Journal of Photochemistry & Photobiology, B: Biology 172 (2017) 102–108

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Novel glycosylated mycosporine-like amino acid, 13-*O*-(β-galactosyl)-porphyra-334, from the edible cyanobacterium *Nostoc sphaericum*-protective activity on human keratinocytes from UV light

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ARTICLE INFO

Keywords:
Mycosporine-like amino acid
Nostoc sphaericum
Cyanobacterium

ABSTRACT

A UV-absorbing compound was purified and identified as a novel glycosylated mycosporine-like amino acid (MAA), 13-*O*-β-galactosyl-porphyra-334 (β-Gal-P334) from the edible cyanobacterium *Nostoc sphaericum*, known as "ge xian mi" in China and "cushuro" in Peru. Occurrence of the hexosylated derivative of shinorine (hexosyl-shinorine) was also supported by LC-MS/MS analysis. β-Gal-P334 accounted for about 86.5% of total MAA in *N. sphaericum*, followed by hexosyl-shinorine (13.2%) and porphyra-334 (0.2%). β-Gal-P334 had an absorption maximum at 334 nm and molecular absorption coefficient was 46,700 at 334 nm. Protection activity of β-Gal-P334 from UVB and UVA + 8-methoxypsoralen induced cell damage on human keratinocytes (HaCaT) was assayed in comparison with other MAA (porphyra-334, shinorine, palythine and mycosporine-glycine). The UVB protection activity was highest in mycosporine-glycine, followed by palythine, β-Gal-P334, porphyra-334 and shinorine in order. β-Gal-P334 had highest protection activity from UVA + 8-methoxypsoralen induced cell damage followed by porphyra-334, shinorine, mycosporine-glycine and palythine. We also found an antioxidant (radical-scavenging) activity of β-Gal-P334 by colorimetric and ESR methods. From these findings, β-Gal-P334 was suggested to play important roles in stress tolerant mechanisms such as UV and oxidative stress in *N. sphaericum* as a major MAA. We also consider that the newly identified MAA, β-Gal-P334 has a potential for use as an ingredient of cosmetics and toiletries.

1. Introduction

Nostoc is a very common cyanobacteria both in terrestrial and aquatic environments. *Nostoc* is known to form visible macrocolonies. In the natural environment, *Nostoc* periodically repeats between dessicated and rehydrated status [11,28]. During the dessicated phase *Nostoc* experiences various stresses such as desiccation, heat, UV, and oxidation [25,29]. Therefore *Nostoc* has developed different mechanisms to tolerate these stresses.

As one of the mechanisms to tolerate UV, *Nostoc* produces mycosporine-like amino acids (MAAs, [7]). MAAs are a series of compounds having an aminocyclohexenone or aminocyclohexenimine structure. MAAs have an absorption maximum within the UVB (280 to 320 nm) and UVA (320 to 400 nm) region [6,22]. MAAs can absorb harmful UV in sunlight and dissipate the absorbed UV energy as heat [8,9]. MAAs are widely distributed to aquatic organisms such as fishes, shellfishes, crustaceans, corals and algae [6,22]. MAAs are thought to act as a natural sunscreen in these organisms. Several unique MAAs, glycosylated MAAs, have been identified from *Nostoc commune* [5,16,18,19,25,26].

Nostoc sphaericum is an aquatic *Nostoc* cyanobacterium and also makes macrocolonies [3]. *N. sphaericum* is consumed as food in China (ge xian mi) and in Peru (cushuro). In this study, we investigated MAAs in *N. sphaericum* and found a new glycosylated MAA, 13-*O*-β-galactosyl-

Abbreviations: MAA, Mycosporine-like amino acid

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http://dx.doi.org/10.1016/j.jphotobiol.2017.05.019
Received 26 January 2017; Received in revised form 13 May 2017; Accepted 14 May 2017
Available online 15 May 2017
1011-1344/

porphyra-334 as a major MAA. We evaluated its antioxidant activity and UV protection activity on human keratinocyte HaCaT cells in comparison with other known MAAs (porphyra-334, shinorine, palythine, mycosporine-glycine).

2. Materials and Methods

2.1. Nostoc sphaericum

Nostoc sphaericum was purchased at a local market in Huanuco, Peru in 2009. The cells were cultured in nitrogen-free *Nostoc* medium containing 40 mg K_2HPO_4, 70 mg $MgSO_4{\cdot}7H_2O$, 60 mg $Na_2SiO_3{\cdot}7H_2O$, 36 mg $CaCl_2{\cdot}2H_2O$, 4.8 mg $FeSO_4{\cdot}7H_2O$, 1 mg EDTA·2Na and 1 ml trace-metal mix A5 solution in 1 l of distilled water. The trace-metal mix A5 solution contained 2.86 g H_3BO_3, 1.8 g $MnCl_2{\cdot}4H_2O$, 222 mg $ZnSO_4{\cdot}7H_2O$, 390 mg $Na_2MoO_4{\cdot}2H_2O$, 80 mg $CuSO_4{\cdot}5H_2O$ and 50 mg $Co(NO_3)_2{\cdot}6H_2O$ in 1 l of distilled water. The cells were grown in flasks at a temperature of 22 ± 1 °C and continuous illumination of 30 μmol/m^2/s with air-bubbling. The cells were harvested by centrifugation and immediately dehydrated by freeze-drying. The nucleotide sequences of the 16S rRNA, *nrtP* (nitrate/nitrite transporter) and *petH* (ferredoxin-$NADP^+$ oxidoreductase, FNR) genes of *N. sphaericum* were determined by PCR direct sequencing as described previously [1] and have been deposited in the GenBank/DDBJ/EMBL databases with the following accession numbers: AB775902 (16S rRNA), AB775903 (*nrtP*) and AB775904 (*petH*).

2.2. Purification of UV Absorbing Substance

N. sphaericum powder (10 g) was extracted with 200 ml of 80% aqueous ethanol in the dark at 4 °C over night using a magnetic stirrer. The extract was vacuum-filtered through filter paper (Advantec Toyo No. 5C). Extraction was repeated twice. The filtrate was evaporated using a rotary evaporator, and the residual aqueous suspension was delipidated with n-butanol. The water layer was evaporated to dryness and redissolved in water and applied onto a 2.5 × 15 cm activated charcoal column (Wako Pure Chemical Industry, Osaka, Japan) preconditioned with water. The column was eluted with aqueous ethanol solution under a gradient condition (0–80% ethanol). Every 10 ml of eluate was fractionated. Fractions having maximum absorption between 300 and 350 nm were gathered and evaporated to dryness and redissolved in 10 ml water. Subsequently a UV absorbing substance was purified by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: column, 250 × 4.6 mm i.d. Develosil C8 (3 μm, Nomura Chemical, Aichi, Japan); solvent, 0.05% acetic acid, 5% methanol in water (0.7 ml/min); detection, 310 nm. The HPLC purification step was repeated two times.

2.3. Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

MS and MS/MS spectra of the UV absorbing substance purified from *N. sphaericum* were obtained using a Bruker micrOTOF-QII spectrometer equipped with an ESI interface and Dionex UltiMate 3000 LC. Liquid chromatography conditions were as follows: column, 250 × 3 mm i.d. Capcell Pak C8DD (3 μm, Shiseido, Tokyo, Japan); solvent, 4.75% acetonitrile in water containing 50 mM formic acid; flow rate, 0.4 ml/min. Mass spectrometric analysis was carried out at the following conditions: ionization, electrospray ionization; nebulizer, 1.6 bar; dry heater, 200 °C; dry gas, 10 l/min; ionization voltage, 4500 V; ionization mode, positive mode; scan range, *m/z* 50–3000.

2.4. Nuclear Magnetic Resonance (NMR) Analysis

NMR analysis was carried out using a Bruker AVANCE-III (^{1}H: 800 MHz) spectrometer with a cryogenic probe (5 mm CPTCI ^{1}H-^{13}C/^{15}N/D Z-GRD). β-Gal-P334 was dissolved in 0.6 ml of D_2O (99.8%D, Wako, Osaka, Japan). Acetone-d_6 was used as an internal standard (^{1}H: 2.22 ppm, ^{13}C: 30.9 ppm). Various NMR spectra for ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC were determined at 298 K and calibrated by standard pulse sequences using 90° pulse length. The NMR data was processed by Topspin 3.0 software.

2.5. UV Absorption Spectrum Measurement

Ultraviolet absorption spectrum of the UV absorption substance was measured using a V-560 spectrophotometer (JASCO, Tokyo, Japan) between 200 and 400 nm.

2.6. HPLC Analysis of Mycosporine-like Amino Acids

Dried sample (0.1 g) was extracted three times with 5 ml each of 80% aqueous methanol. Extracts were made up to 25 ml with distilled water and passed through a SepPak C18 cartridge (Waters, MA) to remove the lipids. The eluates were diluted five times with water and applied onto HPLC. HPLC conditions were as follows: detection, 310 nm; column, 250 mm × 3 mm i.d. Capcell Pak C8DD (3 μm, Shiseido, Tokyo, Japan); solvent, 0.1% acetic acid, 5% methanol in distilled water (0.4 ml/min). Peak identification was done by comparing retention time with authentic standards (palythine, porphyra-334, shinorine, mycosporine-glycine) purified as previously reported [20]. Shinorine and glycosylated shinorine was not separated in this HPLC condition, thus glycosylated shinorine and shinorine were quantified as shinorine. To separate glycosylated shinorine and shinorine, another system was used as follows; column, 250 mm × 2 mm i.d. Develosil C30 UG3 (Nomura Chemical); solvent 50 mM formic acid, 2.85% methanol in water (0.2 ml/min).

2.7. UV Protective Activity

Human keratinocyte cell line, HaCaT was obtained from the German Cancer Research Center. The cells were cultured in Dulbecco's Modified Eagle Medium (High glucose, Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine plasma (Life Technologies Japan, Tokyo, Japan). HaCaT cells were inoculated into 96 well microtiter plate (Sumitomo Bakelite Inc., Tokyo, Japan) at 12,500 cells/100 μl/well. Twenty-four hours later, medium was changed to Dulbecco's phosphate buffered saline (PBS, Ca, Mg free). For measurement of UVB protection activity, the cells were irradiated with UVB (50 mJ/cm^2) with a bank of two UVB lamps (GL15E, Sankyo Denki, Kanagawa, Japan). In case of UVA protection activity measurement, the cells were irradiated with UVA (1 J/cm^2) with a bank of two black light-blue lamps (FL15BLB, Toshiba, Tokyo, Japan) in the presence of 100 ng/ml of 8-methoxypsoralen (8-MOPS). 8-MOPS was added as sensitizer because cytotoxicity of UVA was far less than UVB [17]. Samples were dissolved in PBS and applied at 0, 10, 100, and 1000 μM, respectively. Palythine, porphyra-334, shinorine, mycosporine-glycine were also applied to this assay for comparison. After irradiation, PBS was removed and the cells were washed once with PBS, and the medium changed. The number of cells was measured using Cell Counting Kit-8 (Wako Pure Chemical Company, Osaka, Japan) after 48 h culture. UV protective effect was expressed as EC_{50} (μM) value. EC_{50} was calculated from the survival curve of the UV irradiated cells assuming the number of cells of non-irradiated cells as 100%. Cells were used for UV protection assay within 15 passages. All determinations were carried out in duplicate at different concentrations.

2.8. Measurement of Antioxidant Capacity

Radical scavenging activity of 13-β-Gal-P334 was measured with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate in a colorimetric assay [16,21]. Decolorization of the ABTS