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Identification of Four Different Chlorophyll Allomers of *Nostoc* Sp. by Liquid Chromatography-Mass Spectrometer (LC-MS)

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Abstract

Cyanobacteria (Blue Green Algae) differ from other types of bacteria in that they have chlorophyll a, which other photosynthetic bacteria don't have. In this study, liquid chromatography-mass spectrometer (LC-MS) has been used for identification of the four different allomers of chlorophyll (Chlorophyll α, HO-chlorophyll α, HO-lactone-chlorophyll α and MeO-lactone-chlorophyll a) from *Nostoc* sp. The differences in mass spectrometric fragmentation of Extracted ion *chromatogram* can be used as a diagnostic tool for the assignment of the configuration of four different chlorophyll allomers. This case is the first documented of identification of four different chlorophyll a allomers from Nostoc sp. in Iran.

Keywords: chlorophyll α; allomers; Nostoc; liquid chromatography-mass spectrometer;

Introduction

All plants, algae, and cyanobacteria which photosynthesize contain chlorophyll "α". Cyanobacteria contain only one form of chlorophyll, chlorophyll a, a green pigment. In addition, they contain various yellowish carotenoids, the blue pigment phycobilin, and, in some species, the red pigment phycoerythrin. The combination of phycobilin and chlorophyll produces the characteristic blue-green color from which these organisms derive their popular name. Because of the other pigments, however, many species are actually green, brown, yellow, black, or red [1]. The oxidation of the chlorophyll molecule by molecular triplet oxygen in alcoholic solutions causes the replacement of the atom of hydrogen of C-13 located in the isocyclic ring by oxygen or an oxygen-containing species [2]. This reaction named allomerization may occur by both enzymatic and chemical pathways, forming MeO-Lacton chla, OH-chlα and MeO-chla as major products [3]. The acid hydrolysis of the phytol alcohol in the chlorophyll molecule is accompanied by the loss of Mg and products pheophorbides. The excision of phytol without separation from Mg is a specific reaction catalysed by the endodogenous enzyme chlorophyllase and which results in chlorophyllides [4, 5]. In Iran, algological studies are still scarce and limited to phylogenetic of genes encoding proteins involved in bioactive compounds biosynthesis in paddy fields and fresh water regions [6., 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18], Therefore, The objectives of this study was to develop a liquid chromatography-mass spectrometer)LC-MS) method for determination of Chlorophyll a and its derivatives in *Nostoc* sp. moreover we extend the use of MS/MS^2 in the designation of allomers of chlorophyll a configuration to identification of four different allomers during the methanolic allomerisation reactions of chlorophyll α.

Experimental Methods

Strain cultivation and preparation of extract

Nostoc sp. ASN studied in this research was collected from paddy fields in Golestan province of Iran in 2010. It was grown at a photon irradiance of 15 µmol m-2 s-1 in modified Z8IX medium for 21 days (Figure 1). The cells were harvested by centrifuge for 10 min at 10,000 g. After being lyophilized, the biomass was used for identification of the four different chlorophyll allomers of chlorophyll a. The extract for the chlorophyll a analysis was prepared from 1 ml of frozen culture. The microtube containing the culture was placed in a water bath (room temperature, 23°C), allowing the culture to thaw. Subsequently, it was supplemented with 300 mg of glass beads (disruptor beads, 0.5 mm, Scientific Industries) and 2 µl of 50% (v/v) formic acid (Fluka, Sigma-Aldrich) (final concentration of 0.1% (v/v) formic acid). Proper cell disruption was achieved by placing the microtube into a homogeniser (FastPrep®-24 instruments, MP Biomedicals) for 15 s at a speed of 6.5 ms-1. The homogenised mixture was centrifuged at 10 000 G for 5 min. Twenty micro litres of the supernatant were diluted with 9 volumes of acetonitrile (E CHROMASOLV®, Sigma-Aldrich), equalling to a final volume of 200 µl. The extract was analysed subsequently. For the preparation of methanol extracts, 236 mg of wet biomass were freeze-dried (Edwards's lyophilisator). The dry cell mass was measured to be 7.3 mg. A microtube containing the latter amount of dried cells, 300 mg of glass beads (disruptor beads, 0.5 mm, Scientific Industries) and 1 ml of methanol (LC-MS grade, Fischer Scientific) was placed into a homogeniser (FastPrep®-24 instrument, MP Biomedicals) for 15 s at a speed of 6.5 $ms^{-1.}$ The mixture was centrifuged as described above. The supernatant was stored refrigerated at 4°C

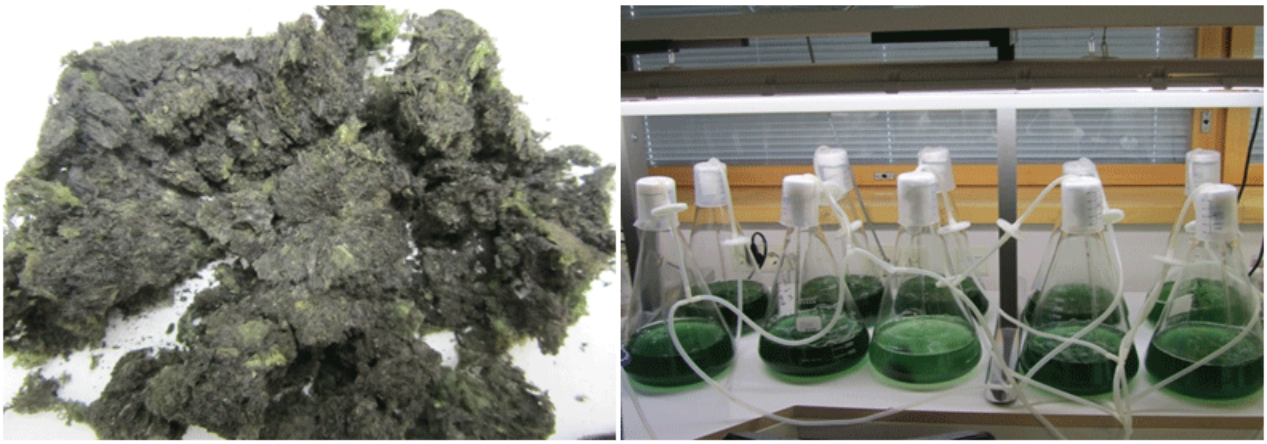

Figure 1: Cultivation of Nostoc sp. ASN in modified Z8IX medium (right fig) and freeze-dried biomass (left fig)..

until chemical analyses were performed [19].

Chemical Analysis

Nostoc sp. ASN cell extracts were analysed by liquid chromatography mass spectrometry (LC-MS) using an Agilent 1100 Series LC/MSD Trap XCT Plus System (Agilent Technologies). The sample was injected into the Luna C18 column (150X10 mm, 5 mm, Phenomenex) in batches (1 ml). The mobile phase A consisted of formic acid (0.1 percent) (Fluka, Sigma Aldrich, Steinheim, Germany) and mobile phase B consisted of Isopropyl alcohol. The injection volume of each sample was 10 µl. Setting of parameters for LC-MS has been shown as a (Table 1).

Results and Discussions

The results of analysis by LC-MS showed the allomerization of chlorophyll a by forming MeO-Lacton chl a, OH-chl a and MeO-chl an as major products. The acid hydrolysis of the phytol alcohol in the chlorophyll molecule is accompanied by the loss of Mg and produces chlorophyllides (Figure 2) (Table 2). Chromatogram in (Figure 3) showed mass spectrum of Total ion *chromatogram* (TLC) and Extracted ion *chromatogram* (EIC) of chlorophyll a molecule after separating of magnesium ion. The results of MS/MS^2 fragmentation pattern of two times protonated chlorophyll a allomers showed that in addition to magnesium, the phytol structure of chlorophyll molecule a has been isolated (Figure 4). Mass-to-charge (m/z) ratios is according to ionizing molecules and then sorting and identifying the ions (Figure 4).

Table 1: LC-MS/MS Instrument Parameter and Feature Details.

Dry Temp (°C)	350
Dry Gas (l/min)	8
Nebulizer (psi)	30
Capillary (V)	-5000
Skimmer (V)	85
Cap Exit (V)	300
Oct DC (V)	11.5
Oct 2 DC (V)	4.1
Trap Drive	144
Oct RF (Vpp)	300
Lens 1 (V)	-6.4
Lens 2 (V)	-76.4
Scan Range (m/z)	200-2200
Polarity (pos/neg)	Pos
Auto MS2	on
M/MS frag Ampl (V)	0.7

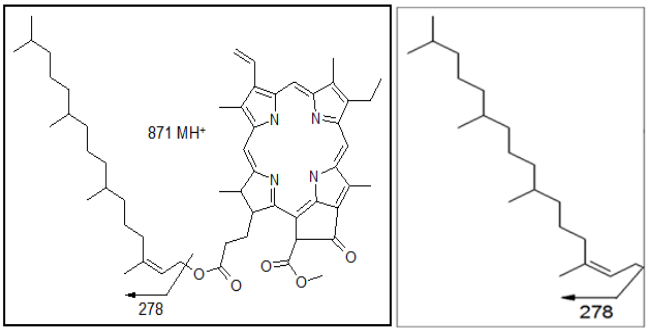

Figure 2: The structure of chlorophyll a molecule without magnesium ion (right fig) and hydrolysis of the phytol tail in the chlorophyll molecule (left fig).

Table 2: molecular weight of protonated molecular ion' (MH+) of different chlorophyll *a* allomers before and after of separation of magnesium ion.

	Molecular weight in natural condition	Molecular weight of Mg	Molecular weight without of magnesium ion
Chlorophyll a	893	22	871
HO-chlorophyll a	909	22	887
HO-lactone-chlorophyll a	925	22	903
MeO-lactone-chlorophyll a	939	22	917

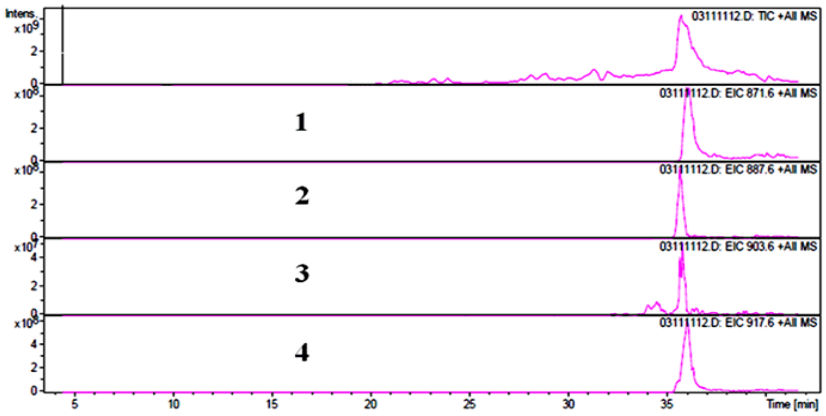

Figure 3: Total ion *chromatogram* (TLC) and Extracted ion *chromatogram* (EIC) of different chlorophyll α allomers (1, Chlorophyll α 871.6 m/z); (2, HO-chlorophyll a 887.6 *m/z*); (3, HO-lactone-chlorophyll α 903.6 6/903 *m/z*) and (4, MeO-lactone-chlorophyll α 917.6 *m/z*) of the ASN_M strain. The x-axis represents retention time (min), and the y-axis represents signal intensity. Intensity is measured in counts per second (cps).

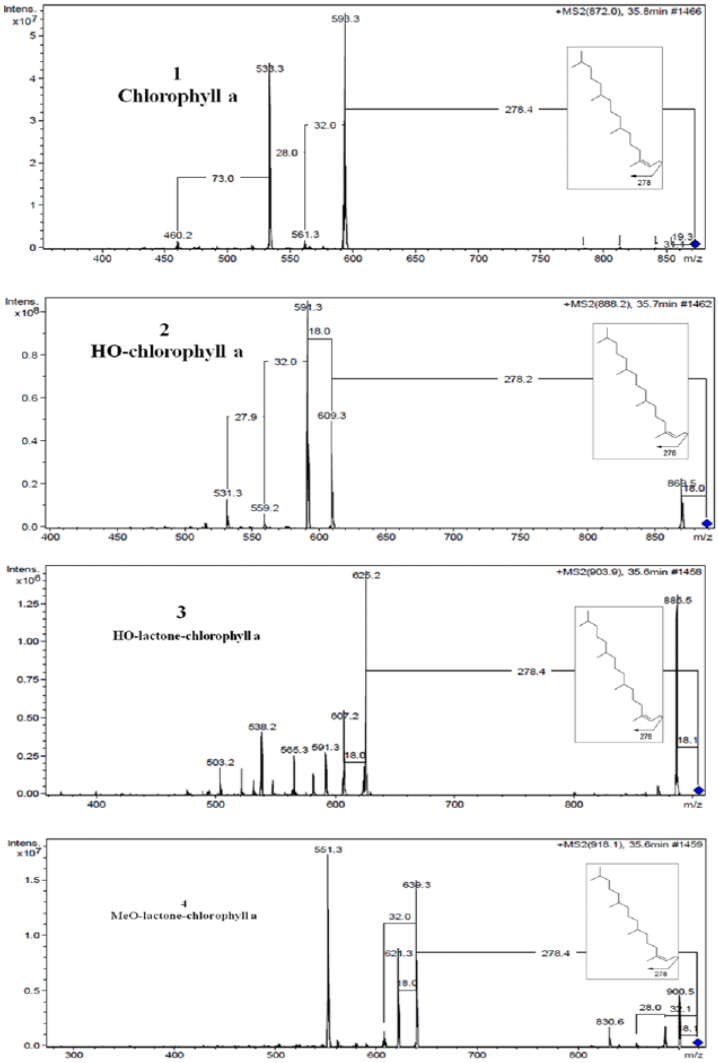

Figure 4: MS/MS^2 fragmentation pattern of protonated chlorophyll a allomers (chlorolhyll α; 872 *m/z*), (HO-chlorophyll α; 8882.2 *m/z*); (HO-lactone-chlorophyll α; 903.9 *M/Z*) and (MeO-lactone-chlorophyll α; 1/918 *m/z*) of the ASN_M strain. *m/z*= Mass-to-charge ratios, The intensity of the ion on the y-axis is given as counted ions per second (cps) and the mass-to-charge ratio (*m/z*) on the x-axis

Liquid chromatography is a fundamental separation technique in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for nonvolatile and thermally fragile molecules, liquid chromatography can safely separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins. Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (m/z) ratios. Two key components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions. Several different types of ion sources are commonly used for LC/MS. Each is suitable for different classes of compounds. Several different types of mass analyzers are also used [20].

Conclusion

Here, we employed LC-MS to separate and identify the allomers of chlorophyll a produced in the *Nostoc* strain. Moreover, we extend the use of MS/MS^2 in the designation of allomers of chlorophyll a configuration to identification four different allomers during the methanolic allomerisation reactions of chlorophyll a. This research is the first documented of isolation four different chlorophyll a allomers of Nostoc sp. by liquid chromatography-mass spectrometer)LC-MS) in Iran.

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