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New Bromoperoxidase from Coralline Algae that Brominates Phenol Compounds

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Bromoperoxidases were found in coralline algae (Corallinaceae, Rhodophyta) collected from seasides in Japan, and high enzyme activities were observed in *Corallina officinalis*, *Corallina pilulifera* and *Amphiroa zonata*. The optimum pHs of the enzymes from coralline algae were around 6.0. The enzymes were specific for I^- and Br^-, and did not act on Cl^- and F^-. The enzymes purified from *Corallina pilulifera* and *Amphiroa ephedraea* catalyzed the brominations of phenol and *o*-hydroxybenzyl alcohol in the presence of Br^- and H_2O_2 to form the same product, 2,4,6-tribromophenol.

Haloperoxidases catalyze the halogenation of various compounds.[1] The chloroperoxidase purified from *Caldariomyces fumago*, has been studied since 1959[2,3]; it introduces iodine, bromine and chlorine atoms into organic compounds through an electrophilic substitution[4,5] or addition mechanism.[6] In our previous paper,[7] we also described an addition reaction catalyzed by the *C. fumago* enzyme: halogenation of several 2,3-unsaturated carboxylic acids such as *trans*-cinnamic acid and its derivatives.

Antibacterial halocompounds like bromophenols have been isolated from many marine algae.[8～11] Enzymes that halogenate organic compounds have been found in marine algae,[12,13] and bromoperoxidases have been purified from *Rhodomela larix*,[13] *Rhipocephalus phoenix*,[14] *Penicillus lamourouxii*[14] and *Penicillus capitatus*.[14,15]

In the course of our study of haloperoxidases, we found coralline algae with high bromoperoxidase activity. This paper describes some properties of bromoperoxidases of coralline algae including the bromination of phenol derivatives by the enzymes.

MATERIALS AND METHODS

Collection of algae. Various algae including 42 species of 34 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) and Hakodate (Hokkaido) in Japan from March to June in 1984. The samples, in ice-cold boxes, were immediately sent to our laboratory at Kyoto University, 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 ($\varepsilon = 19{,}900\ M^{-1}\ cm^{-1}$ 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 (U) 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.[16] The standard assay mixture contained 100 μmol of potassium phosphate buffer (pH 6.0), 5 μmol 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 *et al.*[17] The reaction mixture consisted of 150 μmol of potassium phosphate buffer (pH 7.0), 60 μmol 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.*[18] with bovine serum albumin as a standard.

Enzymatic reaction for the isolation of products from phenol compounds. The reaction mixture for the isolation of the products from phenol or *o*-hydroxybenzyl alcohol consisted of 10 mmol of potassium phosphate buffer (pH 6.0), 4 mmol of potassium bromide, 2 mmol of hydrogen peroxide, 2 mmol of each substrate and 100 units of bromoperoxidase in a total volume of 100 ml. The reaction was started by adding 0.5 mmol of hydrogen peroxide at 10-min intervals, and the mixture was kept at 30°C for 2 hr. A blank test was also performed without enzyme.

Reaction mixture analysis. The reaction mixture was extracted with the same volume of ethyl acetate and samples of the ethyl acetate layer (10～20 μl) were put on silica gel thin layer chromatography (TLC) plate. TLC was done with the following solvent systems (by volume): (a) benzene–*n*-hexane, 7 : 3, (b) benzene–ethyl acetate, 9 : 1, (c) benzene, (d) benzene–ethyl acetate–methanol, 4 : 1 : 1. The products and substrates were detected by ultraviolet-light-absorbing spots.

Product analysis. ^{1}H-NMR spectra were recorded with a JEOL-FX100 (100 MHz) spectrometer with tetramethylsilane as a reference. In Beam-electron impact mass spectrometry (EI-MS) was performed at 20 eV with a JEOL JMS-DX 300 mass spectrometer.

Chemicals. o-Hydroxybenzyl alcohol was bought from Aldrich Chemical Co., Inc., U.S.A. and *p*-hydroxybenzaldehyde from Nakarai Chemicals Ltd., Japan. Thin layer chromatoplates (TLC plates, Silica gel 60 F_{254}) were supplied by Merck Japan Ltd., Japan. DEAE-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Sweden. All other reagents used in this study were commercial products of analytical grade.

RESULTS

Distribution of bromoperoxidase in algae

As shown in Table I, bromoperoxidase activities were measured for 42 algal samples, including 34 genera, which were collected from the seasides of Japan. All the coralline algae 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 pilulipera*. Detectable activities were observed in *Gelidium amansii, Hypnea chararoides, Neodilsea yendoana* and *Codium fragile* except coralline algae. Figure 1 shows photographs of eight coralline algae with high enzyme activities.

pH optima of bromoperoxidases of coralline algae

The activity 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.

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_3^-. The results are given in Table II. 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 II. The maximum activities of all these enzymes were with bromide ion 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 cat-

TABLE I. BROMOPEROXIDASE ACTIVITIES OF VARIOUS ALGAE

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

Bromoperoxidase activity was not detected in the following algae: [Phaeophyta] *Colpomenia bullosa, Colpomenia sinuosa, Ecklonia kurome, Endarachne binghamiae, Papenfussiella kurome, 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.

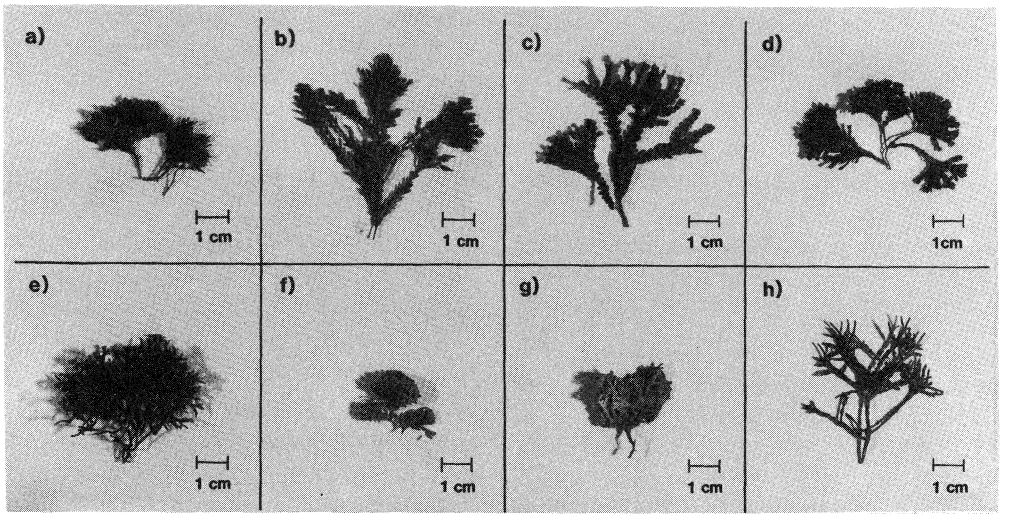

FIG. 1. Photographs of Coralline Algae Collected in Japan.

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

alase activity was detected in crude extracts of the eight coralline algae.

Partial purification of the enzymes from C. pilulifera and A. ephedraea

All of the purification was done at 0～5°C unless otherwise specified.

TABLE II. PROPERTIES OF BROMOPEROXIDASES OF EIGHT CORALLINE ALGAE

Algae	Halide specificity	Optimum Br^- conc. (mM)	Optimum H_2O_2 conc. (mM)
Corallina pilulifera	I^-, Br^-	≧75	0.5 ——5.0
Corallina officinalis	I^-, Br^-	≧75	0.5 ——4.0
Amphiroa zonata	I^-, Br^-	≧75	0.5 ——3.0
Amphiroa ephedraea	I^-, Br^-	≧75	0.5 ——4.0
Amphiroa misakiensis	I^-, Br^-	≧75	0.25——5.0
Bossiella cretacea	I^-, Br^-	≧75	0.75——4.0
Calliarthron yessoense	I^-, Br^-	≧75	0.5 ——4.0
Alatocladia modesta	I^-, Br^-	≧75	0.5 ——5.0

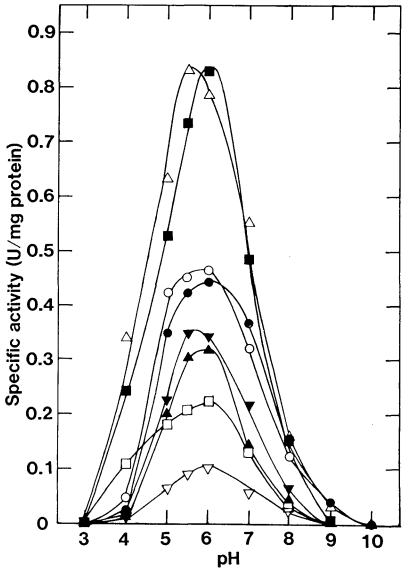

FIG. 2. pH Optima of Bromoperoxidases from Coralline Algae.

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

Step 1. Crude extract preparation. Each of the frozen algae (about 330 g wet weight) was suspended in 150 ml of 0.1 M 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 × *g*, 20 min).

Step 2. Ammonium sulfate precipitation. The crude extract was brought to 80% saturation with ammonium sulfate. After gentle stirring for 12 hr, the precipitate was harvested by centrifugation (9,000 × *g*, 20 min). The precipitate was dissolved in a minimal volume of 50 mM potassium phosphate buffer (pH 6.0), and dialyzed against 10 liters of the above buffer containing 50 mM ammonium sulfate. After dialysis, the precipitate was removed by centrifugation (9,000 × *g*, 20 min).

Step 3. DEAE-Sepharose column chromatography. The enzyme solution was put on a DEAE-Sepharose column (4.4 × 20 cm) equilibrated with the dialysis buffer. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of ammonium sulfate to 1.0 M (Fig. 3). Fractions with high enzyme activity were pooled and concentrated by salting out with ammonium sulfate at 80% saturation. The precipitate was collected by centrifugation (9,000 × *g*, 20 min), dissolved in 50 mM potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer.

By these procedures, 1,960 U of the enzyme were obtained from 1 kg of *C. pilulifera* (yield, 27%) and 580 U of the enzyme from 0.45 kg of *A. ephedraea* (yield, 57%). The enzymes were used for the bromination of phenol derivatives in the following experiments.

Enzymatic bromination of phenol compounds

The bromoperoxidases of *C. pilulifera* and *A. ephedraea* acted on phenol and *o*-hydroxybenzyl alcohol, but *p*-hydroxybenzaldehyde did not serve as a substrate of the enzymes. Figure 4 shows the thin layer chromatograms of the products from phenol (A)

and *o*-hydroxybenzyl alcohol (B) by the enzymes of *C. pilulifera* and *A. ephedraea*. Both enzymes synthesized the same products from either phenol or *o*-hydroxybenzyl alcohol.

Isolation and identification of the product from phenol

To isolate and identify the product from phenol, the reaction was done using the enzyme of *C. pilulifera*. After the reaction, 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 × 70 cm column) and eluted with solvent (b). The product was further purified by TLC with solvent (c). The product was extracted from the spot band (Rf=0.82) with ethyl acetate, concentrated under reduced pressure and rechrmatographed by TLC with solvent (a). The ethyl acetate extract from the spot band (Rf=0.68) provided 50 mg of faint yellowish powder: mp 86~

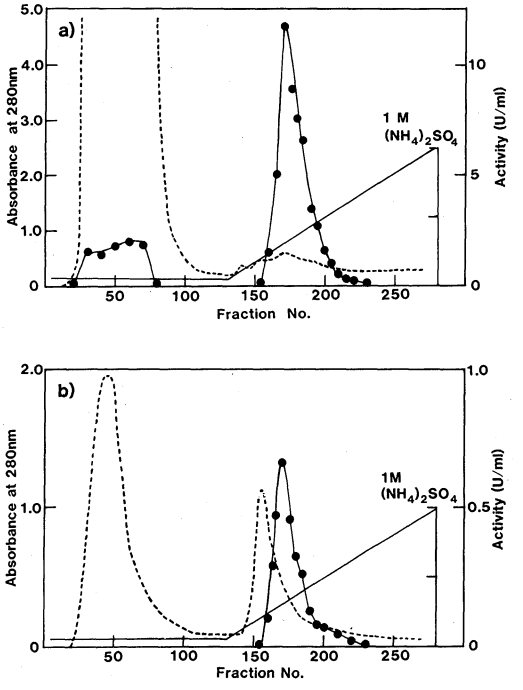

FIG. 3. DEAE-Sepharose Column Chromatography of Bromoperoxidase.

a) bromoperoxidase of *Corallina pilulifera*; b) bromoperoxidase of *Amphiroa ephedraea*. The flow rate was approximately 500 ml per hr, and 18 ml fractions were collected. ----, absorbance at 280 nm; ●—●, enzyme activity; ——, concentration of $(NH_4)_2SO_4$.

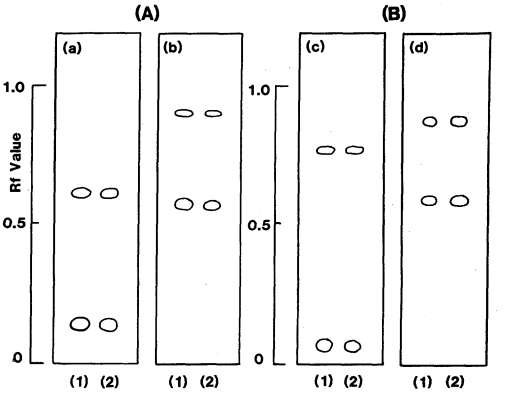

FIG. 4. Thin Layer Chromatograms of Products from (A) Phenol and (B) *o*-Hydroxybenzyl Alcohol.

The reaction was carried out using the enzymes of (1) *Corallina pilulifera* and (2) *Amphiroa ephedraea*. TLC plates were developed with solvent systems, (a) to (d), as described in MATERIALS AND METHODS. In all cases, the upper spots show the products and the lower spots the substrates.

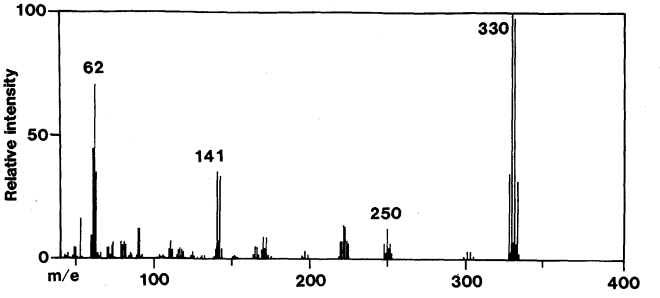

FIG. 5. Mass Spectra of the Products from Phenol and *o*-Hydroxybenzyl Alcohol (EI-MS).

The products had identical spectra.

87°C, ^{1}HNMR ($CDCl_3$) δ 5.88 (1H, s, –OH) 7.58 (2H, s, ArH), EI-MS *m/e* (M^+ 328, 330, 332, 334). The EI-MS spectra of the product (Fig. 5) had molecular ion peaks split into 1:3:3:1 distribution due to the isotopes of three bromine atoms. The NMR spectra and melting point (lit. 87~89°C)[19] were identical with authentic 2,4,6-tribromophenol. Hence, the product was identified as 2,4,6-tribromophenol.

Isolation and identification of the product from o-hydroxybenzyl alcohol

For the isolation and identification of the product from *o*-hydroxybenzyl alcohol as well, the reaction was done under the same conditions as for phenol. 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 × 70 cm column) with solvent (d) as an eluent. The product was put on TLC plates and developed with benzene, and then extracted with ethyl acetate from the spot band (Rf=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}HNMR ($CDCl_3$) δ 5.88 (1H, s, –OH) 7.58 (2H, s, ArH), EI-MS *m/e* (M^+ 328, 330, 332, 334). The melting point, NMR spectra and EI-MS spectra were identical with the product from phenol and 2,4,6-tribromophenol. Hence, the product from *o*-hydroxybenzyl alcohol was identified as 2,4,6-tribromophenol. It indicated that the hydroxymethyl group was also replaced by bromine in the enzyme reaction.

DISCUSSION

Haloperoxidase is an interesting enzyme that halogenates many compounds including substituted phenols,[4,20] β-diketones,[3] alkene,[6] alkyne,[21] steroids[22,23] and several α,β-unsaturated carboxylic acids.[7] Because of the natural occurrence of halocompounds, haloperoxidase has been of interest in recent years. The enzyme has been reported in only two microorganisms, *Caldariomyces fumago*[2] and Streptomyces phaeochromogenes,[24] and a few algae including *Penicillus*,[14,15] *Rhipocephalus*[14] and *Rhodomela*.[13] This paper is the first report 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.[25] Thus, *Corallina pilulifera* seems to be an excellent source of bromoperoxidae.

The properties of the enzymes extracted from the eight coralline algae shown in Table I were quite similar. The optimal pHs of the enzymes were around 6.0. The chloroperoxidase of *C. fumago* and bromoperoxidases of *Rodomela larix* and *Penicillus capitatus* have their maximum activities in more acidic pHs, pH 2.75 for *C. fumago*,[2] pH 4.4 for *R. larix*,[13] and pH 4.0 for *P. capitatus*.[14]

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.[15]

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. capitatua*.[14] Compared with these other enzymes, the enzymes of coralline algae seem to be very resistant to the inhibitory effect of peroxide.

Manley *et al.* suggested that brominated phenols were synthesized from L-tyrosine in cell-free homogenates of *Odonthalia floccosa* (Rhodophyceae).[12] Ahern *et al.* also reported that brominated compounds were detected in the products from *p*-hydroxybenzyl alcohol by

the enzyme of *R. larix*.[13] But the pathway of synthesis of bromophenols in algae is not known. We have shown that phenol compounds such as phenol and *o*-hydroxybenzyl alcohol were suitable substrates of bromoperoxidase from coralline algae, so the participation of bromoperoxidase in the production of bromophenols in nature is possible. The physiological function of halocompounds in marine organisms and ocean environment is unknown. Detailed properties of the enzyme of *C. pilulifera* will be reported elsewhere.

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