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BROMOPEROXIDASE IN *CORALLINA PILULIFERA* IS REGULATED BY ITS VANADATE CONTENT

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Key Word Index—*Corallina pilulifera*; Rhodophyta; bromoperoxidase; metal enzyme; vanadium; iron; regulation.

Abstract—Seasonal changes in bromoperoxidase activity in coralline algae (Corallinaceae) are responsible for the production of volatile halogenated compounds. SDS-polyacrylamide gel electrophoresis (PAGE) of a crude protein extract showed that the concentration of this enzyme was almost constant throughout the year. Therefore, the enzyme activity *in vivo* changed seasonally due to a structural alteration. To elucidate this, the metal content of this enzyme at different states of activity was measured. The results revealed that the enzyme activity is controlled by the incorporation of vanadate ions, less than 1.2 mol mol enzyme^{-1}, in the active site of the enzyme.

INTRODUCTION

Recent studies have revealed the existence of some types of non-heme haloperoxidase: vanadium-containing algal bromoperoxidases from *Corallina* [1–4] and *Ascophyllum* [5–8], the bacterial chloroperoxidase having no essential metals from *Pseudomonas* and *Streptomyces* [9–11], and the cobalt-containing bromoperoxidase from *Ps. putida* [12]. Although these haloperoxidases have been interesting as novel metal or non-metal enzymes, their structure in the following areas remains unclear: the organization of metal ions in the enzyme, the co-existence of other metals such as iron [2, 12], the relatively low and varying content of metal ions which do not coincide with the number of subunits of the enzyme [12, 13], and their reaction mechanisms, especially in non-metal enzymes [14].

Bromoperoxidase from *Corallina pilulifera*, a representative of coralline algae, was first characterized by Itoh *et al.* and it was classed as a non-heme iron bromoperoxidase because of its high iron content [2]. However, recent studies have shown that the enzyme contains another essential metal for bromination activity, vanadium [13, 15].

This enzyme is responsible for the emission of volatile bromomethanes such as bromoform and dibromomethane from the marine environment [16]. The rate of bromomethane emission is related to bromoperoxidase activity, and there are clear seasonal activity changes in the activity of this enzymes in *C. pilulifera*. Therefore, we aimed to clarify the mechanism regulating this enzyme *in vivo*. This paper shows that bromoperoxidase activity is regulated by the incorporation of vanadate ions into the active site of the enzyme *in vivo*.

RESULTS

Relationship between bromoperoxidase activity changes and its concentration in vivo

SDS-PAGE was used to determine bromoperoxidase concentrations in the crude *C. pilulifera* extracts. As shown in Fig. 1a, bromoperoxidase concentrations in the crude samples were nearly constant throughout the year. To measure the quantity of the enzyme, a calibration curve was made with known concentrations of purified bromoperoxidase using a laser densitometer. A linear curve was obtained in the range from 1 μg to 10 μg of purified enzyme (Fig. 2). According to this simple method, the bromoperoxidase content of the crude extracts from samples taken in different seasons, were calculated (Fig. 1b). The seasonal bromoperoxidase activity changes in *C. pilulifera* living in a temperate sea (the Sea of Japan, 36°N, 136°E) varied by a factor of 20, even though its concentration was almost constant throughout the year, within 0.1 and 0.2 mg g^{-1} of wet algae. Conversely, the total soluble protein concentration (7.5–16 mg g^{-1} in wet algae throughout the year) varied by a factor of 2 (Fig. 1b). The results indicated that the change in bromoperoxidase activity was not dependent on its concentrations *in vivo*.

Relationship between bromoperoxidase activity in algae and its specific activity

We could not directly measure the specific activities of the enzyme *in vivo*. Therefore, firstly we purified the enzyme from stock algal samples which showed different enzyme activities even though the same purification

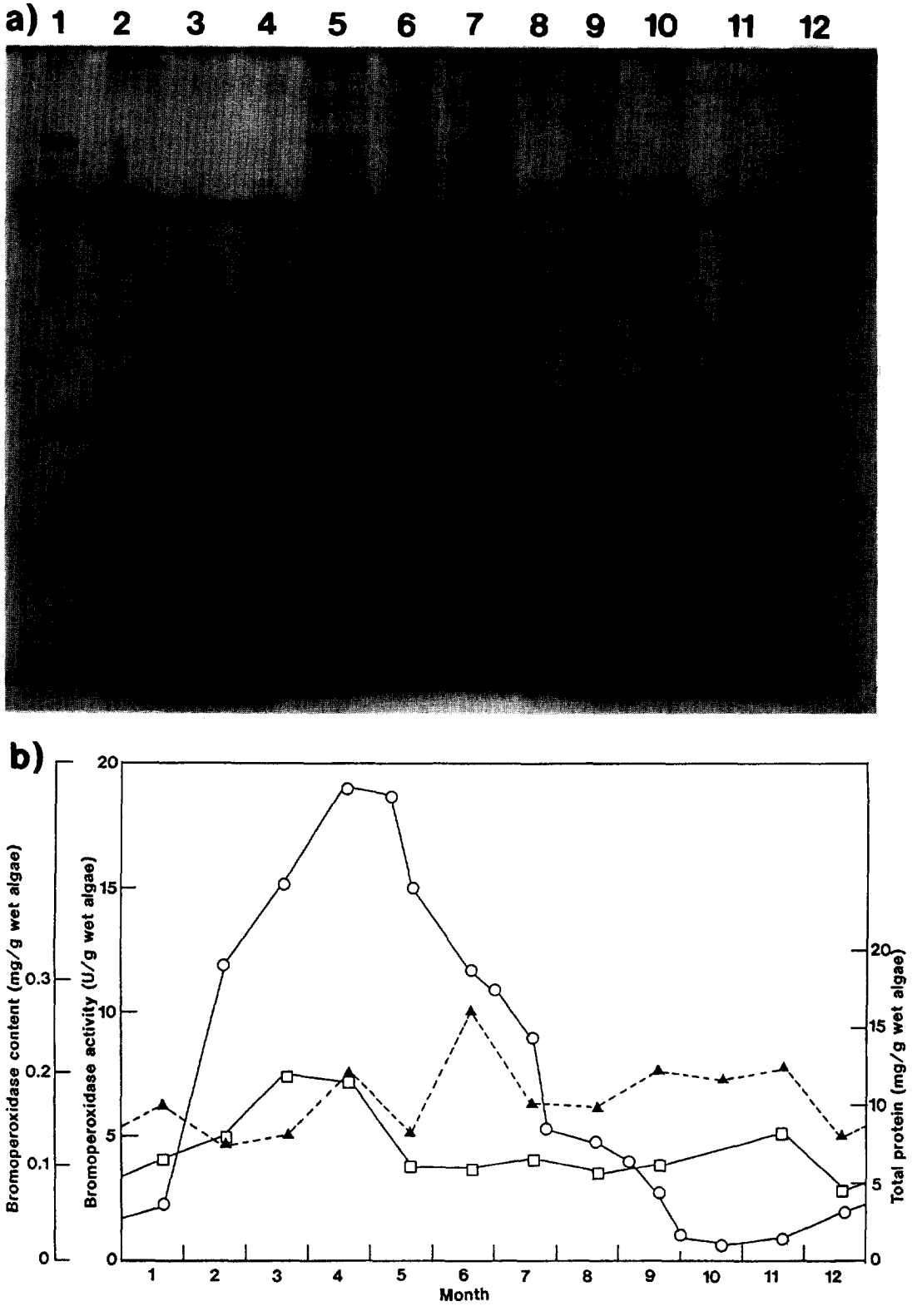

Fig. 1. (a) Photograph of a SDS-PAGE gel of seasonal crude bromoperoxidase samples from *C. pilulifera*, and (b) the correlation between bromoperoxidase activity (○), total protein concentration (▲), and bromoperoxidase concentrations determined by SDS-PAGE (□). Lanes 1 and 12: marker standard proteins, phosphorylase (M_r = 97 400); bovine serum albumin (66 200); ovalbumin (45 000); carbonic anhydrase (31 000); soybean trypsin inhibitor (21 500); lysozyme (14 400). Lanes 3–11: *ca* 100 μg of crude extract from *C. pilulifera* from May, June, July, August, September, November, December 1992, January, February to March 1993, were also run on the SDS-PAGE gel. The subunit M_r of bromoperoxidase (64 000) is indicated by an arrow in (a). The data of April 1993 was omitted from (a).

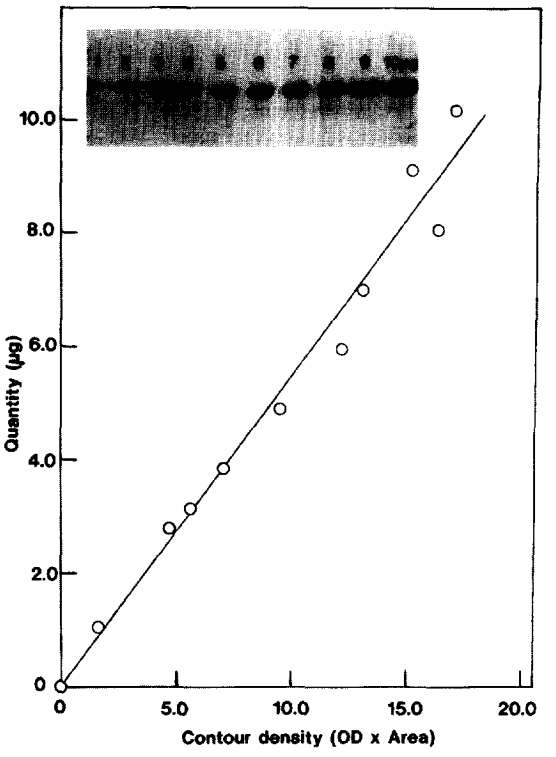

Fig. 2. Calibration curve for the determination of bromoperoxidase concentration in crude extracts by SDS-PAGE. The protein concentration was calculated from the density of each band on the gel. Known data points were obtained using the completely purified bromoperoxidase from 1 to 10 μg.

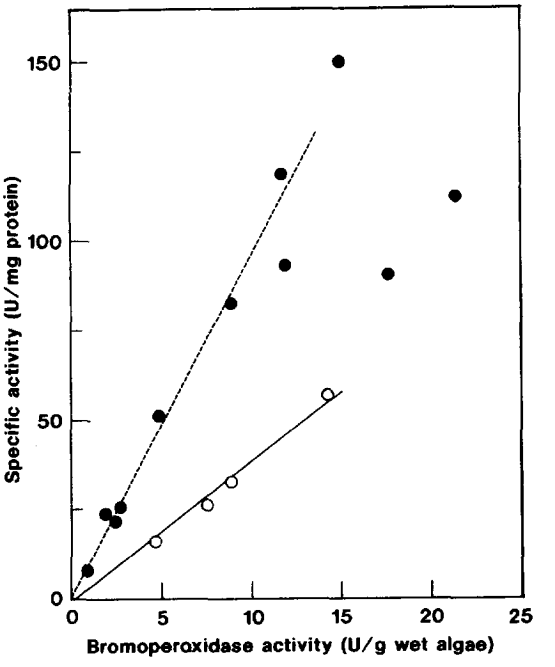

Fig. 3. Relationship between the specific activity and the bromoperoxidase activity in algae. The bold line (—○—) indicates the specific activities of the purified enzyme samples, and the broken line (- -●- -) indicates those calculated from the data in Fig. 1.

procedures were used [1]. The bold line in Fig. 3 shows that high specific activity of the enzyme was found in algal samples having high enzyme activity, and there was a strong linear correlation between them. Secondly, we calculated the specific activities from the data regarding the activity and bromoperoxidase content of the algae in Fig. 1. The results are shown as a broken line in Fig. 3. Although the line was rather irregular over the 10 units g^{-1} wet algae mark, it was almost linear below 10 units g^{-1} wet algae. By comparing both lines, we found that the specific activities of the enzymes decreased about one third during the purification process. These results clearly indicate that the seasonal change in bromoperoxidase activity in *C. pilulifera* is due to changes in specific activity.

Metal contents of bromoperoxidases with different specific activities

To elucidate what causes the varied activity of purified bromoperoxidases obtained in different seasons, we checked the vanadate and ferric ion content of the enzymes. The vanadium content of the purified enzyme correlated linearly with the specific activities of the enzymes in the range from 0.4 to 1.2 mol/mol $enzyme^{-1}$ (Fig. 4a). Ferric iron contents, which ranged between 7.1 and 18.9 mol/mol $enzyme^{-1}$, roughly correlated with the specific activity (Fig. 4b). The iron content was about 20 times higher than the vanadium content in each sample. As vanadate ions are essential for bromoperoxidase activity, it was concluded that the specific activity is regulated by the incorporation of vanadate ions in the active site, and ferric ions have unknown cooperative effects.

DISCUSSION

Seasonal changes in bromoperoxidase activity in *Corallina pilulifera* is regulated by its specific activity. As shown in Fig. 1, the enzyme content per wet alga was almost constant throughout a year in spite of dramatic changes in its activity. Most proteins in organisms are generally regulated by protein synthesis and degradation mechanism. However, this mechanism does not seem to be of much importance for bromoperoxidase in *C. pilulifera*.

Vanadate ions (V) are essential for the bromination activity of nonheme bromoperoxidases in *C. pilulifera*. Therefore the vanadium content was thought to regulate its activity. Measurements of the vanadium content in the enzymes with varied specific activity revealed that the specific activity was strictly controlled by the vanadium content. However, its concentrations were lower than 1.2 mol of vanadium per mol of dodecameric enzyme (<*ca* 40 units mg $protein^{-1}$). Although Krenn *et al.* reported 4 mols of vanadium per mol of purified enzyme in *C. pilulifera* (*ca* 20 units mg $protein^{-1}$) [13] by ESR measurement, we did not observe such high vanadium concentrations. The

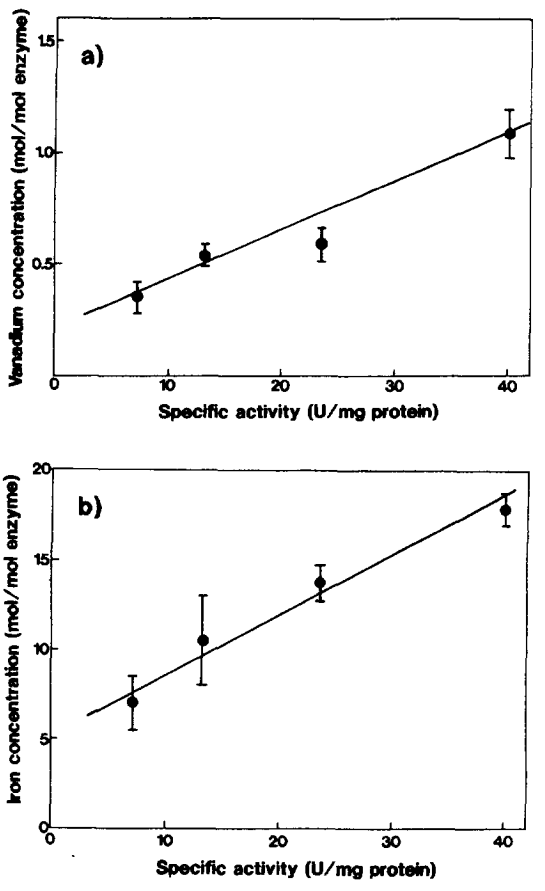

Fig. 4. Relationship between metal ion concentrations and specific activities of bromoperoxidase. Vanadium (a) and iron (b) contents were measured by the HPLC method. Data points represent the mean value of three samples. The bars indicate the deviations.

specific activity of the purified enzyme was calculated to have about 30% of its initial activity *in vivo*. Loss of vanadate ions during the purification process may explain this decrease. Purification generally yields about 20% of the enzyme from the crude extract [1]. The role of ferric iron in bromoperoxidases is still unclear, in spite of its high concentrations. However, it might assist the incorporation of vanadium into the active site of apo-bromoperoxidases, or undergo unknown redox reactions with vanadate ions, considering the relationship between its content and the specific activity. Our experiments clarified the ambiguous data previously obtained for the content of vanadium and iron in bromoperoxidases from *C. pilulifera* [2, 13]. However, the mechanisms regulating the specific activity of the enzyme remain unclear. There might be a low M_r regulatory compound which can prevent the correct coordination of vanadate ion into the active site. Another theory is that modification of the enzyme protein structure prevents the correct coordination of vanadate ions within the apo-enzyme.

EXPERIMENTAL

Samples of *Corallina pilulifera* Postels et Ruprecht were collected from the intertidal zone of Tojinbo (36°N, 136°E, Fukui Prefecture, Japan) on the coast of the Sea of Japan, between May 1992 and April 1993. The samples were kept in cold sea water and were brought to our laboratory within 2 hr. After removing epiphytic plants and animals, algae were used for experiments.

The bromoperoxidase activity was measured as described in the previous paper, on the basis of the change of monochlorodimedone to monobromomonochlorodimedone [1]. The assay mixture consisted of 150 μmol of KPi buffer (pH 6), 30 μmol KBr, 3 μmol of H_2O_2, 90 nmol of monochlorodimedone and a suitable amount of enzyme soln in a total vol. of 1.5 ml. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the conversion of 1 μmol of monochlorodimedone in 1 min at 25°.

Each sample of algae was washed and then dried between paper towels. Wet alga (1 g) was ground up with sea sand (40–80 mesh) in a chilled mortar, and this mixture was extracted with 4 ml of 20 mM KPi buffer (pH 6.5). After centrifugation (8000 *g*, 20 min), the supernatant was used as a crude enzyme extract, or it was concentrated further by ultrafiltration.

Bromoperoxidase was purified from the stocked samples of *C. pilulifera* which were collected from the same site as mentioned above, according to the procedure of ref. [1]. The purity of the enzyme was checked by SDS-PAGE (10 or 12% slab gels) according to the method described in ref. [17].

Bromoperoxidase concns in the crude enzyme extracts were measured by SDS-PAGE (12% slab gels) after staining the protein with Coomassie Brilliant Blue G-250. Protein bands on the PAGE gel were analyzed by computer-assisted laser densitometer with the UltroScan XL system (Pharmacia). A linear calibration curve was obtained in the range from 1 to 10 μg of the purified enzyme (Fig. 2).

General protein concns were determined by the method described in ref. [18], with bovine serum albumin as a standard.

Quantitative analysis of the vanadium and iron content of the enzyme was done by two different methods; inductively coupled plasma emission spectrometry (ICP-AES) using a SPS-4000 ICP spectrometer (Seiko Instruments Co., Tokyo) and HPLC-spectrometry of chelated complexes of azo dye with metal ions [19]. Details of both methods were described in previous papers [2, 12]. We obtained similar results for the V and Fe contents of the enzyme using either of the two methods. Therefore, we generally used the HPLC-spectrometry method because it is easier.

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