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Occurrence of bromoperoxidase in the marine green macro-alga, *ulvella lens*, and emission of volatile brominated methane by the enzyme

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Abstract

Bromoperoxidase activity was detected in the marine green macro-alga, *Ulvella lens*, which is used to induce the larval metamorphosis of sea urchin in aquaculture in Japan. The enzyme activity was enhanced 8.5- and 2.2-fold by the addition of cobalt and vanadium ions to the reaction mixture, respectively. The volatile halogenated compounds dibromomethane and tribromomethane were formed in the reaction mixture when the enzyme was incubated with oxaloacetate, hydrogen peroxide and potassium bromide. These results suggest that dibromomethane, which was reported to be released by *U. lens* and play an important role as the inducer of larval settlement and metamorphosis, is produced by bromoperoxidase in the alga.

Keywords: ulvella lens; Marine macro-alga; Green alga; Haloperoxidase; Bromoperoxidase; Volatile halogenated compounds; Sea urchin

1. Introduction

Many species of marine macro-algae contain a variety of halogenated secondary metabolites (Niedleman & Geigert, 1986). A halogenating enzyme, haloperoxidase (Niedleman & Geigert, 1986; Franssen, 1994), is considered to participate in their syntheses in the presence of halides and hydrogen peroxide. Among them, two bromoperoxidases from the red alga, *Corallina pilulifera* (Itoh, Izumi & Yamada, 1985, 1986; Izumi, Ohshiro & Wever, 1997) and the brown alga, *Ascophyllum nodosum* (Wever, Plat & de Boer, 1985), have been extensively studied, and were found to be non-heme enzymes containing a unique prosthetic group, vanadium (Krenn, Izumi, Yamada & Wever, 1989; de Boer, van Kooyl, Tromp, Plat & Wever, 1986b). Recently we succeeded in cloning the gene of bromoperoxidase (BPO) from *C. pilulifera* and expressing it in *Escherichia coli* (Shimonishi et al., 1998). Additionally, there are a few reports concerning haloperoxidases from green algae. A heme prosthetic group was reported for enzymes from the green algae, *Penicillus capitatus* (Manthey & Hager, 1981), *P. lamourouxii* (Barden & Corbett, 1980), and *Rhipocephalus phoenix* (Barden & Corbett, 1980). It was reported that BPO from the green alga, *Halimeda* sp., was a non-heme enzyme (Butler & Walker, 1993), but its detailed properties were not reported.

The biological halogenation by algal haloperoxidases is considered to lead to the emission of the volatile halogenated compounds such as CH_3I, CH_3Br, CH_3Cl and CH_2Br_2 (Wever, 1988; Wever, Tromp, Krenn, Marjani & van Toi, 1991; Collén, Ekdhal, Abrahamsson & Pedersén, 1994; Itoh & Shinya, 1994; Itoh, Tsujita, Ando, Hisatomi & Higashi, 1997), and these compounds are recognized as substrates that destroy the ozone layer. It has also been demonstrated

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Table 1
Partial purification of BPO from *U. lens*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold	Yield (%)
Cell-free extract	570	6.21	0.011	1	100
DEAE-Sepharose	258	5.38	0.021	1.91	86.6
Phenyl-Toyopearl	68.9	4.00	0.058	5.27	64.4
1st Toyopearl HW-55	6.95	0.48	0.069	6.27	7.73
2nd Toyopearl HW-55	6.72	0.46	0.068	6.18	7.41

that they are involved in the defense mechanism (allelopathy) of the alga (Franssen, 1994). Such compounds are one of several types of allelochemicals produced by algae. For instance, the red alga, *Neodilsea yendoana* produces a polyunsaturated fatty acid which inhibits growth of the foliaceous green alga, *Monostroma oxyspermum* (Suzuki, Wakana, Denboh & Tatewaki, 1996), and *C. pilulifera* released volatile halomethanes which had suppressive effects on the development of a brown alga, *Laminaria angustata* sporelings (Denboh, Suzuki, Mizuno & Ichimura, 1997). In addition, a volatile halogenated compound, dibromomethane, was reported to induce larval settlement and metamorphosis of the sea urchin, *Strongylocentrotus nudus* (Taniguchi, Kurata, Maruzoi & Suzuki, 1994a; Taniguchi, Kurata & Suzuki, 1994b). It was noted that this inducer was released from several algae, including the green alga, *Ulvella lens*.

The present study reports BPO activity in *U. lens*, partial purification of the enzyme from the green alga, and the formation of volatile brominated compounds by the partial purified enzyme in the presence of oxaloacetate, hydrogen peroxide and potassium bromide.

2. Results and discussion

2.1. Partial purification of bromoperoxidase

It was difficult to extract proteins from the green alga, *U. lens*; repeated operation of a Dyno-mill was required to disrupt the cells, and led to the extraction of only 570 mg protein from 500 g (wet weight) algae. Further operation did not improve the extraction efficiency of protein from the algae. The specific activity of *U. lens* enzyme in cell-free extract (0.011 units/mg protein) was 30- and 90-fold less than those of *C. pilulifera* (Itoh et al., 1985) and *A. nodosum* (Wever et al., 1985), respectively, which were reported to be high producers of BPO. A typical partial purification is summarized in Table 1.

2.2. Effect of metal ions on enzyme activity

The spectrum of the partially purified enzyme (3.7 mg-protein/ml) did not show any absorption peaks other than at 280 nm; sodium azide did not inhibit enzyme activity (data not shown), suggesting that the enzyme did not have a heme prosthetic group. Various metal ions were added to the reaction mixture to examine their effects on BPO activity. As shown in Table 2, 1 mM cobalt and vanadium ions enhanced the enzyme activity 8.5- and 2.2-fold, respectively. On the contrary, other metal ions, Ni^{2+}, Zn^{2+}, Cu^{2+} (1 mM), and Fe^{3+} (0.1 mM), inhibited the enzyme activity, suggesting that a metal might be involved in the catalytic site. Although several vanadium-dependent BPOs were found in marine macro-algae such as *C. pilulifera* (Krenn et al., 1989), *A. nodosum* (de Boer et al., 1986b), *C. officinalis* (Sheffield, Harry, Smith & Rogers, 1993) and *Laminaria saccharina* (de Boer, Tromp, Plat, Krenn & Wever, 1986a), there are no reports of cobalt-dependent haloperoxidases from marine macro-algae. It was reported that a bacterium, *Pseudomonas putida*, produced BPO containing cobalt ion (Itoh, Morinaga & Kouzai, 1994) and the metal ion stimulated enzyme activity. The results obtained in this study may indicate that BPO from *U. lens* is a non-heme protein and cobalt-dependent. However, further study is needed to examine the role of cobalt and vanadium for BPO activity.

2.3. Volatile halide production from oxaloacetate by BPO from U. lens

Some organic acids, especially keto acids, are recog-

Table 2
Effect of metal ions on BPO activity

Compound	Conc. (mM)	Relative activity (%)
None		100
$CaCl_2$	1	87
$MgSO_4$	1	96
$MnSO_4$	1	78
$CoSO_4$	1	857
$NiCl_2$	1	26
$ZnCl_2$	1	39
$CuCl_2$	1	52
$FeCl_3$	0.1	30
$NaVO_3$	1	221

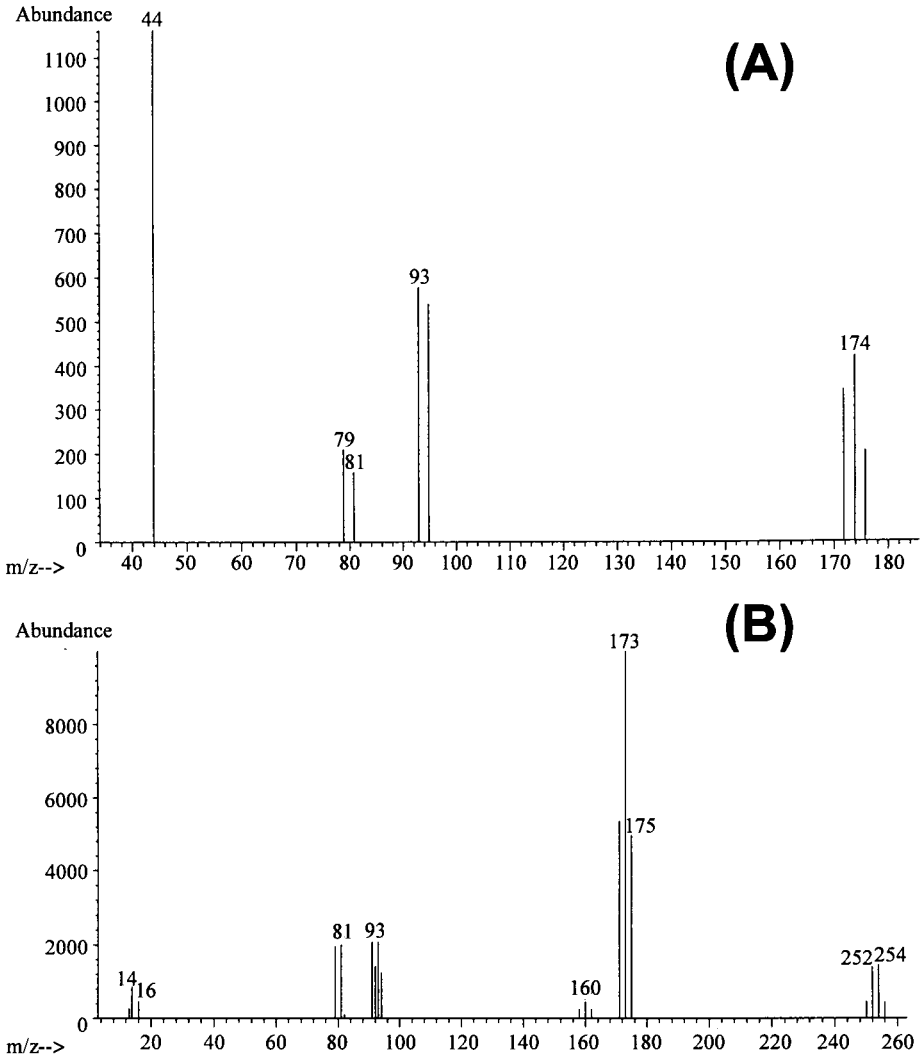

Fig. 1. GC-Mass spectra of the volatile compounds produced by BPO from *U. lens*. The mass spectra of the peaks at: (A) 13.8 min and (B) 19.3 min separated by GC.

nized as substrates for volatile halomethane formation by BPO. It was shown that a marine red alga, *Bonnemaisonia hamifera*, produced bromoform and dibromomethane from 3-oxooctanoic acid (Theiler, Cook & Hager, 1978). The simpler keto acids including oxaloacetate and 2-ketoglutarate were shown to serve as the substrate of the purified BPO from *C. pilulifera*, and bromomethane was released (Itoh & Shinya, 1994). In the present report, the formation of volatile halomethanes was examined using the partially purified BPO preparation from *U. lens* according to the method used for the *C. pilulifera* enzyme (Itoh & Shinya, 1994). When the enzyme reaction was performed with oxaloacetate as a halogen acceptor, the GC chromatogram of volatile products showed new peaks (retention time; 13.8 and 19.3 min) which did not appear in control lacking BPO. The GC-MS spectra of the new peaks are shown in Fig. 1. The peaks with retention time at 13.8 and 19.3 min corresponded to dibromomethane and tribromomethane, respectively. In this experiment, the enzyme from *U. lens* catalyzed the halogenation reaction without the presence of added cobalt or vanadium in the reaction mixture. As shown in Table 2, the enzyme from *U. lens* was active without added cobalt or vanadate. In the case of *C. pilulifera*, the purified, vanadium-dependent BPO was active without adding vanadate to the reaction mixture (Itoh et al., 1985; Krenn et al., 1989). The enzymes of these algae may contain some metal(s) required for activity, which are not removed by the purification procedures.

Thus, these data demonstrate that BPO from *U. lens* could produce volatile halomethane from oxaloacetate; volatile halomethane was previously reported to induce larval settlement and metamorphosis of sea urchin by Taniguchi et al. (1994a, 1994b).

3. Experimental

Ulvella lens was grown on transparent, colorless acrylic resin plates (580 × 300 × 0.5 mm) for approximately two months at the Hokkaido Aquaculture Development Authority, Kayabe-gun, Hokkaido, in early spring, then scraped by spatulas and stored at −20°C until use. Enzyme purification was performed below 15°C. Algal cells (500 g, wet weight) were suspended in 500 ml of 50 mM Tris-SO_4 buffer (pH 7.4) and disrupted by ϕ 0.5 mm glass beads through a Dyno-mill homogenizer (Willy A. Bachofen, Basel, Switzerland) five times. The cell-free extract was obtained by centrifugation at 12,000 × *g* for 30 min, and dialyzed against 50 mM Tris-SO_4 buffer (pH 7.4). The dialyzed enzyme solution was applied onto a DEAE-Sepharose Fast Flow column (ϕ 3.6 × 30 cm) equilibrated with 50 mM Tris-SO_4 buffer (pH 7.4). The column was washed with the same buffer, and the enzyme was eluted with the same buffer containing 0.8 M NaCl at a flow rate of 60 ml/h. The active fractions were combined, dialyzed against 50 mM Tris-SO_4 buffer (pH 7.4) containing 0.8 M $(NH_4)_2SO_4$, and applied onto a Phenyl Toyopearl column (ϕ 1.8 × 19 cm) equilibrated with the dialysis buffer. The column was washed with the same buffer and eluted with a linear $(NH_4)_2SO_4$ gradient (from 0.8 to 0 M) in 50 mM Tris-SO_4 buffer (pH 7.4), using a flow rate of 10 ml/h. The active fractions were combined and concentrated by ultrafiltration. The concentrated enzyme was applied to a Toyopearl HW-55 gel filtration column (ϕ 3.6 × 30 cm) equilibrated with 50 mM Tris-SO_4 buffer (pH 7.4) containing 0.1 M KCl at a flow rate of 3 ml/h. The combined active fractions were concentrated by ultrafiltration and re-applied to a Toyopearl HW-55 column.

Enzyme activity was determined using monochlorodimedone (MCD, Sigma, St. Louis, USA) as described (Yamada, Itoh, Murakami & Izumi, 1985) with slight modifications. The assay mixture contained 20 mM KBr, 60 μM MCD, 2 mM H_2O_2, 100 mM Tris-SO_4 buffer (pH 7.4) and enzyme in a final volume of 1 ml. The decrease in the absorbance of MCD at 290 nm was measured at 40°C, using a molar extinction coefficient of 19.9. One unit of enzyme activity was defined as the amount of protein catalyzing the conversion of 1 μmol of MCD per min. The activity for each column chromatography fraction was qualitatively measured using phenol red as described previously (de Boer et al., 1987). The assay mixture was the same as described above except that 50 μM phenol red was added instead of MCD. The purple color was detected due to the formation of bromophenol blue by the bromination of phenol red. Protein determination was performed according to the method of Bradford (1976) using bovine serum albumin as a standard protein.

The enzymatic reaction to release the volatile halogenated compounds was carried out according to the procedure described previously (Itoh & Shinya, 1994). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 10 mM KBr, 1 mM oxaloacetic acid, 1 mM H_2O_2 and 0.0023 units/ml BPO from *U. lens* in a total volume of 30 ml. A vial (30 ml headspace) containing the reaction mixture was allowed to stand for 2 h at 20°C. A control was performed without BPO. The gas phase was analyzed by the headspace method using a GC-MS instrument. Analysis of the products was performed with a Hewlett Packard gas chromatograph (HP5890-II) fitted with a capillary column (Rtx-volatiles, 60 m: 0.25 mm id: 1 μm) and a Hewlett Packard mass spectrometer (HP-5971A).

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References

Barden, D. G., & Corbett, M. D. (1980). *Biochem. J*, *187*, 205.
Bradford, M. (1976). *Anal. Biochem*, *72*, 248.
Butler, A., & Walker, J. V. (1993). *Chem. Rev*, *93*, 1937.
Collén, J., Ekdhal, A., Abrahamsson, K., & Pedersén, M. (1994). *Phytochemistry*, *36*, 1197.
de Boer, E., Plat, H., Tromp, M. G. M., Wever, R., Franssen, M. C. R., van der Plas, H. C., Meijer, E. M., & Schoemaker, H. E. (1987). *Biotechnol. Bioeng*, *30*, 607.
de Boer, E., Tromp, M. G. M., Plat, H., Krenn, G. E., & Wever, R. (1986a). *Biochim. Biophys. Acta*, *872*, 104.
de Boer, E., van Kooyl, Y., Tromp, M. G. M., Plat, H., & Wever, R. (1986b). *Biochim. Biophys. Acta*, *869*, 48.
Denboh, T., Suzuki, M., Mizuno, Y., & Ichimura, T. (1997). *Bot. Mari*, *40*, 249.
Franssen, M. C. R. (1994). *Biocatalysis*, *10*, 87.
Itoh, N., Izumi, Y., & Yamada, H. (1985). *Biochem. Biophys. Res. Commun*, *131*, 428.
Itoh, N., Morinaga, N., & Kouzai, T. (1994). *Biochim. Biophys. Acta*, *1207*, 208.
Itoh, N., Izumi, Y., & Yamada, H. (1986). *J. Biol. Chem*, *261*, 5194.
Itoh, N., & Shinya, M. (1994). *Mar. Chem*, *45*, 95.
Itoh, N., Tsujita, M., Ando, T., Hisatomi, G., & Higashi, T. (1997). *Phytochemistry*, *45*, 67.
Izumi, Y., Ohshiro, T., & Wever, R. (1997). In D. B. Janssen, K. Soda, & R. Wever, *Mechanisms of Halogenation and Dehalogenation* (p. 69). Dordrecht: Kluwer Academic Publishers.

Krenn, B. E., Izumi, Y., Yamada, H., & Wever, R. (1989). *Biochim. Biophys. Acta*, *998*, 63.

Manthey, J. A., & Hager, L. P. (1981). *J. Biol. Chem*, *256*, 11232.

Niedleman, S. L., & Geigert, J. (1986). *Biohalogenation — Principles, Basic Roles and Applications*. Chichester: Ellis Horwood.

Sheffield, D. J., Harry, T., Smith, A. J., & Rogers, L. J. (1993). *Phytochemistry*, *32*, 21.

Shimonishi, M., Kuwamoto, S., Inoue, H., Wever, R., Ohshiro, T., Izumi, Y., & Tanabe, T. (1998). *FEBS Lett*, *428*, 105.

Suzuki, M., Wakana, I., Denboh, T., & Tatewaki, M. (1996). *Phytochemistry*, *43*, 63.

Taniguchi, K., Kurata, K., Maruzoi, T., & Suzuki, M. (1994a). *Fish. Sci*, *60*, 795.

Taniguchi, K., Kurata, K., & Suzuki, M. (1994b). *Kagaku to Seibutsu (in Japansese)*, *32*, 434.

Theiler, R., Cook, J. C., & Hager, L. P. (1978). *Science*, *202*, 1094.

Wever, R. (1988). *Nature*, *335*, 501.

Wever, R., Plat, H., & de Boer, E. (1985). *Biochim. Biophys. Acta*, *830*, 181.

Wever, R., Tromp, M. G. M., Krenn, B. E., Marjani, A., & van Toi, M. (1991). *Environ. Sci. Technol*, *25*, 446.

Yamada, H., Itoh, N., Murakami, S., & Izumi, Y. (1985). *Agric. Biol. Chem*, *49*, 2961.