ARTICLE
doi: 10.2306/scienceasia1513-1874.2006.32(s1).031

ScienceAsia 32 Supplement 1 (2006): **31-35**

Isolation and Characterisation of Vanadium Bromoperoxidases from Thai Red Alga *Gracilaria tenuistipitata*

Jirasak Kongkiattikajorn[a]* and Pintip Ruenwongsa[b]

[a] School of Bioresources and Technology, King Mongkut' s University of Technology Thonburi, Thailand.
[b] Department of Biochemistry, Mahidol University, Thailand.

* Corresponding author, E-mail: jirasak.kon@kmutt.ac.th

ABSTRACT: Many species of marine macro-algae contain a variety of halogenated secondary metabolites. A halogenating enzyme, haloperoxidase is considered to participate in their syntheses in the presence of halides and hydrogen peroxide. In this study, two enzymes which were isolated and characterised as bromoperoxidases (BPOI and BPOII), with vanadium-dependent activity, have been purified from the red alga *Gracilaria tenuistipitata* collected in the Eastern Thailand coast, at Ban Laem Sok beach in Trad province. The relative molecular masses were 70 kDa for BPOI and 48 kDa for BPOII as determined by gel filtration. The following kinetic parameters have been determined from a steady-state analysis of the oxidation of bromide by H_2O_2: BPOI, pH_{opt} = 5.5, $K_m(Br^-)$ = 2.17 x 10^{-4} M, $K_m(H_2O_2)$ = 1.0 x 10^{-4} M, K_m(MCD) = 2.94 x 10^{-5} M; and BPOII, pH_{opt} = 7.0 , $K_m(Br^-)$ = 4.72 x 10^{-5} M, $K_m(H_2O_2)$ = 2.47 x 10^{-5} M, K_m(MCD) = 8.52 x 10^{-4} M. These bromoperoxidases are thermostable, as also observed for other vanadium bromo- and chloro-peroxidases.

KEYWORDS: vanadium, bromoperoxidase, kinetic, *Gracilaria*.

INTRODUCTION

The number of cases in which vanadium has been found to occur in living systems, and appears to play a significant role, has increased in recent years[1]. One case which received attention in the last few years is that of the vanadium haloperoxidases, a new class of enzymes that contain vanadium (V), as vanadate, in the active site and catalyze halogenation reactions of several substrates. These enzymes are found in many brown, in some red, and in one green marine alga[2] and in the lichen *Xantoria parietina*[3]. Recently, vanadium dependent haloperoxidases were also found in some fungi[4]. All the vanadium haloperoxidases isolated to date share some common features: they are composed of one or more subunits of relative molecular mass around 67 kDa; they can be inactivated by dialysis against EDTA at low pH; their activity is only restored by addition of vanadium (as vanadate) and they seem to have similar coordination of vanadium in the active site[5]. The isolation of more than one form of vanadium haloperoxidase has been reported for *Ascophyllum nodosum*[6], a brown seaweed. It is also possible to purify more than one form of the enzyme[7] from the brown seaweeds *Sacchoriza polyschides* and *Pelvetia canaliculata*.

The present report on the isolation and characterization of haloperoxidases from *Gracilaria tenuistipitata* collected in the Eastern Thailand coast, at Ban Laem Sok beach in Trad province, is a contribution for the study of the role of vanadium in these enzymes.

MATERIAL AND METHODS

Collection of Algae

The seaweeds (*G. tenuistipitata*) were collected at low tide from the Eastern Thailand coast, at Ban Laem Sok beach in Trad province. After collection, the algae were transported to the laboratory, thoroughly washed with distilled water, chopped and stored frozen until required.

Enzyme Purification

The concentrated cell free extract was brought to 25% saturation with solid ammonium sulfate. The solution was stirred overnight at 4°C and centrifuged at 16,000 x *g* for 30 min. The supernatant was brought to 0-60% saturation with solid ammonium sulfate, stirred for 2 h at 4°C and centrifuged at 16,000 x g. After centrifugation, the pellet was resuspended in 250 ml cold 100 mM Tris-(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) buffer, pH 7.0. Any remaining undissolved precipitate was removed by centrifugation at 16,000 x g for 15 min and the resuspended pellet was dialyzed against 100 mM Tris-HCl buffer pH 7.0. The dialyzed ammonium sulfate fraction was applied to a 5x20 cm DEAE-cellulose DE52 column equilibrated with 100 mM Tris-HCl pH 8.5 buffer. The column was washed with 500 ml of 100 mM Tris-HCl pH 8.5 buffer

followed by a linear gradient of 1000 ml of 0–1,000 mM NaCl in 100 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 Sephadex G-200 equilibrated and eluted with 100 mM Tris-HCl pH 7.0 buffer. Fractions containing activity were pooled and concentrated. The concentrated enzyme solution was applied to a second Sephadex G-200 (1.7×75 cm) column equilibrated and eluted with the same buffer. All the active fractions were pooled and submitted to a second chromatographic separation on a fast protein liquid chromatography (FPLC) system with a Mono Q HR 5/5 column. The column was equilibrated with 20 mM Tris-HCl (pH 8.0) and the protein eluted with a gradient of 1 M NaCl in the same buffer. The protein was eluted at 0.25-0.3 M NaCl. Further purification was carried out with a gel filtration Superose 12 (Pharmacia). The elution was carried out with a solution of 50 mM Tris-HCl (pH 9.0). The purified enzyme was stored at -20°C. Protein content was determined by the method of Lowry *et al.* with bovine serum albumin (BSA) as a standard[8].

Enzyme Assay

Enzyme activity of bromoperoxidase was measured by the bromination of monochlorodimedone (2-chloro-5,5-dimethyl-1,3-cyaclohexanedione) (MCD) according to literature[9]. The standard assay mixture contained 0.1 M phosphate buffer (pH 6.0), 2 mM H_2O_2, 50 mM KBr. The decreases of the absorbance of MCD (ε_{290} =20.1 mM^{-1} cm^{-1}) were measured at 25°C. Kinetic data were obtained under steady state conditions by changing the concentrations of reactants. Thermal stability of bromoperoxidase was evaluated by measuring enzyme activity at 25°C after incubating the protein molecule between 25°C and 80°C for 10 min.

Molecular Mass Determination

The relative molecular mass was determined by FPLC/gel filtration chromatography on Superose 12 (Pharmacia) with a mobile phase of 50 mM Tris–HCl (pH 9.0). Standard proteins from Pharmacia (ribonuclease A, Mr 13.7 kDa; chymotrypsinogen, Mr 25 kDa; ovalbumin, Mr 43 kDa; aldolase, Mr 158 kDa; and catalase, Mr 232 kDa) were used for the calibration of the column.

Metal Ion Requirement

Influence of vanadium pentoxide (V_2O_5) on bromoperoxidase activity was determined by the purified enzyme dialyzed against free ion distilled water (three times, 6 h each time) and incubated with 5 mM V_2O_5 for 24 h at 4°C. After incubation the reactivity of the treated enzyme was determined under assay conditions.

Effect of Inhibitors

For the effect of ethylene diamine tetra acetic acid (EDTA), the purified enzyme was dialyzed against free ion distilled water with 1 mM EDTA (three times, 6 h each time). After dialysis the reactivity of the dialysed enzyme was determined under assay conditions. Effect of inhibitors, sodium azide and potassium cyanide (NaN_3 and KCN) on inactivation of the enzyme was determined by treatment of the enzyme with 1 mM inhibitors. The residual activity of the treated enzyme was determined by assaying the enzyme under standard conditions.

Results

The first purification step of the crude extract was achieved by fractional precipitation of proteins using the $(NH_4)_2SO_4$ method. In the $(NH_4)_2SO_4$ method, the supernatant was brought to 0-60% w/v saturation with ammonium sulfate. After centrifugation at 18,000 rev./min for 15 min, the precipitate was dissolved in an initial volume of 0.02 M phosphate buffer of pH 7.4. Bromoperoxidase activity and protein values were determined for both phases. The same experiments have also been repeated for the crude extract. The highest BPO activity value for the bottom phase was obtained using 60% $(NH_4)_2SO_4$. The partially purified bromoperoxidase sample was fractionated on a column of DE52-cellulose equilibrated with 0.02 M phosphate buffer pH 7.4. Weakly bound proteins were initially eluted from the column with 20 mM phosphate buffer, pH 7.4. Subsequently the more strongly bound proteins were eluted with a linear gradient of NaCl solution 0.2-1.0 M. The activity in the recovered fraction showed a 3.14 and 17.61-fold increase in specific activity, for BPOI and BPOII, respectively. The fractions which displayed bromoperoxidase activity were pooled and dialysis to remove salt in the fractions and were applied to a MonoQ column of FPLC. The proteins were eluted by a linear gradient of NaCl solution 0.2-1.0 M.

The pooled fractions which displayed bromo peroxidase activity were concentrated and were then applied to a Superose 12 column which had been equilibrated with 0.02 M phosphate buffer pH 7.4. The enzyme was eluted with this buffer. The results of these purification procedures are summarized in Table 1. The highest specific bromoperoxidase activity observed after the Superose 12 gel column step was 61.86 and 330.58 mU/mg for BPOI and BPOII, respectively. The two purified haloperoxidases show bromoperoxidase activity, but the specific activity of BPOII is 5.34 fold of that of BPOI (specific activities: BPOI 61.86 mU/mg; BPOII 330.58 mU/mg). The purified

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	1,932.30	402.35	4.80	100.00	1.00
0-60% $(NH_4)_2SO_4$	1892.95	351.85	5.38	97.96	1.12
DE52					
BPOI	228.31	15.13	15.09	11.82	3.14
BPOII	581.52	6.88	84.52	30.10	17.61
MonoQ					
BPOI	151.20	3.87	39.07	7.77	8.14
BPOII	337.22	1.97	171.18	17.45	35.66
Superose12					
BPOI	103.92	1.68	61.86	5.38	12.89
BPOII	271.08	0.82	330.58	14.03	68.87

enzymes are colourless and their absorption spectrum in the visible range is featureless.

The K_m values for H_2O_2 and Br^- determined by Lineweaver-Burk plots, for BPOI are larger than those for BPOII, showing that BPOI has a lower affinity for the two substrates while the K_m values for MCD, for BPOI is less than that for BPOII, showing that BPOI has higher affinity for the substrate (Table 2).

Table 2. Kinetic parameters of bromoperoxidases from *G. tenuistipitata*.

Enzyme	$K_m(H_2O_2)$(M)	K_m(MCD)(M)	$K_m(Br^-)$(M)
BPOI	1.00×10^{-4}	2.94×10^{-5}	2.17×10^{-4}
BPOII	2.47×10^{-5}	8.52×10^{-4}	4.72×10^{-5}

Properties of the Enzymes

The properties of the enzymes are summarized in Table 3. The molecular weight of the purified enzymes as determined by FPLC/gel filtration was 70 and 48 kDa. The effect of the 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 showing an optimal activity at pH 5.5 and bromoperoxidase activity showed 50% of maximal activity at pH 4.5 and 9.0. The enzymes were stable in a pH range of 5.0 – 9.5 and 5.5 - 9.0 after 24 h preincubation at 4 °C, for BPOI and BPOII, respectively. Studies of the effect of the temperature on the bromoperoxidase were also performed; the activity increased with the temperature to reach an optimum at 50°C and 45 °C, for BPOI and BPOII, respectively. The enzymes were stable at 45 °C for 30 min incubation and 20% of maximal activity was detected at 60 °C for 30 min incubation.

Table 3. Properties of bromoperoxidase.

Property	BPOI	BPOII
Molecular weight	70kDa	48kDa
Optimum pH	5.5	7.0
pH stability	5.0-9.5 for 24 h at 4°C	5.5-9.0 for 24 h at 4°C
Optimum temperature	50°C	45°C
Temperature stability	45 °C for 30 min	45 °C for 30 min
Metal ion requirement	Required	Required
Inhibitor	EDTA, NaN_3, KCN	EDTA, NaN_3, KCN

Influence of metal ions on the activity of the enzyme revealed that the bromoperoxidase requires metal ions for its activity. The treated bromoperoxidase with EDTA showed inhibition for 1.0 mM of EDTA. Incubation with 1.0 mM EDTA treated enzyme showed that V^{5+} could restore and activate the activity of the treated enzyme (Table 4), indicating that the bromoperoxidase needs metal ions for expressing full activity. The metal ion could play a role in the protein folding or in the catalysis. Most of bromoperoxidase are known to be metal ion dependent enzymes. However, NaN_3 and

Table 4. Effect of EDTA, V^{5+}, NaN_3 and KCN on bromoperoxidase.

Ion (1 mM)	Bromoperoxidase activity BPOI (%)	Bromoperoxidase activity BPOII (%)
Dialysed enzyme	100	100
Dialysed enzyme + EDTA	0	0
Control (no addition)	207	220
Dialysed enzyme + V^{5+}	250	350
Control + NaN_3	8.5	7.0
Control + KCN	5.5	4.3

KCN, which are heme inactivating reagents, could inhibit reactivity of both BPOI and BPOII.

Discussion

The purification of enzymes extracted from marine algae is a difficult task, since the composition of the raw material is complex and the presence of polyphenols and tannins in the extracts complicates the process. In our studies with *G. tenuistipitata* we extracted, purified and characterized two enzymes. Previously, the presence of more than one HPO in the same algae was observed only in *A. nodosum*, one located inside the thallus (bromoperoxidase I), particularly around the conceptacles, and the other (bromoperoxidase II) present at the thallus surface[6]. These results suggest that the presence of more than one isoenzyme in algae may be a common feature, as is found in terrestrial plants. The existence of different isoforms of haloperoxidases may be the result of an adaptation to environmental conditions; for instance, salinity, light conditions or presence of other marine organisms or substances[10]. It is not yet known whether the two bromoperoxidases extracted from *A. nodosum* originate from the same gene. As in *G. tenuistipitata*, the two bromoperoxidases differ in their binding to substrates, which implies different enzyme kinetic patterns.

The K_m values for Br^- and H_2O_2, determined by Lineweaver-Burk plots, for BPOI are larger than those for BPOII, showing that BPOI has a lower affinity for the two substrates (Table 2). The K_m value for Br^- of BPOI and K_m value for MCD of BPOII were the highest among three reactants, suggesting that relatively high Br^- and MCD concentration are required for BPOI and BPOII, respectively, under steady state conditions.

The molecular weight of purified algal peroxidase is similar to the molecular weight of several previously characterized bromoperoxidases. All have molecular weights between 64 and 97 kDa.

The thermal stability of *G. tenuistipitata* enzymes was also investigated. Both enzymes remain active up to 45°C (Table 3). The thermal stability has also been studied for the haloperoxidases from the red algae *Corallina officinalis*[11], *Ceramium rubrum*[12], the brown algae *Phyllariopsis brevipes*[13] and *A. nodosum*[14]. These studies indicate that the thermal stability of V-HPO is generally high. The high stability of these enzymes will be due to the compact packing of a-helices shown in the X-ray crystal structure of chloroperoxidase[15].

The pH optima in bromination reactions were almost equal (pH 5.5 and 7.0) to other bromoperoxidases. For the BPOI enzyme, an appreciable difference of enzymatic activity was observed, around pH 5.5. This was already observed by Sheffield et al., for the bromoperoxidase from *C. pilulifera*[11] . Maximal activity of BPOII was found at pH 7.0 (Table 3). The difference is less than that in case of the two bromoperoxidases (I and II) extracted from *A. nodosum* (for which the optimal pH values are 6.0 and 7.3, respectively). Interestingly, the optimum pH for BPOI and BPOII are the same as for bromoperoxidase I and II from *A. nodosum*, which confirm the similarities between these enzymes. These results, together with the studies of deactivation/reaction, seem to imply a difference in the bonding of the vanadium in these two enzymes.

When sodium azide or potassium cyanide was included in the reaction mixture, no bromide oxidation was observed, which is consistent with the presence of the effect on the prosthetic group in the catalytic mechanism.

By dialysis with phosphate buffer in the presence of EDTA the enzymes were inactivated due to removal of the prosthetic group (Table 4). A similar behaviour has also been observed for the enzyme extracted from brown algae of the Laminariaceae (bpo1). Since vanadium uptake (as vanadate) depends on the polypeptide chain and on the bonding of this species to the active site, the difference may be due to different conformations of the protein near the active site, which may alter the binding of vanadate. We note also, that the values of specific haloperoxidase 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.

However, the X-ray crystal structure of azide-bound chloroperoxidase from the fungus *Curvularia inaequalis* indicated that a hydrogen vanadate (V) is in a trigonal bipyramidal geometry with the metal coordination to three oxygens in the equatorial plane, to the OH group at one apical position, and to the 2 nitrogen of a histidine at the other apical position[15]. Although the presence of V in *G. tenuistipitata* bromoperoxidase was qualitatively indicated by inactivation due to dialysis. According to literature[16-18], bromoperoxidases as isolated were partially lacking in V, similar as in the present *G. tenuistipitata* enzyme. It is not known whether V was partly eliminated from the protein molecules during preparation or whether bromo peroxidases are present both in the holo and apo forms in natural systems.

In conclusion, the two bromoperoxidases from *G. tenuistipitata* are vanadium enzymes, while the enzymes show characteristics similar to those of other haloperoxidases, especially to bromoperoxidases of *A. nodosum*, low molecular mass, and higher affinity for hydrogen peroxide and bromide. These particular results are associated with the habitat of the species. A more complete study on the origin of these isoenzymes,

their location in the plant and their structure, and a comparison with haloperoxidase of the same algae collected at other sites, will enable further elucidation of the role played by these enzymes. For application studies such as immobilization and syntheses of various organohalogen compounds, the stabilities of bromoperoxidase against temperature is very favorable.

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