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Purification and Characterization of a Monohalomethane-producing Enzyme *S*-adenosyl-L-methionine: Halide Ion Methyltransferase from a Marine Microalga, *Pavlova pinguis*

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A monohalomethane-producing enzyme, *S*-adenosyl-L-methionine-dependent halide ion methyltransferase (EC 2.1.1.-) was purified from the marine microalga *Pavlova pinguis* by two anion exchange, hydroxyapatite and gel filtration chromatographies. The methyltransferase was a monomeric molecule having a molecular weight of 29,000. The enzyme had an isoelectric point at 5.3, and was optimally active at pH 8.0. The K_m for iodide and SAM were 12 mM and 12 μM, respectively, which were measured using a partially purified enzyme. Various metal ions had no significant effect on methyl iodide production, suggesting that the enzyme does not require metal ions. The enzyme reaction strictly depended on SAM as a methyl donor, and the enzyme catalyzed methylation of the I^-, Br^-, and Cl^- to corresponding monohalomethanes and of bisulfide to methyl mercaptan.

Key words: *S*-adenosyl-L-methionine; halide ion methyltransferase; marine microalga; monohalomethane; *Pavlova pinguis*

Considerable amounts of halomethanes such as CH_3Cl, CH_3Br, CH_3I, $CHBr_3$, CH_2Br_2, and $CHClBr_2$ occur in natural sources. These ubiquitous gasses were found in marine environments at high concentrations.[1–7] Based on the measurements of these gasses in coastal air and seawater, many researchers proposed that these gasses observed in the ocean were almost a natural source.[1] It has been reported that some kelps living in coastal seawater accumulate and produce a variety of halomethanes.[3,4,7,8] Consequently, the marine environment has been recognized as a main source of natural halogenated compounds.

The mechanism producing di- and trihalomethanes, such as CH_2Br_2, $CHBr_3$, and $CHClBr_2$, involves a bromoperoxidase reaction in marine macroalgae. The enzyme produces di- and trihalomethanes by its reaction with some organic ketoacids: oxalacetate, phosphoenol pyruvate, pyruvate, itaconate, and 2-ketoglutarate, and halide ions and hydrogen peroxide.[5,9] The enzyme catalyzes a two-electron oxidation of halogen, producing hypohalous acid, which can react with biologically constitutive organic acids or natural fluvic acid.[10,11] The reaction mediated by the enzyme was suggested to be the main route for the atmospheric di- and trihalomethanes.[5]

On the contrary, the origin of naturally occurring monohalomethanes, which play a pivotal role in the effect of halogens on atmospheric chemistry, have been discussed for a long time, especially focusing on the conservation of the ozone layer. In early research studies, the gasses were reported in marine environments at high concentrations.[1,4,6] In particular, methyl iodide (CH_3I) in seawater in an Irish *Laminaria digitata* kelp bed was 1000 times higher than that of pelagic Atlantic Sea water.[1] Hence, the ocean had been considered a main source of the monohalomethanes. The proposed but not verified natural monohalomethane-producing mechanisms are as follows: a nucleophilic reaction of Cl^- with CH_3I in seawater,[12] the decompositon of sulfonium salts such as dimethylsulfoniopropionate (DMSP) with halide ions,[13] and another abiotic mechanism.[14] Wuosmaa and Hager reported that a chloride methyltransferase from the marine red alga, *Endocladia muricata*, could carry out the transfer of a methyl group from *S*-adenosyl-L-methionine to a halide as follows.[15]

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Abbreviations: SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosylhomocysteine; HMT, halide ion methyltransferase; DMSP, dimethylsulfoniopropionate

$$X^- + SAM \dashrightarrow CH_3X + SAH$$

The enzyme has been found in a variety of organisms, such as higher plants,[16-18] macroalgae,[15,19] and a white-rot-fungus.[20,21]

Itoh *et al.* reported that a marine phytoplankton, *Pavlova gyrans*, evolves CH_3Br and CH_3I at a high level, found a novel SAM: halide ion methyltransferase (HMT) in the cell-free extract, and characterized the enzyme.[19] They also reported other high CH_3I-producing macroalgae, *Papenfussiella kuromo* and *Sargassum horneri*, and estimated the global emission rates of monohalomethanes from these marine organisms.[5,19] However, purification of the enzyme could not be accomplished because of its instability and the very small quantity in the crude extract. Therefore, the detailed properties of the enzyme remained unclear.

According to a further investigation of various marine microalgae, we found that HMT activity occurred in other *Pavlova* species. In particular, *Pavlova pinguis* CCAP 940/2 had a high level and stable HMT activity. In this paper, we report the purification and characterization of a CH_3I-producing enzyme, HMT, from the marine microalga *P. pinguis*.

Materials and Methods

Chemicals. *S*-Adenosyl-L-methionine and *S*-adenosylhomocysteine were bought from Sigma-Aldrich, USA. KI and authentic CH_3I were from Nacalai Tesque, Japan. DEAE-Toyopearl was from Tosoh, Japan. Authentic CH_3Cl and CH_3Br were obtained from Supelco, USA. SDS-PAGE molecular weight standards (LMW Kit E) and carrier ampholite were purchased from Amersham Pharmacia Biotech, USA. Dimethylsulfoniopropionate (DMSP) was synthesized by the methods of Challenger *et al.*[22] The pI marker kit was form Oriental Yeast, Japan. All other chemicals were of the highest purity available.

Microalga cultivation. *P. pinguis* (CCAP 940/2) was purchased from the CCAP (Culture Collection of Algae and Protozoa), UK. The alga was cultured in 20 ml of natural sea water containing Marine Enrichment Basal Medium (Sigma-Aldrich) at 20°C 200 $\mu E/m^2/s$ (12 h light and 12 h dark) for 10 days. These cultured algae were transferred into 3 liters of the medium mentioned above with sterile air bubbling (ca. 25 ml/min) with a filter (0.2 μm). Subsequent large-scale culture was done for 10 days in a 20-liter vessel with the 3-liter culture. The final cell concentration was $2\text{–}5 \times 10^7$ cells/ml-medium.

Measurement of SAM: halide ion methyltransferase activity. Because I^- was the most available substrate among halides, it was used as the halide ion substrate throughout the purification procedure. Formation of methyl iodide was assayed with a Shimadzu gas chromatograph GC-14A with an ECD (electron capture detector) (Kyoto, Japan), and methyl chloride, methyl bromide, and methyl mercaptan were measured with a Shimadzu QP-5000 GC-mass spectrometer (MS) (quadruple polar type). The enzyme activity was assayed in a 10-ml mixture containing 0.2 μM SAM, 20 mM halides, or 20 mM $(NH_4)_2S$ for bisulfide methylation. The enzyme reaction was started by the addition of 300 μl of enzyme solution. The mixture was incubated in a vial sealed with a silicon septum, then shaken at 170 rpm at 30.0°C for 30 min. The head space gas was drawn and injected into a DB-624 capillary column (0.3 mm × 30 m, 3 μm film, J & W Scientific, CA, USA) GC-ECD, or a DB-VRX capillary column (0.25 mm × 60 m, 1.4 μm film, J & W Scientific, CA, USA) in GC-MS. The carrier gas flow rate was 0.28 ml/min (GC-ECD) or 7.9 ml/min (GC-MS). The products were measured by peak area and identified by comparison of their retention times with those of authentic methyl halides, which were used to calibrate the instrument. The detection limits of the GC-ECD were 10 ng/ml for CH_3I, and those of the GC-MS were 50 ng/ml for CH_3Cl, 20 ng/ml for CH_3Br, and 50 ng/ml for CH_3SH. The column temperature was 50°C for CH_3I, 50°C for CH_3Br, 40°C for CH_3Cl, and 50°C for CH_3SH. The data presented here for the properties of HMT was the average of at least each triple measurements.

Buffers. The buffers used were as follows: MES (pH 6.5); Tris-HCl (pH 8.0); potassium phosphate (KPi) buffer (pH 7.8); HEPES (pH 7.0). All buffers contained 10% (v/v) glycerol and 1 mM KBr unless stated otherwise.

Purification of HMT. All manipulations were done at 4°C unless stated otherwise. The algal cells cultivated on a large scale were harvested by centrifugation at 10,000 × *g* for 10 min. Thirty-two grams (wet weight) of the algae were suspended in 300 ml of 20 mM MES buffer (pH 6.5) with 1 mM dithiothreitol (DTT). The cell suspension frozen by liquid nitrogen was disrupted with a mortar and pestle, extracted with 300 ml of MES buffer, and centrifuged at 10,000 × *g* for 20 min. To remove phenolic compounds, 10% (w/v) polyvinylpolypyrrolidone was added to the recovered supernatant. The polysaccharides existing in the crude extract were then removed by 10 mM $BaCl_2$ treatment.[23] After centrifugation at 10,000 × *g* for 40 min, the resulting supernatant was dialyzed against the 20 mM MES buffer containing 1 mM DTT. The enzyme solution was put on a DEAE-Toyopearl anion exchange column (25 mm × 220 mm) equilibrated with the same buffer. The column was washed with the same buffer, and then the enzyme was eluted with a 0 to 0.4 M NaCl linear

gradient. The active fractions were pooled and concentrated to a proper volume with Centriprep-10 (Millipore, Japan). After dialysis against 50 mM Tris-HCl buffer, the enzyme solution was applied to FPLC (Amersham, Pharmacia Biotech, Japan) with a Poros HQ/L (7.5 mm × 100 mm, PerSeptive Biosystems, MA, USA) equilibrated with 50 mM Tris-HCl buffer. After collecting the fractions with the activity, the solution was concentrated and dialyzed against 50 mM KPi buffer. The enzyme solution was then put on a Poros HQ/M column (4.6 mm × 100 mm, PerSeptive Biosystems, MA, USA) equilibrated with the KPi buffer. The HMT active fractions passing through the column were collected and concentrated. The enzyme solution was put on a Hiload Superdex 200 pg column (16 mm × 600 mm) equilibrated with the same buffer, and the elution was done at a flow rate of 0.5 ml/min. The enzyme fractions were pooled and dialyzed against 10 mM KPi buffer (pH 6.0), and put on a HAP-C-BEADS hydroxyapatite column (16 mm × 100 mm, Sangi, Japan) equilibrated with the same buffer. The column was washed with the buffer, and the enzyme was then eluted with a linear gradient of 10 to 1000 mM KPi buffer at a flow rate of 3 ml/min. The enzyme fractions were collected and dialyzed against 50 mM HEPES buffer, put on the Poros HQ/M column which had been equilibrated with same buffer. The enzyme was eluted with a linear gradient of 0 to 500 mM NaCl at a flow rate of 3 ml/min. The active fractions were pooled and dialyzed against 50 mM HEPES buffer; then the enzyme was again put on the Poros HQ/M column, which had been equilibrated with HEPES buffer. The enzyme was eluted with a linear gradient of 0 to 500 mM NaCl at a flow rate of 3 ml/min.

Molecular weight measurement and electrophoresis. The molecular weight of the native HMT was measured by gel filtration on a HiLoad Superdex 200 pg column (16 mm × 600 mm) calibrated with the following markers: cytochrome *c* (MW 12,500), chymotrypsinogen A (MW 25,000), ovalbumin (MW 43,000), albumin (MW 68,000), aldolase (MW 158,000), and catalase (MW 240,000). For each purification step, the denatured enzyme samples were analyzed by SDS-PAGE using 12% (w/v) gels,[24] and stained by Coomassie Brilliant Blue R-250 or a silver staining kit, Silvest Staine (Nacalai Tesque, Japan). The molecular weight of the denatured enzyme was compared with the migration distance of standard proteins. Isoelectronic focusing on acrylamide gel was done by the method of Itoh *et al.*[25] The electrophoresis was done using a 5% gel containing 2% (w/v) Ampholines (9 (pH 3.5–10):1 (pH 3.5–5)). The pI of the enzyme was estimated by comparison with the migration of standard proteins.

Protein assay. Protein concentration was estimated by measuring the absorbance at 280 nm or by using the Bio-Rad Protein Assay kit with bovine serum albumin (Sigma-Aldrich) as the standard protein according to the manufacturer's protocol.

Results

Purification of HMT from P. pinguis

The purification of HMT from *P. pinguis* is summarized in Table 1. The HMT was purified to homogeneity through the seven steps of column chromatography. Addition of $(NH_4)_2SO_4$ to the enzyme solution caused almost complete inactivation; therefore, this step was avoided for the enzyme concentration. Although we tested an *S*-adenosylhomocysteine-ligand or a dye-ligand affinity chromatography in a preliminary experiment, it was not effective for purification of HMT. The HMT showed no interaction with the hydrophobic matrices. One mM KBr and 10% glycerol were required to maintain HMT activity during purification. However, the HMT activity still decayed to about 30% of the original value after each anion exchange column chromatography. After DEAE-Toyopearl column chromatography, the concentrated active fraction was put on the Poros HQ/L column in Tris buffer. Subsequently, the HMT was passed through the Poros HQ/M gel matrix with KPi buffer, where the contaminating proteins were adsorbed to the gel. The resulting active fractions were filtered on a Superdex 200 pg gel column, in which the three protein peaks were observed (Fig. 1(a)). The active fraction was further purified by hydroxyapatite column chromatography. Poros HQ/M column chromatography using HEPES buffer was duplicated with an FPLC system (Fig. 1(b)). The enzyme samples from the various purification steps were put through SDS-PAGE and are displayed in Fig. 2. Finally, HMT

Table 1. Purification of Halide Ion Methyltransferase from *Pavlova pinguis*

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude extract	355.75	52669	148.1	100.0	1
PVPP	323.65	44989	139.0	85.4	0.9
$BaCl_2$	280.30	53321	190.2	101.2	1.3
DEAE-Toyopearl	66.83	17821	266.7	33.8	1.8
1st Poros HQ/L	19.51	6178	316.5	11.7	2.1
2nd Poros HQ/M	10.20	3753	367.8	7.1	2.5
Superdex 200 pg	3.26	972	298.0	1.9	2.0
Hydroxyapatite	0.86	354	410.0	0.7	2.8
3rd Poros HQ/M	0.08	81	983.7	0.2	6.6
4th Poros HQ/M	0.02	30	1789.0	0.1	12.1

[a] One unit of halide ion methyltransferase is defined as the amount of enzyme that catalyzes the conversion of 1 pmol of substrates under the assay conditions. The HMT activity was measured by methyl iodide production.

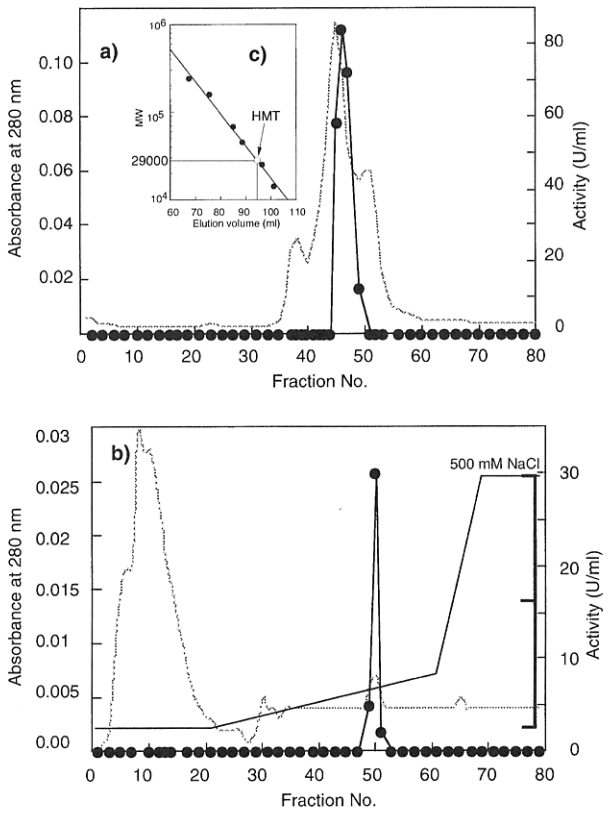

Fig. 1. Elution Profile of HMT from Superdex 200 pg (a) and 4th Poros HQ/M with HEPES Buffer (b).

Symbols: solid line, concentration of NaCl in the elution buffer, HMT activity (●); dashed line, the absorbance at 280 nm of the protein. The insert (c) identifies the molecular weight markers used for calibration.

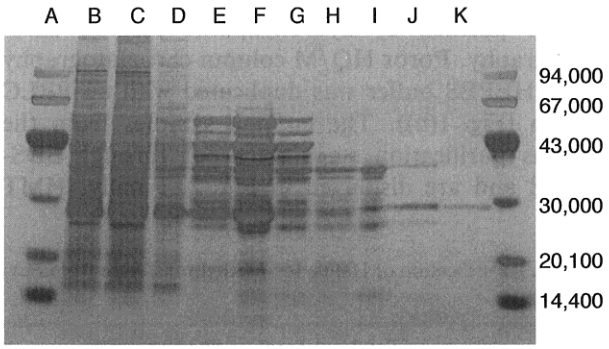

Fig. 2. SDS-PAGE (12%) of the HMT on Various Purification Steps.

Lane A, molecular weight marker; *lane B*, crude extract (5.3 μg); *lane C*, polyvinylpolypyrrolidone treatment (5.7 μg); *lane D*, 10 mM $BaCl_2$ treatment (5.4 μg); *lane E*, DEAE-Toyopearl (5.5 μg); *lane F*, 1st Poros HQ/L on Tris buffer (7.5 μg); *lane G*, 2nd Poros HQ/L on KPi buffer (5.6 μg); *lane H*, Superdex 200 pg (3.4 μg); *lane I*, Hydroxyapatite (2.1 μg); *lane J*, 3rd Poros HQ/M on HEPES buffer (0.5 μg); *lane K*, 4th Poros HQ/M on HEPES buffer (0.3 μg). The gel was stained with a silver staining kit.

from *P. pinguis* was purified approximately 12-fold with a yield of 0.06%. According to the SDS-PAGE and gel filtration chromatography (Fig. 1(c)) of the purified enzyme, the molecular weight of HMT was estimated to be 29,000, consisting of a monomeric protein. The pI of the purified enzyme was estimated to be 5.3 (data not shown).

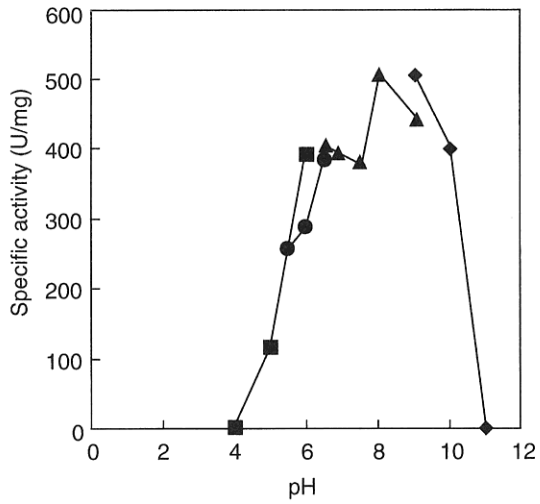

Fig. 3. Effects of pH on the HMT Activity.

The enzyme activities were measured in the following 50 mM buffers: citrate (■), MES (●), Tris-HCl (▲), glycine-NaOH (◆). The halide ion methyltransferase activity was expressed by the amounts of methyl iodide production.

Effects of pH on HMT activity

The pH dependency of HMT activity is shown in Fig. 3. The maximum halide methyltransferatse activity was observed at pH 8.0. The enzyme activity rapidly declined below pH 5 and above 10; however, it had fairly high activities around pH 8.0.

Effects of various compounds on HMT activity

The purified HMT was not obtained in an amount sufficient for biochemical and kinetic analyses. They were done using a partially purified HMT sample which was obtained after Superdex 200 pg chromatography. As shown in Table 2, various metal ions did not affect the HMT activity, suggesting that the HMT probably does not require such metal ions. Halide ion methyltransferases from higher plants,[18] fungi,[20] marine macroalgae,[15] and halide/bisulfide methyltransferase from cabbage (*Brassica oleracea*)[17] did not show a dependency on any metal ions. HMT was similar to those halide ion methyltransferases on this point. Sulfhydryl reagents including *p*-CMB had no effect on the enzyme activity, indicating that the SH group does not participate in the halide methylation.

The halide/bisulfide methyltransferase can catalyze the methylation of not only halide ions but also bisulfide.[17,26,27] Attieh *et al.* reported that the enzyme from cabbage can catalyze a methylation of bisulfide (or thiocyanate) with SAM to yield methyl mercaptan or methyl thiocyanate;[26,27] and the enzyme was

Table 2. Effects of Various Compounds on the Halide Ion Methyltransferase Activity

Compound	Concentration (mM)	Relative activity[1] (%)
none	—	100
$CaCl_2$	1	119
$BaCl_2$	1	102
$MgSO_4$	1	75
$MnCl_2$	1	100
$CoCl_2$	1	82
$NiCl_2$	1	62
$ZnCl_2$	1	86
$FeCl_3$	1	103
EDTA	1	84
	5	68
2-Mercaptoethanol	1	84
Dithiothreitol	5	72
	1	84
α,α'-Dipyridyl	1	110
	5	102
p-Chloromercurybenzoate	0.5	70
Monoidoacetate	1	84
	5	72
NaN_3	1	73
	5	68

[1] Enzyme activity are expressed as the percentage of methyl iodide production under the standard asasy conditions.

named thiol methyltransferase.[27] To test whether bisulfide (HS^-), thiocyanide (SCN^-), and cyanide (CN^-) act as a methyl acceptor of HMT, the influence of the three ions on the enzyme was examined. As shown in Table 3, SCN^- and HS^- showed a significant inhibition of methyl iodide production and CN^- a weak inhibition. On the contrary, halide ions did not have significant effects of the CH_3I-producing reaction of HMT up to 100 mM. These results imply that HMT may prefer those anions to halides as its methyl acceptor.

Substrate specificity and kinetic analysis of HMT

The methyl donor specificity of HMT is shown in Table 4. The enzyme had a high specificity for SAM. Other methyl donors, including dimethylsulfoniopropionate (DMSP), betaine, L-methionine, and L-methylmethionine, which are known to be physiological methyl donors, did not serve as a substrate. The methyl donor specificity of HMT was similar to those of chloride methyltransferases from fungi,[20] plants,[17,18] and algae.[15,19] Furthermore, HMT catalyzed the conversions of Cl^-, Br^-, and I^-, using them as methyl acceptors for the corresponding monohalomethane (Table 4). The reaction rates of each product were 2.5 for CH_3Cl, 16.7 for CH_3Br, and 148.1 pmol/min/mg-protein for CH_3I. The enzyme also produced CH_3SH from HS^- and SAM, and the production rate was 155.7 pmol/min/mg-protein. A weak non-enzymatic reaction was observed for CH_3I and CH_3SH; therefore, the value was subtracted from the enzymatic reaction.

Table 3. Effects of Monovalent Anions on Halide Ion Methyltransferase Activity

Anion	Relative activity (%)	
	10 mM	100 mM
F^-	113.3[1]	133.3
Cl^-	121.0	129.0
Br	116.5	112.7
SCN^-	40.4	8.1
SH^-	15.7	0.0
CN^-	85.7	0.0
$HCOO^-$	109.4	125.5

[1] Enzyme activity are expressed as the percentage of methyl iodide production under the standard assay conditions. Each of the monovalent anions was added to the standard assay mixture at the concentration indicated.

Table 4. Substrate Specificity of Halide Ion Methyltransferase

Acceptor	Methyl donor	Production rate of methyl compounds (pmol/min/mg-protein)
Cl^- [a]	*S*-Adenosyl-L-methionine	2.5
Br^- [a]	*S*-Adenosyl-L-methionine	16.7
I^- [a,b]	*S*-Adenosyl-L-methionine	148.1
HS^- [a]	*S*-Adenosyl-L-methionine	155.7
I	*S*-Methylmethionine-Cl	0.0
I^-	*S*-Methylmethionine-I	0.0
I	L-Methionine	0.0
I^-	Dimethylsulfoniopropionate	0.0
I	Betainine	0.0

[a] Those anion methylation reactions were measured by GC-MS under 100 mM anion and 300 μM SAM for 200 min, 30°C, using the crude extract.

[b] The reaction was measured by GC-ECD with standard assay conditions.

The apparent K_m for iodide ion calculated from the Lineweaver-Burk plot was 12 mM, and that for SAM was 12 μM (Fig. 4). Under the assay conditions we adopted, the Michaelis constants for Cl^-, Br^-, and HS^- were not defined because of the low sensitivities of the three products on GC-MS. The HMT activity was not inhibited at high iodide concentrations, which was observed for the halide ion methyltransferase from *Pavlova gyrans*.[19]

Discussion

We previously showed that a marine, microalga, *Pavlova gyrans*, and a few macroalgae have monohalomethane producing activity, and we estimated the environmental contribution of those organisms to tropospheric chemistry.[5,19] However, the macroalgal HMT was too unstable to be purified. By screening for a more stable enzyme, we found that the HMT in *Pavlova pinguis* was more stable than *P. gyrans* and that its stability was increased by the addition of 1 mM Br^- ions and 10% glycerol in the buffer. Based on these findings, we attempted the purification of

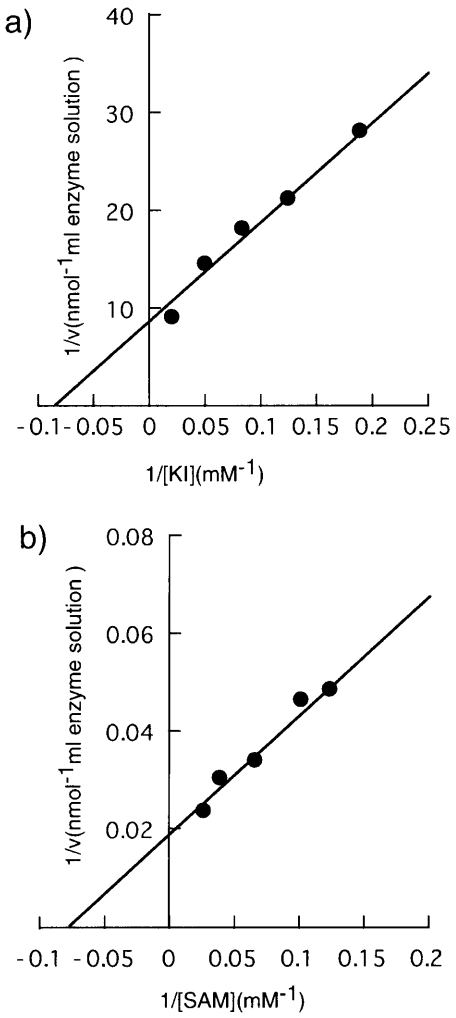

Fig. 4. Lineweaver-Burk Plots for the Reaction the HMT from *P. pinguis*.

The halide methylation was measured with (a) varying amounts of KI as indicated at 2 μmol SAM, and (b) SAM as indicated at 0.2 mmol KI, in a total volume of 10 ml. The enzyme reaction started by the addition of 200 μl of enzyme solution (0.05 mg of protein).

HMT from *P. pinguis*. However, the purification was difficult because of the very low content of HMT in the crude extract and the limitations of suitable column matrices. The HMT activity was only 1,650 units per g of *P. pinguis* cells, which was approximately 0.07% of that of halide/bisulfide methyltransferase in cabbage.[17] In addition, HMT became unstable as the purification steps proceeded. Therefore, we did the purification with a careful combination of seven column chromatographies and successfully purified the enzyme to homogeneity. Gel filtration column chromatography was efficient in removing the contaminant proteins which could not be separated by anion exchange chromatographies (Fig. 2, *lane H*).

The K_m estimated for SAM (12 μM) is similar to those of *P. gyrans* (63 μM),[19] the marine red alga, *Endocladia muricata* (16 μM),[15] cabbage (30 μM),[17] the white-rot fungus, *Phellinus pomaceus* (4.5 μM),[20] and a halophytic plant *Batis maritima* (30 μM).[28] The molecular weight of HMT (29,000) was also similar to those of other HMTs from *E. muricata* (20,000–25,000), cabbage (28,000), and *B. maritima* (29,000).[15,17,18] No dependency of HMT activity on metal ions was reported for those originating from terrestrial plants[17] and fungi.[20] Although the primary structure of the enzyme is not clear, some amino acid sequences were analyzed using the digested HMT (data not shown). Using this information, we are trying to clone the cDNA gene.

Many researchers reported that the major atmospheric monohalomethanes, especially CH_3I, originated from the marine environment[1-8] for a long time. However, recent research studies revealed that the evolution of gasses including CH_3Cl and CH_3Br is not mainly from the ocean.[16,21,29-32] This knowledge suggests that the major biogenic monohalomethanes originate from terrestrial organisms.[33] However the remaining amounts of monohalomethanes, especially CH_3I, are emitted from marine environments,[34] mainly from marine macroalgae and microalgae.[35-37] Therefore monohalomethanes produced by such marine algae are partly related to the atmospheric concentrations of those gasses. In this study, we could not detect a monohalomethane production from the intact algal cells (data not shown). Biogenic di- and trihalomethanes formation by marine macroalga shows a dramatic alteration through the year,[9] which is depending on the bromoperoxidase activity *in vivo*. Itoh *et al.* showed that the seasonal variation is regulated by the specific activity of the enzyme.[38] The monohalomethane-producing activity of *P. pinguis* may be controlled by similar regulation mechanism. Furthermore, a taxonomic distribution in marine microalgae having the HMT activity has not been well known until now. Therefore, it was rather difficult to accurately estimate the amounts of biogenic monohalomethanes from the marine environment.

As shown in Table 3, the CH_3I-producing acitivity was not inhibited by other halide ions (up to 100 mM), and the activity of the enzyme showed higher preference for $I > Br > Cl$ (Table 4), suggesting that the physiological role of this enzyme is to produce methyl iodide. Other halide methyltransferases has also shown similar tendencies; $Cl < Br < I$.[21] Halide methyltransferases catalyze a nucleophilic substitution reaction of the halide anions with SAM. The reaction was proposed to proceed in an ordered bi-bi mechanism.[17] Initially, the electron of SAM bound at the active site is withdrawn, and then the stimulated SAM increased the positive charge on the methyl moiety. The positively charged methyl group is attacked by nucleophic compounds such as halide, bisulfide, and so on. Consequently, the specificity of halide methyltran-

sferases depends on the electrophilicity for SAM at the active site. Ni *et al.* suggested that the chloride methyltransferase from *B. maritima* may be related to a salt-tolerant system with Cl^- excretion.[18,28] Although our purified enzyme could produce CH_3Cl, the production rate was very low compared with methyl iodide. The result suggests that the HMT is not responsible for Cl^- excretion from internal tissue. In cabbage, halide/bisulfide methyltransferase probably plays a role in detoxifying monovalent anions, such as SCN^-, HS^-, and aromatic thiols, in the degradation of glucosinolate.[26,27] The HMT of *P. pinguis* also catalyzed the methyl transfer reaction to HS^- to form CH_3SH, which was same reactivity for I^-. The methylation of CN^- and SCN^- could not be detected due to the detection limits. However, the inhibitions of methyl iodide production by CN^- and SCN^- (Table 3) suggested that those anions might serve as methyl acceptor substrates. Thus, the physiological role of HMT is closely allied to cabbage enzyme, thiol methyltransferase.

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