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BROMINATION OF PHENOLS USING AN ALGAL BROMOPEROXIDASE

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Key Word Index—*Corallina vancouveriensis*; Rhodophyta; enzymes; phenols; biological halogenation; bromoperoxidase.

Abstract—A crude acetone powder preparation from the red alga *Corallina vancouveriensis* has been found to serve as a convenient source of bromoperoxidase. This acetone powder (CVAP) catalyses the bromination of phenols in the presence of H_2O_2 and bromide. Conversion of substrate to product(s) is usually rapid and in high yield. The enzyme in the form of CVAP exhibits significantly greater stability than either the cell-free extract or purified bromoperoxidase. CVAP may be useful as a general source of bromoperoxidase and may extend the utility of this enzyme as a chemoenzymatic reagent.

INTRODUCTION

Many species of marine algae are known to contain enzymes known as bromoperoxidases [1]. In the presence of H_2O_2, bromide and an appropriate organic substrate, these enzymes will catalyse an electrophilic-type substitution or addition to the organic molecule. Bromoperoxidases from marine algae (and also bacteria) have recently come under intense scrutiny, as many have been found to contain either vanadium or a 'non-haem iron' as metal cofactors [2, 3]. In some cases, algal bromoperoxidase may be involved in the biosynthesis of halogenated natural products. However, bromoperoxidases have also been found in algae which are not known to produce halometabolites, suggesting a broader biological role for these enzymes.

In addition to general interest in the biochemical and bioinorganic aspects of bromoperoxidases, another area is in the potential application of these enzymes as 'chemoenzymatic reagents'. Enzymatic halogenation has a number of potential practical advantages over chemical halogenation methods. For example, in contrast to most common techniques of chemical bromination, enzymatic halogenation may be carried out in aqueous buffers, uses reagents that are relatively easy to handle and minimizes use of organic solvents and production of toxic wastes. Thus, at very least, enzymatic halogenation offers a 'cleaner' alternative to the production of brominated organic molecules. However, in order to be practical, chemoenzymatic reagents must be easy to prepare, relatively inexpensive and must allow some level of control or selectivity with specific substrates.

Several reports of the reactions of bromoperoxidases with organic molecules have appeared [4, 5]. However, none describes a method involving a generally useful preparation of a bromoperoxidase as a chemoenzymatic reagent. Herein, we report that an acetone powder of the red alga, *Corallina vancouveriensis*, may be used conveniently to catalyse electrophilic bromination of a variety of phenols. In general, the reactions mimic chemical electrophilic aromatic substitution reactions of the same substrates, but benefit by being performed under enzymatic, rather than chemical, conditions.

RESULTS AND DISCUSSION

Preparation of crude acetone powder

Corallina vancouveriensis was collected in La Jolla, CA and kept frozen from the time of collection until processed. The preparation of acetone powder involved repeated homogenization of the algae with a slurry of dry ice–acetone, filtration and re-homogenization of the solid residue. This process was continued until the green pigment in the acetone filtrate was significantly reduced in colour (usually six–seven blending/filtering cycles, total of *ca* 600 ml acetone).

The *C. vancouveriensis* acetone powder (CVAP), is a pale pink, free-flowing powder and *ca* 60 g of acetone powder is obtained from 100 g of frozen algae. When tested against the standard assay for haloperoxidases, using monochlorodimedone (MCD) as substrate [6], CVAP was found to have significant activity (see below).

Stability study

CVAP retained a very high level of bromoperoxidase activity if stored at −4°. For example, in July 1993, batches of CVAP prepared in the summers of 1991, 1992

and 1993 were compared for bromoperoxidase activity using the MCD assay. The highest overall activity was observed in the original batch of CVAP (prepared in July 1991) which was stored for 2 years at −4° without any special precautions (Fig. 1). Lower activity values for the other two years are not likely to be a reflection of loss of enzymatic activity on storage, but rather a variation of bromoperoxidase concn that is observed from one collection to another. Even working from the same site, we have thus far not been able to find a clear correlation between the season of collection or maturity of the plant and the amount of bromoperoxidase activity observed.

Reactions with phenols

For the purposes of this study, one reaction protocol was used for all substrates investigated. Table 1 gives the results obtained with a number of phenols. In each case, it is important to note that control reactions were performed where one of the necessary constituents (H_2O_2, NaBr, or CVAP) was omitted from the reaction soln. In all cases shown, the controls showed no new product formation. The reactions were allowed to proceed for 6–24 hr; the length of time used depended on the observed conversion to product, which was monitored by TLC or HPLC. Additionally, different batches of CVAP converted substrates to products at slightly different rates, but the products were the same. At the concns used, most of the substrates dissolve fairly readily in the aq. buffer upon vigorous stirring. However, even the substrates that did not dissolve immediately eventually went into soln as the reaction proceeded.

Role of hydrogen peroxide

The rate of addition of the H_2O_2 was found to play an important part in the brominating ability of CVAP. If an aliquot of H_2O_2 is added all at once, the rate of product formation is relatively low and slow (Fig. 2). However, as the same total amount of H_2O_2 is added in smaller aliquots which are added over intervals of time, the rate of product formation and overall yield is significantly higher. If present at too high concns, H_2O_2 is believed to irreversibly oxidize the metal cofactor found in these enzymes. The rate of addition of H_2O_2 has been observed to have an effect on the enantioselectivity of chloroperoxidase in the formation of epoxides with alkene substrates [7].

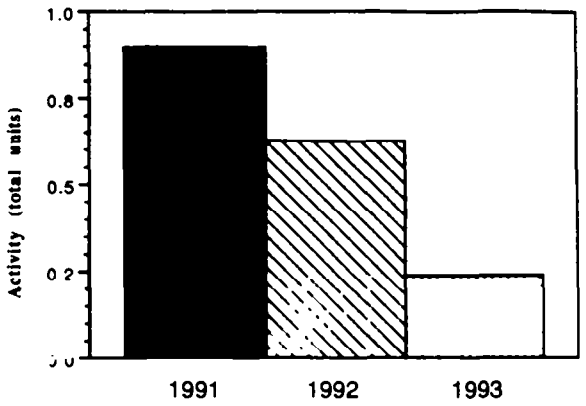

Fig. 1. Relative stability of CVAP prepared in June or July of 1991, 1992 or 1993: MCD activities measured in June 1993.

CVAP vs cell-free extracts

For comparative purposes, we have also performed reactions of the cell-free extract and of purified bromoperoxidase [Okuda, unpublished results] from *C. vancouveriensis* with some of the substrates using the same reaction conditions. We observed that there was little difference between the products formed using any of the three enzyme sources. Apparently, under the conditions of the bromoperoxidase reactions, other enzymes that may be present in the crude prepns do not have significant catalytic activity.

EXPERIMENTAL

CVAP preparation. Corallina vancouveriensis was collected from San Diego, CA and frozen immediately upon

Table 1. Substrates and products obtained by reaction of CVAP with various phenols. The reaction conditions are described in the text and are not optimized for this study

Substrate	Products	% Products*	% Unreacted substrate	Time (hr)
Phenol	2,4,6-Tribromophenol	65	35	24
o-Hydroxybenzyl alcohol	2,4,6-Tribromophenol	78	20	24
m-Hydroxybenzyl alcohol	3-Hydroxy-2,4,6-tribromobenzyl alcohol 4-Bromo-3-hydroxybenzyl alcohol	18 34	48	24
p-Hydroxybenzyl alcohol	2,4,6-Tribromophenol	60	40	6
Methyl salicylate	3,5-Dibromo-2-hydroxymethyl benzoate	91	5	10
Thymol	2,4-Dibromo-3-isopropyl-6-methyl phenol 2,5-Dibromo-3-isopropyl-6-methyl phenol	56 31	9	24

*Determined by HPLC of total extractable products. Minor products account for remaining per cents.

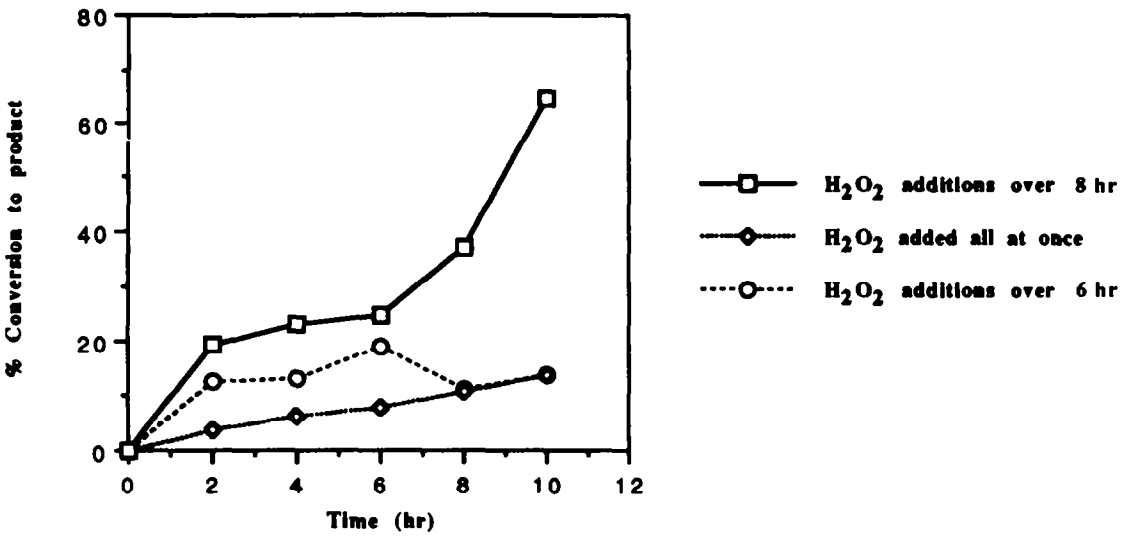

Fig. 2. Effect of the rate of H_2O_2 addition on product formation of 3,5-dibromo-2-hydroxymethyl benzoate (methyl salicylate as substrate).

collection. A voucher specimen is deposited in the Carl Sharsmith Herbarium Collection at San Jose State University. The prepn of the Me_2CO powder consists of blending the frozen algae with ~100 ml of dry ice–Me_2CO, then filtering the suspension. The solid residue is returned to the blender with fresh dry ice–acetone, blended and re-filtered. This cycle of blending and filtering is repeated until the green colour of the Me_2CO extract diminishes and the solid residue becomes a pink, free-flowing powder. Approximately 50–60 g of CVAP are obtained from 100 g of wet algae. The CVAP is stored frozen at −4° until used.

Enzyme assay. The standard assay for haloperoxidase activity was used with some modification. The assay soln consisted of 0.08 mM MCD, 100 mM NaBr, 15 mM H_2O_2 in 75 mM Pi buffer, pH 5.5. For direct measurements of enzyme activity, the assay is initiated by the addition of a measured aliquot (e.g. 25 μl) of the enzyme soln and monitoring by the change in *A* at 290 mm using a diode-array spectrometer. For measurement of bromoperoxidase in CVAP, 100 mg was placed in 500 ml of the MCD assay soln (as indicated above) and stirred at room temp. At measured intervals of time, an aliquot was removed, quickly filtered to remove solid particles and the filtrate *A* measured at 290 nm. One unit of bromoperoxidase activity is defined as the amount of enzyme necessary to convert 1 μM of MCD to product in 1 min at 25°.

Reactions and work-up. In a typical reaction protocol, 1 mM of a substrate is dissolved or suspended in 500 ml of 50 mM Pi buffer, pH 5.5 and 50 mM NaBr and 0.5 g CVAP added to the reaction soln. The reaction is initiated by addition of 5 mM H_2O_2. In some cases, H_2O_2 was added all at once or added in aliquots at regular intervals. The reaction is stirred at room temp. and monitored by TLC or HPLC (silica gel, hexane–EtOAc). For every substrate, 3 controls were prepd, where one of the reagents (NaBr, H_2O_2 or CVAP) was eliminated. The controls were subjected to the same experimental conditions as the reaction mixt. In some cases, an aliquot (e.g. 20 ml) was removed from the reaction and control solns at specific intervals, and immediately extracted with solvent for HPLC or TLC analysis of reaction turn-over.

When completed, the reaction solution is filtered *in vacuo* and the filtrate extracted using either hexane–EtOAc (1:1), or EtOAc if TLC shows the products to be relatively polar. The organic products are then purified by a combination of CC (EM Kieselgel 60; gradient from hexane to EtOAc), prep. TLC (silica gel 60A, hexane–EtOAc, 3:7), or HPLC (Alltech Econosil, hexane–EtOAc, 3:1).

Product data. All products were obtained from reactions of substrates (Table 1) under standard CVAP reaction conditions. Structural assignments were supported by calculations using standard tables [8].

2,4,6-Tribromophenol (prepd from CVAP reaction with *o*- and *p*-hydroxybenzyl alcohol), IR cm^{-1} ($CHCl_3$): 3500, 3076, 2908, 1501, 1380, 1306, 1254, 854. LR EIMS 70 eV, *m/z* (rel. int.): 334 (27), 332 (83), 330 (83), 328 (27), 294 (9), 292 (17), 252 (9), 250 (8), 89 (100); HR EIMS *m/z* obs $[M]^+$ 329.7722, $C_6H_3OBr_3$ (calcd mass 329.7713 for $C_6H_3O^{79}Br_2^{81}Br$). ^1H NMR ($CDCl_3$): δ5.89 (1H, *s*), 7.59 (2H, *s*). ^{13}C NMR ($CDCl_3$): δ110.2, 112.6, 134.0, 148.8.

3-Hydroxy-2,4,6-tribromobenzyl alcohol (prepd from reaction of CVAP with *m*-hydroxybenzyl alcohol), IR cm^{-1} ($CHCl_3$): 3581, 3449, 1441, 1433, 1379, 1298, 1183, 1021. LR EIMS 70 eV, *m/z* (rel. int.): 364 (25), 362 (76), 360 (78), 358 (27), 283 (36), 281 (78), 279 (51), 252 (17), 174 (96), 172 (100), 143 (18); HR EIMS *m/z* obs $[M]^+$ 359.7811, $C_7H_5O_2Br_3$ (calcd mass 359.7839). ^1H NMR ($CDCl_3$): δ4.97 (2H, *s*), 6.03 (1H, *s*), 7.74 (1H, *s*). ^{13}C NMR ($CDCl_3$): δ109.9, 113.4, 115.2, 134.9, 138.5, 149.4.

4-Bromo-3-hydroxybenzyl alcohol (prepd from reaction of CVAP with *m*-hydroxybenzyl alcohol), LR EIMS 70 eV, *m/z* (rel. int.): 204 (36), 202 (38), 187 (97), 185 (100),

123 (11), 95 (11); HR EIMS *m/z* obs $[M]^+$ 201.9636, $C_7H_7O_2Br$ (calcd mass 201.9629). ^1H NMR ($CDCl_3$): δ4.69 (2H, *s*), 5.06 (1H, *s*), 6.62 (1H, *dd*, $J = 3.0$, 8.5 Hz), 7.09 (1H, *d*, $J = 3.0$ Hz), 7.37 (1H, *d*, $J = 8.5$ Hz).

2,4-Dibromo-3-isopropyl-6-methylphenol (prepd from CVAP reaction with thymol). IR cm^{-1} ($CHCl_3$): 3500, 2959, 2913, 2863, 1663, 1651, 1595, 1301, 894. ^1H NMR ($CDCl_3$): δ1.15 (6H, *d*, $J = 2.9$ Hz), 2.21 (2H, *s*), 3.10 (1H, *septet*), 6.57 (1H, *br s*), 7.26 (1H, *s*). ^{13}C NMR ($CDCl_3$): δ18.8, 21.4, 27.8, 130.2, 136.5, 145.5, 154.8, 178.5, 184.7.

2,5-Dibromo-3-isopropyl-6-methylphenol (prepd from CVAP reaction with thymol), IR cm^{-1} ($CHCl_3$): 3499, 2955, 2912, 2861, 1462, 1391, 1387, 1158, 879, 642. ^1H NMR ($CDCl_3$): δ1.23 (6H, *d*, $J = 2.8$ Hz), 2.51 (3H, *s*), 3.24 (1H, *septet*), 5.45 (1H, *br s*), 7.31 (1H, *s*). ^{13}C NMR ($CDCl_3$): δ22.2, 23.8, 28.0, 114.0, 114.9, 128.1, 134.1, 134.8, 148.5.

3,5-Dibromo-2-hydroxymethyl benzoate (prepd from CVAP reaction with Me salicylate), IR cm^{-1} ($CHCl_3$): 3250–3100 (*br*), 2945, 1680, 1600, 1445, 1420, 1320, 1234, 1166, 971. LR EIMS 70 eV *m/z* (rel. int.): 312 (14), 310 (30), 308 (15), 280 (47), 278 (100), 276 (51), 252 (6), 250 (11), 248 (5), 198 (10), 197 (10), 143 (10), 141 (9); HR EIMS *m/z* obs $[M]^+$ 307.8677, $C_8H_6O_3Br_2$ (calcd mass 307.8683). ^1H NMR ($CDCl_3$): δ3.99 (3H, *s*), 7.82 (1H, *d*, $J = 2.5$ Hz), 7.92 (1H, *d*, $J = 2.5$ Hz), 11.38 (1H, *s*). ^{13}C NMR ($CDCl_3$): δ53.1, 110.7, 112.3, 114.4, 131.5, 140.9, 157.3, 169.1

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