Vanadium-dependent bromoperoxidases from *Gracilaria* algae

Tuangporn Suthiphongchai · Patcharee Boonsiri · Bhinyo Panijpan

Abstract Red algae from the Gulf of Thailand were examined for haloperoxidatic activity. Six species, *Gracilaria changii*, *G. edulis*, *G. firma*, *G. fisheri*, *G. salicornia*, and *G. tenuistipitata*, showed bromoperoxidatic activity. Duplicate polyacrylamide electrophoretic gels showed enzyme activity patterns developed by phenol red staining for bromoperoxidatic activity and by 3,3′-diaminobenzidine staining for peroxidatic activity. All algae gave isoenzymic bromoperoxidatic activity bands and peroxidatic activity bands, but there were peroxidatic and bromoperoxidatic activity bands that did not correspond. The bromoperoxidatic activity of the crude enzyme extracts as well as previously dialyzed enzyme solutions was enhanced significantly by incubation with vanadium pentoxide. The three purified bromoperoxidases from *G. fisheri* contained vanadium, and their relative activities corresponded to the ratio of vanadium to enzyme. In addition, they were not inhibited by H_2O_2. These data confirm that the enzymes are vanadium bromoperoxidases.

Keywords Bromoperoxidase staining · Red algae · V-BPO

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Abbreviations

Apo-BPO	Apo-bromoperoxidase
BPO	Bromoperoxidase
DAB	3,3′-Diaminobenzidine
HPO	Haloperoxidase
GfL	Low-molecular-weight bromoperoxidase from *G. fisheri*
GfH	High-molecular-weight bromoperoxidase from *G. fisheri*
MCD	Monochlorodimedone
U	Unit of enzyme activity (μmole MCD decrease.min^{-1})
V-Apo	Vanadium-reactivated apo-bromoperoxidase
V-BPO	Vanadium bromoperoxidase

Introduction

Haloperoxidases are the principal enzymes reported to catalyze the halogenation reaction to synthesize a fascinating number of halogenated compounds that are widespread in nature (Neidleman and Geigert 1986), from bacteria (Neidleman 1975) to algae (Fenical 1975) to mammals. These compounds, some very useful as pesticides or as pharmaceutical active agents, are believed to provide selective environmental protection against predators or microbial colonization (Ohsawa et al. 2001; Pereira et al. 2003). Because of its ability to catalyze the halogenation of diverse halogenated compounds, the haloperoxidase has attracted the interest of the pharmaceutical and chemical industries (Neidleman and Geigert 1986; Alaee et al. 2003; Butler and Carter-Franklin 2004; Cardozo et al. 2007). The general reaction is as follows:

$$AH + H_2O_2 + X^- + H^+ \xrightarrow{HPO} AX + 2H_2O$$
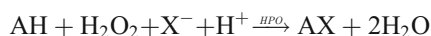
where AH = organic substrate, X⁻ = halide ions: Cl⁻, Br⁻, I⁻, and HPO = haloperoxidase.

In general, haloperoxidases are hemoproteins containing ferriprotoporphyrin IX as prosthetic group. Heme-containing bromoperoxidases (BPOs) are also found in some marine algae, for instance, the red algae *Cystoclonium purpureum* (Pedersen 1976) and *Rhodomela larix* (Ahern et al. 1980). However, a nonheme BPO was first discovered in the brown alga *Ascophyllum nodosum* and proved to have vanadium (V) as the prosthetic group (de Boer et al. 1986). This enzyme can be inactivated by dialyzing against EDTA at low pH and specifically reactivated by vanadium (Vilter 1984). Several nonheme vanadium BPOs (V-BPOs) have since been isolated from brown algae *Fucus distichus* and *Macrocystis pyrifera* (Soedjak and Butler 1991) and the *Laminariaceae* (Almeida et al. 2001), and red algae *Corallina pilulifera* (Krenn et al. 1989), *C. officinalis* (Yu and Whittaker 1989), *C. vancouveriensis* (Everett et al. 1990), *Gracilaria fisheri* (Suthiphongchai et al. 1994; Kongkiattikajorn and Pongdam 2006) and others. These V-BPOs have been shown to catalyze halogenation and oxidation of a variety of organic substrates such as alkenes, aromatics, and thiophene (Itoh et al. 1988), bromination and cyclization of terpenes, and brominative oxidation of indoles (Butler and Carter-Franklin 2004). These vanadium enzymes are more resistant to azide, cyanide and hydrogen peroxide than the heme-containing ones, pointing to some advantages with respect to commercial applications of the former.

Here we developed a staining method for BPO that can distinguish BPO from peroxidase (PO) activity and used it in a survey of the presence of BPOs in *Gracilaria* red seaweeds and for staining BPO isozymes in nondenaturing polyacrylamide gel electrophoresis (ND-PAGE). Six species of *Gracilaria* algae were found to possess several BPO isoforms with different band patterns. We have purified three isoenzymes of BPO from *G. fisheri* and showed that they are V-BPO with high resistance to hydrogen peroxide.

Methods

Algal screening and extraction of bromoperoxidases. Algae of the genus *Gracilaria* were collected from eastern and southern coasts of Thailand, namely *Gracilaria changii* (Xia & Abbott) Abbott, Zhang & Xia from Ao Lane and Lam Sok, Trad Province (GC1 and 2); *G. edulis* (Gmelin) Silva and *G. irregularis* Abbott from Ao Lane; *G. firma* Chang & Xia and *G. salicornia* (C. Agardh) Dawson from Lam Tian; *G. fisheri* (Xia & Abbott) Abbott, Zhang & Xia 1 and 2 from Pattani Province and culture pond in the Rayong Province; and *G. tenuistipitata* Chang & Xia from Pattani Province (Lewmanomont 1993). The algae were washed and stored frozen at −20°C.

The algae were screened for BPO activity at the sites of collection by using agarose microscopic slides containing 50 μM phenol red, 50 mM KBr, and 2.4 mM H_2O_2 in 0.1 M phosphate buffer pH 5.8.

The seaweeds were extracted by homogenization at one volume of 20 mM Tris-HCl pH 8.0 per gram of seaweed wet weight in a Waring blender. After centrifugation at 4,000 g, 4°C for 30 min, the pink supernatant was collected as a crude extract.

Assay for bromoperoxidatic activity. The bromoperoxidatic activity was measured by following the initial rate for 1 min in the conversion of monochlorodimedone (MCD) to monobromomonochlorodimedone at A_{290nm} (Hager et al. 1966) at 25°C. The assay mixture contained 30 μM MCD, 4 mM KBr, and 1.6 mM H_2O_2 in 0.1 M phosphate buffer pH 5.8; the reaction was started by addition of enzyme extract. The enzyme activity unit (U) was in μmole MCD.min⁻¹.

The enzyme activity was also measured by using phenol red as substrate. The reaction was determined by following the increase in A_{592nm} due to brominated products (de Boer et al. 1987) for 10 min in the reaction mixture containing 30 μM phenol red, 4 mM KBr, and 1.6 mM H_2O_2 in 0.1 M phosphate buffer pH 5.8 at 25°C.

Assay for protein. Protein concentration was determined by Coomassie Brilliant Blue using bovine serum albumin as standard (Bradford 1976).

Activation of enzyme extracts and apoenzymes by vanadium. The crude enzymes at an activity of about 0.2–0.3 U (MCD).mL⁻¹ were activated by incubating with 50 μM vanadium pentoxide (V_2O_5) in Tris-HCl pH 8.0 at 4°C for 2 days. V_2O_5 was first dissolved in 0.1M NaOH; in this condition vanadium would exist in VO_4^{3-} form (Greenwood and Earnshaw 1984). The pH was then adjusted to 8 before addition to the enzyme solution to yield a final concentration of 50 μM V_2O_5, which was equivalent to 0.1 mM vanadate. At this low concentration, vanadium mainly exists as protonated orthovanadate (HVO_4^{2-}, $H_2VO_4^-$) (Rehder 1991). To obtain the deactivated apo-bromoperoxidases (Apo-BPO), these vanadium-activated enzymes (V-BPO) were dialyzed against 1 mM EDTA in citrate phosphate buffer pH 3.8 (Vilter 1984), followed by water, and finally against 20 mM Tris-HCl pH 8.0. For reactivation of the deactivated enzymes, the apoenzymes were re-incubated with 50 μM V_2O_5 for 4 days at 4°C to obtain vanadium-reactivated apoenzyme (V-Apo).

Resistance to azide and cyanide. The enzyme activity of algal extracts was determined in the reaction mixture containing various concentrations of azide or cyanide.

Nondenaturing polyacrylamide gel electrophoresis. (ND-PAGE) ND-PAGE was performed on the slab gel of 5–15% gradient according to the modified method of Davis (1964). The gel was stained for bromoperoxidatic activity; the staining solution contained 80 µM phenol red, 50 mM KBr and 2.4 mM H_2O_2 in 0.1 M phosphate buffer pH 5.8. The PO staining was carried out in a solution containing 0.5 mM 3,3′-diaminobenzidine (DAB) and 1 mM H_2O_2 with or without 10 mM KBr in 0.1 M phosphate buffer pH 5.8 (Olsen and Little 1979).

Purification of bromoperoxidases from G. fisheri. The crude extract of *G. fisheri* was brought to 60% saturation with ammonium sulfate. The precipitated protein was recovered by centrifugation and redissolved in 20 mM Tris-HCl buffer pH 8.0. After dialysis against the same buffer, the dialysate was loaded onto a column of diethylaminoethyl (DEAE) cellulose (Whatman) and eluted by a gradient of 0–0.8 M NaCl in that buffer. Fractions containing enzyme activity were pooled separately. Peak 2 and peak 3 were further purified by using Mono-Q column and Superose 12 column sequentially to obtain one major peak.

Determination of vanadium in the enzymes. The purified enzyme species were incubated with 50 µM V_2O_5 for 5 days, then the unbound vanadium was removed by centrifuge through Sephadex G25. After hydrolysis with 6 M HCl at 110°C for 20 h, vanadium content in the vanadium-activated enzymes and purified enzymes was determined by atomic absorption spectrometer model 3100 (Perkin Elmer) with HGA600 graphite furnace at 318.4 nm irradiated by vanadium hollow-cathode lamp (Buchet JP. 82).

SDS polyacrylamide gel electrophoresis. Purified proteins from *G. fisheri* were analyzed by 10% SDS-PAGE under reducing conditions according to the method of Laemmli (1970). Protein was stained with 0.2% Coomassie Brilliant Blue R250.

Results

Screening of algae for bromoperoxidatic activity. Algae along the eastern and southern parts of the Gulf of Thailand were screened on the spot for bromoperoxidatic activity by immersing the plant tissues in agar coated on microscopic slides containing bromide, H_2O_2 and phenol red. Algae possessing substantial bromoperoxidatic activity would render the yellow color (due to phenol red) surrounding the algal tissues blue (due to brominated phenol red) as shown in Fig. 1. For this type of screening, it was found that *G. changii* from Lam Sok (GC2), Trad Province, and *G. fisheri* (GFis) from Pattani and Rayong Provinces produced blue color within 20 min. As for *G. tenuistipitata* (GT), *G. edulis* (GE), *G. salicornia* (GS), *G. firma* (GFir) and *G. changii* from Ao Lane (GC1), Trad Province, the blue color appeared after 1–2 h, whereas no color change was observed after 3 h for *G. irregularis* (GI).

Extracts of the separately collected algae were later studied spectrophotometrically to confirm the haloperoxidatic activity. Only bromoperoxidatic activity (using only bromide and iodide ions) was found, and no chloroperoxidatic activity was found over a wide pH range; the substrates used were phenol red (for I⁻, Br⁻, and Cl⁻) and MCD (for Br⁻ and Cl⁻). Using MCD as substrate, crude extracts from GC, GE, GFis, and GT were found to contain about 0.15–0.3 $U.g^{-1}$ algal wet weight, whereas those of GFir and GS had lower activity at about 0.05 U/g wet weight.

Vanadium activation of bromoperoxidases. Enzyme extracts of the algae were adjusted to about 0.2–0.3 activity $U.mL^{-1}$ before incubation with vanadium pentoxide to determine activation by vanadium ion. After 48-h incubation at 4°C, nearly all algal extracts were activated (Fig. 2). The two GFis were activated up to eight times their extract activities giving them the highest enzyme activity per wet weight. There was some variation across different batches of algae; for instance, the range of vanadium activation of GFis was 4- to 10-fold the native enzyme, and for GC it was 1.7- to 3-fold. This variation could occur due to

Fig. 1a, b Screening of algae containing bromoperoxidases by using agar slides. The agar slides contained 0.05 mM phenol red, 50 mM KBr and 2.4 mM H_2O_2 in phosphate buffer pH 5.8. **a** Algae showing substantial bromoperoxidase activity. **b** Algae containing low or no bromoperoxidase
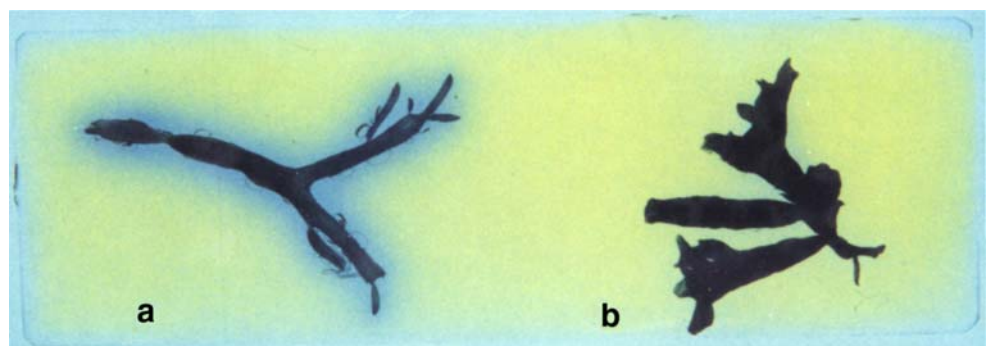
seasonal variation as has been reported by Itoh et al. (1996) who described that seasonal variation of BPO activity of *C. pilulifera* is regulated by vanadium.

Upon dialyzing against 1 mM EDTA at low pH, these activated enzyme extracts became deactivated (Fig. 2). Most of the dialyzed enzyme extracts, upon re-incubation with vanadium, became clearly reactivated. GC2, GFir and two of the GFis were reactivated to about 100% of their vanadium-activated activities before dialysis. However, the GT activity was lost after dialysis and was apparently not restored. Tris-HCl alone, vanadium in Tris buffer, and also vanadium incubated with heated enzyme extract were used as negative controls.

Bromoperoxidase isoenzyme patterns on native electrophoretic gel. Nondenaturing electrophoretic gels of vanadium-incubated algal extracts were subjected to phenol red staining for bromoperoxidatic activity (Fig. 3b). All the above algal samples showed two to three major isoenzyme bands after 2–3 min staining and several minor bands after prolonged incubation. Different species of algae showed different isoenzyme patterns. Nearly all algal extracts gave BPO bands at Rf of about 0.78, albeit at different intensities. Samples of the same species from different locations, i.e., GC and GFis, showed very similar isoenzyme patterns.

It is well known that haloperoxidases show PO activity (Neidleman and Geigert 1986). Indeed, the use of PO staining for chloroperoxidatic and bromoperoxidatic activities is common. We thus stained duplicate electrophoretic gels using DAB for PO activity. It was found that all BPO bands correspondingly showed PO activity (Fig. 3). However, there were PO bands that did not correspond to any BPO bands even after overloading the gels, e.g., PO of GT
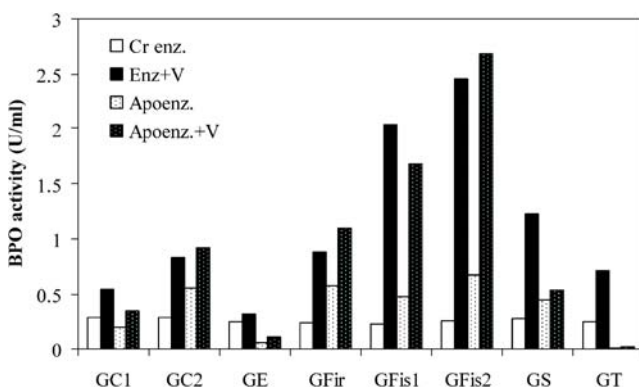
Fig. 2 Effects of vanadium on the activation of bromoperoxidase and apo-bromoperoxidase. *Cr enz.* Crude enzyme extracts, *enz+V* V-BPO (enzyme incubated with 0.1 mM vanadium for 48 h), *Apoenz.* apoenzyme (V-BPO dialyzed against EDTA), *Apoenz.+V* V-Apo (apoenzyme incubated with 0.1 mM vanadium for 4 days). All experiments were performed with crude extracts from various *Gracilaria* species with an activity of about 0.2–0.3 $U.mL^{-1}$

at Rf 0.65, 0.80 and 0.83, as shown by the black arrows in Fig. 3a. Moreover, these latter PO activity bands did not require bromide ions to exhibit enhanced intensity. On the contrary, PO bands that corresponded with BPO bands needed the bromide ion for more intense PO staining (data not shown).

Purification of bromoperoxidases. Three BPO isoenzymes were purified from *G. fisheri*. The reddish color of phycobiliprotein, the major soluble protein in red algal extract, was one of the major problems in purification. It required more than one ion exchange column to eliminate this protein. The results of purification are summarized in Table 1.

Three enzyme activity peaks, minor peak 1, middle peak 2, and major peak 3, were eluted from the first DEAE column pH 8.5. This step eliminated a great deal of reddish protein from peak 2 and peak 3. However, the minor peak 1 was eluted at nearly the same time as phycobiliprotein with low specific activity. Thus, no further purification of these was pursued.

Peak 2 was further purified by MonoQ 10/10 pH 8.0, from which two peaks of the enzyme were eluted. Based on Superose, peak 1 from MonoQ was a low-molecular-weight protein of 70 kDa (GfL1) corresponding to the band at Rf 0.78 of native electrophoretic gel, whereas peak 2 was a high-molecular-weight protein of about 560 kDa (GfH) corresponding to the band at Rf 0.16. The major peak 3 was further purified by a sequence of second DEAE, MonoQ, and Superose 12 chromatography to obtain one major peak. The molecular weight calculated by Superose 12 was 70 kDa (called GfL2) corresponding to the band at Rf 0.78.

Molecular properties. Three purified BPOs from Superose 12 were characterized. From the SDS-PAGE study, the large BPO (GfH) suggested an oligomeric structure with a subunit of 68 kDa because it gave rise to a 68 kDa band in SDS gel. The small enzymes, GfL1 and GfL2, also showed the same molecular size of 68 Da on SDS gel. The optical spectra of these three enzymes showed no Soret band (Fig. 4), indicating nonheme enzymes.

Vanadium activation and determination. All three purified enzymes were activated by vanadium, and GfH was highly activated to about 7–10 times its purified activity. The molar ratio of vanadium to protein was determined in vanadium-activated enzyme. GfL1 and GfL2 contained about 1 mole of vanadium to 1 mole of enzymes, and GfH contained about 1 mole of vanadium to 1 mole of enzyme subunit. The vanadium content of the purified enzyme was determined and compared to the enzyme activity as the percentage of the vanadium-activated activity. The amount of vanadium content in these three enzymes correlated quite

Fig. 3a, b Nondenaturing poly-acrylamide gel electrophoresis (5–15%) of eight algal samples. **a** Peroxidase staining by 3,3′-diaminobenzidine in the presence of Br⁻ ion. **b** Bromoperoxidase staining by phenol red. Enzyme load=0.8 A_{590}/10 min of brominated phenol red. *Black arrows* show PO activity bands with no BPO activity
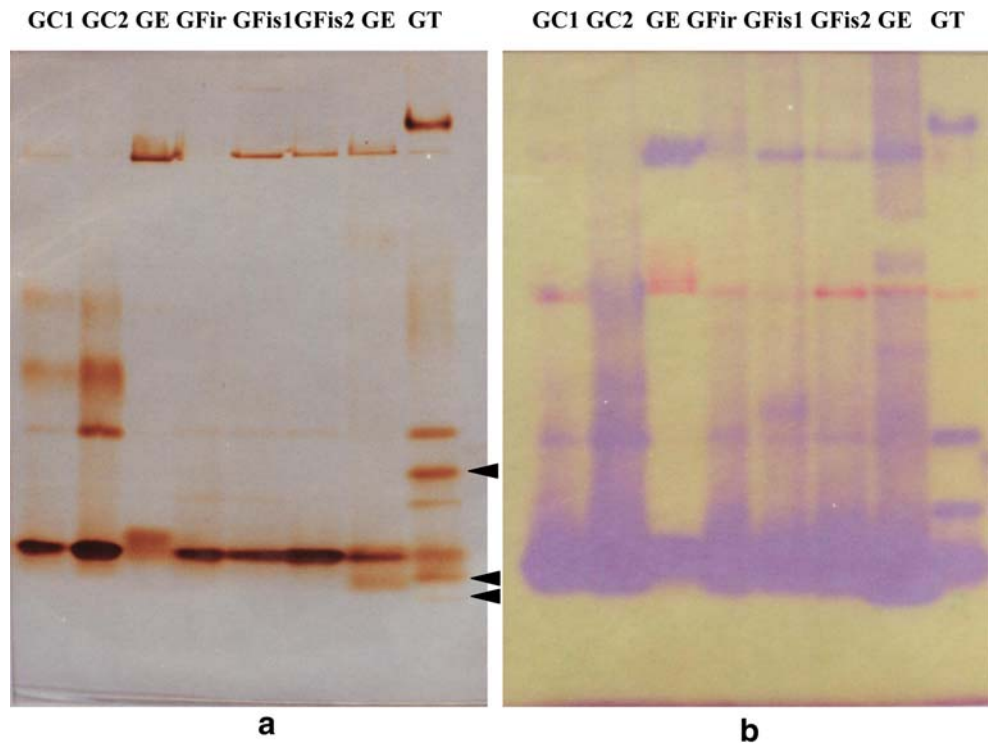
well with the enzyme activity, for instance GfH contained about 0.18 mole of vanadium to 1 mole of its subunit, and its activity was about 15% of that of vanadium-activated (Table 2).

Effects of azide, cyanide and high concentration of hydrogen peroxide on BPO activities. Generally V-BPO exhibits high chemical stability against azide, cyanide and H_2O_2, which inhibit heme-containing HPO; therefore we monitored the activity of our enzymes in the presence of these chemicals. All three enzymes were still fully active at high concentrations of H_2O_2 up to more than 10 mM

(Fig. 5a). Surprisingly, all three were inhibited by cyanide (Fig. 5b). As for azide, the high-molecular-weight enzyme, GfH, was more resistant (about 30% inhibition by 0.1 mM azide) than the low-molecular-weight enzymes, GfL1 and 2 (nearly 100% inhibition by 0.1 mM azide) (Fig. 5c).

Discussion

The use of agar-coated slides containing phenol red, H_2O_2, and bromide has proved to be very effective in field

Table 1 Purification of BPOs from *G. fisheri* in Tris-HCl

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U.mg^{-1})	Purification fold	% Yield
Crude enzyme	907.5	1,430	1.6	1	100
$(NH_4)_2SO_4$ precipitation	888.1	1,660	1.9	1.2	116.1
DEAEI Peak 1	18	74	4.1	2.6	5.2
DEAEI Peak 2	5.9	70.7	12.0	7.6	4.9
MonoQI Peak 1	1.7	31.2	18.4	11.6	2.2
SuperoseI (GfL1)	0.056	11.08	197.9	125.6	0.8
MonoQI Peak 2	2.2	22.1	10.1	6.4	1.5
SuperoseI (GfLi)	0.87	13.7	15.7	10.0	1.0
DEAEI Peak 3	44.8	1,260	28.1	17.8	88.1
DEAEII	21	855	40.7	25.8	59.8
MonoQI	5.5	525.3	95.5	60.6	36.7
MonoQII	2.9	421.9	145.5	92.3	29.5
SuperoseII (GfL2)	0.35	109	311.4	197.6	7.6
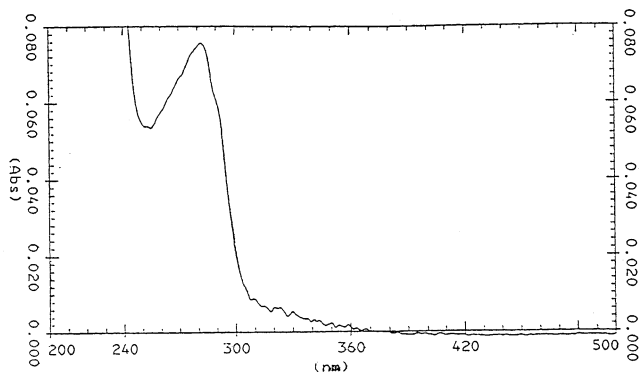
Fig. 4 UV and visible spectrum of bromoperoxidase (GfL1) from *G. fisheri* shows no Soret band in the visible range

screening for algae with relatively high BPO activity. The slides, kept in cool and moist closed slide boxes, are usable several weeks after preparation, making them convenient for use in an extensive field survey, especially in out-of-the-way places.

The spectrophotometric assays of enzyme extracts using phenol red and/or MCD as substrates in the presence of bromide and chloride over a wide pH range showed that the bromide ion and not the chloride ion is used in the haloperoxidatic reaction; i.e., the activity is confined to that of bromoperoxidation. MCD has the advantage of giving only one product but suffers from being detectable only in the UV range, whereas phenol red gives colored products but can be brominated more than once giving multiple products.
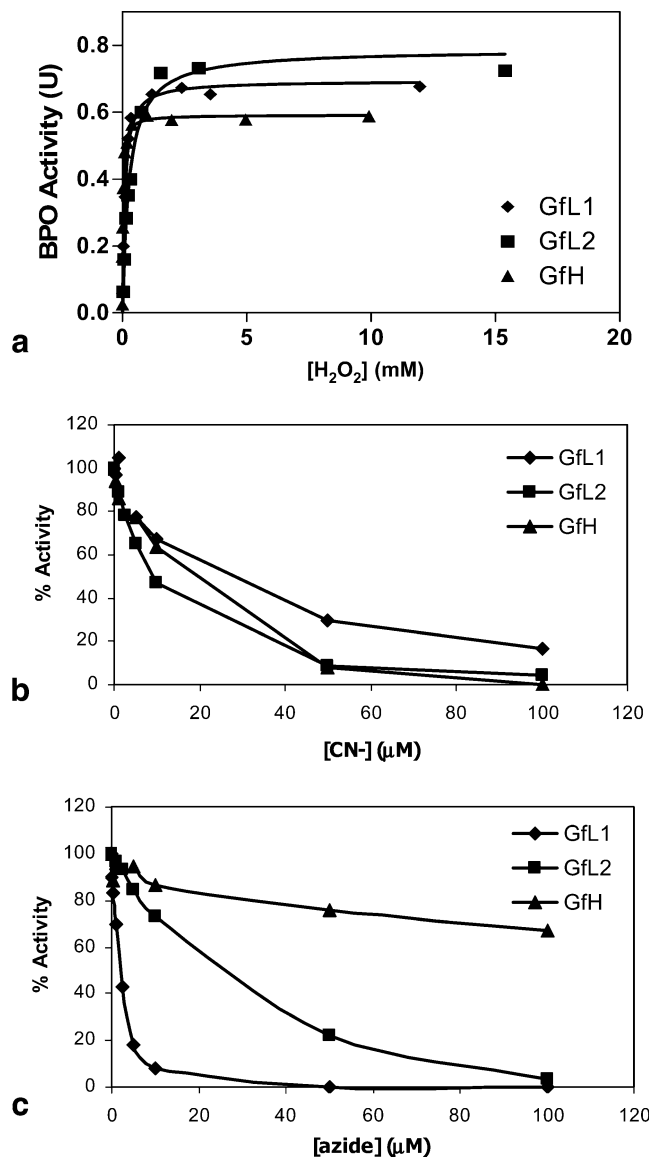
The activation of bromoperoxidatic activity in the crude extracts by vanadium ion indicates that at least some BPOs were nonheme and required vanadium for their activities, similar to some known vanadium-BPOs (Almeida et al. 2001). Moreover, the role of vanadate was also demonstrated in vivo. Addition of vanadate and bromide to in vivo microplantlet suspension culture of red alga *Ochtodes secundiramea* turned on biosynthesis of halogenated monoterpene (Polzin et al. 2003). Some enzyme molecules must exist in the apoenzyme form in nature to be activatable by vanadium in a way similar to that of blood transketolase activation by thiamine pyrophosphate (Vimokesant et al.

Table 2 Relationship between vanadium content and BPO activity of purified BPOs

Bromoperoxidase	V-to-protein ratio (mole/mole)	% Activity[a] of V-enzyme
GfH	0.18	15
GfL1	0.60	68
GfL2	0.59	53

[a] Activity of purified enzyme calibrated as % activity of vanadium-activated enzyme

1982). Dialysis of vanadium-activated enzymes with EDTA at low pH probably caused some holoenzyme population to become apoenzyme, which could be reactivated to a different extent by incorporation of vanadium ion to the vacant active site. Reactivation of the apoenzyme by vanadium ion and not by several other transition metal ions, i.e., Cu^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, or Co^{2+}, nor by iron-protoporphyrin IX as showed in *G. fisheri*, points to vanadium as the cofactor (Suthiphongchai et al. 1994; Kongkiattikajorn and Pongdam 2006). The fact that the spectra of three purified isoenzymes from *G. fisheri* showed the absence of the Soret band (due to heme) and that vanadium was detected in the purified enzymes and the

Fig. 5a–c Effects of H_2O_2, cyanide, and azide on bromoperoxidatic activity. Bromoperoxidase activity was determined in the presence of various concentrations of H_2O_2 (**a**), cyanide (**b**) and azide (**c**) using MCD as substrate

ratio of vanadium to enzymes was related to their activities also supports that these BPO isoenzymes are nonheme enzymes having vanadium as the cofactor.

Similar to other V-BPO, our enzymes were resistant to H_2O_2. However, they were less resistant to azide and cyanide than other algal nonheme vanadium BPOs found previously, which were not inhibited by azide (Wever et al. 1985; Krenn et al. 1987), but our enzymes were still more resistant than heme-HPO, such as that from *R. larix*, which was completely inhibited by 1.3 μM azide (Ahern et al. 1980). This relative resistance property may be exploited commercially. Note that the high-molecular-weight enzyme, GfH, containing multimeric subunits, was more resistant to azide than that of low-molecular-weight monomeric subunit ones (GfL1 and 2). This may be explained at least partly by the relative inaccessibility of a high percentage of its surface, which is in contact with neighboring subunits as has been shown in X-ray crystal structure of other multimeric V-BPOs (Butler and Carter-Franklin 2004).

The use of phenol red and bromide ion for BPO staining and DAB for peroxidase staining provides us with a way to see the bands for BPO activity as distinct from general peroxidase bands. As can be seen from comparison and contrast of band positions and intensities, the GFis and GCs from different locations gave very similar patterns, confirming the anatomical classification done previously (Lewmanomont 1993) as well as showing some divergence due to geographical conditions. As regards PO existing as minor activity bands only in GT and not in other algae, it may be deduced that peroxidase activity per se may not play a major role in peroxidation in red algae. Perhaps this role is taken up by the haloperoxidases that function as synthetic and defensive tools in algae (Pereira et al. 2003). The dependence of peroxidatic activity of BPO on the bromide ion is very interesting in that the scheme for BPO's peroxidatic activity has to account for the role of bromide.

Until recently, it was still a common practice to stain haloperoxidatic activity on gels by using peroxidase reagents. However, the ability to distinguish between the haloperoxidatic activity and peroxidatic activity in tissues and extracts by phenol red and DAB as shown here should provide more information about more specific enzymatic activity. Moreover, BPO staining suffers less from false positives than peroxidase staining in general, e.g., peroxidatic oxidation of organic substrates due to heme and catalase is not reflected in BPO activity, and phenol red has to be brominated, not just oxidized, to yield the blue color.

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