Bromoperoxidases from *Penicillus capitatus*, *Penicillus lamourouxii* and *Rhipocephalus phoenix*

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The isolation and purification of bromoperoxidases from three marine subtropical green algae is described. In the presence of KBr and H₂O₂, each halide-specific enzyme catalyses the bromination of monochlorodimedone (2-chloro-5,5-dimethylcyclohexane-1,3-dione) to bromochlorodimedone (2-bromo-2-chloro-5,5-dimethylcyclohexane-1,3-dione). The enzymes also catalyse the oxidation of pyrogallol, *o*-phenylenediamine and I[−] to I₃[−]. Preliminary characterization of these enzymes reveals acidic pH optima, high thermal stability, sensitivity to higher H₂O₂ concentrations, and apparent molecular weights ranging from 48 000 to 60 000.

Halogenated organic molecules occur in a variety of marine organisms including sponges (Schmitz & Gopichand, 1978), micro-organisms (Anderson *et al.*, 1974; Wratten *et al.*, 1977), coelenterates (White & Hager, 1977) and algae (Fenical, 1975; Gonzalez *et al.*, 1976; McConnell & Fenical, 1978; Howard & Fenical, 1978). Marine algae, particularly the Rhodophyta, are known to be rich in chlorinated, brominated and iodinated compounds. The Phaeophyta or brown algae produce predominantly iodinated molecules (Fenical, 1975).

Halogen specificity in marine organisms appears to vary between the phyla and may be due to either selective halide ion permeability or enzyme selectivity (Fenical, 1975). Murphy & O'hEocha (1973) suggested the use of a terrestrial fungal enzyme chloroperoxidase (chloride–hydrogen peroxide oxidoreductase, EC 1.11.1.10) as a model for marine halogenating enzymes. A search of many marine phyla has revealed similar enzymes in marine creatures and that some are halogen-selective. Recent reports have described labile chloroperoxidase-like activity in a subtropical marine sponge (Baden & Corbett, 1979) and a bromoperoxidase activity in a temperate marine red alga (Theiler *et al.*, 1978). Our interest in such halogenating enzymes is the result of our discovery that chloroperoxidase possesses a unique capability to oxidize arylamines selectively to the corresponding nitroso compound (Corbett *et al.*, 1978, 1979).

The present paper describes the discovery and protein purification of bromoperoxidases from three marine subtropical Chlorophyta: *Penicillus capitatus* (Lamarck), *Penicillus lamourouxii* (Decaisne) and *Rhipocephalus phoenix* (Ellis et Solander). This discovery is noteworthy because it is the first report of bromoperoxidases from marine green algae. The results of our preliminary studies also illustrate and support the hypothesis of Murphy & O'hEocha (1973) of the similarity between chloroperoxidase and marine halogenating enzymes.

Experimental

All chemicals were of reagent grade. Chloroperoxidase (1400 units/mg) was purchased from Sigma Chemical Company and was used without further purification. One unit of chloroperoxidase converts 1 μ mol of monochlorodimedone (2-chloro-5,5-dimethylcyclohexane-1,3-dione) into dichlorodimedone (2,2-dichloro-5,5-dimethylcyclohexane-1,3-dione)/min at pH 2.75 and 25°C (Morris & Hager, 1966). *P. capitatus*, *P. lamourouxii* and *R. phoenix* were collected at a depth of 1–3 m of water off-shore of Big Pine Key, FL, or Soldiers Key, FL, U.S.A.

Assay methods

Protein was determined by the method of Bradford (1976). All halogenation assays were conducted in 0.1 M-potassium phosphate buffer, pH 4.5, at 22°C, except where noted, with a Perkin–Elmer Coleman 124 DB spectrophotometer equipped with a cuvette carousel and constant-temperature circulator. Halogenating activity was initially measured

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over a 5 min time span in 2 ml volume by the method of Morris & Hager (1966), with monochlorodimedone (0.14 mM) as substrate in the presence of H_2O_2 (2 mM) and halide (30 mM) and 0.25 ml of enzyme preparation.

Activity measurements with respect to pH were performed by mixing 1 ml of 0.2 M-potassium phosphate buffer (pH 3–9) with 1 ml of monochlorodimedone, H_2O_2 and halide solution prepared at twice the final assay concentration described above. Activity with respect to H_2O_2 concentration (0.01–10 mM) was examined at each respective optimal pH in an analogous manner by mixing 1 ml of the factor being permuted with 1 ml of the other factors. Both KCl and KBr were investigated as potential halogenating ions, ranging in concentration from 0.005 to 0.5 M, at each optimal pH and H_2O_2 concentration. When the optimal pH and H_2O_2 and halide concentrations had been determined, each parameter was varied while holding the other two constant to ensure optimal conditions. The purification scheme was assayed at each step utilizing optimal conditions.

Utilizing the optimal assay mixtures, activity with respect to temperature was measured from 18 to 45°C. In addition, temperature-stability studies were performed by incubating portions of each purified bromoperoxidase in pH 7.4 phosphate buffer at 15, 25, 30, 37, 41, 45 or 50°C for 1 h. These samples were then cooled and assayed at 22°C under optimal assay conditions.

Assays for the oxidation of *o*-dianisidine were conducted in 2.5 ml final volume and contained 1.5 μ mol of *o*-dianisidine, 5 μ mol of H_2O_2 , 100 μ mol of potassium phosphate buffer (pH 4.5) and 0.25 units of enzyme preparation. Peroxidase assays with chloroperoxidase contained 0.3 units of chloroperoxidase. The rate of oxidation of *o*-dianisidine was determined spectrophotometrically at 460 nm over a 5 min time span. Additional compounds assayed as potential substrates (including the λ_{max} of their respective oxidation products) were pyrogallol (420 nm), gallic acid (425 nm), *o*-phenylenediamine (600 nm) and 4-hydroxybenzaldehyde (600 nm).

Sephadex gel chromatography

Molecular-size chromatography was performed on a Sephadex G-100 column (0.8 cm $\times$ 25 cm) calibrated with Blue Dextran, aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A. Eluted fractions of 1 ml volume were collected and assayed for protein and, where appropriate, for halogenating activity.

Results

Purification of bromoperoxidase

Specimens were washed with filtered sea water

and then water, and the rhizoids were removed and discarded. The stalks and tufts were stored frozen at -20°C .

Step 1 (extraction). All homogenization procedures were conducted at 2–5°C in 0.1 M-potassium phosphate buffer (pH 7.4), containing 0.1% Triton X-100. Frozen specimens were homogenized (1 g/ml of buffer) in a Waring blender, a Mikrotek Tissuemizer and finally ground in a Potter–Elvehjem homogenizer. Centrifugation at 10000g for 30 min yielded particulate-free supernatant solutions.

Step 2 $[(NH_4)_2SO_4]$ concentration. Solid $(NH_4)_2SO_4$ was added to each supernatant solution to 50% (w/v) saturation and the resulting insoluble materials were collected by centrifugation. The supernatants were discarded and the pellets were resuspended in 200 ml of 0.1 M-potassium phosphate buffer (pH 7.4). Each suspension was allowed to reach equilibrium at 4°C overnight, after which time centrifugation was repeated. The resulting pellets were inactive, but contained 25% (*R. phoenix*, *P. capitatus*) and 50% (*P. lamourouxii*) of the total protein obtained from the initial 50% $(NH_4)_2SO_4$ precipitation. The values expressed in Tables 1–3 are those for the active supernatant only.

Step 3 $[(NH_4)_2SO_4]$ fractionation. Solid $(NH_4)_2SO_4$ was added to each supernatant to reach 25% (w/v) saturation. After a 1 h equilibration time at 4°C, insoluble materials were again removed by centrifugation. $(NH_4)_2SO_4$ fractionation was repeated, increasing the concentration to 35%, 45%, 55% and 65% saturation. Insoluble materials recovered after each addition were redissolved in 100 ml of phosphate buffer. After $(NH_4)_2SO_4$ fractionation, at least 70% of the *o*-dianisidine-oxidizing activity was removed from the haloperoxidase activity in each case and at least 50% of the protein was removed.

Step 4 (TEAE-cellulose fractionation). The crude bromoperoxidases were loaded on TEAE-cellulose columns (2 cm $\times$ 7 cm) and were washed with 25 ml of 0.1 M-potassium phosphate buffer (pH 7.4). The bromoperoxidases were then eluted from their respective columns with 25 ml portions of 0.1, 0.2, 0.3, 0.4 and 0.5 M-NaCl in buffer. Protein in each fraction was recovered by precipitation in 50% $(NH_4)_2SO_4$. Fractions were resuspended in 25 ml of phosphate buffer and were exhaustively dialysed in the cold against water (molecular-weight exclusion, 18000).

Step 5 (TEAE-cellulose gradient). Each fraction was centrifuged to remove insoluble materials, and the resulting supernatant solutions were loaded on analytical TEAE-cellulose columns (1.2 cm $\times$ 22 cm). The bromoperoxidases were then eluted with an NaCl gradient (0.1–0.55 M, 300 ml) in phosphate buffer. Protein in each eluted fraction (5 ml) was

Table 1. *Purification of R. phoenix bromoperoxidase*

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
50% (NH ₄) ₂ SO ₄ -precipitated fraction*	200	108	1.2 × 10 ⁵	1110	100
(NH ₄) ₂ SO ₄ fractionation (35–45%)	100	21	9.2 × 10 ⁴	4380	77
TEAE-cellulose (0.3–0.5 M-NaCl eluent)	25	9	4.5 × 10 ⁴	5000	38
TEAE-cellulose gradient (0.3 M-NaCl eluent gradient)	10	2.2	3.1 × 10 ⁴	14 100	26
Gel disc electrophoresis	10	0.52	2.9 × 10 ⁴	56 000	24

* Prepared from 391 g of plant material.

Table 2. *Purification of P. capitatus bromoperoxidase*

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
50% (NH ₄) ₂ SO ₄ -precipitated fraction	200	152	2.0 × 10 ⁵	1320	100
(NH ₄) ₂ SO ₄ fractionation (25–45%)	100	77	1.9 × 10 ⁵	2470	95
TEAE-cellulose (0.3–0.5 M-NaCl eluent)	25	20	9.6 × 10 ⁴	4800	48
TEAE-cellulose gradient (0.31 M-NaCl eluent gradient)	10	3.7	3.8 × 10 ⁴	10 300	19
Gel disc electrophoresis	10	1.1	3.1 × 10 ⁴	28 200	15.5

* Prepared from 1210 g of plant material.

Table 3. *Purification of P. lamourouxii bromoperoxidase*

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
50% (NH ₄) ₂ SO ₄ -precipitated fraction*	200	240	1.2 × 10 ⁵	500	100
(NH ₄) ₂ SO ₄ fractionation (0–25%)	100	112	1.7 × 10 ⁵	1520	142
TEAE-cellulose (0.2–0.3 M-NaCl eluent)	25	16.5	6.3 × 10 ⁴	3820	52
TEAE-cellulose gradient (0.28 M-NaCl eluent gradient)	10	8.7	6.0 × 10 ⁴	6900	50
Gel disc electrophoresis	10	1.3	5.2 × 10 ⁴	40 000	43

* Prepared from 1190 g of plant material.

precipitated in 50% (NH₄)₂SO₄, centrifuged, re-suspended in 10 ml of phosphate buffer and dialysed against water.

Step 6 (electrophoresis). Analytical and preparative 1.5% polyacrylamide-gel disc electrophoresis was performed at pH 9.0 (Tris/borate) by the method of Davis (1964). Protein (100 µg) was loaded on analytical gels (total gel volume, 1 ml) and electrophoresis was carried out at a current of 3 mA/tube. Electrophoresis of pooled active fractions (100 µg/tube) was used as one criterion of purity. Preparative gels (40 ml gel volume) separated up to 2.5 mg of each bromoperoxidase. Preparative electrophoresis was performed at a current of 40 mA/gel. After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue by the method of Maurer (1971). Parallel unstained gels were sliced into 3 mm fractions and

were eluted with 10 ml of buffer. These fractions were assayed for both protein and halogenating activity.

The final specific activity achieved for each bromoperoxidase after gel disc electrophoresis was 5.6 × 10⁴ units/mg of protein for *R. phoenix*, 2.8 × 10⁴ units/mg of protein for *P. capitatus* and 4.0 × 10⁴ units/mg of protein for *P. lamourouxii*. One unit of bromoperoxidase activity is the amount of enzyme required to convert 1 µmol of monochlorodimedone into bromochlorodimedone (2-bromo-2-chloro-5,5-dimethylcyclohexane-1,3-dione) at 22°C and 0.14 mM-monochlorodimedone, in the presence of optimal concentrations of H₂O₂ and KBr, and at the optimal pH for each enzyme. Tables 1–3 summarize the course of purification as described above.

Molecular weights estimated by Sephadex G-

100-gel chromatography for each enzyme reveal the *Penicillus* sp. enzymes to be comparable at mol.wt. approx. 50000 and the *R. phoenix* enzyme to be slightly larger (mol.wt. 60000; see Fig. 1). Calculation from four separate determinations show *R. phoenix* contains 307 ± 19 units/g of plant tissue, *P. capitatus* contains 165 ± 25 units/g of plant tissue and *P. lamourouxii* contains 100 ± 15 units/g of plant tissue.

Polyacrylamide-gel electrophoresis and assay of protein fractions for brominating activity revealed the bromoperoxidases from these three plants to be electrophoretically distinct from one another. Their

Fig. 1. Sephadex G-100-gel chromatography of Chlorophyta bromoperoxidases

Molecular-size chromatography was performed on a Sephadex G-100 column (0.8 cm $\times$ 25 cm). The column was calibrated with Dextran Blue, aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A. Fractions (1 ml) were collected and assayed for protein as described in the text. Halogenation assays were also performed as described in the text. Sample volume of the bromoperoxidases was 0.1 ml or 200 units.

migration with respect to Bromophenol Blue migration was 0.66 for *R. phoenix*, 0.67 for *P. capitatus* and 0.55 for *P. lamourouxii*.

Each purified bromoperoxidase exhibited a constant specific activity when rechromatographed either by linear NaCl gradient ion-exchange procedures (TEAE-cellulose) or by Sephadex G-100 molecular-sieve techniques. Also the enzyme activity/protein ratio in each case was constant through-

Fig. 2. Determination of temperature effects on the bromination of monochlorodimedone

Assays for the bromination of monochlorodimedone were carried out in 0.1 M-phosphate buffer under conditions of optimum pH, bromide concentration and peroxide concentration. The decrease in A_{275} was measured spectrophotometrically as described in the text. The rate of bromination was shown, in each case, to increase with increasing temperature. Symbols: $\blacktriangle$, *R. phoenix*; $\blacksquare$, *P. capitatus*; $\bullet$, *P. lamourouxii*.

Table 4. Comparison of bromoperoxidases from *R. phoenix*, *P. capitatus* and *P. lamourouxii*

	<i>R. phoenix</i>	<i>P. capitatus</i>	<i>P. lamourouxii</i>
Optimal pH	5-6	4	4
Optimal $[H_2O_2]$ (mM)	0.25	0.125	0.125
Optimal $[KBr]$ (mM)*	30	30	15
Activity (units†/g of plant material)	307 ± 19	165 ± 25	100 ± 15
Specific activity (units†/mg of protein)	5.6×10^4	2.8×10^4	4.0×10^4
T_n ‡	3.5×10^6	1.7×10^6	2.2×10^6
Apparent E_a (joules)	73 200	35 900	31 200
Apparent M_w	6.0×10^4	5.2×10^4	4.8×10^4
Relative migration on gel disc electrophoresis	0.66	0.67	0.55

* None exhibit any chlorinating activity.

† μ mol of monochlorodimedone converted into bromochlorodimedone per min (at 22°C; 0.14 mM-monochlorodimedone; optimal H_2O_2 , KBr and pH).

‡ Turnover number, μ mol of monochlorodimedone converted into bromochlorodimedone per min/ μ mol of enzyme (at 22°C; 0.14 mM-monochlorodimedone; optimal H_2O_2 , KBr and pH).

out each profile, further suggesting homogeneity. Pooled active fractions from each disc-gel electrophoresis migrated as a single band on analytical disc gels.

The haloperoxidases from these three chlorophyta are compared in Table 4. Each exhibits an acidic pH optimum with respect to monochlorodimedone-halogenating activity. These enzymes are bromide-selective and possess no activity in the presence of KCl. The optimal halide concentration used by Morris & Hager (1966) for chloroperoxidase is near optimal for these enzymes at 15–30 mM. The optimal H_2O_2 concentration, in all cases, is approx. 0.1–0.25 mM. Peroxide concentrations in excess of 2 mM completely inhibited the *P. capitatus* enzyme. The *R. phoenix* enzyme is also sensitive to concentrations of peroxide in excess of 1 mM. The *P. lamourouxii* enzyme, although sensitive to H_2O_2 , remains partially active in the presence of 10 mM- H_2O_2 .

In seeking a temperature optimum for these enzymes, it was discovered that all are remarkably heat-stable, withstanding temperatures of 60°C for 1 h or more with no loss in activity. The rate of monochlorodimedone bromination was markedly increased at higher assay temperatures (see Fig. 2).

Discussion

When algal supernatant solutions were incubated in the presence of *o*-dianisidine and H_2O_2 , a rapid oxidation of the substrate was evident in each case. The oxidation of *o*-dianisidine in these crude extracts was impossible to quantify, owing to turbidity, but assay mixtures turned dark-brown or black within seconds of substrate addition.

For *P. capitatus* and *R. phoenix*, the bromination of monochlorodimedone was likewise evident in crude supernatant incubations. Bromoperoxidase activity was observed in *P. lamourouxii* preparations only after precipitation and concentration by $(\text{NH}_4)_2\text{SO}_4$ fractionation.

It appears that these Chlorophyta contain large amounts of uncharacterized peroxidases that are unrelated to brominating activity. Throughout each purification, the *o*-dianisidine-oxidizing activity decreased until the purified enzymes possessed less than 1 unit/mg of protein. In fact, of the standard peroxidase assay substrates, few were of any value in measuring bromoperoxidase activity. None of the three purified enzymes will oxidize *o*-dianisidine, guaiacol, gallic acid or hydroxybenzaldehyde, but will oxidize pyrogallol, *o*-phenylenediamine and I^- to I_3^- .

The results presented in Table 4 show that these enzymes are similar to chloroperoxidase (Morris & Hager, 1966). Chloroperoxidase exhibits a halogenation optimum at pH 2.75. In the case of

chloroperoxidase, this rather low optimum is thought to be related to the enzyme's ability to bind halide. The halide optimum found by Morris (1965) was dependent on the peroxide concentration, and vice versa. The optima determined were 20 and 2 mM respectively.

The three bromoperoxidases we have purified exhibit a pronounced sensitivity to H_2O_2 . We believe that this is due to an irreversible inhibition of the enzyme. Dialysis or the addition of halide does not restore activity. There is no apparent interdependence between the halide and peroxide concentrations.

The optimal halide concentration of 20 mM for chloroperoxidase activity is very similar to that determined for the bromoperoxidases. Peroxide, on the other hand, is readily utilized at 5–10% of the concentration required by chloroperoxidase. If the kinetics of peroxide and halide binding prove to be like those for chloroperoxidase, one can infer roughly equivalent halide-binding constants for the bromoperoxidases. The observed H_2O_2 optima may be dependent not only on binding, but also on oxidative inactivation of the active enzymes. Our finding of irreversible loss of enzyme activity mentioned above strengthens our inferences of oxidative inactivation by peroxide.

These bromoperoxidases will not chlorinate monochlorodimedone as will chloroperoxidase. The enzymes are very active brominating enzymes, however, when compared with chloroperoxidase. The theoretical maximum specific activity for chloroperoxidase is 2100 units/mg (Morris, 1965). With optimal assay conditions for monochlorodimedone bromination, the theoretical turnover number for pure chloroperoxidase is about $9 \times 10^4 \text{ min}^{-1}$. Incubation under optimal assay conditions for each purified bromoperoxidase yielded turnover numbers ranging from 1.7×10^6 to $3.5 \times 10^6 \text{ min}^{-1}$, showing that they are from 20 to 40 times more active enzymes. Higher temperatures, as discussed below, further increased the activities. However, the fact that halogenation reactions of this type occur in the presence of these purified proteins indicates an indisputable similarity with chloroperoxidase, rather than with the plant peroxidases horseradish peroxidase and Japanese-radish peroxidase.

Of all the peroxidases described, only chloroperoxidase and myeloperoxidase possess the capability of oxidizing Cl^- . The inability of our peroxidases to oxidize Cl^- , coupled with the ability to oxidize Br^- and I^- reflect a parallel with the mammalian enzyme lactoperoxidase (Morrison & Schonbaum, 1976). Thus our three bromoperoxidases appear intermediate in halonium ion-generating ability. Because our three enzymes cannot chlorinate monochlorodimedone, we postulate that they are incapable of generating enzyme-bound

chloronium ion. We have no direct data to support this hypothesis. However, on the basis of electrochemical potential, and on reports of Cl^- binding but the absence of chloronium generation, i.e. with respect to lactoperoxidase (Kimura & Yamazaki, 1978), it seems more plausible than a site-specific 'selectivity' hypothesis.

The enzymes are remarkably heat-stable. The marine algae *Penicillus* sp. and *Rhizocephalus* sp. are shallow-water species, and as such are exposed to a wide range of temperatures. It is not surprising to us that these enzymes resist denaturation at 50–60°C. Although it is generally true that the rate of reaction increases with increasing temperature, an Arrhenius plot will often show some deviation from linearity either with changes in mechanism, or at high temperature, with enzyme denaturation. Not only did these enzymes withstand increased temperatures, but they displayed increased activity at these temperatures. One can assume, therefore, that these enzymes are active *in vivo* throughout the year in subtropical waters, and they are more active at higher temperatures. The energies of activation calculated in Table 4 are obviously apparent E_a values, representing the rate-limiting step in the halogenation reaction. At this stage in our investigations, the phenomena are far too complex for the rate-limiting step to be interpreted.

On the basis of the results of preliminary dithionite reduction experiments (Deeb & Hager, 1964) and difference spectra, we at present believe these three bromoperoxidases are haemoproteins. In active oxidized form the *P. capitatus* enzyme exhibits an absorbance maximum at 410 nm and when reduced with excess dithionite, the absorbance maximum shifts to 431.5 nm. Similarly, the *R. phoenix* enzyme shows an absorbance shift from 405 nm in the oxidized form to 422 nm in dithionite-reduced form. The absorbance maximum of *P. lamourouxii* shifts from 407 nm to 423 nm on reduction.

Chloroperoxidase (mol.wt. 42 000), horseradish peroxidase (mol.wt. 39 800) and Japanese-radish peroxidase (mol.wt. 55 700) are all similar in size to these enzymes. However, the molecular sizes determined by Sephadex G-100 chromatography for the chlorophyte bromoperoxidases must be interpreted with caution, as haloperoxidases contain various amounts of covalently bound carbohydrate (Morris & Hager, 1966). Polyacrylamide disc gels stained for glycoprotein with thymol and H_2SO_4 (Racusen, 1979) subsequent to electrophoresis showed qualitatively that the *R. phoenix* enzyme is indeed a glycoprotein. Gels containing the electrophoresed *P. capitatus* or *P. lamourouxii* enzymes failed to react and consequently the carbohydrate content is uncertain. Quantification of physical similarities, including verification of molecular weight, carbo-

hydrate content, amino acid composition and haem content, still require analytical investigation.

Conclusions

We have purified three distinct bromoperoxidases from subtropical marine Chlorophyta. All three, like the terrestrial fungal enzyme chloroperoxidase, exhibit acidic pH optima for the bromination of monochlorodimedone. The enzymes are 20–40 times more active than chloroperoxidase. Like many other plant peroxidases, a markedly high thermal stability was noted. Preliminary molecular-weight estimates by Sephadex G-100 chromatography show similarities to other peroxidases in the range 48 000–60 000.

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