Biochemical and spectroscopic analysis of the effect of UV on the pigmentation of the red algae *Gracilaria dentata*, *Hypnea musciformis* and *Centroceras clavulatum*

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Abstract

Red algae are multicellular organisms that belong to the family Rhodophyceae. Majority of them are found at a depth of 40 m, where only short-wavelength visible light penetrates in any significant intensity and can be absorbed by red algae. The objective of this study was to determine the sensitivity of *Gracilaria dentata*, *Hypnea musciformis* and *Centroceras clavulatum* to components of solar spectrum and their survival. Specifically, effects of UV-A, UV-B and PAR on pigmentation of *Gracilaria dentata*, *Hypnea musciformis* and *Centroceras clavulatum* were investigated under laboratory conditions. Thalli of the algae were exposed either to unfiltered solar radiation or solar radiation filtered through WG 295, WG 320, and GG 400 cut-off filters. Sucrose gradient ultracentrifugation revealed that all the organisms had allophycocyanin as accessory pigment in addition to phycoerythrin and phycocyanin. The phycoerythrin occurred in monomers, trimers, and hexamers. Results from SDS-PAGE analyses of the protein profile of the organisms revealed a loss of high molecular weight proteins and that of low molecular weight proteins (α and β monomers), indicating a breakdown of the phycobilisomal complex and impaired energy transfer from accessory pigments to the reaction centres of photosystems. Although the photosynthetic pigments of the organisms were drastically degraded, *Hypnea musciformis* appeared to be more resistant compared to *Gracilaria dentata* and *Centroceras clavulatum*. The SDS-PAGE analyses clearly indicated that organisms exposed to unfiltered solar radiation and PAR+UV-A+UV-B were destroyed as shown by the polypeptide bands intensities. *Hypnea musciformis*, the least bleached, could be used as a reference resistant organism for future studies. The adverse effects of the various components of UV radiation on photosynthetic pigmentation and composition of phycobiliproteins of the red algae indicate the potential deleterious effects of UV-radiation on marine organisms.

Keywords: Solar radiation, Red algae, Proteins, Photosynthetic pigments, SDS-PAGE

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Introduction

Red algae are mainly marine and inhabit coastal waters as intertidal and sub-tidal organisms. They occur as sessile organisms except in a few areas. Red algae as a source can be used in the preparation of biodiesel, bioethanol, biobutanol and hydrogen gases, (Raja et al., 2013), and could be used as good antioxidant properties, which play a major role to fight against various diseases like cancer, chronic inflammation, atherosclerosis and cardiovascular disorder and ageing processes (Pooja, 2014). The most powerful water-soluble antioxidants found in red algae are polyphenols and phycobiliproteins (Plaza et al., 2008). Agars are also extracted from red seaweeds such as Gracilaria, which are used in the food industry and in laboratory media culture (Raja et al., 2013).

The red algae that occur in the sub-tidal zones are found at depths as far as 40 m; and are found in any area as the deepest dwelling seaweeds. Thus, the thalli of deep dwelling red algae are entirely submerged at considerable distance from the surface of the sea. Only short wavelengths of light penetrate to such depths in any significant intensity. These wavelengths can be absorbed by red algae.

Solar ultraviolet-B radiation (UV-B; 280–320 nm) reaching the Earth’s surface has been increasing because of the depletion of the stratospheric ozone layer (den Outer et al., 2005). Increasing levels of the atmospheric CO₂ and UV-B radiation, because of industrial activities, may cause unprecedented changes in marine ecosystems (Beardall et al., 2009). Solar UV radiation damages DNA (Rastogi et al., 2011) and proteins (Bouchard et al., 2005), reduces P uptake (Hessen et al., 2012) and can alter morphology (Schmidt et al., 2010) of photosynthetic organisms.

Phycobilisomes are supra-molecular pigment aggregates, which serve as the primary light-harvesting antennae in red and blue-green algae. The three main classes of phycobiliproteins are phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC). Exposure of Nostoc species to UV results in dramatic increases in PE fluorescence emission, indicating accumulation of PE in the phycobilisome rods (Wang et al., 2008). UV radiation (UVR) does not affect the content of Chl a in Gracilaria lemaneiformis (Xu and Gao, 2010), whereas in the intertidal red macro-alga, Porphyra umbilicalis, Chl a was decreased by 65-67% while carotenoids were decreased by 75-82% with UV exposure (Aguilera et al., 1999). This shows that different red algae respond differently to UVR. Carotenoids act as general radical-trapping antioxidants (Burton and Ingold, 1984). However, they can react with ascorbic acid and thereby neutralize singlet state oxygen (Jialal et al., 1991), and confer direct protection to photosystem II reaction centres against photooxidation (Tefler et al., 1991). They also quench the triplet state of chlorophyll a, another major source of unwanted energy for the intracellular production of singlet-state oxygen (Moore et al., 1982). Certain carotenoids participate in the xanthophyll cycle, a set of reactions that play a role in preventing photoinhibition by dissipating excess light energy (Demmig et al., 1987).

The effects of UV-B on photosystem II activity and protein synthesis may weaken the ability of cells to cope with additional environmental stress. For example, the repair of PAR-induced photoinhibition requires substantial increases in protein synthesis in order to rebuild the damaged photosynthetic reaction centres (Vincent, 1990). The xanthophyll cycle is also inhibited by UV-B radiation, probably via the impairment of the de-epoxidase enzyme and of plastoquinone reduction by photosystem II. Thus, the exposure of cells to UV-B may reduce their capacity for protection as well as recovery from photoinhibition by bright light in the PAR waveband (Pfündel et al., 1992). Although environmental thresholds and determinants of abundance of microalgae such as light and temperature are important, we lack a nuanced understanding of how changes to any of those influences, either alone or in combination, might affect them in the future. Therefore, the aim of this study was to examine the effects of the various components of solar radiation on pigmentation and phycobiliprotein composition of three red algae, Gracilaria dentata, Hypnea musciformis and Centroceras clavulatum. This should enhance our understanding of the possible effects of UVR on marine planktonic organisms in our sub-region.

Material and Methods

Collection and culture of organisms

The organisms used were Gracilaria dentata (Guiry, 2014; Agardh, 1852), Hypnea musciformis [Wulfen] (Lamouroux, 1813) and Centroceras clavulatum [C. Agardh] (Montagne, 1846) from Biriwa, which is about 10 km to the East of Cape Coast, in the Central Region of Ghana with geographical coordinates 5° 9’ 27” North, 1° 8’ 36” West. These organisms were collected on three different sampling periods in the...
year (February, May and September) from their natural habitat in the intertidal zone during low tides. The samples were placed in polythene bags containing seawater and washed thoroughly with seawater to get rid of all extraneous materials. Seawater was brought to the laboratory and autoclaved to reduce bacterial or microbial activities, and some inoculated with the samples. The organisms were identified using available literature on the classification of red algae (John and Asare, 1975; Lawson and John, 1982).

**Exposure of organisms to solar radiation**
A mass of 1 g of the washed/cleaned samples were weighed using an electronic balance (Sartorius) and place in eight Petri dishes wrapped with carbon paper to prevent the effect of ambient light on the pigmentation and phycobilins before exposure. The Petri dishes were labelled; and the organisms spread out in each Petri dish before exposing them to sunlight. The Petri dishes were then placed on ice to prevent overheating of the organisms. The samples were exposed to solar radiation between 10:00 and 14:00 hours at the rooftop of the Faculty of Science building, University of Cape Coast. Small amounts of seawater were sprinkled to keep them moist at 15-minute intervals. The organisms were exposed to unfiltered solar radiation and photosynthetically active radiation (PAR) using GG400, WG320, and WG 295 filters. The GG 400 filter allows only radiation with wavelengths greater than 400 nm to pass through, thus all UV radiation was removed. The WG 320 filter allows only radiation with wavelength greater than 320 nm to pass through thereby effectively removing all UV-B. Similarly, the WG 295 filter allows only radiation with wavelength greater than 295 nm to pass through, hence, effectively removes UV-C.

**Measurement of solar radiation**
The amount of solar radiation reaching the organisms during the experimental period was determined with a radiometer RM-12. The fluence rates for the components of solar radiation were UV-A (315-400 nm), UV-B (280-315 nm) and visible radiation (400-700 nm). The intensity of the solar irradiance was measured in watts per square meter using Gossen Mavolux digital luxmeter at 30-minute intervals on each sampling date for four hours.

**Sample extraction and sucrose gradient centrifugation**
A buffer solution of 0.5 M KH₂PO₄ and 0.5 M K₂HPO₄, pH of 7, was used to extract the crude pigments. Fresh samples of the organisms were washed thoroughly with seawater. The wet biomass (1.0 g) was ground using a mortar and pestle to break the cells and 10 mL of buffer added. Triton X-100 (20 µL) and PMSF (20 µL) were added to the mixture using a micropipette, to solubilize the membranes. The resulting mixture was left to stand for 20 minutes, and then centrifuged at 6000 rpm for 10 minutes to remove the cellular debris. The supernatant was siphoned using a Pasteur micropipette and filled, carefully free of bubbles, into a test-tube. The supernatant of the crude extracts was subjected to step sucrose gradient centrifugation to separate the phycobiliproteins (phycobilins) using concentrations of 10%, 25% and 40% sucrose in phosphate buffer (pH 7.5). A volume (1 mL) of 40% sucrose in phosphate buffer was transferred into centrifuge tubes using a micropipette, followed by 1 mL of 25% and then 1 mL of 10% sucrose in the phosphate buffer. A volume (1 mL) of the crude extracts of each sample was then introduced on top of the step density sucrose gradient. The centrifuge tubes were placed in a swinging bucket RPS 56T-146 rotor and then centrifuged for 2 hr at 40,000 rpm at 5 °C in a Hitachi 80P-7 automatic preparative ultracentrifuge. The resulting fractions were retrieved from the tubes using a 1-mL syringe and subjected to absorption spectra and electrophoretic analyses.

**Determination of absorption spectra**
The absorption spectra for the control of isolated protein fractions were determined between a range of 400 to 700 nm at interval of 5 nm to determine the peaks of the major photosynthetic pigments present in each fraction. The absorption values for fraction II of each sample were determined from 550 to 630 nm at the same intervals.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**
SDS-polyacrylamide gel electrophoresis was carried according to the method described by (Laemmli, 1970). A vertical system, using a resolving gel of 15.5 x 12.5 cm and 12.5%T and 5%T stacking gel was used to separate proteins.

**Statistical analysis**
A mass of 1.0 g of each organism was weighed and placed in a Petri dish at three sampling dates with each Petri dish containing four pieces of 1.0 g of the...
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organism. The Petri dishes were then randomly covered with the 3 filters, leaving one uncovered to receive unfiltered solar radiation. Unirradiated sample served as control, whereas the components of solar radiation served as treatments. The data obtained was analyzed using analysis of variance (ANOVA) and Duncan’s multiple range tests was used to separate the means (Ott, 1993; Duncan, 1955).

**Results**

**Sucrose gradient centrifugation**
The various fractions obtained from crude extracts of the samples using sucrose gradient centrifugation contained chlorophyll $a$, carotenoids, and phycobiliproteins, which comprised phycoerythrin, phycocyanin and allophycocyanin. The crude extracts of the samples were separated into three coloured bands referred to as fractions I, II III, (from top to bottom) on the 10 - 40 % sucrose gradient. For *Gracilaria dentata*, the absorption spectra of fractions I, II and III are shown in Figure 1, with the maximum peaks of wavelength indicating the photosynthetic pigments. The absorption spectrum of fraction I had a maximum at 410 and 665 nm indicating chlorophyll $a$ and 495 nm corresponding to carotenoids. Fraction II had the maxima at 565 and 615 nm indicating phycoerythrin and phycocyanin, as well as at 495 nm corresponding to carotenoids. In contrast, fraction III had absorption maxima at 495 nm, 565 nm, 615 nm and 650 nm corresponding to carotenoids, phycoerythrin, phycocyanin and allophycocyanin, respectively.

For *Centroceras clavulatum*, it showed three distinct coloured fractions with various maximum peaks (Figure 1). The absorption spectrum of Fraction I had a maximum at 410 nm and 665 nm corresponding to chlorophyll $a$ and at 500 nm indicating carotenoids, whereas Fraction II had 570 nm and 615 nm indicating phycoerythrin and phycocyanin and 500 nm corresponding to carotenoids. Fraction III also had an absorption maximum peak at 570 nm, 615 nm and 650 nm that corresponded to phycoerythrin, phycocyanin and allophycocyanin respectively, and again at 500 nm indicating carotenoids.

![Figure-1. Absorption spectra of sucrose gradient fractions of crude extracts of *Centroceras clavulatum* (A) and *Gracilaria dentata* (B). Values are means of 4 replicates.](image-url)

Generally, there were reductions in the absorption spectra for fraction II in all the samples for the unfiltered solar radiation, PAR+UV-A+UV-B, PAR+UV-A and PAR only, with prolonged exposure to solar radiation (Figure 2). Meanwhile, the reduction was greatest under unfiltered solar radiation followed by the PAR+UV-A+UV-B; PAR+UV-A and least under PAR only. After 4 hr of solar irradiation, the peak absorbance could not be traced for all the samples exposed to the various treatments. For PAR only and PAR+UV-A+UV-B, the peak absorbance could not be traced after 3 hr of exposure to solar irradiation and for PAR+UV-A, the peak absorbance could not be traced after 2 hr. However, for unfiltered solar irradiation the peak absorbance disappeared just after 1 hr of exposure to solar irradiation (Figure 2).
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Figure 2. Absorption spectra of phycoerythrin and phycocyanin of sucrose gradient fraction II of Gracilaria dentata after different times of exposure to PAR+UVA; PAR only; PAR+UVA+UVB; and Unfiltered solar irradiation.

SDS-PAGE Analysis
The gel electrophoresis of Fraction III of Hypnea musciformis revealed 13 protein bands of molecular weights 11.8, 14.3, 17.9, 20.8, 26.1, 29.3, 35.4, 41.1, 46.0, 47.8, 49.7, 53.6, 67.2, 75.2, 81.1, 87.5, 94.4, 105.7, and 114.0 kDa (Figure 3). The protein bands, however, disappeared with increasing exposure time. In samples exposed to unfiltered solar radiation, the protein bands disappeared just after 2 hr of exposure. Protein bands of lower molecular weights exposed to PAR+UV-A+UV-B disappeared after 4 hr of exposure, and there was loss of the 11.8, 14.3, 17.9, 20.8, 26.1, and 29.3 kDa protein bands in all the radiation types just after 1 hr of exposure.

Figure 3. SDS PAGE (12.5% T) of Fraction III of Hypnea obtained by sucrose gradient separation of photosynthetic pigments showing (a) Control (b) PAR + UVA + UVB, 4hrs (c) PAR + UVA + UVB, 2hrs (d) PAR + UVA, 1hr (e) UF, 4hrs (f) UF, 2hrs (g) PAR + UVA, 3hrs (h) PAR, 2hrs (i) PAR, 4hrs (j) PAR, 1hr (k) PAR + UVA, 2hrs.

Crude extracts of the control (unirradiated) sample of Centroceras clavulatum showed phycobiliprotein bands of 28.2, 32.8, 42.7, 57.8, 75.2, 94.4, 113.9 and 122.9 kDa. The 28.2 and 32.8 kDa bands disappeared completely in all the treated samples after 2 hr of exposure. In addition to the gradual loss of the phycobiliprotein bands, loss of heavier molecular weight bands (50 kDa and above) corresponding to linker proteins also occurred with UV irradiation (Figure 4).

Figure 4. SDS PAGE (12.5% T) of crude extract of Centroceras exposed to various treatments (a) UF, 2hrs (b) UF, 4hrs (c) PAR + UVA + UVB, 2hrs (d) control (e) PAR + UVA + UVB, 4hrs (f) PAR + UVA, 1hr (g) PAR + UVA, 2hrs (h) PAR + UVA, 4hrs (i) PAR, 1hr (j) PAR, 3hrs
Discussion

For the fractions obtained from the sucrose gradient centrifugation of the pigment extracts, the absorption spectra indicated that, apart from phycoerythrin and phycocyanin, allophycocyanin was also present in all the organisms investigated. These findings corroborate with report by (MacColl and Guard-Friar, 1987). Studies have shown that phycobilisomes contain a considerable and still growing number of minor polypeptides or linker proteins that either carry a chromophore or are uncoloured. The linker proteins function mainly as structural proteins which are involved in regulating the phycobilisomes quaternary structure, as well as in optimizing the energy transfer by modifying the light-absorption properties of the phycobiliproteins (Glauser et al., 1989). Phycobilisomes are the photosynthetic antennae of cyanobacteria and red algae and are highly structured complexes and often form ordered arrays of the cytoplasmic thylakoid surface (Gantt, 1986). They also absorb light strongly over a wide spectral range covering most of what is described as the green hole of chlorophyll $a$ absorption, and transfer the excitation efficiently to the reaction centre located within the photosynthetic membrane (Holzwarth, 1987). The linker polypeptides also introduce a vectorial specificity into the hexameric biliprotein complexes that determine location and function within the phycobilisome. These linker polypeptides modulate the spectroscopic properties of hexameric biliproteins by interactions with the $\beta$-84-chromophores located near the central channel occupied by these molecules (Schirmer et al., 1985).

Fraction III (phycoerythrin, phycocyanin and allophycocyanin) of Hypnea showed phycobiliprotein bands of heavier molecular weight (50 kDa and above), which agrees with reports by (Cohen-Bazire and Bryant, 1981) that some other large polypeptides with molecular weight (70 kDa -100 kDa) are possible linkers between the phycobilisome and thylakoid because they are present in phycobilisome and thylakoids. Such large polypeptides have been found in many phycobilisomes of cyanobacteria (Williams et al., 1980) and in red algae (Koller et al., 1978). According to Sinha et al. (1995), the electrostatic interactions between the basic linker polypeptides and acidic biliproteins significantly stabilize the phycobilisomes assembly and promote the unidirectional highly efficient energy flow both within as well as from the phycobilisomes to chlorophylls of the thylakoid membrane. Studies by Bogorad (1975) have shown that phycobiliprotein content and composition in cyanobacteria and red algae are indeed controlled by both light intensity and light quality. The absorption of visible solar radiation by biliproteins is very sensitive to the aggregation state of the protein and the changes in these spectra with protein concentration are frequently large (Hattori et al., 1965).

Drastic decline in absorption peaks of the phycobilin-containing fractions vividly showed drastic loss of the phycobiliproteins with prolonged exposure to UV irradiation, which appeared to be faster in phycoerythrin than in allophycocyanin. This agrees with works done with Porphyra umbilicalis (Aguilera et al., 1999), Gracilaria lemaneiformis (Xu and Gao, 2010) and Hypnea musciformis (Schmidt et al., 2012). The first step in phycobilisome disintegration is a breakdown of the supramolecular structure, and UV irradiation first causes the high molecular mass aggregates to breakdown into hexamers ($\alpha\beta$)$_{9}$, which further disintegrate into trimers ($\alpha\beta$)$_{3}$ and eventually to monomers ($\alpha\beta$) (Glazer, 1982). In addition, (Fisher and Håder, 1992a) describes the degradation kinetics of the phycobilins during UV irradiation as disintegration from the outside toward the core; and the disassembly of the phycobilisomes may from damage to the linker proteins. Exposure to UV radiation induces a general decrease in protein concentration in algal systems (Fisher and Håder, 1992b). Obviously, the strong visible component of sunlight also plays a decisive additional role in the destruction, as observed in Anabaena variabilis (Nultsch and Agel, 1986).

It was observed in samples of Hypnea musciformis exposed to PAR+UV-A, that there was gradual decrease of phycoerythrin, phycocyanin and allophycocyanin (Fraction III), with increasing exposure time, and a corresponding increase in phycoerythrin and phycocyanin (Fraction II). This indicated heavier proteins were being degraded to lighter ones. Sinha et al. (1995) have found a close correlation in the appearance (intensity) of the protein bands in the sucrose gradient and the measured absorption spectra confirming the degradation.

Analysis of the fractions of phycobiliproteins and crude extracts of photosynthetic pigments by SDS-PAGE indicated that the protein bands declined in intensity with prolonged exposure to UV irradiation. Almost every protein band showed a general and massive decrease of some components.
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The rapid loss of 17.9 kDa and 20.8 kDa (Figure 3) indicated the loss of α and β-subunits after prolonged exposure to solar irradiation. The basic building block of the biliproteins is a monomer composed of two different polypeptides, α and β, with molecular weights between 17 and 22 kDa, and composed of about 160-180 amino acid residues (Zuber, 1978). Redlinger and Grannt (1982) have indicated that the molecular weights of the linker polypeptides are usually between 25 and 120 kDa. The SDS-PAGE analysis showed that there were decreases in polypeptide bands of molecular weights of 28.2, 75.2, 94.4 and 122.9 kDa.

The absorption spectroscopy and SDS-PAGE showed that photobleaching in Hypnea was gradual and took a very long time before the pigments are bleached out. The sucrose gradient ultracentrifugation revealed that all the organisms had phycoerythrin, phycocyanin and allophycocyanin as the accessory pigments of photosynthesis. In addition, the phycobiliproteins were separated into several bands identified as phycobilin aggregates (hexamers and trimers). With increasing UV-B irradiation the heavier phycobilin aggregates were broken into smaller components (monomers). Also, photobleaching of these accessory pigments occurred and resulted in a serious decline in photosynthetic activity and in primary productivity as a whole and this agrees with the work done using the red algae Grateloupia lanceolata (Huovinen et al., 2006).

Bacteria are one of the major sources of contamination and probably inhibit growth of red algae by secreting toxic factors and interfering with algal metabolisms hence the need to autoclave the sea water to prevent potential limitation of this study. Another possible limitation was transportation of red algae to the research laboratory which sometimes takes a longer time before getting a public vehicle and this affects the reading of absorbance. To address this limitation the departmental vehicle was used to collect the samples so as not to delay.

Conclusion

Benthic microalgae (BMA) are responsible for a significant proportion of estuarine and coastal primary production, especially on intertidal flats where higher plants or macroalgae are absent. Using biochemical and spectroscopic analyses, this study has revealed that Centroceras clavulatum was bleached most by UV, followed by Gracilaria dentata and Hypnea musciformis was bleached the least. SDS-PAGE analyses also indicated that the samples exposed to unfiltered solar radiation and PAR+UV-A +UV-B were damaged the most since the intensity of the polypeptide bands was lowest in these samples with the loss of many protein bands with time. Since Hypnea musciformis exhibited resistance to solar radiation, especially UV radiation, it could be used as a standard check for UV resistance while the disappearance of other two red algae could serve as early warning signs of UV radiation in marine waters. Increased incidence of solar radiation, as a result of depletion of the ozone layer, could have serious consequences for marine algae. The depletion of red algae by UV radiation could result in the loss of some natural sources antioxidants, agar, and biofuels. This could affect health delivery, confectionery, and laboratory research work.

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Contribution of Authors

Danquah A: Conceived, designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript
Galyuon IKA: Supervised research and approved final draft
Phares CA and Otwe EP: Helped in write up and final draft preparation