Mycosporine-like amino acids in the marine red alga
Gracilaria cornea — effects of UV and heat

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Abstract

Ultraviolet (UV)-absorbing mycosporine-like amino acids (MAAs) were separated from a marine red alga Gracilaria cornea using HPLC. The isolated MAAs were identified as porphyra-334 and/or shinorine by comparing them with various standards. No in vivo induction of MAAs was detected in G. cornea even when the organism was grown for 4–5 days either in the presence of UV-A and UV-B only or in combination with photosynthetically active radiation. In vitro absorption properties of MAAs were unaffected when irradiated with UV-B or subjected to heat treatment (75 ± 2°C) for up to 6 h. In comparison to MAAs, other pigments such as chlorophyll a (436 and 665 nm), carotenoids (475 nm) and phycocyanin (618 nm) were severely affected by UV-B irradiation. The results indicate a highly stable nature of MAAs against the environmental stress factors like UV-B and heat. The SDS-PAGE protein profile of G. cornea showed a gradual decrease in the intensity of protein bands at 20 kDa (α and β subunits of phycocyanin) but at the same time a gradual increase in the intensity of protein bands at 26 kDa (phycoerythrin), showing the phenomenon of chromatic adaptation (changes in pigmentation in photosynthetic organisms in response to light quality), when the organism was grown in the presence of UV plus PAR. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Continued depletion of the stratospheric ozone layer, mainly due to anthropogenically released atmospheric pollutants such as chlorofluorocarbons (CFCs) is responsible for the increase in solar ultraviolet-B (UV-B; 280–315 nm) radiation reaching the Earth’s surface (Blumthaler and Ambach, 1990; Crutzen, 1992; Kerr and McElroy, 1993; Lubin and Jensen, 1995). In addition to the Antarctic ozone hole where UV radiation has been detected down to a depth of 70 m (Smith et al., 1992), ozone depletion has also been reported in the north polar region (Hoffman and Deshler, 1991). Biologically effective doses of UV-B radiation penetrate deep into the water column (Smith and Baker, 1979) and may thus affect the aquatic...
ecosystems (Häder et al., 1995). The penetration of UV-B strongly depends on the optical properties of the water column. The depth of water required to remove 90% of the solar radiation at 310 nm varies from about 20 m in the clearest oceanic waters to a few centimeters in brown humic lakes and rivers (Kirk, 1994). All plant, animal and microbial groups appear to be susceptible to UV-B, but to a highly variable extent. UV-B is a small (<1% of total energy) but highly active component of the solar spectrum which has the potential to cause wide ranging effects, including destruction of proteins, DNA and other biologically relevant molecules, chronic depression of key physiological processes, acute physiological stress, and consequently the productivity of ecosystems (Karentz et al., 1991a; Vincent and Roy, 1993; Bothwell et al., 1994; Williamson, 1995; Sinha and Häder, 1996).

Certain organisms have developed mechanisms counteracting the damaging effects of UV-B. Besides repair of UV-induced damage of DNA by photoreactivation and excision repair (Britt, 1995; Kim and Sancar, 1995) and accumulation of carotenoids and detoxifying enzymes or radical quenchers and antioxidants that provide protection by scavenging harmful radicals or oxygen species (Mittler and Tel-Or, 1991; Middleton and Teramura, 1993), an important mechanism to prevent UV-B-induced photodamage is the synthesis of UV-absorbing compounds.

Phenylpropanoids, mainly flavonoid derivatives, located in the epidermis have been reported to protect higher plants by absorbing harmful UV radiation (Tevini et al., 1991; Kootstra, 1994). Mycosporine-like amino acids (MAAs) and the cyanobacterial sheath pigment, scytonemin, are thought to accomplish a similar function in lower organisms (Garcia-Pichel and Castenholz, 1991; Karentz et al., 1991b; Garcia-Pichel et al., 1993; Ehling-Schulz et al., 1997; Xiong et al., 1997). MAAs have been identified in a number of taxonomically diverse organisms such as fungi (Favre-Bonvin et al., 1976), marine heterotrophic bacteria (Arai et al., 1992), cyanobacteria (Garcia-Pichel et al., 1993; Karsten and Garcia-Pichel, 1996), eukaryotic algae (Carreto et al., 1998; Karentz et al., 1991a; Karsten et al., 1998), marine invertebrates (Karentz et al., 1991b; Shick et al., 1992), fish (Dunlap et al., 1989) and a wide variety of other marine organisms (Karentz et al., 1991a; Dunlap and Yamamoto, 1995; McClintock and Karentz, 1997; Carefoot et al., 1998; Dunlap and Shick, 1998).

Since macroalgae are sessile, they are restricted to their growth site and simultaneously exposed to elevated levels of PAR and UV radiation in their natural habitat. They can not avoid radiation stress by migration to less affected areas like some microalgae. We tested the hypothesis that macroalgal communities might have developed mechanisms to counteract the damaging effects of radiation stress. Further the hypothesis that UV-B radiation at doses comparable to those in natural radiation has a significant impact on pigmentation and protein of this organism was tested.

2. Materials and methods

2.1. The organism

The test organism *Gracilaria cornea* J. Agardh (Rhodophyta) was brought from Gran Canaria, Spain, where it has been routinely cultivated for the last 6–7 years. In nature, it normally grows free or attached to small rocks and coral fragments at low tide levels. The algae is light red: rust colored, up to 30 cm tall and very rubbery. It does not have leaves and tends to divide by fragmentation. The organism was grown in artificial sea-water (33 ppt; Instant Ocean, Sarrebourg, France and Mentor, Ohio, USA) supplemented with nitrate (9.8 μM l⁻¹) and phosphate (3.1 μM l⁻¹) and illuminated with fluorescent light (12 ± 2 W m⁻²) at a temperature of 20 ± 2°C for a 12 h photoperiod.
2.2. Radiation source

The organisms were transferred to a transparent container (20 × 20 × 8.5 cm) filled up to 3.5 cm with artificial sea water and placed on a rotary shaker to warrant uniform exposure. The algae were irradiated simultaneously under artificial radiation of ultraviolet-B (UV-B; 280–315 nm), ultraviolet-A (UV-A; 315–400 nm) and fluorescent light (PAR; 400–700 nm), in the following referred to as UV + PAR (Fig. 1). UV-C irradiation was eliminated with 295 nm cut-off filters (Ultraphan, Digefra, Munich, Germany). UV-B irradiation was provided by a Philips Ultraviolet-B TL 40 W/12 (Holland) tube with its main output at 312 nm. The irradiation was adjusted to 1.0 W m⁻². UV-A irradiation was provided by a UV-A-340 tube (Q-Panel, Cleveland, Ohio, USA) with its main output at 340 nm. The irradiation was fixed at 1.0 W m⁻². The source of visible light were OSRAM L 36 W/32 Lumilux de luxe warm white and Radium NL 36 W/26 Universal white (Germany) tubes, the irradiance of which was fixed at 12 W m⁻². When required, either a 395 nm (Ultraphan, UV Opak, Diefra, Munich, Germany) or a 320 nm (Montagefolie No. 10155099, Folex, Dreieich, Munich, Germany) UV filter was used to produce only the PAR and UV-A + PAR waveband, respectively. Alternatively, experiments were carried out with a transilluminator (Bachofer, Reutlingen, Germany) producing exclusively UV-B and UV-A (no PAR), with its main output at 312 nm (Fig. 1); the irradiance of this light source was fixed at 2.0 W m⁻² (referred to as UV). The irradiances of the light sources were measured with a double monochromator spectroradiometer (OL 754, Optronic Laboratories, Orlando, FL, USA).

2.3. Extraction and partial purification of mycosporine-like amino acids (MAAs)

Aliquots of 0.5 g (fresh weight) of G. cornea were homogenized and extracted in 5 ml of 20% (v/v) aqueous methanol (HPLC grade) by incubating at 45°C for 2.5 h. After centrifugation at
5000 g; the supernatant was lyophilized and redissolved in 2 ml of 100% methanol, vortexed for 2–3 min and centrifuged at 10 000 × g for 10 min. Thereafter the supernatant was evaporated to dryness at 45°C and the extract redissolved in 2 ml of 0.2% acetic acid. The samples were filtered through 0.2 μm pore-sized mikro-spin filters in order to partially purify the MAAs. These partially purified MAAs were separated by HPLC.

2.4. Extraction of crude pigments

Fresh G. cornea (0.5 g) was homogenized with a mortar and pestle in 5 ml of 100% methanol and kept overnight in a refrigerator at 4°C. The resulting suspension was centrifuged, and the supernatant was used for the in vitro UV irradiation experiments.

2.5. High performance liquid chromatography (HPLC)

HPLC analysis of partially purified MAAs was performed with a HPLC (Merck Hitachi; Interface D-7000, UV-Detector L-7400, Pump L-7100, Darmstadt, Germany) equipped with a LiChrospher RP 18 column and guard (5 μm packing; 250 × 4 mm I.D.). The sample was injected with a Hamilton syringe into the HPLC column through a Rheodyne injection valve equipped with a sample loop. The wavelength for detection was 330 nm at a flow rate of 1.0 ml min⁻¹ and a mobile phase of 0.2% acetic acid. Identification of MAAs was done by comparing the absorption spectra and retention times of several standards such as Devalaearea ramentacea, Porphyra saldanhae, Bostrychia radicans and a supralitoral lichen.

2.6. UV and heat treatment

Purified MAAs or crude pigment extracts of G. cornea were transferred into a quartz cuvette (an optical path length of 10 mm, 2 mm thickness) and irradiated under UV. Absorption and fluorescence spectra of the samples were recorded at regular time intervals. For heat treatment, MAAs were transferred in a 2 ml Eppendorf tube, incubated in a water bath at 75 ± 2°C, and absorption spectra were recorded at defined time intervals.

2.7. Absorption and fluorescence spectroscopy

Absorption spectra of samples were measured at regular intervals in a single beam spectrophotometer (DU 70, Beckman, Palo Alto, USA). The raw spectra were transferred to a microcomputer and treated mathematically and statistically using the software provided by the manufacturer. Fluorescence emission spectra were recorded simultaneously with a spectrofluorometer (RF-5000, Shimadzu, Kyoto, Japan) at room temperature.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of G. cornea, irradiated with UV + PAR for up to four days was carried out in a vertical system (2001, Pharmacia) with gels of 155 × 130 mm, 1.5 mm thick, using the method described by Laemmli (1970), with a gradient (5–20%) T in the resolving gel. The electrophoresis was run initially at 300 V and 30 mA for 1 h. The power was increased to 500 V and 60 mA as soon as the samples had run into the resolving gel. Gels were stained with Coomassie brilliant blue R 250 and dried in a gel dryer (Bio-Rad). Samples were run along with standard SDS molecular weight (SDS-7; approx. mol. wt. ranging from 14.2 to 66 kDa) markers (Sigma Technical Bulletin, 1996). The protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

2.9. Statistics

Results are expressed as the mean values of three replicates where appropriate and the statistical significance of the means was tested with two-way ANOVA with a significance level of P < 0.05.

3. Results

Mycosporine-like amino acids extracted from G. cornea were purified by HPLC and identified according to their retention times, absorption spectra and cochromatography with standards.
Fig. 2. High performance liquid chromatographic separation of the mycosporine-like amino acids (MAAs) from *G. cornea*. LiChrospher RP 18 column and guard; mobile phase 0.2% acetic acid; flow rate 1.0 ml min$^{-1}$; detection by absorbance at 330 nm. 1 and 2, mycosporines with a retention time of 2.6 and 4.9, respectively, spectra of which are presented in Fig. 3.

Two MAAs with retention times of 2.6 and 4.9 min (Fig. 2) and absorption peaks at 332 and 334 nm (Fig. 3), respectively, were identified as porphyra-334 and/or shinorine. There was no in vivo
induction retardation of the MAAs in G. cornea even after 4–5 days of irradiation with any of the light sources either alone or in combination. The effect of heat was assayed by incubating MAAs in a water bath at 75 ± 2°C. Absorption spectra were taken at regular intervals of 1 h. Absorption properties of the MAAs were not significantly different (P < 0.05) from that of the control even after 6 h of heat treatment (Fig. 4).

Absorption spectra of crude extracts of G. cornea showed 6 main peaks at 265 (unknown compound), 334 (MAAs), 436 (chlorophyll a) 475 (carotenoids), 618 (phycocyanin) and 665 nm (chlorophyll a) (Fig. 5). There was a significant decrease (P < 0.05) in the absorbance at all peaks except at 334 nm after only 1 h of UV irradiation and a further bleaching with increasing UV irradiation time. After 6 h of irradiation all peaks, except at 334 nm had more or less disappeared. There was no effect on the 334 nm (MAAs) peak even after 6 h of UV irradiation. A significant decrease (P < 0.01) in the absorbance of MAAs could be recorded only after 24 h of continuous UV irradiation, but the complete elimination of the MAAs peak did not occur even after 72 h of UV irradiation (Fig. 5).

Fluorescence excitation at the 436 nm peak of the crude extract of G. cornea resulted in an emission at 670 nm, which first showed an increase (P < 0.05) in fluorescence and subsequently a slight shift towards shorter wavelengths after 0.5 h of UV irradiation (Fig. 6). A gradual increase (P < 0.05) in fluorescence with a further shift towards shorter wavelengths after 3 h of UV irradiation. The fluorescence returned to its initial level but with an obvious shift (P < 0.01) towards shorter wavelengths after 4 h of UV irradiation. Thereafter, there was a gradual and steady decline (P < 0.01) in fluorescence followed by a further shift towards shorter wavelengths with increasing duration of UV irradiation (Fig. 6). Fluorescence excitation at the 618 nm peak of the crude extract of G. cornea resulted in an emission at around 668 nm, which also first showed an increase (P < 0.05) in fluorescence and subsequently a slight shift towards shorter wavelengths after 0.5 h of UV irradiation (Fig. 7). Thereafter, there was a gradual and steady decline in fluorescence (P < 0.01) followed by a further

Fig. 4. Absorption spectra of the purified MAAs of G. cornea with increasing time to heat (75 ± 2°C) treatment.
Fig. 5. Absorption spectra of the methanolic extract of *G. cornea* with increasing UV exposure time. Note the predominant mycosporine-like amino acids peak at 334 nm.

Fig. 6. Fluorescence emission spectra of the *G. cornea* following increasing exposure time to UV when excited at 436 nm. Shift towards shorter wavelengths with increasing UV irradiation time (Fig. 7).

Changes in the total protein profile of *G. cornea* were investigated by SDS-PAGE analysis follow...
Fig. 7. Fluorescence emission spectra of the *G. cornea* following increasing exposure time to UV when excited at 618 nm.

Fig. 8. Vertical SDS-PAGE (gradient 5–20% T) protein profile of *G. cornea* following UV plus PAR irradiation for 4 days. Lanes 1 and 7: marker proteins (66.0, albumin bovine; 45.0, albumin egg; 36.0, glyceraldehyde-3 phosphate; 29.0, carbonic anhydrase; 24.0, trypsinogen; 20.1, trypsin inhibitor; 14.2, \( \alpha \)-lactalbumin), lane 2: unirradiated control; lane 3: 1 day; lane 4: 2 days; lane 5: 3 days and lane 6: 4 days of UV plus PAR irradiation. Equal amount of proteins were loaded in each well.

4. Discussion

The data obtained in the present study support the hypothesis stated in the introduction and showed the presence of UV-absorbing compounds, MAAs, in the test organism *G. cornea*, which are highly persistent to UV and heat stress and thus may play a vital role as a first line of defense against these environmental stress factors in their natural habitats. Many organisms isolated from marine, freshwater or terrestrial habitats contain MAAs. A few of them have been identified while most still have to be characterized chemically. MAAs such as asterina-330, porphyra-334, shinorine and mycosporine-gly are
common in diverse type of organisms (Karentz et al., 1991b). It is evident from the present investigation that the organism possesses fairly high amounts of UV-absorbing MAAs even without UV induction. While in the present study we did not attempt to chemically characterize the MAAs isolated from *G. cornea*, its spectral and other characteristics are fully comparable to those of porphyra-334 and/or shinorine. The occurrence of high concentrations of MAAs in marine organisms exposed to high levels of solar radiation has been proposed to provide protection as a UV-absorbing sunscreen (Dunlap and Yamamoto, 1995; Karsten et al., 1998), but there is no conclusive evidence for the exclusive role of MAAs as sunscreen. It is possible that they play more than one role in the cellular metabolism of all or some organisms (Castenholz, 1997).

Studies with cyanobacteria have shown that MAAs prevent 3 out of 10 photons from hitting cytoplasmic targets. Cells with high concentrations of MAAs are ≈ 25% more resistant to UV radiation centered at 320 nm than those with no or low concentrations (Garcia-Pichel et al., 1993). Our results indicate that MAAs in *G. cornea* are highly stable against UV and heat stress. But they failed to provide complete protection to the photosynthetic pigments, chlorophyll and the accessory light harvesting pigments such as phycocyanin, from UV induced photobleaching when the crude pigment extract containing high concentrations of MAAs were subjected to UV irradiation. It is pertinent to mention that the bleaching of chlorophyll and carotenoids is caused by photooxidation processes initiated by UV and visible light absorption of endogenous chromophores. The persistence of MAAs to oxidative processes as observed in the present study may be due to the fact that only oxo-carbonyl MAAs have antioxidant properties whereas the imino-MAAs (porphyra-334 and shinorine) present in *G. cornea* are oxidatively inert. The MAAs in *Nostoc commune* have been shown to be extracellular and linked to oligosaccharides in the sheath (Böhm et al., 1995). These glycosylated MAAs represent perhaps the only known example of MAAs that are actively excreted and accumulated extracellularly and therefore act as a true screen (Ehling-Schulz et al., 1997). There may be physiological limitations to the accumulation of osmotically active compounds such as MAAs within the cell, and it seems probable that the maximal specific content of MAAs in the cell is regulated by osmotic mechanisms which is reflected by the fact that field populations of halotolerant cyanobacteria contain unusually high concentration of MAAs (Oren, 1997).

The results on changes in the absorption and fluorescence properties of chlorophyll and phycocyanin, following UV irradiation, are in accordance with earlier reports (Gerber and Häder, 1995; Sinha et al., 1997). The initial increase in the fluorescence of chlorophyll following UV irradiation might be due to an accumulation of excitation energy in the antenna pigments caused by a decrease of PS II activity. The shift in the fluorescence emission of chlorophyll seems to be due to a decrease in the fluorescence of chlorophyll molecules which emit at longer wavelengths and an increase in the fluorescence of the substances such as the antenna pigments or pigments from the core complex which emit at shorter wavelengths (Gerber and Häder, 1995). Similarly, UV-induced changes in the fluorescence properties of phycocyanin followed by a shift towards shorter wavelengths are indicative of an impaired energy transfer from the phycobiliproteins to photosystems. The results indicate that pigmented proteins are one of the main targets of UV (Sinha et al., 1997). The SDS-PAGE protein profile of *G. cornea* after UV + PAR irradiation shows a loss in the 20 kDa (phycocyanin) and simultaneously an increase in the 26 kDa (phycoerythrin) proteins. This could be attributed to a phenomenon known as chromat adaptation (changes in pigmentation in photosynthetic organisms in response to light quality) by which an organism tries to cope with changes in its light environment (Tandeau de Marsac, 1977). The change in character of the light harvesting complex in response to the different wavelengths of light allows the cells to use the incident light efficiently (Grossman et al., 1994). The accessory light harvesting pigments phycocyanin and phycoerythrin may operate as a second line of defense against photodamage and help the organism sur-
vive in highly irradiated environments. Phycobilisomes are designed to funnel radiant energy specifically to PS II at wavelengths where Chl a molecules do not absorb, hence optimizing the energy capture and the colonization of environments with different light regimes (Tandeau de Marsac, 1977).

We conclude that the studied macroalga *G. cornea* has certain properties such as the presence of UV-absorbing compound, MAAs, and the capacity to vary its phycobiliprotein ratio that may enable it to survive excessive irradiances in the natural habitats. Irrespective of the question whether the UV-B protective properties of MAAs and phycobiliproteins are a novel function or whether they are synthesized or accumulated due to UV-B irradiation, the presence of these compounds in an organism may provide protection to the internal organelles and components from the full impact of incident UV-B radiation.

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**References**


