fraction. The yield of these glycans released by Endo H was found to be much lower than expected compared to the major glycan released by Endo H. This observation indicates the possible existence of structures with positions that interfere with Endo H activity. In addition, to verify that the glycans released after Endo H were derived from the same N-glycans released after PNGase F digestion, a comparison of analyses of their masses by MS indicated that they possess the same glycan features.

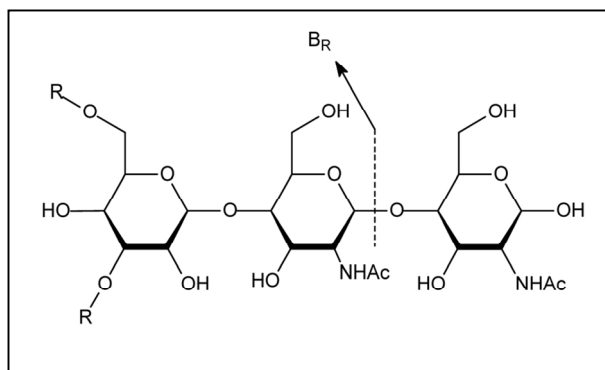
To obtain a more detailed analysis, unlabeled oligosaccharides released by PNGase F were subjected to negative mode MS/MS [47]. The negative ion MS/MS spectra were typical of neutral glycans run as phosphate adducts (phosphate was the anion used to ionize the compounds) [47]. Spectra were interpreted according to published data [49-52]. All spectra contained a major ion 259 mass units below that of the molecular ion and consistent with a $^{2,4}A$ fragmentation (Domon and Costello [53] nomenclature) of the core HexNAc (Scheme 1, loss of 161 mass units and the phosphate adduct) following abstraction of the 3-proton by the phosphate. This mass loss showed no substitution of the core GlcNAc.



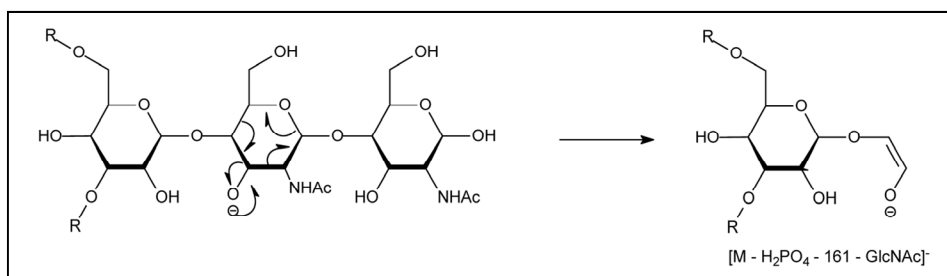
Scheme 1. Fragmentation mechanism in the GlcNAc ring of the chitobiose core

A second ion, 60 mass units below this ion, was also present in all compounds and corresponds to a B_R cleavage (the subscript is used here to refer to the “reducing terminus”) (Scheme 2) consistent with a $\beta(1\rightarrow4)$ -linkage.

The spectra of the compounds weighing 1991 and 2153 Da contained an additional ion, 203 mass units below that of the $^{2,4}A_R$ ion, corresponding to a similar cleavage of the penultimate GlcNAc (Scheme 3).



Scheme 2. Fragmentation mechanism between the two GlcNAc residues of the chitobiose core



Scheme 3. Fragmentation mechanism in the penultimate GlcNAc of the chitobiose core

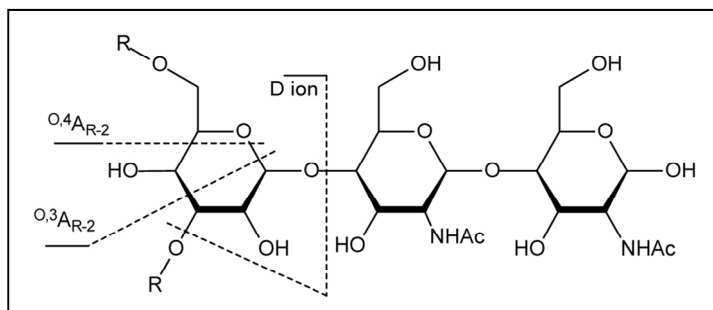
The spectra of compounds weighing 2123 and 2285 Da, that had an extra xylose residue did not contain this ion, suggesting that the xylose was attached to the 3-oxygen of the penultimate GlcNAc, blocking the abstraction of a proton at this site and accounting for the absence of the $^{2,4}A_{R-1}$ ion [47].

Normally, xylose is found attached to the 2-position of the branching mannose. However, the negative ion MS/MS spectrum of $[Man]_2[GlcNAc]_2[Xyl]_1[Fuc]_1$ from horseradish peroxidase, which contains such a 2-linked xylose, contained an abundant ion corresponding to the $^{2,4}A_{R-1}$ fragment (m/z 677) consistent with the 3-proton being available for abstraction [47]. Thus, it appears that the compounds with two xylose residues have one xylose attached to the 3-position of the penultimate GlcNAc residue [47].

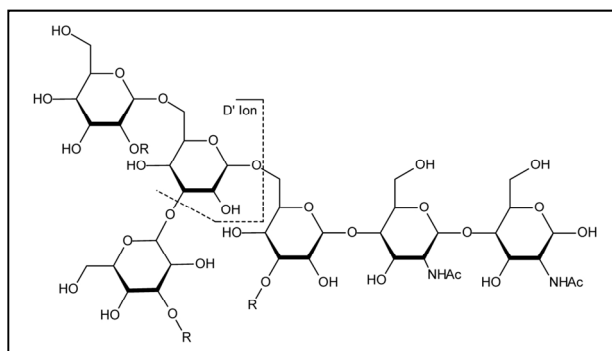
The negative ion MS/MS spectra of all four compounds were virtually identical. The group of ions at m/z 1131, 1113, 1059, and 1029 (weak) are similar to those from high-mannose glycans and correspond to D, $[D-18]^-$, $^{O,3}A_{R-2}$, and $^{O,4}A_{R-2}$, respectively (Scheme 4)[47].

The similarity of these ions to those in the high-mannose glycans again suggests no xylose substitution on the core mannose. The mass of the D ion, which contains the 6-antenna, indicated a composition of $[Hex]_4[MeHex]_2[Xyl]_1$ leaving after subtraction of the core GlcNAc residues. The similarity of the spectra to those of the high-mannose glycans suggests a similar topology, and therefore, the two branches of the 6-antenna contain Hex-

MeHex and Xyl-Hex-MeHex compositions. The composition of the ion at m/z 631 appears to be $[\text{Hex}]_2[\text{MeHex}]_1[\text{Xyl}]_1$, which is consistent with that of a D' ion [the linkage (3 and 6) around the mannose attached to the 6-position of the core mannose is the same as that of the core mannose itself, Scheme 5].



Scheme 4. Fragmentation mechanism between the branching Mannose and the penultimate GlcNAc of the core core (D , O_3A_{R-2} and O_4A_{R-2} ions)



Scheme 5. Fragmentation mechanism in the 6-branch mannose, creating the D' ion.

To further elucidate the glycan structures, each of the 2AB-labeled glycans were also analyzed by positive MS/MS. The results of the positive MS/MS spectra were in good agreement with those of the negative spectra, indicating that each of the N-glycans possesses the same core structure with a composition comprising $[\text{MeMan}]_2[\text{Man}]_4[\text{Xyl}]_1[\text{GlcNAc}]_2$ [47]. It can also be suggested that the glycans with the additional xylose residues (2026, 2188 Da) are attached to the penultimate GlcNAc. The major 2AB Endo-H-released glycan was also analyzed by positive MS/MS, indicating the existence of two isomers in the fractions. The positive MS/MS spectra also indicated that different isomers exist in two of the glycan features [47].

Based on a combination of the two MS/MS spectra, the following structures were suggested [47] (Table 2):

cell-wall glycoprotein production and its N-glycosylation may help us understand the biosynthesis process and function of the polysaccharide. As a result, in addition to N-glycan structure determination of the 66-kDa cell-wall glycoprotein, the effect of growth conditions along with the starvation of sulfate, nitrogen and calcium or the enrichment of sulfate were also tested on the composition and structure of the N-glycan moieties. Prior to the experiments, *Porphyridium* sp. cells were cultivated in accordance with the treatment conditions as follows: The sulfate-enriched growth medium contained four-fold sulfate concentration compared to the original medium (ASW). Sulfate starvation cultures were cultivated for five cycles (five days each) in a medium containing 1/100 of the sulfate concentration of the regular medium. Cells subjected to starvation of either nitrogen or calcium were cultivated in a deficient medium free of nitrate or calcium (without KNO_3 or CaCl_2 , respectively). The *Porphyridium* sp. control cells were cultivated in ASW medium.

After two weeks of growth, all of the cultures (sulfate enrichment or sulfate and nitrogen starvation, or regular medium), which were in the stationary phase, were centrifuged and the supernatant, which contained the polysaccharide, was isolated and dialyzed and concentrated to a final concentration of 0.3 w/v. The amount of cell-wall proteins within the concentrated polysaccharide (1.7 ml) was determined for each of the treatments by Lowry analysis [54]. To isolate the 66-kDa glycoprotein, the concentrated polysaccharide in the different treatments (1.7 ml) was run through SDS-PAGE electrophoresis, and the N-glycans were released by PNGase F following 2AB labeling. The NP-HPLC results for the N-glycans released from the 66-kDa protein were compared between the different treatments. In each NP-HPLC chromatogram, the molar ratios between the sugar features were determined with Empower HPLC software, which calculates the area under each peak, an indication of the sugar molar rate. The fluorescence rate was calibrated to mole amounts using 2-AB calibration standards. Each experiment was repeated twice.

Polysaccharide protein amounts produced under sulfate or nitrogen starvation treatments were 90% less than under the control or sulfate enrichment condition. Accordingly, the N-glycan amounts measured within the 66-kDa glycoproteins produced under these starvation conditions were also low (50 pmol compared to 500 pmol in the control and 450 pmol under the sulfate enrichment conditions). In addition, there was no difference compared to the control in either the cell-wall protein or N-glycan amounts measured within the 66-kDa protein produced under calcium starvation conditions. The NP-HPLC chromatogram of the 2AB- labeled N-glycans released from the 66-kDa glycoprotein, which were separated from algal cultures grown in the different treatments, are shown in Figure 3. The molar rate percentage of the various peaks detected in the different treatments are described below (Table 3). There is no significant change between the NP-HPLC chromatogram and the molar ratio of the different glycans produced under the sulfate enrichment, calcium starvation or control condition. However, an additional N-glycan feature was detected in the algae grown under sulfate enrichment conditions (designated in *, Figure 3A). It is interesting to note that under sulfate starvation conditions, the largest N-glycans were not found in the NP-HPLC (peaks 6 and 7, Figure 3). Similar to the findings under sulfate starvation, the largest N-glycan was not detected in the NP-HPLC chromatogram of the

nitrogen starvation chromatogram (peak 7, Figure 3B). Therefore, the effects of nitrogen and sulfate starvation on N-glycosylation of the 66-kDa protein were found to be similar. These observations are in agreement with former studies [55-57], where it was reported that in both starvation conditions, the cells directed most of their energy toward the synthesis of cell-wall polysaccharide, an activity that is probably important for its survival. The decrease in 66-kDa protein production and in its N-glycan composition in both starvation conditions was expected, because under these conditions, the cells inhibit protein synthesis to the benefit of polysaccharide production. Although amounts of the glycoprotein under these starvation regimes are much lower than for the control, it is still being produced, just not at levels observed in the control cells, a finding that hints at the protein's vitality to cell survival. Since polysaccharide compositions in the sulfate/nitrogen deficient conditions (particularly the increased methyl hexose amounts) were found to differ from that of the control [57], the 66-kDa protein's role in polysaccharide production cannot be ruled out (i.e., it could be part of a specific polysaccharide process that does not occur under these starvation conditions).

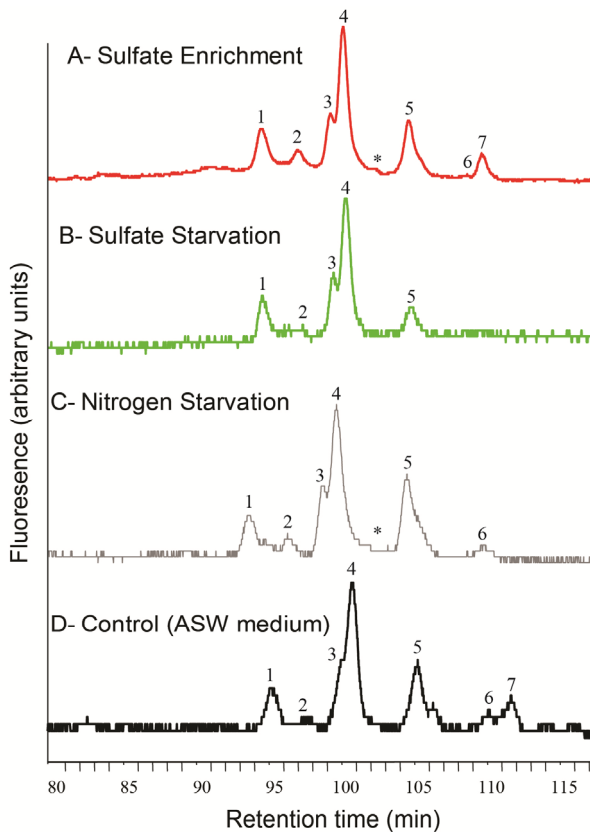


Figure 3. NP-HPLC chromatograms of N-glycans released from the 66-kDa glycoprotein produced in different treatments: A – sulfate enrichment, B – Sulfate starvation, C – Nitrogen starvation, D – Control/ASW medium

Since polysaccharide quantity in the medium was also found to be affected by growth phase (thinnest in logarithmic phase vs. thicker in stationary phase), cell-wall protein production and the 66-kDa glycoprotein N-glycans were studied as described above. In contrast to the nitrogen/sulfate starvation treatments, no difference was observed between the two phases of growth either in cell-wall protein production or in the 66-kDa N-glycan chromatograms. This observation lends credence to the hypothesis suggested by Ramus [58], i.e., in the stationary phase, polysaccharide production is not increased, but rather, its level of production exceeds its dissolution into the medium. If polysaccharide production were actually increasing throughout the stationary phase, then we would expect the corresponding increased energy consumption to be at the expense of protein production, as found in the algae grown under the sulfate/nitrogen starvation conditions. That was not the case here, where no difference in the cell-wall glycoprotein amount was observed between these two growth phases.

Treatment	Molar rate percentage of peak**							
	1-1894.70 Da	2- n.d	3- 2026.75 Da	4- 2056.76 Da	n.d - *	5- 2188.8 Da	6- n.d	7- n.d
Sulfate Enrichment	13.67	8.09	12.95	37.6	1.56	20.86	Little	5.28
Sulfate Starvation	16.59	1.64	18.21	49.59	n.f	13.97	n.f	n.f
Nitrogen Starvation	11.62	3.62	13.32	39.86	n.f	28.83	2.75	n.f
Calcium starvation	11.70	4.96	8.65	41.31	n.f	20.54	4.07	8.76
Logarithmic phase	13.81	4.08	12.23	35.42	n.f	24.74	2.55	7.17
Control	13.01	4.32	12.93	35.08	n.f	25.68	2.21	6.77

** Each peak matches its GU value in line with those shown in the NP-HPLC (in Figure 3). The molar rate was calculated by Water Empower software.

n.f- not found

n.d- not defined

Table 3. Molar rate percentage of the different N-glycans released from the 66-kDa glycoprotein that was isolated from the polysaccharide produced under different treatments.

5. Significance

Several years of intensive, multidisciplinary research have been directed at red microalgae, particularly *Porphyridium* sp. Among the various chemicals produced by *Porphyridium* sp., sulphated polysaccharides have perhaps garnered the most attention because of their potentially high value in biotechnological applications [2-4, 59-60]. However, little attention

has been devoted to elucidating the glycosylation process in red microalgae. To date, our study is the first to report the structures of several N-glycans from a specific red microalga species, *Porphyridium* sp. [47]. This knowledge is important for both basic and applied research. An understanding of the way in which the sugar moieties of glycoproteins are bound to the microalgal proteins will elucidate glycosylation pathways, in the process revealing the enzymes involved, and it will contribute to an understanding of the role(s) of the sugar moieties in microalgal glycoproteins. The findings of this study will thus facilitate the identification of glycan biosynthetic components, thereby making an invaluable contribution to a comprehensive understanding of N-glycosylation in red microalgae. Since the N-glycan structures within the cell-wall glycoprotein were found to be novel, one particularly intriguing research direction will be to test whether these glycosylation structures are unique to the formation of the cell-wall polysaccharide. Alternatively, perhaps they are part of the general glycosylation process in these red microalgae cells exclusively or in a variety of red microalgae species.

Importantly, the technology for growing this species in controlled environments, both in small-scale laboratory facilities and in large-scale, semi-industrial systems, is already well-developed. A stable chloroplast transformation system [62], and recently, a nuclear transformation system, have been developed [63], the latter of which has paved the way for the expression of foreign genes in red algae and has far-reaching biotechnological implications. A growing number of scientists around the world are building a novel assortment of pharmaceutical products using algae as cell factories [64-66]. However, although they are well suited to the large-scale production of recombinant proteins, algae have not been extensively utilized for protein expression [66-67]. There are a number of advantages in cultivating algae as a platform for producing therapeutic proteins. Relatively simple and cheap to grow, algae are also amenable to cultivation under a variety of growth conditions. In addition, they are energy efficient, have a minimal negative impact on the environment, and they are easy to collect and purify. It is, therefore, of the utmost importance to evaluate the glycans attached to any recombinant protein expressed in any system. Since glycosylation may affect the biological role(s) of proteins or elicit an immunogenic response, knowledge of the structure of the microalgal N-glycans is essential for these applications. Moreover, knowledge of glycosylation patterns in algae will enable us to evaluate the potential of red microalgae species, particularly of *Porphyridium* sp., to be used as hosts and as potential alternatives to other plant-derived, transgenic therapeutic proteins. Furthermore, to fully exploit the inherent biotechnological potential of algae, it is important to initiate an overarching research program on the glycosylation pathways in algae that will include in-depth study of the enzymes involved. On the basis of the results toward elucidation of N-glycosylation pathways in red microalgae, we will be able to suggest glycosylation pathway manipulations to produce therapeutic proteins with ideal glycosylation patterns. In addition, the study can provide information about the evolutionary status of the red microalgae, since the N-glycans of the red microalgae combine not only the structural features of eukaryotes and prokaryotes, they also contain additional elements (e.g., the O-methylhexose and the pentose modifications) never before reported in other organisms.

6. Suggested biochemical processes of N-glycosylation

Our study to elucidate 66-kDa glycoprotein N-glycan structures found that those released from the 66-kDa protein possess oligomannose topology. The oligomannose topology may imply the existence of a conserved N-glycosylation pathway in red microalgae that takes place in the ER – which is common to eukaryotic organisms – and that includes the building of the N-glycan on the lipid substrate-dolichol-phosphate and its transfer to the protein. The results of other studies, such as that by Fishcer [68], also hint at the existence of this conserved pathway. Supporting evidence is based on homology searches for N-glycosylation protein sequences using the TBLASTN function on the algae DNA scaffold contigs database. Homologs were found for all N-glycosylation protein sequences in the ER pathways in the algae, thus suggesting that the pathway is conserved in *Porphyridium* sp. as it is in other organisms (animals, plants, yeast, etc.).

All the N-glycans investigated seem to go through the same intermediate glycan feature within their glycosylation biosynthesis, that probably have similar basic form based on the Man-9 topology structure constructed along the pathways typical of the ER. However, other enzymes, not typical to N-glycosylation pathways investigated so far, are involved in this pathway (e.g., the xylose, mannose, and methylated tranferase enzymes). However, we do not know at what stage the methyl and xylose groups were added to the mannoses during biosynthesis. In addition, we do not know if the glycan is assembled by incorporation of methyl-mannose rather than plain mannose or where the methyl groups are added to the intact high-mannose glycans.

If the methylated mannoses were incorporated into the assembled core oligosaccharide (parallel to $[Glc]_3[GlcNAc]_2[Man]_9$) via the same conserved pathway in the ER, the following mechanism can be suggested. The assembled core oligosaccharide (containing methylated groups) is transferred onto a nascent polypeptide imported into the ER (because of its signal sequence). This step is probably catalyzed by an enzyme complex (oligosaccharide transferase). Following Glucosidase I and II actions, the 3 glucose residues are cleaved from the end of the 6-branch, which initiates a process called glycan-mediated chaperoning. The last sugar that is removed in the ER is a mannose that is trimmed by an α -1,2-mannosidase through the action of ER mannosidase I (ManI, scheme 6), an enzyme that is also normally active in N-glycosylation processes, creating Man-8. However, this enzyme seems to be partially activated, as it does not cleave all the mannose residues. This phenomenon can be explained based on the methyl group structure of the oligosaccharide, which may interfere with enzyme cleavage. As a result, two different glycoprotein structures exit the ER (Figure 4):

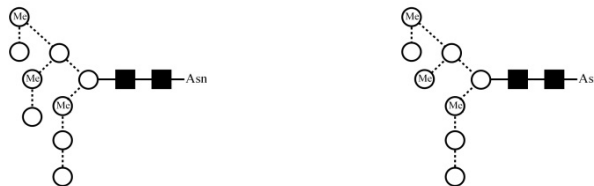
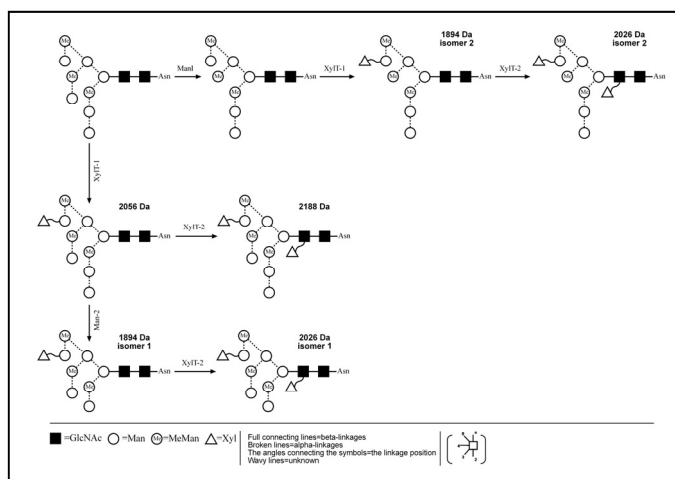


Figure 4. Two suggested intermediate features that are leaving the ER, Followed the ER they are getting their final structures within the Golgi apparatus (GA) by different enzymes.

Other changes are probably made in the GA by various xylose transferases (XylT) and a specific mannosidase (Man-2, scheme 6), the latter of which may only be able to remove the terminal mannose on the 3-antenna of the mannose-9 analog. Moreover, this enzyme may be a Golgi endomannosidase [69-70] that specifically cleaves the α 1-2 linkage between the glucose-substituted mannose residue and the more internal portion of its polymannose branch, leading to the formation of the [Man]₈[GlcNAc]₂ (Man 8A) isomer [71].

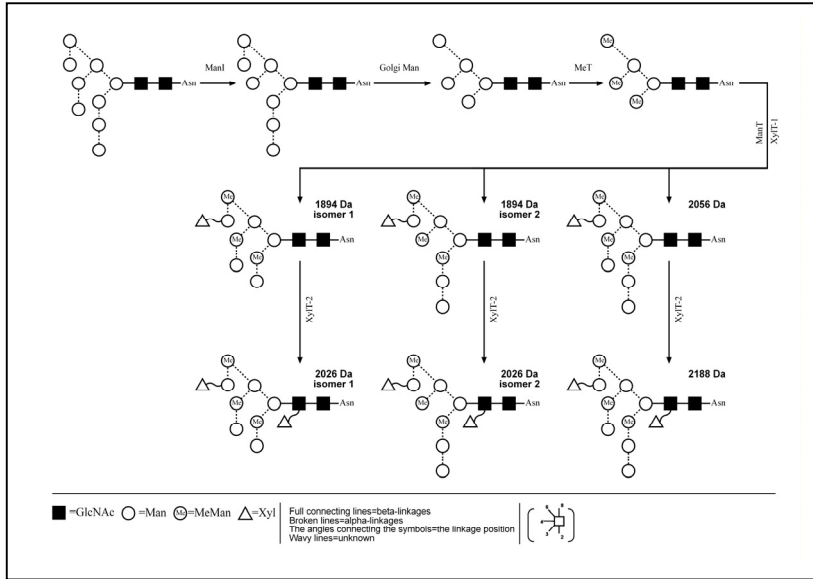
Based on an assumption about N-glycosylation of the 66-kDa protein, namely, that processes occurring in the red microalgal ER are conserved as in eukaryote cells, a scheme for the mechanism of 66-kDa protein N-glycosylation is presented (Scheme 6).



Scheme 6. Suggested mechanism of 66-kDa protein N-glycosylation, after formation of the basic core structure [GlcNAc]₂[MeMan]₉.

Another mechanism for N-glycosylation in red microalgae may be suggested based on the assumption that mannose methylation takes place in the GA, after mannose incorporation into the assembled ER core oligosaccharide. The following mechanism (Scheme 7) is based on the additional assumption that the conserved ER pathway of red microalgae functions much the same as in most eukaryotes, including synthesis of a lipid-linked oligosaccharide, transfer of glucose trimming in the ER, and subsequent cycles of glucose re-addition and removal involved in protein-folding quality control. After core oligosaccharide construction in the ER (following mannosidase I (ManI) cleavage), the ER oligosaccharide is further modified in the GA. The pathway present in the Golgi probably includes the cleavage of 3 mannose sugars by an α 1,2-mannosidase to produce [Man]₅[GlcNAc]₂, the known substrate for N-acetylglucosaminyltransferase I, which adds a single N-acetylglucosamine (GlcNAc) sugar onto the terminal of the 1,3-mannose in the mammalian glycosylation pathway. However, the structures found in this study indicate that the [Man]₅[GlcNAc]₂ is the substrate for the methyl-transferase (MeT) enzyme. Following the addition of methyl groups to the non-reducing end of the substrate, more changes occur, including the addition of mannose and xylose residues to the oligosaccharide mediated by specific transferases

(xylose transferases designated as XylT-1 and XylT-2, and mannose transferases as ManT, scheme 7).



Scheme 7. Suggested mechanism of 66-kDa protein N-glycosylation, after formation of the typical eukaryote core structure comprising $[\text{GlcNAc}]_2[\text{MeMan}]_9$ and based on the assumption that mannose methylation takes place in the GA.

In both the suggested glycosylation pathways, xylose transferases, novel in N-glycosylation pathways, play prominent roles in the glycosylation. Since the ER pathway is probably conserved, it may be assumed that mannose methylation and xylose addition take place in the GA. Moreover, these novel enzymes are probably identical to those involved in the cell-wall polysaccharide biosynthesis that occurs in the GA.

A previous study of the evolutionary conservation of genes that participate in the N-glycosylation pathway in *Porphyridium* sp. showed that the protein sequences had relatively high similarity (40%) to orthologous sequences from red and green algae, diatoms, mammals and yeast [68]. These data are indicative of the extent of conservation of the N-glycosylation pathway and of its general importance in eukaryotes, particularly in photosynthetic organisms. The phylogenetic status of the algae can also be discussed based on the structure of the bodies involved in the N-glycosylation, e.g., ER and Golgi. The Golgi bodies and ER of red microalgae have not been extensively studied. Ultrastructural studies of these cells have produced little information. Both the smooth and rough ER appear to be present, although typically not in large quantities, in red algal unicells that have been studied. One characteristic shared by all unicells studied is a smooth ER system lining the entire interior of the plasma membrane [72-75]. The wide region between this peripheral ER system and the plasma membrane is 100-150 nm and free of major organelles (including ribosomes), but it does appear to contain a fibrous substance [76]. At irregular intervals, tubules arise at

right angles from the ER toward the plasma membrane. Some appear to fuse with the plasma membrane, suggesting direct communication between the ER and the cell exterior [72]. Evans et al. [77] suggested that this system may play a role in mucilage production.

The presence of structures indicative of a eukaryotic organism may also imply that the ER-based glycosylation occurs similar to how it does in other eukaryote organisms. In addition, all red algae also contain a typical eukaryotic GA, comprising 4 to 15 cisternae [72] that are especially prominent during sporogenesis. GA numbers, size and morphology may vary with the cell cycle or culture conditions [75], i.e., in the logarithmic phase of growth of *Porphyridium*, the Golgi bodies are larger and more numerous (due to its higher number of dictyosomes, which are larger and have distended cisternae) than in stationary phase cells [75, 78]. Whereas in most eukaryotes, the cis-Golgi is associated with the ER, in red microalgae it may be involved with other bodies. Some red microalgal ultrastructure micrographs show a thin line of an apparently fibrillar substance between the forming face of the Golgi body and its associated organelles [79], representing a possible cytoskeleton element, such as actin. Moreover, it may be responsible for maintaining the associations between dictyosomes and other organelles.

Golgi involvement in the N-glycosylation pathway has yet to be elucidated. However, some reports have been published about the relationship between cell-wall polysaccharide biosynthesis and the Golgi. The GA of *Porphyridium* sp. [12, 58, 80] and of other red microalgae species [77, 81] were found to be involved in the synthesis of the cell-wall polysaccharide. Polysaccharide synthesis in *P. aerugineum* and *Rhodella reticulata* and their subsequent packaging into vesicles takes place in the Golgi [77, 82]. The vesicles are transported, fuse with the plasma membrane, and then secrete their contents on the cell surface [12, 58, 77]. Involvement of the GA in polysaccharide biosynthesis may indicate the existence of an unusual algal glycosylation process, i.e., enzymes responsible for polysaccharide biosynthesis also act on other glycol-substrates, in our case N-glycans.

The immunological natures of the additions unique to the red microalgal polysaccharide, including methylated and xylose residues, need to be determined. Xylose residues are found in N-glycans from plants [83], insects [84], molluscs [85], and rarely in parasitic helminths [86], but not normally in mammals [47]. In addition, the position and linkage of xylose (attached to the 2-position of the core branching mannose) is the same in all the organisms mentioned above. In this study, we found, for the first time, a xylose residue attached to the mannose of the 6-antenna and 1→3-linked to the penultimate GlcNAc of the core. These xylose residues are attached to a different monosaccharide (and in a different linkage position) than known glycans. Therefore, it is not known whether the xylose residues reported here have allergenic natures similar to those of the xylose residues found in other known glycans [87-88]. In addition, we also do not know how the additional methyl groups affect the protein and its immunogenic response.

The many remaining questions about N-glycosylation in the cell wall of red microalgae prevent the full potential of *Porphyridium* sp. to serve as a host for therapeutic protein production from being realized. For example, is not known whether the unusual N-glycan structures are typical specifically to the 66-kDa glycoprotein (that is part of the polysaccharide) or whether they

represent glycosylation structures characteristic of all algal N-glycosylation processes. Therefore, microalgal potential as a protein production machine cannot be evaluated without additional and extensive research, preferably with a multidisciplinary approach.

Author details

Oshrat Levy-Ontman

Department of Chemical Engineering, Sami Shamoon College of Engineering, Beer Sheva, Israel

Acknowledgement

I would like to thank Prof S. Arad for her support of this research.

7. References

- [1] Arad (Malis) S (1988) Production of sulfated polysaccharides from red unicellular algae. In: Stadler T, Mollion J, Verduzet MC, editors. *Algal Biotechnology—An Interdisciplinary Perspective*. London: Elsevier Applied Science. pp. 65–87.
- [2] Arad (Malis) S (1999) Polysaccharides of red microalgae. In: Cohen Z, editor. *Chemicals from Microalgae*. London: Taylor and Francis. pp. 282–291.
- [3] Arad (Malis) S, Levy-Ontman O (2010) Red microalgal cell-wall polysaccharides: biotechnological aspects. *Curr. Opin. Biotech.* 21: 358–364.
- [4] Arad (Malis) S, Levy-Ontman O (2012) Sulfated Polysaccharides in the Cell Wall of Red Microalgae. In: Sabu T, Dominique D, Christophe C, Jyotishkumar P, editors. *Structure, Properties and Applications (Chapter 2) Biopolymer-based materials: from blends and composites to gels and complex networks*. Verlag: Wiley-VCH.
- [5] Percival EP, Foyle RAJ (1979) The extracellular polysaccharides of *Porphyridium cruentum* and *Porphyridium aerugineum*. *Carbohydr. Res.* 72: 165–176.
- [6] Geresh S, Arad (Malis) S (1991) The extracellular polysaccharides of the red microalgae: chemistry and rheology. *Bioresourc. Technol.* 38: 195–201.
- [7] Geresh S, Adin I, Yarmolinsky E, Karpasas M (2002) Characterization of the extracellular polysaccharide of *Porphyridium* sp.: molecular weight determination and rheological properties. *Carbohydr. Polym.* 50: 183–189.
- [8] Geresh S, Lupescu N, Arad (Malis) S (1992) Fractionation and partial characterization of the sulfated polysaccharide of *Porphyridium*. *Phytochemistry* 31: 4181–4186.
- [9] Heaney-Kieras J, Chapman, DJ (1976) Structural studies on the extracellular polysaccharide of the red alga *Porphyridium cruentum*. *Carbohydr. Res.* 52: 169–177.
- [10] Arad (Malis) S, Kolani R, Simon-Berkovitch B, Sivan A (1994) Inhibition by DCB of cell-wall polysaccharide formation in the red microalga *Porphyridium* sp. (Rhodophyta) *Phycologia* 33: 158–162.
- [11] Lupescu N, Arad (Malis) S, Geresh S, Bernstein M, Glazer R (1991) Structure of some sulfated sugars isolated after acid hydrolysis of the extracellular polysaccharide of *Porphyridium* sp. a unicellular red alga. *Carbohydr. Res.* 210: 349–352.

- [12] Ramus J (1986) Rhodophytes unicells: biopolymer physiology and production. In: Barclay WR, McIntosh RP, editors. *Algal Biomass Technology*. Berlin: J. Cramer. pp. 51–55.
- [13] Ramus J (1973) Cell surface polysaccharides of the red alga *Porphyridium*. In: Loewus F, editor. *Biogenesis of Plant Cell Wall Polysaccharides*. New York : Academic Press. pp. 333–359.
- [14] Ucko M, Geresh S, Simon-Berkovitch B, Arad (Malis) S (1994) Predation by a dinoflagellate on a red microalga with a cell wall modified by sulfate and nitrate starvation. *Mar. Ecol. Prog. Ser.* 104: 293–298.
- [15] Geresh S, Dubinsky O, Arad (Malis) S, Christiaen D, Glaser R (1990) Structure of 3-O-(alpha-D-glucopyranosyluronic acid)-L-galactopyranose, an aldobiouronic acid isolated from the polysaccharides of various unicellular red algae *Carbohydr. Res.* 208: 301–305.
- [16] Geresh S, (Malis) Arad S, Levy-Ontman O, Zhang W, Tekoah Y, Glaser R (2009) Isolation and characterization of poly- and oligosaccharides from the red microalga *Porphyridium* sp. *Carbohydr. Res.* 344: 343–349.
- [17] Gloaguen V, Ruiz G, Morvan H, Mouradi-Givernaud A, Maes E, Krausz P, Strecker G (2004) The extracellular polysaccharide of *Porphyridium* sp. an NMR study of lithium-resistant oligosaccharidic fragments. *Carbohydr. Res.* 339: 97–103.
- [18] Eteshola E, Gottlieb M, Arad (Malis) S (1996) Dilute solution viscosity of red microalga exopolysaccharide *Chem. Eng. Sci.* 51: 1487–1494.
- [19] Eteshola E, Karpasas M, Arad (Malis) S, Gottlieb M (1998) Red microalga exopolysaccharides: 2. Study of the rheology, morphology and thermal gelation of aqueous preparations. *Acta. Polym.* 49: 549–556.
- [20] Ramus J, Kenney BE (1989) Shear degradation as a probe of microalgal exopolymer structure and rheological properties. *Biotechnol. Bioeng.* 34: 1203–1208.
- [21] Gourdon D, Lin Q, Oroudjev E, Hansma H, Golan Y, Arad S, Israelachvili J (2008) Adhesion and stable low friction provided by a subnanometer-thick monolayer of a natural polysaccharide. *Langmuir* 24: 1534–1540.
- [22] Arad (Malis) S, Rapoport L, Moshkovich A, van-Moppes D, Karpasas M, Golan R, Golan Y (2006) Superior biolubricant from a species of red microalga. *Langmuir* 22: 7313–7317.
- [23] Gasljevic K, Hall K, Chapman D, Matthys EF (2008) Drag-reducing polysaccharides from marine microalgae: species productivity and drag reduction effectiveness. *J. Appl. Phycol.* 20: 299–310.
- [24] Gasljevic K, Matthys EF (2007) Ship drag reduction by microalgal biopolymers: A feasibility analysis. *J. Ship. Res.* 51: 326–337.
- [25] Arad (Malis) S, Keristovsky, G, Simon B, Barak Z, Geresh S (1993) Biodegradation of the sulphated polysaccharide of *Porphyridium* by soil bacteria. *Phytochemistry* 32: 287–290.
- [26] Matsui MS, Muizzuddin N, Arad S, Marenus K (2003) Sulfated polysaccharides from red microalgae have anti-inflammatory properties *in vitro* and *in vivo*. *Appl. Biochem. Biotechnol.* 104: 13–22.

- [27] Tannin-Spitz T, Bergman M, van-Moppes D, Grossman S, Arad (Malis) S (2005) Antioxidant activity of the polysaccharide of the red microalga *Porphyridium* sp. J. Appl. Phycol. 17: 215–222.
- [28] Minkova K, Michailov Y, Toncheva-Panova T, Houbavenska N (1996) Antiviral activity of *Porphyridium cruentum* polysaccharide. Pharmazie 51: 3-7.
- [29] Talyshinsky M, Souprun Y, Huleihel M (2002) Anti-viral activity of red microalgal polysaccharides against retroviruses. Cancer Cell Int. 2: 1-8.
- [30] Huleihel M, Ishanu V, Tal J, Arad (Malis), S (2002) Activity of *Porphyridium* sp. polysaccharide against herpes simplex viruses *in vitro* and *in vivo*. J. Biochem. Biophys. Meth. 50: 189-200.
- [31] Huleihel M, Vladimir Z, Tal J, Arad S (2001) Antiviral effect of red microalgal polysaccharides on *Herpes simplex* and *Varicella* viruses. J. Appl. Phycol. 13: 127-134.
- [32] Arad (Malis) S, Ginzberg A, Huleihel M (2006) Antiviral activity of sulfated polysaccharides of marine red algae. In: Fingerman M, editor. Recent Advances in Marine Biotechnology: Biomaterials from Aquatic and Terrestrial Organisms. Enfield : Science Publishers Inc. pp. 37-62.
- [33] Fabregas J, García D, Fabregas J, Garcia D, Fernandez-Alonso M, Rocha AI, Gómez-Puertas P, Escribano JM, Otero A, Coll JM (1999) *In vitro* inhibition of the replication of *haemorrhagic septicaemia* virus and African swine fever virus by extracts from marine microalgae. Antiviral Res. 44: 67-73.
- [34] Dvir I, Geva O, Chayoth R, Sod-Moriah U, Shany S, Arad S, Madar Z (1996) Polysaccharides and algal biomass as new sources of dietary fibers and their physiological effects in rats. FASEB J. 10: 3012-3012.
- [35] Dvir I, Chayoth R, Sod-Moriah U, Shany S, Nyska A, Stark AH, Madar Z, Arad (Malis) S (2000) Soluble polysaccharide and biomass of red microalga *Porphyridium* sp. alter intestinal morphology and reduce serum cholesterol in rats. Br. J. Nutri. 84: 469-476.
- [36] Dvir I, Stark AH, Chayoth R, Madar Z, Arad (Malis) S (2009) Hypocholesterolemic effects of nutraceuticals produced from the red microalga *Porphyridium* sp. Nutrients 1: 156–167.
- [37] Raghavendran HR, Srinivasan P (2008) Effect of crude sulphated polysaccharide from brown algae against acetaminophen-induced toxicity in rats. Can. J. Physiol. Pharmacol. 86: 660-666.
- [38] Shrestha, RP, Weinstein Y, Bar-Zvi D, Arad SM (2004) A glycoprotein noncovalently associated with cell-wall polysaccharide of the red microalga *Porphyridium* sp. (Rhodophyta). J. Phycol. 40: 568-580.
- [39] Shrestha RP (1999) A non covalently bound cell-wall glycoprotein of the red microalga *Porphyridium* sp.: characterization and functions. Ph.D. Thesis, Submitted to Ben-Gurion University of the Negev, Israel.
- [40] Simon-Bercovitch B, Bar-Zvi D, and Arad (Malis) S (1999) Cell-wall formation during the cell cycle of *Porphyridium* sp. (Rhodophyta). J. Phycol. 35: 78-83.
- [41] Simon-Bercovitch B. (1997) Cell-wall formation in the red microalga *Porphyridium* sp. Ph.D. Thesis, Submitted to Ben-Gurion University of the Negev, Israel.
- [42] Ucko, M, Shrestha RP, Mesika P, Bar-Zvi D, Arad (Malis) S (1999) Glycoprotein moiety in the cell wall of the red microalga *Porphyridium* sp. (Rhodophyta) as the

- biorecognition site for the *Cryptocodinium cohnii*-like dinoflagellate. J. Phycol. 35: 1276-1281.
- [43] Gravel P (1996) Identification of glycoproteins on nitrocellulose membranes using lectin blotting. In: Walker JM, editor. The protein protocols Handbook. Totowa: Humana Press. pp.779-794.
- [44] Van Damme, EJM, Peumans WJ, Pusztai A, Bardocz S (1998) Handbook of plant lectins: properties and biomedical application. Chichester: John Wiley and Sons. pp. 452.
- [45] Shibuya N, Goldstein IJ, Van Damme EJM, Peumans WJ (1988) Binding properties of mannose specific lectin from the snowdrop (*Galanthus nivalis* Bulb.) J. Biol. Chem. 263: 728-734.
- [46] Küster B, Wheeler SF, Hunter AP, Dwek RA, Harvey DJ (1997) Sequencing of N-linked oligosaccharides directly from protein gels: In-gel deglycosylation followed by matrix-assisted laser desorption/ionisation mass spectrometry and normal-phase high performance liquid chromatography. Anal. Biochem. 250: 82-101.
- [47] Levy-Ontman O, (Malis) Arad S, Harvey DSJ, Parsons TB, Fairbanks A, Tekoa Y. (2011) Unique N-glycan moieties of the 66-kDa cell wall glycoprotein from the red microalga *Porphyridium* sp. J. Biol. Chem. 286: 21340-21352.
- [48] Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB (1995) Nonselective and efficient fluorescent labelling of glycans using 2-amino benzamide and anthranilic acid. Anal. Biochem. 230: 229-238.
- [49] Harvey DJ (2005) Fragmentation of negative ions from carbohydrates: Part 1; Use of nitrate and other anionic adducts for the production of negative ion electrospray spectra from N-linked carbohydrates J. Am. Soc. Mass Spectrom. 16: 622-630.
- [50] Harvey DJ (2005) Fragmentation of negative ions from carbohydrates: Part 2, Fragmentation of high-mannose N-linked glycans. J. Am. Soc. Mass Spectrom. 16: 631-646.
- [51] Harvey DJ (2005) Fragmentation of negative ions from carbohydrates: Part 3, Fragmentation of hybrid and complex N-linked glycans. J. Am. Soc. Mass Spectrom. 16: 647-659.
- [52] Harvey DJ, Royle L, Radcliffe CM, Rudd PM, Dwek RA (2008) Structural and quantitative analysis of N-linked glycans by MALDI and negative ion nanospray mass spectrometry. Anal. Biochem. 376: 44-60.
- [53] Domon B, Costello CE (1988) A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of Glycoconjugates. Glycoconj. J. 5: 397-409.
- [54] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
- [55] Arad (Malis) S, (Dahan) Friedman O, Rotem A (1988) Effect of nitrogen on polysaccharide production in *Porphyridium* sp. Appl. Environ. Microbiol. 54: 2411-2414.
- [56] Adda M, Merchuk JC, (Malis) Arad S (1986) Effect of nitrate on growth and production of cell-wall polysaccharide by the unicellular red alga *Porphyridium*. Biomass 10: 131-140.
- [57] Ucko M, Geresh S, Simon-Berkovitch B, Arad (Malis) S (1994) Predation by a dinoflagellate on a red microalga with a cell wall modified by sulfate and nitrate starvation. Mar. Ecol. Prog. Ser. 104: 293-298.

- [58] Ramus J (1972) The production of extracellular polysaccharide by the unicellular red alga *Porphyridium aeruginosum*. J. Phycol. 15: 97-111.
- [59] Arad (Malis) S, Richmond A (2004) Industrial production of microalgal cell-mass and secondary products-species of high potential: *Porphyridium* sp. In: Richmond A, editor. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Carlton: Blackwell Science. pp. 289-297.
- [60] Lapidot M, Shrestha RP, Weinstein Y, Arad (Malis) S (2010) Red microalgae: from basic known-how to Biotechnology. In: Seckbach J, Chapman D, editors. Red algae in the genomic age, vol. 13 of Cellular Origins, Life in Extreme Habitats and Astrobiology series. Dordrecht: Springer. pp. 11-19.
- [61] Matsui MS, Muizzuddin N, Arad S, Marenus K (2003) Sulfated polysaccharides from red microalgae have anti-inflammatory properties *in vitro* and *in vivo*. Appl. Biochem. Biotechnol. 104: 13-22.
- [62] Lapidot M, Raveh D, Sivan A, Arad (Malis) S, Shapira M (2002) Stable chloroplast transformation of the unicellular red alga *Porphyridium* sp. Plant Physiol. 129: 7-12.
- [63] Plesser E (2009) Molecular characterization of the Sulfotransferase from the red microalga *Porphyridium* sp. PhD thesis, Ben-Gurion University of the Negev, Beer-Sheva, Israel.
- [64] Giddings G, Allison G, Brooks D and Carter A (2000) Transgenic plant as factories for biopharmaceuticals. Nature Biotechnol. 18: 1151-1155.
- [65] Walmsley AM, Arntzen CJ (2003) Plant cell factories and mucosal vaccines. Curr. Opin. Biotechnol. 14: 145-150.
- [66] Rasala BA, Muto M, Lee PA, Jager M, Cardoso RM, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnol. J. 6: 719-33.
- [67] Franklin SE, Mayfield SP (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin. Biol. Ther. 5: 225-235.
- [68] Fisher M (2011) Elucidation of the N-Glycosylation Pathway in Red Microalgae *Porphyridium* sp. Ph.D. Thesis, Submitted to Ben-Gurion University of the Negev, Israel.
- [69] Lubas WA, Spiro RG (1987) Evaluation of the Role of Rat Liver Golgi Endo- α -D-mannosidase in Processing N-Linked Oligosaccharides. J. Biol. Chem. 262: 3775-3781.
- [70] Lubas WA, Spiro RG (1988) Evaluation of the role of rat liver Golgi endo- α -D-mannosidase in processing N-linked oligosaccharides. J. Biol. Chem. 263: 3990-3998.
- [71] Moore SEH, Spiro RG (1990) Demonstration that Golgi endo- α -D-mannosidase provides a glucosidase-independent pathway for the formation of complex N-linked oligosaccharides of glycoproteins. J. Biol. Chem. 265: 13104-13112.
- [72] Pueschel CM (1990) Cell structure. In: Cole KM, Sheath RG, editors. Biology of the Red Algae. Cambridge: Cambridge University Press. pp. 7-41.
- [73] Patrone LM, Broadwater ST, Scott JL (1991) Ultrastructure of vegetative and dividing cells of the uni-cellular red algae *Rhodella violacea* and *Rhodella maculata*. J. Phycol. 27: 742-753.

- [74] Scott J, Broadwater S, Gabrielson P, Thomas J, Saunders B (1992). Ultrastructure of vegetative organization and cell division in the unicellular red alga *Dixoniella grisea* gen. nov. (Rhodophyta) and a consideration of the genus *Rhodella*. J. Phycol. 28: 649-660.
- [75] Seckbach J (1994) Evolutionary pathways and enigmatic algae: *Cyanidium caldarium* (Rhodophyta) and related cells. In: Broadwater ST, Scott JL, editors. Ultrastructure of unicellular red algae. Dordrecht: Kluwer Academic Publishers. pp. 215-230.
- [76] Broadwater ST, Scott JL, Garbary DJ (1992) Cytoskeleton and mitotic spindle in red algae In: Menzel D, editor. The cytoskeleton of the Algae. Boca Raton: CRC Press: pp. 93-112.
- [77] Evans LV, Callow ME, Pervical E, Fareed V (1974) Studies on the synthesis and composition of extracellular mucilage in the unicellular red alga *Rhodella*. J. Cell Sci. 16: 1-21.
- [78] Ramus J, Robins DM (1975) The correlation of Golgi activity and polysaccharide secretion in *Porphyridium*. J. Phycol. 11: 70-74.
- [79] Alley CD, Scott JL (1977) Unusual dictyosome morphology and vesicle formation in tetrasporangia of the marine alga *Polysiphonia denudate*. J. Ultrastruct. Res. 58: 289-298.
- [80] Keidan M, Friedlander M, Arad (Malis) S (2009) Effect of Brefeldin A on cell-wall polysaccharide production in the red microalga *Porphyridium* sp. (Rhodophyta) through its effect on the Golgi apparatus. Phycologia 21: 707-717.
- [81] Gantt E, Conti SF (1965) The ultrastructure of *Porphyridium cruentum*. J. Cell Biol. 26: 365-381.
- [82] Ramus J (1976) Cell surface polysaccharides of the red alga *Porphyridium*. Plant and cell wall polysaccharides pp.333-357
- [83] Lerouge P, Cabanes-Macheteau M, Rayon C, Fitchette-Lainé AC, Gomord V, Faye L (1998) N-glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol. Biol. 38: 31-48.
- [84] Altmann F, Staudacher E, Wilson IB, Marz L (1999) Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj. J. 16: 109-123.
- [85] Kamerling JP, Vliegenthart JFG. (1997) Hemocyanins. In: Montreuil J, Vliegenthart JFG, Schachter H, editors. Glycoproteins II. Amsterdam: Elsevier Science B.V. pp. 123-140.
- [86] Khoo KH, Chatterjee D, Caulfield JP, Morris HR, Dell A (1997) Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences. Glycobiology 7: 663-677.
- [87] Garcia-Casado G, Sanchezmonge R, Chrispeels MJ, Armentia A, Salcedo G, Gomez L (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. Glycobiology 6, 471-477.
- [88] Van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L, Lerouge P (2000) $\alpha(1,2)$ -xylose and $\alpha(1,3)$ -fucose residues have a strong contribution in IgE binding to plant glycoallergens. J. Biol. Chem. 275, 11451-11458.