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STUDIES ON
THE HALOGENATION REACTIONS CATALYZED BY
THE HALOPEROXIDASES FROM
CALDARIOMYCES FUMAGO
AND CORALLINA PILULIFERA

NOBUYA ITOH

1987

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INTRODUCTION

Enzyme-catalyzed halogenation is a common biological phenomenon. Various halometabolites, including chloramphenicol and pyrrolnitrin, have been isolated from microbial sources and identified (1). In the marine environment, many halogenated compounds such as bromophenols have been found in marine plants (2-4). In mammals, enzymatic halogenations are important in the biosynthesis of the thyroxine hormone and in biological defense mechanisms during the oxidative destruction of microorganisms by the phagocytes (5).

The few detailed studies of the enzymes participating in the biological formation of halometabolites have been limited to those on chloroperoxidase of the fungus Caldariomyces fumago (6-8) and thyroid peroxidase involved in the synthesis of thyroxine (5). Chloroperoxidase of C. fumago catalyzes the sequential halogenation, at C-2 of 1,3-cyclopentanedione, yielding the antibiotic caldariomycin (6). Hager and co-workers examined the enzymatic properties (9), kinetic mechanism (10), and reaction mechanism (11) of the chloroperoxidase. In the chloroperoxidase reaction, a halide anion (X^- , $X:Cl, Br, I$) is activated to the halonium cation (X^+) through hydrogen peroxide-dependent oxidation, and then the halonium cation is transferred to a halogen acceptor molecule. There have been several reports concerning chloroperoxidase substrates such as β -keto acids (7), cyclic β -diketones (9), and steroids (12-15), as well as substituted phenols such as tyrosine (16) and anisole (17), thiols (18), and thiazoles

(19). Recently, Neidleman and co-workers showed that the enzyme catalyzes the halogenation of gaseous alkenes to yield α,β -halohydrins, which are easily converted to α,β -epoxides (20). The enzyme also catalyzes the halogenation of alkynes (21) and cycloalkanes (22), yielding halogenated ketones and halohydrins, respectively. Thus, the application of the enzymatic halogenation to chemical processes has generated new interest in haloperoxidases. However, a systematic evaluation of substrate specificity of chloroperoxidase has not been performed. This prompted the author to study new substrates of chloroperoxidase of C. fumago. Several α,β -unsaturated carboxylic acids, and nucleic bases and related compounds were found to be suitable chloroperoxidase substrates. Therefore, the products obtained from these substrates were examined to systematize the substrate specificity of the enzyme (Chapter I). The results indicated that chloroperoxidase reaction can be explained in terms of molecular halogen (X_2) addition chemistry in water, which is released from the enzyme in the presence of the halide anion and hydrogen peroxide (Chapter I).

Haloperoxidases are divided into three groups according to the specificity for the halide anion: chloroperoxidase, bromoperoxidase, and iodoperoxidase. Myeloperoxidase can catalyze the oxidation of all halide ions except F^- (23). Iodoperoxidase, which is represented by thyroid peroxidase, does not activate Cl^- and Br^- (23). Recently, several bromoperoxidases, which are specific for I^- and Br^- , have been found in and isolated from marine algae such as Rhodomela (24),

Rhipocephalus (25), and Penicillus (25, 26). These enzymes are considered to participate in the synthesis of halocompounds in the marine environment. Indeed, Theiler et al. have suggested that marine organisms may account for a significant portion of the halogenated hydrocarbons in the marine environment (27). van Pee and Lingens detected bromoperoxidase activity in the chloramphenicol-producing actinomycete, Streptomyces phaeochromogenes (28), and they purified the enzyme from Pseudomonas aureofaciens (29). The author examined systematically the distribution of bromoperoxidase in microorganisms, and the algae collected from seashores in Japan. Bromoperoxidase activities were observed in the algae belonging to the coralline family (Corallinaceae, Rhodophyta) (Chapter II, Section 1). Corallina pilulifera, which exhibited high enzyme activity, was therefore chosen for the purification of the enzyme. The author succeeded in obtaining a homogeneous enzyme preparation, and characterized the enzymatic properties (Chapter II, Section 2). The enzyme of C. pilulifera was a different type of haloperoxidase (nonheme type) from other haloperoxidases (heme type) including the chloroperoxidase of C. fumago, bromoperoxidases of Rhodomela, Rhipocephalus, and Penicillus, lactoperoxidase, and myeloperoxidase. (Chapter II). Recently, another nonheme bromoperoxidase was reported in a brown alga, Ascophyllum, which contains vanadium as a prosthetic group (30, 31). Thus, the author first clarified halogenation mechanism of nonheme bromoperoxidase of C. pilulifera (Chapter II, Section 3). The enzyme catalyzed the Br⁻-dependent catalase reaction when a

suitable halogen acceptor was absent. The enzyme could use many organic compounds as halogenation substrates. Therefore, the author systematized the substrate specificity of bromoperoxidase of C. pilulifera and examined regio- and stereospecificities of the enzyme halogenation (Chapter II, Section 4). Finally, the author investigated the immobilization of bromoperoxidase and applied it to halogenation processes (Chapter II, Section 5).

CHAPTER I

CHLOROPEROXIDASE OF CALDARIOMYCES FUMAGO

Section 1. Chloroperoxidase-catalyzed Halogenation of trans-Cinnamic Acid and Its Derivatives

Chloroperoxidase (EC 1.11.1.10) is a unique mold enzyme that has been well studied by Hager and co-workers since 1959 (1,2). Its most unusual property is the ability to catalyze the peroxidative synthesis of the carbon-halogen bond in a large number of substrates in the presence of Cl^- , Br^- or I^- and hydrogen peroxide. These substrates included β -ketoacids (7), cyclic β -diketones (10), steroids (12-15), substituted phenols such as tyrosine (16) and anisole (17), thiols (18) and thiazole (19).

The enzyme also catalyzes the following peroxide-dependent N-demethylation of anilines (32) and antipyrine (33), N-oxidation of 4-chloroaniline to 4-chloronitrosobenzene (34) and C-oxidation of indole to 2-oxoindole (35). Recently, Neidleman and co-workers showed that gaseous alkenes were converted to α,β -halohydrins by the enzyme (20). Chloroperoxidase also catalyzes the halogenation of alkynes and cycloalkanes, yielding β -halogenated ketones and α,γ -halohydrins, respectively (21,22). The chloroperoxidase catalyzed halogenation seems to involve a halonium cation (X^+) or hypohalous acid ($\text{HO}^- \text{X}^+$) as an intermediate of the reaction (11,20), but the reaction mechanism is not yet clarified.

In a study of its stereoselectivity, Kollonitsch et al. showed that the enzyme catalyzed reactions of cis- and trans-propenylphosphonic acid and gave the respective threo- and erythro-chlorohydrins as the racemates (36). Recently, Ramakrishnan et al. reported that the enzyme did not show any appreciable stereoselectivity on any of the following substrates; methionine, 2-methyl-4-propyl-cyclopentane-1,3-dione, 2-exo-methylbicyclo [2,2,1] hept-5-ene-2-endo-carboxylic acid and bicyclo [3,2,0] hept-2-en-6-one (37).

In the studies of chloroperoxidase catalyzed halogenation, the author found that trans-cinnamic acid and its derivatives can be utilized as substrates of chloroperoxidase, and identified the reaction products from these substrates.

This section describes the substrate specificity of the enzyme toward various 2,3-unsaturated carboxylic acids and derivatives, the stereoselectivity and the possible reaction mechanism of the enzyme on trans-cinnamic acid and its derivatives.

MATERIALS AND METHODS

Chemicals trans-Cinnamic acid, trans-4-hydroxycinnamic acid, 4-nitrocinnamic acid, trans-cinnamaldehyde and trans-crotonic acid were purchased from Nakarai Chemicals Ltd., Japan. trans-Crotonamide, trans-cinnamamide, methylacrylate and acrylonitrile were supplied by Tokyo Kasei Co., Japan. trans-4-Chlorocinnamic acid, trans-4-methoxycinnamic acid and trans-

3,4-dimethoxycinnamic acid were purchased from Aldrich Chemical Co. Inc., U.S.A. 1-Bromo-2-phenylethylene (β -bromostyrene) and acrolein were supplied by Wako Pure Chemical Industries Ltd., Japan, and 2-chloroacrylic acid by Sigma Chemical Co., U.S.A. Thin layer chromatoplates (TLC plates, Silica gel 60 F₂₅₄), and high performance thin layer chromatoplates (HPTLC plates, RP-8 F₂₅₄) were purchased from Merck Japan Ltd., Japan. Other chemicals used were reagent grade.

Preparation of Chloroperoxidase Caldariomyces fumago CMI 89362 was obtained from Commonwealth Mycological Institute, England. Stock cultures were maintained on potato dextrose agar slants (pH 5.6) and transferred at 4-month intervals. Inocula were prepared by excising the mold mycelial mats into 2 cm² pieces and homogenizing in 10 ml of the medium (see below) in a sterilized Potter-Elvehjem homogenizer for about 3 min. Then, the mycelium suspension was transferred to 500 ml of the medium (pH 4.0) containing 4% fructose, 0.2% NaNO₃, 0.2% KCl, 0.2% KH₂PO₄, 0.1% MgSO₄·7H₂O and 0.002% FeSO₄·7H₂O in a 2-liter shaking flask, and cultured for 3 weeks at 28°C with shaking. After cultivation, the mold mycelium was filtered off with a Buchner funnel. The filtrate containing chloroperoxidase was then concentrated 10-fold with an evaporator at 35°C. Ethanol (precooled at -20°C) was added to this preparation to a concentration of 45% in an ice bath with stirring. After 30 min, the black precipitate was removed by centrifugation (10,000 x g, 15 min). Ethanol (-20°C) was added to the supernatant to 65%, and stirred in an ice bath for 1-2 h.

The precipitate was collected by centrifugation (10,000 x g, 15 min), and then dissolved in 20 mM potassium phosphate buffer (pH 4.0). This enzyme preparation was stocked at -20°C before use. The above procedures provided 49,200 units of chloroperoxidase from 2 liters of cultured broth (67,000 units) with a 73% yield.

Enzyme Assay The enzyme assay of chloroperoxidase was carried out according to the method of Hager et al.(1) except that the reaction was started by adding hydrogen peroxide. The decrease in absorbance at 278 nm was followed at 25°C. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the conversion of 1 μmol of monochlorodimedone to dichlorodimedone ($\epsilon=12,200 \text{ M}^{-1} \text{ cm}^{-1}$) in 1 min at 25°C.

Reaction Conditions of 2,3-Unsaturated Carboxylic Acids and their Derivatives by Chloroperoxidase

The enzymatic reaction mixture contained 1 mmol potassium phosphate buffer (pH 3.0), 200 μmol KCl or KBr, 200 μmol hydrogen peroxide, 20 units of chloroperoxidase and 20-100 μmol of the following substrates in a total volume of 10 ml in a 30-ml flask: acrylic acid, acrylamide, acrolein, methylacrylate, acrylonitrile, 2-chloroacrylic acid, trans-crotonic acid, trans-crotonamide, fumaric acid, trans-cinnamamide, trans-cinnamaldehyde (100 μmol), trans-cinnamic acid, trans-4-hydroxycinnamic acid (50 μmol), trans-4-methoxycinnamic acid, trans-3,4-dimethoxycinnamic acid, 4-nitrocinnamic acid and trans-4-chlorocinnamic acid (20 μmol). The reaction was started by adding hydrogen peroxide and continued for 60 min at 30°C. Insoluble substrates such

as 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 4-nitrocinnamic acid and 4-chlorocinnamic acid were previously dissolved in warmed ethanol, then poured and suspended in the reaction mixture. The reaction was carried out in the same conditions as above except for the constant stirring. The control test was performed without the enzyme.

Reaction Mixture Analysis For the following volatile substrates: acrylic acid, acrylamide, acrolein, acrylonitrile, 2-chloroacrylic acid, methylacrylate, crotonic acid and crotonamide, aliquots of reaction mixture (2 μ l) were subjected to a gas chromatography equipped with a coiled column (2.0 m x 3 mm) packed with Porapak PS (80-100 mesh). As a carrier, nitrogen gas was used at a rate of 50 ml/min. For each substrate, the following injection and column temperatures were used, respectively: 240°C, 200°C for acrylic acid ($\underline{r_t}$:retention time=2.2 min), 250°C, 220°C for acrylamide ($\underline{r_t}$ =3.0 min), 180°C, 140°C for acrolein ($\underline{r_t}$ =3.2 min), 230°C, 180°C for acrylonitrile ($\underline{r_t}$ =1.2 min), 200°C, 170°C for 2-chloroacrylic acid ($\underline{r_t}$ =4.2 min), 190°C, 170°C for methylacrylate ($\underline{r_t}$ =2.2 min), 250°C, 220°C for crotonamide ($\underline{r_t}$ =5.7 min) and 250°C, 210°C for crotonic acid ($\underline{r_t}$ =2.2 min). For 2-chloroacrylic acid, the 1.0 m x 3 mm column was used. After reaction, the decrease of the substrate and the reaction products were monitored by gas chromatography.

For the non-volatile substrates: fumaric acid, cinnamic acid, cinnamamide, cinnamaldehyde, 4-hydroxycinnamic acid, 4-methoxycinnamic acid, 4-chlorocinnamic acid, 4-nitrocinnamic

acid and 3,4-dimethoxycinnamic acid, aliquots of the reaction mixture (10-20 μ l) were applied to the silica gel thin layer chromatography (TLC) plate. TLC was performed with one of the solvent systems containing a small amount of acetic acid (by volume): (A) benzene, (B) *n*-hexane, (C) *n*-hexane:ethyl acetate; 7:3, (D) *n*-hexane:ethyl acetate; 1:1, (E) *n*-hexane:ethyl acetate; 2:3, (F) *n*-hexane:ethyl acetate; 3:7, (G) *n*-hexane:ethyl acetate; 1:4 (for cinnamaldehyde), (H) ethyl acetate (for cinnamamide, 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 4-chlorocinnamic acid, 4-nitrocinnamic acid), (I) ethyl acetate:methanol; 9:1, (J) ethyl acetate:ethanol; 4:1 (for cinnamic acid), (K) ethyl acetate:methanol; 4:1 (for 4-hydroxycinnamic acid), (L) ethyl acetate:ethanol; 1:4, (M) methanol (for fumaric acid) and (N) methanol:H₂O; 4:1. The products and substrates on the chromatograms were detected by ultraviolet-light or by spraying 5% sulfuric acid and heating for several minutes.

Analysis of the Products NMR spectra were measured with a JEOL-FX 100 (100 MHz) spectrometer with tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as a reference. Gas chromatography-mass spectrometry (GC-MS) was measured with the glass column (1 m x 4 mm) packed with Silicone OV-1 (3%, 80-100 mesh) at 20 eV with a Hitachi M-80 mass spectrometry. In beam-electron impact mass spectrometry (EI-MS) and field desorption mass spectrometry (FD-MS) were carried out at 20 eV with the same instrument. Analytical gas chromatography was performed using the glass column (1 m x 3 mm) packed with

Silicone OV-1 (3 %, 80-100 mesh). N₂ gas was used as a carrier. Infrared spectra were measured with a Shimadzu IR-27G spectrometer. Optical rotation was measured with a Perkin-Elmer 241 MC polarimeter.

Isolation of Products 1 and 2 from trans-Cinnamic Acid The reaction mixture contained 30 mmol potassium phosphate buffer (pH 3.0), 210 mg (1.4 mmol) cinnamic acid, 7.5 mmol KBr and 600 units of enzyme solution in a total volume of 300 ml. The reaction was started by adding 3.75 mmol H₂O₂. After 15 min, another 3.75 mmol of H₂O₂ was added to the mixture, and the reaction was further continued for 45 min at room temperature. Then product 1 was extracted with ether from the reaction mixture. The combined ether extract was dried with Na₂SO₄ and evaporated. The product was further purified by TLC with solvent (B). The product was extracted with ether from the spot layer (R_f=0.73) on TLC plate. The ether extract yielded 24 mg of product 1 as a yellowish oil: ¹HNMR (CDCl₃) δ 6.75 (1H, d, J=14.2) 7.12 (1H, d, J=14.2) and 7.25-7.35 (5H, m, ArH), GC-MS m/z (M⁺ 184,182) 184, 182, 103, 77.

From the aqueous solution saturated with NaCl, product 2 was extracted with ethyl acetate. The ethyl acetate extract was evaporated, and the product was purified by TLC with solvent (G). From the spot layer (R_f=0.15) on TLC plate, product 2 was extracted with solvent (L), dried with Na₂SO₄ and evaporated. Product 2 (220 mg) was crystallized from ethanol-chloroform: [α]_D²³=0° (c=1, CH₃OH), ¹HNMR (D₂O) δ 4.48 (1H, d, J=7.8) 5.06 (1H, d, J=7.8) and 7.4-7.5 (5H, m, ArH),

GC-MS (TMS treatment) m/z (M^+ 388, 390) 373, 375, 282, 284, 309, 179, 147, 73, IR 3500 cm^{-1} (-OH), 1620 cm^{-1} (carbonyl).

Isolation of Products 3 and 4 from trans-4-Methoxycinnamic Acid

4-Methoxycinnamic acid was dissolved in 3 ml of warmed ethanol, and then poured to the reaction mixture. The reaction mixture consisted of 40 mmol potassium phosphate buffer (pH 3.0), 143 mg (0.8 mmol) 4-methoxycinnamic acid, 4 mmol KBr and 1,000 units of enzyme solution in a total volume of 400 ml. The reaction was started by adding 2 mmol H_2O_2 , and performed with constant stirring. After 15 min, another 2 mmol H_2O_2 was added, and the reaction was continued for 45 min. Then the reaction mixture was saturated with NaCl, from which product 3 was extracted with ethyl acetate. The combined ethyl acetate extract was dried with Na_2SO_4 and evaporated. The product was further purified by TLC with solvent (H). Product 3 was extracted with ethanol from the spot layer ($R_f=0.16$) on TLC plate. Evaporation of the extract to dryness gave 40 mg of product 3 as a white powder: $[\alpha]_D^{23}=0^\circ$ ($c=1$, CH_3OH), $^1\text{HNMR}$ (CD_3OD) δ 3.76 (3H, s, $-\text{OCH}_3$) 4.33 (1H, d, $J=7.3$) 4.90 (1H, d, $J=7.3$) 6.81 (2H, m, ArH) 7.36 (2H, m, ArH), EI-MS m/z (M^+ 212) 212, 167, 150, 135, 121, 77, IR 3500 cm^{-1} (-OH), 1580 cm^{-1} (carbonyl).

From the spot layer ($R_f=0.92$) on the TLC plate, product 4 was extracted with ethyl acetate. After concentration, the product was further purified by TLC with solvent (C), and extracted from the spot layer ($R_f=0.7$) with ethyl acetate. Evaporation of the extract provided 74 mg of product 4 as a

syrup: $[\alpha]_D^{23}=0^\circ$ ($c=1$, CH_3Cl), $^1\text{HNMR}$ (CD_3OD) δ 3.77 (3H, s, -
OCH₃) 4.94 (1H, d, $J=4.9$) 5.96 (1H, d, $J=4.9$) 6.88 (2H, m, ArH)
7.36 (2H, m, ArH), GC-MS m/z (M^+ 312, 310, 308) 312, 310, 308,
295, 293, 292, 214, 212, 149, 137, 109, 77.

Isolation of Products 5 and 6 from trans-4-Hydroxycinnamic Acid

The reaction mixture contained 20 mmol potassium phosphate buffer (pH 3.0), 246 mg (1.5 mmol) 4-hydroxycinnamic acid, 7.5 mmol KBr, 7.5 mmol H_2O_2 and 1,000 units of enzyme solution in a total volume of 200 ml. The reaction was started by adding H_2O_2 , and continued for 60 min in the same manner as described for trans-cinnamic acid. After reaction, 4-hydroxycinnamic acid was observed to be converted to two products having R_f values of 0.9 and 0.16 on TLC with solvent (I).

First, the reaction mixture was saturated with NaCl, and the products were extracted with ethyl acetate. The combined extract was evaporated and purified by TLC with solvent (I). The product with a higher R_f value was extracted from the TLC spot layer (R_f=0.9) with ethyl acetate, and rechromatographed with solvent (E). Product 5 was extracted from the spot layer (R_f=0.82) on TLC plate with ethyl acetate, dried with Na_2SO_4 and evaporated to give a yellowish syrup (29 mg): $^1\text{HNMR}$ (CD_3OD) δ 6.69 (1H, d, $J=13.7$) 6.73 (2H, m, ArH) 7.01 (1H, d, $J=13.7$) 7.12 (2H, m, ArH), GC-MS m/z (M^+ 200, 198) 200, 198, 119, 91. The product with a lower R_f value was also purified, but it could not be obtained as a stable compound.

The enzymic reaction was also performed in the same manner using KCl instead of KBr. After reaction, two spots of

products ($R_f=0.88$ and 0.18) were detected on TLC with solvent (K). But, the product with a lower R_f value was unstable, so it was not chased. The ethyl acetate extract from the reaction mixture was evaporated, and further purified by TLC with solvent (F). From the spot layer ($R_f=0.76$) on TLC plate, the product was extracted with ethyl acetate and then dried with Na_2SO_4 . Evaporation of the extract yielded 9.5 mg of product 6 as a yellow oil: $^1\text{H NMR}$ (CDCl_3) δ 6.48 (1H, d, $J=13.7$) 6.77 (1H, d, $J=13.7$) 6.78 (2H, m, ArH) 7.18 (2H, m, ArH), GC-MS m/z (M^+ 156, 154) 156, 154, 119, 91.

Isolation of Products 7, 8, 9, 10, 11 and 12 from trans-3,4-Dimethoxycinnamic Acid trans-3,4-Dimethoxycinnamic acid (167 mg) was dissolved in 3 ml of warmed ethanol, and then poured to the reaction mixture. The reaction mixture consisted of 40 mmol potassium phosphate buffer (pH 3.0), 167 mg (0.8 mmol) 3,4-dimethoxycinnamic acid, 4 mmol KBr, 4 mmol H_2O_2 and 1,000 units of enzyme solution in a total volume of 400 ml. The reaction was carried out in the same manner as described for 4-methoxycinnamic acid with constant stirring. After reaction, the products were extracted from the reaction mixture with chloroform. The combined extract was dried with Na_2SO_4 and evaporated. The products were purified by TLC with solvent (F).

Two spot bands ($R_f=0.87$ and 0.76) were observed on the chromatogram. Product 7 ($R_f=0.87$) was extracted with ethyl-acetate, and further purified by TLC with solvent (D). The product was extracted with ethyl acetate from the spot layer

($R_f=0.82$) on TLC plate. Evaporation of the extract resulted in 23 mg of product 7 as a yellowish syrup: $[\alpha]_D^{23}=0^\circ$ ($c=1$, CHCl_3), $^1\text{HNMR}$ δ 1.97 (1H, brs, -OH) 3.80 (3H, s, -OCH₃) 3.83 (3H, s, -OCH₃) 5.27 (1H, brs) 5.96 (1H, m) 6.91 (1H, m, ArH) 7.09 (1H, m, ArH), GC-MS m/z (M^+ 422, 420, 418, 416) 422, 420, 418, 416, 340, 338, 336, 260, 258, 247, 245, 231, 229, 138, 107. Product 8 ($R_f=0.76$) was also purified in the same way as for product 7 and obtained as a yellowish syrup (32 mg): $[\alpha]_D^{23}=0^\circ$ ($c=1$, CHCl_3), $^1\text{HNMR}$ δ 2.92 (1H, d, $J=3.4$, -OH) 3.89 (3H, s, -OCH₃) 3.91 (3H, s, -OCH₃) 5.00 (1H, t) 5.77 (1H, d, $J=5.4$) 6.8-7.0 (3H, m, ArH), GC-MS m/z (M^+ 342, 340, 338) 342, 340, 338, 180, 167, 151, 138, 107.

The aqueous solution treated with chloroform, was then saturated with NaCl, from which the product was extracted with ethyl acetate. The combined extract was concentrated and the product was further purified by HPTLC with solvent (N). Methanol extract of product 9 from the spot layer ($R_f=0.78$) on HPTLC plate was dried with Na_2SO_4 and evaporated to dryness. Product 9 was obtained as a yellowish powder (20 mg): $[\alpha]_D^{23}=0^\circ$ ($c=1$, CH_3OH), $^1\text{HNMR}$ ($\text{DMSO}-d_6$) δ 3.75 (3H, s, -OCH₃) 3.77 (3H, s, -OCH₃) 4.30 (1H, d, $J=9.3$) 4.76 (1H, d, $J=9.3$) 6.89 (2H, m, ArH) 7.03 (1H, m, ArH), EI-MS and FD-MS m/z (M^+ 306, 304,) FD-MS 306, 304 EI-MS 260, 258, 245, 243, 231, 229, 180, 151, 108, IR 3500 cm^{-1} (-OH), 1720 cm^{-1} (carbonyl).

The reaction was carried out in the same manner using KCl instead of KBr. 3,4-Dimethoxycinnamic acid was observed to be

converted to at least three products, which were detected as three spots ($R_f=0.08$, 0.91 and 0.97) on TLC with solvent (H). But, the product with the lowest R_f value, possibly corresponding to product 9 obtained from the reaction mixture with KBr, could not be isolated due to the small amount. The products were extracted with chloroform from the reaction mixture, and the combined extract was dried with Na_2SO_4 and concentrated. The products were subjected to TLC and developed with solvent (G). Two spot layers ($R_f=0.82$ and 0.62) were observed. From the spot layer ($R_f=0.82$) on TLC plate, the products were extracted with ethyl acetate and evaporated. The products were rechromatographed with solvent (A). Products 10 and 11 ($R_f=0.59$ and 0.47) were independently extracted with ethyl acetate from the spot layers. Evaporation of the extract gave 27 mg of product 10 as a yellowish syrup: $^1\text{HNMR}$ (CDCl_3) δ 3.88 (6H, s, $-\text{OCH}_3$) 6.55 (1H, d, $J=13.7$) 6.84 (2H, s, ArH) 7.13 (1H, d, $J=13.7$), GC-MS m/z (M^+ 234, 232) 234, 232, 217, 191, 189, 153, 125. In the same way, 5 mg of product 11 was obtained as a yellowishsh syrup: $^1\text{HNMR}$ (CDCl_3) δ 3.89 (6H, s, $-\text{OCH}_3$) 6.49 (1H, d, $J=13.7$) 6.79 (1H, d, $J=13.7$) 6.83 (3H, brs, ArH), GC-MS m/z (M^+ 200, 198) 200, 198, 185, 183, 157, 155, 119, 91.

The ethyl acetate extract from the spot layer ($R_f=0.62$) on TLC plate provided 41 mg of product 12 as a yellowish syrup: $[\alpha]_D^{23}=0^\circ$ ($c=1$, CHCl_3), $^1\text{HNMR}$ (CDCl_3) δ 2.96 (1H, brs, $-\text{OH}$) 3.88 (3H, s, $-\text{OCH}_3$) 3.89 (3H, s, $-\text{OCH}_3$) 4.92 (1H, brs) 5.79 (1H, d, $J=5.9$) 6.8-7.0 (3H, m, ArH), GC-MS m/z (M^+ 250) 250, 198, 180, 167, 152, 139, 124, 108.

Chemical Reaction of trans-Cinnamic Acid with Bromine The biomimetic reaction of trans-cinnamic acid with bromine was examined under similar conditions to the chloroperoxidase reaction. The reaction mixture consisted of 15 mmol potassium phosphate buffer (pH 3.0), 0.7 mmol (105 mg) trans-cinnamic acid, 1.88 mmol KBr and 3.75 mmol H₂O₂ in a total volume of 149 ml. Bromine (297 mg, 1.85 mmol as a molecular bromine) dissolved in 1 ml of ethanol was added to the reaction mixture over 10 min at room temperature with mild stirring. After 50 min, the product was extracted with ether from the reaction mixture. The ether extract was analyzed by gas chromatography. Then the aqueous solution was saturated with NaCl, from which the product was extracted with ethyl acetate and further purified in the same way as described for trans-cinnamic acid in the chloroperoxidase reaction. By this method, 50 mg of a white powder was obtained: ¹HNMR (D₂O) δ 4.48 (1H, d, J=7.8) 5.06 (1H, d, J=7.8) 7.4-7.5 (5H, m, ArH).

Synthesis of erythro-2-Bromo-3-hydroxy-3-phenylpropionic Acid

This acid was prepared from trans-cinnamic acid and hypobromous acid according to the modified method of Berner et al. (16). trans-Cinnamic acid (2.5 g) and potassium hydroxide (1.4 g) were dissolved in 35 ml water and stood at 4°C. On the other hand, 2.7 g bromine and 7.5 g sodium carbonate were dissolved in 40 ml cold water. The latter solution was mixed with the former solution and reacted for 30 min at 4°C. Next, about 11 ml of 12 N HCl was gradually added to the solution until the pH was 4.0. The solution was filtered through a Buchner funnel,

and the filtrate was saturated with NaCl. From this solution, the product was extracted with ethyl acetate. The combined extract was evaporated and dissolved in methanol. The product was purified in the same manner as described for trans-cinnamic acid. By this method, 0.25 g of erythro-2-bromo-3-hydroxy-3-phenylpropionic acid was obtained as a white powder: $[\alpha]_D^{23}=0^\circ$ (c=1, CH₃OH), ¹HNMR (D₂O) δ 4.48 (1H, d, J=7.8) 5.06 (1H, d, J=7.8) and 7.4-7.5 (5H, m, ArH).

Synthesis of trans-1-Bromo-2-phenylethylene This compound was also synthesized from trans-cinnamic acid in the same manner as erythro-2-bromo-3-hydroxy-3-phenylpropionic acid. trans-Cinnamic acid (2.5 g) and potassium hydroxide (2.3 g) were dissolved in 35 ml of cold water. The solution of 2.7 g bromine and 4.8 g sodium hydroxide in 40 ml cold water was poured to the first solution, and allowed for 30 min at 4°C. About 10 ml of 12 N HCl was added to the solution with the constant stirring. From this solution, the product was extracted with hexane, dried with Na₂SO₄ and concentrated by evaporation. Then the product was applied to the silica gel chromatography using hexane as eluent. The fractions were combined, and evaporation of the eluate provided 0.5 g of trans-1-bromo-2-phenylethylene as a yellowish oil: ¹HNMR (CDCl₃) δ 6.75 (1H, d, J=14.2) 7.12 (1H d, J=14.2) and 7.25-7.35 (5H, m, ArH).

RESULTS

Substrate Specificity of Chloroperoxidase toward Various 2,3-Unsaturated Carboxylic Acids and their Derivatives

With Br^- present as a halide, chloroperoxidase catalyzed the bromination of trans-cinnamic acid, 4-hydroxycinnamic acid, 4-methoxycinnamic acid and 3,4-dimethoxycinnamic acid, but the other 2,3-unsaturated carboxylic acids tested were not suitable as a substrate for the enzyme (Table I).

TABLE I
Reaction products from various 2,3-unsaturated carboxylic acids by chloroperoxidase-catalyzed halogenation
Chloroperoxidase was inactive toward the following substrates: acrylic acid, acrylamide, acrolein, methylacrylate, acrylonitrile, 2-chloroacrylic acid, *trans*-crotonic acid, *trans*-crotonamide, fumaric acid, *trans*-cinnamamide, *trans*-cinnamaldehyde, 4-nitrocinnamic acid, and *trans*-4-chlorocinnamic acid.

Substrate	Halide	Product	
<i>trans</i> -Cinnamic acid (210 mg)	Br^-	<i>trans</i> -1-Bromo-2-phenylethylene (24 mg)	(1)
		(±)- <i>erythro</i> -2-Bromo-3-hydroxy-3-phenylpropionic acid (220 mg)	(2)
<i>trans</i> -4-Methoxycinnamic acid (143 mg)	Br^-	(±)-2,3-Dihydroxy-3-(4-methoxyphenyl)propionic acid (40 mg)	(3)
		DL-1,1-Dibromo-2-hydroxy-2-(4-methoxyphenyl)ethane (74 mg)	(4)
<i>trans</i> -4-Hydroxycinnamic acid (246 mg)	Br^-	<i>trans</i> -1-Bromo-2-(4-hydroxyphenyl)ethylene (29 mg)	(5)
	Cl^-	<i>trans</i> -1-Chloro-2-(4-hydroxyphenyl)ethylene (9.5 mg)	(6)
<i>trans</i> -3,4-Dimethoxycinnamic acid (167 mg)	Br^-	DL-1,1-Dibromo-2-hydroxy-2-(3,4-dimethoxy-5-bromophenyl)ethane (23 mg)	(7)
		DL-1,1-Dibromo-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane (32 mg)	(8)
		(±)-2-Bromo-3-hydroxy-3-(3,4-dimethoxyphenyl)propionic acid (20 mg)	(9)
	Cl^-	<i>trans</i> -1-Chloro-2-(3,4-dimethoxy-5-chlorophenyl)ethylene (27 mg)	(10)
		<i>trans</i> -1-Chloro-2-(3,4-dimethoxyphenyl)ethylene (5 mg)	(11)
		DL-1,1-Dichloro-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane (41 mg)	(12)

When Cl^- was used as a halide, the enzyme acted on only 4-hydroxycinnamic acid and 3,4-dimethoxycinnamic acid. Hence, the enzyme reaction with Br^- as a halide probably occurs on 3-substituted acrylic acids by electron-releasing aromatic groups such as phenyl, 4-hydroxyphenyl, 4-methoxyphenyl and 3,4-dimethoxyphenyl. However, other acrylic acid derivatives, in which 3-position hydrogen was replaced by one of the following groups: methyl, 4-nitrophenyl and 4-chlorophenyl, were inert. As the halogenation of chloroperoxidase is electrophilic, this phenomenon was conceivable. The electron density of π -bond adjacent to carboxylic group is affected by the substituent

group at 3-position. Hence, the order of the substrate reactivity towards electrophile may be as follows: 3,4-dimethoxycinnamic acid, 4-hydroxycinnamic acid, 4-methoxycinnamic acid, cinnamic acid, 4-chlorocinnamic acid, 4-nitrocinnamic acid, crotonic acid, acrylic acid and fumaric acid. Chloroperoxidase was found to be inert toward cinnamaldehyde and cinnamamide with Br^- as a halide. Therefore, the polarization of π -bond electron was reduced as the carboxylic group was replaced by formyl or amide group.

Identification of Products 1 and 2 from trans-Cinnamic Acid

The GC-MS spectra of product 1 showed the molecular ions of 1-bromo-2-phenylethylene (m/z 182 and 184) split into a 1:1 distribution by the bromine isotopes of m/z 79 and 81. Strong ion peak at m/z 103 indicated the fragment of C_8H_7^+ (Fig. 1b). The NMR spectra also provided the evidence that product 1 was trans-1-bromo-2-phenylethylene from the high J value (14.2) of vinyl protons. Figure 1 shows the gas chromatograms of product 1, synthetic trans-1-bromo-2-phenylethylene and authentic 1-bromo-2-phenylethylene. The last was a mixture of cis- and trans-forms (1:8). Product 1 showed the same retention time of trans-form. From these data, product 1 was identified as trans-1-bromo-2-phenylethylene.

The GC-MS analysis of trimethylsilylated product 2 showed the ion peaks (m/z 375 and 373; $\text{M}^+ - \text{CH}_3$) split into a 1:1 distribution, and other peaks (m/z 309; $\text{M}^+ - \text{Br}$, 179; $\text{M}^+ - \text{Br} \cdot \text{CH} \cdot \text{COOTMS}$), as shown in Fig. 2a. These fragment ion peaks corresponded to $\phi \cdot \text{CH}(\text{OTMS})\text{CH} \cdot \text{Br}(\text{COOTMS})$. The NMR spectra showed

two methine protons ($\delta=4.48$ and 5.06 , $J=7.8$) and five protons of the benzene ring, and were identical with the synthetic ($\pm$)-

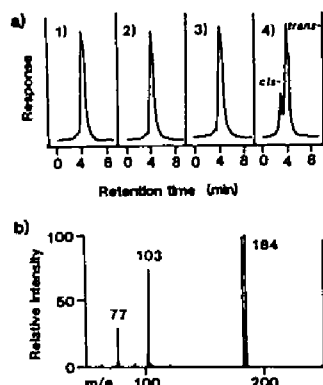


FIG. 1. a, gas chromatograms of 1-bromo-2-phenylethylene synthesized from *trans*-cinnamic acid: 1) produced by chloroperoxidase reaction, 2) synthesized by NaOBr reaction, 3) synthesized by biomimetic bromine reaction, 4) authentic sample (*cis*- and *trans*-form mixtures). b, GC-MS analysis of the above compounds. All compounds showed completely identical spectra.

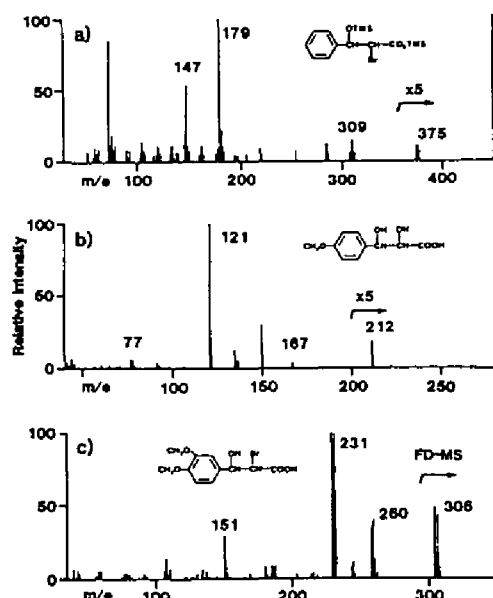


FIG. 2. Mass spectra of (a) ($\pm$)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid (trimethylsilylated by trimethylchlorosilane and pyridine, GC-MS), (b) ($\pm$)-2,3-dihydroxy-3-(4-methoxyphenyl)propionic acid (EI-MS), and (c) ($\pm$)-2-bromo-3-hydroxy-3-(3,4-dimethoxyphenyl)propionic acid (EI-MS and field desorption-mass spectrometry).

erythro-2-bromo-3-hydroxy-3-phenylpropionic acid. The optical rotation of product 2 was 0° in CH_3OH . IR spectra revealed the presence of a carbonyl group (Fig. 3a). From these data, product 2 was identified as ($\pm$)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid.

Identification of Products 3 and 4 from *trans*-4-Methoxycinnamic Acid

The EI-MS analysis of product 3 showed the molecular ion peak (m/z 212; M^+), and other fragment ion peaks (m/z 167; $M^+ - \text{CO}_2\text{H}$, 150; $M^+ - \text{OH} \cdot \text{CO}_2\text{H}$), corresponding to $\text{MeO} \cdot \phi \cdot \text{CH}(\text{OH}) \cdot \text{CH}(\text{OH}) - \text{COOH}$ (Fig.2b). The NMR spectra of product 3 indicated three methyl protons ($\delta=3.76$), two methine protons ($\delta=4.94$ and

5.96, $J=4.9$) and four protons of the benzene ring. The optical rotation of product 3 in CH_3OH was 0° . IR spectra showed the presence of a carbonyl group (Fig. 3b). These data suggested that product 3 is $(\pm)$ -2,3-dihydroxy-3-(4-methoxyphenyl)-propionic acid.

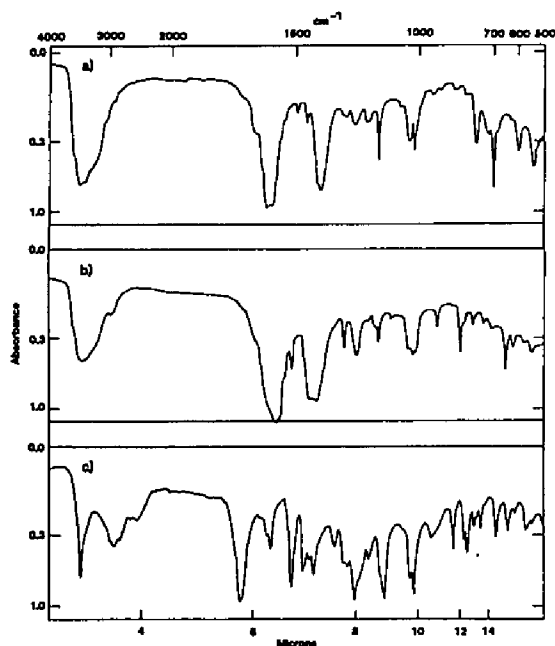


FIG. 3. Infrared spectra (KBr pellet) of (a) $(\pm)$ -erythro-2-bromo-3-hydroxy-3-phenylpropionic acid, (b) $(\pm)$ -2,3-dihydroxy-3-(4-methoxyphenyl)propionic acid, and (c) $(\pm)$ -2-bromo-3-hydroxy-3-(3,4-dimethoxyphenyl)propionic acid.

The GC-MS spectra of product 4 exhibited the molecular ion peaks (m/z 312, 310 and 308) in a ratio of 1:2:1 due to the isotopes of two bromine atoms, and fragment ion peaks (m/z 295, 293 and 291; $M^+-\text{OH}$, 214 and 212; $M^+-\text{OH}\cdot\text{Br}$, 137; $M^+-\text{Br}_2\cdot\text{CH}\cdot\text{OH}$). The NMR spectra showed three methyl protons ($\delta=3.77$), two methine protons ($\delta=4.94$ and 5.96 , $J=4.9$) and four protons of the benzene ring. The optical rotation of product 4 was 0° . These data gave the evidence that product 4 was DL-1,1-dibromo-2-hydroxy-2-(4-methoxyphenyl)ethane.

Identification of Products 5 and 6 from trans-4-Hydroxycinnamic Acid

The GC-MS spectra of product 5 showed the molecular ion peaks (m/z 200 and 198) split into a 1:1 distribution, and other ion peaks (m/z 119; M^+ -Br, 91; M^+ -Br·CO), corresponding to $HO\cdot\phi\cdot CH=H\cdot Br$. The NMR spectra indicated two vinyl protons ($\delta=6.69$ and 7.01 , $J=13.7$), and four protons belonging to the benzene ring. The high J value of vinyl protons demonstrated that the compound was trans-form alkene. From these data, product 5 was identified as trans-1-bromo-2-(4-hydroxyphenyl)ethylene.

The GC-MS spectra of product 6 showed the molecular ion peak (m/z 154 and 156), and fragment ion peaks (m/z 119; M^+ -Cl, 91; M^+ -Cl·CO). The NMR spectra in $CDCl_3$ disclosed one singlet proton ($\delta=5.14$) for -OH group, two vinyl protons ($\delta=6.48$ and 6.77 , $J=13.7$), and four protons of the benzene ring. These data suggested that product 6 is trans-1-chloro-2-(4-hydroxyphenyl)ethylene.

Identification of Products 7, 8, 9, 10, 11 and 12 from trans-3,4-Dimethoxycinnamic Acid

The GC-MS analysis of product 7 exhibited the molecular ion peaks (m/z 422, 420, 418 and 416) in a ratio of 1:3:3:1 due to the isotopes of three bromine atoms, and other fragment ion peaks (m/z 340, 338 and 336; M^+ -HBr, 260 and 258; M^+ -Br₂, 247 and 245; M^+ -Br₂·CH, 231 and 229; M^+ -Br₂·C₂H₅). The NMR spectra of product 7 indicated one proton ($\delta=1.97$) for -OH group, six methyl protons ($\delta=3.80$ and 3.83), two methine

protons ($\delta=5.27$ and 5.96), and two protons for the benzene ring. From the NMR spectra, the 5-position hydrogen of the benzen ring was found to be substituted by bromine. The optical rotation was 0° , so the product was a racemate. Product 7 was identified as DL-1,1-dibromo-2-hydroxy-2-(3,4-dimethoxy-5-bromophenyl)ethane.

The GC-MS spectra of product 8 showed the molecular ion peaks (m/z 342, 340 and 338) in a ratio of 1:2:1, and the other ion peaks (m/z 180; M^+-Br_2 , 167; $M^+-Br_2\cdot CH$, 150; $M^+-Br_2\cdot CH\cdot OH$). The NMR spectra also provided the evidence that product 8 was 1,1-dibromo-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane. The optical rotation was 0° in $CHCl_3$. From these data, product 8 was identified as DL-1,1-dibromo-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane.

The FD-MS analysis of product 9 showed the molecular ion peaks (m/z 306 and 304) split into a 1:1 distribution due to the isotopes of one bromine atom. The EI-MS spectra of product 9 revealed the ion peaks (m/z 260 and 258; M^+-CO_2H , 245 and 243; $M^+-CH_3\cdot CO_2H$, 231 and 229; $M^+-C_2H_5\cdot CO_2H$), as shown in Fig.2c. The NMR spectra of product 9 showed six methyl protons ($\delta=3.83$ and 3.85), two methine protons ($\delta=4.30$ and 4.76 , $J=9.8$), and three protons of the benzene ring. The optical rotation was 0° in CH_3OH . IR spectra disclosed the presence of a carbonyl group (Fig. 3c). From these data, product 9 was identified as ($\pm$)-2-bromo-3-hydroxy-3-(3,4-dimethoxyphenyl)propionic acid.

The GC-MS spectra of product 10 showed the molecular ion

peak (m/z 232 and 235) and other ion peaks (m/z 217 and 219; M^+-CH_3 , 189 and 191; $M^+-CH_3\cdot CO$, 153; $M^+-Cl\cdot CH_3\cdot CO$). The NMR spectra revealed six methyl protons ($\delta=3.88$), two vinyl protons ($\delta=6.55$ and 7.13 , $J=13.7$) and two protons of the benzene ring. From these data, product 10 was identified as trans-1-chloro-2-(3,4-dimethoxy-5-chlorophenyl)ethylene.

The GC-MS analysis of product 11 disclosed the molecular ion peak at m/z 198 and 200, and other fragment ion peaks (m/z 183 and 185; M^+-CH_3 , 155 and 157; $M^+-CH_3\cdot CO$, 119; $M^+-HCl\cdot CH_3\cdot CO$). The NMR spectra exhibited six methyl protons ($\delta=3.89$), two vinyl protons ($\delta=6.49$ and 6.79 , $J=13.7$) and three protons of the benzene ring. The above data showed product 11 to be trans-1-chloro-2-(3,4-dimethoxyphenyl)ethylene.

The GC-MS analysis of product 12 demonstrated a molecular ion peak at m/z 255, 257 and 259, corresponding to 1,1-dichloro-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane. Other fragment ion peaks (m/z 167; $M^+-Cl_2\cdot C_2H_4$, 139; $M^+-Cl_2\cdot C_2H_4\cdot CO$, 124; $M^+-Cl_2\cdot C_3H_7\cdot CO$) were also observed. The NMR spectra of product 12 showed one hydroxyl proton ($\delta=2.96$), six methyl protons ($\delta=3.88$ and 3.89), two methine protons ($\delta=4.92$ and 5.79) and three protons of the benzene ring. The optical rotation was 0° in $CHCl_3$. From these data, product 12 was identified as DL-1,1-dichloro-2-hydroxy-2-(3,4-dimethoxyphenyl)ethane.

The results of identification of the obtained products are summarized in Table I.

Comparison of Chloroperoxidase Reactions with Chemical Haloge-

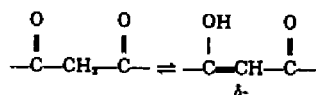
nating Reagents

The reactions of trans-cinnamic acid with chemical halogenating reagents were examined. Molecular bromine in potassium phosphate buffer (pH 3.0) acted on trans-cinnamic acid and converted it to two products, which were detected as two spots on TLC with solvent (J). The NMR spectra and R_f value (0.22) of one product were identical with those of synthetic (+)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid. The other product having a higher R_f value (0.90) was identified as trans-1-bromo-2-phenylethylene from the GC-MS analysis. Thus, the products obtained from trans-cinnamic acid through the chemical reaction with molecular bromine were the same as those in the chloroperoxidase reaction. 2,3-Dibromo-3-phenylpropionic acid, a molecular bromine adduct of trans-cinnamic acid, was not produced in the conditions tested. Molecular bromine is known to be in equilibrium with tribromide ion (Br_3^-) and hypobromous acid (HOBr) in water. Under acidic conditions, the amount of hypobromous acid was assumed to be markedly small, but it was considered to be the active species for the formation of bromohydrin compound. The formation of erythro-2-bromo-3-hydroxy adduct from trans-cinnamic acid suggested that trans-addition of hypobromous acid occurred via bromonium cation-intermediate. It was shown that hypobromous acid reaction on trans-cinnamic acid under alkaline conditions gave erythro-2-bromo-3-hydroxy-3-phenylpropionic acid (38,39). It was found that trans-cinnamic acid was converted to trans-1-bromo-2-phenylethylene under strong acidic conditions by decarboxylation. The chloroperoxidase reaction was similar

to those of molecular bromine and hypobromous acid in respect to reaction products and stereoselectivity.

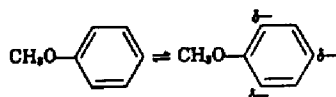
DISCUSSION

Chloroperoxidase catalyzes the formation of carbon-halogen bond in a large number of substrates. In this study, several 2,3-unsaturated carboxylic acids were found to serve as substrates of chloroperoxidase. Also it was clarified that the substrate specificity of the enzyme toward various 2,3-unsaturated carboxylic acids was related to the nucleophilicity of π -bond of substrates, by testing substrates with different states of electron density of π -bond. Hager et al. established that the halogenation agent in chloroperoxidase-catalyzed reaction is electrophilic (17). From the results the author obtained and other studies, the intermediate in the enzyme reaction was assumed to be halonium cation (X^+). The wide substrate specificity of chloroperoxidase can be accounted for by the electrophilic reaction of halonium cation. The compounds, which have an activated methylene group such as 1,3-diketones (7, 10),



or substituted phenols (16, 17), alkenes (20) and alkynes (21) having enough π -electrons, were all catalyzed by chloroperoxidase. The ortho-para orientations of halogenation of anisole (17) is well explained on the basis of π -electron

localization.

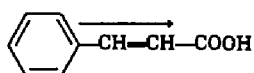


However, for 2,3-unsaturated carboxylic acids, the electron density of π -bond is reduced by the adjacent electron-attracting carboxyl group. Therefore, acrylic acid, crotonic acid, etc., were inert. In contrast, cinnamic acid, 4-methoxycinnamic acid, 4-hydroxycinnamic acid and 3,4-dimethoxycinnamic acid, which have electron-releasing groups, overcome the effect of carboxyl group. Therefore they could serve as a substrate of chloroperoxidase. These data led me to speculate on the possible substrates for the halogenation of chloroperoxidase. The halogen acceptors of chloroperoxidase reaction should be a molecule having carbon atoms of which the electron density is higher than a definite level.

The first intermediate in the enzymatic reaction has not yet been determined. Hager et al. detected the formation of molecular halogen in the presence of halide ion and hydrogen peroxide without substrate (11). They proposed the enzyme-ion(III)-hypohalite complex as the enzymatic halogenating intermediate (11). Neidleman et al. suggested that hypohalous acid was an intermediate on the basis of halohydrin formation from alkenes by chloroperoxidase reaction (20). From the biomimetic reaction of trans-cinnamic acid with bromine, it was found that the product was 2-bromo-3-hydroxy adduct. Bromine is known to be in equilibrium with molecular bromine, tribromide ion and hypobromous acid in water. There-

fore, the formation of bromohydrin, not dibromo adduct, from 2,3-unsaturated carboxylic acids or alkenes was likely. Also, the active species for the formation of halohydrin was assumed to be hypohalous acid. The author could not clarify the first intermediate of enzyme reaction, but it is clear that molecular bromine showed the same reaction in water as chloroperoxidase. Hence, the molecular bromine formed by the enzyme reaction was immediately converted to hypobromous acid in water, acting on the double bond of various substrates.

The chloroperoxidase reaction of trans-cinnamic acid with Br^- and H_2O_2 was similar to the chemical reaction using hypobromous acid. The formation of 2-bromo, not 3-bromo, compound was understandable, since the polalization of π -electron of trans-cinnamic acid favoured the observed orientation of addition.



The chemically and enzymatically formed 2-bromo-3-hydroxyphenyl propionic acid were both ($\pm$)-erythro forms. This supports a reaction mechanism of trans-addition of hypobromous acid which occurs equally from the both sides of π -bond of trans-cinnamic acid via typical bromonium ion-intermediate (Fig. 4). Kollonitsch et al. also reported that erythro- and threo-chlorohydrins were obtained as racemates from trans- and cis-prophenylphosphonic acid, respectively (36). The author could not observe any feasible stereoselectivity of chloroperoxidase-dependent halogenation toward trans-cinnamic acid and its derivatives (Table I). These results were well understood

from the reaction mechanism of chloroperoxidase.

A proposed mechanism of the chloroperoxidase reaction on each of the substrates is depicted in Fig. 4.

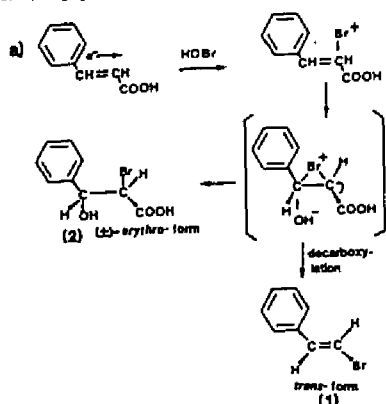
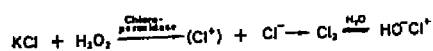
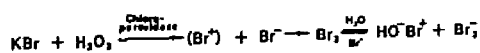


FIG. 4a

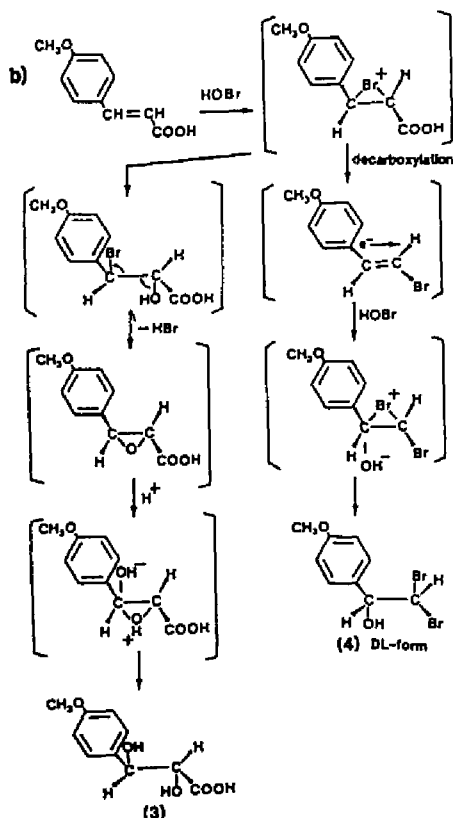


FIG. 4b

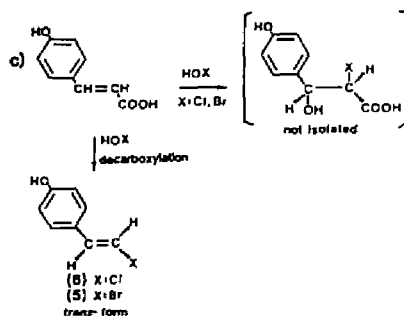


FIG. 4c

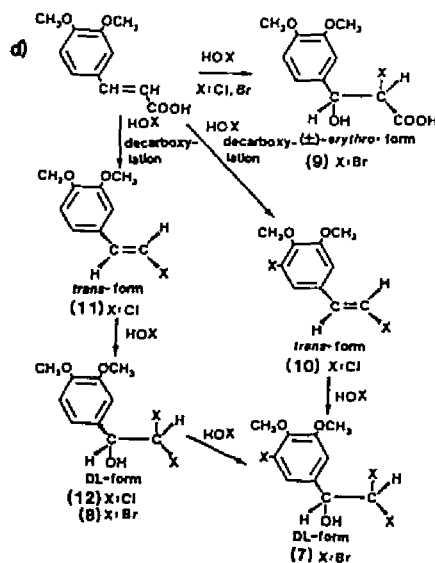


FIG. 4d

FIG. 4. Proposed reaction mechanism of chloroperoxidase reaction on each of the following substrates; (a) *trans*-cinnamic acid, (b) *trans*-4-methoxycinnamic acid, (c) *trans*-4-hydroxycinnamic acid, and (d) *trans*-3,4-dimethoxycinnamic acid.

From trans-cinnamic acid, trans-1-bromo-2-phenylethylene (1) was also produced as a by-product, which was probably formed from the bromonium cation intermediate by decarboxylation under acidic conditions (Fig. 4a). As shown in Table I, similar β -halogeno-styrene derivatives (5, 6, 10 and 11) were obtained from other substrates.

The reason why the dihydroxy compound (3) was obtained from 4-methoxycinnamic acid is not clear. It was assumed that the unstable 2-hydroxy-3-bromo adduct produced by the enzyme reaction was spontaneously converted to 2,3-epoxy compound, then it was hydrolyzed to form 2,3-dihydroxy compound in the reaction conditions. The author also detected the formation of the decarboxylated halohydrin compound (4) from 4-methoxycinnamic acid. It was considered that hypobromous acid again acted on the trans-1-bromo-2-(4-methoxyphenyl)ethylene formed by the decarboxylation of the bromonium cation-intermediate, as shown in Fig. 4b.

For the halogenation of 4-hydroxycinnamic acid with Br^- or Cl^- as a halide, only decarboxylated β -halogeno-styrenes were obtained as stable products. They were also presumed to form from the bromonium cation-intermediate in a similar way to products 1 and 4 (Fig. 4c). The author could not isolate the polar products, maybe corresponding to 2-bromo-3-hydroxy or 2,3-dihydroxy adduct, from 4-hydroxycinnamic acid. Probably these products decomposed through spontaneous oxidation in the purification steps.

When 3,4-dimethoxycinnamic acid was used as a substrate, not only double bond but also C-5 carbon of the benzene ring

was halogenated. The high electron density at C-5 of the benzene ring could be the reason, since its hydrogens at C-3 and C-4 were substituted by electron-releasing methoxy groups (Fig. 4d).

In conclusion, it was demonstrated that the chloroperoxidase-catalyzed halogenation is the same as the chemical reaction with molecular halogen or hypohalous acid in respect to reaction products and stereoselectivity. And the various kinds of the reaction products depends on the chemical properties of substrate in the reaction conditions.

SUMMARY

Several 2,3-unsaturated carboxylic acids, such as trans-cinnamic acid and its derivatives, were found to be halogenated by chloroperoxidase of Caldariomyces fumago. Cinnamic acid, 4-hydroxycinnamic acid, 4-methoxycinnamic acid and 3,4-dimethoxycinnamic acid were suitable substrates of chloroperoxidase, and were converted to 2-halo-3-hydroxycarboxylic acid, 2,3-dihydroxycarboxylic acid, decarboxylated halohydrin or decarboxylated halocompound. However, 4-nitrocinnamic acid and 4-chlorocinnamic acid having electron-attracting groups did not serve as substrates of the enzyme. The enzyme also did not act on acrylic acid, acrylamide, crotonic acid, fumaric acid, etc. The enzymatic reactions were also compared with the chemical reactions of molecular halogen and hypohalous acid. From these data, the enzymatic reactions of chloro-

Section 2. Chloroperoxidase-catalyzed Halogenation of Nitrogen-containing Aromatic Heterocycles

Hager and co-workers presented information on the enzymic properties (9), kinetic mechanism (10) and reaction mechanism (11) of the chloroperoxidase. In the haloperoxidase reaction, a halide anion (X^- , X ; Cl, Br, I) is activated to the halonium cation (X^+) through hydrogen peroxide dependent oxidation, and then the halonium cation is transferred to a halogen acceptor molecule. There have been many reports concerning chloroperoxidase substrates, as follows; β -keto acids (7), cyclic β -diketones (10), steroids (12-15), substituted phenols such as tyrosine (16) and anisole (17), thiols (19), thiazoles (19), alkenes (20), alkynes, cycloalkanes (21, 22), and several α, β -unsaturated carboxylic acids (Section 1). The wide substrate specificity and nonstereospecificity of the chloroperoxidase reaction can be explained in terms of molecular halogen (X_2) addition chemistry in water, which is released from the enzyme in the presence of the halide anion and hydrogen peroxide (Section 1).

In order to apply haloperoxidases to the chemical halogenation process, the author studied the enzymatic halogenation of various aromatic heterocycles with chloroperoxidase. It was found that several nucleic bases were utilized as substrates by the enzyme. Then, the author identified the reaction products derived from these substrates, and compared the enzymatic reactions to the chemical reactions.

This section describes the new substrates, reaction mechanism and enzyme stability of the chloroperoxidase, and also its ability as a biochemical halogenating reagent are discussed.

MATERIALS AND METHODS

Chemicals Cytosine, uracil, thymine, cytidine, adenosine, and guanosine were purchased from Kohjin Co., Japan. 5-Chlorouracil, 5-iodouracil, 5-bromocytidine, 2'-deoxyuridine, purine, pyrimidine and pyrazole were obtained from Nakarai Chemicals Ltd., Japan. 5-Bromocytosine, 5-bromouracil, 5-bromo-2'-deoxyuridine, 8-bromoguanosine and 4-iodopyrazole were obtained from Sigma Chemical Co., U. S. A. and Aldrich Chemical Co., U. S. A. Cellulofine GC-700, a gel filtration medium, was purchased from Seikagaku Kogyo Co., Japan, DEAE-Sephadex CL-6B and Sephadex LH-20 from Pharmacia Fine Chemicals, Sweden, and Amberlite XAD-2 from Rohm & Hass Corp., U. S. A. Thin layer chromatoplates (TLC plates; Silica Gel 60 F₂₅₄) were obtained from Merck Japan Ltd. Chlorine gas was obtained by the reaction of conc. HCl with potassium permanganate. The gas was passed through water and conc. sulfuric acid to remove HCl and water, respectively. Other reagents used were all of analytical grade.

Preparation of chloroperoxidase Chloroperoxidase was obtained from Caldariomyces fumago CMI 89362 grown on the fructose-salts medium as described in Section 1. The enzyme

preparation after ethanol treatment was stocked and used for the halogenation reactions with substrates. Further purification was carried out to obtain a homogeneous enzyme preparation. The enzyme solution from the above step, which had been dialyzed against 20 mM potassium phosphate buffer (pH 6.0), was subjected to DEAE-Sepharose column chromatography (3.5 x 28 cm). After washing the loaded column with the buffer, the enzyme was eluted with a linear gradient of KCl, from 0 to 0.6 M, in the buffer. The active fractions were collected and concentrated to about 20 ml by evaporation at 35°C under reduced pressure. The enzyme solution was applied on a Cellulofine GC-700 column (2.1 x 110 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.0). Following elution with the buffer, the active fractions were collected. This rapid purification method (94 % yield, 75,000 units) gave an enzyme with an A_{410}/A_{280} purity ratio of 1.28. The enzyme solution was used for the halogenation intermediate analysis of the chloroperoxidase reaction.

Enzyme Assay Chloroperoxidase activity was measured spectrophotometrically according to the methods described in Section 1.

Enzymatic Reaction The reaction mixture for the chloroperoxidase reaction comprised 1 mmol of potassium phosphate buffer (pH 3.0), 0.2 mmol of KCl, KBr or KI, 0.2 mmol of hydrogen peroxide (final concentration), 0.1 mmol of each substrate (20 μ mol for guanosine) and 40 units of chloroperoxidase in a total volume of 10 ml, in a 30-ml flask. The

reaction was kept going by adding 0.1 mmol of hydrogen peroxide at 30-min intervals, and allowed to proceed for 3 h at 30°C. In all cases, a control run was done without the enzyme.

Reaction Mixture Analysis Aliquots of a reaction mixture (5-10 μ l) were subjected to analytical high performance liquid chromatography (HPLC). HPLC was performed with a Shimadzu LC-5A system equipped with an M & S pack C18 column (reversed phase column, 4.6 x 150 mm; M & S Instruments Inc., Japan) at the flow rate of 1.0 ml/min, using the following solvent systems: a) 0.2 M potassium phosphate buffer (pH 6.0): methanol, 9:1 (by volume); b) 0.2 M potassium phosphate buffer (pH 6.0):methanol, 17:3; and c) 0.2 M potassium phosphate buffer (pH 6.0):methanol, 4:1. Unless otherwise indicated, the decrease of the substrate and the formation of the product were measured at 254 nm on HPLC, except in the case of pyrazole at 230 nm. The substrates showed the following retention times ($\bar{x}_t$): uracil, 2.3 min; cytosine, 2.0 min; thymine, 3.7 min; cytidine, 2.4 min; 2'-deoxyuridine, 3.6 min; adenine, 4.6 min; purine, 4.5 min (solvent a); guanosine, 3.1 min; adenosine, 6.4 min (solvent b); and pyrazole, 3.9 min (solvent c).

Simultaneously, aliquots of the reaction mixture (5-10 μ l) were applied to a silica gel plate, which was developed with one of the following solvent systems (by volume): a) n-butanol:ethanol:H₂O, 2:1:1 (for cytosine, uracil, thymine and guanosine); b) ethanol (for adenosine); c) methanol (for cytidine and adenine); and d) n-butanol:methanol, 4:1 (for 2'-

deoxyuridine). The products and substrates on the chromatograms were detected with ultraviolet light or iodine vapor.

For volatile substrates such as pyridine and pyrimidine, aliquots of the reaction mixture (2 μ l) were subjected to gas chromatography on a coiled column (2 m x 3 mm) packed with KOH-Carbowax 20M (1 + 0.4%, 60-80 mesh). As a carrier, nitrogen gas was used at a flow rate of 50 ml/min. For each substrate, the following injection and column temperatures were used, respectively: 170°C and 90°C for pyridine (r_t =1.0 min), and 200°C and 100°C for pyrimidine (r_t =1.2 min).

Analysis of the Products NMR spectra were measured with a JEOL-FX 100 (100 MHz) spectrometer with tetramethylsilane as a reference. Gas chromatography-mass spectrometry (GC-MS) was performed on a glass column (1 m x 4 mm) packed with Silicone OV-1 (3%, 80-100 mesh) at 20 eV in a Hitachi M-80 mass spectrometer. In-beam electron impact-mass spectrometry (EI-MS) was carried out at 20 eV with the same instrument. Confirmation of the identification of the products was performed by co-chromatography of authentic standards on HPLC and TLC, whenever they were available.

Isolation of the Products Cytosine (222 mg, 2 mmol) was dissolved in a reaction mixture comprising 20 mmol potassium phosphate buffer (pH 3.0), 4 mmol KBr, 4 mmol hydrogen peroxide (final concentration) and 800 units of chloroperoxidase in a total volume of 200 ml. The reaction was kept going by adding 2 mmol of hydrogen peroxide at 30-min intervals, and

continued for 3 h at 30°C. In further experiments, all reactions were initiated and kept going by the addition of half volume of hydrogen peroxide at 30-min intervals, and continued for 3 h at 30°C. After the reaction, the pH of the reaction mixture was adjusted to 6.5, and then the mixture was evaporated to dryness. The product was extracted with a 90 % ethanol solution, evaporated and then dissolved in a 0.05 N HCl solution. This solution was applied on a Dowex 50x8 (H-form) column (1.7 x 23 cm), which was eluted with 1 N HCl. The product was rechromatographed on the Dowex 50x8 column. This procedure afforded 210 mg of a white powder: $^1\text{HNMR}$ ($\text{DMSO}-d_6$) δ 8.25 (1H, s), EI-MS m/z (M^+ 191, 189) 161, 149, 147, 121, 119, 110, 93, 83, 67.

Isolation of the product from uracil was carried out in a slightly different manner from in the case of cytosine. The reaction mixture comprised 2 mmol (224 mg) uracil, 20 mmol potassium phosphate buffer (pH 3.0), 5 mmol KBr, 4 mmol hydrogen peroxide and 1,000 units of chloroperoxidase in a total volume of 200 ml. After the reaction, the mixture (adjusted to pH 7.0) was evaporated, and the product was extracted with a 80% ethanol solution. Following evaporation of the extract to dryness, the product was dissolved in 20% methanol, applied on a Sephadex LH-20 column (2 x 70 cm) and then eluted with 20% methanol. This chromatography was repeated once more under the same conditions. This procedure afforded 40 mg of a white product: $^1\text{HNMR}$ ($\text{DMSO}-d_6$) δ 7.83 (1H, s), EI-MS m/z (M^+ 192, 190) 149, 147, 122, 120, 106, 104, 93, 91, 81, 79.

The reaction mixture for thymine comprised 1 mmol (126 mg) thymine, 10 mmol potassium phosphate buffer (pH 3.0), 2.5 mmol KBr, 2 mmol hydrogen peroxide and 600 units of chloroperoxidase in a total volume of 100 ml. After incubation, the solution was subjected to Amberlite XAD-2 column chromatography (1.5 x 70 cm). Following washing of the column with water, the product was eluted in a stepwise manner with 20% and 40% methanol solutions. The product obtained in the above step was further purified by preparative HPLC on a YMC D-ODS-5 column (reversed phase column, 20 x 250 mm; Yamamura Chemical Lab. Co., Japan) using 15% methanol as the solvent. The product was unstable under dry conditions, so it was stored in a solution until analysis: EI-MS m/z (M^+ 224, 222) 207, 205, 179, 177, 163, 161, 153, 151, 143, 136, 134, 126, 115, 108, 106, 89.

Cytidine (243 mg, 1 mmol) was dissolved in a reaction mixture comprising 10 mmol potassium phosphate buffer (pH 3.0), 2 mmol KBr, 2 mmol hydrogen peroxide and 800 units of chloroperoxidase in a total volume of 100 ml. After the reaction, the solution (adjusted to pH 7.0) was applied on an Amberlite XAD-2 column (3.2 x 30 cm). The column was washed with water, and then the product was eluted in a stepwise manner with 25% and 50% methanol solutions. Evaporation of the fractions gave 220 mg of a white product: ^1H NMR ($\text{DMSO}-d_6$) δ 3.55-3.75 (2H, brm, 5'-H) 3.75-4.05 (3H, brm, 2'-, 3'- and 4'-H) 5.71 (1H, d, 1'-H) 8.40 (1H, s, 6-H), EI-MS m/z (M^+ +1 324, 322) 242, 240, 231, 229, 220, 218, 192, 190, 175, 173,

147, 133, 120, 110, 95.

The product from 2'-deoxyuridine was isolated from a reaction mixture comprising 1 mmol substrate (114 mg), 2 mmol KBr, 2 mmol hydrogen peroxide and 800 units of chloroperoxidase in a total volume of 100 ml. The HPLC analysis data showed that three products ($\bar{r}_t$ =5.1, 7.8 and 10.2 min) were formed. After the reaction, the mixture was subjected to Amberlite XAD-2 column (1.5 x 70 cm) chromatography. Following washing of the column with water, the products were separately eluted with a linear gradient, 0 to 50% of methanol. Then, the three products were separately purified by preparative HPLC on the same column as above with 10% methanol. But two products ($\bar{r}_t$ =5.1 and 7.8 min, on analytical HPLC) were unstable on drying, so they were dissolved in water until analysis. The fractions containing the last product ($\bar{r}_t$ =10.2, on analytical HPLC) gave 10 mg of a white powder: $^1\text{H NMR}$ (DMSO- d_6) δ 2.03-2.23 (2H, m, 2'-H) 3.50-3.70 (2H, m, 5'-H) 3.70-3.90 (1H, m, 4'-H) 4.10-4.30 (1H, m, 3'-H) 6.11 (1H, t, 1'-H) 8.40 (1H, s, 6-H), EI-MS m/z (M^+ 308, 306) 192, 190, 149, 147, 122, 120, 117, 97.

Pyrazole (136 mg, 2 mmol) was dissolved in reaction mixture comprising 20 mmol potassium phosphate buffer (pH 3.0), 6 mmol KCl, 4 mmol hydrogen peroxide and 800 units of chloroperoxidase in a total volume of 200 ml. After incubation, the mixture was applied on an Amberlite XAD-2 (3.2 x 30 cm) column, and the column was washed with water. The product was eluted in a stepwise manner with 50%, 75% and 100% methanol. The fractions containing the product were collected and concentrated by evaporation. The product was further

purified by Sephadex LH-20 column chromatography (1.6 x 35 cm) with 20% methanol as eluent. Evaporation of the fractions gave 15 mg of a faint brownish product: $^1\text{H NMR}$ (CDCl_3) δ 7.57 (2H, s, 3- and 5-H), GC-MS m/z (M^+ 104, 102) 77, 75.

RESULTS

Substrate Specificity of Chloroperoxidase for Nitrogen-containing Aromatic Heterocycles

The author tested twelve substances, *i.e.*, six pyrimidine bases, four purine bases, pyridine and pyrazole, as substrates for the chloroperoxidase reaction. The enzyme acted on cytosine, uracil, adenine, cytidine, adenosine, guanosine and pyrazole when KCl was used as the halide. The enzyme also catalyzed the bromination of cytosine, uracil, thymine, cytidine, 2'-deoxyuridine, guanosine and pyrazole, and the iodination of uracil and pyrazole. However, nonsubstituted-pyrimidine, -purine and -pyridine were not utilized as substrates. It was apparent that the substitution of hydrogens in pyrimidine and purine bases by amino or carbonyl groups was necessary for the halogen acceptor of the chloroperoxidase reactions.

Identification of Products from Cytosine

The EI-MS spectrum of the brominated product showed molecular ion peaks (m/z , 191 and 189) split into a 1:1 distribution by the bromine isotopes of m/z 81 and 79, which

corresponded to bromocytosine. The NMR spectrum of the product showed a one single peak of a methine proton (8.25 ppm), and this indicated that the 5-position hydrogen was replaced by bromine. HPLC analysis data with a standard sample proved that the product was 5-bromocytosine.

The chlorinated products from cytosine could not be identified because they were unstable.

Identification of Products from Uracil

The chlorinated compound showed the same retention time ($r_t=3.9$) on HPLC and the same R_f value (0.74) on TLC (solvent a) as standard 5-chlorouracil. Therefore, it was identified as 5-chlorouracil.

On EI-MS analysis, the brominated product indicated the molecular ions of bromouracil (m/z , 192 and 190). The NMR spectrum of the product and HPLC analysis confirmed that the product was 5-bromouracil.

The iodinated compound was found to be completely identical with authentic 5-iodouracil on HPLC ($r_t=6.4$ min) and TLC ($R_f=0.80$, solvent a) analyses.

Identification of Product from Thymine

Figure 1a shows the EI-MS spectrum of the brominated product derived from thymine by the enzyme reaction. In the spectrum, molecular ion peaks at m/z 224 and 222, and other fragment ion peaks (m/z , 207 and 205, M^+-OH ; 179 and 177, $M^+-CH\cdot OH\cdot NH$; 136 and 134, $M^+-CH\cdot OH\cdot NH\cdot CO\cdot NH$; 126, $M^+-OH\cdot Br$) were observed. These ion peaks corresponded to a bromohydrin compound derived from thymine. The analysis data of fragment

ion peaks gave the proof of the additions of bromine at the 5-position and a hydroxyl group at the 6-position of thymine. From these results, the product was supposed to be 5-bromo-6-hydroxy-5,6-dihydrothymine.

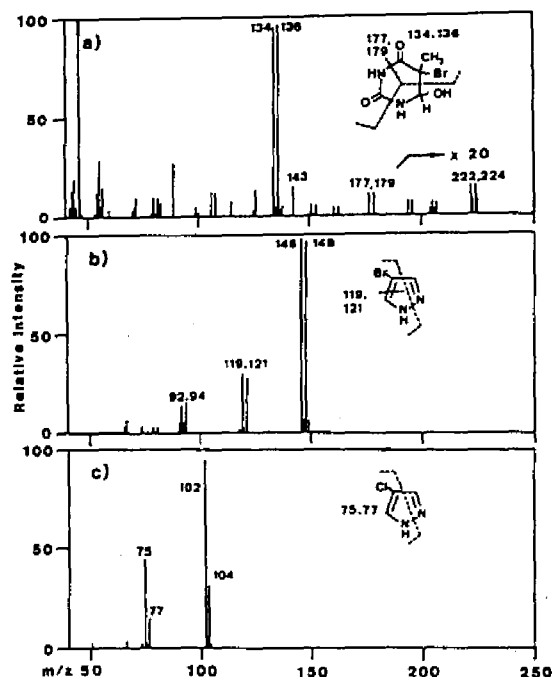


FIGURE 1: Mass spectra of (a) 5-bromo-6-hydroxy-5,6-dihydrothymine (EI-MS), (b) 4-bromopyrazole (GC-MS), and (c) 4-bromopyrazole (GC-MS) through the haloperoxidase reactions.

Identification of Products from Cytidine

The EI-MS spectrum of the product obtained on chloroperoxidase bromination showed molecular ion peaks (m/z , 324 and 322; M^++1), and strong peaks at m/z 192 and 190 due to the fragment ion of bromocytosine. The NMR spectrum and HPLC analysis data confirmed that the product was 5-bromocytidine.

The author could not isolate the chlorinated compounds from the reaction mixture because of their unstability.

Identification of Products from 2'-Deoxyuridine

2'-Deoxyuridine was brominated to yield three products, as shown in Fig. 2c. But the author could not clarify the structures of the first two products because they were unstable on drying. On EI-MS analysis, the last product showed molecular ion peaks at m/z 308 and 306, that corresponded to bromo-2'-deoxyuridine. The NMR spectrum of the product was the same as that of standard 5-bromo-2'-deoxyuridine. HPLC analysis also supported this identification.

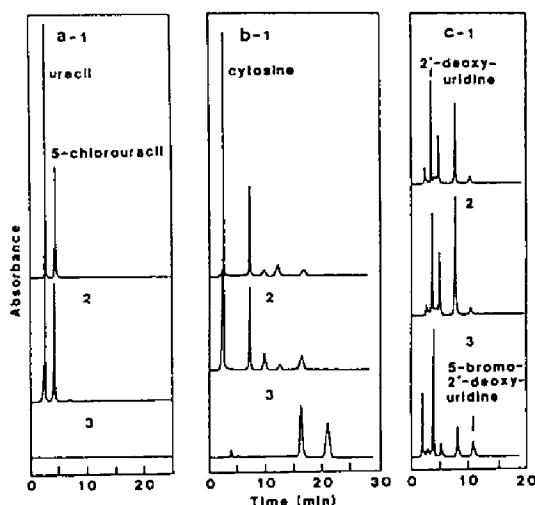


FIGURE 2: HPLC elution profiles of the products obtained on chloroperoxidase-catalyzed chlorination and chlorine gas reaction with (a) uracil and (b) cytosine and chloroperoxidase-catalyzed bromination and molecular bromine reaction with (c) 2'-deoxyuridine. The enzymatic reaction conditions were given under Experimental Procedures. The chlorine gas reaction was performed by bubbling chlorine gas into solutions containing 5 mmol of potassium phosphate buffer (pH 3.0) and 0.5 mmol of each substrate in a total volume of 50 mL. The molecular bromine reaction was performed by the addition of bromine (24 mg, 150 μ mol as Br_2 in 0.5 mL of ethanol) over 10 min, with gentle stirring, to a solution comprising 2 mmol potassium phosphate buffer (pH 3.0 or 6.0) and 0.2 mmol of 2'-deoxyuridine in a total volume of 20 mL. Detection of the product on HPLC was carried out at various wavelengths, in the range of 205–254 nm. The elution profiles shown in this figure were obtained at 254 nm for cytosine and uracil and at 230 nm for 2'-deoxyuridine. (a-1 and b-1) Chloroperoxidase reaction (chlorination); (a-2 and b-2) chlorine gas reaction for 5 s; (a-3) chlorine gas reaction for 2 min; (b-3) chlorine gas reaction for 30 s; (c-1) chloroperoxidase reaction (bromination); and (c-2 and c-3) molecular bromine reaction at pH 3.0 and 6.0, respectively.

Identification of Products from Adenine, Adenosine and Guanosine

Chloroperoxidase catalyzed the chlorination of adenine and adenosine to give unknown compounds, which were very unstable and gradually reverted to the substrate in a few hours. So, the author did not attempt to isolate these products.

In the chlorination reaction of guanosine, the substrate was completely consumed and decomposed into unknown substances.

The brominated compound derived from guanosine was found to

be completely identical with authentic 8-bromoguanosine on HPLC and TLC analyses.

Identification of Products from Pyrazole

Figure 1c shows the GC-MS spectrum of chlorinated product derived from chloroperoxidase reaction with pyrazole. The molecular ion peaks at m/z 104 and 102 with a 3:1 distribution were due to the isotopes of the chlorine atom of m/z 37 and 35. This suggested that the product had one chlorine atom in the pyrazole ring. In the NMR spectrum, no existence of spin-spin coupling of protons was observed. This indicated that the 4-position hydrogen of pyrazole was substituted by chlorine. Hence, the product was identified as 4-chloropyrazole.

Table I: Reaction Products from Various Nitrogen-Containing Heterocycles ^a

substrate	chloroperoxidase, halide		
	Cl ⁻	Br ⁻	I ⁻
cytosine	not identified (6.6, 9.1, 11.5)	5-bromocytosine (3.8)	nr
uracil	5-chlorouracil (3.9)	5-bromouracil (4.5)	5-iodouracil (6.4)
thymine	slight conversion	5-bromo-6-hydroxy-5,6-dihydrothymine (9.3)	nr
adenine	not isolated (7.6)	nr	nr
cytidine	not isolated (6.1, 9.2)	5-bromocytidine (6.1)	nr
2'-deoxyuridine	nr ^b	two unstable products (5.1, 7.8) 5-bromo-2'-deoxyuridine (10.2)	nr
adenosine	not isolated (7.8)	nr	nr
guanosine	decomposition	8-bromoguanosine (11.0)	nr
pyrazole	4-chloropyrazole (13.4)	4-bromopyrazole (17.8) minor product (24.2)	4-iodopyrazole (27.5)

^a Pyridine, pyrimidine, and purine were not used as substrates for chloroperoxidase. The retention time (min) on HPLC is shown in parentheses, and the solvent system is described in "Materials and Methods".

^b nr, no reaction.

GC-MS analysis of the brominated product showed molecular ion peaks at m/z 148 and 146 split into a 1:1 distribution, that corresponded to bromopyrazole, as shown in Fig. 1b. The single peak at 7.60 ppm and the lack of spin-spin coupling in

the NMR spectrum were similar to in the case of 4-chloro-pyrazole. Therefore, the product was identified as 4-bromo-pyrazole.

The iodinated products was found to be completely identical with standard 4-iodopyrazole on HPLC analysis.

The results as to the identification of the products are summarized in Table I.

Comparison of the Chloroperoxidase Reaction with Chemical Halogenating Reagents

The chloroperoxidase-catalyzed chlorinations of uracil and cytosine were compared with the molecular chlorine reactions. Figure 2 shows the results of HPLC analysis of the products obtained from uracil and cytosine after bubbling of molecular chlorine for several seconds into solutions containing each substrate. The analysis data were identical with those for the chloroperoxidase reaction. However, cytosine was converted into unknown products on further incubation with chlorine (Fig. 2b-3). Uracil decomposed into unknown substances, which did not absorb light in the ultraviolet region, on long time reaction with molecular chlorine (Fig. 2a-3).

Figure 2c shows the results of HPLC analysis of the products derived from 2'-deoxyuridine through the molecular bromine reaction, at pH 3.0 and 6.0, and the chloroperoxidase reaction. Molecular bromine acted on 2'-dexoyuridine to give the same products as in the case of chloroperoxidase bromination. The similarity of molecular chlorine and bromine

reactions to the chloroperoxidase halogenations was coincident with the results of a previous study (Section 1).

Stability of Chloroperoxidase under the Reaction Conditions.

Molecular halogens are known to be very active chemical species. Therefore, it was thought that molecular halogen released in the chloroperoxidase reaction mixture may cause damage to the chloroperoxidase itself. Figure 3 shows the

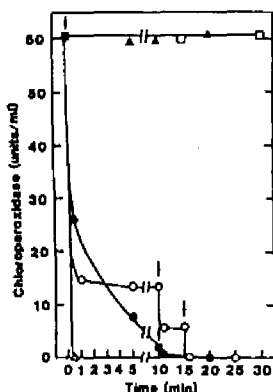


Fig. 3. Stability of the chloroperoxidase in reaction mixture without a halogen acceptor and on exposure to molecular bromine. The chloroperoxidase (80 μg) in 1.0 ml of 0.1 M potassium phosphate buffer (pH 3.0) was incubated with the following additives, and then the remaining activities were measured: □, 20 μmol KBr or KCl; Δ, 5 μmol H₂O₂; Δ, 0.6 μmol Br₂; ○, 20 μmol KBr and 0.15 μmol H₂O₂ (the arrows indicate the additions of 0.05 μmol H₂O₂); ●, 20 μmol KCl and 2 μmol H₂O₂ (the arrows indicate the additions of 1 μmol H₂O₂).

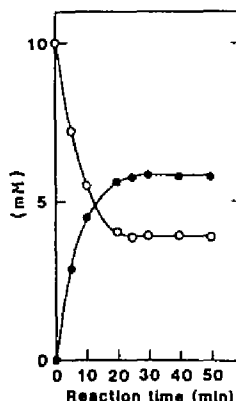


Fig. 4. 5-Bromocytosine formation through chloroperoxidase-catalyzed bromination. The reaction mixtures comprised 1 mmol potassium phosphate buffer (pH 3.0), 0.4 mmol KBr, 0.26 mmol H₂O₂, and 10 units of chloroperoxidase in a total volume of 10 ml. The reaction was started and continued by adding 20 μmol H₂O₂ at 4-min intervals. ○, Cytosine concentration; ●, 5-bromocytosine formed.

remaining chloroperoxidase activity in the presence of halide ions and hydrogen peroxide without a halogen acceptor or on exposure to molecular bromine. Surprisingly, the chloroperoxidase rapidly lost its activity during the reaction. It became apparent that molecular bromine irreversibly denatured chloroperoxidase.

Production of 5-Bromocytosine by the Chloroperoxidase reaction

Figure 4 shows the formation of 5-bromocytosine from

cytosine through the enzyme-catalyzed bromination. The reaction was continued for 50 min by feeding hydrogen peroxide at 4-min intervals. The bromination of cytosine by the enzyme did not completely proceed. This phenomenon was considered to be due to the irreversible denaturation of the enzyme.

DISCUSSION

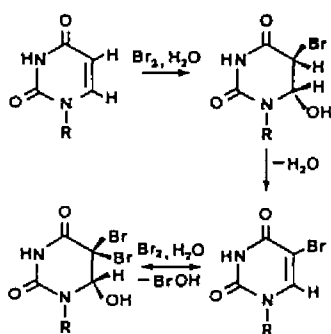
This is the first study concerning the enzymatic halogenations of nucleic bases and related compounds. Halogenated nucleic bases, nucleosides and nucleotides are known to be anticancer or antiviral agents, which inhibit the synthesis of DNA or RNA in a living cell system (40, 41). In the field of microbiology, some halogenated compounds have been isolated as biologically active compounds (42). The results suggested the possibility of the participation of haloperoxidases in the syntheses of these compounds, and the application of haloperoxidases to the enzymatic halogenation process. Table I summarizes the substrate specificity of the chloroperoxidase of Caldariomyces fumago for nucleic acid-related substances.

In Section 1, the author revealed that the chloroperoxidase reaction is the same as that of molecular bromine using trans-cinnamic acid and its derivatives, and systematized the substrates of the chloroperoxidase. The results indicated that the substrate specificity of chloroperoxidase depends on the nucleophilicity of the substrate. As shown in Fig. 2, there is no doubt that the chloroperoxidase reactions are

identical with those of molecular chlorine and bromine in the case of nitrogen-containing heterocycles. Therefore, the substrate specificity of the chloroperoxidase toward these nitrogen-containing aromatic heterocycles are likely. The abilities of pyridine, pyrimidine and purine as nucleophiles are low because π -electron density is reduced by the electronegative nitrogen atom. In addition, the coordination of electrophilic molecular halogen to the nitrogen atom increases the electronegativity of nitrogen atom, which results in less reactivity toward electrophiles. However, the presence of an electron-attracting group in the heterocycles reduces the electron density of the nitrogen atom. So, the coordination of molecular halogen to nitrogen atom hardly occurs. The substrate specificities of the chloroperoxidase can be explained in this way.

Substitution of hydrogen at 5-position of uracil and cytosine and at the 4-position of pyrazole by halogen are likely, because of the general resonance. Bromination of cytosine and uracil by bromine in an aqueous solution was examined in detail by Taguchi and Wang (43) and Tee and Berks (44), respectively. The first step in the bromination reaction is the formation of unstable bromohydrin derivatives, which are subsequently converted to 5-bromocytosine or 5-bromouracil derivatives. They reported that in the presence of excess bromine, 5,5-dibromo-6-hydroxy-5,6-dihydrocytosine or a similar uracil derivative is produced. However, these products also readily revert to 5-bromocytosine or 5-bromo-

uracil derivatives through the nucleophilic or electrophilic elimination of HOBr. On enzymatic bromination of thymine, the author isolated unstable 5-bromo-6-hydroxy-5,6-dihydro-thymine. The formation of this product could be explained in the same manner. In the case of the bromination of 2'-deoxyuridine, unstable products (Fig. 2c) were produced, which probably corresponded to 5-bromo-6-hydroxy-5,6-dihydro- or 5,5-dibromo-6-hydroxy-5,6-dihydro-derivatives.



The EI-MS spectra of the compounds (data not shown) indicated the presence of fragment ion peaks of 5-bromo-2'-deoxyuridine and the absence of molecular ion peaks. The retention times of these products on HPLC supported the existence of a hydroxyl group in the molecular structures of the products.

As to the chlorination reaction, the author isolated only 5-chlorouracil and 4-chloropyrazole. Adenine and adenosine were observed to be converted into unknown compounds. But they were not isolated because they were unstable. The chemical chlorinations of cytosine and uracil were complicated. It was found that long time reaction led to the formation of two products from cytosine, which showed long retention times on HPLC with a reversed phase column (Fig. 2b-3). Hence,

these products cannot be explained in terms of the formation of chlorohydrin derivatives. In the case of uracil, disappearance of the products was observed. This indicated the decomposition of the ring structure of uracil. The saturation at the 5- and 6-positions of the pyrimidine residue probably led to straining of the plane structure of the molecule, and the cleavage of the ring would occur instead of elimination of HOCl.

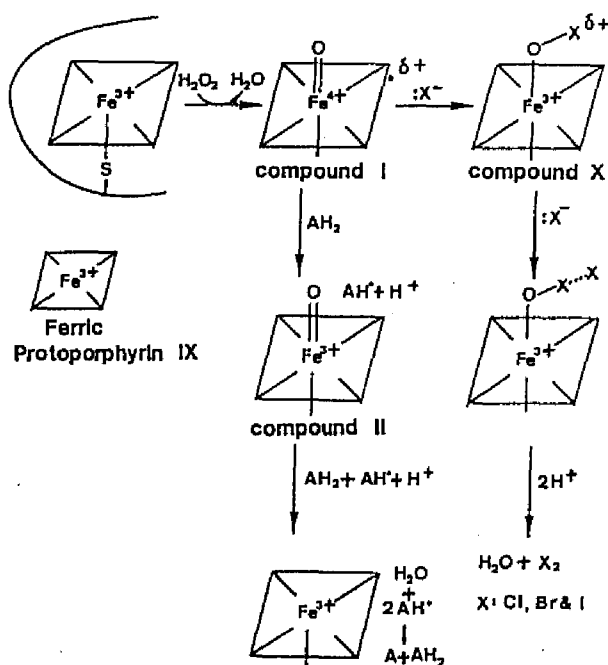


Fig. 5. Reaction mechanism of chloroperoxidase of *C. fumago*.

From the viewpoint of application of chloroperoxidase to the enzymatic process, it has some disadvantages. Figure 4 shows that the chloroperoxidase-catalyzed bromination of cytosine stopped within a short time. This phenomenon was considered to be due to denaturation of the enzyme. Molecular

bromine probably acts on several amino acid residues such as cysteine, histidine, tryptophan and tyrosine (5), and also on coenzymes e.g. NAD⁺, riboflavin, CoA, pyridoxal, etc. Therefore, there is little possibility of using the chloroperoxidase with other enzyme systems, for example, the hydrogen peroxide generating system of oxidases.

Figure 5 illustrates the reaction mechanism of chloroperoxidase-catalyzed halogenation on the basis of the author's and other researchers' studies of the enzyme (11).

SUMMARY

The enzymatic halogenation of nitrogen-containing aromatic heterocycles catalyzed by chloroperoxidase of Caldariomyces fumago was studied. The enzyme catalyzed the chlorination of uracil and pyrazole, the bromination of cytosine, uracil, thymine, cytidine, 2'-deoxyuridine, guanosine and pyrazole, and the iodination of uracil and pyrazole, to yield the respective halogenated products. The results of product and halogenation intermediate analyses suggested that the halogenation reaction of the chloroperoxidase involves the formation of a molecular halogen and its release into the solution. On the basis of the results, the author discussed the ability of the enzyme as a halogenating reagent.

CHAPTER II

BROMOPEROXIDASE OF CORALLINA PILULIFERA

Section 1. Distribution of Bromoperoxidase in Algae and Its Immunological Properties

Enzyme-catalyzed halogenation is a common biological phenomenon. Previously, various antibacterial halocompounds like bromophenols were isolated from many marine algae and identified (3, 4). Furthermore, some bromoperoxidases, which are specific for I^- and Br^- , have been found in and isolated from marine algae such as Rhodomela larix (24), Rhipocephalus phoenix (25), Penicillus lamourouxii (25), Penicillus capitatus (26), and Ascophyllum nodosum (30, 31). The enzymes in algae probably participate in the synthesis of halogenated compounds. In the course of the studies of haloperoxidases, the author found coralline algae with high bromoperoxidase activity.

This section deals with the distribution of bromoperoxidase in coralline algae (Corallinaceae, Rhodophyta), and its some properties and immunological heterogeneity.

MATERIALS AND METHODS

Collection of Algae Various algae including 59 species of 47 genera were collected from shallow water (0.5-2.0 m deep)

on the shores of Suzuka, Mie Prefecture, Miyazu, Kyoto Prefecture, Shirahama, Wakayama Prefecture (temperate sea), Hakodate, Hokkaido (subarctic sea), and Sesoko island, Okinawa Prefecture (subtropical sea) in Japan during 1984 to 1985. The samples, in ice-cold boxes, were immediately sent to my laboratory, and their bromoperoxidase activity was assayed. The rest of the algal samples were stored frozen at -20°C .

Crude Enzyme Preparation Each sample of alga was briefly washed with cold water and dried by pressing between paper towels. Five hundred milligrams of alga was disrupted with sea sand (40-80 mesh) in a chilled mortar and extracted with 4 ml of 50 mM potassium phosphate buffer (pH 6.5).

Enzyme Assay Bromoperoxidase activity was spectrophotometrically assayed at 25°C by measuring the change in absorbance at 290 nm due to the change of monochlorodimedone ($\epsilon = 19,900 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 6.0) to monobromomonochlorodimedone. The standard assay mixture contained 100 μmol of potassium phosphate buffer (pH 6.0), 20 μmol of potassium bromide, 2 μmol of hydrogen peroxide, 60 nmol of monochlorodimedone and a suitable amount of enzyme solution in a total volume of 1.0 ml. The reaction was started by adding hydrogen peroxide. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol of monobromomonochlorodimedone in one min under the standard assay conditions.

The oxidation of iodide was measured at 350 nm by a modification of the method of Hosoya (45). The standard assay

mixture contained 100 μ mol of potassium phosphate buffer (pH 6.0), 5 μ mol of potassium iodide, 400 nmol hydrogen peroxide and a suitable amount of enzyme solution in a total volume of 1.0 ml. A blank test was done without enzyme to compensate the oxidation of iodide by hydrogen peroxide.

Catalase activity was measured at 240 nm by a modification of the method of Beers and Sizer (46). The reaction mixture consisted of 150 μ mol of potassium phosphate buffer (pH 7.0), 60 μ mol of hydrogen peroxide and a suitable amount of enzyme solution in a total volume of 3.0 ml.

Protein Measurement Protein concentrations were measured using the absorbance at 280 nm or by the method of Lowry et al. (47) with bovine serum albumin as a standard.

Purification of Bromoperoxidase and Chloroperoxidase Bromoperoxidase was purified from Corallina pilulifera (Section 2) and chloroperoxidase from Caldariomyces fumago (Chapter I, Section 2).

Antiserum Preparation Antibodies were elicited by injection of 3.0 mg of purified bromoperoxidase of C. pilulifera into a young female rabbit. The antigen was dissolved in physiological saline containing 10 mM potassium phosphate buffer (pH 7.0), homogenized with an equal volume of complete Freund's adjuvant (Difco). After 3 weeks, the booster injection of 2.0 mg antigen homogenized with an equal volume of incomplete Freund's adjuvant (Difco) was administered. On the 7th day after the booster injection, blood was collected from an ear

vein, and allowed to clot. The serum was centrifuged at 600 x g for 10 min and the supernatant was stored at -20°C.

Immunodiffusion Analysis Double-diffusion was performed on microscope slides which were coated with 1% agarose (Difco) in 10 mM Tris-H₂SO₄ buffer (pH 8.0) containing 0.01% sodium azide following Ouchterlony's method (48). The center well was charged with 10 µl of 4 times diluted antiserum in 10 mM potassium phosphate saline buffer (pH 8.0), and the peripheral wells were filled with 15 to 20 µl of crude extracts containing 0.02-0.8 units of enzyme or with 10 µl (0.05 unit) of the purified bromoperoxidase. Blank serum was used to perform the control experiments. The Ouchterlony plates were placed in a high humidity chamber at room temperature and examined for precipitin lines at several hours intervals. The plates were washed with 10 mM sodium phosphate saline buffer (pH 8.0) and then stained with 0.5% Amido Black 10B in methanol/acetic acid (9:1) for several hours. After washing with methanol/acetic acid (9:1), the plates were photographed.

Immunotitration Analysis The purified bromoperoxidase and crude extracts each containing 0.1 unit of the enzyme were incubated with various volumes of 10 times diluted anti-bromoperoxidase serum at 37°C for 30 min. Diluted blank serum was used as a control. After brief centrifugation, the bromoperoxidase activity in the supernatant solution was assayed.

RESULTS

Distribution of Bromoperoxidase in Algae

As shown in Table I and II, bromoperoxidase activities were found in 20 algae of different species of 59 samples collected from the seashores in Japan. Comparing the results in Table I with those in Table II, it appears that the frequency of the existence of the enzyme in the algae from subtropical sea area is higher than that of from the temperate or subarctic seas in Japan. All the coralline algae including Corallina, Amphiroa, Bossiella, Calliarthron, Alatocladia, Mastophora and Jania tested contained some of the enzyme. High specific activities were observed in Amphiroa zonata and Amphiroa ephedraea, and high total activities in Corallina officinalis and Corallina pilulifera. Figure 1 shows photographs of eight coralline algae with high enzyme activities.

Table I. Bromoperoxidase activities of various algae collected from temperate and subarctic seas

Algae	Collection site	Total activity (U/g wet algae)	Specific activity (U/mg protein)
[Rhodophyta]			
<i>Corallina pilulifera</i>	(Shirahama)	11.7	0.54
<i>Corallina officinalis</i>	(Hakodate)	19.3	0.56
<i>Amphiroa zonata</i>	(Shirahama)	9.3	0.83
<i>Amphiroa ephedraea</i>	(Shirahama)	5.5	0.77
<i>Amphiroa misakiensis</i>	(Shirahama)	2.4	0.22
<i>Bossiella cretacea</i>	(Hakodate)	5.6	0.32
<i>Calliarthron yessoense</i>	(Hakodate)	3.2	0.34
<i>Alatocladia modesta</i>	(Hakodate)	0.9	0.10
<i>Gelidium amansii</i>	(Miyazu)	0.6	—
<i>Hypnea choraroides</i>	(Shirahama)	0.2	—
<i>Neodilsea yendoana</i>	(Hakodate)	0.1	—
[Chlorophyta]			
<i>Codium fragile</i>	(Shirahama)	0.5	—

Bromoperoxidase activity was not detected in the following algae: [Phaeophyta] *Calpomenia bullosa*, *Calpomenia sinuosa*, *Ecklonia kurame*, *Endarachne binghamiae*, *Papenfussiella kurame*, *Padina arborescens*, *Sargassum hemiphyllum*, *Sargassum horneri*, *Sargassum thunbergii*, *Scytosiphon lomentarius*, *Spatoglossum pacificum*, *Undaria pinnatifida* (Miyazu), *Alaria crassifolia*, *Costaria costata*, *Laminaria japonica* (Hakodate). [Chlorophyta] *Chaetomorpha moniligera*, *Cladophora opaca*, *Enteromorpha intestinalis*, *Enteromorpha linza*, *Ulva pertusa* (Miyazu). [Rhodophyta] *Carpopeltis flabellata*, *Chondria crassicaulis*, *Chondrus crispus*, *Grateloupia filicina*, *Grateloupia okamurai*, *Polysiphonia urceolata*, *Schizymenia dubyi* (Miyazu), *Porphyra tenera* (Suzuka), *Rhodoglossum japonicum*, *Rhodymenia pertusa* (Hakodate). Collection site is shown in parenthesis.

Table II. Bromoperoxidase activities of various algae collected from subtropical sea

Algae	Total activity (U/g wet algae)	Specific activity (U/mg protein)
[Rhodophyta]		
<i>Bostrychia tenella</i> (Vahl) J. Agardh	0.16	0.04
<i>Galaxaura fastigiata</i> Decaisne	0.60	0.25
<i>Gelidiella acerosa</i> (Forsskal) Feldmann et Hamel	1.07	0.22
<i>Jania decussato-dichotoma</i> Yendo	0.64	0.40
<i>Mastophora rosea</i> (C. Agardh) Setchell	0.28	0.08
[Chlorophyta]		
<i>Chlorodesmis comosa</i> Bailey et Harvey	0.96	0.48
<i>Codium repens</i> (Crouan) Vickers	0.16	0.08
<i>Halimeda incrassata</i> Lamouroux f. <i>typica</i> Barton	0.24	0.30

Bromoperoxidase activity was not found in the following algae :

[Phaeophyta] *Padina australis* Hauck, *Turbinaria ornata* J. Agardh,
[Chlorophyta] *Caulerpa brachypus* Harvey, *Caulerpa racemosa* var.
clavifera f. *macrophyta* W. V. Bosse, *Cladophoropsis sundanensis*,
Codium adhaerens (Cabrera) C. Agardh, *Dictyosphaeria cavernosa*
(Forsskal) Boergesen (Yamada and Tanaka 1938), [Rhodophyta] *Digenea simplex* (Wulfen) C. Agardh, *Galaxaura fasciculata* Kjellm.

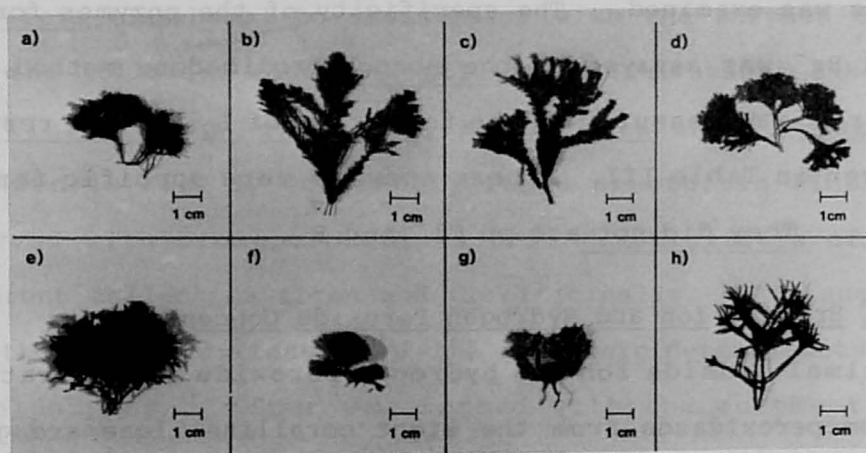


FIG. 1. Photographs of Coralline Algae Collected in Japan.

a) *Corallina pilulifera*; b) *Corallina officinalis*; c) *Calliarthron yessoense*; d) *Alatocladia modesta*; e) *Amphiroa ephedraea*; f) *Amphiroa zonata*; g) *Amphiroa misakiensis*; h) *Bossiella cretacea*.

pH Optima of Bromoperoxidases of Coralline Algae

The activities of the bromoperoxidases were measured as a function of pH for the coralline algal extracts, as shown in Fig. 2. The enzymes from these eight coralline algae all had pH optima near 6.0.

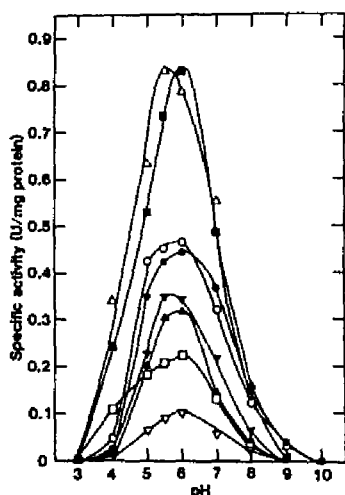


FIG. 2. pH Optima of Bromoperoxidases from Coralline Algae.

The enzyme activity was measured in 0.1M 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). ■—■, *Amphiroa zonata*; △—△, *Amphiroa ephedraea*; ○—○, *Corallina officinalis*; ●—●, *Corallina pilulifera*; ▼—▼, *Calliarthron yessoense*; △—△, *Bossiella cretacea*; □—□, *Amphiroa misakiensis*; ▽—▽, *Alatocladiu modesta*.

Halide Ion Specificity

The halide ion specificity of the eight coralline algal extracts was examined. The specificity of the enzymes for F^- , Cl^- and Br^- was assayed by the monochlorodimedone method, and that for I^- was measured by the formation of I_2 . The results are given in Table III. These enzymes were specific for Br^- and I^- . They did not act on Cl^- and F^- .

Optimal Bromide Ion and Hydrogen Peroxide Concentrations

Optimal bromide ion and hydrogen peroxide concentrations for bromoperoxidases from the eight coralline algae are given in Table III. The maximum activities of all these enzymes were with bromide ions at higher concentration than 75 mM.

The optimum hydrogen peroxide concentration was approximately 0.25-5.0 mM in all cases. Hydrogen peroxide concentrations above 5 mM inhibited these enzyme activities. No appreciable catalase activity was detected in crude extracts of the eight coralline algae.

Table III. Properties of bromoperoxidases of eight coralline algae

Algae	Halide specificity	Optimum Br ⁻ conc. (mM)	Optimum H ₂ O ₂ conc. (mM)
<i>Corallina pilulifera</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 5.0
<i>Corallina officinalis</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 4.0
<i>Amphiroa zonata</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 3.0
<i>Amphiroa ephedraea</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 4.0
<i>Amphiroa misakiensis</i>	I ⁻ , Br ⁻	≥ 75	0.25 — 5.0
<i>Bossiella cretacea</i>	I ⁻ , Br ⁻	≥ 75	0.75 — 4.0
<i>Calliarthron yessoense</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 4.0
<i>Alatocladia modesta</i>	I ⁻ , Br ⁻	≥ 75	0.5 — 5.0

Comparative Immunological Properties of Bromoperoxidases in Algae

To clarify the homology of the bromoperoxidases in marine algae, immunodiffusion techniques were carried out using the antiserum prepared against the purified enzyme of *Corallina pilulifera* (Takahama) to cross-react with the enzymes derived from the following algae: *C. pilulifera* (Shirahama, Hakodate), *C. officinalis*, *A. ephedraea*, *B. cretacea* and *C. yessoense*. As shown in Fig. 3, completely fused precipitin lines were observed with bromoperoxidases from *C. pilulifera* of three different collection sites and *C. officinalis*. This suggested that there is no difference of the antigenic determinants among these enzymes. Spur was formed with the enzyme from *B. cretacea* and incomplete fusion was observed with the enzyme from *A. ephedraea*, which indicated that the bromoperoxidases in these algae are, in part, immunologically identical to that of

C. pilulifera. When the enzymes of C. yessoense and chloroperoxidase of a fungus, Caldariomyces fumago, were tested, no precipitin lines were formed.

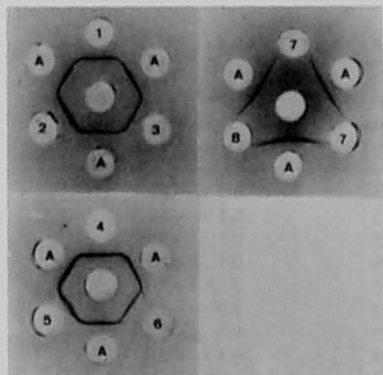


Fig. 3. Double-immunodiffusion analysis of bromoperoxidases from several marine algae and chloroperoxidase from Caldariomyces fumago. A, the purified bromoperoxidase from C. pilulifera (0.05 unit, Takahama); the crude extracts of bromoperoxidase from the following algae: 1, C. pilulifera (0.2 unit, Shirahama); 2, C. pilulifera (0.7 unit, Hakodate); 3, C. pilulifera (0.13 unit, Takahama); 4, C. officinalis (0.8 unit); 5, A. ephedraea (0.12 unit); 6, B. cretacea (0.05 unit); 7, C. yessoense (0.02 and 0.06 unit); 8, chloroperoxidase (6.7 unit) from C. fumago.

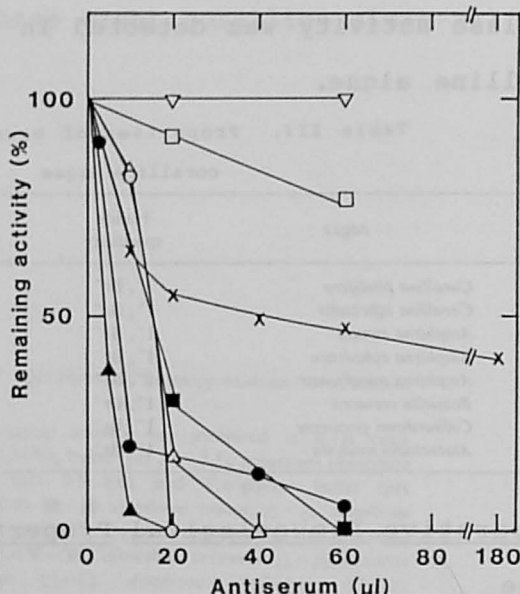


Fig. 4. Immunotitration of crude extracts of several marine algae with the anti-bromoperoxidase serum of C. pilulifera. The each crude extract containing 0.1 unit of bromoperoxidase was incubated with various volumes of anti-bromoperoxidase serum (10 times diluted) at 37°C for 30 min. After brief centrifugation, the enzyme activity in the supernatant solution was assayed. O, C. pilulifera (Takahama); ●, C. pilulifera (Hakodate); △, C. pilulifera (Shirahama); ▲, C. officinalis; ×, A. ephedraea; □, A. zonata; ■, B. cretacea; ▽, C. yessoense, M. rosea, J. decussato-dichotoma, Gelidium amansii, Codium fragile, Hypnea chararoides, Bostrychia tenella, Chlorodesmis comosa, Codium repense and Gelidiella acerosa.

Immunotitration analysis was also carried out to ensure the immunological properties of the enzymes in various marine algae. When bromoperoxidases from C. pilulifera, C. officinalis and B. cretacea were incubated with the antiserum of C. pilulifera, the enzyme activities were completely inhibited (Fig. 4). The enzyme activities of A. ephedraea and A. zonata were partially inhibited. In contrast, there was no

significant decrease of the enzyme activity in C. yessoense, M. rosea, J. decussato-dichotoma (coralline algae) and the other marine algae which do not belong to coralline family. This indicated that the enzymes from these algae did not share any antigenic determinants with the bromoperoxidase of C. pilulifera.

DISCUSSION

Haloperoxidase has been reported in two microorganisms, Caldariomyces fumago (Chapter I) and Pseudomonas aureofaciens, (29) and some algae including Penicillius (25, 26), Rhipocephalus (25), Rhodomela (24), and Ascophyllum (30, 31). This is the first study that coralline algae contain large amounts of bromoperoxidase. More than 10 units of the enzyme per g of wet alga was observed in Corallina officinalis and Corallina pilulifera. Coralline algae are widely distributed in the seashores of the world, not only in Japan but also southern Australia, western North America and eastern South Africa. Corallina pilulifera occurs in all the costal areas in Japan (49). Thus, Corallina pilulifera seems to be an excellent source of bromoperoxidase.

The properties of the enzymes extracted from the eight coralline algae were quite similar. The optimal pHs of the enzymes were around 6.0. The chloroperoxidase of C. fumago and bromoperoxidases of Rhodomela larix and Penicillius capitatus have their maximum activities in more acidic pHs, pH 2.75 for

C. fumago (9), pH 4.4 for R. larix (24) and pH 4.0 for P. capitatus (25).

As for optimal bromide ion concentration, the enzymes of coralline algae were most active at higher concentration than 75 mM. But Hager et al. reported that the enzyme of P. capitatus was not saturated with bromide ion even at concentrations above 1.0 M (26).

With the enzymes of coralline algae, the optimum H_2O_2 concentration was approximately between 0.25-5.0 mM. This value is higher than those for the other reported haloperoxidases, 0.25 mM for R. phoenix and 0.125 mM for P. capitatus (25). Compared with these other enzymes, the enzymes of coralline algae seem to be very resistant to the inhibitory effect of peroxide.

The bromoperoxidases derived from coralline algae had similar enzymatic properties. Therefore, from the evolutionary point of view, the immunological properties of enzymes in coralline algae are interesting. Immunodiffusion and immunotitration analyses showed that the enzymes of C. pilulifera obtained from different collection sites in Japan and Corallina officinalis were immunologically homologous, and the enzymes of Bossiella cretacea and Amphiroa ephedraea were partially identical to that of C. pilulifera. In contrast, the enzymes from some other coralline algae such as Calliarthron yessoense, Alatocladia modesta, Mastophora rosea, and several other algae did not share any antigenic determinants with the enzyme of C. pilulifera. From these results, it was concluded that the bromoperoxidases in coralline algae

can be divided into three immunological types: 1) C. pilulifera, C. officinalis; 2) A. ephedraea, A. zonata, B. cretacea; and 3) M. rosea, C. yessoense, J. decussato-dichotoma. Considering the peculiar physicochemical properties of the bromoperoxidase of C. pilulifera (Section 2), it is assumed that the bromoperoxidases in coralline algae have evolved independently from other algal haloperoxidases.

SUMMARY

Bromoperoxidases were found in coralline algae (Corallinales, Rhodophyta) collected on seashores in Japan, and high enzyme activities were observed in Corallina officinalis, Corallina pilulifera and Amphiroa zonata. The enzymes derived from coralline algae had similar enzymatic properties: optimum pH, halide ion specificity, optimum H_2O_2 and Br^- concentrations. To clarify the homology of the enzymes, the immunological properties were studied using the antiserum prepared against the enzyme of Corallina pilulifera. The enzymes from C. pilulifera of different collection sites and Corallina officinalis were immunologically homologous. The enzymes of Bossiella cretacea and Amphiroa ephedraea were partially identical to that of C. pilulifera. In contrast, the enzymes from some other coralline algae such as Calliarthron yessoense, Alatocladia modesta, Mastophora rosea, and several other algae did not share any antigenic determinants with the enzyme of C. pilulifera. It was concluded that the

bromoperoxidases in coralline algae can be divided into at least three groups based on the immunological type.

Section 2. Purification and Characterization of Bromoperoxidase of Corallina pilulifera

Chloroperoxidase of Caldariomyces fumago, which is able to catalyze the halogenation of many compounds (1), has been well investigated since 1959 (9, 10, 11). Bromoperoxidase which is specific for I^- and Br^- has been found in several marine algae such as Rhodomela (24), Rhipocephalus (25) and Penicillus (25, 26), and is considered to participate in the occurrence of halocompounds in marine environment. Pée and Lingens also reported the purification of the enzyme from Pseudomonas aureofaciens (29). The above haloperoxidases are all typical hemoproteins.

In the previous section, the author described that various coralline algae contained large amounts of bromoperoxidase, and clarified their immunological properties. The present section describes the purification and characterization of bromoperoxidase from Corallina pilulifera, which is distinct from other haloperoxidases.

MATERIALS AND METHODS

Chemicals DEAE-Sepharose CL-6B and Sepharose 6B were purchased from Pharmacia Fine Chemicals, Sweden, and Cellulofine GC-700m from Seikagaku Kogyo Co., Japan. SDS-gel electrophoresis calibration proteins 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. Analytical reagent grade hydrogen peroxide, standard iron and magnesium solutions, and Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt) was purchased from Wako Pure Chemical Industries, Japan, formamidinesulfinic acid from Aldrich Chemical Co. Inc., U.S.A., and *o*-dianisidine and standard iodine solution by Nakarai Chemicals, Japan. Super-pure reagents of potassium iodide, potassium chloride and potassium fluoride were obtained from Merck Japan, Ltd. Carrier ampholyte was supplied by LKB, Sweden. NaOBr solution was prepared by dissolving 120 mg of Br₂ in 3 ml of 1N NaOH, followed by dilution to 100 ml with distilled water.

Collection of Alga Corallina pilulifera was collected from shallow waters (0.5-1.0 m deep) at the shores of Shirahama (Wakayama Prefecture) and Takahama (Fukui Prefecture), Japan, during 1984 to 1985, and stored frozen at -20°C before use.

Crude Extract Preparation Each of frozen algae (ca. 330 g of wet weight) was suspended in 150 ml of potassium phosphate buffer (pH 6.5) and disrupted with a Hitachi VA 895 blender mixer for 15 min. After disruption, the debris was removed by centrifugation (8,000 x g, 20 min).

Enzyme Assay Bromoperoxidase activity was assayed by measuring the change in absorbance at 290 nm due to the change of monochlorodimedone ($\epsilon=19,900 \text{ M}^{-1} \text{ cm}^{-1}$) to monobromomono-chlorodimedone (Section 1). Catalase activity was measured

by the modified method of Beers and Sizer (46). Peroxidase activity was measured with o-dianisidine at 25°C (50). O₂ concentration in the reaction mixture was monitored with a Beckmann Fieldlab oxygen analyzer equipped with a Beckmann 39533 polarographic oxygen sensor. Before analysis, the reaction mixture was saturated with nitrogen gas, and the reaction was carried out in a closed system with constant stirring by a magnetic stirrer. Throughout this section, 1 unit of enzyme activity is equal to the amount of enzyme that converted 1 μ mol of substrate in 1 min at 25°C. The detailed assay conditions is described in Section 1.

Protein Determination The protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry et al. (47) with bovine serum albumin as a standard.

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 (51). The samples were run at 3.0 mA per gel at 4°C. The gels were stained for protein with Coomassie brilliant blue G-250, and destained in methanol/acetic acid/H₂O (1:2:7). Staining of gels for enzyme activity was carried out by incubation in 10 mM potassium phosphate buffer (pH 6.0) containing 0.5% pyrogallol, 7.5 mM hydrogen peroxide and 7.5 mM KBr in the dark at room temperature. Staining of gels for glycoprotein was performed by the periodic acid oxidation and Schiff stain method

developed by Signoret and Crouzet (52). SDS-gel electrophoresis was performed in 10% polyacrylamide slab gel using a Tris-glycine buffer system described by King and Laemmli (53). Molecular weight of the subunit of the enzyme was estimated from the relative mobility of standard proteins.

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.

Isoelectric Focusing The isoelectric point of the enzyme was determined as described by Vesterberg (54) 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 (55). 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 mg/ml 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)) was used as samples. Centrifugation proceeded for 48 h at 25°C at 3,000 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 x 60 cm, Toyosoda Co., Japan) at the flow rate of 0.3 ml/min using 50 mM potassium 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 enzyme were negatively stained with 2% uranyl acetate (UA, pH 4.5) or 2% phosphotungstic acid (PTA, adjusted to pH 7.0 by KOH). A drop of enzyme solution (0.1-0.2 mg 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% UA or PTA 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 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.

Amino Acid Composition Amino acid analyses were performed according to the method of Spackman *et al.* (56) 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 h, 48 h and 72 h at 110°C. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (57). Tryptophan content was estimated by the method of Spies and Chambers (58) with authentic tryptophan as a standard.

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. (59). The enzyme solutions, diluted to about 150 μ g and 100 μ g per 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, and measured again. Buffer was used as the controls. 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 (60) and Siegel et al. (59) with sodium sulfide as a standard. Standard solutions of sodium sulfide were prepared as described by King and Morris (60), 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 protein per ml were measured with a 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 1mM EDTA or o-phenanthroline. The following assay conditions were employed for qualitative analysis; cooling gas 14 l/min, plasma gas 2 l/min, sample gas 0.5 l/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.

Instrumental Analysis Proton nuclear magnetic resonance (^1H NMR) spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. For recordings of paramagnetically shifted proton NMR spectra, typically 50K transients were accumulated to obtain the Fourier transformed spectrum with 8K data points and 6.0- μs 90° pulse. The enzyme sample was dialyzed against 5 mM potassium phosphate buffer (D_2O , pH 6.0), before measurement.

Electron spin resonance (ESR) spectra were measured at 77K with a JEOL JES-FE3X ESR spectrometer. Before analysis, the ESR samples were dialyzed against 50 mM potassium phosphate buffer (pH 6.0) containing 1 mM EDTA and then against the buffer, and frozen in liquid nitrogen.

Circular dichroism (CD) spectra were recorded at room temperature in a 10 mm light-path cuvette with a JASCO J-500 C. spectropolarimeter equipped with an electromagnet (1.5 T).

RESULTS

Purification of Enzyme

The enzyme was prepared from 2.5 kg of wet weight of the

alga collected at Shirahama or Takahama. Unless otherwise indicated, all purification steps were carried out at 0-5°C, using potassium phosphate buffer.

Step 1. Ammonium sulfate precipitation : Solid ammonium sulfate was added to the crude extract to give 80% saturation, and stirred for 15 h. The precipitate was collected by centrifugation (9,000 x g, 20 min), and the pellet was dissolved in 0.1 M of the buffer (pH 7.0) and dialyzed overnight against 20 liters of the same buffer containing 0.1 M KCl. After dialysis, the precipitate was removed by centrifugation (9,000 x g, 20 min).

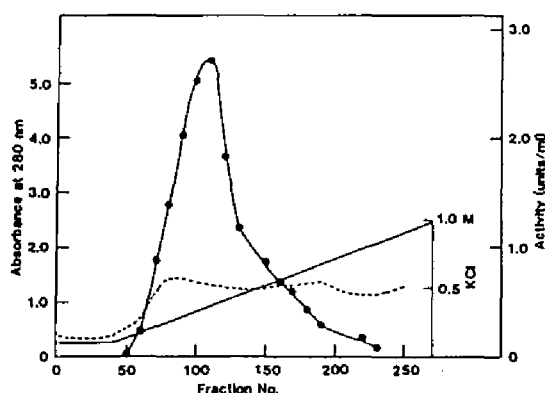


Fig.1. First DEAE-Sephacel CL-6B column chromatography of bromoperoxidase. The flow rate was approximately 500 ml per hr, and 16-ml fractions were collected. (---○---), absorbance at 280 nm; (●---●), enzyme activity; (—) concentration of KCl.

Step 2. First DEAE-Sephacel chromatography : The enzyme solution was applied to the column of DEAE-Sephacel CL-6B (5 x 30 cm) previously equilibrated with the above dialysis buffer. The column was washed well with the same buffer and the adsorbed enzyme was eluted with 0.1 M of the buffer (pH 7.0) containing a linear gradient of KCl from 0.1 to 1.0 M. Figure

1 shows the elution pattern of DEAE-Sepharose column chromatography. The fractions which showed high enzyme activity were pooled. Ammonium sulfate was added to the solution up to 80% saturation. After stirring for 3 h, the precipitate was collected, dissolved in 0.1 M of the buffer (pH 6.5) and dialyzed against 10 liters of the same buffer.

Step 3. Second DEAE-Sepharose chromatography : The enzyme solution was applied to the DEAE-Sepharose CL-6B column (5 x 25 cm) which had been equilibrated with 0.1 M of the buffer (pH 6.5), and the loaded column was washed well with the same buffer. A linear concentration gradient of ammonium sulfate was developed by mixing the equilibrium buffer with 0.8 M ammonium sulfate. Active 12-ml fractions were harvested and concentrated by salting out with ammonium sulfate at 80% saturation. The precipitate was collected by centrifugation (12,000 x g, 20 min), dissolved in 10 mM of the buffer (pH 7.0) and dialyzed against 0.1 M of the buffer.

Step 4. First Sepharose 6B chromatography : Each 50-ml portion of the enzyme solution was placed on a gel filtration column (4 x 75 cm) of Sepharose 6B equilibrated with 0.1 M of the buffer (pH 7.0). After elution with the buffer, the 5-ml fractions with high enzyme activity were pooled and concentrated by salting out with ammonium sulfate at 80% saturation. The precipitate was collected by centrifugation (12,000 x g, 20 min) and dissolved in the same buffer.

Step 5. Cellulofine GC-700 chromatography : The enzyme solution from Step 4 was applied to a gel filtration column of Cellulofine GC-700m (2.7 x 110 cm) which had been equilibrated

with 0.1 M of the buffer (pH 7.0). The enzyme was eluted with the buffer, and the 5-ml fractions with high enzyme activity were combined. Ammonium sulfate was added to the solution up to 80% saturation, and the precipitate was collected by centrifugation (12,000 x g, 20 min) and dissolved in the same buffer.

Step 6. Second Sepharose 6B chromatography : The enzyme solution was subjected to a second Sepharose 6B column chromatography (2.7 x 110 cm) under the similar conditions described in Step 4. The active fractions were combined, and concentrated with an ultrafiltration hollow fiber I-5-P (Asahi Kasei Co., Japan).

The overall purification achieved was approximately 90-fold with a yield of 16%. The enzyme solution thus obtained was designated as the purified enzyme. The results of the purification are summarized in Table I.

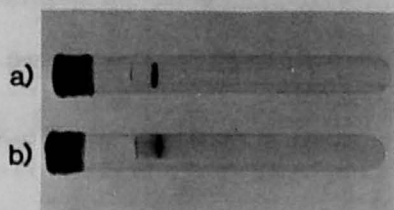
Table I. Summary of Purification of Bromoperoxidase from *C. pilulifera*

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	32,180	8,120	0.3	100.0
Ammonium sulfate precipitation (80%)	11,530	7,090	0.6	87.3
First DEAE-Sepharose chromatography	774	2,825	3.7	34.8
Second DEAE-Sepharose chromatography	362	2,930	8.1	36.1
First Sepharose 6B chromatography	102	2,505	24.6	30.8
Cellulofine GC-700 chromatography	67	1,642	24.5	20.2
Second Sepharose 6B chromatography	49	1,298	26.5	16.0

Purity of Enzyme

Upon polyacrylamide disc gel and SDS-polyacrylamide slab gel electrophoreses (Fig. 2A and 2B) the purified enzyme was shown to migrate as a single band when it was stained either for protein (Fig. 2A-a) or for bromoperoxidase activity (Fig. 2A-b). A brown band was observed when the gel was stained for the enzyme activity which coincided with the protein band. It is known that pyrogallol is one of the usual substrates of peroxidase. But, the omission of potassium bromide in the incubation mixture gave no color development on the gel. Hence, it was obvious that coloration on the gel was due to the bromide-dependent enzymic reaction, but not to peroxidase activity of the enzyme.

A) Disc gel electrophoresis



B) SDS-slab gel electrophoresis

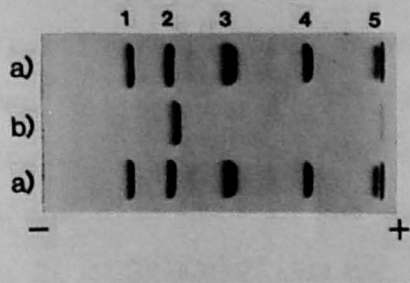


Fig. 2. Polyacrylamide disc gel (A) and SDS-polyacrylamide slab gel (B) electrophoreses of purified bromoperoxidase. (A) About 30 μ g of purified enzyme was subjected to electrophoresis. a, The gel was stained for protein. b, The gel was stained for enzyme activity. (B) a, The following marker proteins 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, About 30 μ g of purified enzyme was subjected to electrophoresis.

Molecular Weight and Subunit Structure

Equilibrium ultracentrifuge was employed to determine the molecular weight of the enzyme. The linearity of the plot as $\log f_r$ (fringe shift) versus r^2 demonstrated that the purified enzyme was homogeneous. The molecular weight was estimated to be $790,000 \pm 20,000$ 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. 2B). These data indicated that the enzyme of C. pilulifera consists of twelve subunits of identical molecular weight.

Figure 3a shows the electron micrograph of uranyl acetate (UA)-negatively stained image of the enzyme. UA-staining was superior to phosphotungstic acid (PTA)-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 x 15-16 nm), may represent a view perpendicular to the symmetry axis. From an analysis of these images, it was concluded that the complete structure of the enzyme was a dodecad aggregate composed of two hexagons face to face, as schematically illustrated in Fig. 3b.

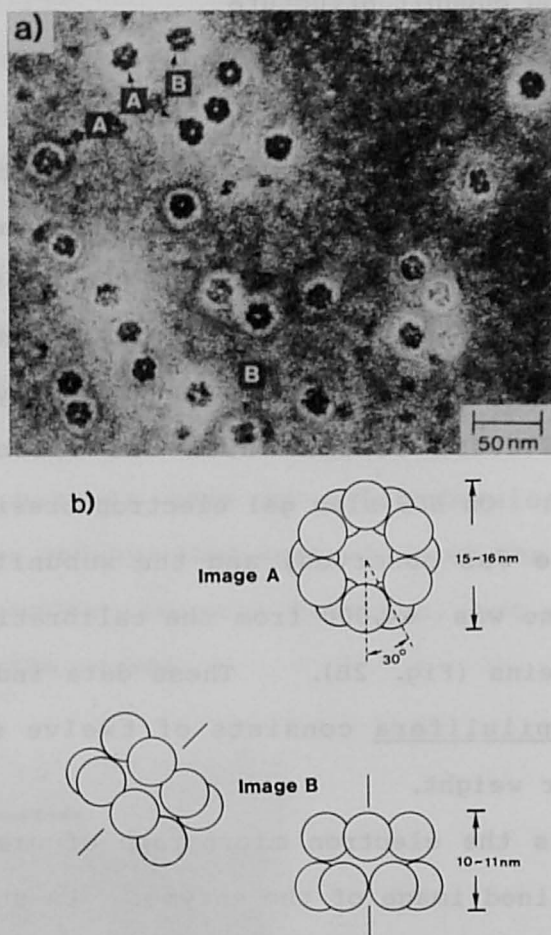


FIG. 3. Electron micrograph of the bromoperoxidase (a) and schematic model of the subunit structure of the enzyme (b). The enzymes were negatively stained with uranyl acetate and observed at 80 kV with a magnification of 80,000. The photograph was reproduced reversely in black and white to obtain a clear image.

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 activity, and that the isoelectric point of the enzyme was estimated to be 3.0.

The results of amino acid analysis of bromoperoxidase are presented in Table II. 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.

Table II. 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 $\pm$ 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 (58).

^c Cysteine was determined as cysteic acid after performic acid oxidation (57).

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 μ g of the enzyme, led me 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 (Shirahama enzyme) was 26.5 $\mu\text{mol}/\text{min}/\text{mg}$ 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 mM and 400 mM (Fig. 4a). 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. 4b).

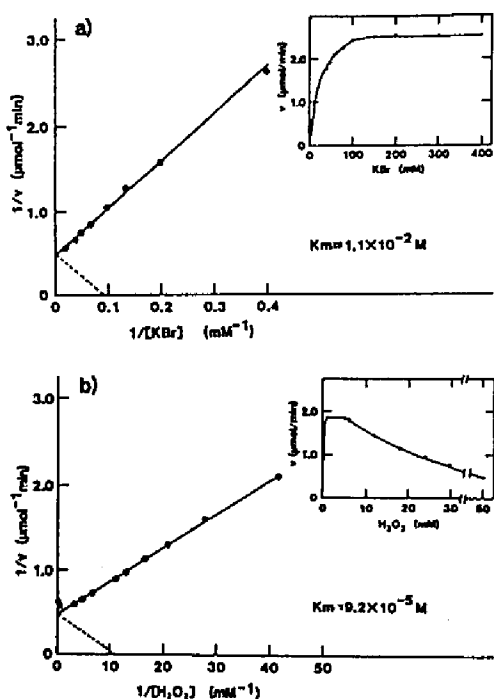


FIG. 4. Lineweaver-Burk plot for the bromination of monochlorodimedone catalyzed by the enzyme. The reactions were done at 25 °C in the reaction mixture containing 54 ng of the enzyme, 80 nmol of monochlorodimedone, 100 μmol of potassium phosphate buffer (pH 6.0), and varying amounts of potassium bromide 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.

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 with those obtained for other coralline algal samples (Section 1).

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 (Shirahama enzyme) exhibited only 0.0036 unit of peroxidase activity (Table III). 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 (Fig. 2A-b).

The enzyme lacked catalase activity as assessed by measurements at three different concentrations of 5, 10, and 20mM H_2O_2 varying the enzyme from 0.5 to 2.0 units per 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.

Table III. Peroxidase and catalase activities of bromoperoxidases

reaction	Origin	
	Shirahama	Takahama
	specific activity (unit/mg protein)	
bromination of monochloro- dimedone	26.5	4.9
catalase activity	0	0
peroxidase activity (o-dianisidine)	0.0036	0.0007

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 IVa). 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 inhibitions of β -mercaptoethanol, dithiothreitol, hydroxylamine and sodium azide were not observed (Table IVb). Therefore, the inhibitions of these compounds appeared to be reversible and not due to damage of the enzyme protein or active site.

Table IV
Effect of various compounds on the bromoperoxidase reaction

Compound	Concentration	Relative activity	
		A*	B*
	<i>mM</i>	<i>%</i>	
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	

* Each compound was added to the reaction mixture.

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

The effect of fluoride ions on the kinetics of the bromination of monochlorodimedone was examined in detail (Fig. 5). The kinetic data indicated that the inhibition of fluoride ions was definitely uncompetitive over the range of concentration between 1.25 mM 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.

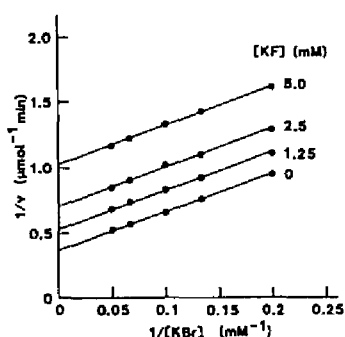


FIG. 5. Double-reciprocal plots of reaction velocity against potassium bromide in the presence of several concentrations of potassium fluoride. The reactions were done at 25 °C in the reaction mixture containing 54 ng of the enzyme, 60 nmol of monochlorodimedone, 2 μ mol of hydrogen peroxide, 100 μ mol 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.

Absorption Spectra

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 nm and 700 nm (Fig. 6). The visible absorption decreased by adding sodium dithionite. No significant absorption bands corresponding to heme or flavin was observed in the spectrum. Exposure of the native enzyme to sodium azide (final concentrations of 10 and 20 mM) and potassium cyanide (final concentrations of 50 and 100 mM) did not affect its spectrum. These results indicate that bromo-

peroxidase of C. pilulifera is completely different from reported haloperoxidases, which are hemoproteins.

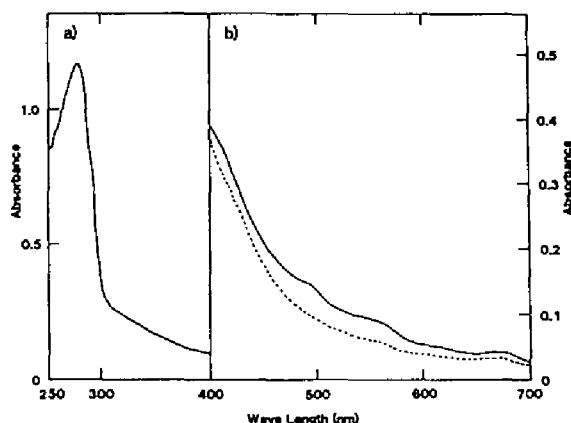


Fig. 6. Absorption spectra of bromoperoxidase. The spectra of the native enzyme (—) was recorded in 0.1 M potassium phosphate buffer (pH 7.0). The spectra of the dithionite reduced enzyme (---) was measured in the same buffer. Enzyme concentrations ; (a) 1.4 mg protein per ml, (b) 4.2 mg protein per ml.

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 (61). 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. Figure 7 showed that the enzyme contained iron and magnesium. Other metals such as calcium, selenium, vanadium, molybdenum, and copper were not observed.

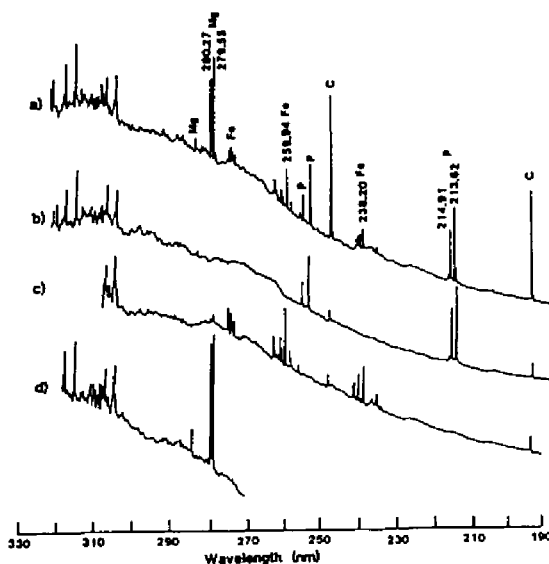


FIG. 7. Argon plasma emission spectrometry of the enzyme samples. a, the enzyme samples dialyzed against 5 mM potassium phosphate buffer (pH 7.0) in the presence or absence of 1 mM of EDTA or o-phenanthroline. b, dialysis buffer containing 1 mM EDTA or o-phenanthroline, or neither. c, standard iron solution containing $2 \mu\text{g}$ of ferric ion/ml of 0.1 N HCl. d, standard magnesium solution containing $0.5 \mu\text{g}$ 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.

Plasma emission spectroscopy of two enzyme preparations (Shirahama and Takahama) revealed that the enzyme had 2.3 ± 0.2 iron atoms and 1.6 ± 0.1 magnesium atoms, and 14.0 ± 0.5 iron atoms and 0.7 ± 0.1 magnesium atoms per molecule of the enzyme, respectively (Table V). Exhaustive dialysis of the enzyme samples against the buffer containing 1mM 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 non-heme iron center of the enzyme differs from [4Fe-4S] or [2Fe-2S] cluster (62, 63).

Table V. Differences in metal contents of bromoperoxidases from two different collection sites

properties	Shirahama (Pacific Ocean)	Takahama (Japan Sea)
specific activity (units/mg protein)	26.5	4.9
iron content (mol/mol enzyme) (dodecameric enzyme)	2.3 ± 0.2	14.0 ± 0.5
magnesium content (mol/mol enzyme) (dodecameric enzyme)	1.6 ± 0.1	0.7 ± 0.1
$E_{280}^{1\%}$	8.05	11.89

Valency State of Nonheme Iron

Incubation of the native enzyme (Takahama) with 2 mM formamidinesulfinic acid, a mild reducing agent, for about 2 h under anaerobic conditions caused the enzyme's complete inactivation. When it was reoxidized thereafter by bubbling with air, its activity returned almost to the original level (Fig. 8). Addition of 2 mM sodium dithionite caused a similar change, but enzyme activity was not restored by oxidation with air.

When the enzyme was incubated with 2 mM Tiron which is a chelating agent specific for ferric ion (64), changes in spectra of the enzyme were observed, which indicated the formation of a chelated complex (Fig. 9a). Absorbance at 450 nm was increased, with a concomitant decrease in enzyme activity (Fig. 9b).

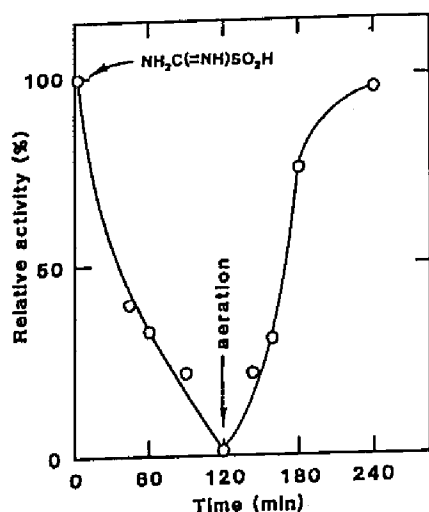


Fig. 8. Effect of formamidinesulfonic acid on enzyme activity. The enzyme sample (1.3 mg protein/ml in 50 mM potassium phosphate buffer, pH 6.0) was anaerobically incubated with 2 mM formamidinesulfonic acid at 25°C. After 2 h, air was passed through the sample to reoxidize the enzyme.

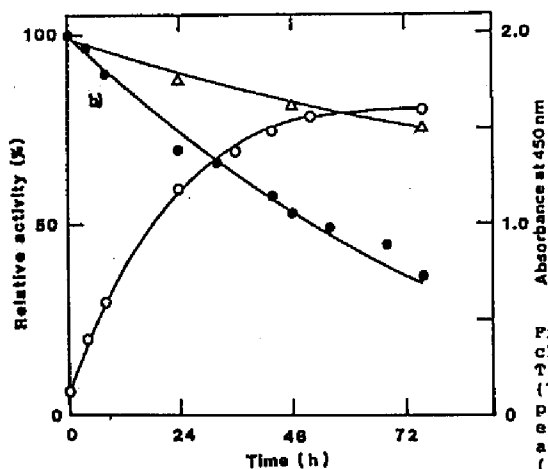
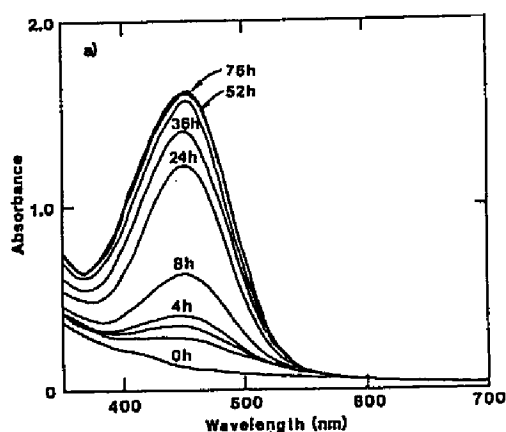


Fig. 9. Enzyme spectroscopic and activity changes following incubation with Tiron. Tiron (2 mM) was added to the enzyme sample (1.3 mg protein/ml in 50 mM Tris-HCl buffer, pH 8.0), and spectroscopic change (a) and enzyme activity (b) were monitored for 76 h at 25°C. Activity of Tiron-treated enzyme (●), activity of non-treated enzyme (△), absorbance at 450 nm of Tiron-treated enzyme (○).

Incubation of the enzyme with a ferrous ion chelating agent, *o*-phenanthroline, had no effect on the enzyme activity, but incubation with *o*-phenanthroline under reducing conditions completely inhibited it (Fig. 10). These data showed that nonheme iron of bromoperoxidase was ferric.

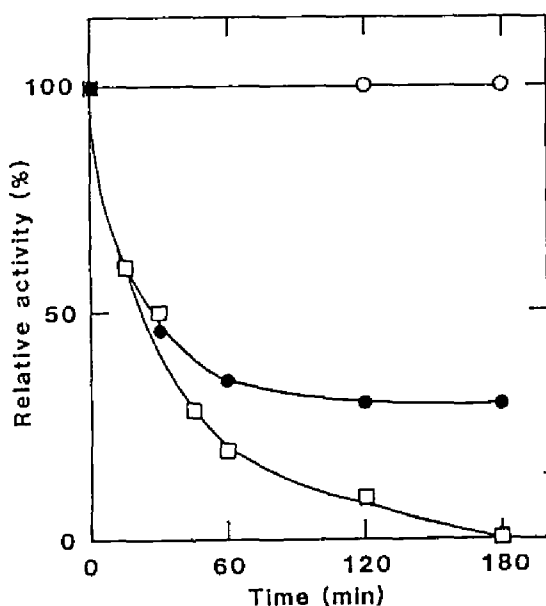


Fig. 10. Effect of *o*-phenanthroline on enzyme activity. The enzyme sample (1.3 mg protein/ml in 50 mM Tris-HCl buffer, pH 8.0) was treated at 25°C with the following compounds and the remaining activity was measured: 1 mM *o*-phenanthroline (○); 1.5 mM formamidesulfinic acid (●); 1 mM *o*-phenanthroline and 1.5 mM formamidesulfinic acid (□).

Instrumental Analyses

As described above, the enzyme's nonheme iron tightly bound to the polypeptide residues, and no decrease in iron content was observed after prolonged incubation with EDTA or *o*-phenanthroline. It was also found that the enzyme contained no acid-labile sulfide. In the CD spectra of native and reduced enzyme samples, there were no characteristic peaks corresponding to [2Fe-2S] and [4Fe-4S] clusters, which are common in nonheme iron proteins (Fig. 11) (63, 64). These spectra were rather similar to that of rubredoxin, whose nonheme iron forms

complexes with sulfur donor ligands of 4 cysteine residues (65).

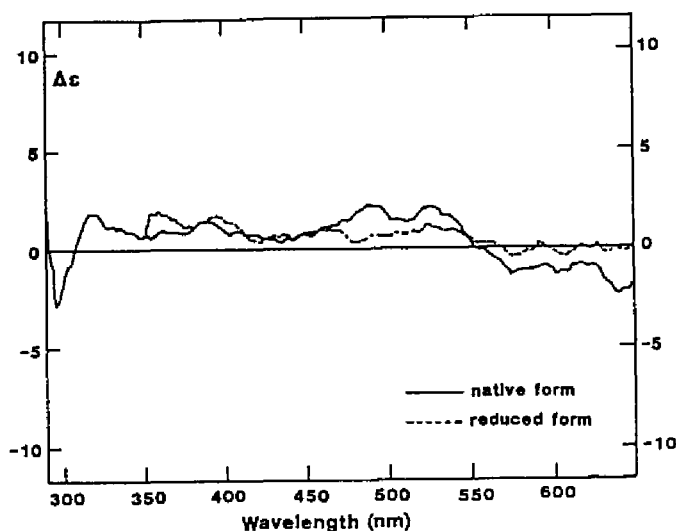


Fig. 11. CD spectra of native and reduced enzymes. The spectra were recorded in a 1-cm light path cuvette at room temperature. The sample solution contained 50 mM potassium phosphate buffer (pH 7.0) and 3.63 mg/ml (4.5 μ M) of enzyme. After recording the spectrum of the native enzyme, dithionite (ca. 3 mM) was added to the sample solution, and the spectrum of the reduced enzyme was again measured.

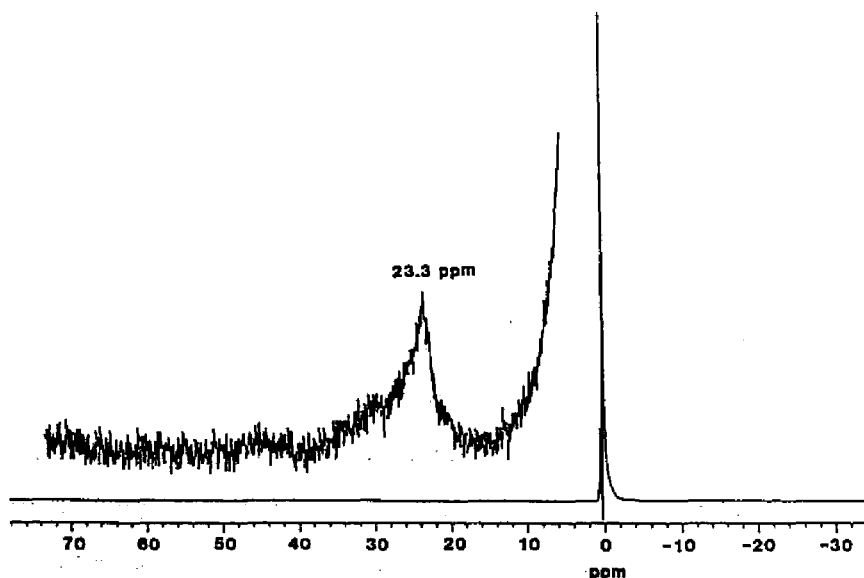


Fig. 12. Enzyme proton NMR spectrum. NMR spectrum was measured by use of a sample containing 5 mM potassium phosphate/ D_2O buffer (pH 7.0) and 11.4 mg/ml (14.1 μ M) of enzyme at 23°C.

As shown in Fig. 12, only a broad peak at 23.3 ppm, caused by protons in paramagnetic ferric ion field was observed in proton NMR spectrum of the enzyme. Further information concerning the ferric ion ligands could not be obtained from the proton NMR spectrum because of the enzyme's high molecular weight (790,000).

No ESR-active ferric ions were observed in native enzyme under the conditions tested (77K). Addition of 2 mM H_2O_2 , with or without 20 mM KBr to the enzyme caused no change in the ESR spectrum.

DISCUSSION

Coralline algae, which show high bromoperoxidase activity, are widely distributed from the tropical to polar seas in the world, and C. pilulifera is found in all coastal areas in Japan (Section 1). The bromoperoxidase of C. pilulifera comprised 1% of the total protein in the crude extract as calculated from the results in Table I.

The enzyme has been successfully purified from the crude extract of C. pilulifera by the combination of anion exchange chromatography and gel filtration 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

62,212 (amino acid composition), and the dodecad aggregate-image of the enzyme by electron microscope analysis. The molecular weight of 880,000 estimated by 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 (66, 67). From the image B of Fig. 3a, one hexagon probably faces the other hexagon at 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. 3b. 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 P. lamourouxii, 60,000 of bromoperoxidase from R. phoenix (25), and 42,000 (monomer) of chloroperoxidase of C. fumago (9). Pée and Lingens reported that the procaryotic bromoperoxidase from Pseudomonas aureofaciens is a dimeric enzyme with a molecular weight of 155,000-158,000 (29).

The optimum pH of the enzyme was 6.0. 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 (9), and bromoperoxidase of P. capitatus (26). Many peroxidases and chloroperoxidase of C.

fumago (9) are known to contain substantial levels of carbohydrate, but the enzyme had no carbohydrate residues.

Manthey et al. 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 ions (26). 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) (10). 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. 4). 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 (25). 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 anti-thyroid agents such as thiouracil and thiourea (68). The enzyme of C. fumago acts on these compounds to yield the disulfide. These products may arise via intervention of a sulphenyl halide ($-SX$, X:halide) (68). Therefore, the inhibition

mechanism of the enzyme reactions of C. pilulifera by these sulfhydryl compounds seems to identical to that of the enzyme of C. fumago by antithyroid agents.

The inhibition of enzymatic halogenation by fluoride ions 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 (26, 69), the enzyme of C. pilulifera exhibited much lower peroxidase activity and no catalase activity itself in the absence of halide ions. Pée and Lingens reported that procaryotic bromoperoxidase of P. aureofaciense has high catalase activity as well as peroxidase activity and low halogenating activity (29). 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 others heme iron as a prosthetic group.

The light brown of the concentrated enzyme solution was due to the ferric ions (Fe^{3+}) bound to the polypeptide residues of the enzyme. The enzyme rapidly lost its activity during reduction by formamidinesulfinic acid, but recovered it following subsequent oxidation by air (Fig. 8). This suggested that the valency state of nonheme iron of the enzyme was trivalent (Fe III). Tests on the enzyme's complex formation, using Tiron and o-phenanthroline supported this suggestion. Inability to detect ESR-active ferric ions was probably due to the rapid relaxation time at 77K of the enzyme's nonheme iron electrons.

Tiron is known to form chelated complexes with free ferric ions in a short time (64); Kojima et al. reported that pyrocatechase, an enzyme which contains Fe (III), lost its activity in about 60 min when incubated with Tiron (70). Thus, the slow rate of complex formation of bromoperoxidase with Tiron (Fig. 9) suggested that the enzyme's ferric ions were strongly bound to the polypeptide residues at the active site, which prohibited easy complex formation with metal chelating agents. That the enzyme did not lose its activity following prolonged incubation with EDTA or the treatment with 6 M urea for several hours is in accord with this proposal. The enzyme was found to contain no acid-labile sulfide, a finding which was supported by the analysis data of CD spectrum of the enzyme. The similarity of the CD spectra of the enzyme to ferric-rubredoxin implied the enzyme ligands properties that ferric ion directly bound to 4 cysteine residues of the enzyme. The results of amino acid analysis of the enzyme, which found 4 cysteine residues/enzyme subunit, are not in conflict with this speculation. It was unsuccessful to measure the enzyme's resonance Raman spectrum because of the obstacle fluorescence depending on small amount of red dye covalently bound to the enzyme. Thus, the complete ligands properties of the ferric ion of the enzyme have not yet been clarified. Other possible ferric ion ligands are tyrosine phenolate and histidine imidazole anions.

The role of magnesium of the enzyme is obscure. It was speculated that magnesium ions bind to carboxylic residues of

the enzyme and contribute to the conformational stabilization of the enzyme. Recently, Haschke et al. reported that calcium ions are needed for the conformational maintenance of horseradish peroxidase (71).

The enzyme isolated from C. pilulifera collected at Shirahama (Pacific Ocean, temperate sea) contained 2.3 ± 0.2 iron atoms and 1.6 ± 0.1 magnesium atoms/molecule of dodecameric enzyme. The enzyme purified from algae at a different collection site, Takahama (Japan Sea, temperate sea) was found to be differed from the Shirahama enzyme in its iron and magnesium contents (14 ± 0.5 iron and 0.7 ± 0.1 magnesium atoms/molecule of enzyme), but the other physicochemical and immunological properties (Section 1) were identical. It was speculated that the algal growing conditions, e. g., growing season, day-light, tide movement, mineral and nutrient compositions, and temperature of the sea water, probably affected the metal content of the enzyme, resulting in different enzyme activities (Table V). Seasonal changes in bromoperoxidase activity has been reported in Rhodomela latrix (24). Ferric ions was essential for the enzyme activity of C. pilulifera. However, the reason for the low specific activity of the Takahama enzyme in spite of the high ferric ion content is unclear (Table V). Compared with the Shirahama enzyme, the magnesium content of that from Takahama was low, so, magnesium ions may play a very important role in conformational maintenance of the enzyme, which in turn would affect its specific activity. Other factors resulting in low enzyme specific activity such as protease digestion of the enzyme toward the

end of the growing season are possibilities. The author have not yet checked the seasonal variations of algal bromoperoxidase activity at any one collection site.

The haloperoxidases of C. fumago, P. aureofaciense and some marine algae including Penicillius 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. The author proposes a classification of haloperoxidases into two groups; one is a heme type (H type) and the other is a nonheme type (NH type) haloperoxidase.

SUMMARY

Bromoperoxidase was purified from the crude extract of Corallina pilulifera to be homogeneous upon polyacrylamide disc gel and SDS-polyacrylamide gel electrophoreses. The enzyme had a molecular weight of approximately 790,000, and was composed of twelve subunits of identical molecular weights (Mr: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. The enzyme had no heme- or flavin-like compounds as a prosthetic group. Plasma emission

spectroscopy revealed that the enzyme contains iron and magnesium. The enzyme lost its activity when reduced with formamidinesulfinic acid, and recovered it when oxidized by air. Incubation of the enzyme with ferric or ferrous ion chelating agents indicated that its nonheme iron was ferric. Analyses of CD and proton NMR spectra suggested that the ferric ion tightly bound to cysteine, histidine or tyrosine residues of the enzyme. Hence, bromoperoxidase of Corallina pilulifera was distinct from other haloperoxidases and many peroxidases, which are hemoproteins.

Section 3. Halogenation Mechanism of Bromoperoxidase

It was found that the enzyme of Corallina pilulifera is a new type of haloperoxidase containing nonheme iron as a prosthetic group (NH type) (Section 2), instead of the usual heme iron (H type), which is seen in chloroperoxidase of Caldariomyces fumago (EC 1.11.1.10) (9, 10, 11), bromoperoxidases of Rhodomela (24), Ripocephalus (25), Penicillius (25, 26) and Pseudomonas (29), as well as in iodoperoxidase (EC 1.11.1.8) (72). Recently, another nonheme (NH type) bromoperoxidase has been reported in a brown alga, Ascophyllum nodosum, which contains vanadium as a prosthetic group (30, 31).

Halogenation by the H type chloroperoxidase of the fungus C. fumago, is caused by release of molecular halogen into the reaction mixture by the enzymatic reaction (Chapter I). The halogenation intermediate of the enzyme of C. pilulifera is a halonium cation (X^+), not a radical, because it produces 2,4,6-tribromophenol from phenol (Section 4). No catalase and only slight peroxidase activities exhibited by bromoperoxidase of C. pilulifera (Section 2), imply that its reaction mechanism is quite different from that of H type haloperoxidase. However, halogenation mechanism of NH type haloperoxidase has not yet been clear.

This section deals with the halogenation mechanism of nonheme bromoperoxidase of C. pilulifera, and clarifies the differences between the reaction mechanisms of heme and nonheme haloperoxidases.

MATERIALS AND METHODS

Chemicals NaOBr solution was prepared by dissolving 120 mg of Br₂ in 3 ml of 1N NaOH, followed by dilution to 100 ml with distilled water. Other reagents used in this section were all commercial products of analytical grade.

Collection of Algae and Enzyme Purification C. pilulifera was collected from shallow water (0.5-1.0 m deep) on the shores of Takahama (Fukui Prefecture), Japan, in April, 1985, and frozen at -20°C until use.

The enzyme was purified from crude extracts of C. pilulifera as described in Section 2.

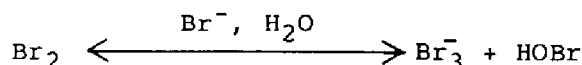
Enzyme Assay Bromoperoxidase, catalase and peroxidase activities were measured as described in Section 1. O₂ concentration in the reaction mixture was monitored with a Beckmann Fieldlab oxygen analyzer equipped with a Beckmann 39533 polarographic oxygen sensor as in the same manner described in Section 2. One unit of enzyme activity is equal to the amount of enzyme that converted 1 μmol of substrate in 1 min at 25°C.

RESULTS

Analysis of Brominating Intermediate of the Enzyme Reaction

Bromine is known to form a tribromide ion complex, which absorbs light in the ultraviolet region (72), and hypobromous

acid in an aqueous solution.



Libby *et al.* (11) reported the formation of molecular bromine from bromide ions through the chloroperoxidase reaction.

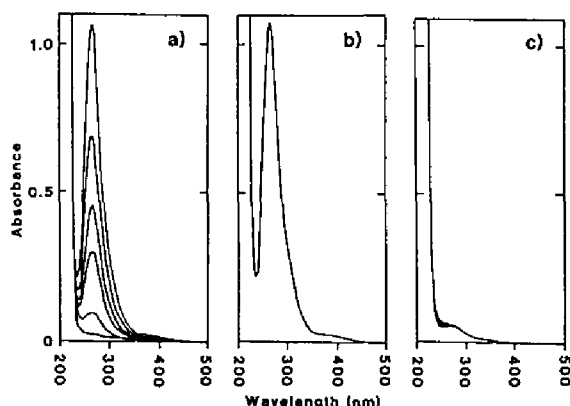


FIGURE 1. Comparison of the absorption spectra of (a) the chloroperoxidase- and (c) bromoperoxidase-generated products with that in the case of (b) molecular bromine in an aqueous solution. (a) The reaction mixture comprised 0.1 mmol of potassium phosphate buffer (pH 3.0), 0.1 mmol of KBr, 0.5 μmol of H_2O_2 , and 0.2 unit of chloroperoxidase in a total volume of 1.0 mL. The reaction was initiated by adding H_2O_2 , and the spectrum was measured at 2-min intervals at 25 $^\circ\text{C}$. (b) The solution comprised 0.1 mmol of potassium phosphate buffer (pH 3.0 and 6.0), 0.1 mmol of KBr, and 60 (pH 3.0) or 90 nmol of bromine (pH 6.0) in a total volume of 1.0 mL. (c) The reaction mixture comprised 0.1 mmol of potassium phosphate buffer (pH 6.0), 0.1 mmol of KBr, varying amounts of H_2O_2 , from 50 nmol to 1 μmol , and 0.2, 0.4, or 0.8 unit of bromoperoxidase in a total volume of 1.0 mL. The measurements were performed in the same manner as described in (a).

Figure 1 shows the ultraviolet spectra of the enzymatic products of chloroperoxidase and bromoperoxidase compared with those in the case of a bromine solution under the same conditions. In the case of chloroperoxidase, the formation of tribromide ions was obvious (Fig. 1a). For bromoperoxidase, the formation of tribromide ions was carefully investigated through measurements at different hydrogen peroxide concentrations with various enzyme concentrations from 0.2 to 0.8 units/ml of the mixture. But no appreciable generation of

tribromide ions in the reaction mixture was observed (Fig. 1c).

Stability of Enzyme under the Reaction Conditions.

Molecular halogens are very active chemical species. Therefore, if molecular halogen was released in the enzyme reaction mixture, it should cause damage to the enzyme itself as in the case of chloroperoxidase (Chapter I, Section 2). Figure 2 shows the remaining bromoperoxidase activity in the presence of halide ions and hydrogen peroxide without a halogen acceptor or on exposure to molecular bromine. Bromoperoxidase was stable for at least 3 hours' reaction.

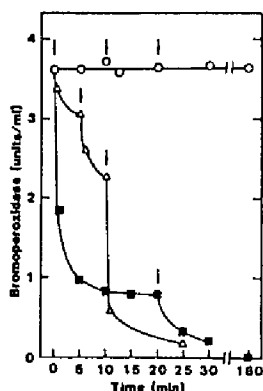


Fig. 2. Stability of bromoperoxidase in reaction mixture without a halogen acceptor and on exposure to molecular bromine. The bromoperoxidase (720 μ g) in 1.0 ml of 0.1 M potassium phosphate buffer (pH 6.0) was incubated with the following additives, and then the remaining activities were measured: (O) 20 μ mol of KBr and 20 μ mol of H_2O_2 (the arrows indicate the additions of 5, 5, and 10 μ mol of H_2O_2); (Δ) 3.0 μ mol of Br_2 (the arrows indicate the additions of 0.6, 1.2, and 1.2 μ mol of Br_2 , in that order); ($\blacktriangle$) 3.0 μ mol of Br_2 (the arrows indicate the additions of 2.4 and 0.6 μ mol of Br_2 , in that order).

It became apparent that molecular bromine irreversibly denatured the enzyme. That no bromoperoxidase activity was lost during the incubation with bromide ions and hydrogen peroxide suggested that the enzyme did not release active species such as molecular bromine into the solution.

Br⁻-dependent Catalase Reaction

Catalase activity of the enzyme was assayed by spectrophotometric measurement of the decrease in H_2O_2 concentration

at 240 nm, and oxygen electrode measurement of the formation of O_2 . The enzyme showed no catalase activity in the absence of bromide. However, in the presence of bromide it decomposed 2 mol of H_2O_2 to give 1 mol of O_2 , as shown in Table I.

Table I. Bromoperoxidase-catalyzed reactions of C. pilulifera

Br^- -dependent catalase activity was measured using an oxygen electrode at 25°C. The reaction mixture comprised 1.3 mmol potassium phosphate buffer (pH 6.0), 0.26 mmol KBr, 65 μ mol H_2O_2 , and 0.3 units of enzyme in a total volume of 13 ml. The reaction was started by adding enzyme. A control run was done without enzyme.

reaction	specific activity (μ mol/min/mg protein)	K_m (M)
catalase activity ($-Br^-$)	0	
Br^- -dependent catalase activity	10.5 (H_2O_2 decomposition)	1.0×10^{-2} (for KBr)
	5.1 (O_2 evolution)	1.1×10^{-4} (for H_2O_2)
bromination of monochlorodimedone	4.9	1.0×10^{-2} (for KBr)
	0 (O_2 evolution)	9.2×10^{-5} (for H_2O_2)
peroxidase activity (o-dianisidine)	0.0007	

In the presence of monochlorodimedone, a good acceptor of bromonium cations, no O_2 formation was observed. The specific activity of the Br^- -dependent catalase reaction was just twice that of the bromination of monochlorodimedone. The K_m values for KBr and H_2O_2 of both reactions were identical. Therefore, it was thought that the enzyme's Br^- -dependent catalase and bromination reactions would proceed by the same

mechanism.

Catalase Reaction in the Presence of Monochlorodimedone

The rate of O_2 formation from H_2O_2 was carefully measured at different monochlorodimedone concentrations (Fig. 3). It was found that O_2 evolution occurred only after the complete consumption of monochlorodimedone, indicating that monochlorodimedone was brominated preferably by the bromination intermediate (Br^+). After the consumption of monochlorodimedone, the bromination intermediate was then able to act on H_2O_2 .

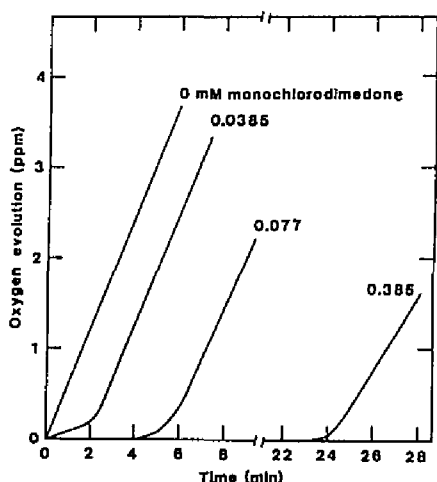


Fig. 3. Effect of monochlorodimedone on the Br^- -dependent catalase reaction of the enzyme. The evolution rate of O_2 was monitored in the same manner as described in Table I.

Comparison of the Enzyme Bromination Reaction with the NaOBr Reaction

NaOBr ($HOBr$) is known to oxidize H_2O_2 to yield O_2 as follows: $NaOBr + H_2O_2 \longrightarrow NaBr + O_2 + H_2O$ (73-75). Supposing that the reaction intermediate of bromoperoxidase were NaOBr ($HOBr$), and that it was released into the reaction mixture, then the enzymic and chemical reactions of NaOBr should show the same rate of O_2 evolution in the presence of several halogen acceptors. Bromoperoxidase of C. piluli-

fera catalyzes the bromination of not only monochlorodimedone but also of many other organic compounds, such as substituted phenols, heterocycles and substituted alkenes (Section 4). The author compared the O_2 formation rate of the enzyme reaction with that of the NaOBr reaction in the presence of either monochlorodimedone, phenol or cytosine. The rate of feeding NaOBr solution into the reaction mixture was adjusted until the O_2 formation rate was the same as in the enzymic reaction in the absence of halogen acceptor.

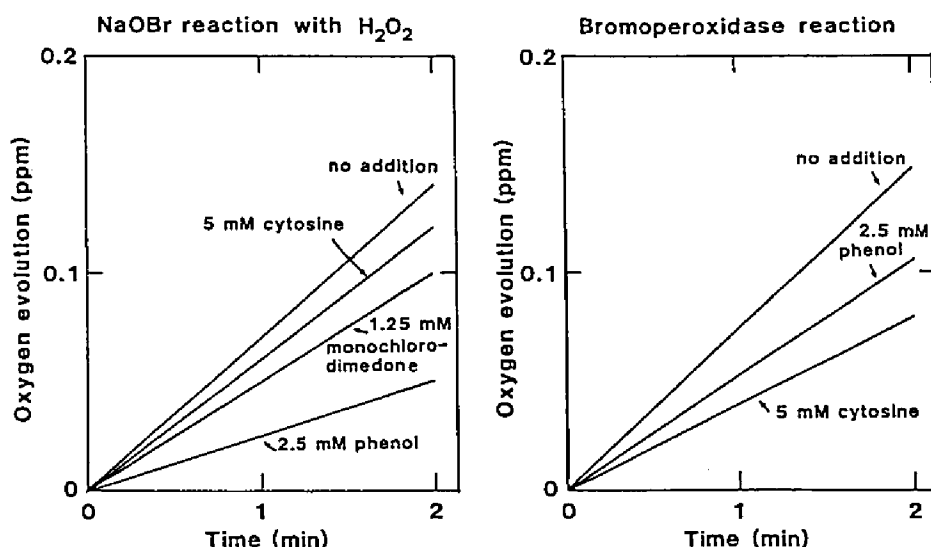


Fig. 4. Comparison of the O_2 evolution rates by enzymic and NaOBr reactions in the presence of some halogen acceptors. The enzyme reaction was done as described in Table I. In the case of NaOBr reaction, NaOBr solution was injected into the reaction mixture without enzyme at a flow rate of 67 μ l/min.

As shown in Fig. 4, in the NaOBr reaction, O_2 formation was observed at high concentrations of monochlorodimedone (1.25 mM), a result completely different from that obtained with the enzymic reaction (Fig. 3). The rates of O_2 formation in the enzymic reaction in the presence of phenol or cytosine also differed from those of the NaOBr reaction. Thus, it was

obvious that the enzyme possessed some affinity for monochlorodimedone and cytosine. These data suggested that bromination reaction of the enzyme was not due to the release of NaOBr or molecular bromine into the reaction mixture, but to the active site bromination.

Kinetics of O_2 Formation by the Enzyme Reaction in the Presence of Phenol

The effects of the presence of the halogen acceptor, phenol, on the kinetics of O_2 formation were examined in detail (Fig. 5). The kinetic data indicated that phenol's inhibition of O_2 formation was competitive, with a K_i value calculated from the plot data to be 3.8×10^{-5} M. The results revealed that H_2O_2 and phenol competitively reacted with the bromination intermediate (Br^+OH^-) formed at the enzyme's active site.

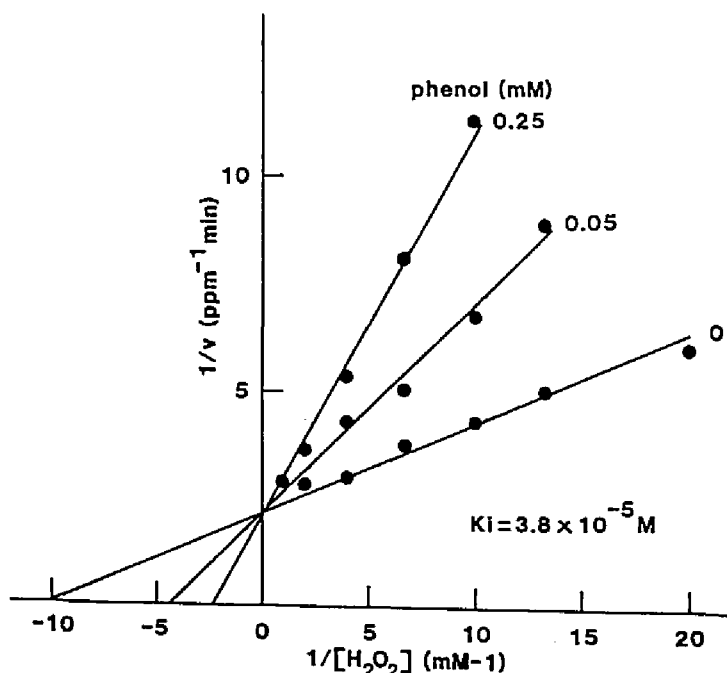


Fig. 5. Double-reciprocal plots of O_2 formation rate against H_2O_2 concentration in the presence of various concentrations of phenol. The reaction was performed as described in Table I.

DISCUSSION

Bromoperoxidase of C. pilulifera catalyzes the peroxidative oxidation of halide ions (X^-) to yield halonium cations (X^+). On this point, C. pilulifera nonheme bromoperoxidase halogenation is the same to those of usual heme haloperoxidases.

Iodoperoxidase and chloroperoxidase (H type) are known to show the halide-dependent catalase activity yielding singlet oxygen, 1O_2 (73-76), but they show catalatic activities even in the absence of halide. Bromoperoxidase of C. pilulifera catalyzed an absolutely Br^- -dependent catalatic reaction. The chemical equivalence of H_2O_2 consumption and O_2 formation in the Br^- -dependent catalase reaction suggested that one molecule of H_2O_2 was used in the oxidation of Br^- to Br^+ , the latter then reacting with another molecule of H_2O_2 to yield one molecule of O_2 . This proposal is based on the observations that the halogenation intermediate of C. pilulifera bromoperoxidase was a halonium cation (X^+), and that hypobromous acid (Br^+OH^-) catalyzed the oxidation of H_2O_2 to O_2 . However, in the presence of the halogen acceptor, monochlorodimedone, the active intermediate (Br^+OH^-) was used only in the bromination of that, and no detectable O_2 was produced (Table I and Fig. 3). In addition, the bromination rate of monochlorodimedone was the same as that of O_2 formation in Br^- -dependent catalase reaction. Therefore, it was concluded that the enzymic bromination proceeded as shown in the formulae in Fig. 6.

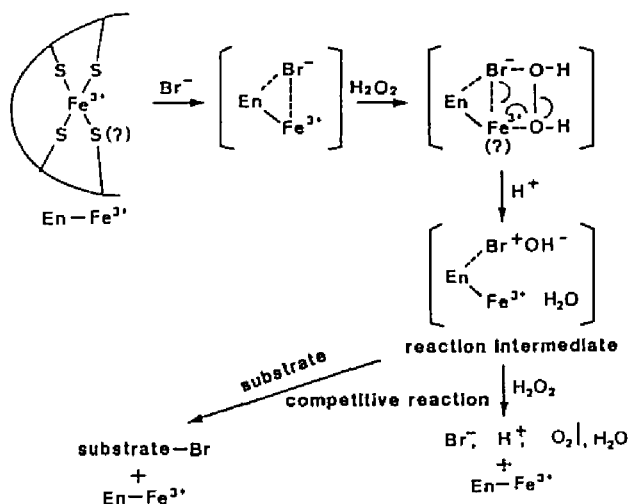
On the other hand, the differences in the O_2 formation rates between the HOBr chemical and enzymatic reactions in the

presence of halogen acceptors, showed that the bromoperoxidase-catalyzed bromination occurred at the enzyme's active site, and was not dependent on release of molecular bromine or HOBr into the reaction mixture. The result was in fair agreement with the finding that no formation of tribromide ions was observed in the reaction mixture (Fig.1). On this point, C. pilulifera nonheme bromoperoxidase halogenation is quite different from the usual heme haloperoxidases. The results in Fig. 4 also showed that the enzyme had some affinity toward the halogen acceptors, resulting in different rates of bromination from those shown by the NaOBr reaction.

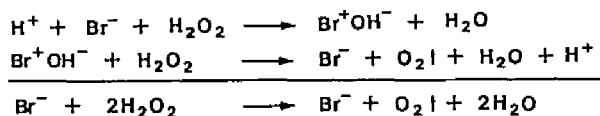
As shown in Fig. 5, O_2 formation by bromoperoxidase catalytic reaction was competitively inhibited by phenol, indicating that both phenol and H_2O_2 reacted on the same bromination intermediate. Lack of formation of O_2 in the presence of monochlorodimedone (Fig. 4) was probably due to a high enzyme affinity toward it. In Section 2, the author showed that inhibition of monochlorodimedone bromination by fluoride ions was uncompetitive over a concentration range of fluoride ions from 1.25 to 5.0 mM. This phenomenon can easily be understood from the enzyme's reaction mechanism. Fluoride ions probably bind to the Br^+OH^- intermediate to give BrF_3 . The reactivity of BrF_3 as an electrophile is lower than that of Br^+OH^- because of its high binding energy.

The reaction mechanism of nonheme (NH type) bromoperoxidase of C. pilulifera is summarized in Fig. 6. The differences between the halogenation mechanisms of heme (Chapter I, Section

2) and nonheme haloperoxidases became clear.



Bromide-dependent catalase reaction



Bromination reaction



Fig. 6. Reaction mechanism of bromoperoxidase of C. pilulifera.

The characteristic of the bromoperoxidase reaction in terms of no catalase reaction, minimal peroxidase activity and stability under the reaction conditions (Fig. 2) are clearly explained by its reaction mechanism.

SUMMARY

The halogenation mechanism of nonheme bromoperoxidase of Corallina pilulifera was studied. The enzyme catalyzed

Br^- -dependent catalase reactions to yield one mol of O_2 from 2 mols of H_2O_2 . No O_2 evolution was observed when bromination reaction of monochlorodimedone occurred. From these results, together with other knowledge of this enzyme (Section 2, 4), it was concluded that it activated bromide anion (Br^-) to bromonium cation (Br^+) using one molecule of H_2O_2 , and this Br^+OH^- formed at the active site then decomposed another H_2O_2 to yield O_2 in the absence of halogen acceptors (substrate). When substrate was present in the reaction mixture, it and H_2O_2 competitively reacted with the reaction intermediate (Br^+OH^-) to give brominated products. This mechanism could reasonably explain the characteristic reactions catalyzed by nonheme bromoperoxidase of C. pilulifera.

Section 4. Substrate Specificity and Regio- and Stereospecificities of Bromoperoxidase-catalyzed Halogenation

In previous sections, the author described the distribution (Section 1), enzymatic properties (Section 2), and reaction mechanism (Section 3) of bromoperoxidase derived from coralline alga (Corallinaceae, Rhodophyta). The bromoperoxidase of Corallina pilulifera is clearly distinguishable from the usual haloperoxidases because it has nonheme iron as a prosthetic group (Section 2). In addition, the enzyme was found to be stable under normal reaction conditions (Section 3), thus, making it suitable for use in halogenation processes.

This section describes the substrates and the regio- and stereospecificities of this enzyme's halogenation reaction. From the results, the author also discusses its ability as a halogenating agent and its biological roles in algae.

MATERIALS AND METHODS

Chemicals o-Hydroxybenzyl alcohol, 1-methoxynaphthalene, 2-bromothiophene and 3-bromothiophene were purchased from Aldrich Chemical Co. Inc., U. S. A. Acetylene dicarboxylic acid and styrene were supplied by Tokyo Kasei Co., Japan, and p-bromoanisole, acrylic acid, trans-crotonic acid, trans-cinnamyl alcohol and cyclohexene by Nakarai Chemicals Ltd., Japan. Anisole, o-bromoanisole and β -bromostyrene were purchased from Wako Pure Chemical Industries Ltd., Japan. Thin layer

chromatoplates (TLC plates; Silica Gel 60F₂₅₄) were obtained from Merck Japan Ltd., and Sephadex LH-20 from Pharmacia Fine Chemicals, Sweden, and Amberlite XAD-2 from Rohm & Hass Corp. cis-Propenylphosphonic acid was synthesized according to the methods of Glamkowski et al.(77). All other reagents used were of analytical grade.

Preparation of Bromoperoxidase Bromoperoxidase was obtained from stock samples of Corallina pilulifera collected on the coast at Takahama (Fukui Prefecture, Japan) in October, 1985, as described in Section 1. Unless otherwise indicated, partially purified enzyme produced by DEAE-Sephadex chromatography was used throughout the experiments.

Enzyme Assay Bromoperoxidase activity was assayed spectrophotometrically as described in Section 1.

Enzymatic Reaction The reaction mixture comprised 1 mmol potassium phosphate buffer (pH 6.0), 0.4 mmol KBr or KI, 0.2 mmol (final concentration) hydrogen peroxide, 0.1 mmol of each substrate (0.05 mmol for trans-cinnamic acid) and 12.5 units of bromoperoxidase in a total volume of 10 ml in a 30-ml flask. The reaction was started by addition of 0.1 mmol H₂O₂ at 1-h intervals and was allowed to proceed for 3 h at 30°C. Insoluble substrates such as styrene, cyclohexene, 1-methoxynaphthalene were previously suspended in the reaction mixture by ultrasonication. In all cases, a control experiment was done without enzyme.

Reaction Mixture Analysis After reaction, decrease in substrate and formation of product were analyzed by gas chromatography (GC), high performance liquid chromatography (HPLC) or silica gel thin layer chromatography (TLC), depending on the nature of the substrate.

For volatile substrates, aliquots of the reaction mixture (2 μ l) were subjected to GC on a coiled column (2.0 x 3 mm or 1.0 x 3 mm) packed with Silicone OV-1 (3%, 80-100 mesh) or Porapak PS (80-100 mesh). As a carrier, nitrogen gas was used at a flow rate of 50 ml/min.

HPLC was performed with a Shimadzu LC-5A system equipped with an M & S pack C18 column (reversed phase column, 4.6 x 150 mm, M & S Instruments Inc., Japan) at a flow rate of 1.0 ml/min using the following solvent systems: a) 0.2 M potassium phosphate buffer (pH 6.0):methanol at a 19:1 ratio (by volume), b) 0.2 M potassium phosphate buffer (pH 6.0):methanol; 9:1, c) 0.2 M potassium phosphate buffer (pH 6.0):methanol; 4:1, d) 50 mM potassium phosphate buffer (pH 6.0):methanol; 1:1, e) 25 mM potassium phosphate buffer (pH 6.0):methanol; 3:7, f) 50 mM phosphoric acid, and g) 0.01 N HCl.

TLC was performed with one of the solvent systems described in Table I, after applying the reaction mixture (10-20 μ l) to the TLC plate. The product and substrate were detected on the chromatogram by UV light, or by spraying sulfuric acid (5% or concentrated) or 5% phosphomolybdic acid (in methanol) followed by heating for several minutes, or by spraying 5% KMnO_4 .

The detailed analysis conditions for each substrate and

product are summarized in Table I.

Table I. Analysis of substrates and products

substrate	HPLC wavelength (nm)	solvent	R _t		column length (m)	GC temperature (°C)		R _t		TLC solvent	detection	R _f	
			sub. a	pro. b		inj. c	col. d	sub. e	pro. f			sub. g	pro. h
acetylene dicarboxylic acid	230	g	2.2	-	-	-	-	-	-	90% MeOH	UV or KMnO ₄ (5%)	0.58	-
acrylic acid	205	c	3.6	-	Porapak PS (2)	240	200	2.2	-	-	-	-	-
anisole	225	e	5.3	7.6 (g-form) 10.9 (p-form)	Silicone OV-1 (1)	180	80	2.4	15.1 (g-form) 14.1 (p-form)	benzene: hexane (1:1)	UV or sulfuric acid	-	0.71 (p-form)
trans-cinnamyl alcohol	-	-	-	-	Silicone OV-1 (1)	240	140	2.3	6.2	hexane: ethyl acetate (1:1)	UV or sulfuric acid	0.58	0.43
trans-cinnamic acid	220	d (nonvolatile product)	15.1	11.2	Silicone OV-1 (1)	140	110	-	3.5	-	-	-	-
trans-crotonic acid	205	b	2.7	-	Porapak PS (2)	250	210	2.2	-	-	-	-	-
cyclohexene	-	-	-	-	Silicone OV-1 (1)	200	95	-	2.2	benzene: ethyl acetate (7:3)	phospho- molybdic acid (5% in EtOH)	-	0.7
1-methoxy-naphthalene	-	-	-	-	Silicone OV-1 (1)	190	150	2.0	6.9	hexane: benzene (3:1)	UV or sulfuric acid	0.5	0.59
phenyl acetylene	-	-	-	-	Silicone OV-1 (2)	240	120	0.8	7.0	-	-	-	-
propargyl alcohol	205	a	3.2	-	-	-	-	-	-	-	-	-	-
cis-propenyl-phosphonic acid	210	g	3.3	2.9	-	-	-	-	-	-	-	-	-
pyrrole	230	d	5.0	2.3 2.7 25.3 26.0	-	-	-	-	-	-	-	-	-
styrene	-	-	-	-	Silicone OV-1 (1)	240	110	1.2	5.2	hexane: ethyl acetate (7:1)	UV or sulfuric acid	-	-
thiazole	230	d	5.9	-	-	-	-	-	-	-	-	-	0.65
thiophene	-	-	-	-	Silicone OV-1 (2)	160	80	-	2.5 (2-bromo- form) 2.8 (3-bromo- form)	-	-	-	-

monochloro- dimedone	290	c	5.9	-	Silicone 200 OV-1 (1)	200	120→200 3.8 (4°C/min)	4.8 (main) 6.8 8.8	ethyl acetate: nitro- benzene: phenyl- ethanol hydrazine (7:3:1)	0.5	0.65 (main)
phenol		-				-			benzene: n-hexane (7:3)	0.14	0.68
p-hydroxy- benzyl alcohol		-				-			benzene	0.10	0.80

^a substrate, ^b product, ^c injection temperature, ^d column temperature, ^e The substrate and product were extracted with the same volume of ethyl acetate, and applied to a TLC plate. After developing the TLC plate with solvent, 0.05% 2,4-dinitrophenylhydrazine in 50% ethanol and 50% 2N HCl were sprayed onto the chromatogram. After drying the plate, 6% NaOH in 5% methanol were sprayed onto it, and spots detected.

Table II. Large-scale reaction conditions for the isolation of products

compound	contents of reaction mixture ^a				total volume (ml)
	substrate (mmol)	bromoperoxidase (units)	H ₂ O ₂ (mmol)	KBr (mmol)	
phenol	2	100	2	4	100
p-hydroxybenzyl alcohol	2	100	2	4	100
anisole	2	200	6	8	200
1-methoxy- naphthalene	1	150	6	8	200
cyclohexene	2	200	8	8	200
styrene	3.3	150	9	12	300
trans-cinnamyl alcohol	1	50	3	4	100
trans-cinnamic acid	1.5	200	12	12	300
cis-propenyl- phosphonic acid	2	50	4	4	100

^a Throughout the experiments, 0.1 M potassium phosphate buffer (pH 6.0) was used. Each half of the indicated amount of hydrogen peroxide was added to the reaction mixture at 1-h intervals, and the mixture was kept at 30°C for 3 h.

Instrumental Analysis of the Products ^1H NMR spectra were measured with a JEOL-FX 100 (100 MHz) spectrometer with tetramethylsilane as a reference. Gas chromatography-mass spectrometry (GC-MS) was performed using a glass column (1 m x 4 mm) packed with Silicone OV-1 (3%, 80-100 mesh) at 20 eV with a Hitachi M-80 mass spectrometer. In-beam electron impact-mass spectrometry (EI-MS) was carried out at 20 eV with the same instrument. Infrared spectra were measured with a Shimadzu IR-27G spectrometer. Optical rotation was measured with a Perkin-Elmer 241 MC polarimeter.

Isolation of Products The large-scale reaction conditions for each substrate are shown in Table II. After reaction, each product was purified to homogeneity by the following procedures.

1. Phenol substrate The product was extracted twice with 150 ml of ethyl acetate each time from the reaction mixture. The combined ethyl acetate layer was dried with Na_2SO_4 and evaporated. The product was subjected to the silica gel chromatography (1 x 70 cm column) and eluted with benzene-ethyl acetate (9:1). The product was further purified by TLC using benzene. The product was extracted from the spot band ($R_f=0.82$) with ethyl acetate, concentrated under reduced pressure and rechromatographed by TLC with benzene-*n*-hexane (7:3). The ethyl acetate extract from the spot band ($R_f=0.68$) provided 50 mg of a faint yellowish powder: mp 86-87°C, ^1H NMR (CDCl_3) δ 5.88 (1H, s, -OH) 7.58 (2H, s, ArH), EI-MS m/z (M^+ 334, 332, 330, 328) 253, 251, 249, 224, 222, 220, 176,

174, 143, 141.

2. o-Hydroxybenzyl alcohol substrate The product was extracted twice with 150 ml of ethyl acetate each time. The ethyl acetate layer was dried with Na_2SO_4 , evaporated, and the product was subjected to silica gel chromatography (1 x 70 cm column) using benzene-ethyl acetate-methanol (4:1:1) as eluent. The product was put on TLC plates and developed with benzene, and then extracted with ethyl acetate from the spot band ($R_f=0.8$). The product was rechromatographed by TLC under the same conditions. By this method, 53 mg of faint yellowish powder was obtained: mp 86-87°C, $^1\text{HNMR}$ (CDCl_3) δ 5.88 (1H, s, -OH) 7.58 (2H, s, ArH), EI-MS m/z (M^+ 334, 332, 330, 328) 253, 251, 249, 224, 222, 220, 176, 174, 143, 141.

3. Anisole substrate The product was extracted twice with ethyl acetate from the reaction mixture. The combined extracts were dried with Na_2SO_4 , and then evaporated. The product was subjected to silica gel chromatography (2.2 x 77 cm) and was eluted with benzene-hexane (1:1). Evaporation of the fractions gave 11 mg of product as a clear oil: $^1\text{HNMR}$ (CDCl_3) δ 3.78 (3H, s, $-\text{OCH}_3$) 6.78 (2H, m, ArH) 7.38 (2H, m, ArH), GC-MS m/z (M^+ 188, 186) 173, 171, 145, 143, 141, 131, 119.

4. 1-Methoxynaphthalene substrate The product extracted from the reaction mixture with ethyl acetate was evaporated to dryness. The sample was redissolved in methanol and

subjected to Sephadex LH-20 column (2.2 x 70 cm) chromatography and was eluted with methanol. The product was further purified by silica gel column (1.1 x 75 cm) chromatography using benzene-hexane (1:3) as eluent. The fractions were concentrated by evaporation to yield 53 mg of purified product as a clear oil: $^1\text{H NMR}$ (CDCl_3) δ 3.99 (3H, s, $-\text{OCH}_3$) 6.88 (1H, d, $J=7.9$) 7.4-7.7 (2H, m, ArH) 7.66 (1H, d, $J=7.9$) 8.1-8.4 (2H, m, ArH), GC-MS m/z (M^+ 238, 236) 223, 221, 195, 193, 126, 114.

5. Cyclohexene substrate Following extraction of the product with ethyl acetate from the reaction mixture, it was purified by silica gel column (1.1 x 75 cm) chromatography using hexane-ethyl acetate (7:3) as eluent. The fractions were collected and evaporated to dryness, providing 37 mg of product as a clear oil: $^1\text{H NMR}$ (CDCl_3) δ 1.1-2.5 (8H, m) 2.54 (1H, brs, $-\text{OH}$) 3.59 (1H, m, H_{1a}) 3.90 (1H, ddd, $J_{1a,2a}=9.7$, $J_{2a,3a}=11.2$ and $J_{2a,3e}=4.4$, H_{2a}), GC-MS m/z (M^+ 180, 178) 134, 132, 99, 81, 59.

6. Styrene substrate The product was extracted with ethyl acetate and the volume of the extracts was then reduced by evaporation. The sample was applied to a silica gel column (2.2 x 75 cm) and was eluted with hexane-ethyl acetate (7:3). The fractions were combined and evaporated to give 279 mg of product as a faint brownish oil: $[\alpha]_D^{23}=0^\circ$ ($c=2$, CH_3OH), $^1\text{H NMR}$ (CDCl_3) δ 2.65 (1H, d, $-\text{OH}$) 3.4-3.7 (2H, m) 4.8-5.0 (1H, m) 7.37 (5H, brs, ArH), GC-MS m/z (M^+ 202, 200) 185, 183, 120,

7. trans-Cinnamyl alcohol substrate After reaction, the reaction mixture was directly subjected to Amberlite XAD-2 column (3.2 x 35 cm) chromatography. The product was eluted in a stepwise manner with 25, 50, 75 and 100% methanol solutions, and the fractions were then pooled and concentrated by evaporation. The product was purified by preparative HPLC on a YMC D-ODS-5 column (reversed phase column, 20 x 250 mm, Yamamura Chemical Lab. Co., Japan) using 35% methanol as solvent. The combined fractions were evaporated to dryness, which provided 150 mg of an oily white product: $[\alpha]_D^{23}=0^\circ$ (c=2, CH₃OH), ¹HNMR (CDCl₃) δ 2.5 (1H, brs, -OH) 3.0 (1H, brs, -OH) 3.8-4.0 (2H, m) 4.2-4.4 (1H, m) 5.05 (1H, d, $J=5.9$) 7.39 (5H, brs, ArH), EI-MS m/z (M⁺ 232,230) 214, 184, 182, 150, 133, 107.

8. trans-Cinnamic acid substrate After reaction, the solution was adjusted to pH 3.0 with 6N HCl, and was then applied to an Amberlite XAD-2 column (3.2 x 40 cm). After washing the column with water, the product was eluted in a stepwise manner with 20, 40, 60 and 80% methanol solutions. Following evaporation of the fractions, the product was further purified by silica gel column (2.2 x 70 cm) chromatography using ethyl acetate-ethanol (4:1) as eluent. The combined fractions were evaporated to dryness under a vacuum. By these procedures, 40 mg of product were obtained as a white powder: $[\alpha]_D^{23}=0^\circ$ (c=2, CH₃OH), ¹HNMR (D₂O) δ 4.48 (1H, d, $J=7.8$) 5.06 (1H, d, $J=7.8$) 7.4-7.5 (5H, m, ArH).

9. cis-Propenylphosphonic acid substrate Following adjustment of the reaction mixture to pH 3.0 with 6N HCl, the volume of the solution was reduced by evaporation. The sample was then applied to a Dowex 50x8 (H⁺ form) column (2.5 x 30 cm), from which it was eluted with water. The column fractions were pooled and reduced by evaporation. The product was purified by preparative HPLC on the same column as described above using 0.01 N HCl as solvent. Evaporation of the combined fractions to dryness provided 120 mg of product as a white syrup: $[\alpha]_D^{23}=0^\circ$ (c=2, CH₃OH), ¹HNMR (DMSO-d₆) δ 1.20 (3H, d, J=6.4, -CH₃) 3.88 (1H, dd, J=12.2, 3.4) 3.8-4.1 (1H, m), EI-MS m/z (M⁺-15 205, 203) 176, 174, 156, 158, 149, 123, 122, 121, 120, 105, 82, 80, 57.

10. Pyrazole substrate After incubation, the mixture was applied on an Amberlite XAD-2 (3.2 x 30 cm) column, and the column was washed with water. The product was eluted in a stepwise manner with 50%, 75% and 100% methanol solutions. The fractions containing the product were collected and concentrated by evaporation. The product was further purified by Sephadex LH-20 column chromatography (1.6 x 35 cm) with 20% methanol as eluent. Evaporation of the fractions gave 10 mg of a white product: ¹HNMR (CDCl₃) δ 7.60 (2H, s, 3- and 5-H), GC-MS m/z (M⁺ 148, 146) 121, 119, 94, 92.

RESULTS

Identification of Products

1. Phenol The EI-MS spectra of the product had molecular ion peaks split into a 1:3:3:1 distribution due to the isotopes of three bromine atoms. The NMR spectrum and melting point (lit. 87-89°C) were identical with authentic 2,4,6-tribromophenol. Hence, the product was identified as 2,4,6-tribromophenol.

2. o-Hydroxybenzyl alcohol The melting point, NMR spectrum and EI-MS spectrum were identical with the product from phenol and 2,4,6-tribromophenol. Hence, the product was identified as 2,4,6-tribromophenol. It indicated that the hydroxymethyl group was also replaced by bromine in the enzyme reaction.

3. Anisole The main product from anisole showed the same retention times as authentic p-bromoanisole on GC and HPLC. The NMR and GC-MS spectra confirmed this identification. From the GC and HPLC analyses, the minor product was decided to be o-bromoanisole. The o-/p- ratio of the products is detailed in the following section.

4. 1-Methoxynaphthalene GC-MS analysis of the product showed bromonaphthalene molecular ion peaks (m/z , 238, 236) split into a 1:1 distribution by the bromine isotopes of m/z 81 and 79 (Fig. 1a). The NMR spectrum disclosed three singlet protons (δ = 3.99) for $-OCH_3$, and six protons belonging to a naphthalene ring (δ = 6.88, 7.4-7.7, 7.66 and 8.1-8.8). The coupling of H_2 - and H_3 -protons (J = 7.9) of the naphthalene ring indicated that the hydrogen at 4-position was replaced by bromine. From these data, the product was identified as 1-methoxy-4-bromonaphthalene.

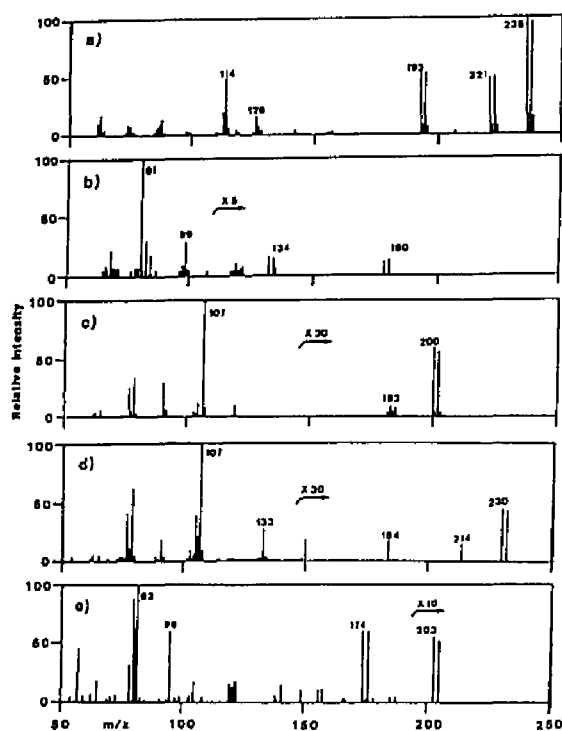


Fig. 1. Mass spectra of (a) 1-methoxy-4-bromonaphthalene (GC-MS), (b) trans-1-hydroxy-2-bromocyclohexane (GC-MS), (c) DL-1-bromo-2-hydroxy-2-phenylethane (GC-MS), (d) (±)-1,3-dihydroxy-2-bromo-3-phenylpropane (GC-MS), and (e) (±)-threo-1-bromo-2-hydroxypropylphosphonic acid (EI-MS).

5. Cyclohexene Analysis of the product by GC-MS suggested that the product was a bromohydrin compound of cyclohexene (m/z M^+ 180, 178) (Fig. 1b). The NMR spectrum in $CDCl_3$ showed one singlet proton ($\delta=2.54$) for -OH, eight methylene protons ($\delta=1.1-2.5$), and two methine protons ($\delta=3.59$ and 3.90). Detailed analysis of the two methine protons indicated that a H_2 -proton coupled with two axial and one equatorial protons (Fig. 2) because the coupling constants (J value) of the H_2 -proton were 9.7, 11.2 (for axial and axial) and 4.4 (for axial and equatorial). Thus, the bromohydrin derivative of cyclohexene was in the trans-form, and was decided to be trans-1-hydroxy-2-bromocyclohexane.

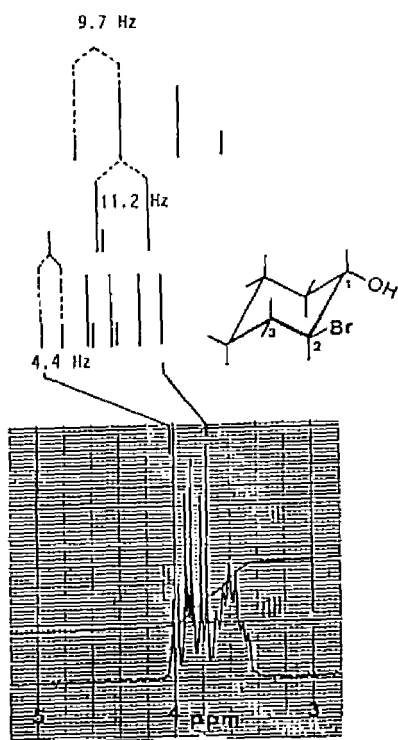


Fig. 2. Analysis of NMR spectrum of trans-1-hydroxy-2-bromocyclohexane. The other part of the spectrum ($\delta=0-3$, 5-10 ppm) was omitted.

6. Styrene The GC-MS spectrum of the product showed molecular ion peaks at m/z 202 and 200, corresponding to a styrene bromohydrin product (Fig. 1c). The NMR spectrum in $CDCl_3$ exhibited one -OH proton ($\delta=2.65$), two methylene protons ($\delta=3.4-3.7$), one methine proton ($\delta=4.8-5.0$) and five protons of a benzene ring. The analysis data also disclosed that the bromine had bound to the β -position, and the -OH group to the α -position of styrene. The optical rotation of the product was 0° .

Thus, the product was decided

to be DL-1-bromo-2-hydroxy-2-phenylethane.

7. trans-Cinnamyl alcohol In the EI-MS spectrum of the product (Fig. 1d), molecular ion peaks of m/z 232 and 230, corresponding to a bromohydrin product of trans-cinnamyl alcohol were observed. Analysis of the product's NMR spectrum indicated the existence of two -OH protons ($\delta=2.5$ and 3.0), two methylene protons ($\delta=3.8-4.0$), two methine protons ($\delta=4.2-4.4$ and 5.05) and five protons of a benzene ring. The data also suggested that -OH had bound to the adjacent carbon of the benzene ring. The optical rotation of the product was 0° .

Therefore, the product was identified as ($\pm$)-1,3-dihydroxy-2-bromo-3-phenylpropane.

8. trans-Cinnamic acid trans-Cinnamic acid was converted into two products, one volatile, the other nonvolatile. The volatile product was directly analyzed in the reaction mixture by GC and GC-MS, and found to be trans- β -bromostyrene. The NMR spectrum of the isolated nonvolatile product was identical to that of ($\pm$)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid. Enzymatic bromination of trans-cinnamic acid by the bromoperoxidase was the same as the reaction by the chloroperoxidase of C. fumago as described in Chapter I, Section 1.

9. cis-Propenylphosphonic acid The EI-MS spectrum of the product exhibited no molecular ion peaks, corresponding to a bromohydrin product of the substrate. But analysis of fragment ion peaks indicated the existence of m/z 205 and 203; $M^+ - CH_3$ and m/z 176 and 174; $M^+ - CH_3 \cdot COH$ (Fig. 1e). These fragment ion peaks were clearly caused by 1-bromo-2-hydroxypropylphosphonic acid. The NMR spectrum showed three methyl protons ($\delta=6.4$) and two methine protons ($\delta=3.88$ and $3.8-4.1$), thus supporting the above identification. Kollonitsch et al. reported that threo-halohydrin was obtained as racemates from cis-propenylphosphonic acid by the reaction catalyzed by chloroperoxidase of C. fumago (36). The retention time for the product on HPLC was identical to that formed by chloroperoxidase bromination, and its optical rotation was 0° . Thus, the product was found to be ($\pm$)-threo-1-bromo-2-hydroxypropylphosphonic acid.

10. Pyrazole GC-MS analysis of the product showed molecular ion peaks at m/z 148 and 146 split into a 1:1 distribution, that corresponded to bromopyrazole. The single peak at 7.60 ppm and the lack of spin-spin coupling in the NMR spectrum indicated that the 4-position hydrogen of pyrazole was substituted by bromine. Therefore, the product was identified as 4-bromopyrazole.

The iodinated products obtained through the enzyme reactions was found to be completely identical with standard 4-iodopyrazole on HPLC analysis.

11. Cytosine The product obtained through the bromoperoxidase reaction was completely identical with the product of the chloroperoxidase reaction (Chapter I, Section 2), and was identified as 5-bromocytosine.

12. Uracil The brominated product of the reaction was the same as that of the chloroperoxidase reaction (Chapter I, Section 2). Therefore, it was decided to be 5-bromouracil.

The iodinated compound formed through the bromoperoxidase reaction was found to be completely identical with authentic 5-iodouracil on HPLC and TLC analyses.

13. Cytidine The product obtained through the bromoperoxidase reaction was the same as that in the case of the chloroperoxidase reaction and an authentic 5-bromocytidine (Chapter I, Section 2).

14. Thiophene The reaction mixture containing the product derived from thiophene was directly analyzed by GC using authentic compounds, and it was identified as 2-bromothiophene. GC-MS analysis showed molecular ion peaks at m/z 164 and 162,

and a fragment ion peak at m/z 83; M^+-Br , confirming the identification.

15. Monochlorodimedone Monochlorodimedone is a most suitable substrate for measurement of the activity of C. pilulifera bromoperoxidase (Section 3). Hager et al. reported that the major product of chloroperoxidase bromination of monochlorodimedone was monobromomonochlorodimedone (10). Due to the similarity of the bromination reactions catalyzed by bromoperoxidase and chloroperoxidase, both producing bromonium cations by peroxidative oxidation of bromide anions, the product of the bromoperoxidase reaction with monochlorodimedone was presumed to be monobromomonochlorodimedone. However, the author observed by both TLC and GC-MS the formation of different products (Fig. 3). Comparison by GC-MS of the fragment ion peaks of the main product (Fig. 3b-2) with authentic dimedone (Fig. 3b-3) suggested that the product contained no halogen atoms. Monochlorodimedone showed molecular ion peaks (m/z 176, 174) split into a 1:3 distribution due to the different isotopes of the chlorine atom (m/z 37, 35). This implied that in the bromoperoxidase reaction the product was initially converted into monobromomonochlorodimedone, but that spontaneous elimination of halogen atoms and decomposition through cleavage of the ring structure then took place to give unknown products. It was unsuccessful to isolate the product because of its instability.

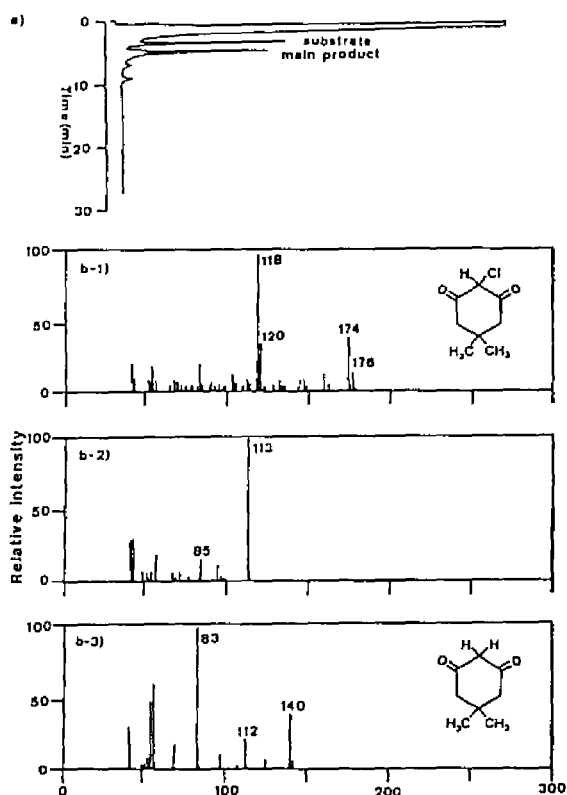


Fig. 3. Analysis of the product from monochlorodimedone. Gas chromatogram of the reaction mixture (a), and GC-MS of monochlorodimedone (b-1), unknown product (b-2) and standard dimedone (b-3).

Substrate Specificity Bromoperoxidase of *C. pilulifera* was found to catalyze bromination of a range of organic compounds, as summarized in Table III. These compounds generally consisted of cyclic β -diketones, substituted phenols, substituted alkenes and nitrogen- or sulfur-containing heterocycles.

Monochlorodimedone, a cyclic β -diketones, is a most suitable substrate for bromoperoxidase (Section 3).

In the case of substituted phenols, phenol itself was the substrate most readily brominated by this enzyme. According to rough estimations of the product yields (Table III), enzyme reactivity for these compounds decreased in the order; phenol > *o*-hydroxybenzyl alcohol > 1-methoxynaphthalene > anisole.

The results illustrated that enzyme reactivity depended upon the nucleophilic nature of each substrate.

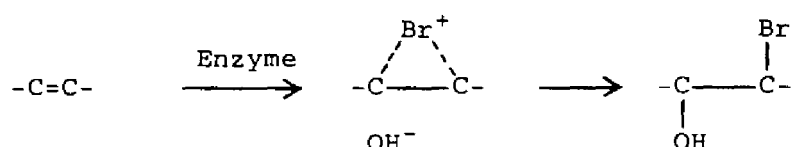
Table III. Reaction products from various substrates in bromoperoxidase-catalyzed halogenation

Substrate ^a	Product	
	Halide	
	Br ⁻	I ⁻
phenol (188 mg)	2,4,6-tribromophenol (50 mg)	nt ^b
<u>o</u> -hydroxybenzyl alcohol (250 mg)	2,4,6-tribromophenol (53 mg)	nt
anisole (216 mg)	<u>p</u> -bromoanisole, <u>o</u> -bromoanisole (11 mg)	nt
1-methoxynaphthalene (158 mg)	1-methoxy-4-bromonaphthalene (53 mg)	nt
cyclohexene (164 mg)	<u>trans</u> -1-hydroxy-2-bromo- cyclohexane (37 mg)	nt
styrene (208 mg)	DL-1-bromo-2-hydroxy-2- phenylethane (279 mg)	nt
<u>trans</u> -cinnamyl alcohol (134 mg)	(±)-1,3-dihydroxy-2-bromo- 3-phenylpropane (150 mg)	nt
<u>trans</u> -cinnamic acid (222 mg)	<u>trans</u> -β-bromostyrene, (±)-erythro-2-bromo-3-hydroxy -3-phenylpropionic acid (40 mg)	nt
<u>cis</u> -propenyl- phosphonic acid (244 mg)	(±)-threo-1-bromo-2-hydroxy- propylphosphonic acid (120 mg)	nt
cytosine	5-bromocytosine	slight conversion
cytidine	5-bromocytidine	nr ^c
uracil	5-bromouracil	5-iodouracil
pyrazole (34 mg)	4-bromopyrazole (10 mg)	4-iodopyrazole
thiophene	2-bromothiophene	nt
monochloro- dimedone	decomposition	nt

^a Acrylic acid, trans-crotonic acid, acetylene dicarboxylic acid, propargyl alcohol and thiazole were not used as substrates of bromoperoxidase, and cis-2-butene-1,4-diol, phenyl acetylene and pyrrole were converted to unknown products.

^b nt, not tested. ^c nr, no reaction.

Substituted alkenes, including cyclohexene, styrene, trans-cinnamyl alcohol, trans-cinnamic acid and cis-propenyl-phosphonic acid were also good substrates. On the other hand, acrylic acid and trans-crotonic acid, which each has an electron-attracting carboxylic group adjacent to the double bond, were inert. This was also observed in the chloroperoxidase-catalyzed bromination reaction (Chapter I, Section 1). Halohydrin formation proceeds through a halonium cation intermediate, as follows:



The reactivity of the enzyme toward alkynes was very low. Phenyl acetylene was found to be only slightly converted to unknown compounds, which could not be isolated and identified. Propargyl alcohol and acetylene dicarboxylic acid were inert to the enzyme.

The enzyme also catalyzed the bromination and iodination of nitrogen- and sulfur-containing heterocycles, such as cytosine, uracil and thiophene. The bromoperoxidase showed a similar tendency of substrate specificity to that of the chloroperoxidase (Chapter I, Section 2). When the bromination reactions of the two haloperoxidases were compared, a difference between the chloroperoxidase and bromoperoxidase became obvious. The bromoperoxidase did not utilize 2'-deoxyuridine and guanosine as a substrate.

The results in Table III demonstrated the wide substrate specificity of C. pilulifera bromoperoxidase, and it was

concluded that it had no rigid substrate restriction sites. Consideration of the enzyme's bromination at the active site and its high affinity for monochlorodimedone and cytosine (Section 3) suggests that its substrate specificity is determined not only by the nucleophilic nature of each substrate but also by the enzyme's affinity for that substrate.

Regiospecificity The ortho-para orientations of enzymatically and chemically (molecular bromine) brominated anisole were examined in detail. The para-/ortho-ratio produced by enzymatic bromination was identical to those produced by chemical reactions, as shown in Table IV. Of course, the conversion was rather low when H_2O_2 existed in the reaction mixture, since H_2O_2 decomposes HOBr to give O_2 , H_2O and Br^- (Section 3). Within the limits of the experiment, the enzyme showed no regiospecificity toward anisole.

Table N. Regiospecificity of the enzyme reaction for anisole

halogenating reagent ^a	ratio of o-/p-bromo-anisole		conversion (%)	
	with H_2O_2	without H_2O_2	with H_2O_2	without H_2O_2
bromoperoxidase	0.021	-	10	-
NaOBr	0.022	0.021	36	65
Br_2	0.023	0.021	31	60

^a The reaction mixture comprised 1.0 mmol potassium phosphate buffer (pH6.0), 0.1 mmol anisole, 0.2 mmol KBr, and 0.08 mmol Br_2 (24 mg bromine in 0.5 ml ethanol), 0.16 mmol NaOBr (24 mg bromine in 0.6 ml of 1N NaOH) or 25 units of bromoperoxidase in a total volume of 20 ml with or without 0.1 mmol hydrogen peroxide. Chemical reagent was added to the reaction mixture over 10 min at 30°C. Enzymatic reaction was carried out as described in "Materials and Methods". Contents of o- and p-bromoanisoles in the reaction mixture were analyzed by HPLC and calculated from the calibration curves of authentic compounds.

Stereospecificity The stereochemical result of bromoperoxidase-catalyzed bromination of four substrates - styrene, trans-cinnamyl alcohol, trans-cinnamic acid and cis-propenylphosphonic acid - are presented in Table III. In all cases, the enzyme gave products as racemates. From trans-cinnamic acid and cis-propenylphosphonic acid, erythro-form and threo-form bromohydrins were obtained, respectively. The results indicated that bromine transfer occurred with equal facility to both sides of the double bonds of the substrates. Thus, the enzyme did not exhibit appreciable stereospecificity for these substrates.

DISCUSSION

In previous sections, the author reported that the enzyme was distinct from the usual heme type (H type) haloperoxidases in its enzyme properties and halogenation mechanism. The halogenation reaction of H type haloperoxidases, as represented by the chloroperoxidase of C. fumago, proceed by enzymatic release of molecular halogen into the reaction mixture (Chapter I, Section 1 and 2). Thus, the substrates, namely the halogen acceptors, of H type enzymes undergo halogenation with electrophilic molecular halogen or hypohalous acid in water. Non-substrate specificity and nonstereospecificity of chloroperoxidase can be explained in this way.

However, the studies of bromoperoxidase of C. pilulifera have identified an active site bromination for substrate (Sec-

tion 3). The active site of the enzyme possesses a clear affinity for some substrates such as monochlorodimedone and cytosine, and it was presumed, as a result, that it might show regiospecific or stereospecific substrate bromination.

Initially, research work focused on the enzyme's substrates and identification of the products. Table III shows the results of a substrate survey. The active intermediate in the enzyme bromination is Br^+OH^- , which acts on the substrate through electrophilic addition or substitution mechanism (Section 3). The products produced enzymatically from the substrates were in agreement with theoretical concepts of the chemical reaction of bromonium cations. However, the substrate specificity of the enzyme differed from the chemical reaction of Br^+OH^- . From these findings, it was concluded that the enzyme had no appreciable restriction sites to limit the substrate structure, and that the interaction between the enzyme and substrate was due to hydrophobic or electrostatic conditions at the enzyme's active site. In addition, the outcome of the substrate survey (Table III) implied that cyclic compounds were especially good substrates for this enzyme.

Subsequently, the author tested the regiospecific bromination of anisole by the enzyme. The fact that no enzyme regiospecificity was observed showed to reflect the active site's inability to distinguish ortho- and para- sites of anisole. In bromohydrin formation from the prochiral compounds, trans-cinnamic acid, trans-cinnamyl alcohol, styrene, and cis-propenylphosphonic acid, all products were obtained as racemates. The results were closely related to

the wide substrate specificity and lack of regiospecificity of the enzyme. These findings strongly suggest that the enzyme would have no detectable regio- or stereospecificity for other substrates. Of course, as all substrates used in this work had small molecular weight and limited structural properties, possible regio- or stereospecific halogenation of other substances by this bromoperoxidase could not be denied. Considering the wide substrate specificity and high stability of the enzyme under reaction conditions (Section 3), the bromoperoxidase of C. pilulifera has great potential for halogenation of many organic compounds.

From the viewpoint of its physiological function in vivo, the enzyme is presumed to have an important roles in the production of organohalogenated compounds. The variety of halogenated compounds in the marine environment (3, 4) is probably due to the wide substrate specificity of the haloperoxidases. The organohalogenated compounds in algae appear to have antimicrobial or antianimal functions. However, the specific functions of halogenated compounds in sea water and algae have not yet been clarified. Recently, Geschwend et al. suggested that algal haloperoxidases participated in the air pollution of earth by making gaseous halogen compounds such as bromoform and dibromomethane (78). The effect of these halocompounds produced by algae on earth's ecosystem has been obscure. The present section offers biochemical knowledge of one enzyme participating in the halogen cycle of the ocean environment. Further studies are needed to clarify the

ecological cycle of halocompounds.

SUMMARY

Many organic compounds were found to be substrates for halogenation reactions catalyzed by bomoperoxidase of Corallina pilulifera. Anisole, 1-methoxynaphthalene and thiophene were converted to o- and p-bromoanisoles, 1-methoxy-4-bromonaphthalene and 2-bromothiophene, respectively. Regiospecificity of the enzymatic bromination of anisole was tested and found to be the same as in the chemical reaction with NaOBr. The enzyme also acted on substituted alkenes such as styrene, cyclohexene, trans-cinnamic acid, trans-cinnamyl alcohol, and cis-propenylphosphonic acid, to give the respective bromohydrin compounds or decarboxylated bromocompound. These bromohydrin compounds were always mixtures of stereoisomers. In the light of the above findings, enzymatic halogenation catalyzed by the enzyme was also discussed.

Section 5. Immobilized Bromoperoxidase as a Halogenating Biocatalyst

Bromoperoxidase of C. pilulifera catalyzes the halogenation reactions with many organic compounds including phenol derivatives, cyclic β -diketone, nitrogen- or sulfur-containing heterocycles and alkenes (Section 4). In addition, the enzyme is very stable under the reaction conditions compared to the chloroperoxidase of Caldariomyces fumago (Section 3). Therefore, the enzyme of C. pilulifera is suitable for the application to the enzymatic halogenating process.

The use of immobilized enzyme offers several advantages to large-scale process, including effective use of enzyme, improved enzyme stability and easy handling. To perform the halogenating reactor system, the immobilized bromoperoxidase of C. pilulifera has been investigated.

This section deals with the useful immobilization techniques for the bromoperoxidase and the continuous operation of the halogenating biocatalyst system.

MATERIALS AND METHODS

Chemicals κ -Carrageenan were purchased from Sigma Chemical Co., U. S. A., and 1,4-butanediol diglycidylether from Aldrich Co. Ltd., U. S. A. Controlled-Pore Glass (CPG 01400) was obtained from Electro-Nucleonics Inc., U. S. A., and n-octyltriethoxysilane and n-dodecyltriethoxysilane from Tokyo Kasei

Kogyo Co. Ltd., Japan. DEAE-Cellulofine AM and Cellulofine (for affinity chromatography) were supplied by Seikagaku Kogyo Co. Ltd., Japan and Chisso Corp., Japan. All other reagents used were of analytical grade.

Preparation of the Enzyme The enzyme was prepared from stocked samples of C. pilulifera, which were collected on the coast at Takahama (Fukui Prefecture, Japan) in April 1985, as described in Section 1. The enzyme preparation after the DEAE-Sepharose chromatography was used throughout the experiments.

Enzyme Assay Bromoperoxidase activity was measured as described in Section 1.

Immobilization Procedures Unless otherwise indicated, potassium phosphate buffer (pH 6.0) was used throughout the experiments.

1. Covalent binding method: Epoxy-activated Cellulofine was prepared by the treatment of Cellulofine (fine cellulose beads) with epichlorohydrin (79) or 1,4-butanediol diglycidyl ether (80). One milliliter of the enzyme (total 20 units in 10 mM buffer, pH 6.0) was added to the mixture comprised of 5 ml of 0.2 M sodium carbonate buffer (pH 10.3) and 4 g (wet weight) of epoxy-activated Cellulofine. Then the mixture was incubated at 30°C for 20 h with gentle shaking. After incubation, the matrix was filtered and washed with 0.1 M buffer on a glass filter.

2. Ionic binding method: The wet gel of DEAE-Cellulofine AM

(6 g), which had been equilibrated with 0.1 M buffer and filtered with a Buchner funnel, was added to 6 ml of the enzyme solution (total 25 units in 0.1 M buffer). Following incubation for 20 h at 4°C with gentle shaking, the enzyme-adsorbed matrix was collected by filtration and washed with the same buffer on a glass filter.

3. Physical adsorption method: Five milliliters of Controlled-Pore Glass (CPG 01400, 80-120 mesh) were washed with acetone and dried in an oven at 50 °C. Then the CPG was added to the mixture consisting of 9 ml of 95 % (v/v) methanol solution and 1 ml of n-octyltriethoxysilane. In the case of n-dodecyltriethoxysilane, 99 % ethanol was used instead of methanol solution. After keeping the matrix in an oven at 50°C to dryness, the alkylsilane treated CPG was washed with ethanol and then with 0.1 M buffer. The matrix (ca. 1.5 g wet weight) was put in 10 ml of the enzyme solution (total 25 units in the same buffer) and the mixture was kept for 20 h at 4°C with gentle shaking. Then the enzyme adsorbed CPG was washed with the buffer on a glass filter.

4. Entrapment methods in gels: The enzyme was immobilized in photo-crosslinkable resin gel (ENT-2000) according to the method of Fukui et al. (81). One milliliter of enzyme solution (total 20 units in 0.1 M buffer) was poured in the prepolymer slurry comprised of 1.0 g of ENT-2000, 1.5 ml of buffer and 10 mg of benzoin ethyl ether as an initiator. The mixture was transferred onto a glass plate, illuminated for 3 min with near UV-light and formed to a sheet gel. The gel was cut into pieces (ca. 5 x 5 x 0.5 mm) and washed with buffer

before use.

The enzyme was immobilized with the polyurethane prepolymer method (81). Two milliliters of enzyme solution (total 20 units in 0.1 M buffer) was added to the warmed polyurethane prepolymer (PU-6, 1.0 g) and mixed well for a short time. Then the mixture was kept standing for 1 h at 4°C to attain complete gelation. The formed gel was cut into pieces (ca. 2 x 2 x 2 mm) and washed with the same buffer.

The enzyme was entrapped in κ -carrageenan gel according to the method of Tosa et al. (82). The enzyme solution (1.4 ml; total 20 units) which had been dialyzed against physiological saline was warmed at 40°C. On the other hand, κ -carrageenan (135 mg) was dissolved in 2.6 ml of saline at 60°C. The two were mixed well, and the mixture was cooled at around 4°C. Then the gel was kept in 0.1 M cold buffer. The resulting gel was formed to a cubic gel (ca. 2 x 2 x 2 mm). To increase the stability of immobilized enzyme, these cubic gels were treated with hexamethylenediamine (85 mM) and glutaraldehyde (85 mM) by the method of Tosa et al. (82). The hardened gels were washed well with 0.1 M buffer before use.

In all cases, non-adsorbed enzyme or leaked enzyme from matrix was checked by measuring the activity of combined washing solutions. The recovery of enzyme activity of each immobilized system was calculated on the basis of the total amount of enzyme initially added.

Assay and Monitoring Procedures

The enzymatic halogenation reaction was carried out as follows: The reaction mixture in a

50-ml flask contained 1 mmol potassium phosphate buffer (pH 6.0), 0.5 mmol KBr, 0.1 mmol H₂O₂, 25 μ mol monochlorodimedone and immobilized enzyme, which corresponded to 2.0 units of free enzyme in a total volume of 10 ml. The reaction was initiated by addition of hydrogen peroxide, and continued for 30 min at 30°C with shaking (120 strokes/min). When uracil was used as a substrate, the reaction was carried out in the same manner as in the case of monochlorodimedone, except that the reaction mixture contained 50 μ mol uracil and each immobilized enzyme corresponding to 5.0 units of free enzyme. In all cases, a control run was done without hydrogen peroxide.

Aliquots (5-10 μ l) of the reaction solution were drawn at 2.5- or 5-min intervals, and analyzed by high performance liquid chromatography (HPLC) at 290 nm for monochlorodimedone and 254 nm for uracil. HPLC was performed with a Shimadzu LC-5A system equipped with an M & S pack C18 column (reversed phase column, 4.6 x 150 mm; M & S Instruments Inc., Tokyo, Japan) at the flow rate of 1.0 ml/min, using the following solvent systems: a) 0.2 M potassium phosphate buffer (pH 6.0): methanol, 4:1 (by volume) (for monochlorodimedone); b) 0.2 M potassium phosphate buffer (pH 6.0): methanol, 9:1 (for uracil). The substrates and products showed the following retention time ($\underline{r_t}$): monochlorodimedone, 5.9 min; uracil, 2.3 min; 5-bromouracil, 4.5 min. The enzyme activity was calculated from the linear portion of the decrease of the substrate versus time course.

RESULTS AND DISCUSSION

Preparations of Immobilized Enzymes

Several immobilized bromoperoxidases were used for the enzymatic bromination reactions of monochlorodimedone and uracil. Monochlorodimedone is decomposed through monochloro-monobromodimedone by the bromoperoxidase reaction (Section 4). Uracil is converted to 5-bromouracil by the enzyme (Section 4). The results are summarized in Table I.

TABLE I
COMPARISON OF ENZYME ACTIVITIES OF IMMOBILIZED BROMOPEROXIDASE BY VARIOUS METHODS

Matrix	Type of immobilization	Substrate			
		Monochlorodimedone		Uracil	
		Enzyme activity ($\mu\text{mol min}^{-1}$)	Recovery of activity (%)	Enzyme activity ($\mu\text{mol min}^{-1}$)	Recovery of activity (%)
Free enzyme	—	1.00	100	0.32	100
Epoxy-Cellulofine	Covalent binding	0 ^a	0	0 ^a	0
DEAE-Cellulofine	Ionic binding	0.87	87	0.23	72
n-Octyl-CPG	Physical adsorption	0.78	78	0.15	47
n-Dodecyl-CPG	Physical adsorption	0.81	81	0.18	56
ENT-2000	Entrapment	0.54	54	0.21	66
PU-6	Entrapment	0.33	33	0.13	41
κ -Carrageenan	Entrapment	0.41	41	0.15	47
κ -Carrageenan (hardening treatment)	Entrapment	0.10	10	— ^b	—

^a No adsorption.

^b Not determined.

The epoxy-coupling technique is useful if the enzyme can be coupled at basic pH. Since the enzyme of Corallina pilulifera is very stable under alkaline conditions (Section 2), this method was tested. However, the enzyme activity was all found in the supernatant solution, regardless of the chain length of epoxy ligand. This suggested that little

available active amino acid residues such as lysine for epoxy-coupling were present on the surface of the enzyme. The very low content of basic amino acids of the enzyme (Section 2) is probably related to this phenomenon.

The ionic adsorption method for enzyme immobilization is a simple and useful technique. This method was particularly useful for the bromoperoxidase because the enzyme is a highly acidic protein ($pI=3.0$) (Section 2). DEAE-Cellulofine AM was selected as a support material because it is superior in compressive strength. As shown in Table I, the immobilized enzyme on DEAE-Cellulofine had the highest enzyme activity compared to the other immobilization techniques.

The immobilization method by covalent attachment to inorganic supports have been well investigated by Weetall (83). Silanization is a particularly useful method to introduce an active group to inorganic materials (83). However, the adsorption method using alkylated inorganic supports for the enzyme immobilization has not been well studied. Thus, the alkylsilane-treated highly porous glass was chosen as a matrix for immobilization. The bromoperoxidase was readily adsorbed on the surface of these matrices, and the immobilized enzymes exhibited high enzyme activity (Table I).

The entrapment method in gel was considered to be useful for the bromoperoxidase because the enzyme is characterized by high molecular weight ($M_r=790,000$) (Section 2). Until now, a number of different enzymes have been successfully immobilized with photo-crosslinkable resin prepolymer (81, 84, 85). Immo-

bilized bromoperoxidase in ENT-2000 gel showed high enzyme activity, taking into account the hindrance of substrate transport by gel matrix (Table I).

The water-miscible urethane prepolymer method was found to be useful for the immobilization of microbial cells (81, 86). However, for some enzymes, the urethane prepolymer caused a significant loss of activity. That was considered to be due to the isocyanate group in the prepolymer, which reacts not only with water but also with active amino acid residues in the enzyme. The author succeeded in the formation of immobilized bromoperoxidase with PU-6 prepolymer, which retained sufficient enzyme activity (Table I). That probably depends on the special surface circumstances of the enzyme as described above.

The κ -carrageenan method developed by Chibata et al. (87) has been applied to industrial processes. However, immobilized bromoperoxidase with κ -carrageenan showed lower activity than that of ENT-2000. In addition, hardening treatment with hexamethylenediamine and glutaraldehyde of gel was not suitable for the recovery of enzyme activity (Table I).

In all cases, non-enzymatic adsorption of the substrates to the support materials were not observed.

Stability of Immobilized Enzyme on Repeated Use

Figure 1 shows the repeated use of immobilized bromoperoxidase for the bromination reaction of monochlorodimedone. The reaction was successively carried out as described in MATERIALS AND METHODS, except that the reaction time was 10 min.

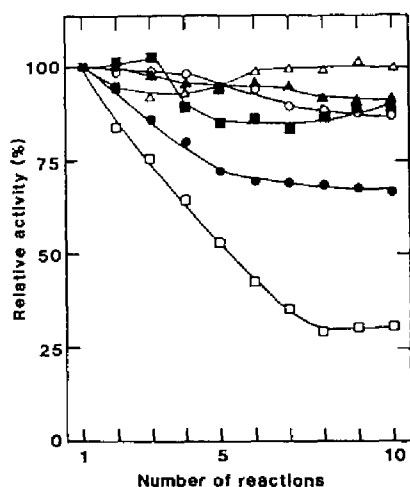


Fig. 1. Repeated use of immobilized bromoperoxidases on the bromination of monochlorodimedone. Each batch was carried out for 10 min with shaking (120 strokes per min). DEAE-Cellulofine (○), *n*-dodecyl-CPG (●), ENT-2000 (△), PU-6 (▲), κ -carrageenan (□) and hardened κ -carrageenan (■).

After each reaction, the immobilized enzyme was washed with 0.1 M potassium phosphate buffer (pH 6.0) for several minutes to eliminate the remaining substrates. The results indicated that the immobilized enzyme with ENT-2000 was most stable upon repeated use. The immobilized enzymes using PU-6, DEAE-Cellulofine and hardened κ -carrageenan gel showed almost similar stabilities. However, the activity of the immobilized enzymes with alkylated highly porous glass and κ -carrageenan diminished gradually with repeated use. That was probably due to the release of the enzyme from the matrix. From the above results together with those in Table I, the author concluded that the immobilization methods using ENT-2000 and DEAE-Cellulofine were suitable for the bromoperoxidase of *C. pilulifera*.

Continuous Operation of Immobilized Enzyme on DEAE-Cellulofine

The operational stability of the immobilized enzyme for the

bromination of uracil was studied as a column reactor system. The adsorption method on DEAE-Cellulofine was adopted for the reactor system on the following reasons: 1) the immobilized enzyme on DEAE-Cellulofine would be more stable in the column system than in the batch reaction system (Fig. 1) in which the matrix would be under mechanical stress. 2) the matrix could be repeatedly used after the decrease of the enzyme activity. 3) the enzyme could be purified by the immobilization procedure.

The cell-free extract of C. pilulifera (total 500 units) was directly applied to a DEAE-Cellulofine packed column (3 x 7.5 cm), which had been equilibrated with 0.1 M potassium phosphate buffer (pH 6.0). After complete washing of the column with buffer, it was used as a brominating reactor.

Experiments were carried out to investigate the influence of resident time (flow rate of the reaction mixture) on the degree of conversion of uracil to 5-bromouracil. It was found that the bromination reaction of uracil was very complicated because the product (5-bromouracil) was further attacked in the enzyme reaction and decomposed to give unknown compounds. As described in the previous section (Chapter I, Section 2), 5-bromouracil is probably converted to 5,5-dibromo-6-hydroxy-5,6-dihydrouracil, which readily reverts to 5-bromouracil and partially decomposes to unknown products by the occurrence of ring cleavage. The bromination reaction of uracil to 5-bromouracil is not an equimolar reaction. Therefore, the complete consumption of uracil needed a long resident time (Fig. 2). In addition, the content of 5-bromouracil was very low under such conditions. The continuous opera-

tion of the reactor was carried out at the flow rate (10 ml/h), which gave the maximum content of 5-bromouracil. As shown in Fig. 3, the half-life of the reactor was found to be 45 days under these conditions. The mechanical stability of the matrix was good and no turbidity was detected in the outlet. However, the occurrence of oxygen bubbles (Section 3) in the column was observed during the reaction. That caused a problem in obtaining a good flow of the reaction mixture. It would be necessary for a large-scale process to eliminate the oxygen bubbles from the reactor system.

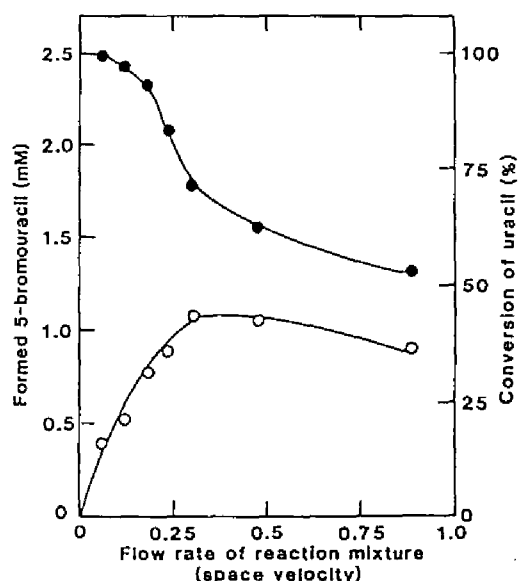


Fig. 2. Effect of flow rate on the bromination reaction of uracil in column reactor system. The reaction mixture comprised 50 mM potassium phosphate buffer (pH 6.0), 10 mM H_2O_2 , 50 mM KBr and 2.5 mM uracil. The column was maintained at 30 °C. Space velocity (SV) was calculated from the equation: $SV = \text{flow rate (ml/hr)} / 33.5 \text{ ml}$ (matrix volume: column volume (50 ml) - void volume (16.5 ml)). Formed 5-bromouracil (O) and conversion of uracil (●).

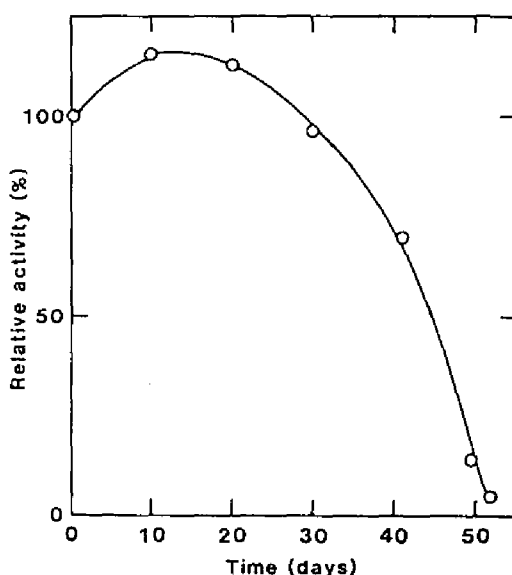


Fig. 3. Operational stability of the halogenating reactor on the bromination of uracil. The reaction was carried out as described in Fig. 2. The flow rate of the reaction mixture was 10 ml/hr (SV=0.3).

In conclusion, the long half-life time of the reactor indicated the possible application of the reactor to industrial processes. The enzyme of C. pilulifera catalyzes the bromination of many organic compounds. Thus, the biocatalyst system using the bromoperoxidase could be applicable to these substrates with minor changes of the conditions as described in the case of uracil.

SUMMARY

The partially purified bromoperoxidase was immobilized on the following matrices: Cellulofine (covalent binding), DEAE-Cellulofine (ionic binding), or alkylsilane treated Controlled-Pore Glass (physical adsorption), and entrapped in the soft gels using photo-crosslinkable resin prepolymer (ENT-2000), polyurethane prepolymer (PU-6), or κ -carrageenan. These different forms of immobilized bromoperoxidase were tested for the bromination reactions of monochlorodimedone and uracil. The immobilization techniques using DEAE-Cellulofine and ENT-2000 were found to be suitable for the bromoperoxidase reaction. The immobilized enzyme on DEAE-Cellulofine showed the highest activity and a half-life of 45 days when it was used for the conversion of uracil to 5-bromouracil.

GENERAL CONCLUSION

This thesis describes the halogenation reactions catalyzed by two haloperoxidases: chloroperoxidase from a fungus Caldariomyces fumago and bromoperoxidase from a red alga Corallina pilulifera. Chloroperoxidase of C. fumago (EC 1.11.1.10) is a unique heme enzyme that introduces halogen-carbon bonds in many organic compounds. Compared with chloroperoxidase, bromoperoxidase of C. pilulifera, which was first isolated by the author, is a quite different haloperoxidase containing nonheme ferric iron as a prosthetic group. Comparison of two haloperoxidases contributed to further clarification of the biological halogenation systems.

In Chapter I, systematic substrate specificity, stereospecificity, and halogenation mechanism of Caldariomyces chloroperoxidase were investigated. On the basis of the research work, the enzyme halogenation could be explained by the molecular halogen or hypohalous acid addition chemistry in water, which was released into the reaction mixture by the enzyme in the presence of halide ions and H_2O_2 . The wide substrate specificity and non-stereospecificity of its reaction was readily understood by its reaction mechanism. However, the enzyme was not suitable for the use in the halogenation processes, because the resulting active molecular halogen denatured the enzyme.

Chapter II described bromoperoxidase distribution in algae, and the purification, characterization and application of Corallina enzyme. The enzyme activity was observed in all

the algae belonging to the coralline family (Corallinaceae) tested. The author first succeeded in obtaining homogeneous bromoperoxidase of C. pilulifera. Compared with the previously reported haloperoxidases, it showed peculiar enzymatic properties: high molecular weight protein (790,000) composed of 12 identical subunits, low pI value (3.0), high affinity for H_2O_2 , high stability under alkaline and reaction conditions, nonheme enzyme, and active site halogenation. This is the first report of the existence of nonheme haloperoxidase in nature. Analyses of halogenation kinetics and intermediates clarified the differences in the reaction mechanisms between nonheme and heme haloperoxidases. No catalase and only slight peroxidase activities of the bromoperoxidase could be explained by its reaction mechanism. Although it showed no regio- and stereospecificities, the potential halogenation of many organic compounds and stability under reaction conditions enabled its use as a halogenating reagent.

Thus, the author have succeeded in clarification of the halogenations of two different haloperoxidases. These results provided new information on the application of haloperoxidases in chemical industry as well as on biological halogenation in nature.

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