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HALOCARBON PRODUCTION AND *IN VIVO* BROMINATING ACTIVITY OF *EUCHEUMA DENTICULATUM*

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Key Word Index—*Eucheuma denticulatum*; Rhodophyta; peroxidase; phenol red; volatile halocarbons; bromoform; oxidative stress.

Abstract—*In vivo* brominating activity by *Eucheuma denticulatum* was compared with its production of volatile halocarbons. The ability of the alga to brominate phenol red showed good correlation ($p < 0.05$, $r = 1.0$) to the production of bromoform ($CHBr_3$) estimated by gas chromatography. The production of tetrabromophenol or bromoform was raised by exposing the algae to mechanical stress (in the form of cutting), increased light or addition of the herbicide DCMU, prior to the measurements. We suggest that the *in vivo* brominating activity of *E. denticulatum* can be used as a simple method to study the physiology of this alga. We also suggest that the activity of the brominating peroxidases is dependent on the intracellular concentration of H_2O_2, possibly produced by the Mehler reaction. Production of singlet oxygen and triplet chlorophyll by photo-oxidative damage may increase halocarbon release. The amount produced and the species of volatile halocarbons released are dependent on the conditions the algae are subjected to prior and during the experiment. Addition of NaCN or NaN_3 inhibited the brominating activity with 95 or 75% of control values.

INTRODUCTION

In a world with limited resources and food supplies, great interest has been shown over the past 30 years in cultivating seaweed. Algal products are used as emulsifiers in many foods and algal species are cultivated for their content of antioxidants, e.g. β-carotene. The marine red alga *Eucheuma denticulatum* contains iota carrageenan which is used as an emulsifier and thickener in many diet food products. Production of volatile halocarbons has been reported from several species of marine algae. Bromoform, methylbromide and methylchloride have been reported from green, brown and red macroalgae [1–4]. CH_2I_2 found in some algal species has been reported to reduce herbivore feeding, and volatile halocarbons may also be involved in epiphytic control [5]. Volatile brominated and iodinated substances have also been shown to be produced by planktonic micro-organisms (K. Abrahamsson, unpublished results).

During photoinhibition, the flow of electrons is inhibited [6] which renders a dissipation of excess exciton energy through fluorescence and singlet oxygen production [7]. Both triplet chlorophyll (Chl3) and singlet oxygen ($O_2$1) are reactive and will, if not quenched by membrane bound carotenoids [8], react with most cell components within diffusion distance [9, 10]. Hydrogen peroxide produced in the thylakoids is mainly scavenged by ascorbate peroxidase in the chloroplast [11], but may also be removed from the cytosol by catalase present in peroxisomes [12]. Algae growing in water can also eliminate H_2O_2 by diffusion [13].

Higher plants and algae have several different forms of peroxidases in the chloroplasts and cell walls. Peroxidases usually perform hydroxylations even though other reactions may occur. Bromoperoxidases can use H_2O_2, bromide and ketones (and other compounds) as substrates forming brominated halocarbons [14]. Addition of external H_2O_2 increased halocarbon production in *Meristiella gelidium* [15].

In this report a spectrophotometric method was used to measure the ability of *E. denticulatum* to brominate phenol red under various physiological conditions to elucidate the physiological factors which trigger halocarbon production. Halocarbon production was also measured by gas chromatography.

RESULTS AND DISCUSSION

Production of tetrabromophenol from phenol red by *E. denticulatum* is not a linear process. Maximum brominating activity (3.4 nmol kg^{-1} s^{-1}) was measured

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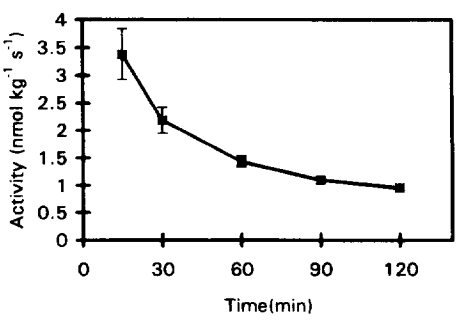

Fig. 1. Brominating activity of *E. denticulatum* was measured as nmol tetrabromophenol kg^{-1} s^{-1} ($n = 3$). 0.3 g fr. wt of algae in 12.5 ml seawater was incubated during 120 min in 600 μmol photon m^{-2} s^{-1}.

during the first 15 min and then the activity decreased to about one third after 120 min (Fig. 1).

Photosynthetic measurements of *E. denticulatum* recorded a compensation point of 52 μmol photon m^{-2} s^{-1} and a light saturation point of 660 μmol photon m^{-2} s^{-1}. An increase of the light intensity from 15 to 600 μmol photon m^{-2} s^{-1} was followed by a subsequent increase in brominating activity (Table 1). The brominating activity of control samples kept in darkness was of the same order of magnitude as the samples subjected to 15 μmol photon m^{-2} s^{-1}. Induction of photoinhibition in the algae by exposing them to high light intensities (2000 μmol photon m^{-2} s^{-1}) prior to incubation in 200 μmol photon m^{-2} s^{-1} did not give any significant change in brominating activity. However, when the algae were subjected to mechanical stress, in the form of cutting, prior to incubation in light (200 μmol photon m^{-2} s^{-1}) the brominating activity increased by 120% ($p = 0.05$) compared with controls subjected to the same light intensity.

The influence of mechanical and physical stress on halocarbon production is important to consider. Grazing animals, high temperature and light are unavoidable when farming alga in the intertidal zone in the tropics. *E. denticulatum* produces H_2O_2 in response to mechanical stress [16]. Our present finding that *E. denticulatum* increases brominating activity in response to mechanical stress (cutting) is in agreement with the results on H_2O_2 production by Collén and Pedersén [16].

Addition of the inhibitor 3-(3,4-dichlorophenyl) 1,1-dimethylurea (DCMU) to the reaction vessel increased the brominating activity by 110% (Table 2). DCMU affects photosynthesis by inhibiting electron transport [17], which increases fluoresence and promotes production of singlet oxygen. Addition of methyl viologen (Paraquat) to a final concentration of 20 μM increased the brominating activity slightly, but addition of both methyl viologen and DCMU reduced the brominating activity by 60%, which may be due to reduction of H_2O_2 levels by methyl viologen radicals [18]. Methyl viologen captures electrons from PSI and donates them to molecular oxygen, producing superoxide. Both DCMU and photo-oxidative damage usually increase both $O_2$1 and Chl3 levels. Halogenated fatty acids are present in algae [19]. It is possible that degradation of such fatty acids will lead to an increased release of volatile hydrocarbons (VHC).

Addition of catalase, which reduces external levels of H_2O_2 [12, 17], did not influence the brominating activity of *E. denticulatum*. This indicates that production of VHC takes place inside the algal cells. Addition of H_2O_2 on the other hand increased the brominating activity by 300% confirming that hydrogen peroxide is important for the regulation of the brominating activity.

Inhibitors such as cyanide and azide inhibit peroxidase activity by binding to the prosthetic group of the enzyme [20, 21]. Vanadium bromoperoxidases have been shown to be more sensitive to cyanide that azide if compared with the haem peroxidases having iron as the prosthetic group [19]. Exposing the algae to 10 μM NaCN or NaN_3 caused a drop in brominating activity by about 95 and 75% of control values respectively. These results indicate that the bromination in *E. denticulatum* is mainly caused by a vanadium bromoperoxidase.

Table 1. Brominating activity in response to increased light and mechanical stress*

Light (μmol photon m^{-2} s^{-1})	Activity (nmol kg^{-1} s^{-1})
0	0.34±0.19
15	0.34±0.10
200	0.94±0.53
600	1.70±0.49
200 + mech. stress	2.25±0.10
200 + photoinhibition	1.22±0.11

*Conversion of phenol red to tetrabromophenol by *E. denticulatum* in response to increasing light intensity and physical stress ($n = 3$).

Table 2. Brominating activity in response to different inhibitors*

	Mean and standard deviation ($n = 3$)	Significance (p)
Paraquat	135.5±12.5	>0.05
DCMU	210±54	0.05
Paraquat +DCMU	39±11	0.05
Cyanide	1.4±4.1	0.05
Azide	25.0±6	0.05
Catalase	125.0±53	>0.05
H_2O_2	435±44	0.05

*Conversion rate of phenol red to tetrabromophenol as percentage of control after 120 min with 0.3 g (fr. wt) of *E. denticulatum* in 12.5 ml seawater incubated in 600 μmol photon m^{-2} s^{-1}. Separate controls were made for each experiment.

Table 3. VHC concentrations measured with a gas chromotograph*

(ng l^{-1})	$CHCl{=}CCl_2$	$CHCl_3$	CH_3I	$CHBr_3$
Blank	2.06	1.83	0.56	2.0×10^3
Control	2.11	1.99	8.97	4.9×10^3
DCMU	5.87	6.61	10.4	187×10^3

*Production of halocarbons by *E. denticulatum*. Concentrations (ng l^{-1}) after incubation during 180 min in 400 μmol photon m^{-2} s^{-1}, with and without the addition of DCMU. Algal concentration was 0.6 g/60 ml seawater. $CHCl{=}CCl_2$ and $CHBr_3$, $n = 2$ and CH_3I and $CHCl_3$, $n = 4$.

Measurements were made with a gas chromatograph to compare the phenol red method with the actual production of halocarbons. Concentrations of $CHCl{=}CCl_2$ and $CHCl_3$ increased three-fold in response to the addition of DCMU compared with both control and blank values, which may indicate that $CHCl{=}CCl_2$ and $CHCl_3$ are only produced when *E. denticulatum* is subjected to severe stress or chemical treatment. *E. denticulatum* shows novel production of both CH_3I and $CHBr_3$, separated from blank values, in response to 400 μmol photon m^{-2} s^{-1}. Addition of DCMU increased bromoform concentration in the samples with algae from 4.9×10^3 ng l^{-1} to 187×10^3 ng l^{-1} (Table 3). Correlation analysis between production of tetrabromophenol and the released amount of $CHBr_3$ when measurements were made with and without DCMU indicates that measurement of bromoperoxidase activity is tentatively a good estimation of $CHBr_3$ production (correlation coefficient 1.0, $p < 0.05$) (Fig. 2).

In vivo production of VHC by *Eucheuma denticulatum* prior shown by Motolera *et al.* [22] and the results presented in Table 3 indicate that production is a natural process. This process is, however, accentuated by physiological stress and the amount of produced halocarbons, and the released species of VHC reflects the conditions the algae have been exposed to prior to stress induction.

Haloperoxidases readily halogenate a wide variety of aromatic compounds, such as phenols and anilines [23]. Haloperoxidases using ketones as substrate produce VHC [14]. Halogenated cell compounds such as membrane lipids and proteins are easily affected by activated oxygen species. Superoxide, $^1O_2$, H_2O_2 and OH·, all very reactive, cause breakdown of different cell components when they are released within the cell [24, 17]. This could lead to release of small halogenated compounds as well as volatile halocarbons without any increase in haloperoxidase activity.

Scavenging of H_2O_2 by ascorbate peroxidase uses ascorbate and NADPH as reductants. Under circumstances of high production of H_2O_2, *E. denticulatum* has to use compounds or enzyme systems other than ascorbate and ascorbate peroxidase to eliminate H_2O_2. We suggest that H_2O_2, produced by the Mehler reaction or pseudocyclic photophosphorylation during oxidative stress causes an increase in the brominating activity of other peroxidases. Quenching of excited chlorophyll molecules and singlet oxygen during photoinhibition through the xanthophyll cycle is also dependent on ascorbate. It is possible that during prolonged photoinhibition the pool of ascorbate is exhausted, and scavenging of H_2O_2 by ascorbate peroxidase is reduced. This in its turn could lead to increased brominating activity by other peroxidases, and increased VHC production.

EXPERIMENTAL

Phenol red measurements. Eucheuma denticulatum, (N. C. Burman) Collins et Hervey, Rhodophyta, collected at Zanzibar, Tanzania but originating from the Philippines, was cultivated in 70 μmol photon m^{-2} s^{-1} (36W Polylux 4000, Thorn) with 12:12 h light period. *Eucheuma denticulatum* was investigated for its brominating activity using a phenol red method described in ref. [25]. *Eucheuma denticulatum* (0.3 g (fr. wt) in 12.5 ml SW) was incubated in 25 μM phenol red during illumination from two light banks of 6

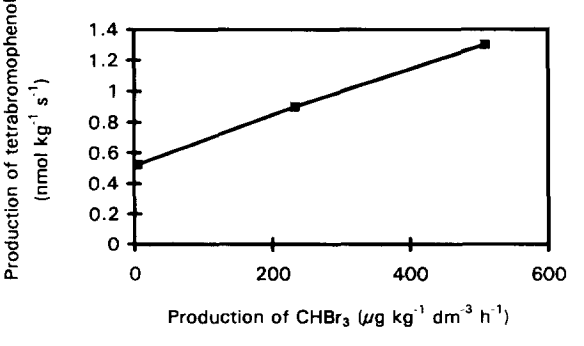

Fig. 2. Concentration of $CHBr_3$ (μg kg^{-1} dm^{-3} h^{-1}) as a function of brominating activity, measured as produced amount of tetrabromophenol (nmol kg^{-1} s^{-1}). Incubation was performed during 180 min in 400 μmol photon m^{-2} s^{-1} with 0.6 ft. wt of algae in 60 ml seawater.

fluorescent tubes each. During each experiment, samples of 0.9 ml were taken at initial time and after 120 min; pH was set to 6.5 with 0.1 ml of 1 M Bis Tris buffer, and A was measured on a Hitachi U-2000 spectrophotometer at 592 nm. The samples were stirred with small magnetic stirrers which did not touch the algal pieces. The temp. was kept at 20°. The brominating activity of the peroxidases was estimated as production of tetrabromophenol in nmol g (fr. wt) kg^{-1} s^{-1}, using an extinction coefficient of 67.4 mM^{-1} cm^{-1} at 592 nm. Samples were taken at 0, 15, 30, 60, 120, 180, 240 min (Fig. 1). Light conditions were investigated by incubation in 0, 15, 200 and 600 μmol photon m^{-2} s^{-1} for 120 min. By subjecting the alga to cutting or exposing it to high light intensity (2000 μmol photon m^{-2} s^{-1} for 20 min), the effect of mechanical stress and photoinhibition was measured (Table 2). To measure the effect of different chemicals on the brominating activity the phenol red reaction was performed with the addition of 400 μM DCMU, 20 μM methyl viologen, 100 μM catalase and 1 mM H_2O_2. DCMU was dissolved in 2 ml of 95% EtOH and diluted to final concn with sea water. Experiments were also performed where cyanide (NaCN) and azide (NaN_3) were added to a final concn of 10 μM.

The compensation point (P_c) and light saturation point (P_{sat}) were estimated by using an Illuminova Light pipette. The light pipette measures O_2 concn and light intensities in a 3 ml cuvette; 0.07 g fr. wt of alga was used in each experiment. The temp. was kept at 20° with a waterbath, and light levels were increased exponentially from 0 to 2000 μmol photon m^{-2} s^{-1} during 20 min. Measurements of light, temp., O_2 concn and O_2 evolution were made every 0.4 sec, and the figures were presented as means of 10 values. All presented data are means from 4 samples. P_c was calculated by using a linear trend line assumption calculated from 20 values around the assumed compensation point for each measurement. P_{sat} was calculated from the secondary derivative of the O_2 concn at max. O_2 evolution.

In order to relate the tetrabromophenol production from phenol red to produced amounts of halocarbons, measurements were made on a GC with EC detection (^{63}Ni), Varian 3400. The compounds were pre-concentrated with a purge and trap technique and trapped in a stainless steel tube filled with Porapak N 80/100 (Alltech Inc.). The trap was cooled to 4° and after 4 min heated to 140° for 2 min enabling the different compounds to desorb. Ultra-pure N_2 used as carrier gas was led through the trap to enable injection into the gas chromatograph. The identities of the individual compounds were verified by comparison with a method based on liquid–liquid extraction as pre-concentration technique [26].

The statistical program used as StatMost 2.01 for Windows. Correlation analysis was done with Spearman rank correlation test and the tests for related samples were made with the Mann–Witney test based on ranked sums. All tests were evaluated with a 95% confidence interval.

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