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Short Communication

Identification and characterization of mycosporine-like amino acid in the cyanobacterium *Scytonema* sp. HKAR-16 by HPLC, Raman spectroscopy, and ESI-MS

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Abstract

The incidence of ultraviolet radiation has increased on the Earth's surface due to depletion of the stratospheric ozone layer which has several detrimental effects on living organisms including cyanobacteria. Mycosporine-like amino acids (MAAs) are eco-friendly natural sun screening biomolecules having prominent photoprotective potentials. Our study provides the identification and characterization of one of these MAAs from the cyanobacterium *Scytonema* sp. strain HKAR-16. The identification and characterization of MAA palythine (λ_{max} = 320 nm; m/z = 245.1) from this strain was performed using ultraviolet-visible spectrophotometer, high-performance liquid chromatography (HPLC) associated with photodiode array (PDA) detector and electrospray ionization-mass spectrometry (ESI-MS) analyses. Further characterization was performed by Raman spectroscopy. To the best of our knowledge, this is the first report regarding the presence of MAA palythine in *Scytonema* sp. HKAR-16. Our results indicate that the cyanobacterium can protect itself by synthesizing MAA palythine in response to injurious UV radiation. This naturally occurring photoprotective compound may have the potential to replace synthetic photoprotectants that can be used by the humans.

Keywords: *Scytonema* sp. HKAR-16, MAA palythine, HPLC, ESI-MS, Raman spectra

1. Introduction

Cyanobacteria are an enormous source of many valuable natural photoproducts with global distribution from tropical to polar environments (Jungblut, Lovejoy, & Vincent, 2010; Ward, Castenholz, & Miller, 2012). They are regarded as widespread biomass producers in aquatic as well as in terrestrial ecosystems (Häder, Helbling, Williamson, & Worrest, 2011). Based on the fossil record, cyanobacteria are known to be the earliest groups of oxygen-evolving microalgae on the Earth during the Precambrian era (2.8 to 3.5 billion years ago). Because of their oxygen-evolving property, they give rise to an oxygen-rich environment for the development of aerobic life forms (Brocks, Logan, Buick, & Summons, 1999; Fischer, 2008). Furthermore, they are a potent source of several natural products of agricultural, medicinal and industrial value (Rastogi & Sinha, 2009).

In addition, over the past few decades, the stratospheric ozone layer has drastically depleted resulting in an increased level of solar UV radiation, especially UV-B radiation (280-315 nm) on the Earth's surface (Manney *et al.*, 2011). UV-B radiation is a highly potent component of the UV radiation that can affect the metabolic processes of all microorganisms (Häder & Sinha, 2005), by directly damaging the nucleic acids and proteins or by concomitant effects on the lipid molecules with the emergence of reactive oxygen species (Rastogi, Richa, Tyagi, & Sinha, 2010).

So far, about forty mycosporine-like amino acids (MAAs) have been reported having an absorption maxima ranging from 309-365 nm (Wada, Sakamoto, & Matsugo, 2015). MAAs are the most prominent photoprotective secondary metabolites (Garcia-Pichel, Wingard, & Castenholz, 1993), produced by many cyanobacteria and several other organisms (Mishra, Pandey, & Sinha, 2019).

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MAAs and their associated group of organic compounds are water-soluble and low molecular weight (<400 Da) molecules composed of the cyclic chromophore (hexenone or hexinimine) associated with a nitrogen substituent such as an amino acid or its imino alcohol (Ahmed *et al.* 2021).

Some of the distinguishing properties of MAAs are strong UV absorption, high molar extinction coefficients (ε) from 28,100-50,000 M^{-1} cm^{-1}, ability to withstand many abiotic stresses such as UV radiation, various temperature and pH values supporting their role as productive natural photoprotectants (Mishra, Pandey, & Sinha, 2019; Mishra *et al.*, 2019; Kumar *et al.* 2021). In addition, it can be exploited in medicinal, cosmetics, and many other promising industries (Rastogi & Sinha, 2009). There is no report so far on the presence of naturally occurring MAA palythine in the cyanobacterium *Scytonema* sp. HKAR-16. Therefore, the main objective of this study was to identify and chemically characterize the MAA palythine.

2. Materials and Methods

2.1 Materials and sample extraction

The cyanobacterium *Scytonema* sp. HKAR-16 was collected from the ancient Lolark Kund (water body), which is located in Varanasi (25°28' N, 82°96' E), Uttar Pradesh, India. The cyanobacterium was identified by *16S rRNA* gene sequencing (GenBank accession No. MW403992). The sample was centrifuged at 7,000 *g* for 10 min and the pellet was dissolved in 2 ml of 100% HPLC-grade methanol overnight at 4 ℃. Thereafter, the diluent was centrifuged at 5,000 *g* for 10 min. Resulting supernatant was subjected to spectroscopic evaluation by ultraviolet-visible/double beam spectrophotometer (U-2600, Shimadzu, Japan). The crude spectrum was taken to a computer to analyze the peaks and these peaks were examined by UV-probe software, Version-2, Japan. Subsequently, methanol was vaporized at 45 ºC and the aliquots were redissolved in water and chloroform (8:2) to obtain water-soluble MAA. It was centrifuged at 10,000 *g* for 5 min and supernatant associated with water-soluble MAA was taken. The sample was filtered through the sterilized microcentrifuge syringe-driven filter (0.2 µm, Axiva Sichem Biotech., New Delhi) for HPLC analyses.

2.2 Identification and characterization of MAA

2.2.1 HPLC-PDA analysis

MAA was analyzed with the help of HPLC equipped with PDA detector (Waters 2998, 515 HPLC pump, Nova-Pak ® C18 reverse-phase (RP) guard column of 4.6 × 150 mm diameter). A 50 µL sample was transferred to the vial and an injection set of 30 µL sample was introduced into the RP guard column of the HPLC *via* Waters 717 Plus autosampler with the help of an autoinjector. The detector was set to 330 nm wavelength and its scanning range was from 250 to 400 nm for individual peaks to be observed by PDA. The mobile phase (1 L double-distilled H_2O + 0.02% v/v acetic acid) was run isocratically at a flow rate of 1 mL min^{-1} in the HPLC apparatus. Here, MAA was identified based on its characteristic λ_{max}/detection wavelength and retention time. After elution, the fraction containing the pure MAA was stored with the help of Fraction Collector (FC III) and this purified fraction of MAA was used for further identification and characterization.

2.2.2 Electrospray ionization-mass spectrometry (ESI-MS)

HPLC purified MAA was utilized to produce protonated molecules by ESI and Amazon SL mass spectrometer (Bruker Daltonics Inc., Bremen, Germany, Model number: 294444.00083) was used to record mass spectra. A 30V cone voltage was found to instigate the generation of $(M + H)^{1+}$ with a mass range of 100 m/z. On the basis of m/z values and previously determined retention times, precursor ions were selected specifically for MS/MS. It was accomplished in manual mode accompanied by fragmentation of the precursor ions by collision-induced dissociation (CID) using helium as the collision gas at 40 V. Capillary voltage (5,500 V) and temperature (300 °C) were other MS settings. Inside an isolation width of 2u, precursor ions were selected and scans were accompanied with changeable RF signal amplitudes. 'Tuning mix' was the external calibration standard electrospray which was used to calibrate the m/z scales of the mass spectra (Agilent Technologies, Santa Rosa, USA). The data was analyzed by the software Data Analysis 4.0 (Bruker Daltonics Inc., Bremen, Germany).

2.2.3 Raman spectra measurement

Raman analysis of the sample was performed at room temperature using Renishaw in via Raman spectrometer (Wotton-under-Edge, UK) equipped with 785 nm laser (excitation at 785 nm). To avoid heating, 785 nm line of diode-pumped solid-state laser conveys the power of 3 mW/mm². From this 3 mW/mm², only 5-10% was used for exciting the Raman spectra and the incident laser beam was concentrated onto the sample with a 50x short-range objective attached to the Leica DM 2500 M microscope. All procedures such as data collection, scanning and processing were performed by a resolution computer with Wire 4.0 software. Absorption spectra were recorded by using Perkin Elmer Ultraviolet-visible Lambda 25 spectrophotometer. Further analysis of Raman spectra was done by using Origin 6.1 software.

3. Results and Discussion

The cyanobacterium *Scytonema* sp. strain HKAR-16 was used in the present study because the organism has a thick mucilaginous sheath showing resistance against harmful UV radiation (Rastogi & Sinha, 2009). In comparison to other cyanobacteria *Scytonema* sp. strain HKAR-16 synthesizes large amount of the photoprotective compound MAA palythine. The absorption spectrum of methanolic extract of the sample showed peaks at 334, 434, 485, 620 and 663 nm. Peak at 334 nm denotes the presence of MAA whereas peaks at 434 and 663 nm show the presence of Chl. *a*. Peaks at 485 nm and 620 nm imply the presence of carotenoids and phycobiliproteins respectively (Figure 1).

Identification and characterization of the photoprotective compound MAA palythine was done by HPLC. HPLC chromatogram showed the peak at a retention

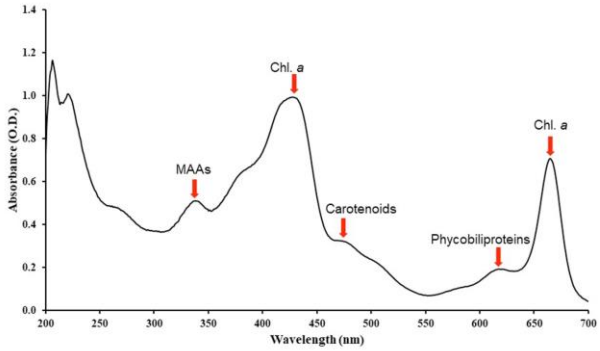

Figure 1. Absorption spectrum of methanolic extract of the cyanobacterium *Scytonema* sp. strain HKAR-16 showing the presence of MAAs, Chl. *a*, carotenoids and phycobiliproteins

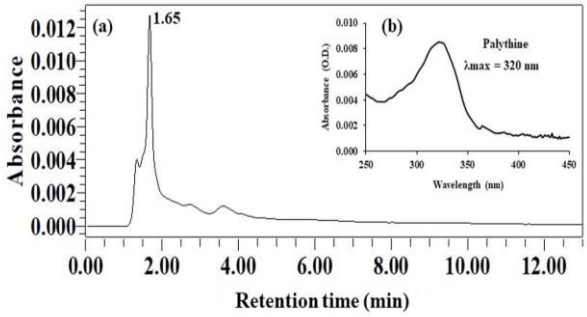

Figure 2. HPLC chromatogram of MAA showing the typical peak at a retention time (RT) of 1.65 min (a) with an absorption maxima (λ_{max}) at 320 nm (b) denoting the presence of MAA palythine in the cyanobacterium *Scytonema* sp. HKAR-16

time (RT) of 1.65 min (Figure 2a) and absorption maxima at 320 nm (Figure 2b) corresponding to the presence of MAA palythine. ESI-MS analysis of the peak revealed an m/z value of 245.1 that confirmed the presence of MAA palythine (Figure 3).

Raman spectroscopy provides structural fingerprints based on inelastic scattering of photons or light. The Raman spectrum (Figure 4) shows signatures at 1,464, 1,350, 1,181, 1,150 and 845 cm^{-1} which were assigned to MAAs (Vítek *et al.*, 2010). The band at 2,893, 1,332, 1,000, 974 and 810 cm^{-1} were also recorded in Raman spectrum showing the presence of MAAs. Raman spectrum could be used for selecting specific peak corresponding to target analyte MAA palythine. The band at 1,464 cm^{-1} shows the medium intensity of δ (CH_2) and δ (CH_3) asymmetric stretch (aromatic ring). The band at 1,000 cm^{-1} shows the strong intensity of the aromatic ring. A band at 2,893 cm^{-1} shows the strong intensity of aromatic C-H stretch.

Cyanobacteria are the prime source of natural photoprotective MAAs, which have several medicinal values such as anti-photoaging, anti-inflammatory, anticancerous, anti-immunosuppressive and are therefore believed to confer protection against harmful UV radiation (Chrapusta *et al.*, 2017). Many other marine resources provide MAAs with several other bioactivities such as antioxidants, cosmeceutical molecules, growth factors, DNA protection, and inhibition of collagenase, hyaluronidase and elastase (Chaves-Peña *et al.*, 2020; Figueroa, 2021). MAAs have the potential to block an overabundance of a biological marker of skin inflammation, consequently defending in case of psoriasis, which is a chronic condition resulting from suppression of the immune system (Lawrence, Long, & Young, 2018). MAA especially palythine acts as an *in vitro* molecular photoprotectant of human keratinocytes (Lawrence, Gacesa, Long, & Young, 2018). MAAs have been reported to be transformed and accumulated into palythine by the activity of *MysH* gene (Chen *et al.*, 2021) A detailed study on biosynthetic pathway of MAAs is required to unlock new possibilities for the production of next-generation natural sunscreens.

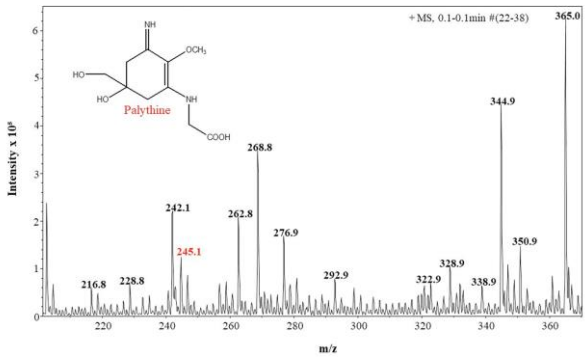

Figure 3. Fragmentation pattern of electrospray ionization-mass spectrometry (ESI-MS) of the HPLC-purified MAA palythine showing a prominent peak at m/z 245.1

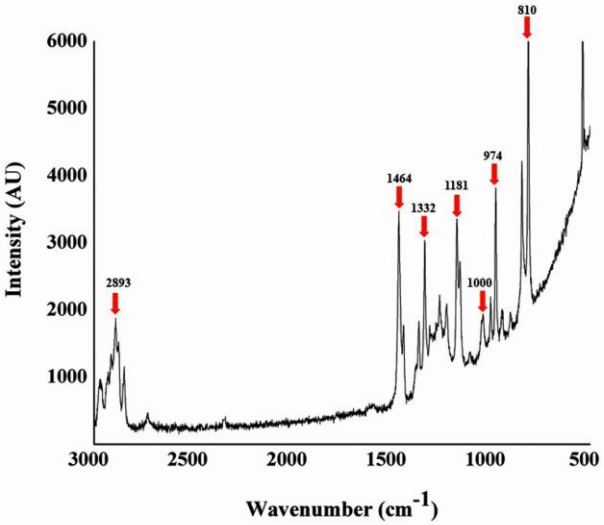

Figure 4. Raman spectrum reveals various bands showing the presence of MAA palythine

4. Conclusions

The cyanobacterium *Scytonema* sp. HKAR-16 seems to be a prime source of natural UV protective compound MAA palythine. The identification and chemical characterization of MAA palythine was done by a combination of techniques such as HPLC (RT = 1.65 min; λ_{max} = 320 nm), Raman spectrum and ESI-MS (m/z = 245.1). Peak at 334 nm as shown by UV-VIS spectrophotometer and

the bands at 2893, 1464, 1332, 1181, 1000, 974, 810 cm^{-1} in Raman spectrum suggested the presence of MAA palythine. Our study has clearly shown the presence of a photoprotective MAA palythine in an ecologically and economically important cyanobacterium *Scytonema* sp. HKAR-16. This strain could be used for the overproduction of natural photoprotective compound for the industrial production of sunscreens.

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