Different regulation of haloperoxidation during agar oligosaccharide-activated defence mechanisms in two related red algae, *Gracilaria* sp. and *Gracilaria chilensis*

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Abstract

The related red seaweeds *Gracilaria* sp. from the eastern Mediterranean and *Gracilaria chilensis* from Chile were similar in their enzymatic inventory for halogenation. In both species, halogenation was dependent upon H2O2 and thus driven by haloperoxidases. These could be inhibited with phosphate and reversibly inhibited with azide and were therefore apparently dependent upon vanadate. Both species generated in the first line bromoform and other brominated halocarbons. Gel electrophoresis under non-denaturating conditions demonstrated that both species expressed halogenating peroxidases. Elicitation of *Gracilaria* sp. with agar oligosaccharides resulted in marked increases in bromination, iodination, and chlorination. Production rates of volatile halocarbons and phenol red bromination both increased by a factor of eight, presumably due to increased availability for haloperoxidases of H2O2 during the oxidative burst response. Elicitation of *Gracilaria* sp. also triggered a release of bromide ions through DIDS-sensitive anion channels, which allowed for some bromination in bromide-free medium. However, this effect was relatively limited. By contrast, agar oligosaccharide oxidation in *G. chilensis* did not increase halogenation. Obviously, agar oligosaccharide oxidation does not provide sufficient amounts of hypohalous acids for such increases, because it does not deliver H2O2 at the active site of vanadium-dependent haloperoxidases. These results correlate with earlier findings that the agar oligosaccharide-elicited oxidative burst controls microorganisms while agar oligosaccharide oxidation does not.

Key words: Bromination, *Gracilaria*, halogenation, haloperoxidase, iodination, seaweed–microbe interactions.

Introduction

Volatile halogenated organic compounds (VHOCs) provide I and Br radicals which react with atmospheric ozone and influence the lifetime of other greenhouse gases. Much of the global production of volatile halocarbons is biogenic, and marine algae play a major role in this process (Gribble, 2000). For example, it has been estimated that marine micro- and macroalgae produce 70% of global bromoform (Carpenter and Liss, 2000; Quack and Wallace, 2003). The production of volatile and non-volatile halogenated organic compounds from macroalgae is thought to involve in some cases methyltransferases, which do not depend upon H2O2 (Manley, 2002), but mainly halogenating peroxidase enzymes, which catalyse the oxidation of halide ions to hypohalous acid and require H2O2. Polyhalogenated compounds may be subsequently formed via the haloform reaction, which results in sequential substitution of hydrogen atoms on a nucleophilic acceptor with halogen atoms. Halogenating peroxidases are usually relatively unspecific with regard to the nucleophilic substrate and generate not only VHOCs, but also halogenated terpenes and phenols (Butler and Carter-Franklin, 2004). Some halogenating peroxidases are true...
haloperoxidases that oxidize only halides. Many others, for example myeloperoxidase (Gaut et al., 2001) and horseradish peroxidase (Ballschmiter, 2003), also oxidize alkyl acids (Jacks, 2005) or other substrates.

Nearly all of the halogenating peroxidases from red (Yu and Whittaker, 1989; Itoh et al., 1996; Bukin et al., 1997), green (Ohshiro et al., 1999), and brown (Weyand et al., 1999; Colin et al., 2003) seaweeds that have been examined so far were non-haem-enzymes, dependent upon vanadium. However, haem-containing halogenating peroxidases have been detected in the red seaweed Cystoclonium purpureum (Pedersen, 1976) and in other red algae (Murphy et al., 2000), and a recent genomic analysis of Chondrus crispus indicates that this red seaweed expresses both a vanadium-dependent halogenating peroxidase and an enzyme similar to haem-containing myeloperoxidase (Collen et al., 2006).

The function of the VHOC’s produced by algae is still under dispute. There is evidence that they may act as a chemical defence against microorganisms (Paul et al., 2006), and they may also be a by-product of the production of other halogenated defence agents such as hypohalous acid (Brochardt et al., 2001; Gaut et al., 2001) or haloterpenes (Sakata et al., 1991). Alternatively, VHOCs may just be a by-product of the removal of toxic reactive oxygen species (ROS; O2•-, H2O2, OH•), which are formed continually during normal metabolism (Manley, 2002) and at particularly high rates when respiratory or photosynthetic electron transport are impaired due to stress.

It has been shown in the past that red seaweeds of the order Gracilariales release ROS when they are exposed to agar oligosaccharides during bacterial degradation of their agar cell wall matrix (Weinberger et al., 1999). The response seemed to be fully comparable to the ‘oxidative burst’, a typical rapid response after plant defence elicitation, which has also been reported from spermatophytes (Apostol et al., 1987) and kelps (Küpper et al., 2002). However, a comparative study of Gracilaria sp. (as Gracilaria conferta) and Gracilaria chilensis revealed that a typical oxidative burst was only triggered in the former species. After agar oligosaccharide perception, a relatively complex signalling chain-activated NADPH-oxidoreductase-catalysed production of ROS at the plasma membrane (Weinberger et al., 2005) and subsequent elimination of associated bacteria (Weinberger and Friedlander, 2000) in Gracilaria sp. By contrast, G. chilensis featured an agar oligosaccharide oxidase, which catalysed production of H2O2 in the outer cell wall (Weinberger et al., 2005). This reaction—which did not require any perception or signalling events—did not affect associated bacteria, although similar amounts of ROS were detected in the medium of both species when agar oligosaccharide had been applied. An additional production of other defence agents in Gracilaria sp., but not in G. chilensis, was apparent.

It was the aim of the present study to investigate the potential role of halogenation in agar-oligosaccharide-activated defence mechanisms in Gracilaria sp. and in G. chilensis and to examine the enzymatic inventory for halogenation in the Gracilariales, which has so far received relatively little attention, despite the worldwide distribution of this group.

Materials and methods

Plant material and cultivation procedures

Gracilaria chilensis C.J. Bird, McLachlan & E.C. Oliveira originated from Caldera, IIIrd region of Chile. The clonal isolates used were CR14 and CS7. A clonal isolate of Gracilaria sp. originated from the eastern Mediterranean (Israel). Gracilaria chilensis and Gracilaria sp. were cultivated in aerated tanks. The water temperature was maintained with heat exchangers at 25 °C in the case of Gracilaria sp. and at 12–17 °C in the case of G. chilensis. Day length was 12 h and the photon flux density was 45 μmol m−2 s−1. During light exposure, a halogen lamp provided Gracilaria sp. every hour for 30 min with additional 80 μmol m−2 s−1. The water was exchanged daily and NH4NO3 (0.5 mM) and HNa2PO4 (0.1 mM) were supplied by weekly pulse feeding. Different individual thalli of the algal material produced were used as replicates in all experiments.

Short-time incubations for elicitations and physiological assays were conducted in Petri dishes on shakers, using autoclaved sea water as medium. MBL’s medium (Bidwell and Spotte, 1985) was made up with distilled water and used in experiments which required a halide composition different from that in sea water (543 mM Cl−, 824 μM Br−, 63 μM F−, 479 mM I−; Bidwell and Spotte, 1985). MBL’s medium is similar to sea water in its content of major ions (Cl−, Na+, Mg2+, SO4 2−, Ca2+, K+, HCO3−) and was supplemented with the trace ions Fe3+, Mn2+, Zn2+, Co2+, and Cu2+ by addition of iron and metal solutions of SFC enrichment medium for red seaweeds (Correa and McLachlan, 1991). The molarity of Cl− in MBL’s medium was reduced to the same extent to which Br−, I−, or F− was added. Plant fresh weight density was generally 50 mg ml−1 and the temperature during incubation was 13 °C with G. chilensis and room temperature with Gracilaria sp.

Chemicals and oligosaccharides

All chemicals and solvents were from Sigma (St Quentin, France) and Merck (Darmstadt, Germany). Pharmacological agents were added from stock solutions prepared with dimethyl sulphoxide or with water in the case of KCN and Na2S. Agar oligosaccharide was produced, stored, and applied as described earlier (Weinberger et al., 2005).

Enzyme assays

Bromination of phenol red to bromophenol blue was used as an indicator for algal brominating activity. Phenol red was added to the medium at 25 μM and brominated phenol red was determined spectrophotometrically after an incubation period of 30 min according to Wever et al. (1991). Peroxidase activities were detected in in-gel assays after crude protein extraction and non-denaturing polyacrylamide gel electrophoresis, as described in Weinberger et al. (2005). For development, the gels were soaked with 25 mM TRIS buffer, pH 7.4, in the presence of 90 μM o-dianisidine and 0.45 mM H2O2. Acetic acid at 1.5 mM and potassium iodide, potassium bromide, or potassium chloride at 10 μM were added in order to reveal peroxidase, iodoperoxidase, bromoperoxidase, or chloroperoxidase activities, respectively.
Quantification of volatile halocarbon production

The kinetics of volatile halogenated compounds released by *Gracilaria* spp. (5 g blotted fresh weight in 100 ml of 0.7-μm-filtered sea water) in response to challenge with agar oligosaccharides were monitored by gas chromatography (Pruvost et al., 1999). Incubation sea-water media from glass bottles were withdrawn directly into glass sampling devices filled previously with ultra-pure nitrogen. This transfer was achieved with a stainless-steel tube. Afterwards the sampling device was inserted in a coupled purge and trap—gas chromatography–electron capture detection system in order to determine VHOC concentrations in incubation sea water. VHOCs were extracted from sea water and trapped at low temperature on a microtrap. Then they were injected at high temperature toward the gas chromatography and separated on a capillary column. During the separation they were detected at the column outlet by the ECD and chromatograms were collected using PC-based software. Measurement precisions varied between 1.4% and 5.7% and detection limits were <0.17 ng l⁻¹ for all the VHOCs.

**Results**

**VHOC emission in Gracilaria sp. and Gracilaria chilensis and effect of agar oligosaccharides**

Nine different volatile halogenated compounds were detected in the medium of *Gracilaria* sp. (Fig. 1, left). They were all derivatives of methane with the exception of iodoethane, which was the least concentrated compound. Brominated compounds accumulated at particularly high rates and CHBr₃, CH₂Br₂, and CH₂BrI were particularly abundant. Over all, 23.7 mol of Br⁻ and 2.9 mol of I⁻ were bound to carbon per mole of Cl⁻. Presence of 30 μM agar oligosaccharide in the medium resulted in a greater production of all the compounds. On average, iodination increased by 5 times, chlorination by 7 times, and bromination by 8 times. The overall rate of carbon halogenation increased from 475 pmol g⁻¹ min⁻¹ without to 3990 pmol g⁻¹ min⁻¹ with the presence of agar oligosaccharide.

With the exception of iodoethane, such marked increases were not observed with *G. chilensis* when 300 μM agar oligosaccharide was present (Fig. 1, right); the overall rate of carbon halogenation increased only slightly from 560 to 663 pmol g⁻¹ min⁻¹. The relative abundance of volatile halocarbons in the medium of *G. chilensis* also differed clearly from that in *Gracilaria* sp. medium. In particular, chlorinated compounds were more abundant than iodinated compounds. Overall, 62.3 mol of Br⁻ and 12.7 mol of Cl⁻ were bound to carbon per mole of I⁻. CH₂ClI was not detected at all and the three least concentrated volatile halocarbons were all iodinated compounds.

**Halogeneration capacity in Gracilaria chilensis and Gracilaria sp.**

Both *Gracilaria* species brominated phenol red when H₂O₂ was present in the medium (Fig. 2). Increasing concentrations of H₂O₂ up to 10 mM resulted in increasing brominating activity. A decreasing brominating activity was observed when H₂O₂ concentrations increased further, and this was correlated with depigmentation and reduced algal survival. The potential for phenol red bromination in sea water was 47 times as high in *Gracilaria* sp. (893±74 nmol g⁻¹ h⁻¹) as in *G. chilensis* (19±1.8 nmol g⁻¹ h⁻¹).

Phenol red bromination was also observed when *Gracilaria* sp. and *G. chilensis* were incubated in MBL’s medium, and increasing concentrations of Br⁻ resulted in increasing production of bromophenol blue. With unelicited *Gracilaria* sp., half-maximal phenol red bromination was observed at a Br⁻ concentration of 125 μM (95% confidence interval: 9–242; Fig. 3). Elicitation of an oxidative burst in *Gracilaria* sp. with agar oligosaccharide increased the maximal response by ~5 times, while the Kₘ remained in the same order of magnitude (169 μM; 95% confidence interval: 66–271). Phenol red bromination was lower in natural sea water than in MBL’s medium with a comparable Br⁻ concentration. Even at a concentration of 0.41 mM Br⁻, phenol red bromination was significantly more intense in MBL’s medium than in sea water (0.81 mM Br⁻) when agar oligosaccharide was

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*Fig. 1.* Accumulation of volatile halogenated compounds in the medium of *Gracilaria* sp. (left) and *G. chilensis* (right). The rate of enrichment during 30 min is indicated for each compound in the medium without (left box and whiskers-plot) and with (right box and whiskers-plot) addition of agar oligosaccharide to the medium. The initial concentration of agar oligosaccharide was 30 μM in the case of *Gracilaria* sp. and 300 μM in the case of *G. chilensis*. Median, minimum, and maximum of n=3 are shown.
not applied (U-test, \( P < 0.005 \), reduction in sea water by 57\%) and weakly significant after elicitation (\( P < 0.08 \), reduction in sea water by 33\%). Similarly, \( G. \text{chilensis} \) also generally exhibited a weaker phenol red brominating

activity in sea water than in MBL’s medium containing 0.41 mM Br\(^-\) (Fig. 4); without any externally supplied source of H\(_2\)O\(_2\) (\( P < 0.02 \), reduction in sea water by 68\%), in the presence of agar oligosaccharide (\( P < 0.08 \), reduction in sea water by 63\%), and in presence of H\(_2\)O\(_2\) (\( P < 0.001 \), reduction in sea water by 59\%). In MBL’s medium, similar maximal rates of phenol red bromination were observed when agar oligosaccharide was absent (56±9 nmol g\(^{-1}\) h\(^{-1}\)) or present (57±11 nmol g\(^{-1}\) h\(^{-1}\)), while direct addition of 5 mM H\(_2\)O\(_2\) increased the maximal rate to 174±47 nmol g\(^{-1}\) h\(^{-1}\).

The inhibitory effect of sea water upon phenol red bromination was examined further with \( G. \text{chilensis} \). In MBL’s medium containing 0.81 mM Br\(^-\), presence of potassium iodide reduced bromination significantly. This inhibitory effect was biphasic, with a reduction by \( \sim 50\% \) in the iodide concentration range between 47 nM (approximate concentration in natural sea water) and 1.6 \( \mu \)M (Fig. 6, left). A considerably higher concentration of potassium fluoride (670 \( \mu \)M) was necessary for a significant inhibition (Fig. 6, right). Phenol red bromination by \( G. \text{chilensis} \) was also affected by PO\(_4\)\(^3-\). A clear inhibition of bromination was observed when 100 \( \mu \)M PO\(_4\)\(^3-\) or more were present in the medium (Fig. 5).

Addition of NaN\(_3\) to the medium of both species under investigation also resulted in an inhibition of phenol red bromination. Similar concentrations were needed for half-maximal inhibition of \( G. \text{trichophylloides} \) (5.1 \( \mu \)M; 95%...
confidence interval: 3.3–9.2) and G. chilensis (3.5 μM; 95% confidence interval: 2.4–5.0). This effect was due, in both species, to substrate inhibition. Presence of 3 μM NaN₃ increased the necessary concentration of Br⁻ that was required for half-maximal bromination by approximately a factor of 10 in Gracilaria sp. (Fig. 7A) and of 2 in G. chilensis (Fig. 7B), while the maximal bromination rate in both species remained unchanged.

Phenol red bromination by Gracilaria sp. after agar oligosaccharide elicitation was partially inhibited in Br⁻-free MBL’s medium when 100 μM DIDS was present in the medium (Fig. 8). Such an inhibitory effect of DIDS was not observed after H₂O₂ was supplied externally.

**Haloperoxidase activity patterns in Gracilaria chilensis and Gracilaria sp.**

Gel electrophoresis under non-denaturing conditions revealed that several peroxidases were present in Gracilaria sp. A relatively sharp band appeared on gels that had been developed in the presence of either KI or KBr, with more intense staining in the presence of KI (Fig. 9, band 1). This band was observed in the absence of acetate, but the presence of acetate resulted in more intense staining. A cluster of isoforms that did not form sharp bands was only observed when KI and acetate were present (Fig. 9, band 2). A third band was only observed on gels that had been developed in the presence of acetate, irrespective of the presence or absence of halides (Fig 9, band 3), and a similar band was also detected on gels that had been loaded with crude protein from G. chilensis (Fig. 9, band 5). A second, relatively weak band was observed with G. chilensis when both KI and acetate were present (Fig. 9, band 4).

**Discussion**

Taken together, the above data show that the oxidative burst signalling induced in Gracilaria sp. by agar oligosaccharides controls the activation of the halocarbon pathway, whereas oxidation of agar oligosaccharides in G. chilensis does not regulate halocarbon emission. This study is the first to quantify an increase in the production of volatile halocarbons by red algae in treatments that simulate a pathogen attack. In sea water without agar oligosaccharides, both Gracilaria species generated VHOCs. With the exception of CH₂ClI—which was only detected in the medium of Gracilaria sp.—both species released the same spectrum of compounds, but in different relative amounts. Brominated compounds were predominant in both species, and CHBr₃ was the most abundant compound, which has already been reported for Gracilaria cornea (Pedersen et al., 1996), as well as for various other algal species (Carpenter and Liss, 2000; Quack and Wallace, 2003). However, Gracilaria sp. released 3 times more iodinated than chlorinated compounds and G. chilensis 13 times more chlorinated than iodinated compounds. As a consequence, the overall mixture of volatile halogenated compounds that were released by both species was not identical. Such differences may either result from different halide or hydrocarbon substrates or...
from distinct enzymes that catalyse the biosynthesis of VHOCs. With the exception of iodoethane, all compounds detected were derivatives of methane and the hydrocarbon source was therefore presumably identical in both species. The content of halides in sea water is relatively constant and can therefore also not account for the observed differences. Kelps have been reported to accumulate substantial amounts of halides (Küpper et al., 1998), which may be released during oxidative stress (Palmer et al., 2005), and differential accumulation of iodine in the Gracilariales might theoretically explain the observed differences in VHOC release. Bromination of phenol red was indeed observed in the present experiments when Gracilaria sp. was incubated in artificial sea water that was free of Br⁻, indicating that an internal source of Br⁻ may be involved. Presence of the anion channel inhibitor DIDS significantly reduced phenol red bromination under such conditions, suggesting that active translocation of Br⁻ over the membrane played a role. However, the amounts of bromophenol blue generated in the absence of external Br⁻ were relatively low. It appears therefore to be improbable that this mechanism contributes to the formation of substantial amounts of VHOC in sea water. Translocation of anions often plays a role in cellular signalling after elicitation. For example, Cl⁻-channels are involved in signal transduction in spermatophytes (Zimmermann et al., 1998). It will be interesting to investigate in the future whether translocation of Br⁻ over the membrane has a similar function in Gracilaria sp.

In conclusion, differences in the availability of halide substrates probably do not primarily account for the observed differences in VHOC release by Gracilaria sp. and G. chilensis. It follows that these differences must be due to other features of the response which affect the global brominating capacity of the species. While exposure to agar oligosaccharides had no obvious effect upon halogenation in G. chilensis, elicitation of Gracilaria sp. resulted in a general up-regulation of the release of all VHOC that were detected—on average by a factor of eight—which suggests that all of these compounds were synthesized via the same pathway or agar oligosaccharide elicitation activated different pathways to the same extent. At least a major part of the halocarbons that were released by Gracilaria sp. was generated by peroxidases. This is indicated by the fact that halogenation was limited by the availability of H₂O₂. Gel electrophoresis with crude protein extracts confirmed that both species contain halogenating peroxidases. These could be fully inhibited with PO₄³⁻, suggesting that they were vanadate-dependent (Soedjak et al., 1991). Correspondingly, inhibition by PO₄³⁻ was also observed with phenol red bromination in G. chilensis. Enzymes that contain haem as a prosthetic
group are irreversibly inhibited by NaN_3, while this compound has been reported to inhibit red algal vanadate-dependent peroxidase reversibly (Itoh et al., 1986). Both Gracilaria species under investigation were in the present study inhibited by NaN_3 due to substrate competition with KBr, which again points to vanadate-dependent peroxidases. Further, a more intense oxidation of α-dianisidine was generally observed in in-gel assays when acetate was present together with halide during the staining procedure, which also points to vanadium-dependent peroxidases rather than haem-peroxidases (Jacks, 2005).

The enzymatic equipment for haloperoxidation in Gracilaria sp. and G. chilensis is similar, but not identical, as indicated by the distinct electrophoretic patterns obtained by in gel assays (Fig. 9). Despite the overall preference of haloperoxidases for I^−, considerably higher amounts of brominated than iodinated compounds were released by both species, which is doubtless due to the fact that Br^− is ~2000 times more abundant in sea water than I^− (Bidwell and Spotte, 1985).

In conclusion, the present data indicate strongly that both Gracilaria sp. and G. chilensis generate VHOCs in the first line via pathways that involve substrate activation by vanadium-dependent haloperoxidases. Different intensities of iodination, bromination, and chlorination in both species may be explained casually by distinct substrate affinities of the enzymes that are present; Gracilaria sp. predominantly expresses one or several iodo-bromoperoxidases, which results in predominant release of brominated and iodinated compounds. Gracilaria chilensis, by contrast, expresses mainly a less selective haloperoxidase, which results in release of more chlorinated and less iodinated compounds.

Agar oligosaccharide elicitation of Gracilaria sp. clearly resulted in an activation of VHOC production, and this effect was not due to increased expression of haloperoxidases (data not shown), but is partly due to increased availability of the limiting substrate, H_2O_2. By contrast, oxidation of agar oligosaccharides in G. chilensis—which generates comparable amounts of H_2O_2—does not lead to increased VHOC emission. One possible explanation is that agar oligosaccharide oxidation takes place in the outer layers of the outer cell wall of G. chilensis, while agar oligosaccharide elicitation of Gracilaria sp. results in formation of ROS at the plasma membrane (Weinberger et al., 2005). As a consequence, major amounts of H_2O_2 that are generated by G. chilensis may immediately be diluted in the medium, while in Gracilaria sp. they need to diffuse through the whole apoplast with higher probabilities of reaching the active site of vanadium haloperoxidase before such dilution. Attempts to localize haloperoxidase in both species may reveal more extracellular isoforms in Gracilaria sp. than in G. chilensis.

The fact that increased halogenation and control of associated agar-degrading microorganisms are correlated—both effects are present after agar oligosaccharide application in Gracilaria sp. and absent in Gracilaria chilensis—supports the idea that halogenated compounds may be relevant for the control of bacteria in the Gracilariales. However, while agar oligosaccharide oxidation only results in production of H_2O_2, other forms of ROS may additionally be generated in the oxidative burst and these potentially also affect bacteria.

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References


