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PURIFICATION OF BROMOPEROXIDASE FROM CORALLINA PILULIFERA

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SUMMARY : Bromoperoxidase was purified from the crude extract of Corallina pilulifera (Corallinaeae, Rhodophyta) and found to be homogeneous upon disc gel electrophoresis by precipitation of ammonium sulfate and sequential column chromatographies of DEAE-Sepharose CL-6B, Sepharose 6B and Cellulofine GC-700m. The purified enzyme did not exhibit optical absorption spectra of a hemoprotein. Therefore, bromoperoxidase of C. pilulifera was completely distinguishable from other haloperoxidases which have heme-irons at the catalytic sites.

Chloroperoxidase of Caldariomyces fumago, which is able to catalyze the halogenation of many compounds (1), has been well investigated since 1959 (2-5). Recently, bromoperoxidase which is specific for I^- and Br^- has been found in several marine algae such as Rhodomera (6), Rhipocephalus (7) and Penicillus (7,8), and is considered to participate in the occurrence of halocompounds in marine environment. Pee and Lingens also reported the purification of the enzyme from Pseudomonus aureofaciens (9). The above haloperoxidases are all typical hemoproteins.

In the previous study, we described that various coralline algae contained large amounts of bromoperoxidase, and demonstrated the bromination of phenol derivatives such as phenol and o-hydroxybenzyl alcohol (10). In the course of our study, we found that the bromoperoxidase of C. pilulifera is a new haloperoxidase containing no heme-like compound as a

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prosthetic group. The present communication describes the purification of bromoperoxidase from C. pilulifera.

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. Other reagents in this study were all commercial products of analytical grade.

Collection of alga : Corallina pilulifera was collected from shallow waters (0.5-1.0 m depth) at the shore of Shirahama (Wakayama Prefecture) in Japan at April in 1984, 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 subjected to disruption as described in the previous paper (10). Then, 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 (ε=19,900 M^{-1} cm^{-1}) to monobromomonochlorodimedone. The detailed assay conditions have been described in the previous paper (10). One unit of the enzyme activity is the amount of the enzyme which changes 1 μmol of substrate in one min at 25°C.

Protein determination : The protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry et al. (11) 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 (12). 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/H_2O (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.

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.

RESULTS

Purification of Enzyme : The enzyme was prepared from 2.5 kg of wet weight of the alga. Unless otherwise indicated, all purification steps were carried out at 0-5°C, using potassium phosphate buffer.

Step 1. Ammonium Sulfate Precipitation : Soid ammonium sulfate was added to the crude extract to give 80% saturation,

and stirred for 15 hr. 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).

Step 2. First DEAE-Sepharose Chromatography : The enzyme solution was applied to the column of DEAE-Sepharose 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 hr, the precipitate was collected, dissolved in 0.1 M of the buffer (pH 6.5) and dialyzed against 10 liters of the same buffer.

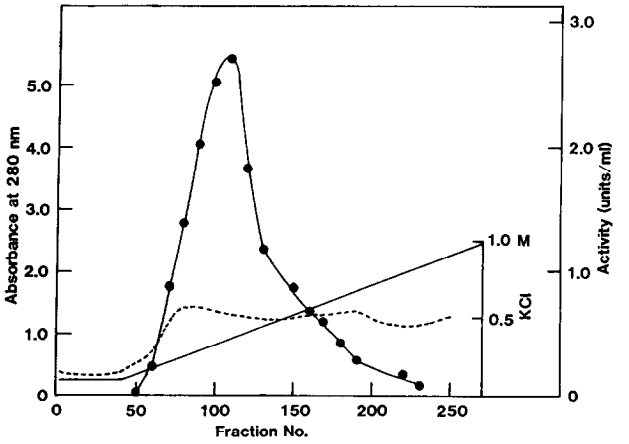

Fig.1. First DEAE-Sepharose CL-6B column chromatography of bromoperoxidase. The flow rate was approximately 500 ml per hr, and 16-ml factions were collected. (- - -), absorbance at 280 nm; (●—●), enzyme activity; (——) concentration of KCl.

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

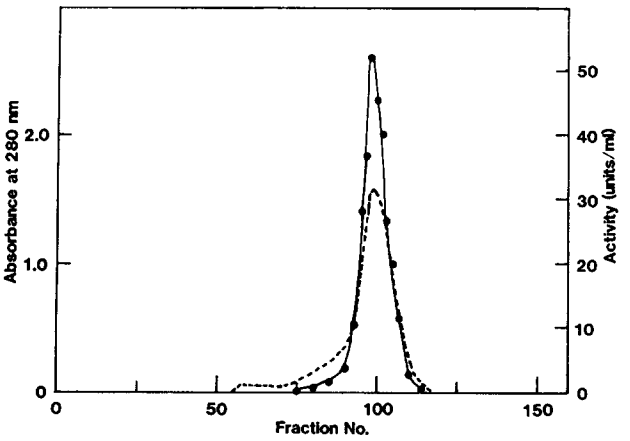

Fig.2. Second Sepharose 6B column chromatography of bromoperoxidase. The flow rate was approximately 50 ml per hr, and 4-ml fractions were collected. (- - -), absorbance at 280 nm; (●—●), enzyme activity.

chromatography (2.7 x 110 cm) under the similar conditions described in Step 4. Figure 2 shows the elution pattern of this chromatography. 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 1.

Purity of Enzyme : Upon polyacrylamide disc gel electrophoresis the purified enzyme was shown to migrate as a single band when it was stained either for protein (Fig. 3a) or for bromoperoxidase activity (Fig. 3b). 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

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

coloration on the gel was due to the bromide-dependent enzymic reaction, but not to peroxidase activity of the enzyme.

Absorption spectra : The concentrated solution of the purified enzyme was a light brown, having an absorption peak at 277 nm. A few broad and weak absorption bands were detected between 390 and 700 nm (Fig. 4). The visible absorption decreased by adding sodium dithionite. No significant absorption bands

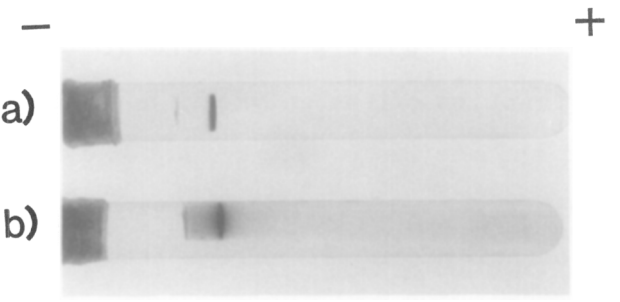

Fig.3. Polyacrylamide disc gel electrophoresis of purified bromoperoxidase. About 30 μg of purified enzyme was subjected to electrophoresis. The gel was stained for protein (a) and for enzyme activity (b).

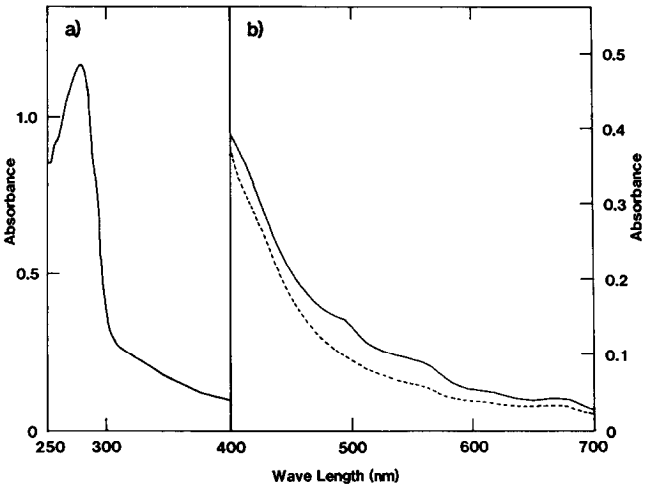

Fig.4. Absorption spectra of bromoperoxidase. The spectra of the native enzyme (———) was recorded in 0.1 M potassium phosphate buffer (pH 7.0). The spectra of the dithionite reduced enzyme (- - -) was measured in the same buffer. Enzyme concentrations ; (a) 1.4 mg protein per ml, (b) 4.2 mg protein per ml.

corresponding to heme or flavin were observed in the visible region. These facts indicate that bromoperoxidase of C. pilulifera has no prosthetic group such as heme and flavin.

DISCUSSION

Coralline algae, which show high bromoperoxidase activity (10), are known to be widely distributed from the tropical to polar seas in the world, and C. pilulifera is found in all coastal areas in Japan. The bromoperoxidase of C. pilulifera comprised 1% of the total protein in the crude extract as calculated from the results in Table 1. Although the physiological function of the enzyme is not known yet, such a high content of the enzyme may imply that the enzyme takes an important role in vivo. The enzyme appears to participate in the synthesis of antimicrobial halogenated organic compounds such as bromophenols (10)

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

chromatography and gel filtration. The purified enzyme did not exhibit optical absorption spectra characteristic of a hemoprotein. The decrease of visible absorption by adding sodium dithionite suggests that the enzyme contains a metal which is involved in the oxido-reduction. The enzyme did not exhibit peroxidase activity in the absence of halide ion. But haloperoxidases from other origins have been reported to possess peroxidase activity (8,13). Procaryotic bromoperoxidase of P. aureofaciens also has high catalase activity as well as peroxidase and low halogenating activity (9). This marked difference between the enzyme of C. pilulifera and other haloperoxidases appears to lie on their catalytic sites, that is, our present enzyme is not a hemopretein, while the others are. Detailed properties of the enzyme will be reported elsewhere.

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