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ORIGINAL ARTICLE

Extracellular secretion of phenolic substances from living brown algae

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Abstract The chemical structures of ultraviolet (UV)-absorbing substances secreted from the healthy living brown algae, *Eisenia bicyclis* and *Ecklonia kurome*, were demonstrated. The living activity of algal cells was critically examined using a confocal laser-scanning microscope after incorporation of fluorescein diacetate (FDA) into the cells. Using thin-layer chromatography (TLC), reversed-phase three-dimensional high-performance liquid chromatography (RP-3D-HPLC) and gas chromatography-mass spectrometry (GC-MS), it was found that the UV-absorbing substances (λ_{max} 265–270 nm) secreted from the living brown algae mainly consisted of the three monomeric bromophenols, 2,4-dibromophenol, 2,4,6-tribromophenol and dibromo-iodophenol, but not phloroglucinol or phlorotannins. The other minor compounds detected in the secretions were as follows: benzothiazole, fatty acids (14:1, 16:0 and 18:0 acids), franesol, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate and squalene. Exudation of phloroglucinol and phlorotannins was ascertained to take place only after the cell death of these algae. These results indicate that, whilst the algae are alive, polymeric phlorotannins are strictly kept within the algal body, and only monomeric bromophenols are secreted into the seawater medium.

Keywords Bromophenols · *Ecklonia kurome* · *Eisenia bicyclis* · Gas chromatography-mass spectrometry · Phlorotannins · Thin-layer chromatography

Abbreviations

FDA	fluorescein diacetate
GC-MS	gas chromatography-mass spectrometry
TLC	thin-layer chromatography
RP-3D-HPLC	reversed-phase three-dimensional high-performance liquid chromatography
UV	ultraviolet
UV-VIS	ultraviolet-visible

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Introduction

In marine chemical ecology, it is well known that living brown algae secrete ultraviolet (UV)-absorbing substances into seawater medium (Fogg & Boalch, 1958; Craigie & McLachlan, 1964; Carlson, 1982; Ragan & Glombitza, 1986; Swanson & Druehl, 2002). Many investigators have thus assumed that living brown algae also secrete phlorotannins directly into seawater on the basis of the following two facts, (1) the secretions are positive for phenolic substances in colorimetric tests using Folin-Denis and Brentamine reagents and (2) phlorotannins are contained commonly in brown algae (McLachlan & Craigie, 1964; Sieburth & Conover, 1965; Carlson & Carlson, 1984; Ragan & Glombitza, 1986; Taniguchi et al., 1992; Jennings & Steinberg, 1994; Sakami, 1996; Swanson & Druehl, 2002). Accordingly, investigators have discussed the allelopathic effects of phlorotannins, e.g., anti-algal (Sieburth & Conover, 1965), antibacterial (Sakami, 1996) and feeding-deterrent activities (Taniguchi et al., 1992), as well as UV protection activity (Swanson & Druehl, 2002) based on this assumption. Phlorotannins of marine algal polyphenols, which have only been found to exist within brown algae, are formed by the polymerisation of phloroglucinol (1,3,5-trihydroxybenzene) (Ragan & Glombitza, 1986; Nakamura et al., 1996) and are accumulated among the vegetative cells of the outer cortical layer of the brown algae (Pellegrini, 1980; Shibata et al., 2004). However, no further verifications have been carried out, and the question of "What are these UV-absorbing secretions?" still awaits clarification.

In this report, we study the chemical structures of UV-absorbing substances secreted from living or dead brown algae, *Eisenia bicyclis* (Kjellman) Setchell and *Ecklonia kurome* Okamura, using thin-layer chromatography (TLC), reversed-phase three-dimensional high-performance liquid chromatography (RP-3D-HPLC) and gas chromatography-mass spectrometry (GC-MS). We also discuss the problem of the secretion of phlorotannins.

Materials and methods

Cultivation of brown algae

The brown algae, *Ecklonia bicyclis* and *Eisenia kurome*, without any visible grazing or other tissue damage, were collected in spring of 2003 from along the coast of the Itoshima peninsula (33° 37′N, 130° 10′E) in Fukuoka Prefecture, Japan. After removal of epiphytes and periphytons from the surface of the thalli, the developed plant (fully developed sporophyte with a forked stipe; ca. 70 cm in length) was placed in glass aquariums with PESI (Tatewaki, 1966) medium (12 L), and cultured in the cultivation chamber (CL-301, Tomy). The ultraviolet level in the chamber was less than minimal erythema dose. The cultivation condition of the brown algae was emulated the environmental condition of the collection site. In the secretion test for living algae, two samples of the developed plant (total weight ca. 400 g) were cultured at 20°C under 20 μmol photon m^{-2} s^{-1} in a 12:12 h light:dark cycle with aeration and stirring. In the exudation test for dead algae, two plants of the algae were frozen by liquid nitrogen and stored at −80°C for 1 week, and then the plants were placed in glass aquariums under the same condition as the living algae. The time course of secretion or exudation of UV-absorbing substances from the brown algae was analysed photometrically (A_{265} and A_{295}).

Microscopic observation

The living activity of the algae was determined using fluorescein diacetate (FDA) staining (Widholm, 1972; Shibata et al., 2002a) with a confocal laser-scanning microscope (Digital Eclipse C1, Nikon). 20% FDA in acetone was added to each culture medium to produce a final concentration of 0.01%. Localisation of phlorotannins was investigated by light microscopy (Optiphoto, Nikon) after vanillin-HCl staining (Shibata et al., 2004).

Extraction of UV-absorbing substances and phlorotannins

The UV-absorbing substances in the culture medium were prepared by a modified method of Folch et al. (1957). Namely, each of the filtered-culture media (1,200 mL) was shaken with 4,800 mL of $CHCl_3$:MeOH (2:1, v/v) in a separating funnel for 3 min, and was partitioned between the upper (hydrophilic) and lower (lipophilic) fractions. The upper fractions were concentrated *in vacuo*, and dissolved in $CHCl_3$:MeOH (1:1, v/v). The lower fractions were concentrated *in vacuo*, and each of the residues was

dissolved in a small amount of $CHCl_3$:MeOH (2:1, v/v). Extraction of the phlorotannins from the thalli of the living brown algae was carried out according to the modified method of Folch et al. (1957) described in previous reports (Nagayama et al., 2002, 2003).

TLC

Samples were spotted onto a TLC plate (Silica Gel 60 F_{254}, 0.25 mm, Merck), which had been activated at 120°C for 5 min before use. The plate was developed with $CHCl_3$:MeOH:water:acetic acid (65:25:4:3, v/v), and 50% H_2SO_4 (Krebs et al., 1969), Folin-Ciocalteu reagent (Krebs et al., 1969), and diphenylamine-aniline (Bailey & Bourne, 1960), were used as the detecting agents for organic compounds, for phenolic compounds, and for sugars, respectively.

RP-3D-HPLC

Samples were analysed by RP-3D-HPLC (Inertsil ODS-3 column, 4.6 mm i.d. × 250 mm, GL Science Co.; MCPD-3600 UV detector, Otsuka Electronics). Elution was performed at a flow-rate of 1.0 mL min^{-1} with a linear gradient from 30% MeOH/water (eluent A) to 100% MeOH (eluent B) for 20 min, and was maintained for 25 min. The photodiode array detector was set a wavelength range of 190–500 nm.

GC-MS

Samples were analysed on a Shimadzu QP-5000 gas chromatograph-mass spectrometer (Shimadzu) equipped with a fused silica capillary column (DB-5, 0.25 mm × 30 m, J&W Scientific). After the column temperature had been maintained at 40°C for 3 min, it was subsequently increased to 140°C at 20°C min^{-1} and then to 300°C at 3°C min^{-1}. The mass spectrometer was operated in the electron ionisation mode under the following conditions: ionisation energy, 70 eV; ion source temperature, 250°C; scan interval, 0.5 s. If necessary, the samples were subjected to methanolysis and/or trimethylsilylation before the analysis.

Results

Microscopic observations of the algae

It should be emphasised that determination of living activity of the algae during cultivation is crucial for this kind of experiment. The algal cells were critically examined periodically with a confocal laser-scanning microscope following FDA staining. Fluorescence developed in the cells of the brown algae deeply after 24 and 48 h (Fig. 1A) and moderately after 72 h of cultivation. Light microscopic observations after staining with vanillin-HCl revealed that phlorotannins had been kept within the vegetative cells of the outer cortical layer of the algae after cultivation for 48 h (Fig. 1A). The dead alga, which was killed with liquid nitrogen and stored at −80°C for 1 week, was immersed in the same medium as living algae for 48 h, and observed by microscopy. No fluorescent cells or cells positive for vanillin-HCl were observed in the dead plants (Fig. 1B).

TLC of secretions from *E. bicyclis* and *E. kurome*

After 48 h of cultivation, the culture media of both living *E. bicyclis* and *E. kurome* became yellowish due to the accumulation of substances secreted from the algae. The ultraviolet-visible (UV-VIS) profiles of the media showed similar profiles, a strong absorption at λ_{max} 265 to 270 nm. The UV-absorbing substances in the culture medium were separated into two liquid phases with $CHCl_3$:MeOH:water (8:4:3, v/v), and each of the divided hydrophilic (H-1 and 2 of living algae, H-3 and 4 of dead algae) and lipophilic fractions (L-1 and 2 of living algae, L-3 and 4 of dead algae) of the secretions and exudates was analysed using TLC (Fig. 2). If phloroglucinol and/or phlorotannins were present in the secretions, then they should be separated into the hydrophilic fraction (Nakamura et al., 1996; Nagayama et al., 2002, 2003; Shibata et al., 2002a,b, 2003, 2004). In the case of living brown algae, even though relatively less polar phenolic compounds were in the L-1 and L-2, spots of phloroglucinol, phlorotannins or other polar phenolic compounds were not detected in the H-1 or H-2 on TLC plates (Fig. 2A). The preparations (T-1 and T-2) from the thalli of 48 h cultured living algae, retained phloroglucinol and phlorotannins (Fig. 2). Antithetically, extracts (H-3 and H-4, and L-3 and L-4) from the media in which the dead algae had been immersed for 48 h, that is, substances which corresponded to phloroglucinol and phlorotannins, were detected (Fig. 2B). In agreement with the preceding report (Shibata et al., 2002a), the exudation of phlorotannins was again found to be confined within

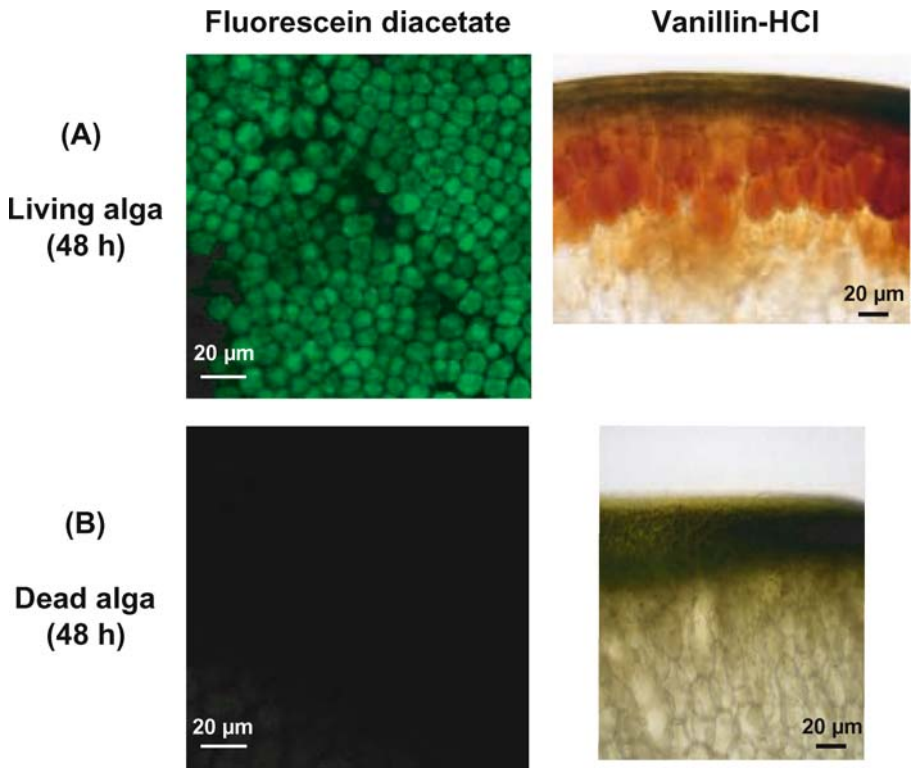

Fig. 1 Microscopic observations of thallus of the cultured brown alga, *E. bicyclis*. (A) Living alga. A typical sample cultured for 48 h is shown. (B) Dead alga. Using a confocal laser-scanning microscope, fluorescence in brown algal cells was observed. Living cells are visualised by FDA staining (green). Localisation of phlorotannins was investigated by light microscopy after vanillin-HCl staining. Phlorotannins in the cells are stained reddish orange.

the media of dead algae by TLC. In the living algae, polar spots positive for diphenylamine-aniline were detected in both H-1 and H-2, respectively (data not shown).

RP-3D-HPLC of hydrophilic fractions of secretions from *E. bicyclis* and *E. kurome*

Further analysis of the hydrophilic fractions (H-1 and H-2) of secretions from living algae, was carried out using RP-3D-HPLC. As shown in Fig. 3, within 30 min a distinct separation of each of the components (phloroglucinol and phlorotannins) in the preparations (T-1 and T-2) of 48 h cultured living algae was obtained by a linear gradient system ranging from 30% MeOH to 100% MeOH. The UV-VIS profiles of phloroglucinol and phlorotannins showed the typical spectrum of phenolic compounds, and their absorption maxima of B-band, λ_{max} 265 (phloroglucinol), λ_{max} 292 (eckol, dieckol and 8,8′-bieckol) and λ_{max} 303 nm (phlorofucofuroeckol A) were observed (Fig. 3). On the other hand, no peaks of phloroglucinol or phlorotannins were detected in any of the chromatograms of H-1 or H-2 (Fig. 3).

GC-MS of lipophilic fractions of secretions from *E. bicyclis* and *E. kurome*

From the results of Fig. 2, it is estimated that the UV-absorbing substances secreted from the living brown algae consist of lipophilic phenolic compounds which are different from phloroglucinol and phlorotannins. Therefore, the phenolic compounds were identified using GC-MS (Fig. 4). *Peak a*: The mass spectral pattern agreed well with that of authentic dibromophenols. The retention time of the compound on a DB-5 capillary column was identical with that of 2,4-dibromophenol ($C_6H_4Br_2O$), but not with that of 2,6-dibromophenol. *Peak c*: The fragmentation pattern clearly suggested that three bromine atoms are present

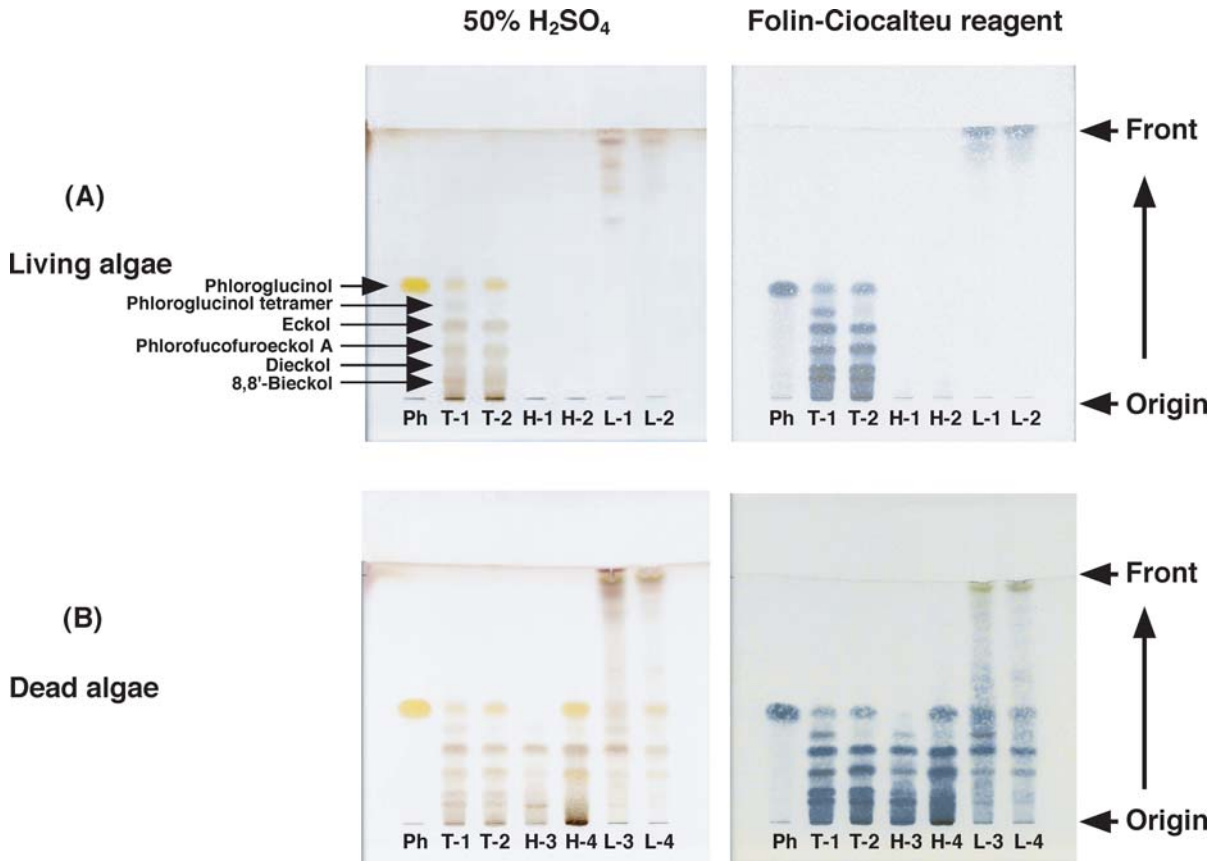

Fig. 2 TLC of secretions or exudates from the brown algae, *E. bicyclis* and *E. kurome*. (A) Secretions from the living algae. (B) Exudates from the dead algae. The plates were sprayed with either 50% H_2SO_4 or Folin-Ciocalteu reagent. Ph, phloroglucinol (20 μg); T-1 and T-2, preparation from the thalli of 48 h cultured living *E. bicyclis* (T-1) and *E. kurome* (T-2); H-1 and H-2, hydrophilic fraction of secretions from living *E. bicyclis* (H-1) and *E. kurome* (H-2); H-3 and H-4, hydrophilic fraction of exudates from 48 h immersed dead *E. bicyclis* (H-3) and *E. kurome* (H-4); L-1 and L-2, lipophilic fraction of secretions from living *E. bicyclis* (L-1) and *E. kurome* (L-2); L-3 and L-4, lipophilic fraction of exudates from dead *E. bicyclis* (L-3) and *E. kurome* (L-4). Each 100 μg was spotted. The preparations (T-1 and T-2) were composed of phloroglucinol, phloroglucinol tetramer, eckol, phlorofucofuroeckol A, dieckol and 8,8'-bieckol

in the molecule. The molecular ion is shifted 78 mass units (which corresponds to one Br atom) higher than that of dibromophenol. The mass spectrum and the retention time agreed well with those of authentic 2,4,6-tribromophenol ($C_6H_3Br_3O$). *Peak d*: The fragmentation pattern was very similar to that of 2,4-dibromophenol, but the molecular ion (*m/z* 376) was 126 mass-units higher than that of dibromophenol (Fig. 5). The characteristic ion *m/z* 127 is detected in the mass spectrum (Fig. 5). This discrepancy can be explained by replacing one hydrogen atom with one iodine atom (127) in the molecule. As a result, the compound was assigned as being dibromo-iodophenol ($C_6H_3Br_2IO$, position of the substituents is not yet determined). This compound was found only in the medium of living *E. bicyclis*, but not in that of *E. kurome*. This is the first reported instance of the identification of dibromo-iodophenol from plant origin. The other minor compounds detected in the gas chromatograms were as follows: 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate (peak b) and squalene (peak h). Benzothiazole (peak i) was detected as a minor compound localised in L-2. Fatty acids (14:1, peak e; 16:0, peak f; 18:0, peak g) and franesol (peak j) were detected in each of the lipophilic fractions of the secretions from brown algae. None of the compounds described above were detected in the seawater medium of the blank test. These results indicated that phenolic compounds secreted from living brown algae consist of monomeric bromophenols.

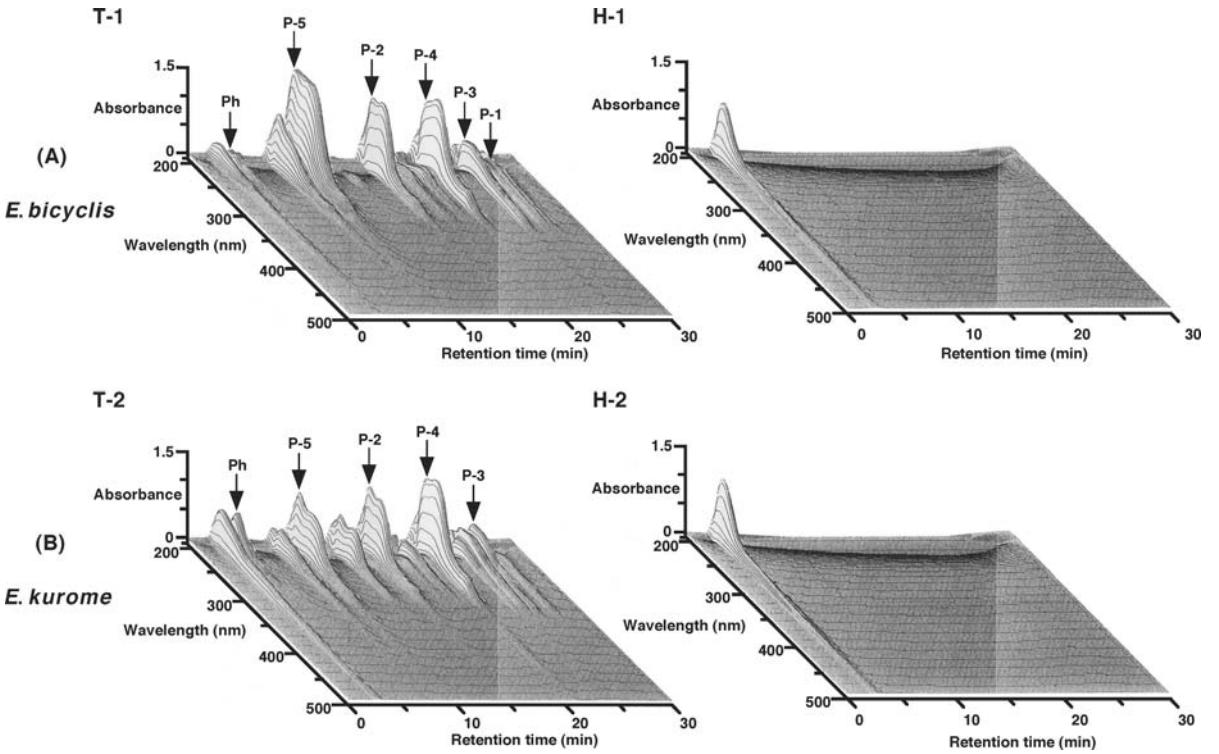

Fig. 3 RP-3D-HPLC of secretions from the brown algae, *E. bicyclis* and *E. kurome*. (A) *E. bicyclis*. (B) *E. kurome*. Conditions for the chromatography are described in the materials and methods. T-1 and T-2, preparation from the thalli of 48 h cultured living *E. bicyclis* (T-1) and *E. kurome* (T-2); H-1 and H-2, hydrophilic fraction of secretions from living *E. bicyclis* (H-1) and *E. kurome* (H-2). Ph, phloroglucinol; P-1, phloroglucinol tetramer; P-2, eckol; P-3, phlorofucofuroeckol A; P-4, dieckol; P-5, 8,8'-bieckol

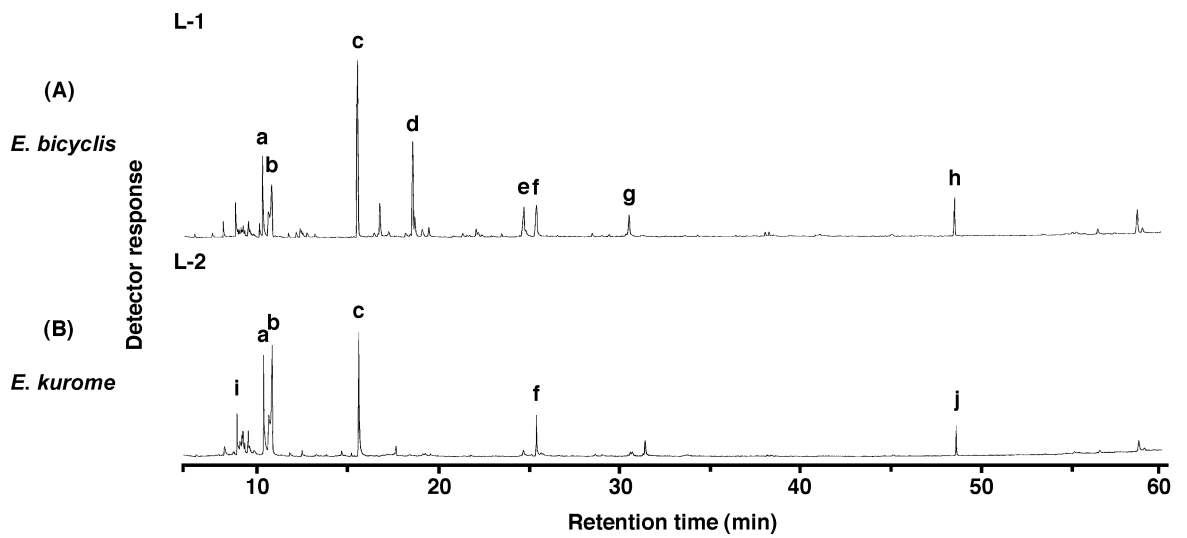

Fig. 4 Gas chromatography of secretions from the brown algae, *E. bicyclis* and *E. kurome*. (A) *E. bicyclis*. (B) *E. kurome*. L-1 and L-2, lipophilic fraction of secretions from living *E. bicyclis* (L-1) and *E. kurome* (L-2). a, 2,4-dibromophenol; b, 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate; c, 2,4,6-tribromophenol; d, dibromo-iodophenol; e, fatty acid (14:1); f, fatty acid (16:0); g, fatty acid (18:0); h, squalene; i, benzothiazole; j, franesol

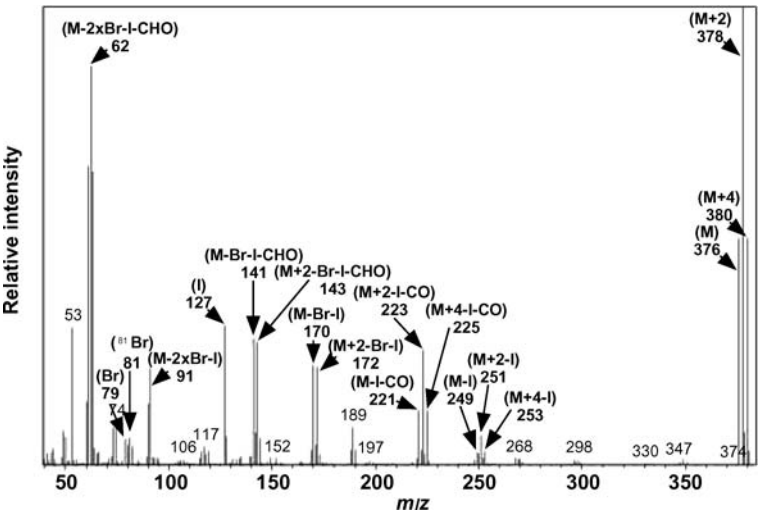

Fig. 5 Mass spectrum of dibromo-iodophenol. The mass spectrometer was operated in the electron ionisation mode under the conditions described in the materials and methods

Discussion

In this report, we identified the chemical structure of UV-absorbing substances from the living brown algae, *E. bicyclis* and *E. kurome*. It has become apparent that the substances are mainly low-polar phenolic compounds obviously different from phloroglucinol and phlorotannins. The compounds were identified as being the three monomeric bromophenols, 2,4-dibromophenol, 2,4,6-tribromophenol and dibromo-iodophenol. In particular, the identification of dibromo-iodophenol is reported for the first time from plant origin. The amounts of bromophenols in the medium increased with time in both the developed plant and the young plant (young sporophyte without a forked stipe). In particular, the secretion rate of bromophenols estimated by UV-absorption was about three times greater in the young plant. It has been reported that halometabolites represented by bromophenols in algae are formed depending upon the photosynthesis of H_2O_2 and that they have a role as a scavenger of H_2O_2 which is toxic to algal cells (Pedersen et al., 1996). Extracellular secretions, that is to say UV-absorbing substances of living brown algae, are known to have some allelopathic effects (McLachlan & Craigie, 1964; Sieburth & Conover, 1965; Carlson & Carlson, 1984; Taniguchi et al., 1992; Jennings & Steinberg, 1994; Sakami, 1996; Swanson & Druehl, 2002). In a study comprising a feeding test with the representative marine herbivorous gastropod, *Turbo cornutus*, bromophenols were found to have a much stronger retarding activity, about one thousand times greater than that of phlorotannins (data not shown). Thus, the secretion of bromophenols is greatly advantageous for the survival of brown algae. In conclusion, brown algae produce two types of phenolic compounds, secretory phenolic compounds comprising bromophenols and storage phenolic compounds comprising phlorotannins, and in addition to their role as a radical scavenger in the cells, the former compounds would seem to act as a chemical defence agent of brown algae against environmental stresses, such as herbivore or pathogen attack.

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