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Seasonal variation in bromophenol content and bromoperoxidase activity in *Ulva lactuca*

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Abstract

Seasonal variation in bromophenol content and bromoperoxidase activity in the green marine alga, *Ulva lactuca*, was studied. Bromophenols were extracted from the alga by simultaneous steam distillation-solvent extraction, followed by identification and quantification by gas chromatography–mass spectrometry. A method for the extraction of bromoperoxidases from the alga was developed, which includes homogenisation in Milli-Q water and addition of glycerol. The results obtained show that both bromophenol content and bromoperoxidase activity exhibit extreme seasonal variation, with high values in summer and low ones in winter.

Keywords: *Ulva lactuca*; Chlorophyta; Algae; Enzyme; Bromophenol; Bromoperoxidase; Bromination

1. Introduction

Several species of marine algae are known to contain simple bromophenols (Whitfield, Helidoniotis, & Drew, 1997) and a large number of other brominated compounds (Neidleman & Geigert, 1986; Gribble, 1992). They are also known to release large amounts of volatile halocarbons, such as bromoform ($CHBr_3$), chloromethane (CH_3Cl), bromomethane (CH_3Br) and iodomethane (CH_3I), into the atmosphere (Gribble, 1992). In addition, many species of marine algae contain bromoperoxidases, enzymes capable of brominating organic substrates in the presence of bromide ion and hydrogen peroxide. This gives the algae the ability to biosynthesise various brominated compounds. Two surveys of marine algae (Hewson & Hager, 1980; Moore & Okuda, 1996) have demonstrated that the majority of red and green marine algae possess bromoperoxidase activity and their characteristics have been reviewed by Butler and Walker (1993).

However, most studies on brominated compounds and bromoperoxidase activities in marine algae have been conducted on algae collected on isolated occasions. In this study, our aim was to investigate if the bromophenol content and bromoperoxidase activity are constant during the year or if a seasonal variation could be detected. The green marine alga, *Ulva lactuca*, was chosen for the study, because it is known to contain high concentrations of bromophenols (Whitfield et al., 1997). A method for the extraction of bromoperoxidases from *U. lactuca* was developed and bromoperoxidase activity and bromophenol content in the alga were measured during a period of over a year.

2. Results and discussion

2.1. Seasonal variation in bromophenol content

The bromophenol content in *U. lactuca* was measured monthly from January 1997 to May 1998. The bromophenols detected were 2-bromo-, 4-bromo-, 2,4-dibromo-, 2,6-dibromo- and 2,4,6-tribromophenol (Table 1), which is in accordance with other investigations on this species (Whitfield et al., 1997). The most abundant compound all year round was 2,4,6-tribromophenol (2,4,6-TBP). However, it showed an extreme seasonal variation, with high concentrations in late summer and low concentrations during the rest of the year. There was also a difference in 2,4,6-TBP content between the summer of 1997 and 1998. In 1997, *U. lactuca* contained high concentrations of 2,4,6-TBP (840 to 1600 ng g^{-1}) from January to April, while in 1998, high concentrations could only be measured during a short period in February (850 ng g^{-1}). The concentrations of the mono- and dibro-

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Table 1
Seasonal variation of bromophenol content in the green alga *U. lactuca* collected in the intertidal zone at Turimetta Head, Sydney, Australia

		Amount of bromophenols in *U. lactuca* (ng/g fr. wt)[a]				
Date	Abbreviation	2-BP	4-BP	2,4-DBP	2,6-DBP	2,4,6-TBP
6.1.97	J-97	0.1	1	13	9	1100
7.2.97	F	0.2	2	11	2	840
4.3.97	M	0.8	16	17	3	1600
3.4.97	A	0.1	8	8	1	1300
1.5.97	M1	3	70	23	5	700
30.5.97	M2	0.4	2	3	3	42
2.7.97	J	3	32	14	8	47
30.7.97	J	0.4	n.d.	2	1	10
28.8.97	A	0.1	n.d.	0.9	1	13
30.9.97	S	0.1	0.4	2	0.7	20
27.10.97	O	0.1	0.2	2	1	25
26.11.97	N	0.1	0.8	2	0.7	42
26.12.97	D	0.1	3	4	n.d.	51
21.1.98	J-98	0.1	1	7	2	300
25.2.98	F	0.1	0.9	6	2	850
24.3.98	M	0.7	2	4	2	58
21.4.98	A	0.2	n.d.	2	1	72
21.5.98	M	0.6	5	4	3	22

[a] 2-BP, 2-bromophenol; 4-BP, 4-bromophenol; 2,4-DBP, 2,4-dibromophenol; 2,6-DBP, 2,6-dibromophenol; 2,4,6-TBP, 2,4,6-tribromophenol.
n.d. means not detected at 0.1 ng g^{-1}.

mophenols were always low and no seasonal trend could be established.

Most investigations concerning identification and quantification of halogenated compounds in algae involved algae collected on isolated occasions. However, there are a few studies regarding the seasonal variation in the production and release of volatile halocarbons by marine algae. For example, a seasonal trend has been detected for the release of $CHBr_3$, CH_2Br_2 and $CHBr_2Cl$ by marine algae on the western coast of Sweden (Klick, 1992), which also showed a high production in summer and a low one in winter.

2.2. Characteristics of bromoperoxidase (BPO)

BPO activity in *U. lactuca* was monitored by the bromination of monochlorodimedone. The optimum bromination conditions were established by measuring the activity at various pH values and at various concentrations of H_2O_2 and bromide. The results are presented in Fig. 1. The pH optimum was 7 regardless of the concentrations of H_2O_2 and bromide. However, at low H_2O_2 concentrations (0.1 and 0.5 mM), the pH optimum appeared to shift towards pH 8.0. The optimum H_2O_2 and bromide concentrations were 2 and 100 mM, respectively; however, the bromide concentration was not as crucial giving high activity between 50 and 200 mM. Assays made in the presence of chloride, instead of bromide, showed no activity, confirming that the enzyme is a bromoperoxidase and not a chloroperoxidase.

2.3. Seasonal variation in BPO activity

The BPO activity in *U. lactuca* was measured monthly from May 1997 to May 1998. The BPO activity also exhibits an extreme seasonal variation with high activity in summer and low in winter (Fig. 2). Similar results have been obtained for the red alga, *Corallina pilulifera* (Tojinbo, Japan), which possesses high BPO activity in spring and early summer, but low activity in winter (Itoh & Shinya, 1994).

It should be noted that the increase in BPO activity in November 1997 did not coincide with an increase in 2,4,6-TBP content in the algae (see Fig. 2, where the 2,4,6-TBP content in the alga is also shown for clarity). These results may indicate that the role of the BPO in *U. lactuca* is not primarily to produce bromophenols but other halogenated compounds. For example, *U. lactuca* is also known to produce large quantities of $CHBr_3$ (Nightingale, Malin, & Liss, 1995; Pedersén, Collén, Abrahamsson, & Ekdahl, 1996). This production could be a way of scavenging H_2O_2 when the algae are exposed to high light intensities (Pedersén et al., 1996).

The results presented in our study should be considered when measuring bromoperoxidase activity or quantifying brominated compounds in marine algae. Measurements

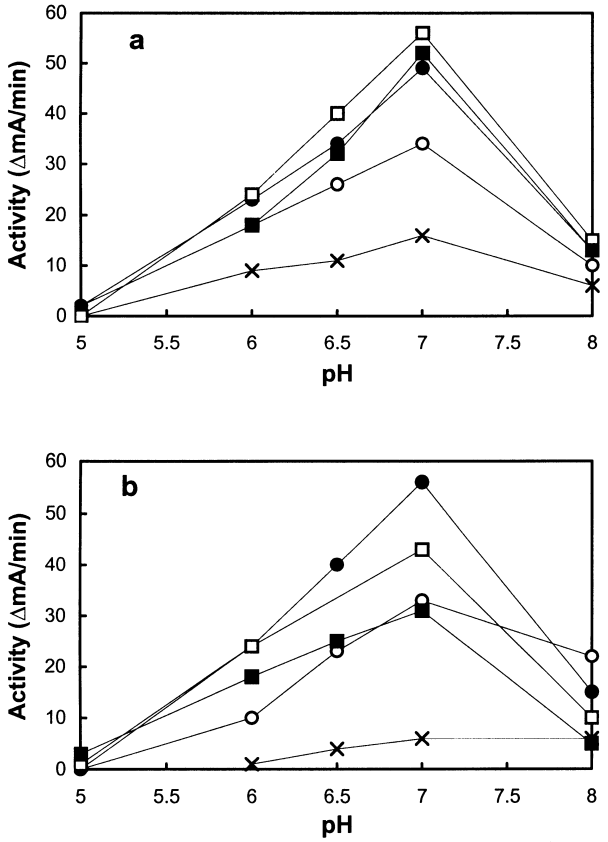

Fig. 1. Variation in BPO activity in *U. lactuca* as determined by (a) pH and KBr concentration (×, 10 mM; ○, 25 mM; ●, 50 mM; □, 100 mM; ■, 200 mM) at 2 mM H_2O_2 and (b) pH and H_2O_2 concentration (×, 0.1 mM; ○, 0.5 mM; ●, 2 mM; □, 3 mM; ■, 5 mM) at 100 mM KBr. Activity is presented as the measured decrease in A min^{-1}.

on algae collected on isolated occasions could give a false picture of their ability to produce brominated compounds. For example, in the survey by Hewson and Hager (1980), *U. lactuca* showed bromoperoxidase activity, while in the survey by Moore and Okuda (1996), no activity was detected. These results could be due to the fact that the algae were collected at different times of the year, although other explanations may also apply.

3. Experimental

3.1. Plant material

The green marine alga *U. lactuca* was collected monthly at Turimetta Head, just north of Sydney, on the eastern coast of Australia. It was collected in the intertidal zone at low tide and transported on ice to the laboratory and stored at −20°C.

3.2. Extraction of bromophenols

The concns of bromophenols were determined according to Whitfield, Shaw, and Svoronos (1994). The method includes simultaneous steam distillation-solvent extraction (SDE) of bromophenols from the algae with subsequent analysis by GC–MS.

3.3. GC–MS

Bromophenols were identified and quantified by GC–MS. The gas chromatograph was equipped with a non-polar bonded phase capillary column (HP-5 trace analysis (5% Ph 95% Me siloxane) 25 m × 0.2 mm × 0.33 μm film thickness). He was used as carrier gas at a constant velocity of 30 cm s^{-1}. Injection temperature was 250°C and 1 μl of sample was injected with a split ratio of 1:20. The column temperature was initially held at 40°C for 2 min, then programmed from 40 to 280°C at 20°C min^{-1}, before holding this temperature for 20 min. The MS detector was operating in the EI mode (70 eV at 170°C). Bromophenols were quantified in the selected ion monitoring mode against calibration curves of each compound with 2,6-dibromophenol-d_3 as internal standard. Positive identifications were based on matching R_ts with those of authentic compounds and the appearance of the correct isotopic ratios of the selected ions. Target ions were as follows: 2,6-dibromophenol-d_3, *m/z* 255, 257; monobromophenols, *m/z* 172, 174; dibromophenols, *m/z* 250, 252, 254; tribromophenol, *m/z* 330, 332.

3.4. Preparation of crude enzyme extract

Samples of frozen algae were thawed and cleaned from contaminating sand, other algae or animals. They were patted dry between paper tissues and cut into small pieces with a pair of scissors. Cut algae (4 g) were homogenised on ice for 4 min in 20 ml of Milli-Q H_2O with an Ultra Turrax homogeniser. The slurry was squeezed through eight layers of cheesecloth and glycerol (2 g) was added to an aliquot of the filtrate (8 g). This crude enzyme extract was stored at −6°C until BPO activity was measured within a day of extraction. All extractions were done in duplicate.

Other extraction methods were tested but only gave reduced BPO activity. Homogenisation in various buffers, such as 0.2 M Tris (pH 8.3, adjusted with H_2SO_4), 0.2 M HEPES (pH 7.5, adjusted with KOH) and 0.2 M PIPES (pH 6.8, adjusted with KOH) gave much lower activity. Experiments where algae were homogenised in Milli-Q H_2O to disrupt the cells, followed by the addition of the different buffers, improved the extraction of BPO compared with direct homogenisation in the buffer. However, homogenisation in Milli-Q H_2O followed by the addition of glycerol proved to be by far the most effective method. The use of frozen algae also improved the extraction efficiency compared with extraction of fresh algae.

3.5. Bromoperoxidase assay

Crude enzyme extracts were tested for their BPO activity with the standard assay using mono-

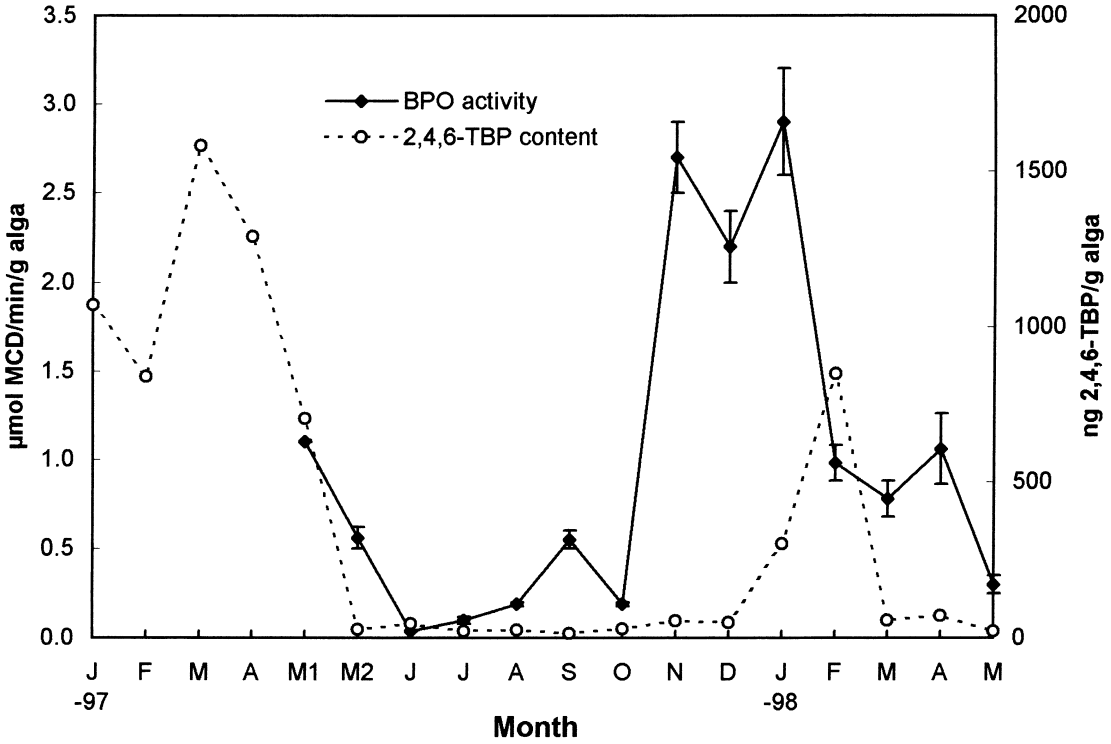

Fig. 2. Seasonal variation in BPO activity in *U. lactuca* from May 1997 to May 1998. The 2,4,6-TBP content in the alga from January 1997 to May 1998 is also shown for comparison. Collection dates and abbreviations are presented in Table 1.

chlorodimedone (MCD) as substrate (Soedjak & Butler, 1990). Bromination of MCD was followed spectrophotometrically as a decrease in absorbance at 290 nm using the extinction coefficient of MCD of 20,000 M^{-1} cm^{-1}. The assay was performed at room temperature in 0.1 M K–Pi buffer (pH 7), in the presence of 0.2 M Na_2SO_4, 100 mM KBr, 50 µM MCD, 2 mM H_2O_2 and 25 µl of crude enzyme extract, in a total volume of 2 ml. BPO activity in samples was calculated as the amount of MCD that could be brominated per min per g of alga (µmol MCD min^{-1} g^{-1} alga) and the decrease in absorbance (ΔA) between 1 and 2 min was used for the calculation. The assay was also performed at various pH values, Br^- concns and H_2O_2 concns, in order to establish optimum bromination conditions. The buffers used for the various pHs were 0.1 M citric acid for pH 4 and 5 (pH adjusted with KOH) and 0.1 M K–Pi for pH 6, 6.5, 7 and 8. The optimum conditions were then utilised when investigating the seasonal variation of BPO activity in *U. lactuca*. To establish if the enzyme was a chloroperoxidase or a bromoperoxidase, the optimised assay was also repeated in the presence of 100 mM KCl, instead of KBr.

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