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Bromoperoxidase from a Marine Red Macro-alga, *Corallina pilulifera*

Abstract

We studied the distribution of bromoperoxidase in marine algae. As a result, we selected the red alga, *Corallina pilulifera*, as a high producer of the enzyme. The enzyme was purified to homogeneity and characterized. The enzyme had a molecular mass of 790 kDa and was composed of 12 subunits which gave hexagonal molecular shapes of the enzyme. The enzyme was the non-heme haloperoxidase and contained a small amount of vanadium (4 moles V/mole of enzyme) as well as iron (III) and magnesium. When vanadate (VO_4^{3-}) was added to the dialyzed enzyme which had lost 77% of its brominating activity, the activity increased proportionally to the added amounts of vanadate. EPR analysis revealed that the enzyme as isolated was found to contain vanadium. From these results, it was concluded that vanadium was a prosthetic group of the enzyme. We also studied the application of the enzyme in the production of various halogenated compounds, such as the conversion of phenol to tribromophenol, anisole to *o*- and *p*-bromoanisoles, 1-methoxynaphthalene to 1-methoxy-4-bromonaphthalene and thiophene to 2-bromothiophene.

Introduction

Many physiologically active, halogenated compounds have been found in marine algae. Haloperoxidases, which catalyze the following halogenating reaction: $AH + H_2O_2 + H^+ + X^- \rightarrow AX + 2H_2O$ (where AH is a nucleophilic substrate, and X = Cl, Br, I), are known to be involved in the biosynthesis of these halometabolites. Presently marine biotechnology is attracting considerable attention from both applied and basic sciences. Therefore, we have studied the distribution of haloperoxidases in marine macro-algae and purified and characterized the bromoperoxidase from a red alga, *Corallina pilulifera.*

Distribution of bromoperoxidase activities in marine macro-algae

Bromoperoxidase activities were measured for over 60 macro-algal samples, including 34 genera of Rhodophyta, Chlorophyta and Phaeophyta, which were

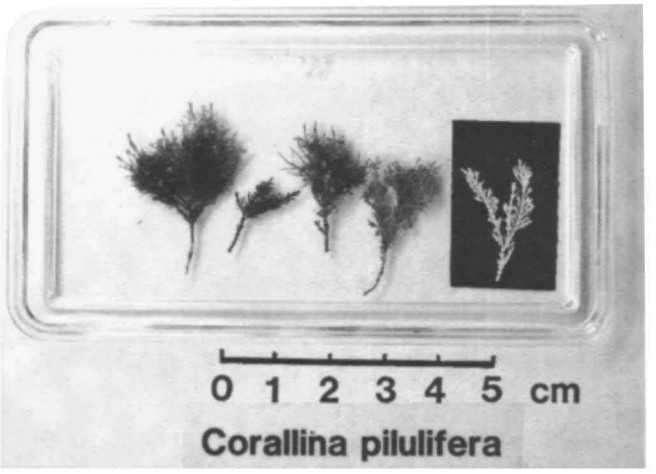


Fig. 1. The marine red macro-alga, *Corallina pilulifera*, the highest bromoperoxidase producer

collected from the seasides of Japan (Yamada *et al.*, 1985; Itoh *et al.*, 1987b). All the coralline algae (8 species) tested showed relatively high enzyme activities. We selected *Corallina pilulifera* for the following experiments not only because this alga was widely found on seahores of any place in Japan but also because it could be collected abundantly and easily (Fig. 1).

Purification and characterization of bromoperoxidase from *C. pilulifera*

Bromoperoxidase was purified from the crude extract of *C. pilulifera* to complete homogeneity (Itoh *et al.*, 1985). From the result that the overall purification was 36-fold, the content of the enzyme in the alga was found to be as much as 3% of the total protein of the crude extract. The enzyme had a molecular mass of about 790 kDa and was composed of twelve subunits of identical molecular mass of 64 kDa. A hexagonal molecular shape of the enzyme was observed by electron microscopy (Itoh *et al.*, 1986) (Fig. 2a) and the complete structure of the enzyme was concluded to be a dodecad aggregate composed of two hexagons face to face, as schematically illustrated in Fig. 2b.
Table 2 summarizes the properties of the enzyme (Itoh *et al.*, 1986; Krenn *et al.*, 1989). The enzyme was specific for I^- and Br^-, and inactive toward Cl^- and F^-. The enzyme was found to contain Fe^{3+}, Mg^{2+} and V5+ (Fig. 3), among which V^{5+} (vanadate, VO_4^{3-}) played a role as a prosthetic group. As shown in Table 3, there was a good correlation between vanadium content and specific activity, whereas no correlation between iron content and specific activity was observed (Krenn *et al.*, 1989). Ferric ion markedly shortened the time required for the full activation of the apo-enzyme by vanadate (Izumi *et al.*, 1992). We also studied the application of the enzyme in the production of various halo-

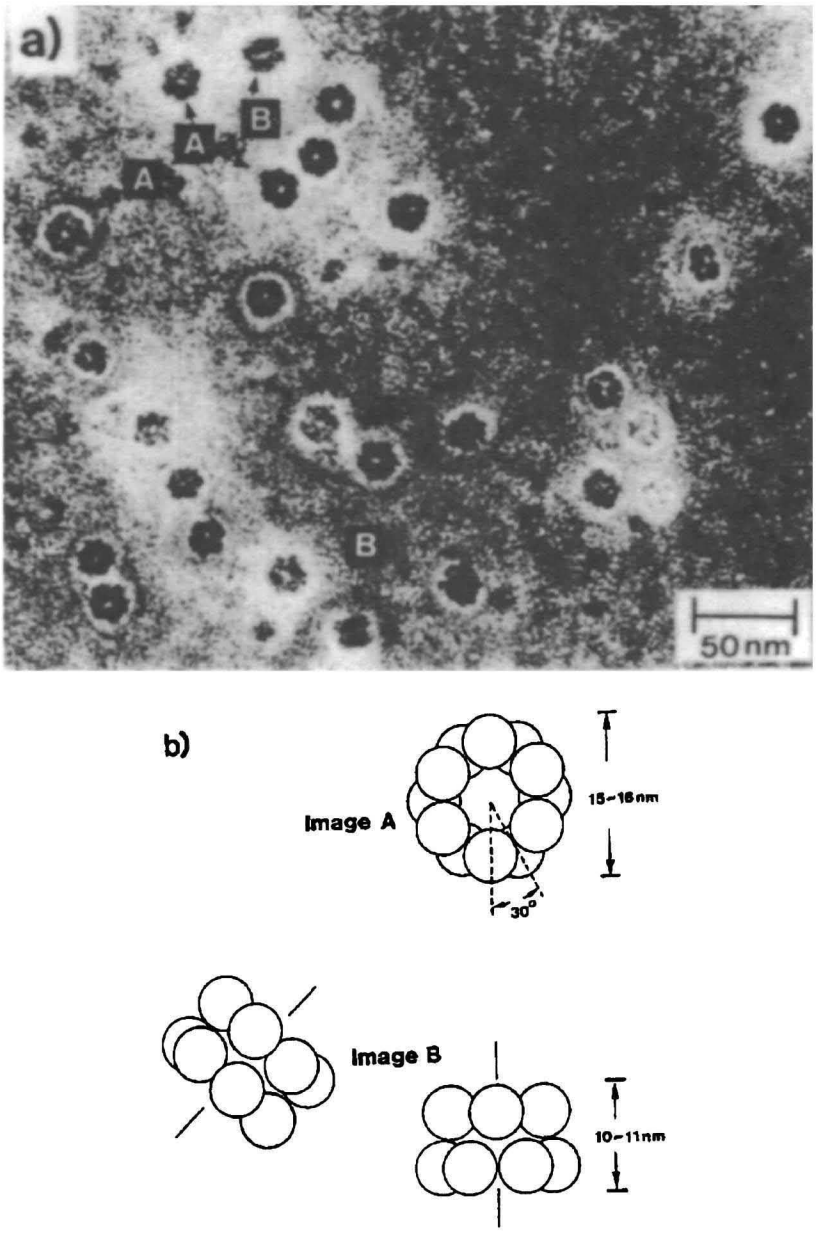


Fig. 2. Electron micrograph (a) and schematic model of the subunit structure of the bromoperoxidase of *Corallina pilulifera*

Table 1. Purification of bromoperoxidase from *Corallina pilulifera.*

Procedure	Protein (mg)	Total activity[a] (Units)	Total activity[b] (Units)	Specific activity[a] (U/mg)	Specific activity[b] (U/mg)
Crude extract	5,933	4,158	7.954	0.7	1.3
Ammonium sulphate (80%)	2,754	1,356	5,965	0.5	2.2
DEAE column (1)	378	973	5,481	1.9	10.7
DEAE column (2)	189	348	5,250	1.8	27.8
Sepharose 6B column	96	317	4,347	3.3	45.2
Cellulofine GC-700	41	149	1,930	3.6	47.1

The enzyme activity in the various fractions was assayed directly (a) and after incubation with vanadate (1 mM, 25°C) (b).

Table 2. Properties of the bromoperoxidase from *Corallina pilulifera.*

Molecular mass	790 kDa
Subunit structure	64 kDa × 12
Isoelectric point	3.0
Carbohydrate residue	–
Halide specificity	Br^-, I^-
Optimum pH	6.0
Km for H_2O_2	9.2×10^{-5} M
Km for Br^-	1.1×10^{-2} M
Catalase activity	+ (halide-dependent)
Peroxidase activity (*o*-dianisidine)	–
Prosthetic group	vanadium (V^{5+})
Metal ions	Fe^{3+}, Mg^{2+}

Table 3. Metal analysis and specific activity of *Corallina pilulifera.*

	Before dialysis	After dialysis[a]
Specific activity (U/mg protein)	20.6	4.8
Vanadium content (mol/mol enzyme)	4.0	0.9
Iron content (mol/mol enzyme)	22	13

[a]dialysis against citrate/phosphate buffer (pH 3.8) containing 1 mM EDTA

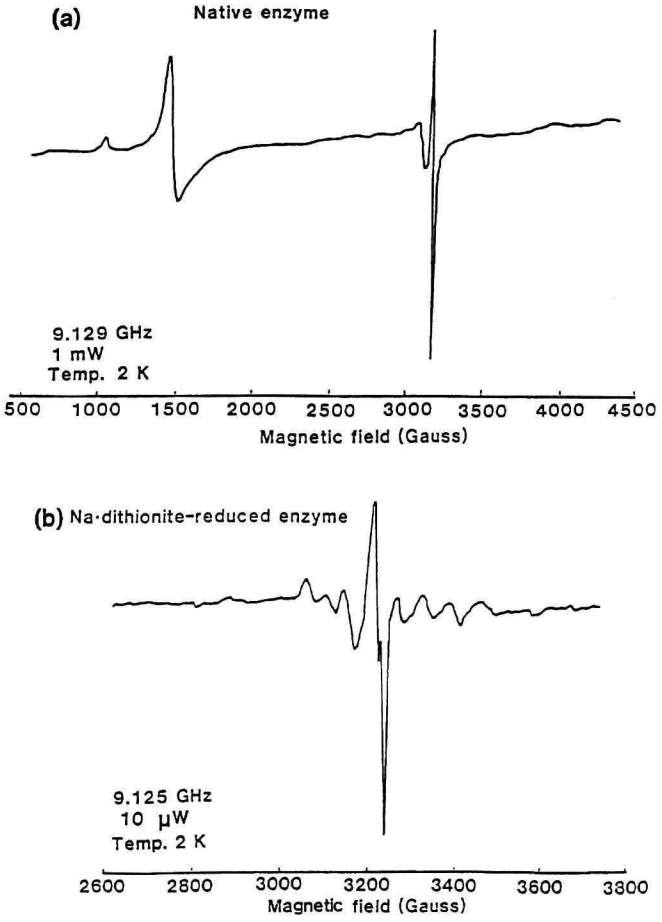
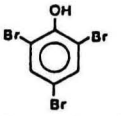
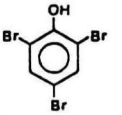
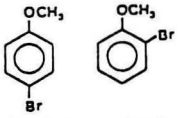
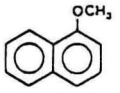
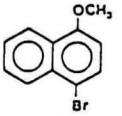
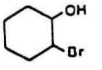
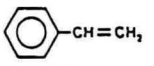
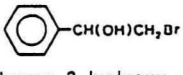
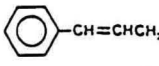
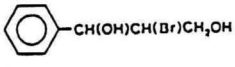
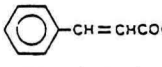
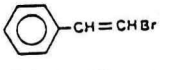
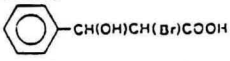


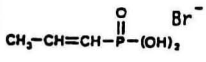
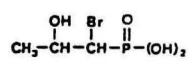
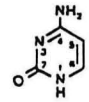
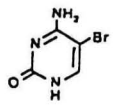
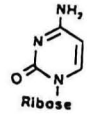
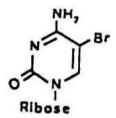
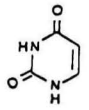
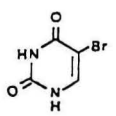
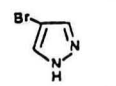
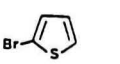
Fig. 3. EPR spectra of bromoperoxidase before (a) and after reduction (b) by dithonite.

genated aromatic compounds, such as the conversion of phenol to tribromophenol, anisole to *o*- and *p*-bromo-anisoles, 1-methoxynaphthalene to 1-methoxy-4-bromonaphthalene and thiophene to 2-bromothiophene as shown in Table 4 (Itoh *et al.*, 1987d, 1988; Izumi *et al.*, 1989). The immobilization techniques using DEAE-Cellulofine and ENT-200 were also found to be suitable for the bromoperoxidase reaction (Itoh *et al.*, 1987a). The immobilized enzyme on DEAE-Cellulofine showed a half-life of 45 days when it was used for the conversion of uracil to 5-bromo-uracil. Thus, the reactor system using the bromoperoxidase could also be used to convert the substrates shown in Table 4 with minor changes of the reaction conditions.

We have recently succeeded in cloning the gene of the enzyme, and the molecular genetical studies of the enzyme will be described elsewhere.

Table 4. Production of various halogenated compounds by bromoperoxidase.

Substrate	Halide	Product
 phenol	Br^-	 2,4,6-tribromophenol
 o-hydroxybenzyl alcohol	Br^-	 2,4,6-tribromophenol
 anisole	Br^-	 p- and o-bromoanisole
 1-methoxy-naphthalene	Br^-	 1-methoxy-4-bromo-naphthalene
 cyclohexene	Br^-	 trans-1-hydroxy-2-bromocyclohexane
 styrene	Br^-	 DL-1-bromo-2-hydroxy-2-phenylethane
 trans-cinnamyl alcohol	Br^-	 (±)-1,3-dihydroxy-2-bromo-3-phenylpropane
 trans-cinnamic acid	Br^-	 trans-β-bromostyrene
		 (±)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid

Substrate	Halide	Product
 cis-propenyl-phosphonic acid	Br^-	 (±)-threo-1-bromo-2-hydroxypropylphosphonic acid
 cytosine	Br^-	 5-bromocytosine
 cytidine	Br^-	 5-bromocytidine
 uracil	Br^- (I^-)	 5-bromouracil (5-iodouracil)
 pyrazole	Br^- (I^-)	 4-bromopyrazole (4-iodopyrazole)
 thiophene	Br^-	 2-bromothiophene

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