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N-Glycosylation Profiles of the Green Microalgae *Chlorella Zofingiensis*

Yeşil Mikroalg Türü *Chlorella Zofingiensis*'in N-glikozliasyon Profili

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ABSTRACT

Nowadays, the use of microalgae species as raw materials in biopharmaceutical production is on the agenda. The reason behind this idea is that microalgae are cell factories that are able to efficiently utilize carbon dioxide for the production of numerous biologically active compounds. However, there are several problems that remain to be solved in the production of recombinant protein from microalgaes. One of the critical requirements is to produce a bio-compatible N-glycosylation profile from the secreted recombinant proteins. The knowledge about the glycosylation machinery and N-glycan profiles of microalgae spices are quite limited. In the study, it was aimed to characterize N-glycan profiles of a green microalgae, *Chlorella zofingiensis*. To achieve this, photoautotrophically grown *Chlorella zofingiensis* extracts including (glyco-)proteins were enzymatically deglycosylated and labelled with 2-aminobenzoic acid tag. Released N-glycans were purified with a HILIC-based approach and analyzed by MALDI-TOF(/TOF)-MS. The results showed that *C. zofingiensis* included oligomannosidic type N-glycan patterns. In addition, N-glycosylation profiles of *C. zofingiensis* by MALDI-MS revealed that most of the oligomannosidic N-glycans were phosphorylated.

Key Words

Chlorella zofingiensis, microalgae N-glycosylation, MALDI-MS, mannose-6-phosphate.

ÖZ

Günümüzde, mikroalg türlerinin biyofarmasötik üretiminde hammadde olarak kullanımı konusu tartışılmaktadır. .Bu fikrin altında yatan temel neden mikro alglerin, biyolojik olarak aktif çok sayıda bileşenin üretimi için karbon dioksiti etkin bir şekilde kullanabilen hücre fabrikaları olmasıdır. Bununla birlikte, mikroalglerden rekombinant protein üretiminde kullanılması için çözülmesi gereken birkaç sorun mevcuttur. Kritik gereksinimlerden birisi salgılanan rekombinant proteinlerden biyo-uyumlu bir N-glikozilasyon profile üretmektir. Mikroalg türlerinin glikozilasyon makineleri ve N-glikan profilleri hakkındaki bilgiler oldukça sınırlıdır. Bu çalışmada, yeşil bir mikroalg türü olan *Chlorella zofingiensis* 'in N-glikan profilinin karakterize edilmesi amaçlandı. Bu amaçla (gliko-)proteinleri içeren fotootrofik olarak yetiştirilmiş *Chlorella zofingiensis* ekstreleri enzimatik olarak deglikozile edildi ve 2-aminobenzoik asit etiketi ile etiketlendi. Serbest hale getirilen N-glikanlar HILIC bazlı bir yaklaşımla saflaştırıldı ve MALDI-TOF (/TOF)-MS ile analiz edildi. Sonuçlar *C. zofingiensis*'in oligomannozidik türde N-glikan modellerini içerdiğini göstermiştir. Ek olarak, MALDI-MS tarafından *C. zofingiensis*'in N-glikosilasyon profilleri, oligomannosidik N-glikanların neredeyse yarısının fosforilenmiş olduğunu ortaya koymuştur.

Anahtar Kelimeler

Chlorella zofingiensis, mikroalg N-glikozilasyonu, MALDI-MS, mannoz-6-fosfat.

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INTRODUCTION

Biopharmaceuticals can be defined as pharmaceutical components used for therapeutic or in vivo diagnostic purposes, obtained by a method other than direct extraction from a biological source. Today, the roles of biopharmaceuticals in the pharmaceutical industry are rapidly expanding. About half of the new drugs are classified biopharmaceutical, and the majority of them are proteins [1, 2]. Due to the advancements of genetic engineering tools, therapeutic protein production has shifted to recombinant expression systems; thus, the production of these macromolecules in many expression systems, even including mammalian and insect cells [3]. Each of these systems has advantages and disadvantages in terms of cost and efficiency. For instance, proteins obtained by mammalian cell culture have a therapeutically more biocompatible character, but requires a high-cost system for the production of therapeutics on an industrial scale [4].

Microalgae have been recognized as an alternative platform with their promising properties in recombinant protein production. Since microalgae are the feedstock sources of many valuable products such as biofuels, staple foods, vitamins and polyunsaturated fatty acids, its products have a tremendous market value. They are also an environmentally important actor for the treatment of waste water and reducing the dependence to the fossil fuels [5]. Microalgal biomass contains four main groups of molecules, consisting of oils, carbohydrates, proteins and pigments [6]. Microalgae cultures are relatively cheap systems in terms of protein production. Like other plant-based systems, microalgae protein cultures do not contain non-biocompatible contaminations for humans [7]. The potential for yielding products with different variations in the production of recombinant protein as a single-celled organism is low. Furthermore, as a eukaryotic cell, they can form complex structures such as post-translational modifications (PTM) in human proteins by the cellular means that they possess[8]. PTMs, particularly N-glycosylation, should be considered, because their absence or diversity can create significant problems for in vivo applications of the resulting product. Most of the produced recombinant proteins are glycoproteins [9, 10].

N-glycans refer to oligosaccharide chains attached to the nitrogen atom in the asparagine side chain in the amino acid sequence of Asn-X-Ser / Thr (X must not be proline) of proteins. All N-glycans share a common core sugar sequence consisting of three mannose and two N-acetyleglucosamine in the region where they are attached to the amino acid (Figure 1-A). They are classi-

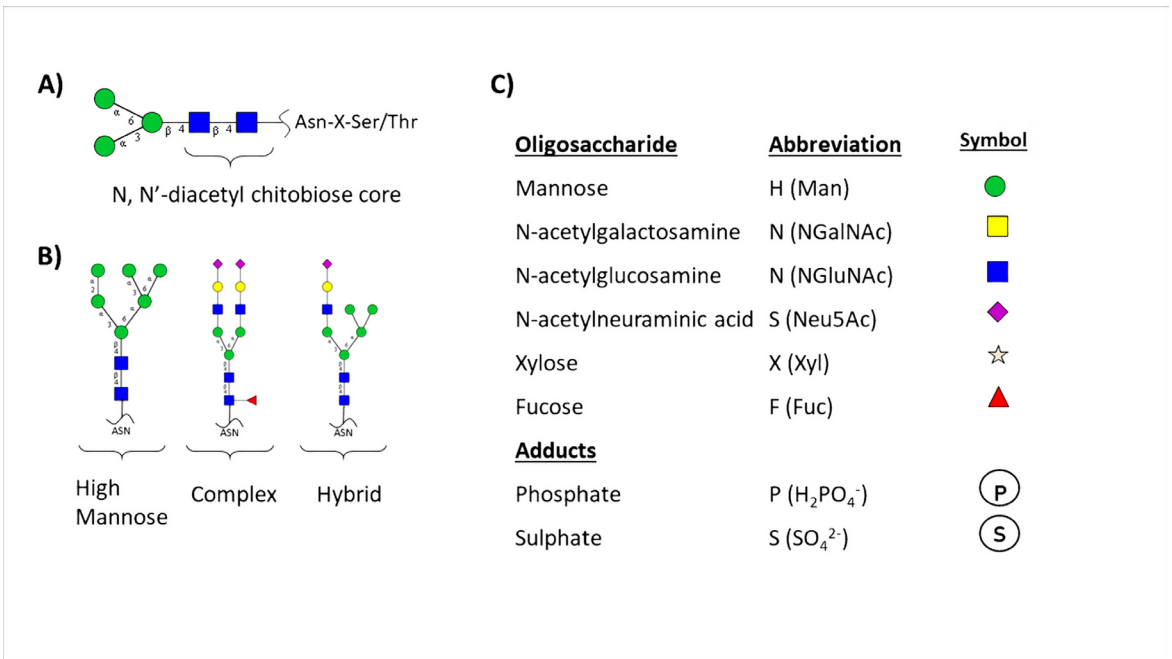

Figure 1. General structures of N-glycans. A) Basic core structure that unique for all N-glycans. B) Type of N-glycans, C) Symbols and abbreviations used for major oligosaccharides and adducts present in N-glycan structures.

fied into three types: first type that have only mannose residues are attached to the core named oligomannose or high mannose, second type that contains "antennae" formed by N-acetylglucosaminyltransferases (GlcNAcTs) are attached to the core named complex and the third type that only mannose residues are attached to the one arm of the core and the other mannose arm contains one or two antennae named hybrid (Figure 1-B). Oligosaccharides and linkages in the content of N-glycan are represented their symbols and abbreviations (Figure 1-C) [11, 12].

N-glycosylation is the most common PTM of mammalian proteins and has various functional roles that affect the properties of the protein such as folding, stability, solubility, and intracellular trafficking [13-15]. Within the immune system, the N-glycans on an immune cell's surface will help dictate that migration pattern of the cell. Therefore, it's expected that the biopharmaceutical proteins do not have any foreign N-glycan patterns to human cells[13]. The expression host significantly changes the N-glycosylation profile of the recombinant protein product. Among all possible expression systems, mammalian cells are the most widely used expression system for the production of biotherapeutic glycoproteins. This is because microalgae, yeast, plants and insect systems produce patterns of immunogenic glycans, while mammalian systems are capable of producing human-like complex glycans [16, 17]. Therefore, the absence of immunogenic or non-human type glycan patterns (β-1-2 Xylose, α-1-3 Fucose and α1-3Gal (alpha-Galactose) etc. [18]) in proteins of a microalga species can provide an advantage for use it as a host in recombinant protein expression.

Chlorella zofingiensis is a fresh water green microalgae. It grows fast and cultured easily. In addition, it can be scaled up both indoors as well as outdoors and provided ultrahigh cell density. *Chlorella zofingiensis* has become a highly preferred species in recent years especially in the production of high-value astaxanthin, lutein and oil. *C. Zofingiensis* is a species of microalgae suitable for growth under autotrophic, mixotrophic and heterotrophic conditions. It can be grown in open ponds, photobioreactors and biofermantors [19-21]. Chlorella cells can produce up to 60% protein, about 20% polysaccharide and oil each [8]. Given the growth rate and protein content, *C. Zofingiensis*, as a eukaryotic microalgae, has the potential to produce complex eukaryotic proteins having PTMs [22]. In recent studies, isolation and characterization of modified phytoene desaturase with genetic engineering from *C. Zofingiensis* culture have been successfully performed [23, 24].

Apart from various studies on N-glycan profiles of microalgae, the literature knowledge on this subject is limited [25-27]. In particular, to the best of our knowledge, a study to determine the N-glycan profile of *C. Zofingiensis* does not exist. In this study, it was aimed to determine the N-glycan profiles of photoautothropically produced *C. Zofingiensis* samples using MALDI-MS-based glycomics approach.

MATERIALS and METHODS

Materials

Trifluoro acetic acid, DL-dithiothreitol (DTT), dimethyl sulfoxide (DMSO) and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Iodoacetamid (IAA), sodium dodecyl sulfate (SDS), 4-nonilphenyl-polyethylene glycol (NP-40) were purchased from Sigma Aldrich (St. Louis, MO). Recombinant Peptide N-Glycasidase F (PNGase F) of Flavobacterium meningosepticum were obtained from Roche Diagnostics (Mannheim, Germany). 2,5-dihydroxy benzoic acid (DHB) were purchased from Bruker Daltonics (Bremen, Germany). Deionized water (R> 18.2 MΩ/cm) was prepared daily by PURELAB Ultra system (Elga Labwater, Ede, The Netherlands) and used throughout the study.

Algal strain and cultivation conditions

Chlorella zofingiensis ***Dönz*** (CCALA 944) was obtained from the Culture Collection of Autotrophic Organisms, Czech Republic. Microalgae were grown photoautotrophically in BG-11 medium, which consisted of: $NaNO_3$ (1.5 g/L); K_2HPO_4 (40 mg/L); $MgSO_4{\cdot}7H_2O$ (75 mg/L); $CaCl_2{\cdot}2H_2O$ (36 mg/L); $NaCO_3$ (20 mg/L); ferric-ammonium citrate (6 mg/L); citric acid (6 mg/L) and 1 mL of microelements composed of H_3BO_3 (2.86 mg/L), $MnCl_2{\cdot}4H_2O$ (1.81 mg/L), $ZnSO_4{\cdot}7H_2O$ (0.22 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (0.39 mg/L), $CuSO_4{\cdot}5H_2O$ (0.08 mg/L), $Co(NO_3)_2{\cdot}6H_2O$ (0.05 mg/L) in 1000 mL acidified water including 1 mL concentrated H_2SO_4 in 1 L distilled water [20]. The bubble column photo-bioreactor (BC-PBR) was manufactured using an acrylic transparent tube with the inner diameter of 10 cm and the wall thickness of 0.5 cm. A diffuser (pore size: 40-100 µm) was placed on the bottom of the reactor. The reactor built in 50 cm length, was 4 L and the working volume was 3.6 L.

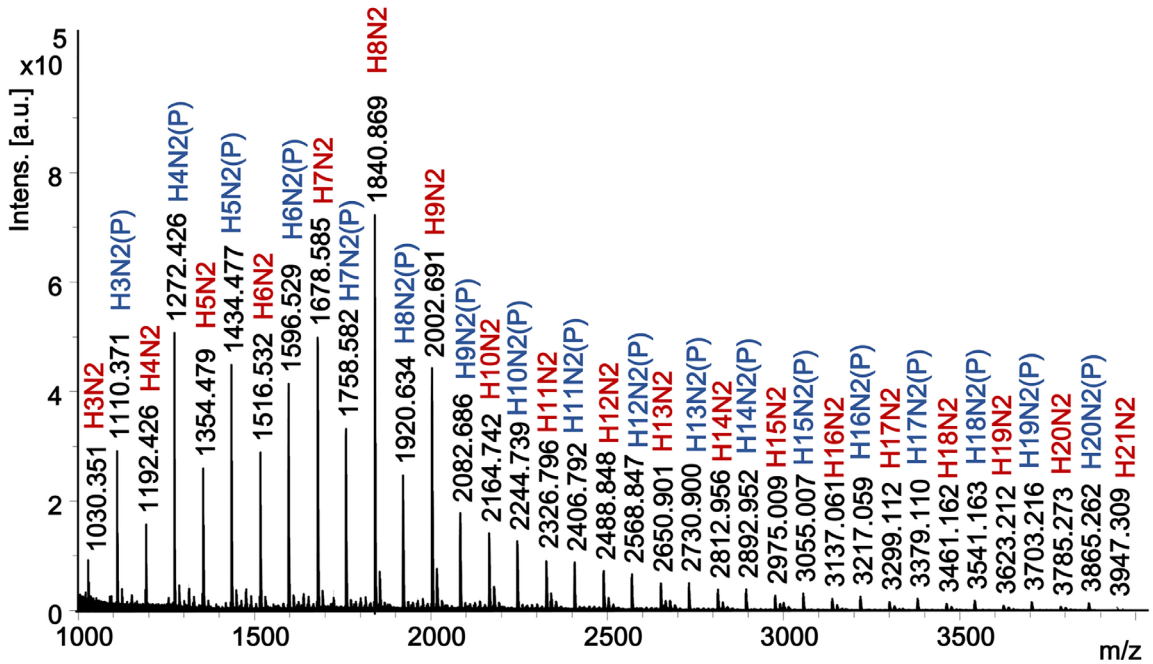

Figure 2. MALDI-MS spectrum of extracts from *C. Zofingiensis* with proposed structures for N-glycan structures. (P: phosphate adducts).

The BC-PBR was illuminated by the white strip-LEDs (HI-LED 5050) placed at a 45º angle along the perimeter of the column and the average light intensity was approximately 175 µmol·m^{-2}·s^{-1} and photoperiod was 16:8 h:h (light: dark). Temperature was maintained at 25ºC. The cultures were bubbled with sterilized-filtered air at a flow rate of 1600 mL·min^{-1}. An autonomous pH-controller regulated the pH-value (6.8) in the suspension by controlling a CO_2-valve to modulate the CO_2 concentration in the aeration stream.

Harvesting the microalgal biomass and preparation of cell protein extract

Microalgal growth was observed daily by UV-visible spectrophotometer (Merck Prove 300) at 680 nm wavelength. The microalgae reaching the exponential growth phase were harvested by centrifugation at 3000 rcf at 4ºC. The pellet was dried in vacuum concentrator at 30°C for 2 hours. Each pellets were redissolved with denaturation buffer (5% SDS (w/v), 0.4 M DTT) to give final concentration of 10 mg/mL.. andwere incubated at 97°C for 1 hour for achieving denaturation.

Enzymatic de-glycosylation, 2-AA Derivatization and purification of N-glycans

200 µL of denatured protein samples were mixed with 100 µL digestion solution (containing 10% NP-40, G1 Reaction Buffer (New England BioLabs), and 10 µL PNGase F (1 Unit/µL)) and the mixtures were incubated at 37°C overnight. Subsequently, the samples were centrifuged and supernatants were collected.

After digestion, released oligosaccharides were derivatized by 2-AA tag. For this purpose, 50 µL of digested sample solutions were mixed with 25 µL of 2-AA label solution (48 mg/mL 2-AA in DMSO containing 15% glacial acetic acid) and gently mixed. Subsequently, 25 µL aliquots of reducing agent solutions (1 M 2-picoline-borane in DMSO) were added, followed by vortex mixing for 2 min. and incubation at 65°C for 2 h.

Labelled N-glycans with 2AA were purified by a cotton based HILIC-SPE method which has previously described with minor modifications [28]. HILIC-SPE microtips were prepared by taking approximately 2 mg of 100% cotton wool into 200 µL polypropylene pipette tips and placed end part of tip using an injector nozzle. HILIC SPE microtip was washed with 5 times with 20 µL

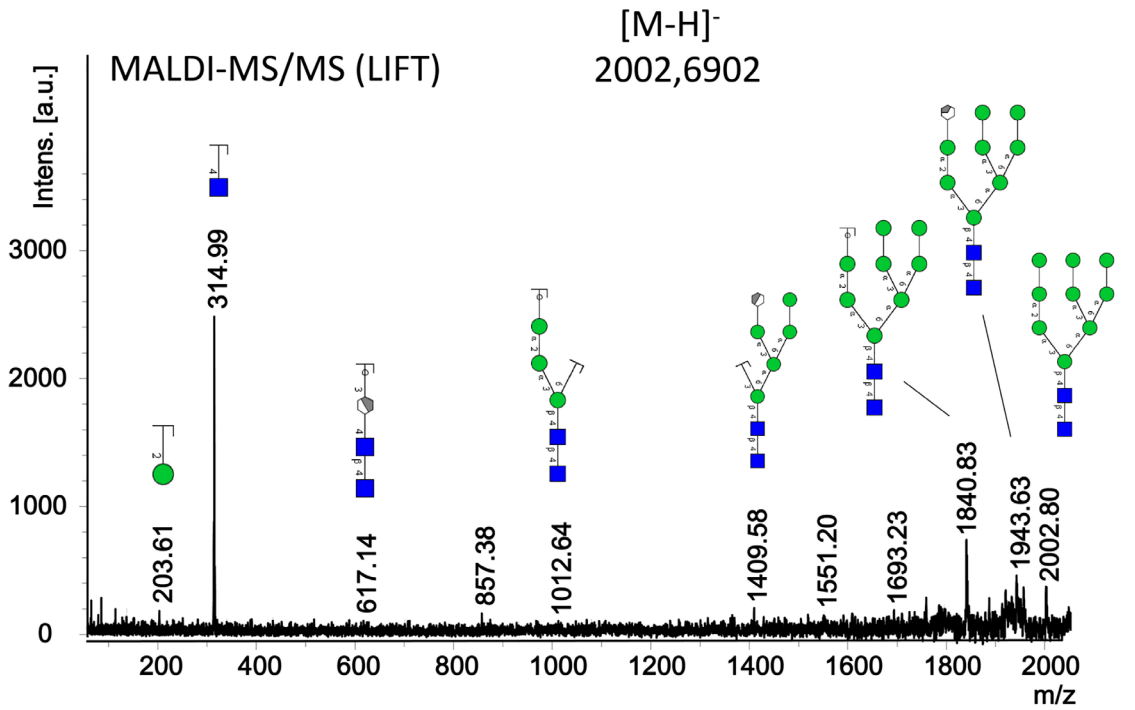

Figure 3. MALDI-MS/MS spectra of an N-glycan structure H9N2 is exemplified.

of deionized water and 5 times with 85% acetonitrile for conditioning. Sample loading solutions were prepared for each samples by adding 15 µL of labelled N-glycans into 85 µL of acetonitrile. Samples were loaded to the microtips by pipette aspirate and dispense movements for 20 times in sample loading solutions. The adsorbed N-glycans washed by 5 times with 20 µL of 85% acetonitrile containing 0.1% TFA and 85% acetonitrile solution, respectively. N-glycans were eluted into 20 µL of deionized water by aspirate and dispense movements of microtip inserted pipette. The purified N-glycan solutions were stored at -20°C until analysis.

MALDI-TOF(/TOF)-MS Analysis of Derivatized N-glycans

MALDI-TOF(/TOF)-MS analysis of 2-AA labeled N-glycans was performed on a Bruker rapifleX™ MALDI Tissuetyper™ (Bruker Daltonik GmbH, Bremen, Germany) equipped with a Smartbeam 3D laser system. Twenty-five kV acceleration voltage was applied with a 160 ns extraction delay. Totally 10.000 shots at 5000 Hz were summed per spectrum. All Spectra were obtained in negative ion and reflectron mode using a random walk pattern in the mass range of 1000-4000 Da. The instrument was externally calibrated with a peptide calibration standard prior to analysis. 2,5-dihydroxy benzoic acid (DHB) (5 mg/mL in 50% acetonitrile solution) was used as matrix in all MALDI-TOF(/TOF)-MS analysis. Matrix solution (1 µL) was applied to dried sample solution (1 µL) spotted on the MTP Anchorchip Target (Bruker™). MS/MS spectra were obtained using the (LIFT-TOF/TOF) "LIFT" in which fragment ions produced by unimolecular decomposition of the precursor ions produced during the MALDI process are isolated using a timed ion gate.

Data Analysis

The obtained data by MALDI-TOF(/TOF)-MS analysis were processed with Flex Analysis v. 4.0 software (Bruker Daltonik Gmbh) by means of the baseline subtraction and throwing peaks have signal/noise ratio less than 3. N-glycans were manually verified to match with 2-AA labelled N-glycan structure by the GlycoWorkbench program found in the Eurocarb database http://www.Eurocarb.org/ [29]. Relative amounts of N-glycans were calculated as the ratio of the area of each N-glycan ion to the summed peak area of the detected total N-glycans. Standard deviations were calculated with the results obtained by preparing four different samples independently.

Table 1. 2-aminobenzoic acid labelled N-glycan compositions detected by MALDI-MS analysis achieved in negative ionization and reflectron mode. The mass peaks used for the internal calibration are indicated in bold. (H = hexose; N = N-acetylexosamine).

N-glycan Composition	m/z meas. (mono)	z	m/z calc. (mono)	Δ MH [Da] (mono)	Relative Amount (%) ± SD	RSD%
H3N2	1030.351	-1	1030.373	-0.022	0.31 ± 0.01	4.64
$[H3N2+ H_2PO_4]^-$	1110.371	-1	1110.340	0.031	2.62 ± 0.18	7.14
H4N2	1192.426	-1	1192.426	0.000	1.48 ± 0.14	8.06
$[H4N2+ H_2PO_4]^-$	1272.426	-1	1272.392	0.034	5.25 ± 0.62	14.05
H5N2	1354.479	-1	1354.479	0.000	3.02 ± 0.34	9.67
$[H5N2+ H_2PO_4]^-$	1434.477	-1	1434.445	0.032	5.59 ± 0.27	4.71
H6N2	1516.532	-1	1516.532	0.000	3.9 ± 0.30	6.92
$[H6N2+ H_2PO_4]^-$	1596.529	-1	1596.498	0.031	5.98 ± 0.35	5.48
H7N2	1678.585	-1	1678.585	0.000	7.61 ± 0.31	3.89
$[H7N2+ H_2PO_4]^-$	1758.582	-1	1758.551	0.031	5.43 ± 0.27	4.75
H8N2	1840.637	-1	1840.637	0.000	12.28 ± 0.49	4.13
$[H8N2+ H_2PO_4]^-$	1920.634	-1	1920.604	0.030	4.54 ± 0.23	4.81
H9N2	2002.691	-1	2002.690	0.001	8.46 ± 0.63	8.23
$[H9N2+ H_2PO_4]^-$	2082.686	-1	2082.657	0.030	3.71 ± 0.17	4.44
H10N2	2164.742	-1	2164.743	-0.001	3.14 ± 0.22	7.91
$[H10N2+ H_2PO_4]^-$	2244.739	-1	2244.709	0.030	3.02 ± 0.11	3.64
H11N2	2326.796	-1	2326.796	0.000	2.34 ± 0.16	7.41
$[H11N2+ H_2PO_4]^-$	2406.792	-1	2406.762	0.030	2.37 ± 0.11	4.38
H12N2	2488.848	-1	2488.849	-0.001	2.02 ± 0.11	6.19
$[H12N2+ H_2PO_4]^-$	2568.847	-1	2568.815	0.032	1.93 ± 0.07	3.39
H13N2	2650.901	-1	2650.902	-0.001	1.52 ± 0.10	6.97
$[H13N2+ H_2PO_4]^-$	2730.900	-1	2730.868	0.032	1.27 ± 0.03	1.82
H14N2	2812.956	-1	2812.954	0.002	1.26 ± 0.08	7.24
$[H14N2+ H_2PO_4]^-$	2892.952	-1	2892.921	0.031	1.34 ± 0.01	0.74
H15N2	2975.009	-1	2975.007	0.002	1.02 ± 0.07	7.86
$[H15N2+ H_2PO_4]^-$	3055.007	-1	3054.973	0.034	1.15 ± 0.03	3.02
H16N2	3137.061	-1	3137.060	0.001	0.86 ± 0.05	6.52
$[H16N2+ H_2PO_4]^-$	3217.059	-1	3217.026	0.033	1.03 ± 0.06	5.73
H17N2	3299.112	-1	3299.113	-0.001	0.69 ± 0.06	9.11
$[H17N2+ H_2PO_4]^-$	3379.110	-1	3379.079	0.031	0.92 ± 0.07	8.56
H18N2	3461.162	-1	3461.166	-0.004	0.56 ± 0.05	10.14
$[H18N2+ H_2PO_4]^-$	3541.163	-1	3541.132	0.031	0.83 ± 0.08	10.57
H19N2	3623.212	-1	3623.218	-0.006	0.46 ± 0.04	9.18
$[H19N2+ H_2PO_4]^-$	3703.216	-1	3703.185	0.031	0.75 ± 0.06	8.92
H20N2	3785.273	-1	3785.271	0.002	0.38 ± 0.03	10.57
$[H20N2+ H_2PO_4]^-$	3865.262	-1	3865.238	0.024	0.69 ± 0.05	8.34
H21N2	3947.309	-1	3947.324	-0.015	0.28 ± 0.03	11.35

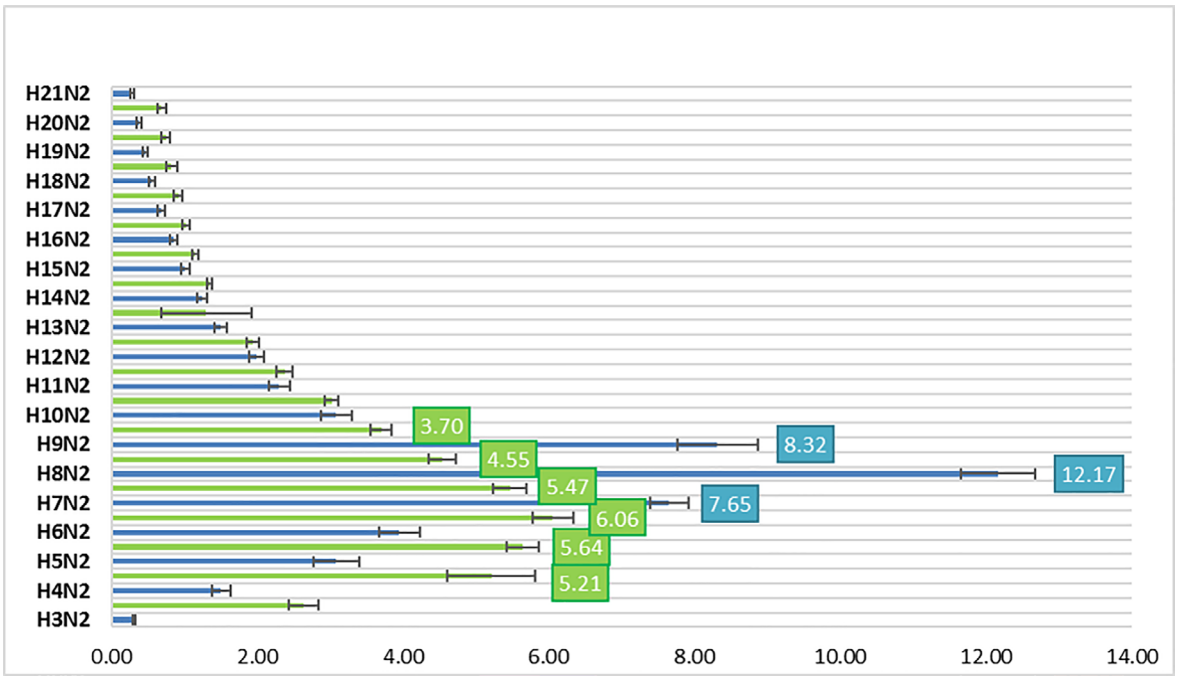

Figure 4. Relative abundances of N-glycans identified in the study. The relative abundances of 4 different samples were averaged and standard deviations are based on multiple analysis.

RESULTS and DISCUSSION

N-glycans were released from the extracted proteins of *C. Zofingiensis* by applying an enzymatic process. Then, the released N-glycan samples were analyzed by MALDI-MS. N-glycan patterns of *C. Zofingiensis* detected by MALDI-MS analysis are shown in Figure 2. Mass peaks of the parent compounds are allowed to define monosaccharide contents of N-glycans. It was determined that the detected mass peaks were consistent with the theoretical masses of 2-AA labeled N-glycans. The identified N-glycan patterns are listed in Table 1**.** Totally thirty-seven N-glycan structures were identified, of which 19 of them were high-mannose containing type of N-glycans including from 2-mannose to 21-mannose. In addition, it was detected that most of the oligomannosidic N-glycans had phosphate groups.

To confirm N-glycan structures of *C. Zofingiensis*, some of the identified N-glycan mass peaks were subjected to MS/MS analysis. An example MS/MS spectrum belonging to H9N2 N-glycan type is shown in Figure 3. The fragments observed in MS/MS spectrum indicated the Hexose losses which confirmed the N-glycan structure H9N2. Most of MS peaks were matched with theoretical masses of 2-AA labelled N-glycans. These findings are confirmed the N-glycan structures detected in the study.

The relative abundances of the each identified N-glycans were calculated by using total area normalization approach. The analyses were achieved for four experimental replicates. The relative standard deviation (RSD) of the relative abundances are ranged from 0.74% to 14.05%. Thus, the results demonstrate that the reported method provides a reproducible analysis for N-glycan samples prepared separately from denatured protein extracts. Major N-glycan patterns were found as H7N2, H8N2 and H9N2 with an average relative abundance 7.65, 12.17 and 8.32, respectively. It was also observed that some of phosphated mannose N-glycan species ([H4N2+ H_2PO_4]$^-$, [H5N2+ H_2PO_4]$^-$, [H6N2+ H_2PO_4]$^-$ and [H7N2+ H_2PO_4]$^-$) were found in relatively high amounts in the N-glycans distribution (Figure 4). It was detected that the relative abundance of all identified oligoman-

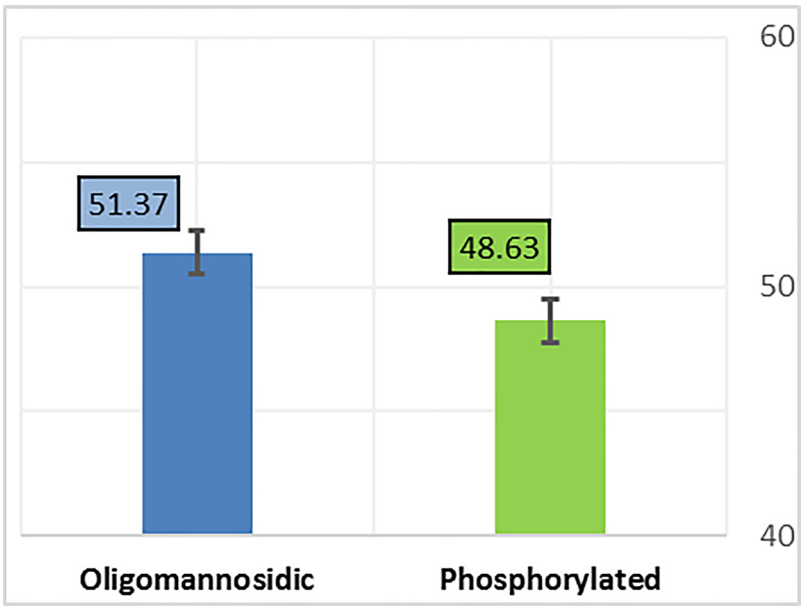

Figure 5. Distributions of abundance of oligomannosidic and phosphorylated species in all identified N-glycan structures.

nosidic N-glycan structures were found to be 51.37% (Figure 5). In addition, phosphorylated oligomannosidic N-glycans were covered 48.63% in total N-glycan pool.

It is known that the N-linked glycosylation pathway in algae is highly similar to other eukaryotic organisms [30]. However, previous studies reveal that different microalgae species are frequently exhibit different N-glycan patterns from each other. Mathieu-Rivet et al. [27] were examined N-glycosylation pathway of the model *Chlamydomonas reinhardtii* which most commonly studied microalgae for biotechnological potential revealed that the N-glycan profile contains mainly oligomannose (Man-2 to Man-5) as well as 6-O-methyl mannose and one or two xylose residues. Similar results were found for a *Porphyridium* sp., a red microalgae was obtained by Levy-Ontman et al. [31]. It was reported the presence of high-mannose type (Man-8, Man-9) with non-characteristic xylose residues and also 6-O-methly-Man-3 type attached to the chitobiose core. Another study conducted for *Chlamydomonas reinhardtii* reveals that in addition to high mannose type, it also has more complex structures containing sialylated and core a1,3-linked fucose residues [25]. Xylose and core a1,3-linked fucose patterns are known to cause carbohydrate-induced cross-reactivity [32]. Unlike these microalgae species, the presence of xylose- and a1,3-linked fucose containing oligosaccharides in *C. zofingiensis* has not been observed.

Conclusion

In the study, we demonstrated that photoautotrophically grown *C. zofingiensis* extracts contain mainly oligomannosodic N-glycans, but they had phosphorylated species. It was found that 51.37% and 48.63% of N-glycans were oligomannosodic and phosphorylated oligomannosidic N-glycan types, respectively. As these microalgae are able to express biocompatible N-glycosylated endogenous proteins, we believe that it is possible to provide polypeptides containing N-glycan structures suitable for biotechnological applications and expression of biologically active therapeutic glycoproteins.

Acknowledgments

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References

1. G. Walsh, Biopharmaceuticals: biochemistry and biotechnology, John Wiley & Sons2013.
2. N.K. Tripathi, A. Shrivastava, Scale up of biopharmaceuticals production. Nanoscale Fabrication, Optimization, Scale-Up and Biological Aspects of Pharmaceutical Nanotechnology, Elsevier2018, pp. 133-172.
3. A.F. Jozala, D.C. Geraldes, L.L. Tundisi, V.d.A. Feitosa, C.A. Breyer, S.L. Cardoso, P.G. Mazzola, L.d. Oliveira-Nascimento, C.d.O. Rangel-Yagui, P.d.O. Magalhães, Biopharmaceuticals from microorganisms: from production to purification, Braz. J. Microbiol., 47 (2016) 51-63.
4. D.C. Andersen, L. Krummen, Recombinant protein expression for therapeutic applications, Curr. Opin. Biotechnol., 13 (2002) 117-123.
5. R. Kothari, A. Pandey, S. Ahmad, A. Kumar, V.V. Pathak, V. Tyagi, Microalgal cultivation for value-added products: a critical enviro-economical assessment, 3 Biotech., 7 (2017) 243.
6. O. Pignolet, S. Jubeau, C. Vaca-Garcia, P. Michaud, Highly valuable microalgae: biochemical and topological aspects, J. Ind. Microbiol. Biotechnol., 40 (2013) 781-796.
7. R. Mócsai, R. Figl, C. Troschl, R. Strasser, E. Svehla, M. Windwarder, A. Thader, F. Altmann, N-glycans of the microalga Chlorella vulgaris are of the oligomannosidic type but highly methylated, Sci. Rep., 9 (2019) 1-8.
8. E. Becker, Micro-algae as a source of protein, Biotechnol. Adv., 25 (2007) 207-210.
9. P. Jacobs, N. Callewaert, N-glycosylation engineering of biopharmaceutical expression systems, Curr. Mol. Med. 9 (2009) 774-800.
10. L. Zhang, S. Luo, B. Zhang, Glycan analysis of therapeutic glycoproteins, MAbs, Taylor & Francis, (2016) 205-215.
11. G.Y. Wiederschain, Essentials of glycobiology, Springer Science & Business Media, (2009).
12. P. Stanley, R.D. Cummings, Structures common to different glycans. Essentials of Glycobiology [Internet]. 3rd edition, Cold Spring Harbor Laboratory Press 2017.
13. H. Lis, N. Sharon, Protein glycosylation: structural and functional aspects, Eur. J. Biochem., 218 (1993) 1-27.
14. P. Ahmad, F. Bano, Posttranslational Modifications in Algae: Role in Stress Response and Biopharmaceutical Production, Protein Modificomics, Elsevier2019, pp. 313-337.
15. M. Aebi, N-linked protein glycosylation in the ER, Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1833 (2013) 2430-2437.
16. D. Ghaderi, R.E. Taylor, V. Padler-Karavani, S. Diaz, A. Varki, Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins, Nat. Biotechnol., 28 (2010) 863.
17. F. Li, J.X. Zhou, X. Yang, T. Tressel, B. Lee, Current therapeutic antibody production and process optimization, Bio. Process. J., 5 (2007) 16.
18. D. Ghaderi, M. Zhang, N. Hurtado-Ziola, A. Varki, Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation, Biotechnol. Genet. Eng. Rev., 28 (2012) 147-176.
19. Z. Zhang, D. Sun, T. Wu, Y. Li, Y. Lee, J. Liu, F. Chen, The synergistic energy and carbon metabolism under mixotrophic cultivation reveals the coordination between photosynthesis and aerobic respiration in *Chlorella zofingiensis*, Algal research, 25 (2017) 109-116.
20. P. Feng, Z. Deng, Z. Hu, L. Fan, Lipid accumulation and growth of *Chlorella zofingiensis* in flat plate photobioreactors outdoors, Bioresour. Technol., 102 (2011) 10577-10584.
21. J. Liu, Z. Sun, H. Gerken, Z. Liu, Y. Jiang, F. Chen, *Chlorella zofingiensis* as an alternative microalgal producer of astaxanthin: biology and industrial potential, Mar. Drugs, 12 (2014) 3487-3515.
22. B. Yang, J. Liu, Y. Jiang, F. Chen, Chlorella species as hosts for genetic engineering and expression of heterologous proteins: progress, challenge and perspective, Biotechnol. J., 11 (2016) 1244-1261.
23. J. Huang, J. Liu, Y. Li, F. Chen, Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *chlorella zofingiensis* (chlorophyta) 1, J. Phycol., 44 (2008) 684-690.
24. J. Liu, Z. Sun, H. Gerken, J. Huang, Y. Jiang, F. Chen, Genetic engineering of the green alga *Chlorella zofingiensis*: a modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker, Appl. Microbiol. Biotechnol., 98 (2014) 5069-5079.
25. T. Mamedov, V. Yusibov, Green algae Chlamydomonas reinhardtii possess endogenous sialylated N-glycans, FEBS Open Bio., 1 (2011) 15-22.
26. O. Levy-Ontman, M. Fisher, Y. Shotland, Y. Weinstein, Y. Tekoah, S. Arad, Genes involved in the endoplasmic reticulum N-glycosylation pathway of the red microalga Porphyridium sp.: a bioinformatic study, Int. J. Mol. Sci., 15 (2014) 2305-2326.
27. E. Mathieu-Rivet, M. Scholz, C. Arias, F. Dardelle, S. Schulze, F. Le Mauff, G. Teo, A.K. Hochmal, A. Blanco-Rivero, C. Loutelier-Bourhis, Exploring the N-glycosylation pathway in Chlamydomonas reinhardtii unravels novel complex structures, Mol. Cell. Proteomics, 12 (2013) 3160-3183.
28. M.H. Selman, M. Hemayatkar, A.M. Deelder, M. Wuhrer, Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides, Anal. Chem., 83 (2011) 2492-2499.
29. A. Ceroni, K. Maass, H. Geyer, R. Geyer, A. Dell, S.M. Haslam, GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans, J. Proteome Res., 7 (2008) 1650-1659.
30. V. Gomord, A.C. Fitchette, L. Menu-Bouaouiche, C. Saint-Jore-Dupas, C. Plasson, D. Michaud, L. Faye, Plant-specific glycosylation patterns in the context of therapeutic protein production, Plant Biotechnol. J., 8 (2010) 564-587.
31. O. Levy-Ontman, S. Arad, D.J. Harvey, T.B. Parsons, A. Fairbanks, Y. Tekoah, Unique N-glycan moieties of the 66-kDa cell wall glycoprotein from the red microalga Porphyridium sp, J. Biol. Chem., 286 (2011) 21340-21352.
32. I.B. Wilson, F. Altmann, Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens: core α1, 3-linked fucose and xylose present in all pollens examined, Glycoconj. J., 15 (1998) 1055-1070.