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Characterization of Nonheme Type Bromoperoxidase in *Corallina pilulifera**

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Bromoperoxidase was purified from the crude extract of *Corallina pilulifera* to be homogeneous upon polyacrylamide disc gel and sodium dodecyl sulfate-polyacrylamide gel electrophoreses according to the procedures previously reported (Itoh, N., Izumi, Y., and Yamada, H. (1985) *Biochem. Biophys. Res. Commun.* 131, 428–435). The enzyme had a molecular weight of approximately 790,000 and was composed of 12 subunits of identical molecular weights (M_r 64,000). Hexagonal molecular shapes of the enzyme were observed by electron microscopy. The isoelectric point of the enzyme was 3.0, and the predominance of acidic amino acids was revealed by amino acid analysis of the enzyme. The enzyme was specific for I^- and Br^- and inactive toward Cl^- and F^-. The optimum pH of the enzyme was 6.0, and the enzyme was stable in a range from pH 5.0 to 11.0. The enzyme had no heme- or flavin-like compounds as a prosthetic group. Plasma emission spectroscopy revealed that the enzyme contains 2.3 ± 0.2 atoms of iron and 1.6 ± 0.1 atoms of magnesium/molecule of protein. Hence, bromoperoxidase of *C. pilulifera* was distinct from other haloperoxidases and many peroxidases, which are hemoproteins.

A diversity of peroxidases, which catalyze the peroxidative oxidation of many compounds, has been reported in animal and plant tissues and microorganisms. Until now, various types of peroxidases including NAD^+ peroxidase (2), $NADP^+$ peroxidase (3), fatty acid peroxidase (4), cytochrome peroxidase (5, 6), catalase (7), peroxidase (8), glutathione peroxidase (9, 10), iodoperoxidase (11), bromoperoxidase (12–16), and chloroperoxidase (17–19) have been investigated. Among them, haloperoxidases are very interesting enzymes that introduce the halogen-carbon bond in many organic compounds (20). Haloperoxidases are divided into three groups according to the specificity for the halide ion: chloroperoxidase, bromoperoxidase, and iodoperoxidase.

Chloroperoxidase, which was found in *Caldariomyces fumago* by Hager and co-workers (17, 18), has been well studied. The enzyme catalyzes the chlorination, bromination, and iodination of various compounds through electrophilic substitution (19, 21) and addition mechanism (22, 23). We also have studied the specificity, stereoselectivity, and reaction mechanism of the chloroperoxidase reaction using *trans*-cinnamic acid substrates (24). Myeloperoxidase can also catalyze the oxidation of all halide ions except F^- (25).

Recently, bromoperoxidase, which is specific for I^- and Br^-, has been reported in several marine algae such as *Rhodomela* (13), *Rhipocephalus*, and *Penicillus* (12). The enzyme is considered to participate in the synthesis of halocompounds in the marine environment (13, 26). Neidleman and Geigert (25) reported that lactoperoxidase catalyzes the bromination reaction. van Pée and Lingens detected bromoperoxidase activity in the chloramphenicol-producing actinomycete, *Streptomyces phaeochromogenes* (15), and they purified the enzyme from *Pseudomonas aureofaciens* (16).

Iodoperoxidase, which is represented by thyroid peroxidase, does not activate Cl^- and Br^-. Horseradish peroxidase oxidizes only I^- (25).

The above haloperoxidases and peroxidases are all heme-containing enzymes except NAD^+ and $NADP^+$ peroxidases, glutathione peroxidase, and fatty acid peroxidase. NAD^+ peroxidase possesses FAD as a prosthetic group (2), and glutathione peroxidase contains four selenium atoms/molecule of the enzyme (10). The detailed properties of $NADP^+$ peroxidase and fatty acid peroxidase have not been investigated.

In the previous study, we found that coralline alga contains a large amount of bromoperoxidase and demonstrated the bromination of phenol derivatives such as phenol and *o*-hydroxybenzyl alcohol with the enzyme (27). We also established the purification procedures for the enzyme from *Corallina pilulifera* and found that the enzyme contains no heme-like compound as a prosthetic group (1). The present communication describes in detail the properties of bromoperoxidase derived from *C. pilulifera*.

MATERIALS AND METHODS

Chemicals—SDS[1]-gel electrophoresis calibration proteins and DEAE-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Sweden, and marker proteins for molecular weight determination on high performance liquid chromatography (HPLC) from Oriental Yeast Co., Japan. Cellulofine GC-700 was purchased from Seikagaku Kogyo Co., Japan. Analytical reagent grade hydrogen peroxide and standard iron and magnesium solutions were supplied by Wako Pure Chemical Industries, and *o*-dianisidine and standard iodine solution by Nakarai Chemicals, Japan. Superpure reagents of potassium iodide, potassium chloride, and potassium fluoride were obtained from Merck Japan Ltd., Japan. Carrier ampholyte was supplied by LKB, Sweden. Other reagents used in this study were all commercial products of analytical grade.

Collection of Alga—C. pilulifera was collected from shallow water (0.5–1.0 m deep) on the shore of Shirahama (Wakayama Prefecture), Japan in April, 1984, and frozen at −20 °C until use.

Purification of the Enzyme—Bromoperoxidase was purified from the crude extract of *C. pilulifera* as previously reported (1). The purified enzyme was homogeneous using polyacrylamide disc gel electrophoresis.

Enzyme Assay—Bromoperoxidase activity was assayed by measuring the change in absorbance at 290 nm due to conversion of monochlorodimedone to monobromomonochlorodimedone (27). Assay of the oxidation of iodide was carried out according to the modified

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

method of Hosoya (28). Catalase activity was measured at 240 nm by the modified method of Beers and Sizer (29). The detailed reaction conditions were described in the previous paper (27). Peroxidase activity was measured with *o*-dianisidine at 25 °C (30). In all cases, 1 unit of enzyme activity is the amount of the enzyme that converts 1 μmol of substrate in 1 min at 25 °C.

Protein Determination—The protein concentration of bromoperoxidase was determined by measuring the absorbance at 280 nm using the equation: $E^{1\%}_{280\ nm} = 8.05$ (data below).

Polyacrylamide Gel Electrophoresis—Analytical disc gel electrophoresis was performed in 7.0% polyacrylamide gel with Tris-HCl buffer (pH 8.9) according to the method of Davis (31). The samples were run at 3.0 mA/gel at 4 °C. The gels were stained for protein with Coomassie Brilliant Blue G-250 and destained in methanol/acetic acid/H_2O (1:2:7). Staining of gels for glycoprotein was performed by the periodic acid oxidation and Schiff stain method developed by Signoret and Crouzet (32). SDS-gel electrophoresis was performed in 10% polyacrylamide slab gel using a Tris-glycine buffer system described by King and Laemmli (33). Molecular weight of the subunit of the enzyme was estimated from the relative mobility of standard proteins.

Isoelectric Focusing—The isoelectric point of the enzyme was determined as described by Vesterberg (34) using Ampholine electrofocusing equipment (110-ml column). A mixture of Ampholine LKB (pH range of 2.5–4.0) and Ampholine LKB (pH range of 3.5–10) (5:1, by volume) was used as the carrier ampholyte. The samples were run at 500 V for 48 h at 4 °C. The column was then attached to a fraction collector, and 1-ml fractions were collected. Absorption at 280 nm, pH, and enzyme activity were measured for each fraction.

Sedimentation Study—The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (35). The experiments were carried out with a Hitachi analytical ultracentrifuge (model 282) equipped with Rayleigh interference optics. Multicell operations were employed in order to perform the experiments on two samples of different initial concentrations (1.0 and 0.7 mg/ml) with the use of the RAM-18SC rotor and a 3-sample centerpiece cell. Protein solutions, which had been dialyzed against the reference solvent (0.1 M potassium phosphate buffer, pH 7.0), were used as samples. Centrifugation proceeded for 48 h at 25 °C at 3000 rpm, and the interference patterns were photographed to compare and ensure that the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined by the use of the synthetic boundary cell.

High Performance Liquid Chromatography—Analytical HPLC was carried out with a Hitachi 638 system equipped with a TSK-Gel G4000sw column (0.75 × 60 cm, Toyosoda Co., Japan) at the flow rate of 0.3 ml/min using 50 mM potasium phosphate buffer (pH 7.5) containing 0.2 M NaCl. The molecular weight of the enzyme was determined from the calibration curve of standard proteins as follows: acyl-CoA oxidase, 600,000; glutamate dehydrogenase, 290,000; lactate dehydrogenase, 142,000; enolase, 67,000; adenylate kinase, 32,000; cytochrome *c*, 12,400.

Electron Microscope Analysis—For electron microscopy, the enzymes were negatively stained with 2% uranyl acetate (pH 4.5) or 2% phosphotungstic acid (adjusted to pH 7.0 by KOH). A drop of enzyme solution (0.1–0.2 mg of protein/ml), which had been dialyzed against 5 mM potassium phosphate buffer (pH 7.0), was applied on an electron microscopic grid covered with a collodion film. The excess solution was blotted off with filter paper, and then a drop of 2% uranyl acetate or phosphotungstic acid solution was added, blotted, and dried. The grid was observed by a JEM-100C or JEM-1200EX electron microscope (JEOL, Japan). The observations were done at magnifications of 50,000, 80,000, or 100,000. The specimens were photographed by exposure on Fuji FG films (Fuji Photo Film Co., Japan).

Dry Weight Extinction Coefficient—A definite volume of concentrated solution of bromoperoxidase, which had been extensively dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and its absorbance measured at 280 nm, was transferred into the separate stainless vessels. The vessels were placed in the oven at 95 °C for 24 h and weighted after cooling at 1-day intervals until constant weights were achieved. The difference of weights of the vessels containing protein solution and buffer control was used to calculate the dry weight of the enzyme sample. From these data, the average of $E^{1\%}_{280\ nm}$ of 8.05 could be calculated.

Amino Acid Composition—Amino acid analyses were performed according to the method of Spackman *et al.* (36) on an automatic amino acid analyzer K101-AS (Kyowa Seimitsu Co., Japan) with authentic materials as a standard. Samples were hydrolyzed in 6 N HCl under a vacuum for 24, 48, and 72 h at 110 °C. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (37). Tryptophan content was estimated by the method of Spies and Chambers (38) with authentic tryptophan as a standard.

Absorption Spectra—Absorption spectra were measured in cuvettes of 1-cm path length with a Shimadzu UV-240 spectrophotometer at 20 °C equipped with a Shimadzu PR-1 computer-controlled graphic recorder.

Flavin Analysis—Flavin analysis of enzyme solution was performed fluorometrically with a Hitachi 204-R spectrophotofluorometer (excitation, 450 nm; emission, 535 nm) by the procedure of Siegel *et al.* (39). The enzyme solutions, diluted to about 150 and 100 μg/ml with 0.1 M potassium phosphate buffer (pH 7.7), were boiled for 3 min, while protected from light, to cause release of flavin. Then, the fluorescence of 2.5-ml aliquots was measured, and following the addition of 0.25 ml of 1 N HCl, measured again. Buffer was used as the control. Standard FMN and FAD solutions in the same buffer were treated in parallel with the enzyme solutions.

Labile Sulfide Analysis—Acid-labile sulfide content of the enzyme samples was measured according to the method of King and Morris (40) and Siegel *et al.* (39) with sodium sulfide as a standard. Standard solutions of sodium sulfide were prepared as described by King and Morris (40), and their concentrations determined iodometrically.

Metal Analysis—All glassware was boiled briefly in 2 N HCl and then exhaustively rinsed with distilled water before use. The enzyme samples containing 1.0–3.0 mg of protein/ml were measured with an ICAP-500 argon plasma emission spectrophotometer (Nippon Jarrell-Ash Co., Japan). Before assays, the enzyme samples were dialyzed against 5 mM potassium phosphate buffer (pH 7.0) with or without 1 mM EDTA or *o*-phenanthroline. The following assay conditions were employed for qualitative analysis: cooling gas, 14 liters/min; plasma gas, 2 liters/min; sample gas, 0.5 liter/min; sample inhalation, 1 ml/min. The spectra were scanned from 400 to 190 nm at a speed of 25 nm/min. Quantitative analyses of iron and magnesium of enzyme samples were also carried out with the same apparatus by measuring the plasma emission spectroscopy at 259.94 nm for iron and 279.55 nm for magnesium. The metal contents of the enzyme samples were determined from the calibration curves of standard solutions.

RESULTS

Molecular Weight and Subunit Structure—Equilibrium ultracentrifugation was employed to determine the molecular weight of the enzyme. The linearity of the plot as log *fr* (fringe shift) *versus* r^2 demonstrated that the purified enzyme was homogeneous. The molecular weight was estimated to be 790,000 ± 20,000 from the slope of the data plotted, assuming a partial specific volume ($\bar{v}$) of 0.74. The molecular weight of the enzyme was determined to be 880,000 by analytical HPLC on TSK Gel G4000sw column which was calibrated with standard proteins. On SDS-slab gel electrophoresis, a single band of the enzyme was observed, and the subunit molecular weight of the enzyme was 64,000 from the calibration curve of the reference proteins (Fig. 1). These data indicated that the enzyme of *C. pilulifera* consists of 12 subunits of identical molecular weight.

Fig. 2*a* shows the electron micrograph of the uranyl acetate negatively stained image of the enzyme. Uranyl acetate staining was superior to phosphotungstic acid staining in displaying the fine structures of the enzymes. In the photograph, two distinct images were observed. In image *A*, the enzyme appears to be hexagonal with a symmetry axis (15–16 nm). Image *B*, which shows a cleavage line bisecting the rectangular figures (10–11 × 15–16 nm), may represent a view perpendicular to the symmetry axis. From an analysis of these images, we concluded that the complete structure of the enzyme was a dodecad aggregate composed of two hexagons face to face, as schematically illustrated in Fig. 2*b*.

Isoelectric Point and Amino Acid Composition—Isoelectric analysis of the purified enzyme revealed that a single protein peak was coincident with the peak of bromoperoxidase activ-

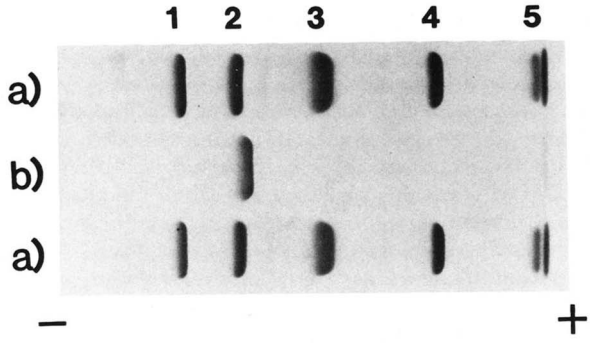

FIG. 1. **SDS-slab gel electrophoresis of the enzyme.** *a*, the following marker proteins are used: *1*, phosphorylase (M_r = 94,000); *2*, bovine serum albumin (67,000); *3*, ovalbumin (43,000); *4*, carbonic anhydrase (30,000); *5*, soybean trypsin inhibitor (20,000). *b*, the purified enzyme (30 μg) was subjected to electrophoresis.

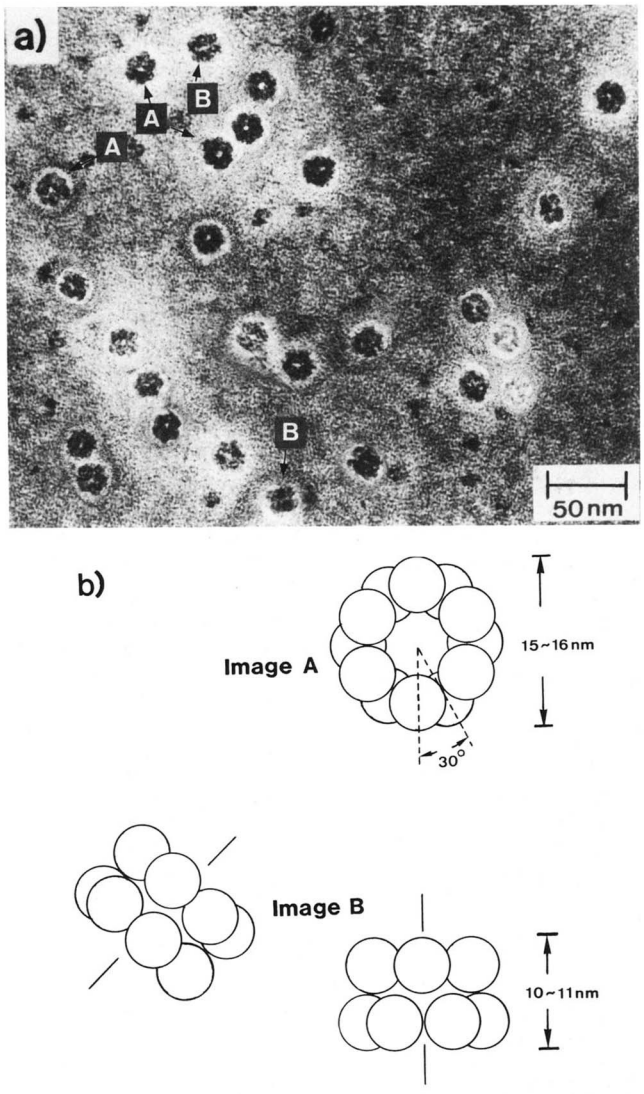

FIG. 2. **Electron micrograph of the bromoperoxidase (*a*) and schematic model of the subunit structure of the enzyme (*b*).** The enzymes were negatively stained with uranyl acetate and observed at 80 kV with a magnification of 80,000. The photograph was reproduced reversely in black and white to obtain a clear image.

ity and that the isoelectric point of the enzyme was 3.0.

The results of amino acid analysis of bromoperoxidase are presented in Table I. The amino acid composition of the enzyme was characterized by the predominance of the acidic amino acids over the basic residues. This resulted in the low pI value (3.0) of the enzyme.

The subunit molecular weight of the enzyme, based on its amino acid composition, could be calculated to be 62,122. The value of the subunit molecular weight of the enzyme was coincident with that (64,000) obtained on SDS-polyacrylamide slab gel electrophoresis. This observation, together with the absence of the periodic acid oxidation-Schiff staining on polyacrylamide, which contained 40 μg of the enzyme, led us to the conclusion that bromoperoxidase of *C. pilulifera* contained no carbohydrate residues.

Enzymatic Reaction and Kinetic Properties—The enzyme catalyzed the oxidation of bromide ions with subsequent carbon-bromine bond formation of monochlorodimedone. The specific activity of the purified bromoperoxidase was 26.3 μmol/min/mg of protein. The omission of hydrogen peroxide or bromide ions in the complete reaction mixture gave rise to no reaction.

The apparent K_m values for bromide ions and hydrogen peroxide of the enzyme were calculated from Lineweaver-Burk plots. The K_m value for bromide ion was 1.1×10^{-2} M. The plot data of bromide ions was linear over a range of concentration between 2.5 and 400 mM (Fig. 3*a*). The apparent K_m value for hydrogen peroxide was 9.2×10^{-5} M, and an inhibition by hydrogen peroxide was observed at concentrations higher than 5.0 mM (Fig. 3*b*).

Halide Ion Specificity—Halide ion specificity of the enzyme for F^-, Cl^-, and Br^- was examined by the monochlorodimedone assay method and that for I^- was by the formation of I_2. The purified enzyme was specific for Br^- and I^-, and the enzyme did not act on Cl^- and F^-. The data were coincident

TABLE I

Amino acid composition of bromoperoxidase

Details of the hydrolyses are given under "Materials and Methods." Except where noted, each value is the average of 24-, 48- and 72-h hydrolysates. Except for those amino acids determined independently and those estimated by extrapolation, the results are consistent to ± 5% or better.

Amino acid	Residues/monomer
Aspartic acid	56
Threonine[a]	24
Serine[a]	33
Glutamic acid	50
Proline	26
Glycine	38
Alanine	49
Valine	32
Methionine	7
Isoleucine	29
Leucine	45
Tyrosine	11
Phenylalanine	28
Lysine	21
Histidine	7
Arginine	25
Tryptophan[b]	4
Half-cystine[c]	4

[a] Values obtained by extrapolating to time zero, assuming first order decay.

[b] Tryptophan was determined by the method described by Spies and Chambers (38).

[c] Cysteine was determined as cysteic acid after performic acid oxidation (37).

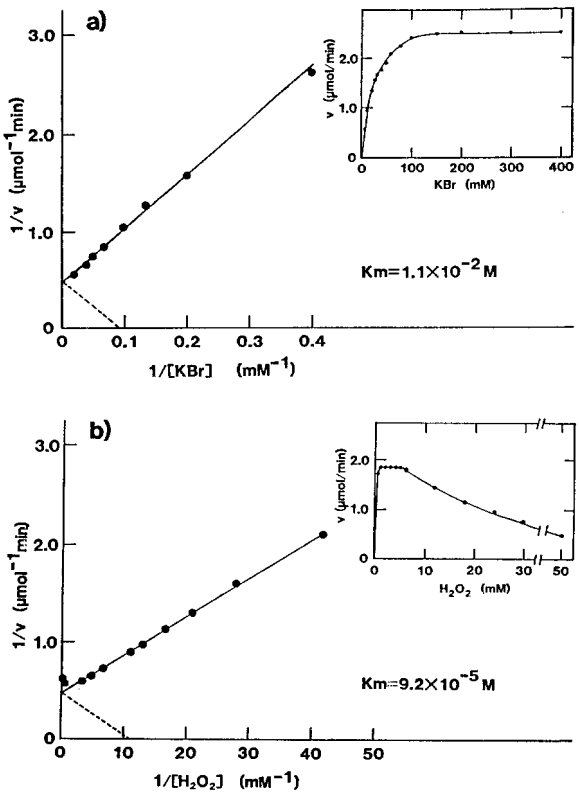

FIG. 3. **Lineweaver-Burk plot for the bromination of monochlorodimedone catalyzed by the enzyme.** The reactions were done at 25 °C in the reaction mixture containing 54 ng of the enzyme, 60 nmol of monochlorodimedone, 100 μmol of potassium phosphate buffer (pH 6.0), and varying amounts of potassium bromide and 2 μmol of hydrogen peroxide (*a*), or varying amounts of hydrogen peroxide and 20 μmol of potassium bromide (*b*) as indicated, in a total volume of 1.0 ml.

with those previously obtained for other coralline algal samples (27).

Peroxidase and Catalase Activities—The peroxidase activity of the enzyme was determined by the *o*-dianisidine method at pH 6.0. In the absence of halide ions, bromoperoxidase slightly catalyzed the oxidation of *o*-dianisidine. One unit of bromoperoxidase exhibited only 0.0036 unit of peroxidase activity. This result was also confirmed by the finding that no color development was observed on gel stained for peroxidase activity using pyrogallol as a substrate without halide ions (1).

The enzyme lacked catalase activity as assessed by measurement at three different concentrations of 5, 10, and 20 mM H_2O_2 varying the enzyme from 0.5 to 2.0 units/ml of reaction mixture at pH 6.0.

Effect of pH and Temperature—The effect of pH on the activity of the purified enzyme was measured in the following buffer (final concentration of 0.1 M): citric acid-K_2HPO_4 buffer (pH 3.0–5.5), potassium phosphate buffer (pH 5.5–8.0), and Tris-glycine buffer (pH 8.0–10.0). The enzyme showed the maximum activity at pH 6.0. The pH stability of the enzyme was also measured by incubation for 2 h at 25 °C in the following buffer (final concentration of 0.1 M): citric acid-K_2HPO_4 (pH 3.0–5.5), potassium phosphate buffer (pH 5.5–8.0), Tris-glycine-NaOH buffer (pH 8.0–11.0), and K_2HPO_4-NaOH buffer (pH 11.0–12.0). The enzyme was stable in a pH range from 5 to 11 and retained 90% of the initial activity after incubation even at pH 12.0, but the enzyme rapidly lost its activity below pH 4.0.

The activity of the enzyme was measured at various temperatures from 20 to 60 °C. The enzyme exhibited the maximum activity at approximately 60 °C. When the enzyme was incubated in 0.1 M potassium phosphate buffer (pH 7.0) at various temperatures for 20 min, it exhibited the following activities: 45 °C, 93%; 50 °C, 80%; 55 °C, 68%; 60 °C, 54%; 65 °C, 4%.

Inhibitors—The effect of various compounds on the bromoperoxidase reaction was examined by adding each compound to the reaction mixture (Table II, A). Metal ions tested here showed no influence on the enzyme activity. The enzyme was strongly inhibited by potassium cyanide, β-mercaptoethanol, dithiothreitol, hydroxylamine, and hydrazine. Sodium azide and potassium fluoride were also inhibitory to the enzyme reaction. However, when the enzyme was assayed after preincubation with these compounds at 25 °C and pH 7.0 for 10 min, the inhibitors of β-mercaptoethanol, dithiothreitol, hydroxylamine, and sodium azide were not observed (Table II, B). Therefore, the inhibitions of these compounds appeared to be reversible and not due to damage of the enzyme protein or active site.

The effect of fluoride ions on the kinetics of the bromination of monochlorodimedone was examined in detail (Fig. 4). The kinetic data indicated that the inhibition of fluoride ions was definitely uncompetitive over the range of concentration between 1.25 and 5.0 mM. The K_i value of fluoride ions was calculated to be 2.6×10^{-3} M from the plot data. Chloride ions (final concentrations of 10 and 20 mM) did not affect the enzyme reaction.

Absorption Spectra—As described in the previous paper (1), a concentrated solution of the purified bromoperoxidase was a light brown with an absorption peak at 277 nm and broad and weak absorption bands between 390 and 700 nm. In addition, no significant absorption bands corresponding to heme or flavin were observed in the spectrum. Exposure of the native enzyme to sodium azide (final concentrations of 10

TABLE II

Effect of various compounds on the bromoperoxidase reaction

Compound	Concentration	Relative activity	
		A[a]	B[b]
	mM	%	
None		100	100
$CaCl_2$	1	99	
$MgCl_2$	1	99	
$MnCl_2$	1	97	
$CoCl_2$	1	97	
$NiCl_2$	1	106	
$ZnCl_2$	0.5	100	
$CuSO_4$	0.1	104	
$FeCl_3$	0.1	105	
$PbCl_2$	0.1	104	
$HgCl_2$	0.1	112	
KCN	0.1	5	
	1	0	83
NaN_3	1	74	99
2-Mercaptoethanol	0.1	0	
	1	0	102
Dithiothreitol	0.1	0	
	1	0	107
Hydroxylamine	1	4	99
Hydrazine	1	9	71
EDTA	1	104	102
α,α′-Dipyridyl	1		96
o-Phenanthroline	1		100
p-Chloromercuribenzoate	1		91
Monoiodoacetate	1	74	

[a] Each compound was added to the reaction mixture.

[b] The enzyme solution was treated with each compound for 10 min at 25 °C, and the residual activity was assayed.

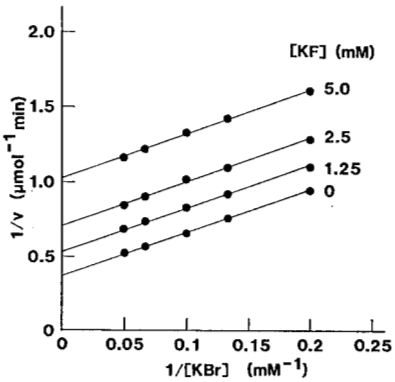

FIG. 4. **Double-reciprocal plots of reaction velocity against potassium bromide in the presence of several concentrations of potassium fluoride.** The reactions were done at 25 °C in the reaction mixture containing 54 ng of the enzyme, 60 nmol of monochlorodimedone, 2 μmol of hydrogen peroxide, 100 μmol of potassium phosphate buffer (pH 6.0), and various amounts of potassium bromide and potassium fluoride as indicated, in a total volume of 1.0 ml.

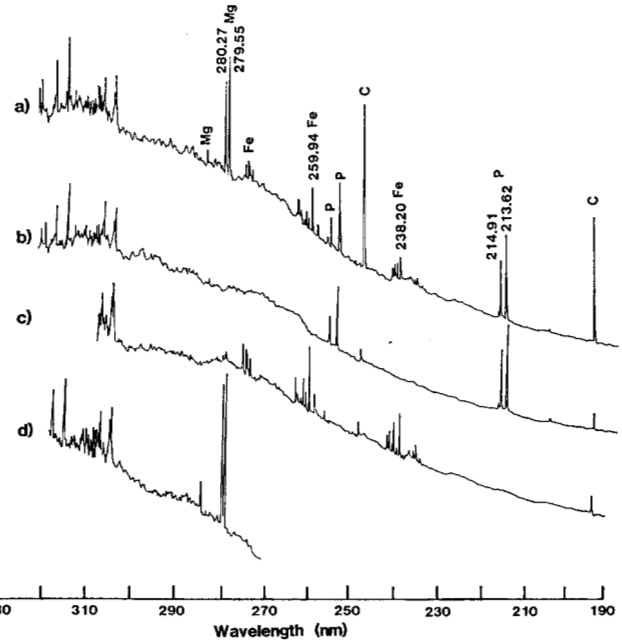

FIG. 5. **Argon plasma emission spectrometry of the enzyme samples.** *a*, the enzyme samples dialyzed against 5 mM potassium phosphate buffer (pH 7.0) in the presence or absence of 1 mM of EDTA or *o*-phenanthroline. *b*, dialysis buffer containing 1 mM EDTA or *o*-phenanthroline, or neither. *c*, standard iron solution containing 2 μg of ferric ion/ml of 0.1 N HCl. *d*, standard magnesium solution containing 0.5 μg of magnesium ion/ml of 0.02 N HCl. The spectra were scanned from 400 to 190 nm at the speed of 25 nm/min, but the unnecessary parts of the spectra were omitted.

and 20 mM) and potassium cyanide (final concentrations of 50 and 100 mM) did not affect its spectrum. These results indicate that bromoperoxidase of *C. pilulifera* is completely different from reported haloperoxidases, which are hemoproteins.

Prosthetic Group—To confirm that the enzyme is not a hemoprotein, the enzyme solution was treated with HCl-methylethylketone for extrication of heme by the method of Teale (41). Then the absorption spectra of the ketoic layer was measured, but no heme was detected.

Analysis of flavin was also performed fluorometrically to ensure that the enzyme contained no flavin compounds such as FMN and FAD.

Qualitative analysis of metals in the concentrated enzyme solutions was done with an argon plasma emission spectroscopy. Fig. 5 showed that the enzyme contained iron and magnesium. Other metals such as vanadium, calcium, selenium, molybdenum, and copper were not observed.

Plasma emission spectroscopy of three enzyme preparations (0.91, 1.50, and 2.10 mg of protein/ml) revealed that the enzyme had 2.3 ± 0.2 atoms of iron and 1.6 ± 0.1 atoms of magnesium/molecule of the enzyme. Exhaustive dialysis of the enzyme samples against the buffer containing 1 mM EDTA or *o*-phenanthroline did not affect the metal contents or enzyme activities. Therefore, iron and magnesium are considered to tightly bind to the polypeptide residues of the enzyme.

Analysis of the enzyme for acid-labile sulfide revealed that the enzyme contained no labile sulfur atom. This suggested that the nonheme iron center of the enzyme differs from the [4Fe-4S] or [2Fe-2S] cluster, which is well known for nonheme iron proteins (42, 43).

The above data led us to the conclusion that bromoperoxidase from *C. pilulifera* is a new type of peroxidase.

DISCUSSION

Coralline algae are characterized by the precipitation of inorganic calcium carbonate in their bodies. These algae are found at the seashores throughout the world, and *C. pilulifera* is found in all coastal areas in Japan. As described in the previous paper (27), the algae including *Corallina*, *Amphiroa*, *Bossiella*, *Calliarthron*, and *Alatocradia* which belong to Corallinaceae exhibited high bromoperoxidase activities. The physiological function of the enzyme in these algae is not yet known, but the enzyme appears to participate in syntheses of antimicrobial organic compounds such as bromophenols. In our laboratory, experiments were carried out using the enzymes of *C. pilulifera* and *Amphiroa ephedraea* to demonstrate the formation of bromophenols from phenol derivatives (27).

The purification of the enzyme from the crude extract of *C. pilulifera* by the combinations of anion exchange chromatography and gel filtration (1) was successful because of the enzyme's high molecular weight and low pI value.

The molecular weight of the purified enzyme was approximately 790,000 by the ultracentrifugal sedimentation equilibrium method. This value seems reasonable, judging from the subunit molecular weight of 64,000 (SDS-gel electrophoresis) or 62,122 (amino acid composition) and the dodecad aggregate image of the enzyme by electron microscope analysis. The molecular weight of 880,000 estimated by the HPLC method appears to be somewhat high. The molecular shapes of dodecad aggregate of two hexagons face to face are similar to those of hemoglobins in earthworms (44, 45). From the image *B* of Fig. 2*a*, one hexagon probably faces the other hexagon at a rotation angle of 30° perpendicular to the axis of the two hexagons. The subunit molecules are packed together as closely as their geometry allows, as illustrated in Fig. 2*b*. The bromoperoxidase of *C. pilulifera* is characterized by its high molecular weight, whereas previously reported haloperoxidases and peroxidases have much lower molecular weight, *e.g.* 40,000 (monomer) of horseradish peroxidase, 48,000 of bromoperoxidase from *Penicillus lamourouxii*, 60,000 of bromoperoxidase from *Rhipocephalus phoenix* (12), and 42,000 (monomer) of chloroperoxidase of *C. fumago* (17). van Pée and Lingens (16) reported that the procaryotic bromoperoxidase from *P. aureofaciens* is a dimeric enzyme with a molecular weight of 155,000–158,000.

The optimum pH of the enzyme was 6.0; however, the other marine algal bromoperoxidases from *Penicillus capitatus*, *P.*

lamourouxii, *R. phoenix*, and *Rhodomela larix* exhibit the maximum activities at a more acidic region of pH 4.0–4.5 (12, 13). One marked property of the enzyme was the high stability in alkaline pH region. However, the enzyme was unstable under acidic conditions (below pH 4.0).

The predominance of glutamic acid and aspartic acid over basic residues in the amino acid composition of the enzyme was similar to that found in horseradish peroxidase, chloroperoxidase of *C. fumago* (17), and bromoperoxidase of *P. capitatus* (14). Many peroxidases and chloroperoxidase of *C. fumago* (17) are known to contain substantial levels of carbohydrate, but the enzyme had no carbohydrate residues.

Manthey *et al.* (14) described that bromoperoxidase of *P. capitatus* could not be used to detect the substrate saturation with bromide ions at very high concentrations because of its low affinity toward bromide ion. However, the enzyme of *C. pilulifera* showed an apparent K_m value of 1.1×10^{-2} M for bromide ions, and this value was similar to that of chloroperoxidase of *C. fumago* for chloride ions (2.8×10^{-2} M) (18). Affinity of the enzyme toward hydrogen peroxide was very strong, and the K_m value was 9.2×10^{-5} M. This value was much lower than that of chloroperoxidase (7.9×10^{-4} M). The enzyme exhibited constant activity in a wide range of hydrogen peroxide concentrations from 0.5 to 5.0 mM (Fig. 3*b*). While the other algal bromoperoxidases have lower optimum hydrogen peroxide concentrations, *i.e.* 0.25 mM for *R. phoenix* and 0.125 mM for *P. capitatus* (12), the enzyme of *C. pilulifera* seemed to possess a high resistance to the inhibitory effect of hydrogen peroxide.

Sulfhydryl compounds like β-mercaptoethanol and dithiothreitol serve as an inhibitor of halogenation reaction of the enzyme of *C. pilulifera*. On the other hand, the reactions of chloroperoxidase of *C. fumago* are inhibited by several antithyroid agents such as thiouracil and thiourea (46). The enzyme of *C. fumago* acts on these compounds to yield the disulfide. These products may arise via intervention of a sulfenyl halide (—SX, X:halide) (46). Therefore, the inhibition mechanism of the enzyme reactions of *C. pilulifera* by these sulfhydryl compounds seems to be identical to that of the enzyme of *C. fumago* by antithyroid agents.

The inhibition of enzymatic halogenation by fluoride ion was not competitive with the halide ion substrate at the active sites. Fluoride ion may bind to the enzyme-bromide-hydrogen peroxide complex.

Compared with the haloperoxidases of other origins (14, 47), the enzyme of *C. pilulifera* exhibited much lower peroxidase activity and no catalase activity itself in the absence of halide ions. van Pée and Lingens (16) reported that procaryotic bromoperoxidase of *P. auerofaciens* has high catalase activity as well as peroxidase activity and low halogenating activity. This marked difference between the enzyme of *C. pilulifera* and other haloperoxidases appears to be due to the differences in the catalytic site, that is the enzyme contains nonheme iron and other haloperoxidases possess heme irons as a prosthetic group.

The light brown of the concentrated enzyme solution was considered to be due to the ferric ions (Fe^{3+}) bound to the polypeptide residues of the enzyme. The bromoperoxidase of *C. pilulifera* catalyzes the bromination of phenol to yield 2,4,6-tribromophenol (27). Thus, the active species of bromination reaction is an electrophile of the bromonium cation (Br^+), not a radical. The ferric ion of the enzyme is probably involved in the oxidoreduction of halide ion and hydrogen peroxide. The reason why the enzyme of *C. pilulifera* contained only 2.3 ± 0.2 iron atoms/molecule of dodecameric enzyme was not clear. However, the similar phenomena are observed in most peroxidases and also bromoperoxidase (16). Since the enzyme contained no acid-labile sulfide, iron atoms probably bind to the cysteine or tyrosine residues of the enzyme. Circular dichroism spectra of the enzyme also support our investigations.[2] Further studies are in progress to detect the active site constitution of the enzyme.

The role of magnesium of the enzyme is obscure. We speculate that magnesium ions bind to carboxylic residues of the enzyme and contribute to the conformational stabilization of the enzyme. Recently, Haschke *et al.* (48) reported that calcium ions are needed for the conformational maintenance of horseradish peroxidase.

The haloperoxidases of *C. fumago*, *P. aureofaciens*, and some marine algae including *Penicillus* and *Rhodomela* may have evolved from classical peroxidases or catalase. However, judging from the above results, it seems that the enzyme of *C. pilulifera* evolved from a different type of enzyme. We propose a classification of haloperoxidases into two groups; one is a heme type (H type) and the other is a nonheme type (NH type) haloperoxidase. Our results provide new information on bromoperoxidase as well as on the classification of peroxidases. The immunochemical property, substrate specificity, stereospecificity, and reaction mechanism of the enzyme will be reported elsewhere.

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