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Characterization of Non-Heme Haloperoxidase from Marine Red Algae, *Gracilaria* sp.

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ABSTRACT

In this study, the haloperoxidase has been isolated from the marine red algae, *Gracilaria* sp. and has been characterized. Haloperoxidase requires vanadium for enzyme activity. The enzyme was purified by ion-exchange and size-exclusion chromatography. The relative molecular mass was 619 kDa, as determined by gel filtration. The UV spectrum of the peroxidase did not show absorbance in the Soret band indicating a non-heme protein, like vanadium-dependent haloperoxidases. Reconstitution experiments in the presence of several metal ions showed that only vanadium completely restored the enzyme activity. The enzyme was also moderately thermostable, keeping full activity up to 50°C for 30 min. It has a pH optimum for bromoperoxidase activity at 6.0 and exhibits activities over a broad pH range from 4.5 to 8.5 It was inhibited by ethylene diamine tetra acetic acid (EDTA). Brominating activity was noticed using monochlorodimedone (MCD) as a substrate. The preliminary steady-state kinetic study was performed and apparent Michaelis–Menten kinetic parameters were determined for the substrate monochlorodimedone. The kinetic parameter has been determined from a steady-state analysis of the bromination: K_m MCD = 2.74×10^{-4}M. Enzyme was inactivated by diafiltration against 100 mM citrate-phosphate pH 3.8 buffer in the presence of 1 mM EDTA, followed by a second diafiltration with 20 mM Tris-HCl (pH 8.0). Reactivation studies were carried out with ferric chloride, manganese sulphate, manganese sulphate, potassium chloride, cobolt sulphate, nigel chloride, cupric chloride, zinc chloride and sodium vanadate in the later buffer. The thermostability of the enzyme was tested by heating the extract at 30-80°C for 30 min and checking the activity (bromide assay). The enzyme from the marine red alga, *Gracilaria* sp. was found to be a non-heme bromoperoxidase.

Key words: vanadium, bromoperoxidase, algae

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 . For the vanadium chloroperoxidase, structural details are available (Messerschmidt and Wever, 1995). The enzymes are found in an increasing number of marine (algae) and terrestrial (lichen and fungi) organisms, and their role in the synthesis of some of the widespread natural halogenated compounds has been proposed (Neidleman and Geigert, 1986). The importance

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of these compounds for diverse pharmaceutical and industrial applications has been documented (Littlechild, 1999). In the brown algae (Phaeophyta), vanadium dependent HPO activities were previously reported in *Ascophyllum nodosum* (L.) Le Jolis, *Fucus serratus* (L.), *Fucus spiralis* (L.), *Fucus vesiculosus* (L.) and *Pelvetia canaliculata* (L.) Decne. Et Thur from Northern Europe and for *Fucus distichus* (L.) from Californian coast. *Ascophyllum nodosum*, the most studied source of vanadium haloperoxidases, contains two bromoperoxidases (Krenn *et al*., 1989b) and shares with *P. canaliculata* the singularity of having an associated endophytic fungus, the ascomycete *Mycosphaerella ascophylli* Cotton. Iodoperoxidase and bromoperoxidase activities have been observed previously in crude extracts of *P. canaliculata* (Wever *et al*., 1991), but they were not further characterized. Although all the vanadium HPO examined so far exhibit common basic features, it is not yet understood which factors determine why some of these enzymes catalyse the oxidation of iodide only and others also the oxidation of bromide and chloride. The objective of this research was to study the properties of the haloperoxidase extracted from red algae collected from their natural habitat the Southern Thailand coast in Songkha province.

MATERIALS AND METHODS

Collection of algae

The seaweeds (*Gracilaria* sp.) were collected at low tide from the Southern Thailand coast, at Kogyo beach in Songkha province, at the end of summer. After collection, the algae were transported to the laboratory, thoroughly washed with distilled water, chopped and stored frozen until required.

Enzymatic assays

Haloperoxidase activity was monitored using total volume of the enzyme reaction 1.0 ml, a mixture of 48 mM monochlorodimedone (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 ($\varepsilon = 1.99 \times 10^4$ M^{-1} cm^{-1} (van Pâe and Lingens , 1985) upon bromination of the MCD enol was monitored over time on a Shimazu UV-Vis spectrophotometer. The control contained enzyme sample in the buffer, MCD and KBr but omitted H_2O_2. One unit of bromoperoxidase is defined as the amount of enzyme required to form 1 μmol of bromochlorodimedone in one minute.

Extraction and purification

Intact fresh algae of 1 kg were washed in 20 mM Tris–HCl (pH 8.5) for 1 h, and the extract was centrifuged (7,000 × g, 20 min) in order to remove sand, cell debris and other impurities. Protein was precipitated with 30-70% acetone. 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 centrifuged (14,000 × g, 20 min).The resulting pellet was collected and resuspended in 50 mM Tris–HCl (pH 9.0) buffer. In order to reactivate the HPO, the extract was dialysed overnight against 20 mM V_2O_5 in 50 mM Tris–HCl (pH 9.0). The dialysed sample (125 ml of 98.2 mg protein) was then applied to an anionic exchange column (DEAE-Toyopearl) (2.8 × 40 cm) and eluted with an increasing linear gradient of 0–1.0 M of (NaCl in 0.2 M Tris–HCl (pH 8.5) at the flow rate of 1.0 ml/min. Fractions of 4 ml were collected and assayed protein and brominating activity. Fractions containing bromoperoxidase activity were pooled separately. Enzyme active fractions were then loaded onto a column (Sephadex G-200 from Pharmacia) (2.6 × 100 cm) and eluted with 50 mM Tris–HCl (pH 8.5) at the flow rate of 1.0 ml/min. Fractions of 2 ml were collected and assayed protein and brominating activity. Fractions containing bromoperoxidase activity were pooled

separately. All steps were carried out at 0–4°C.

Protein content was determined by the method of Lowry *et al*. using bovine serum albumin (BSA) as a standard (Lowry *et al*., 1951).

Enzyme molecular weight determination

Molecular weight of the purified enzyme was determined using gel filtration on a Sephadex G-200 column 1.6 × 90 cm equilibrated with 0.02 M potassium phosphate buffer, pH 8.5. Molecular weight standards (Pharmacia Biotech) were blue dextran (2000 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa).

pH activity assay

To obtain the correct pH, the following buffer was used (Jouve *et al*., 1983): 31 mM citric acid, 28 mM KH_2PO_4, 28 mM H_3PO_4 and 28 mM 5,5-diethylbarbituric acid titrated with 0.2 M NaOH to obtain different pH values in the range from 4.5 to 10. In the measuring cell (1 ml) the pH buffer was mixed with a diluted enzyme sample with 100mM potassium bromide. The mixture was incubated at 25°C for 2 min and the reaction was started by adding H_2O_2 to a final concentration of 8.8 mM. The enzyme activities in different pH were assayed and presented as percentage of relative activity compared to the highest enzyme activity.

pH stability

pH stability of bromoperoxidase was evaluated by measuring enzyme activity between pH values in the range from 4.5 to 10. In the measuring cell (1 ml) the pH buffer was mixed with a diluted enzyme sample with 100mM potassium bromide. The mixture was incubated in the buffer pH values in the range from 4.5 to 10 at 4°C for 24 h and the reaction was started by adding H_2O_2 to a final concentration of 8.8 mM. The enzyme activity was assayed and presented as percentage of relative activity compared to the enzyme at zero time.

Thermal activity

Thermal activity of bromoperoxidase was evaluated by measuring enzyme activity during incubating the enzyme at different temperature. In the measuring cell (1 ml), the buffer pH 6.0 was mixed with a diluted enzyme sample with 100mM potassium bromide. The mixture was incubated between 25 and 70°C and the reaction was started by adding H_2O_2 to a final concentration of 8.8 mM. The enzyme activities in different temperature were assayed and presented as percentage of relative activity compared to the highest enzyme activity.

Thermal stability studies

The purified enzymes were incubated in a thermostated vessel for 30 min at temperatures ranging from 25 to 70°C. Activity was determined after subsequent equilibration at room temperature. The enzyme activity was assayed and presented as percentage of relative activity compared to the enzyme at zero time.

Reactivation studies

The HPO was inactivated at low pH by extensive dialysis for 24–72 h against a citrate–phosphate buffer at pH 3.8, in the presence of 1 mM EDTA, followed by dialysis against 20 mM Tris–HCl (pH 8.0). Reactivation studies with sodium vanadate were carried out in 20 mM Tris–HCl (pH 8.0). The final vanadium concentration for reactivation was 200 mM. The same conditions were used in the reactivation studies with other inorganic salts (ferric chloride, manganese sulphate, manganese sulphate, potassium chloride, cobolt sulphate, nigel chloride, cupric chloride, zinc chloride).

Steady-state kinetic experiments

Kinetic data were obtained under steady state conditions by changing the concentrations

of reactants. These experiments were carried out in 20mM phosphate buffer (pH 6.0) by measuring the oxidative bromination of MCD by H_2O_2 and Br^-. The initial rate of formation of oxidative bromination was determined at constant concentration of hydrogen peroxide (0.8 mM) and the potassium bromide (100mM) concentration and MCD was varied between 0.1 and 10 mM. All measurements were performed at 25.0±0.5 °C.

Steady-state kinetic analysis

The initial rates, v_0 plotted as a function of MCD concentration were fit to a Michaelis–Menten equation. The apparent kinetic parameters K_m^{app} MCD as well as V_{max} were obtained from primary double reciprocal plots.

RESULTS

Purification of bromoperoxidase

Purification of bromoperoxidase from *Gracilaria* sp. gave a 37.9% yield with a specific activity of 315.30 mU/mg and 14.3 purification fold. Purification results are summarized in Table 1, respectively. 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, repeated freezing and thawing led to considerable loss of its activity.

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 619 kDa. The effect of the pH on the bromoperoxidase activity was analyzed by carrying out assays at different pHs as described in materials and methods ranging from 4.5 to 10.0 at 50°C. The result was bell-shape curve showing an optimal activity at pH 6.0 and bromoperoxidase activity showed 50% of maximal activity at pH4.5 and 8.5 (Figure 1). The enzyme was stable over a pH range of 5–10.0 after 24 h preincubation at 4°C (Figure 2). The effect of temperature on bromoperoxidase were also performed; the activity increased with temperature to reach an optimum at 55°C and decreased sharply at higher

Table 1 Summary of purification steps of bromoperoxidase from *Gracilaria* sp.

Purification step	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Cell free crude extract	14,746.6	668	22.1	100	1.0
30-70% Acetone	9,156.8	98.2	93.2	62.1	4.2
DEAE-Toyopearl	6,986.4	43.6	160.4	47.4	7.3
Sephadex G-200	5,589.1	17.7	315.6	37.9	14.3

Table 2 Properties of bromoperoxidase from *Gracilaria* sp.

Characteristics	Value
Molecular weight	619 kDa
Optimum pH	6.0
pH stability	5.0-10.0 for 24 h at 4°C
Optimum temperature	55°C
V^{5+} requirement	Required
K_m	2.74×10^{-4}M
Inhibitor	EDTA

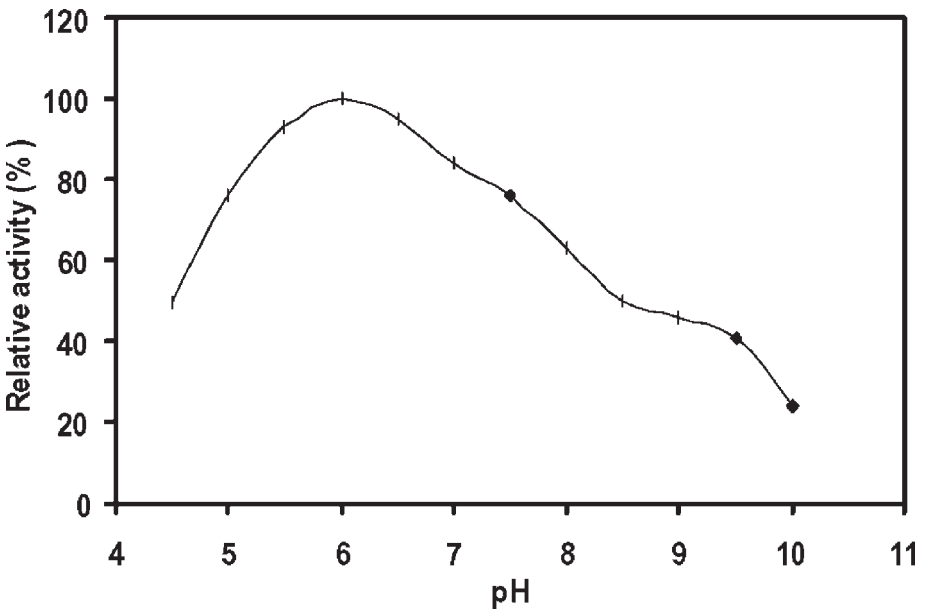

Figure 1 pH-dependence of the bromination activity of the purified bromoperoxidase.Buffers were prepared as described in materials and methods.

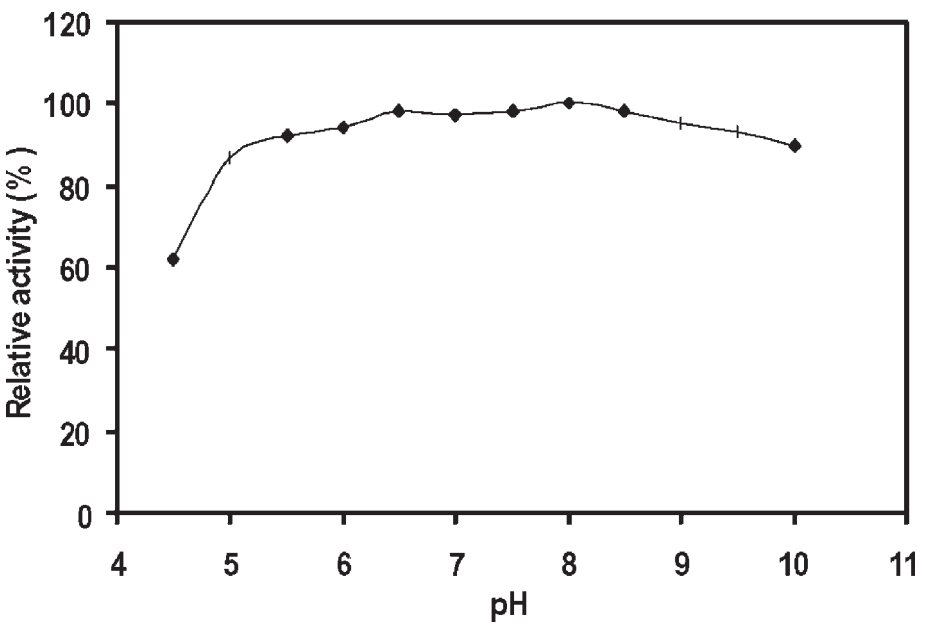

Figure 2 pH-stability of the bromination activity of the purified bromoperoxidase. Buffers were prepared as described in materials and methods.

temperatures (Figure 3). The enzyme was stable at 50°C after 30 min incubation and 20% of maximal activity was detected at 70°C for 30 min incubation (Figure 4). This enzyme followed Michaelis–Menten kinetics with apparent K_m for MCD (at pH 6.0) 2.74×10^{-4}M and a V_{max} of 5.32×10^{-2} U/ml. Influence of metal ions on the activity of the enzyme revealed that the bromoperoxidase requires vanadium for its activity. The treated bromoperoxidase activity was inhibited with 1.0 mM of EDTA. Incubation of different metal ions (ferric chloride, manganese sulphate, manganese sulphate, potassium chloride, cobolt sulphate, nigel chloride, cupric chloride, zinc chloride) with 1.0 mM EDTA treated enzyme as described in materials and methods showed that V^{5+} could restore and activate the activity of the treated enzyme (Table 3), indicating that the bromoperoxidase needs vanadium for expressing full 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 (Almeida *et al*., 2001).

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. 80% of the enzyme is in the apo form when isolated.

DISCUSSION

Vanadate ions (V^{5+}) are essential for the bromination activity of nonheme bromoperoxidases. Therefore the vanadium content was thought to regulate its activity. The present *Gracilaria* 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 pilulirera bromoperoxidase (Itoh *et al*., 1996).

According to literatures (De Boer *et al*., 1986; Soedjak and Butler, 1991), bromoperoxidases as isolated were partly lacked in V similarly as in the present *Gracilaria* 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. In connection with the fact vide supra, we tried to deplete V from bromoperoxidase using EDTA. It is suggested that a portion of bromoperoxidase is present in the apo form. In line with this, it took ca. 24 h for bromoperoxidase (as isolated) to gain the full enzyme activity by treating with V^{5+}

Loss of vanadate ions may occur during the purification process. Purification generally yields about 20% of the enzyme from the crude extract (Itoh *et al*., 1992). However, the mechanisms regulating the activity of the enzyme remain unclear. There might be a low molecular regulatory compound which can prevent the correct coordination of vanadate ion in the active site. Another theory is that modification of the enzyme protein structure prevents the correct coordination of vanadate ions within the apo-enzyme.

CONCLUSIONS

The bromoperoxidase has been isolated from the marine red algae, *Gracilaria* sp.(619kDa) and has been characterized. Bromoperoxidase requires vanadium for enzyme activity. The enzyme activity increased ca. 270% with the action of V^{+5} on the isolated enzyme, since more than 2/3 of the protein molecules were in the apo form. The increase in the enzyme activity was specific to V^{+5}, while Co^{+2}, Fe^{+3}, Mg^{+2}, Mn^{+2}, Ni^{+2}, K^{+1}, Zn^{+2} and Cu^{+2} inhibited the enzyme activity. The

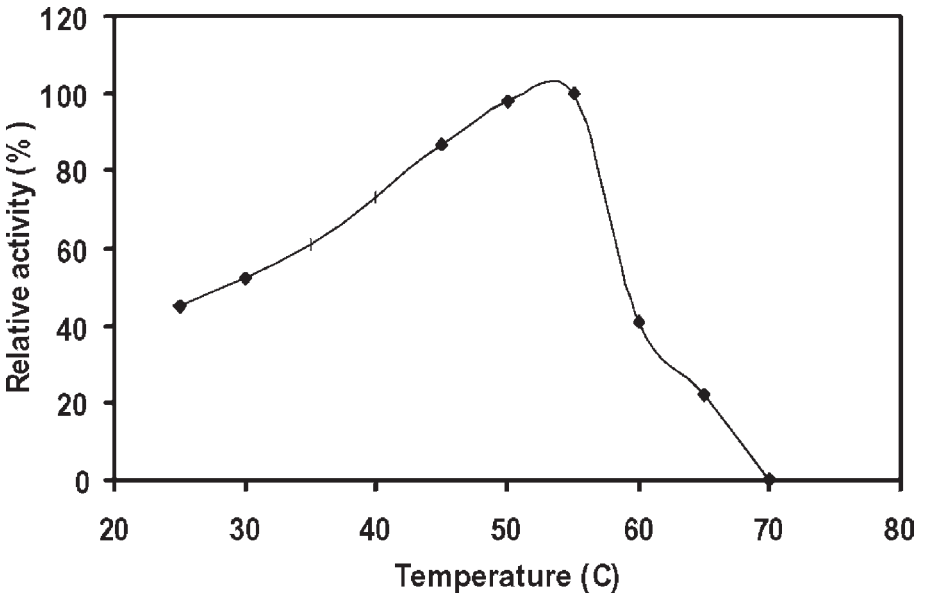

Figure 3 Thermal activity of the bromoperoxidase. Samples of the enzymes and substrates were incubated at the given temperature in buffer pH 6.0 and enzymatic activity was measured as described in materials and methods.

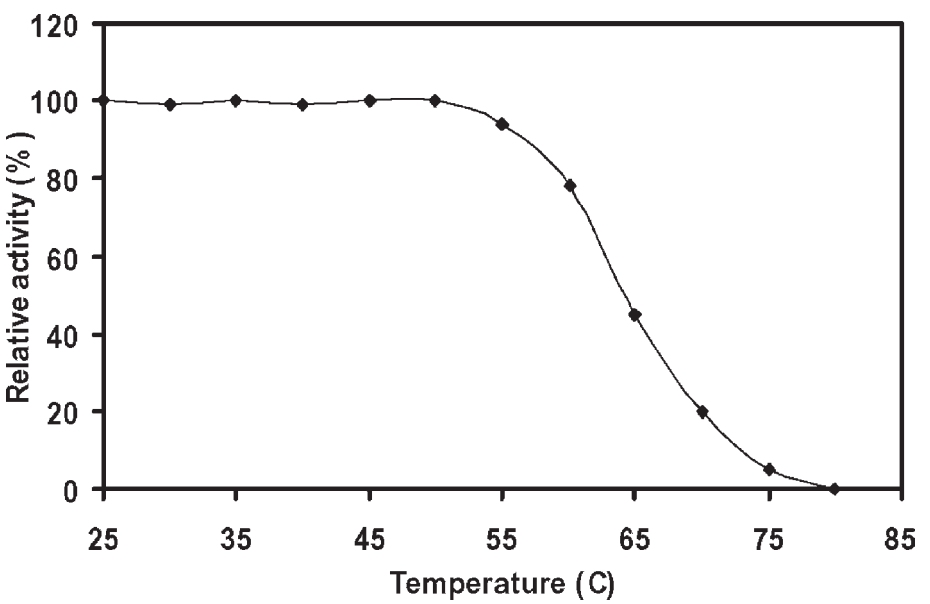

Figure 4 Thermostability of the bromoperoxidase. Samples of the enzymes were incubated in buffer pH 6.0 for 30 min at the given temperature after which enzymatic activity was measured as described in materials and methods.

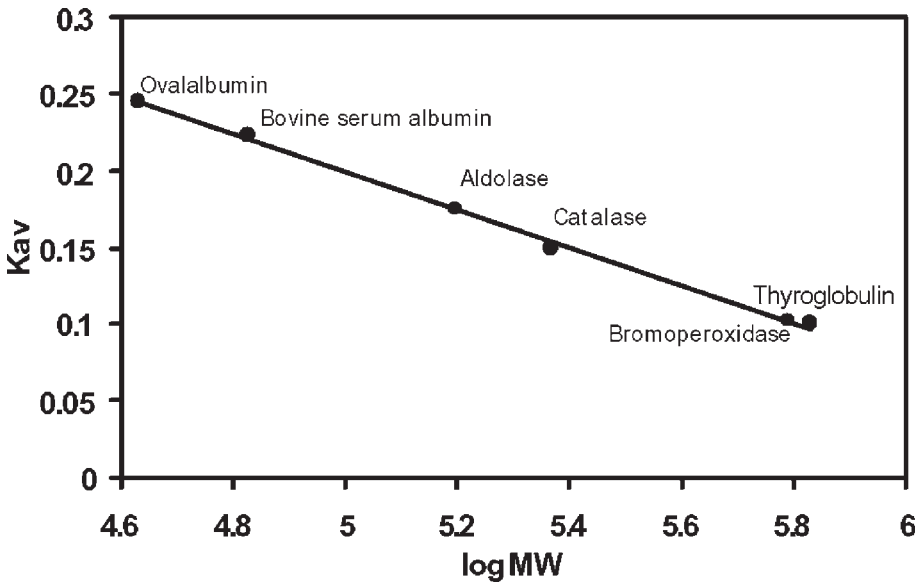

Figure 5 Molecular weight estimation for bromoperoxidase using gel filtration. The K_{av} of bromoperoxidase is compared to that of proteins with known molecular weights.

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

Ion (1 mM)	Bromoperoxidase activity[c](%)
Dialysed enzyme[a]	100
Control[b] (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

[a] The treated bromoperoxidase with1.0 mM EDTA

[b]The native purified enzyme

[c]Percentage of relative activity compared to the dialysed enzyme.

bromoperoxidase exhibited a high thermostability and a high stability in acidic and alkaline pH. This finding opens up the possibility to design activator of the enzyme, thereby potentially producing the brominated products.

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