Biochimica et Biophysica Acta, 616 (1980) 329—339

BBA 69136

NEW BROMOPEROXIDASES OF MARINE ORIGIN

PARTIAL PURIFICATION AND CHARACTERIZATION

TIM J. AHERN [a], G. GRAHAM ALLAN [a,*] and DARRELL G. MEDCALF [b,**]

[a] *College of Forest Resources, AR-10, University of Washington, Seattle, WA 98195 and* [b] *Department of Chemistry, University of Puget Sound, Tacoma, WA 98416 (U.S.A.)*

(Received February 28th, 1980)
(Revised manuscript received July 7th, 1980)

Key words: Bromoperoxidase; p-Hydroxybenzyl alcohol; Halogenation; Marine organism; (Rhodophyta)

Summary

Enzymes capable of catalyzing the bromination of *p*-hydroxybenzyl alcohol by Br^- have been shown to be present in crude homogenates of the alga *Rhodomela larix* (Rhodophyta). There are also indications of such activity in the marine invertebrates *Thelepus setosus* and *Ptychodera flava laysanica*.

Detailed analysis of *R. larix* samples indicated that the activity in this species is greatest in the late spring and summer. After partial purification the enzyme had a pH optimum of approx. 4.4, a temperature optimum around 32°C and was inhibited by NaN_3. This algal bromoperoxidase requires the presence of H_2O_2 and can brominate monochlorodimedon and oxidize iodide, but it cannot oxidize chloride. The enzyme appears to be particulate.

Introduction

Many algae and marine invertebrates have been found to contain brominated compounds which, in some species, can account for as much as 9% of the dry weight of the organism [1]. In recent years, the existence of enzymes capable of halogenating organic compounds have been demonstrated in various algae [2—4]. Indeed, Theiler et al. [3] have suggested that marine organisms may account for a significant portion of the halogenated hydrocarbons found in the

* To whom correspondence should be addressed.
** Present address: General Foods Corporation, Technical Center, 250 North St, White Plains, NY 10625, U.S.A.

marine environment. Many of these have been attributed in the past to industrial waste [5]. The antibiotic activity of metabolites such as the bromophenols of *Rhodomela larix* [6] and the potential of bromoperoxidases for the commercial production of brominated flame retardants have generated new interest in these enzymes.

Materials and Methods

R. larix (Turner) C. Agardh, a red alga, was collected at Point Partridge on the west coast of Whidbey Island in the state of Washington during March, July, and September of 1977 and in January, April, June, August, and November of 1978. The algae were frozen within 3 h of collection and thereafter stored at −10°C.

P. flava laysanica (Spengel), a hemichordate acorn worm, and *T. setosus* (Quatrefages 1865), a marine annelid, Family Terebellidae, were collected at Kahala Beach and Kaneohe Bay, respectively, on Oahu Island, Hawaii, in January of 1977. They were frozen immediately and thereafter stored at −10°C.

3,5-Dibromo-4-hydroxybenzyl alcohol

A stirred solution of *p*-hydroxybenzyl alcohol in 15 ml acetic acid was treated with 5 g Br_2 and allowed to stand for 20 min. The crystals which separated were recrystallized from ethanol, washed with cold ethanol, and dried under reduced pressure to yield the product as irregular, short, thick prisms (1.08 g), m.p. 114—115°C, (Auwers and Daecke [7] give m.p. 116—117°C) NMR ($C^2H_3CO_2{}^2H$) 4.7 (*s*, 2H), 7.6 (*s*, 2H), and 2.2 (*d*, 2H); ultraviolet (EtOH) 208 nm (32 812 $M^{-1} \cdot cm^{-1}$) and 288 nm (1147 $M^{-1} \cdot cm^{-1}$).

Preparation of bromoperoxidases

(a) Crude preparations from worms. A suspension of 15 g worms (approx. 10 in number) in 25 ml 50 mM phosphate buffer (pH 5.4) containing 24% sucrose was homogenized at 4°C in 15 s bursts for a total of 10 min. The slurry was then filtered through cheesecloth. In some cases the product filtrate was dialyzed overnight against 50 mM phosphate buffer (pH 5.4).

(b) Crude preparations from alga. A suspension of 500 g *R. larix* in a 250 ml solution was homogenized and filtered as described above. The filtrate was centrifuged at 10 000 × *g* for 30 min. In some experiments, the 10 000 × *g* pellets were mixed with equal volumes of 2% digitonin solution and homogenized in a Teflon® tissue grinder before recentrifugation at 32 000 × *g* for 30 min.

(c) $(NH_4)_2SO_4$ 0—30% and 30—60% fractions. A 30% saturated $(NH_4)_2SO_4$ solution containing the undialyzed *R. larix* crude preparation was centrifuged at 10 000 × *g* for 30 min. The $(NH_4)_2SO_4$ concentration of the supernatant was increased to 60% saturation and centrifugation was performed as before. Each precipitate was taken up in 50 mM phosphate buffer (pH 5.4), homogenized in a Teflon® tissue grinder and centrifuged at 32 000 × *g* for 30 min. The supernatants obtained were designated the 0—30% and the 30—60% fractions.

(d) Sephadex G-100 fraction. Gel filtration of $(NH_4)_2SO_4$ fractions was

carried out by using Sephadex G-100 columns (44 × 2.5 cm) equilibrated with 50 mM phosphate buffer (pH 5.4). The protein content of fractions was established by the method of Lowry et al. [8] and enzymic activity by the oxidation of iodide (see below). The pooled fractions having oxidative activity constitute the Sephadex G-100 fraction.

Enzyme assay 1: bromination of p-hydroxybenzyl alcohol by crude worm and algal preparations

Crude tissue preparations were stirred for 40 min at 23°C in the dark in 80 ml solutions which consisted of 38 mM phosphate buffer (pH 5.4), 1.25 mM *p*-hydroxybenzyl alcohol, and $NaBr/H_2O_2$ (a, 2.0 : 4.0 mM, or b, 50 : 1 mM). Equal amounts of tissue preparations heated for 20 min at 75°C prior to assay were used in the control experiments. The reaction mixtures were then filtered and extracted with ethyl acetate (3 × 60 ml). The dried (Na_2SO_4) extracts were filtered, concentrated under reduced pressure, transferred to polyethylene vials, and allowed to evaporate to dryness. The samples were quantitatively analyzed for bromine by neutron activation analysis, as described by Schulze [9], and qualitatively analyzed by TLC with *n*-hexane as the solvent.

Alternatively, ethanol solutions containing the samples were layered on cross-linked poly(vinyl pyrrolidone) columns prepared as described by Olsson and Samuelson [10]. The ultraviolet absorption of the eluates was monitored spectrophotometrically. *p*-Hydroxybenzyl alcohol was eluted by using 1 mM aqueous HCl. The brominated derivatives were then eluted from the column with 3 M aqueous acetic acid.

Enzyme assay 2: bromination of p-hydroxybenzyl alcohol by G-100 fractions from R. larix

G-100 fractions from *R. larix* were stirred for 40 min at 23°C in the dark in 160 ml solutions which consisted of 22.5 mM phosphate buffer (pH 2.7, 4.4, 5.4, or 7.0)/1.25 mM *p*-hydroxybenzyl alcohol/50 mM NaBr/1.25 mM H_2O_2. The control experiments contained neither *p*-hydroxybenzyl alcohol nor H_2O_2. The reaction mixtures were then prepared for neutron activation analysis as described in enzyme assay 1.

Enzyme assay 3: bromination of monochlorodimedon

Bromoperoxidase preparations were assayed in 3.1 ml 20 mM phosphate buffer (pH 4.4) containing 160 μM H_2O_2, 5.2 mM NaBr and 78 μM monochlorodimedon synthesized as described by Hager et al. [11]. Enzymic activity was measured by the decrease in monochlorodimedon absorbance at 291 nm (19 700 $M^{-1} \cdot cm^{-1}$) at 22°C.

Enzyme assay 4: oxidation of iodide

Bromoperoxidase preparations were assayed in 3.1 ml 20 mM phosphate buffer (pH 4.4) containing 0.5 mM H_2O_2 and 10 mM KI. The reaction rate was calculated from the increase in absorbance at 350 nm according to the method of Hosoya [12]. Controls containing no enzyme were also run and the difference between the two rates was used to determine the rate of enzyme-catalyzed triiodide complex formation.

Results and Discussion

Enzymic bromination of p-hydroxybenzyl alcohol

Table I demonstrates that a substance with an R_F value much larger than that of the starting material, *p*-hydroxybenzyl alcohol, and comparable to that of a brominated derivative, such as 3,5-dibromo-4-hydroxybenzyl alcohol, was extracted with ethyl acetate from reaction solutions containing crude tissue preparations of *P. flava laysanica*, but not from those containing heated tissue preparations of that organism. Although the exact structure of the compound formed was not determined, the behavior of mono- and dibrominated phenols and benzaldehydes (R_F 48—68) relative to their respectively nonbrominated starting materials (R_F 42—51) on TLC silica gel plates (toluene/ethyl formate/formic acid (50 : 40 : 10, v/v/v)), as observed by Manley and Chapman [4], supports the tentative identification of the compound as a brominated derivative of *p*-hydroxybenzyl alcohol. Neutron activation analyses of these extracts, as well as extracts of similar reaction solutions, containing crude preparations of *R. larix* (collected in the summar) or dialyzed *T. setosus* indicated that 1.6—2.2 μmol Br/g tissue were incorporated into the compounds extractable with ethyl acetate.

In contrast, only 0—0.6 μmol Br/g tissue were incorporated into the reaction solutions containing *R. larix* collected in March, November, or January. The seasonal variation in the brominating activity of *R. larix* is plotted in Fig. 1.

When extracts of reaction solutions containing *R. larix* (collected in the summer) were chromatographically separated on cross-linked poly(vinyl pyrrolidone), it was found that a compound with an ultraviolet absorption maximum (λ_{max} 273 nm) and an elution volume similar to the starting material, *p*-hydroxybenzyl alcohol, was obtainable from both active algal solution extracts and extracts of algal reaction solutions containing 1 mM NaN_3, a general inhibitor of peroxidases. When acetic acid was applied to the column the active extracts yielded 1.2—2.5-times more ultraviolet-absorbing compound than the control extracts. Identification of the compound from the second

TABLE I

R_F VALUES OF *p*-HYDROXYBENZYL ALCOHOL, 3,5-DIBROMO-4-HYDROXYBENZYL ALCOHOL, AND THE ENZYMICALLY SYNTHESIZED *p*-HYDROXYBENZYL ALCOHOL DERIVATIVE

Sample	R_F values *	
p-Hydroxybenzyl alcohol	34	—
3,5-Dibromo-4-hydroxybenzyl alcohol	—	54
Extracts of stirred reaction solutions **		
Crude preparation		
Assay procedure 1a	34	60
Crude preparation		
Assay procedure 1b	34	60
Heated crude preparation (75°C, 20 min)		
Assay procedure 1b	37	—
Heated crude preparation (75°C, 20 min)		
No substrates	—	—

* Developed on 10 cm silica gel TLC plates. *n*-hexane solvent.

** Solutions contained 2 ml crude preparations from the worm *P. flava laysanica*. See assay procedure 1.

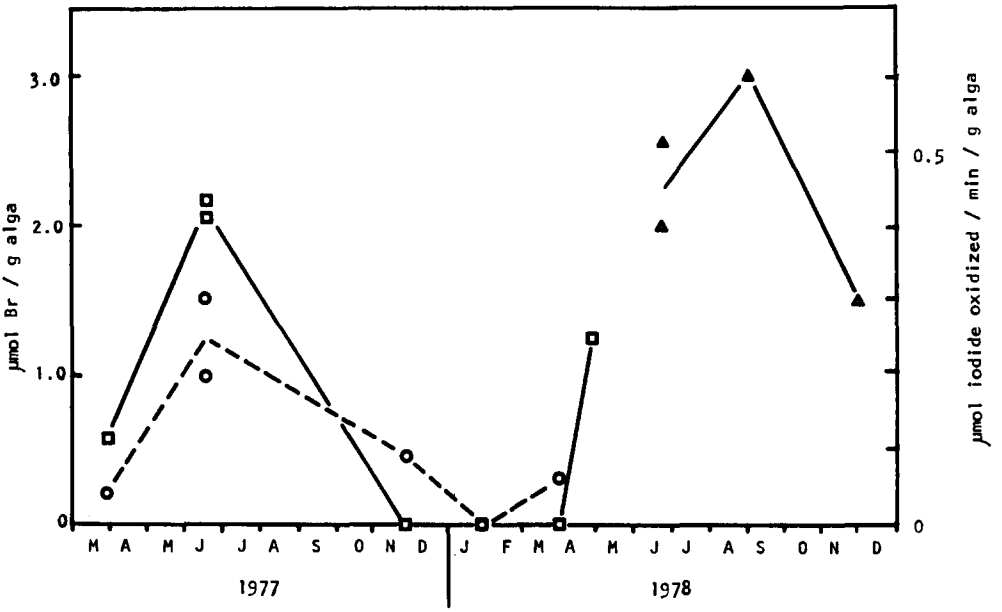

Fig. 1. Seasonality of bromoperoxidative activity in the alga *R. larix*. □———□, *R. larix* crude preparation (1.8 g alga), see Methods, enzyme assay 1b. ○———○, *R. larix* crude preparation (1.8 g alga), see Methods, enzyme assay 1a. Neutron activation values are corrected for bromine found in controls. △———△, *R. larix* 30—60% fraction (approx. 20 µg protein), see Methods, enzyme assay 4.

fraction as a brominated derivative of *p*-hydroxybenzyl alcohol is based on the observation of Olsson et al. [13], that introduction of halogens into aromatic compounds increased their retention volumes upon elution from cross-linked poly(vinyl pyrrolidone). These results, while not conclusive, strongly suggest the enzymic bromination of *p*-hydroxybenzyl alcohol in the presence of crude preparations of the two worms and the alga.

Partial purification of R. larix bromoperoxidase

Throughout these purification steps, enzymic activity was determined by monitoring the conversion of iodide to the triiodide complex. The $(NH_4)_2SO_4$

TABLE II

PARTIAL PURIFICATION OF THE BROMOPEROXIDASE OF THE ALGA *R. LARIX*

Specific activity nmol iodide oxidized/min per mg protein; total units, µmol iodide oxidized/min.

Step	Specific activity	Total units	Yield (percent)	Purification factor (-fold)
Crude preparation, dialyzed	9	5.0	100	1.00
$(NH_4)_2SO_4$ precipitation:				
0—30% fraction, 10 000 × *g* pellet	69	12.9	260	7.7
0—30% fraction, 32 000 × *g* supernatant	78	1.7	32	8.8
30—60% fraction, 10 000 × *g* pellet		17.0	342	
30—60% fraction, 32 000 × *g* supernatant	88	8.3	167	9.9
Gel filtration:				
Sephadex G-100 fraction	139	7.6	153	15.7

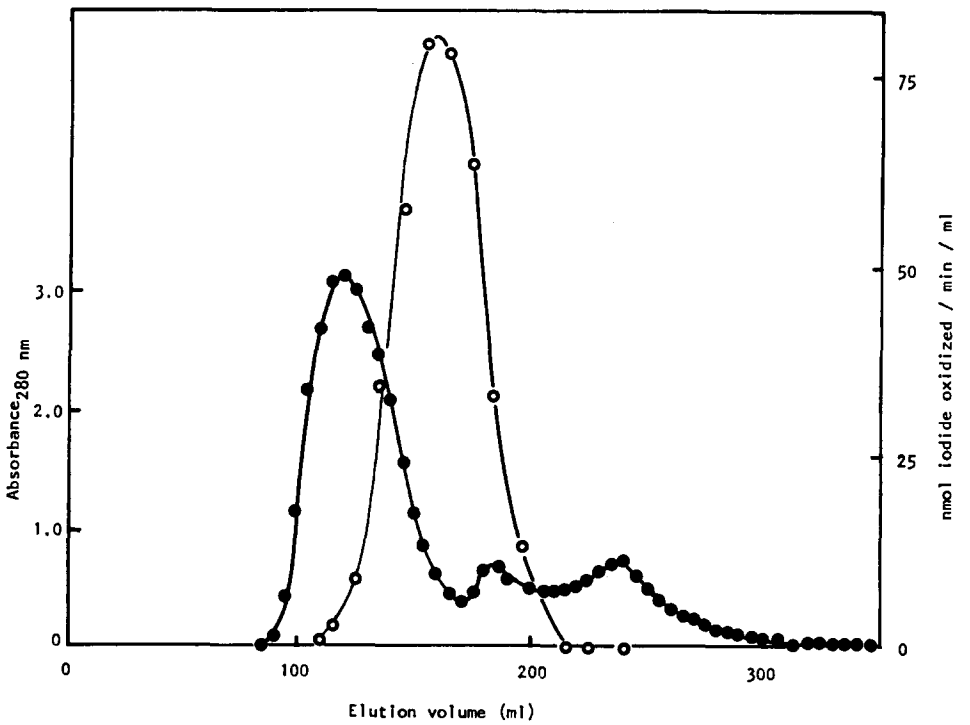

Fig. 2. Fractionation of *R. larix* bromoperoxidase on Sephadex G-100. The 30—60% fraction was layered on the column and eluted with 50 mM phosphate buffer (pH 5.4). •———•, protein was monitored by ultraviolet absorbance; ○———○, enzymic activity of 0.5 ml column eluate aliquots was determined by assay procedure 4.

fractions contained significantly more activity than the crude preparations (Table II). Low enzymic activity in crude preparations of Rhodophytes has been attributed to the presence of sulfate and other inhibitory ions [14]. The elution volume of bromoperoxidase from the Sephadex G-100 column was only slightly larger than the void volume; consequently, maximal activity was found on the shoulder of the peak associated with void volume material (Fig. 2).

Effects of digitonin on enzyme activity and solubility

Treatment with digitonin, a surfactant, increased the activity of the preparations only moderately prior to further centrifugation, but thereafter the activities of the supernatants of the 0—30% fraction and 30—60% fraction were 145 and 83% greater, respectively, than those of fractions not treated with digitonin (Table III). Because of this, and the fact that activity was distributed among any $(NH_4)_2SO_4$ fractions prepared (e.g., 0—25 to 60—80%), we believe that the enzyme is particulate. Pedersen [2] and Theiler et al. [3] came to similar conclusions with regard to other algal haloperoxidases.

Bromoperoxidative activity: assay by spectrophotometric methods

The brominating activity of *R. larix* was calculated from the rate of forma-

TABLE III

THE EFFECT OF DIGITONIN ON THE ACTIVITY AND SOLUBILITY OF *R. LARIX* BROMOPEROXIDASE

Bromoperoxidase fraction	Treatment		Effect	
	Addition of digitonin (final conc. 1%)	Subsequent centrifugation [32 000 X *g* supernatant]	Bromoperoxidative activity *	Increase of activity due to treatment
0—30% saturated $(NH_4)_2SO_4$ precipitate (10 000 X *g* pellet)	no	no	1.38	27%
	yes	no	1.76	
	no	yes	0.66	145%
	yes	yes	1.64	
30—60% saturated $(NH_4)_2SO_4$ precipitate (10 000 X *g* pellet)	no	no	2.49	7%
	yes	no	2.66	
	no	yes	1.54	83%
	yes	yes	2.82	

* μmol monochlorodimedon brominated/min per ml of original bromoperoxidase fraction.

tion of 2-bromo-2-chloro-5,5-dimethyl-1,3-cyclohexanedione (monobromomonochlorodimedon). No loss of absorbance at 291 nm was observed when either enzyme preparation, H_2O_2 or NaBr was omitted from the reaction mixture, and the loss of absorbance was linear for approx. 60 s at pH 4.4. It was found that under optimal conditions of pH, temperature and reactant concentrations, the specific activity of the enzyme for bromination of monochlorodimedon was 2-fold that for iodide oxidation.

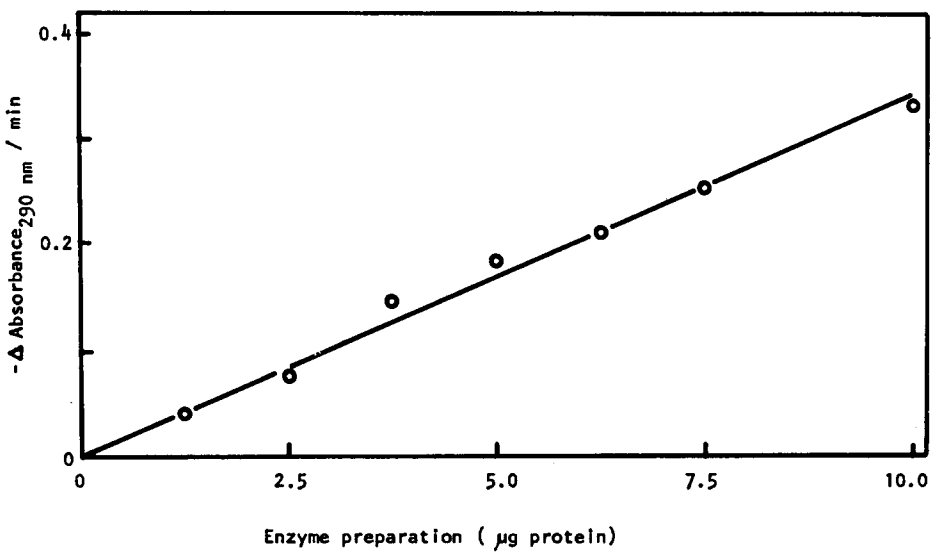

Fig. 3. The effect of *R. larix* enzyme concentration on the rate of bromination of monochlorodimedon. The enzymic activity of the Sephadex G-100 fraction was determined by assay procedure 3.

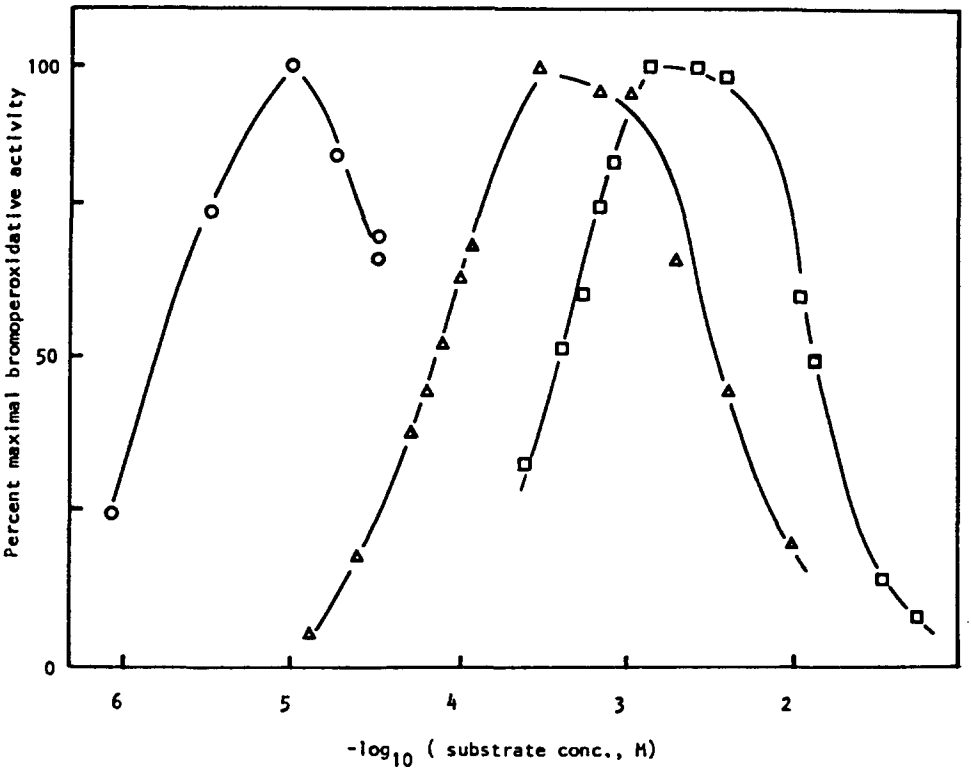

Fig. 4. The effect of substrate concentrations on *R. larix* bromoperoxidative activity. The 30—60% fraction was the enzyme source used. All other reaction conditions were as described in Methods, assay procedure 3, except for the NaBr concentration (1.29 mM). ○———○, monochlorodimedon; △———△, H_2O_2; □———□, NaBr.

The rate of formation of bromochlorodimedon was linear with respect to enzyme concentration in the range of concentrations used (Fig. 3). The data presented in Fig. 4 show that enzymic activity at low concentrations of H_2O_2 or Br^- increased linearly with respect to the log of substrate concentrations, but at higher concentrations of any of the three substrates the enzyme was inhibited. Maximal enzyme activity was attained when Br^-, H_2O_2, and monochlorodimedon concentrations were 1.3, 0.3, and 0.013 mM, respectively.

The temperature profile of bromoperoxidative activity illustrated in Fig. 5 indicates that the maximal activity is approx. 32°C. When the enzyme was subjected to temperatures above 23°C for 20 min prior to the assay, however, a significant diminution of activity was observed. Although dilute, partially purified preparations of membrane or organelle-bound enzymes do not reflect the state of the enzyme in vivo, it is noteworthy that an enzyme preparation from an alga exposed during the day to high air temperatures (approx. 30°C) in the summer months is not stable at temperatures higher than 23°C.

As shown in Fig. 6, the effect of pH on the brominative and oxidative activity of *R. larix* bromoperoxidase is the same, whether the substrate halogenated is *p*-hydroxybenzyl alcohol, monochlorodimedon, or iodide. *R. larix* bromoperoxidase differs from all haloperoxidases heretofore described in that the pH

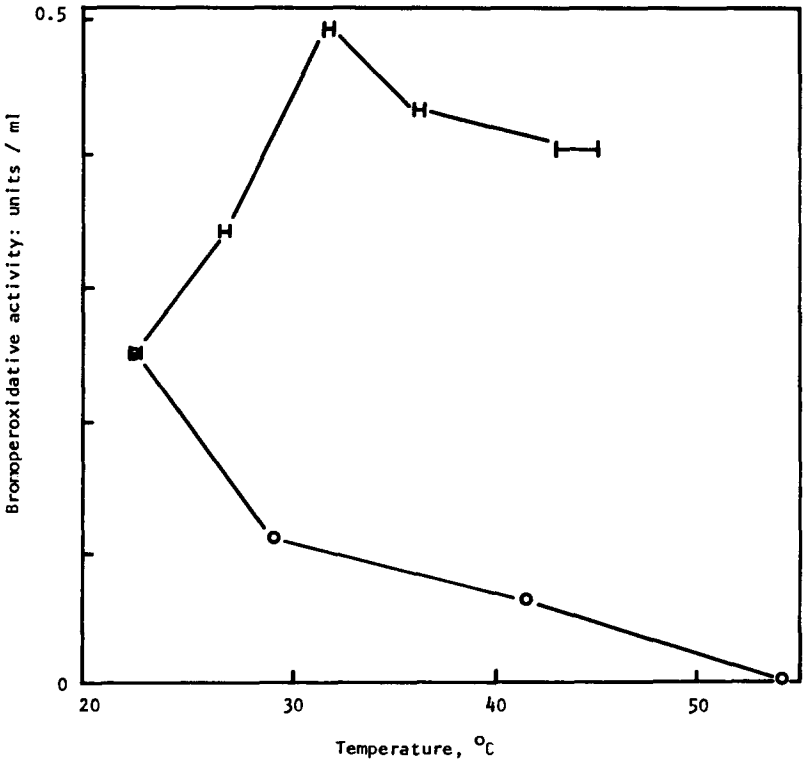

Fig. 5. The effect of temperature on *R. larix* bromoperoxidative activity. The enzymic activity was determined by assay procedure 3. The bars indicate the variation in the temperature of the reaction solutions. ○———○, enzyme was heated to temperature indicated for 20 min prior to reaction.

value (4.4) for optimal activity lies between the halogenation optimum pH of 2.8 for the fungal haloperoxidase [15] and the optimum pH of 5.4 for the red algae, *Cystoclonium purpureum* [2] and *Bonnemaisonia hamifera* [3], and 7.0 for *Odonthalia floccosa* [16].

Seasonality and stability of the algal (R. larix) enzyme

As shown in Fig. 1, bromoperoxidative activity in *R. larix* is highest in late summer and either very low or nonexistent in winter.

Frozen samples of *R. larix* retained 50% of their bromoperoxidative activity after storage at —10°C for 6 months. Crude preparations having low specific activity and low protein concentration lost activity rapidly, as much as 50% in 4 days, when stored at either —10°C or 10°C in 30 mM phosphate buffer at either pH 5.4 or 6.0. However, preparations containing greater than 10 mg protein per ml and having specific activities greater than 0.084 units/mg protein, could be stored frozen for several months without any significant loss in activity.

Halide specificity and inhibition

Bromoperoxidase from *R. larix* has been shown to catalyze the formation of monobromomonochlorodimedon from monochlorodimedon in the presence of bromide but not the formation of dichlorodimedon in the presence of chloride.

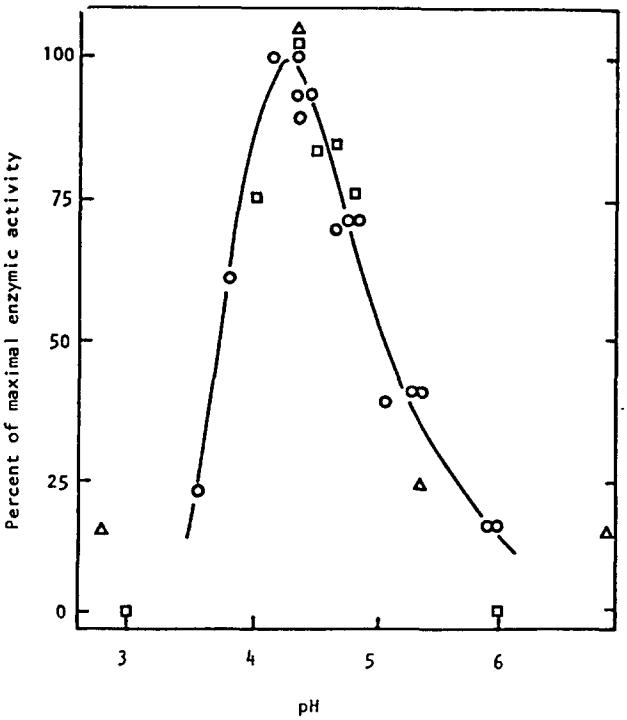

Fig. 6. The effect of pH on the brominative and oxidative activity of *R. larix* bromoperoxidase. Activity was determined by: △———△, the bromination of *p*-hydroxybenzyl alcohol (enzyme assay procedure 2. 100% maximal enzymic activity corresponded to 17.4 μmol bromine incorporated into compounds extractable with ethyl acetate); ○———○, the bromination of monochlorodimedon (enzyme assay procedure 3); and □———□, the oxidation of iodide to the triiodide complex (enzyme assay procedure 4).

It is unclear whether or not the enzyme catalyzes the formation of iodochlorodimedon in the presence of iodide, since under those conditions the possible loss of absorbance at 291 nm is obscured by the increase of absorbance due to the formation of I_3^-. It is of interest that the natural concentration of bromide in sea water, approx. 0.82 mM [18], is within the range in which the velocity of enzyme-catalyzed bromination, v, is sensitive to small changes of bromide concentration (see Fig. 1).

Unlike the fungal chloroperoxidase which catalyzes the chlorination, bromination, and iodination of nucleophilic acceptors [15], *R. larix* bromoperoxidase does not appear to chlorinate such compounds. This probably explains why no chlorinated metabolites have been discovered in *R. larix*, while bromometabolites are reported to account for as much as 9.2% of the dry weight of *R. subfusca* collected in Japan [1], and approx. 5% of the dry weight of *R. larix* found at our collection site [17]. Monobromomonochloro-3,4-dihydroxybenzyl alcohol and dibromomonochloro-3,4-dihydroxybenzyl alcohol have recently been isolated from *R. confervoides* [19], but in quantities so much lower than those of the bromophenols in that algae, that they had escaped detection in previous investigations [20—22].

It is characteristic of heme peroxidases to oxidize I^- [23]. This algal brominase shares this ability, and is subject to 100% inhibition by low concentrations

(1.3 μM) of NaN_3, long recognized as an inhibitor of heme proteins. In addition, the absorption spectra of the fractions, eluted from Sephadex G-100 columns which exhibited bromination and iodide oxidation activity, showed an absorption shoulder at 395—410 nm which corresponds to the prominent Soret band characteristic of porphyrins. Although purer preparations of the enzyme must be obtained to determine the presence or absence of the secondary protoporphyrin absorption bands, we propose that the enzyme is probably a heme peroxidase.

When 19 mM acetate or citrate was substituted for phosphates as buffer, bromination of monochlorodimedon underwent 61 or 57% inhibition, respectively. Thus, they are inappropriate for use as buffers in experiments involving bromoperoxidases.

Acknowledgments

This work was part of the Washington Sea Grant Program (Grant No. 04-7-158-44021) supported by the National Oceanic and Atmospheric Administration of the U.S. Department of Commerce and by matching funds from the resources of the State of Washington. Thanks are due to Professors R.H. Haschke, J.R. Waaland, and B. Weinstein for continued consultation in matters relating to enzymology, phycology, and marine chemistry. The technical assistance of Dr. M.J. Cousin, R.A. Mikels, and W.P. Miller is gratefully acknowledged.

References

1 Kurata, K. and Amiya, T. (1975) Bull. Jap. Soc. Sci. Fish. 41, 657—659
2 Pedersen, M. (1976) Physiol. Plant. 37, 6—11
3 Theiler, R.F., Siuda, J.S. and Hager, L.P. (1978) in Drugs and Food from the Sea, Myth or Reality? (Kaul, P.N. and Sindermann, C.J., eds.), pp. 153—169, University of Oklahoma Press, Norman, OK
4 Manley, S.L. and Chapman, D.J. (1978) FEBS Lett. 93, 97—101
5 Siuda, J.F. and DeBernardis, J.F. (1973) Lloydia 36, 107—143
6 Bhakuni, D.S. and Silva, M. (1974) Bot. Mar. 17, 40—51
7 Auwers, K. and Daecke, S. (1900) Chem. Ber. 32, 3373—3381
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
9 Schulze, W. (1969) in Advances in Activation Analysis (Lenihan, J.M.A. and Thomson, S.J., eds.), pp. 1—36, Academic Press, New York, NY
10 Olsson, L. and Samuelson, O. (1974) J. Chromatogr. 93, 189—199
11 Hager, L.P., Morris, D.R., Brown, F.S. and Eberwein, H. (1966) J. Biol. Chem. 241, 1769—1777
12 Hosoya, T. (1963) J. Biochem. 53, 381—388
13 Olsson, L., Renne, N. and Samuelson, O. (1976) J. Chromatogr. 123, 355—365
14 Wong, K.F. and Craigie, J.S. (1978) Plant Physiol. 61, 633—666
15 Thomas, J.A., Morris, D.R. and Hager, L.P. (1970) J. Biol. Chem. 245, 3129—3134
16 Manley, S.L. and Chapman, D.J. (1979) Plant Physiol. 64, 1032—1038
17 Weinstein, B., Rold, T.L., Harrell, C.E., Jr., Burns, M.W., III. and Waaland, J.R. (1975) Phytochemistry 14, 2667—2670
18 Sverdrup, H.V., Johnson, M.W. and Fleming, R.H. (1942) The Oceans, p. 176, Prentice Hall, New York, NY
19 Pederson, M. (1978) Phytochemistry 17, 291—293
20 Craigie, J.S. and Gruenig, D.E. (1967) Science 157, 1058—1059
21 Glombitza, K.W. and Stoffeln, H. (1972) Planta Med. 22, 391—395
22 Pedersen, M., Saenger, P. and Fries, L. (1974) Phytochemistry 13, 2273—2279
23 Morrison, M. and Schonbaum, G.R. (1976) in Annual Review of Biochemistry (Snell, E.E., ed.), pp. 861—888, Annual Reviews, Inc., Palo Alto, CA