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Vanadium Haloperoxidase from the Red Alga *Gracilaria fisheri*

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ABSTRACT: In this study, we isolated a vanadium-haloperoxidase from a marine red alga, *Gracilaria fisheri* and characterized it. *Gracilaria fisheri*, was collected on the Southern Thailand coast, at Kohyor Beach in Songklha province. The enzyme was purified by homogenization and centrifugation, acetone fractionation, ion-exchange and gel filtration chromatography. Molecular weight was determined by gel chromatography and found to be approximately 615 kDa. The UV spectrum of the peroxidase did not show absorbance in the Soret band indicating a non-heme protein, like a vanadium-dependent haloperoxidase. Reconstitution experiments in the presence of several metal ions confirmed haloperoxidase requires vanadium for enzyme activity and showed that only vanadium completely restored the enzyme activity. The enzyme was moderately thermostable, keeping full activity up to 40°C. Some preliminary steady-state kinetic studies were performed and apparent Michaelis-Menten kinetic parameters were determined for the substrates bromide and hydrogen peroxide.

KEYWORDS: vanadium, bromoperoxidase, algae, *Gracilaria*, haloperoxidase.

INTRODUCTION

The number of cases in which vanadium has been found to occur in biology and appears to play a significant role has increased in recent years. A particular example, which is attracting attention, is the presence of this metal in the active site of a family of haloperoxidases (HPO), a subgroup of peroxidases (EC.1.11.1). For vanadium chloroperoxidase, structural details are available[1]. The enzymes are found in an increasing number of marine (algae) and terrestrial (lichen and fungi) organisms, and a role for them in the synthesis of some of the widespread natural halogenated compounds has been proposed[2]. The importance of these compounds for diverse pharmaceutical and industrial applications has been documented[3].

Marine organisms, particularly seaweeds, produce large quantities of halometabolites, which are held within algal membrane bound vesicles and are thought to result from the catalytic activity of haloperoxidases. These halometabolites probably act as hormones or as repellents in biological defence mechanisms[4]. Siegel and Siegel[5] proposed that an anomalous substrate specificity of haloperoxidases might be the reason why seaweeds are not lignificated. More recently, Kupper *et al.*[6] reported that haloperoxidases might be involved in the uptake of iodide from seawater, a process that, according to these authors, may be related to conditions of oxidative stress. ten Brink *et al.*[7] reported that marine vanadium haloperoxidases were able to catalyse enantioselective sulfoxidation reactions. Despite these many studies, the reason for the selection of vanadium for these enzymes remains elusive since other more common biological metals, e.g. iron (heme) or manganese could also have been used[8]. The vanadium-bromoperoxidase enzymes from red algae, Corallina species; *Corallina officinalis* Linnaeus, *Corallina pilulifera* Postels et Ruprecht; have been shown to be thermostable, stable to organic solvents and also resistant to high concentrations of hydrogen peroxide[9,10]. These features make vanadium-bromoperoxidase enzyme a candidate for use as an industrial catalyst in biotransformation reactions[2,9,11].

The objective of this research was to study the properties of the haloperoxidases extracted from the red alga *Gracilaria fisheri*, collected from the Southern Thailand coast in Songkhla province. The finding in this study that V_2O_5 enhanced activity of bromoperoxidase suggested that the enzyme contained vanadium as might be the prosthetic group in a similar way to bromoperoxidase from other red alga.

MATERIALS AND METHODS

Collection of Algae

The specimens of seaweeds (*Gracilaria fisheri* (Xia & Abbott) Abbott, Zhang & Xia) were collected at low tide from the Southern Thailand coast, at Kohyor Beach in Songkhla province, at the end of summer. After collection, the algae were transported to the laboratory, thoroughly washed with distilled water, chopped and

stored frozen at -20°C until required.

Enzyme Extraction

Each 1,000 g portion of algae was suspended in 1,000 ml of 20 mM Tris–HCl pH 8.5, and processed for five minutes in a Waring homogenizer. The resulting homogenates were pooled and filtered through cheesecloth. The residue was resuspended in 200 ml of the same buffer and homogenized and filtered again. The pooled filtrate was centrifuged for 30 min at 16,000×*g* at 4 °C.

Enzyme Purification

The cell free extract was brought to 30% acetone. The solution was stirred overnight at 4°C and centrifuged at 14,000×*g* for 30 min. The 30% supernatant was brought to 70% saturation with acetone, stirred for 2 h at 4°C and centrifuged at 14,000×*g* as described above. After centrifugation, the pellet was resuspended in 250 ml cold 20 mM Tris–HCl, pH 8.5. After centrifugation (14,000×*g*, 45 min), the precipitate was resuspended in 60% (v/v) ethanol in 20 mM Tris-HCl (pH 8.5), and again centrifuged (14,000×*g*, 20 min). The resulting pellet was collected and resuspended in 20 mM Tris-HCl (pH 8.5) buffer. The acetone fraction was applied to a 5×20 cm DEAE-Toyopearl column equilibrated with 20 mM Tris–HCl (pH 8.5) buffer. The column was washed with 500 ml of 20 mM Tris pH 8.5 buffer followed by a linear gradient of 500 ml of 100–500 mM KCl in 20 mM Tris–HCl pH 8.5 buffer. Fractions containing activity were pooled and concentrated. The concentrated enzyme solution was applied to a 2.7×50 cm column of Sepharose 6B equilibrated and eluted with 20 mM Tris–HCl (pH 8.5) buffer. Fractions containing activity were pooled and concentrated. The concentrated enzyme solution was applied to a second Sepharose 6B (1.7×75 cm) column equilibrated and eluted with the same buffer. All enzyme activity eluted as a single sharp peak. The purified enzyme was stored at 4°C.

Protein content was determined by the method of Lowry *et al.*[12] with bovine serum albumin (BSA) as a standard.

Enzymatic Assays

Haloperoxidase activity was monitored with a reaction mixture containing 48 mM monochloro dimedone (MCD), 8.8 mM H_2O_2, 100 mM KBr and an appropriate amount of enzyme in 100 mM sodium phosphate, pH 6.0 at room temperature. The decrease in absorbance at 290 nm[13] ($\varepsilon = 1.99\times10^4\ M^{-1}cm^{-1}$) upon bromination of the MCD enol was monitored over time on a Shimadzu UV-Vis spectrophotometer. One unit of bromoperoxidase is defined as the amount of enzyme required to form 1 μmol of bromochlorodimedone in one minute.

Enzyme Molecular Weight Determination

The molecular weight of the purified enzyme was determined using gel filtration on a Sepharose 6B column 1.6×90 cm equilibrated with 0.02 M potassium phosphate buffer, pH 7.4. Blue dextran 2,000,000, BSA 66,000, carbonic anhydrase 29,000, cytochrome c 12,400 and aprotinin 6,500 were used as molecular-weight standards.

Absorption Spectra Determination

Bromoperoxidase (15 mg/ml) in 20 mM Tris–HCl buffer pH 7.5 was measured at 25 °C for absorption spectra from 200 to 700 nm in cuvettes of 1-cm path length using a UV–visible recording double beam spectrophotometer (Shimadzu).

Reactivation Studies

The bromoperoxidase was inactivated by the procedures of van Schijndel *et al.*[14]. The enzyme was inactivated at low pH by extensive dialysis for 24 h against citrate-phosphate buffer at pH = 3.8 (0.071 M Na_2HPO_4 and 0.064 M citric acid) in the presence of 1 mM EDTA, followed by dialysis against 20 mM Tris–HCl buffer (pH 8.5). The enzyme was used as dialysed enzyme. The reactivation studies with V_2O_5 were carried out in 20 mM Tris–HCl (pH 8.5). The final vanadium concentration during reactivation was 1 mM for 24 h at 4°C. Reactivation studies were also performed with other metal ions, K^+, Mg^{2+}, Mn^{2+}, Fe^{3+},Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}.

The control (non-dialysed enzymes) was reaction mixture without metal ions. Effect of EDTA on inactivation of the enzyme was determined by treatment of the enzyme with 1mM EDTA. Then the treated enzyme was dialyzed against 20 mM Tris–HCl buffer (pH 8.5) for 24 h at 4°C (dialysed enzyme) and incubated with 1mM of different metal ions for 24 h at 4°C. The residual activity of the treated enzyme was determined by assaying the enzyme under standard conditions.

Results

Purification of the Bromoperoxidase

The results of the purification of haloperoxidase from *G. fisheri* are summarized in Table 1. The yield of purified peroxidase from the algae compared to activity of the initial extracts was 37.9% with a specific activity of 315.30 mU/mg. The specific activity of the combined extracts increased 14.42 -fold. The total amount of purified protein per kilogram of algae was about 17.71 mg. The purified enzyme could be stored in 20 mM Tris–HCl buffer pH 8.5, at -20 °C for more than 4 months without any apparent loss of activity. However,

Table 1. Summary of purification steps of bromoperoxidase.

Purification step	Total activity(mU)	Total protein(mg)	Specific activity(mU/mg)	Yield(%)	Purification(fold)
Cell free crude extract	14,746.59	667.95	22.14	100.00	1.00
30-70% Acetone	9,156.83	98.23	93.21	62.09	4.21
DEAE-Toyopearl	6,986.35	43.57	160.38	47.37	7.24
Sepharose 6B	5,589.12	17.71	315.30	37.90	14.42

repeated freezing and thawing led to considerable loss of its activity.

The volume of crude extract (2 lit) was reduced to 200 ml using acetone fractionation provided a convenient first step and yielded a 4.2 -fold purification. In the case of peroxidase from *G. fisheri*, chromatography on DEAE–Toyopearl resulted in a doubling of the specific activity. Peroxidase activity was eluted from the DEAE– Toyopearl column in several broad peaks centered around 0.2 M NaCl. The active fractions were pooled and concentrated. The active concentrated pool from the previous purification step was applied to a Sepharose 6B gel filtration column. The partially purified enzyme was eluted in three peaks. The second peak, a pale pink color, contained most of the peroxidase activity. The active fraction of the first gel filtration step was lyophilized and redissolved in 20 mM Tris buffer, pH 8.5. The reconstituted sample was further purified on a second Sepharose 6B gel filtration column. The second Sepharose 6B column was longer, narrower, and had a slower flow rate than the first gel filtration column. The bromoperoxidase activity was eluted from this column in a single sharp peak.

Acetone fractionation provided a convenient first step and yielded a 4.21 -fold purification. Ion exchange chromatography had been employed previously as a medium for purification of peroxidases from other sources. In the case of peroxidase from red algae, chromatography on DEAE-Toyopearl resulted in a 1.72 fold of the specific activity. Peroxidase activity was eluted from the DEAE-Toyopearl column in several broad peaks centered around 0.25 M NaCl. The active fractions were pooled and concentrated.

To extract proteins from the red alga, *G. fisheri*; repeated operation of a Waring homogenizer was required to disrupt the cells, and led to the extraction of only 667.95 mg protein from 1,000 g (wet weight) algae. Further operation did not improve the extraction efficiency of protein from the algae. The specific activity of *G. fisheri* enzyme in cell-free extract (22.14 mU/mg protein) was 15 - and 45 -fold less than those of *Corallina pilulifera* Postels et Ruprecht[15] and *Ascophyllum nodosum* (Linnaeus) Le Jolis[16], respectively, which were reported to be high producers of bromoperoxidase.

Properties of the Enzyme

The properties of the enzyme are summarized in Table 2. The molecular weight of the purified enzyme as determined by gel filtration was 615 kDa. The effect of pH on the bromoperoxidase activity was analyzed by carrying out assays at different pHs ranging from 4.5 to 10 at 50 °C. The result was a bell-shaped curve in bromoperoxidase activity showing an optimal activity at pH 6.0 and about 50% of maximal activity at pH 4.5 and 8.5. The enzyme was stable in a pH range of 5–10.0 after 24 h preincubation at 4 °C. Studies of the effect of temperature on the bromoperoxidase were also performed. Enzyme activity increased with temperature to reach an optimum at 55 °C and decreased sharply at higher temperatures. The enzyme was stable at 50 °C for a 30 min incubation and 20% of maximal activity was detected at 70 °C for a 30 min incubation. This enzyme followed Michaelis–Menten kinetics with apparent K_m for MCD (at pH 6.0) of 2.74×10^{-4}M and a V_{max} of 5.32×10^{-2} U/ml.

The absorption spectrum of bromoperoxidase (Figure 1) exhibited an absorption band in the ultraviolet range, centered about 280 nm (Figure 1). In the visible region there was no measurable absorbance in the region of 400–420 nm. Since a characteristic of heme-containing proteins is the Soret band absorbance in this visible region. This result suggests that the bromoperoxidase contains no heme.

Spectrum of Algal Bromoperoxidase

The absorption spectrum of bromoperoxidase was recorded. Profile of tertiary conformational structure was obtained in the absorbance at 275 nm (which is

Table 2. Properties of bromoperoxidase.

Property	Value
Molecular weight	615 kDa
Optimum pH	6.0
pH stability	5.0-10.0 for 24 h at 4°C
Optimum temperature	55°C
Temperature stability	50 °C for 30 min
Metal ion requirement	Required
K_m	2.74×10^{-4}M
V_{max}	5.32×10^{-2} U/ml
Inhibitor	EDTA

related to the aromatic side chains of amino acids of enzyme). The UV absorbance of the enzyme in buffer was determined from 200 to 700 nm (Figure 1). The spectrum does not shows a absorption in the Soret band at 404 nm inconsistent with the presence of a heme group. The enzyme did not show an appreciable absorbance in the Soret band indicating that it is not a heme protein. This is consistent with seawater algal peroxidase, like marine algal peroxidases, being vanadium dependent.

Influence of metal ions on the activity of the enzyme

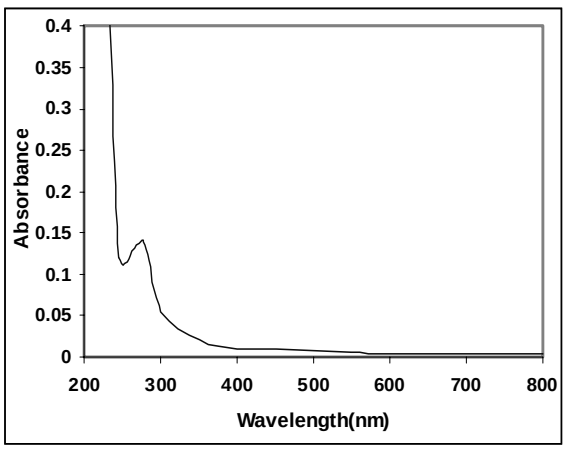

Fig 1. Absorption spectrum of bromoperoxidase in 20 mM Tris–HCl buffer pH 7.5. Bromoperoxidase (15 mg/ml) in 20 mM Tris–HCl buffer pH 7.5 were added to the sample cuvette, and Tris–HCl buffer (20 mM, pH 7.5) was added to the reference cuvette. Spectrum was recorded from 200 to 800 nm on a Shimadzu UV–VIS spectrophotometer.

(Table 3) revealed that the bromoperoxidase requires metal ions for its activity. Treatment of the bromoperoxidase with EDTA showed inhibition for 1.0 mM of EDTA. Incubation of different metal ions with 1.0 mM EDTA treated enzyme showed that V^{5+} could partially restore and activate the activity of the treated enzyme (Table 3), indicating that the bromoperoxidase needs metal ions for expressing full activity.

Enzyme activity was lowered by more than 50% by dialysis against 1 mM EDTA at pH 3.8 in 50 mM phosphate-citrate buffer for 48 h, followed by further dialysis for 24 h against 20 mM Tris–HCl, pH 8.5 at 4°C. Following this treatment, enzyme activity dropped from 315.3 mU to 138.29 mU. Reactivation by dialysis for 24 h against 20 mM Tris–HCl, pH 8.5 containing 1 mM V_2O_5 restored activity to a level of 373.38 mU. A control sample stored at 4°C for the entire period of treatment did not show a drop of activity. Clearly, then, it is possible to inactivate the bromoperoxidase of *G. fisheri* and to reactivate it by the addition of vanadium to the inactivated enzyme.

The intrinsic cofactor is V^{5+} as was ascertained from the additions of a variety of metal ions on the native (as isolated) enzyme (Table 3). Only when V^{5+} was added the enzyme activity was increased ca. 270%, whilst the enzyme activity was decreased by K^+, Mg^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}.

This indicated that not only the present bromoperoxidase is V-containing enzyme but also ca.15.56 % of the enzyme is in the apo form when isolated.

Discussion

Table 3. Influence of various metal ions on reactivity of 1 mM EDTA treated bromoperoxidase.

Ion (1 mM)	Bromoperoxidase activity (%)
Dialysed enzyme	100
Control (no addition)	228
K^+	93
Mg^{2+}	82
V^{5+}	270
Mn^{2+}	97
Fe^{3+}	86
Co^{2+}	97
Ni^{2+}	71
Cu^{2+}	86
Zn^{2+}	84

The red alga, *G. fisheri* contains a vanadium bromoperoxidase. Since the first discovery of vanadium bromoperoxidase in the marine brown alga *Ascophyllum nodosum* (Linnaeus) Le Jolis[17] haloperoxidases have been isolated from various brown algae such as *Laminaria saccharina*(Linnaeus) Lamouroux [18], *Fucus distichus* L. emend. Powell[11], a red seaweed, *Corallina pilulifera* Postels et Ruprecht[19], and a fungus, *Curvularia inaequilis*[14]. Together with heme haloperoxidases isolated from algae such as *Rhodomela larix* (Turner) C. Agardh and *Penicillus capitatus* Lamarck, haloperoxidases are widely distributed in alga and are also in bacteria, plants, and animals. The present *G. fisheri* bromoperoxidase showed a lower activity (315.30 mU/mg) than those of the other marine brown alga enzymes from *Ascophyllim nodosum* (Linnaeus) Le Jolis[20] (126 U/mg), *Laminaria saccharina* (Linnaeus) Lamouroux (615 U/mg) (de Boer et al., 1986), *Fucus distichus*[11] (1580 U/mg) and the specific activity was lower than the enzymes from *Curvularia inaequilis*[14] (7.5 U/mg) and *Corallina pilulifera* Postels et Ruprecht[19]

(21 U/mg) .

In contrast to non-heme haloperoxidases from other alga, brominating activity of bromoperoxidase from *G. fisheri* could already be measured in crude extracts. About 17.71 mg of homogeneous bromoperoxidase could be isolated from 1000 g of algae (wet weight) by acetone fractionation, ion exchange chromatography and gel chromatography.

As observed with other algal non-heme haloperoxidases, total activity increases during the first few purification steps[21]. This is probably due to the removal of a yet unknown inhibitor. However, vanadium that acts as prothetic group of the enzyme might be depleted during the purification process. The specific brominating activity was 315.30 mU (mg protein)$^{-1}$. The results of a typical purification procedure are summarized in Table 1.

The molecular mass determination (Table 2) by gel filtration gave a molecular mass of 615 kDa, which is similar to the values for the vanadium bromoperoxidase from red macroalgae *Corallina officinalis* Linnaeus, a seaweed found on the south Devon coast, has been isolated and characterized[9]. It was suggested to be a homododecamer based on its subunit molecular mass of 64 kDa, and a total molecular mass from equilibrium centrifugation studies of 740 kDa[9]. Itoh *et al.*[10] reported the same molecular mass and the oligomer molecular mass of 790(±20) kDa for the related enzyme from *Corallina pilulifera* Postels et Ruprecht.

However, similar results were found before for bromoperoxidases extracted from other algae[19]. It is likely that the enzyme forms aggregates easily, but it is also possible that the enzyme is tightly bound to alginates[9].

The present *G. fisheri* bromoperoxidase is apparently specific to $V^{5+}$with respect to cofactor (Table 3). Other metals inhibited the enzyme activity to some extent. In contract, in addition to V^{5+}content Fe content correlated with the specific activity of *Corallina pilulifera* Postels et Ruprecht bromoperoxidase[10] .

According to literatures[11,22,23], bromoperoxidases as isolated were partly lacked in V similarly as in the present *G. fisheri* a enzyme. It is not known whether V was partly eliminated from the protein molecules during preparation or bromoperoxidases are present both in the holo and apo forms in natural systems. To deplete V from bromoperoxidase using EDTA, it was not successful in preparing the complete apo enzyme, even though EDTA was treated on bromoperoxidase at various conditions. This strongly suggests that a portion of bromoperoxidase is present in the apo form. Very recently, it has been shown that the amino acid sequences contributing to the active site of the vanadate containing haloperoxidases are conserved in three families of acid phosphatases[24].

As Table 3 shows, the enzyme is reactivated by vanadium (V). The values of activity found, after reactivation, with the other metal ions tested are significantly lower than the values obtained for vanadium (V). This result supports the idea that vanadate is essential for catalytic activity.

The spectrum of the purified enzyme (3.7mg-protein/ml) did not show any absorption peaks other than at 280 nm; 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 bromoperoxidase activity. As shown in Table 3, 1 mM vanadium ions enhanced the enzyme activity 2.7- and 1.2-fold, comparing to the dialysed enzyme and control (no addition), respectively.

On the contrary, other metal ions, K^{+}, Mg^{2+}, Mn^{2+}, Fe^{3+},Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} inhibited the enzyme activity, suggesting that a metal might be involved in the catalytic site. Although several vanadium-dependent bromoperoxidases were also found in marine macro-algae such as *Corallina pilulifera*[19], *Ascophyllum nodosum*[22], *Corallina officinalis*[9] and *Laminaria saccharina* (Linnaeus) Lamouroux[17], there are no reports of cobalt-dependent haloperoxidases from marine macro-algae. However, it was reported that a bacterium, *Pseudomonas putida*, produced bromoperoxidase containing cobalt ion[25] and the metal ion stimulated enzyme activity. Metal ions could play a role in the protein folding or in the catalysis. Most of bromoperoxidase are known to be metal ion dependent enzymes. The results obtained in this study may indicate that bromoperoxidase from *G. fisheri* is a non-heme protein and vanadium-dependent. However, further study is needed to examine the role of vanadium for bromoperoxidase activity.

It was noted that the values of specific bromoperoxidase activity were usually higher after reactivation with vanadate, which means that the vanadate group is not tightly bound at the active site and partial losses during the processes of purification may have occurred. The effect of other metal ions, such as K^{+}, Mg^{2+}, Mn^{2+}, Fe^{3+},Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} on the reactivation of the apo-haloperoxidases was also tested; reactivation was always found to be less of that observed with vanadate.

Vanadate ions are essential for the bromination activity of non-heme bromoperoxidases. Therefore the vanadium content was thought to regulate its activity. Loss of vanadate ions may occur during the purification process. Purification generally yields about 20% of the enzyme from the crude extract[26]. However, the mechanisms regulating the specific activity of the enzyme remain unclear. In the conclusion, we have purified and characterized haloperoxidase from *G. fisheri*. Recent studies have now revealed that this group

of enzymes is better divided into heme-, vanadium-, and metal-free enzymes[3]. In an attempt to classify the enzyme studied here we conclude that in a broad perspective it is a vanadium protein with brominating activity towards monochlorodimedone. This is the report of any such enzyme from an organism which produces a brominated product.

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