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A vanadium-dependent bromoperoxidase in the marine red alga *Kappaphycus alvarezii* (Doty) Doty displays clear substrate specificity

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Abstract

Bromoperoxidase activity was initially detected in marine macroalgae belonging to the Solieriaceae family (Gigartinales, Rhodophyta), including *Solieria robusta* (Greville) Kylin, *Eucheuma serra* J. Agardh and *Kappaphycus alvarezii* (Doty) Doty, which are important industrial sources of the polysaccharide carrageenan. Notably, the purification of bromoperoxidase was difficult because due to the coexistence of viscoid polysaccharides. The activity of the partially purified enzyme was dependent on the vanadate ion, and displayed a distinct substrate spectrum from that of previously reported vanadium-dependent bromoperoxidases of marine macroalgae. The enzyme was specific for Br^- and I^- ions and inactive toward F^- and Cl^-. The K_m values for Br^- and H_2O_2 were 2.5×10^{-3} M and 8.5×10^{-5} M, respectively. The halogenated product, dibromoacetaldehyde, that accumulated in *K. alvarezii* was additionally determined.

Keywords: *Kappaphycus alvarezii* (Doty) Doty; Solieriaceae; Marine algae; Bromoperoxidase; Vanadium-dependent; Dibromoacetaldehyde

1. Introduction

Halogenated compounds are synthesized by several marine algae with the aid of haloperoxidases. These compounds are classified into indoles, terpenes, acetogenins, phenols, and volatile halogenated hydrocarbons (Faulkner, 2000, 2001, 2002). Halogenated marine metabolites generally possess biological antifungal, antibacterial, antiviral and anti-inflammatory activities (Cowan, 1999). Some additionally function as allelochemicals in the marine environment (Sakata et al., 1991; Denboh et al., 1997; Ohsawa et al., 2001).

While the biosynthesis of halogenated marine natural products has intrigued scientists for decades, the mechanism has remained unclear to date (Littlechild et al., 2002). Haloperoxidases generally oxidize a halide anion (X^-) into a halonium cation (X^+) with H_2O_2, and the X^+ and its related species (XOH, X_2, X_3^-) generated acts on many organic substrates as an electrophile (Yamada et al., 1985a; Itoh et al., 1988). The enzymes are divided into three groups, specifically, heme types (eukaryotic heme types and bacterial types containing protoporphylin IX as a prosthetic group) (Morris and Hager, 1966; Manthey and Hager, 1981), bacterial non-metal enzymes (perhydrolase) (van Pee et al., 1997; Kawanami et al., 2002) and eukaryotic vanadium-containing enzymes (Itoh et al., 1986; Ohsawa et al., 2001; Krenn et al., 1989). Bacterial non-metal enzymes are related to the esterases and lipases because they contain the representative catalytic triad (Itoh et al., 2001).

Abbreviations: BPO, bromoperoxidase; MCD, monochlorodimedone.

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Vanadium-containing bromoperoxidases (BPOs) specific for Br^- and I^- are widespread in marine macroalgae such as *Corallina* (Itoh et al., 1987a; Sheffield et al., 1993) and *Ascophyllum* (Vilter, 1984; Krenn et al., 1989; Butler, 1998). Some of these enzymes display low activity towards Cl^- (Soedjak and Butler, 1990), and modification of enzyme can change the halide ion specificity (Ohshiro et al., 2004). Although oxidation of bromide ion occurs at the active site in the case of vanadium-containing enzymes (Dau et al., 1999; Littlechild et al., 2002), BPOs including *Corallina* generally do not display distinct substrate specificity or stereoselectivity (Itoh et al., 1987b, 1988; Soedjak and Butler, 1990). Recent studies show that flavin-dependent halogenases involved in chlorination of secondary metabolites in bacteria catalyze the regioselective chlorination of tryptophan or pyrrole derivatives (Dong et al., 2005; van Pee and Patallo, 2006).

During screening, it was initially observed that specific samples of marine macroalgae belonging to the Solieriaceae family, including *Kappaphycus alvarezi* (Doty) Doty, exhibited significant bromoperoxidase activity (Kamenarska et al., 2006). *K. alvarezii* and its related algae are the basic source of carrageenan (a polysaccharide), which is commonly applied in the food industry and medicine. Owing to high commercial interest, *K. alvarezii* is widely cultivated in the Philippines, Indonesia, Vietnam and Tanzania. Recently, tentative cultivation during the summer season was introduced in Shikoku Island, Southern Japan (Ohno et al., 1994). To date, most investigations on this alga focus on polysaccharide content and its properties (Ohno et al., 1996). Here, we report the properties of this novel BPO displaying distinct substrate specificity and its natural brominated compound was identified in *K. alvarezii*.

2. Results and discussion

2.1. Distribution of BPO activity in marine algae

As shown in the comparative data in Table 1, variable BPO activities were observed in a number of macroalgae belonging to the Solieriaceae family.

To our knowledge, this is the first report on BPO activity in these red algae, except for the previously documented *Eucheuma denticulatum* (Mtolera et al., 1996) and *Meristiella gelidium* (Collen et al., 1994) in the Solieriaceae. These produce volatile halogenated compounds when exposed to stress conditions, such as at an elevated pH and at high light intensities.

The addition of KBr to the crude extracts of *K. alvarezii* during overnight dialysis led to improved stability. Moreover, enhanced stability and elevated activity was observed with increasing concentrations of KBr during dialysis (initial activity, 0.007 U/ml (100%), 0.1 mM KBr, 0.18 U/mg protein (129%); 0.5 mM KBr, 0.037 U/ml (529%); 1 mM KBr, 0.08 U/ml (1142%); and 5 mM KBr, 0.17 U/ml (2428%)), respectively. While the mechanism of activation by KBr is currently unclear, it is speculated that the masked BPO activity is stimulated upon incubation with Br^- ions. KCl did not activate the enzyme. *K. alvarezii* was selected as the BPO source for further experiments, since its specific activity was high and biomass was available by aquaculture. The buffer was supplemented with 5 mM KBr and 0.5 mM $NaVO_3$ throughout the isolation and purification of BPO, unless otherwise specified.

Table 1
BPO activities in marine macroalgae

Alga	Total activity (U/g fresh alga)	Specific activity (U/mg protein)
Rhodohyta		
Solieriaceae		
Kappaphycus alvarezii	1.00	0.14
Eucheuma serra	1.12	0.03
Solieria robusta	0.02	0.0006
Corallinaceae		
Corallina pilulifera	11.7[a]	0.54[a]
Corallina officinalis	19.3[a]	0.56[a]
Amphilroa zonata	9.3[a]	0.83[a]
Phaeophyta		
Ascophyllum nodosum	4.3[b]	0.79[b]
Laminaria saccharina	12.75[c]	0.57[c]

[a] Data from Yamada et al. (1985b).
[b] Data from Wever et al. (1985).
[c] Data from De Boer et al. (1986).

2.2. Enzyme purification

Since BPO of *K. alvarezii* was stable in the presence of Br^- and vanadate ions, all purification procedures were performed at room temperature. BPO was extracted from the alga and partially purified using the procedures described in Section 4. The elimination of polysaccharide from crude enzyme by adding gel-forming agents to the enzyme solution, such as $CaCl_2$ or KCl, led to a drastic loss in enzymatic activity, indicating that BPO was probably bound to polysaccharides. The activities of a number of enzymes were next examined that hydrolyze polysaccharides: Labiase® from *Streptomyces fulvissimus*, mainly containing *N*-acetylmuramoylhydrolase (Ohbuchi et al., 2001), was effective in decreasing the viscosity of crude extracts of *K. alvarezii*, but relatively ineffective in completely removing polysaccharides from the enzyme solution. Thus, only partial purification of the enzyme was achieved. To purify the enzyme to homogeneity, further steps to completely hydrolyze the contaminating polysaccarides are essential, but this may lead to loss of enzyme activity. For this reason, complete purification of the enzyme was not attempted, and most experiments were performed with partially purified enzyme. The results of the purification are summarized in Table 2.

Table 2
Purification of BPO from *K. alvarezii*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	122.5	16.6	0.14	100	1
Labiase treatment/dialysis	66.7	61.5[a]	0.92	370.5	6.6
Octyl-Sepharose	4.2	20.5	4.88	123.5	34.9
QAE-Toyopearl	1.05	3.2	3.05	19.3	21.8

[a] The increase of activity was probably due to the incubation with KBr.

This enzyme preparation indicated some indistinct protein bands on PAGE-gel, and the purity was roughly estimated to be less than 50% from Fig. 1.

2.3. Enzyme properties

BPO of *K. alvarezii* catalyzed the bromination of monochlorodimedone (MCD) **1** in the presence of bromide ion (3.05 U/mg protein, 100%), i.e. the oxidation of Br^- to Br^+ and its related species, but not chlorination or fluorination. The enzyme also displayed strong oxidation activity towards I^- to produce I_2 (2.08 U/mg protein, 68%). Despite variable assay methods and activities, BPO activity for I^- appeared to be comparable with that for Br^-.

The influence of the KBr concentration on BPO activity was determined. A rise in enzymatic activity was observed with increasing KBr concentrations (from 0.5 to 20 mM). However, a decrease in activity (85% at 40 mM) was evident at higher levels of KBr. Using the linear portion of the saturation curve, the K_m value for Br^- was calculated from Lineweaver–Burk plot as 2.5 mM (Fig. 2a).

The influence of the H_2O_2 concentrations was also determined. The K_m value was calculated as 8.5×10^{-5} M (Fig. 2b). Inhibition by H_2O_2 was detected at concentrations higher than 10 mM. The K_m value for Br^- was rather low, compared to that of previously studied BPOs, while that for H_2O_2 was comparable (1.1×10^{-2} M for Br^-, 9.2×10^{-5} M for H_2O_2 reported for *C. pilulifera* BPO (pH 6.0) (Itoh et al., 1986), and 9.4×10^{-3} M for Br^-, 1.04×10^{-4} M for H_2O_2 by *Ascophyllum nodosum* BPO

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of partially purified BPO. About 10 μg of enzyme was subjected to electrophoresis. The gel was stained for protein (a) and for enzyme activity (b). The arrow indicates the position of BPO.

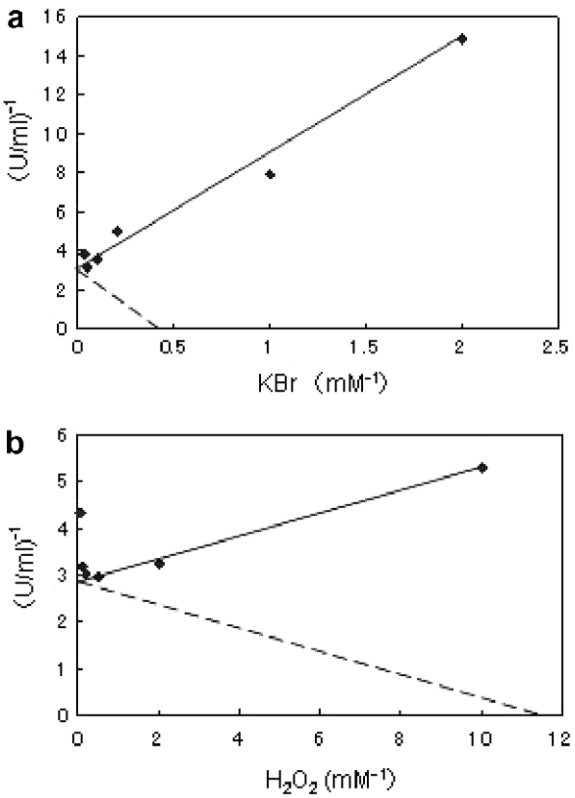

Fig. 2. Lineweaver–Burk plots for the bromination of MCD by BPO. (a) Plot with varying amounts of KBr, (b) plot with varying amounts of H_2O_2. The intersection of dashed line on abscissa indicates $1/K_m$.

(pH 5.9)) (Krenn et al., 1989). Catalase activity, which is common in heme type haloperoxidases, was not observed.

BPO was active over a broad pH range, and the optimum pH was around at 6.5. Higher activity was observed with phosphate buffer at around pH 5.5 (Fig. 3).

When the analysis was performed in acetate or MES buffer between pH 4.0 and 5.5, the activity was twice as low. These finding imply that both pH and type of buffer affects enzymatic activity. The presence of phosphate ions had a positive impact on BPO activity. Although the

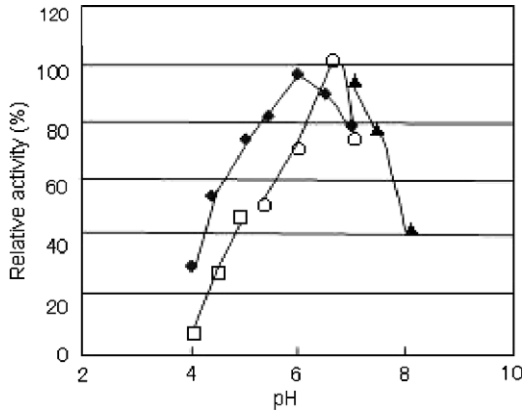

Fig. 3. pH optima of BPO. Enzyme activity was measured in 50 mM acetate buffer (pH 4–5) (□), potassium phosphate buffer (pH 4–7) (◆), MES buffer (pH 5.5–7) (○) and Tris–HCl buffer (pH 7–8) (■).

vanadium-dependent BPOs are known to share similar active sites with some acid phosphatases (Ishikawa et al., 2000; Littlechild et al., 2002), the positive effect of phosphate ions not negative on *K. alvarezii* BPO has been unclear.

The effects of a range of compounds for crude enzyme, described in Section 4, were also determined. $AgNO_3$, *p*-CMB, iodoacetic acid (sulfuhydryl reagents), and Na_2MoO_4, $VOSO_4$, $NaVO_3$, NaCl and EDTA did not affect the enzymatic activity. On the other hand, a number of divalent metal ions increased enzymatic activity ($CoCl_2$ by 33%; $MnCl_2$, 39%; $BaCl_2$, 42%; $NiCl_2$, 47%; $MgCl_2$, 49%; $CaCl_2$, 52%; $CuCl_2$ and $ZnSO_4$, both by 69%).

2.4. Reconstitution of BPO with vanadate ions

A reconstitution process disclosed that the BPO of *K. alvarezii* is a vanadium-dependent haloperoxidase. The initial activity of the enzyme was calculated as 3.06 U/mg protein (100%). After 5 h incubation at pH 2.5 in the presence of Na-EDTA, total loss of enzymatic activity was observed (0%), and thus low pH was necessary to inactivate the enzyme. Overnight incubation with $NaVO_3$ at neutral pH led to a reconstitution of the enzyme and recovery in activity to 1.29 U/mg (42%), while no activity was evident upon incubation in the absence of $NaVO_3$. Moreover, it was observed that addition of 0.5 mM $NaVO_3$ in the buffer solution effectively maintained enzymatic activity.

2.5. Substrate specificity of BPO for organic compounds and stereoselectivity in bromination

Substrate specificity and stereoselectivity are key issues in characterizing haloperoxidases, particularly with regard to its reaction mechanism. This is because the bromination of most organic compounds appear to nonenzymatically proceed with the electrophilic addition/substitution of molecular bromine (Br_2) or hypobromous acid (BrOH), in spite of the fact that bromide ion is oxidized by a vanadium-peroxo complex at the active sites of vanadium-dependent BPOs (Dau et al., 1999). According to the mechanism, many organic compounds are brominated by this enzyme. Indeed, Itoh and colleagues demonstrated that despite evident substrate preference with BPO from *C. pilulifera* (marine red alga), several phenolic and heterocyclic compounds, alkenes, as well as keto acids were substrates of the enzyme (Itoh et al., 1987a, 1988). Butler reviewed an interesting phenomenon of vanadium-dependent *A. nodosum* and *C. officinalis* BPO/CPO, specifically, the active site bromination reaction when indole, but not cytosine, is used as a substrate (Butler, 1998). Accordingly, we examined a number of organic compounds for bromination by BPO from *K. alvarezii,* and compared them with the previous data. Data presented in Fig. 4 reveals clear substrate specificity of the enzyme. Indene (**2**), styrene (**3**), 4-chlorostyrene (**4**), cinnamyl alcohol (**5**), and cyclohexene (**6**) were all brominated to the corresponding bromohydrin compounds by the enzyme, specifically, *trans*-2-bromo-1-indanol (**7**), 2-bromo-1-phenylethanol (**8**), 2-bromo-1-(4-chlorophenyl)-1-ethanol (**9**), 2-bromo-3-phenyl-1,3-propanediol (**10**), and 2-bromo-1-cyclohexanol (**11**). The above products corresponded to those in a previously report, and were thus easily confirmed (Yamada et al., 1985a; Itoh et al., 1988). However, 2-bromo-1-phenylethanol (**8**) from styrene (**3**) and *trans*-2-bromo-1-indanol (**7**) from indene (**2**) were racemic mixtures on chiral HPLC analysis, and therefore no stereoselectivity in bromination was detected for *K. alvarezii* BPO. The enzyme additionally catalyzed the bromination of 3-methyl-2-buten-1-ol (**12**) to 2-bromo-3-metyl-1,3-dihydroxybutane (**13**), which displayed comparable retention time and mass-spectra as the authentic compound. However, bromination of 3-methyl-2-buten-1-ol (**12**) analogues, including 3-methyl-3-butene-1-ol (**14**), 2-methyl-2-propene-1-ol (**15**), and 3-butene-1-ol (**16**), was not catalyzed by *K. alvarezii* BPO. All unreactive compounds contained an aliphatic isolated terminal double bond (Fig. 4).

No brominated products were obtained with anisole (**17**), *o*-hydroxybenzyl alcohol (**18**) and phenol red (**19**), implying that the enzyme catalyzes only specific reactions of addition, and not substitution of phenolic compounds. No brominated compounds (lipophilic, polar or volatile) were detected when phenylpyruvic acid (**20**) was used as a substrate. In addition, no activity was observed on pyruvic (**21**) and oxalacetic (**22**) acids, known precursors of volatile halomethanes for BPO in *C. pilulifera* leading to formation of $CHBr_3$, $CHClBr_2$ and CH_2Br_2 (Itoh and Shinya, 1994). Therefore, it was concluded that *K. alvarezii* enzyme does not utilize α-keto acids or phenolic compounds as a bromination substrate. This is the first report on such clear substrate preference by BPO. Our data strongly suggest that the reaction of *K. alvarezii* BPO proceeds only at the active site, and does not depend on released active brominated species, such as Br^+OH^-. This presumption contradicts the fact that there is no chiral induction of bromination for some substrates. We speculate that the enzyme contains no strict substrate binding site to control the stereoselectivity in a similar manner as *Corallina* BPO. Our finding provides new aspect of vanadium-BPO in marine algae, and possible application of the enzyme to substrate-specific bromination.

2.6. Determination of brominated compounds in K. alvarezii

In view of the existence of BPO in *K. alvarezii*, the algae were analyzed for brominated compounds. *K. alvarezii* emitted an irritant compound, which caused itchiness on skin when disrupted. Consequently, volatile halogenated compounds were directly measured by heating the algae in a head-space bottle. No halogenated compounds, including $CHBr_3$ or CH_2Br_2, were detected by GC–MS. On the other hand, GC–MS analysis of the EtOAc extract of *K. alvarezii* revealed the presence of dibromoacetalde-

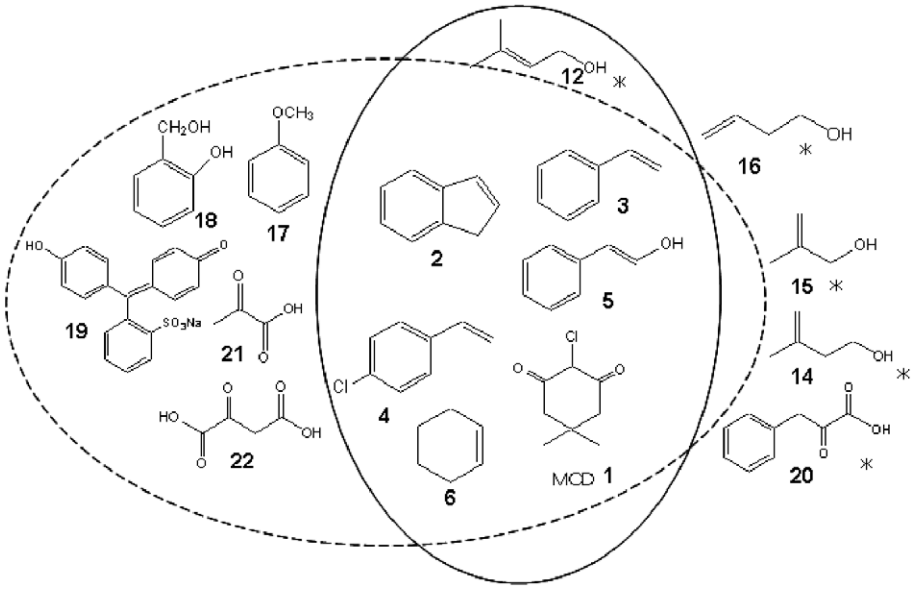

Fig. 4. Summary of substrate spectra of vanadium-dependent BPOs of different origins. Compounds brominated by BPO from *K. alvarezii* are displayed inside a solid circle, and those brominated by the enzyme from *C. pilulifera* inside a broken line. The compounds, that have not been tested by BPO of *C. pilulifera*, are presented with an asterisk.

hyde (**23**) ($C_2H_2OBr_2$) (t_R 14.98 min); EI-MS *m/z* (isotope ratio, rel. int.): 200/202/204 $[M]^+$ (1:2:1, 8), 172/174/176 (50), 120/122 (8), 93(100), which indicated a 97% identity with the authentic compound registered in NIST (National Institute of Standards and Technology, USA) mass spectral library as shown in Fig. 5, and benzaldehyde (t_R 18.78 min); EI-MS *m/z*: 106 $[M]^+$ (100), 105(80). We could not isolate the compounds because of their small amounts and easy vaporization. Dibromoacetaldehyde (**23**), which is toxic and irritant for many organisms, may participate in the defense mechanism of algae as allelochemical against invertebrates and microorganisms. BPO is possibly involved in the biosynthesis of dibromoacetaldehyde (**23**), although the biosynthetic pathway is currently unclear.

In Hawaii islands, *K. alvarezii* is harmful to corals as a result of shadowing or smothering, and causes coral death, due to its high growth rate (Rodgers and Cox, 1999). Dibromoacetaldehyde (**23**) identified in algae by our group may be involved in algal survival in the marine environment by supporting the growth rate. From this view point, they should be more careful in aquaculturing a large amount of *K. alvarezii* in the sea.

3. Concluding remarks

BPO was first identified in *Kappaphycus alvarezi* belonging to the Solieriaceae, which are important industrial sources of carrageenan. The enzyme was dependent on vanadium (V), and displayed a distinct substrate spectrum from that of previously reported BPOs from marine macroalgae. Dibromoacetaldehyde accumulated in *K. alvarezi* was additionally determined.

4. Experimental

4.1. Cultivation of algae

Kappaphycus alvarezii (Doty) Doty ex Silva (Soleriaceae, Gigartinales, Rhodophyta) from the Philippines was cultivated in the subtropical waters during the summer in Kochi, Japan. The cultivation method employed thallus fragments fixed to floating ropes in the sea (Fig. 6).

Voucher-specimen was deposited in the herbarium of Kochi University. Fresh algae cultured were frozen at −20 °C for experiments in the laboratory. Other algal samples were cultured in Kochi.

4.2. Crude enzyme reparations form the algae

Defrosted algae (1–2 g wet weight) were disrupted with sea sand (40–80 mesh) using a mortar and pestle at room temperature, and with the whole them extracted with 10 mM 2-(*N*-morpholino)ethanesulphonic acid–NaOH (MES) buffer (pH 7.0, buffer A) at a ratio of 0.1 g algal sample/0.5 ml buffer at room temperature. Next, the crude extract was centrifuged at 4 °C for 30 min at 10,000*g* to obtain the supernatant.

4.3. Partial purification of the enzyme

All manipulations were performed at room temperature using 10 mM MES buffer, pH 7.0 (buffer A), or 10 mM MES buffer, pH 7.0, plus 5 mM KBr and 0.5 mM $NaVO_3$ (buffer B), unless stated otherwise. Each 30 g (total 120 g wet weight) of algae washed with distilled water were disrupted using a mortar and pestle, extracted with each

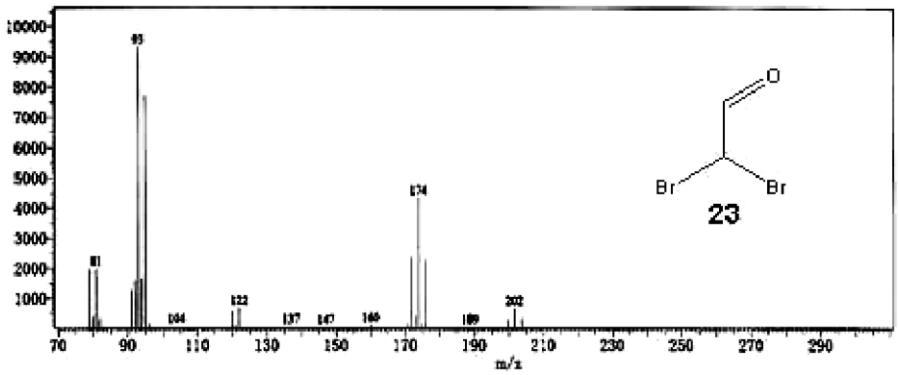

Fig. 5. GC–MS analysis of brominated compound in *K. alvarezii*.

Fig. 6. The photo of *K. alvarezii* cultivated in the sea.

150 ml buffer B (total 600 ml), and centrifuged at 10,000*g* for 30 min to obtain the supernatant as described above. Labiase (0.5 g: more than 5 U of *N*-acetylmuramoylhydrolase), a lysis enzyme obtained from Seikagaku Corp. (Tokyo), was added to the recovered supernatant (350 ml), and incubated at 37 °C for 48 h with gentle shaking to dissolve the polysaccharide and decrease the viscosity. After dialysis against buffer B, the enzyme solution was subjected to freeze-drying and dissolved in distilled H_2O (140 ml). The solution was applied to a Octyl-Sepharose hydrophobic column chromatography (6 by 8 cm) previously equilibrated with buffer B. Following application of the enzyme solution to the column, some non-adsorbed proteins and partially degraded polysaccharides were eluted with buffer B. The enzyme was eluted with a increase in concentrations of sodium cholate (0.2%, 0.5%, 1% and 1.5%) in buffer B (250 ml). Factions showing the activity (1% and 1.5% of sodium cholate) were combined and concentrated to 140 ml with an ultrafiltration unit HHP-62K equiped with a Q0100 polysulfone membrane filter (Advantec Toyo, Tokyo, Japan). Following dialysis against buffer B, the enzyme solution was subjected to a QAE-Toyoperl-550 C column (6 cm by 6 cm) previously equilibrated with buffer B. The column was washed with the same buffer, and the enzyme eluted with a linear gradient of 0.005–1.5 M KBr. Enzyme fractions that displayed activity were combined, concentrated to 5 ml and subjected to dialysis, and used as purified enzyme.

The protein concentration was estimated by measuring the absorbance at 280 nm or by using the bicinchoninic acid (BCA) method (Smith et al., 1985), calibrated with bovine serum albumin as a standard (BCA Protein Assay Kit, Pierce/Thermo Scientific, USA).

4.4. Polyacrylamide gel electrophoresis (PAGE)

Analytical PAGE was performed in 7% polyacrylamide gel with Tris–HCl buffer (pH 8.9) according to the method of Davis (1964). The gels were stained for protein with Coomassie brilliant blue G-250, and staining of gels for enzyme activity was carried out by incubation in 10 mM MES buffer (pH 6.5) containing 0.5% pyrogallol, 10 mM H_2O_2 and 10 mM KBr in the dark at 4 °C.

4.5. Enzymatic activity

Enzymatic activity was determined with the MCD test. Enzyme solution (0.01 ml) was assayed in the reaction mixture containing 10 mM MES buffer, pH 6.5, 60 μM MCD, 20 mM KBr and 10 mM H_2O_2 in a total volume of 1 ml at 25 °C. Enzymatic activity (U/ml) was spectrophotometrically measured by the decrease in absorbance of MCD at 290 nm ($\varepsilon = 19{,}900\ cm^{-1}\ M^{-1}$), according to the bromination of MCD.

Catalase activity was assayed by measuring the decrease in absorbance at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$) of H_2O_2 in a total volume of 1.0 ml (Itoh et al., 2001).

One unit (U) of enzyme activity was defined as the amount of the enzyme that catalyzed the formation of brominated MCD or decomposition of H_2O_2 in 1 min.

4.6. Halide ion specificity

Halide ion specificity of BPO was determined for F^-, Cl^-, Br^- and I^-. The general MCD test was performed by replacing KBr with KF and KCl for F^- and Cl^-. For iodide, the oxidation rate was directly measured. The reaction mixture consisted of 100 mM MES buffer, pH 6.5, 5 mM KI and 2 mM H_2O_2, and 0.005 ml of enzyme solution in a total volume of 1 ml. The reaction rate was

calculated from the increase in absorbance at 350 nm due to triiodide complex (I_3^-) formation, according to the method of Hosoya (1963). A control containing no enzyme was included in the experiments, and the difference between the two rates was used to determine the rate of enzyme-catalyzed triiodide complex formation.

One unit of the enzyme activity was determined as the amount producing 1 μmol of ($[I_2] + [I_3^-]$) per one min from I^-. The equation is expressed as follows: U/ml enzyme solution = $(\Delta A350_{\text{with enzyme/min}} - \Delta A350_{\text{without enzyme/min}})/26 \times (1.3 \times 10^{-3}/[I^-]_{\text{initial}} + 1) \times$ (volume of reaction mixture/volume of enzyme solution).

4.7. K_m values for H_2O_2 and KBr

The general MCD test was performed by successively altering concentrations of each compound, KBr (0.5, 1, 5, 10, 20, 30, 40 mM final concentration) and H_2O_2 (0.1, 0.5, 2, 5, 10, 15, 20 mM final concentration) in the assay mixture. K_m values were calculated from a Lineweaver–Burk plot.

4.8. Inhibitors and activators of the enzymatic activity

Enzymatic reactions were performed in the presence of 1 mM $CuCl_2$, $ZnSO_4$, $NiCl_2$, $BaCl_2$, $CoCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $AgNO_3$, Na_2MoO_4, $VOSO_4$, $NaVO_3$, *p*-chloromercuribenzoic acid (*p*-CMB), iodoacetic acid and EDTA, respectively, using the crude enzyme preparation, which was extracted with buffer A without KBr and $NaVO_3$.

To establish the effects of specific compounds on the stability and activity of BPO, the crude enzyme was dialyzed against 10 mM MES buffer A, in the presence of 5 mM each of the above mentioned compounds, 1 and 5 mM of NaCl, 5 mM of KCl, 0.1, 0.5, 1 and 5 mM KBr for 12 h, and the activity changes were measured.

4.9. Reconstitution of the enzyme with vanadate ion

To remove the prosthetic metal ions from BPO, the pH of the enzyme solution was decreased to 2.5 using 1 M HCl, and 1 mM EDTA/Na (final concentration) was added to the solution. After 5 h incubation (complete loss of enzymatic activity), pH was increased from 2.5 to 6.85. The test solution was divided into two fractions. $NaVO_3$ (5 mM final concentration) was added to reconstitute the enzyme in the second fraction. The two test solutions were dialyzed against 10 mM MES buffer, pH 7.0, plus 1 mM EDTA/Na (final concentration) to remove excess $NaVO_3$ for 24 h. The enzymatic activity of reconstituted enzyme was measured and the degree of reconstitution evaluated.

4.10. Brominating activity and product analysis

To determine the substrates of *K. alvarezii* BPO, 0.05 ml of enzyme solution (total 0.03 U) was added to the reaction mixture consisting of 100 mM MES buffer, pH 6.5, 2 mM H_2O_2 and 2 mM KBr in a total volume of 0.5 ml. As potential substrates, 3-methyl-2-buten-1-ol (**12**), 3-methyl-3-butene-1-ol (**14**), 2-methyl-2-propene-1-ol (**15**), 3-butene-1-ol (**16**), indene (**2**), styrene (**3**), 4-chlorostyrene (**4**), *trans*-cinnamyl alcohol (**5**), cyclohexene (**6**), anisole (**17**), *o*-hydroxybenzyl alcohol (**18**), phenol red (**19**), phenylpyruvate (**20**) (Itoh et al., 1988, 2001),were used at a final concentration of 10 mM. Blank tests without H_2O_2 and KBr were additionally included. After 24 h reaction in a Bioshaker (1250 rpm, Taiteck, Tokyo) at 25 °C, the reaction mixture was subjected to centrifugation for 10 min at 13,000*g*. The supernatant was transferred and extracted with EtOAc (0.5 ml). The EtOAc extract was dried with anhyd. Na_2SO_4 and analyzed by GC–MS. Polar products from phenylpyruvate were also analyzed by GC–MS after conversion to methyl esters using trimethylsilyldiazomethane. The reagent was added drop-wise into the dried and evaporated product mixture until the emission of bubbles stopped.

A QP-2010 GC–MS (Shimadzu) equipped with a DB-VRX (J&W Scientific, 60 m × 0.25 mm i.d., 0.25 μm film thickness) was employed for GC–MS analysis. The column pressure was 130 kPa, and the linear velocity of He gas was 27 cm/s. Samples were injected in a split mode of 1:40 at 180 °C with the following temperature program: 60 °C for 5 min, increased by 10 °C/min to 250 °C and a 10 min hold at 250 °C. Mass spectra were obtained at 70 eV with an electron-impact ion source (EI, 200 °C).

trans-2-Bromo-1-indanol (**7**); retention time (t_R) 28.74 min in GC–MS, EI-MS *m*/*z* (isotope ratio, rel. int.): 212/214 $[M]^+$ (1:1, 4.2), 133 (87), 115 (100), 103/105 (70).

2-Bromo-1-phenylethanol (**8**); t_R 26.14, EI-MS *m*/*z*: 200/202 $[M]^+$ (1:1, 1), 107 (100), 103/105 (6.4).

2-Bromo-1-(4-chlorophenyl)-1-ethanol (**9**); t_R 30.44, EI-MS *m*/*z*: 234/236/238 $[M]^+$ (3:4:1, 4.3), 154 (4), 141/143 (100), 125 (25), 113/115 (80), 103 (32).

2-Bromo-3-phenyl-1,3-propanediol (**10**); t_R 32.19, EI-MS *m*/*z*: 230/232 $[M]^+$ (1:1, 1), 150 (1) 133 (13), 107 (100).

2-Bromo-1-cyclohexanol (**11**); t_R 22.15, EI-MS *m*/*z*: 178/180 $[M]^+$ (10.6), 158/160 (2.1), 132/134 (42), 122/124 (28), 108 (28), 99 (100).

4.11. Halomethane analysis

The method of Itoh and Shinya (1994) was applied for determining the volatile halomethanes from fresh algae. Four samples of *K. alvarezii* (0.5, 1.0, 2.0 and 5.0 g wet weight), washed with cool autoclaved seawater and dried with a paper towel for 2 min, were individually transferred into 20 ml vials, and sealed with silicon rubber septa and aluminum caps. Each vial was maintained at 60 °C for 15 min to ensure transfer of volatile halomethanes into the gas phase. The gas (0.1 ml) was withdrawn and ana-

lyzed by GC–MS. The above GC–MS apparatus was employed for analysis at a column pressure of 130 kPa. Samples were injected in a splitless mode at a temperature program of 40 °C for 5 min, increased by 10 °C/min to 200 °C and a 5 min hold at 200 °C. Mass spectra were obtained at 70 eV with an electron-impact ion source (EI, 200 °C).

In vitro formation of halomethanes by BPO from specific keto acids was measured in a 10.0 ml reaction mixture comprising 50 mM MES buffer, pH 6.5, 10 mM KBr, 10 mM H_2O_2, 2.0 ml of enzyme extract (total 1.2 U), 5 mM pyruvic, phenylpyruvic or oxalacetic acid, respectively, in a total volume of 20 ml within each vial as described above. Reactions were performed for 24 h at 25 °C, followed by 60 °C for 15 min. A control run was performed without the enzyme, KBr or H_2O_2.

4.12. Analysis of the stereoselectivities of bromination

To determine the absolute configuration of the bromohydrine products, analytical HPLC using a Shimadzu LC-10AT system equipped with a Chiracel OB-H (4.6 × 250 mm, Daicel Chemical Industries, Osaka, Japan) was performed for *trans*-2-bromo-1-indanol (**7**) and (*R*,*S*)-2-bromo-1-phenylethanol (**8**). The mobile phase contained *n*-hexane/2-propanol (9:1 w/w) at a flow rate of 0.5 ml/min. The product was spectrophotometrically detected at 220 nm. Retention times of racemic *trans*-2-bromo-1-indanol (**7**) were 12.3 and 16.8 min, while those of (*R*,*S*)-2-bromo-1-phenylethanol (**8**) were 16.6 and 20.5 min, respectively (Kawanami et al., 2002).

4.13. Analysis of brominated compounds in K. alvarezii

Samples of *K. alvarezii* (about 5 g) were extracted with EtOAc, MeOH and $CHCl_3$ (15 ml each), respectively, at 37 °C with stirring at 115 rpm for 48 h. Each extract was evaporated to a small amount, and a portion was subjected to GC–MS analysis (see Section 4.10 for details). Acids isolated with MeOH and $CHCl_3$ were analyzed after conversion to their methyl esters using trimethylsilyldiazomethane, as described above. Similar GC–MS conditions were applied for analysis.

4.14. Chemicals

2-Bromo-3-metyl-1,3-dihydroxybutane (**13**) was synthesized from 3-methyl-2-buten-1-ol (**12**) by bromination with *N*-bromosuccinimide (Hanzlik, 1988). 3-Methyl-2-buten-1-ol (**12**) (430 mg, 5 mmol), water (225 mg, 12.5 mmol) and *N*-bromosuccinimide (1.78 g, 10 mmol) were dissolved in 10 ml of DMSO, and maintained at room temperature for 20 min. To terminate the reaction, excess water was added to the mixture. The product was extracted with CH_2Cl_2, dried with anhydrous Na_2SO_4, and purified by silica gel chromatography using *n*-hexane and EtOAc (4:1). 2-Bromo-3-metyl-1,3-dihydroxybutane (**13**) was obtained with a yield of 78% (714 mg); ^{1}H NMR (CD_2Cl_2) δ (ppm) 1.4 (s, 3H), 1.4 (s, 3H), 4.13 (t, 1H), 4.01 (d, 2H).

Octyl-Sepharose was prepared as follows: Sepharose CL-4B (200 ml) was washed with distilled H_2O, and suspended in 0.6 M NaOH (260 ml). Epichlorohydrin (20 ml) was added to the solution, and stirred for 2 h in a water bath at 40 °C. The mixture was filtered, and the epoxy-activated Sepharose CL-4B was extensively washed with distilled H_2O. Activated Sepharose was resuspended in 0.1 M NaOH (260 ml) containing 20% dioxane (v/v) and 3.4 g of octylamine, and stirred for 20 h in a water bath at 30 °C. Next, octyl-Sepharose was filtered, and completely washed with 0.1 M NaCl containing 35% 1-PrOH (v/v), followed by distilled H_2O. All other chemicals were of analytical grade, and available commercially.

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