Effect of Salts on Enzymatical Production of Dimethyl Sulfide from *Gyrodinium cohnii*

Hajime KADOTA* and Yūzaburō ISHIDA**

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As we reported in the previous paper, unicellular algae of marine origin often produced a large amount of DMS*** during the growth, but the fresh water algae did not produce this compound¹⁾. It was also found that the intracellular precursor of DMS in unicellular marine algae, such as *Gyrodinium cohnii*, was DMPT²⁾.

These facts suggest that the production of DMS during the growth is a characteristic of marine algae, and that DMPT plays a certain important role³⁻⁶⁾ in the metabolism of unicellular algae which live in the water of high salt concentration.

The present work was undertaken in an attempt to clarify the physiological significance of the production of DMS in unicellular algae inhabiting the sea. For this purpose the effect of salts on the production of DMS or the cleavage of DMPT in *G. cohnii* was studied biochemically.

Materials and Methods

Organism. *G. cohnii*, a heterotrophic unicellular alga of marine origin⁷⁾, was used throughout the work. A large amount of DMPT is contained in the cells of this alga.

Culture medium. A chemically defined medium reported by PROVASOLI⁷⁾ was employed.

Substrate for enzymatic experiments. DMPT labeled with ³⁵S (³⁵S-DMPT) was prepared from the cells of *G. cohnii* which was grown in the medium containing Na₂³⁵SO₄. The ³⁵S-labeled cells were harvested by centrifugation and extracted with 5% cold PCA. The extract was passed through an Amberlite IR 120 (H⁺) column, and then eluted with 2N HCl. After removing HCl by evaporation, the solution was passed through an Amberlite IRA 400 (OH⁻) column. The effluent, after acidified with HCl, was further treated with Norit A and evaporated *in vacuo* to a syrup. This syrup was used as a sample of ³⁵S-DMPT, after being dehydrated with ether.

Non-labeled sample of DMPT was synthesized using a method based on that of MAW⁸⁾.

Preparation of cell free extract and crude enzyme. The cells harvested were suspended in an aliquot of 1/10 MCILVAINE buffer (pH 5.1), and disintegrated by use

* Research Institute for Food Science, Kyoto University, Kyoto, Japan.

** Department of Fisheries, Kyoto University, Maizuru, Japan.

*** Abbreviations used are; DMS, dimethyl sulfide; DMPT, dimethyl-β-propiothetin; ACA, acrylic acid; PCA, perchloric acid; pCMB, p-chloromercuribenzoic acid; IAA, iodoacetamide; Hcys, homocysteine; ETG, 2-mercaptoethanol.

of a sonic oscillator. The sonicate was centrifuged at $3,000 \times g$ for 15 min. at 0°C . The supernatant solution thus obtained was used as a preparation of the cell free extract.

To the cell free extract, saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 5.1) was added to 0.25 saturation. The resulting precipitate was discarded by centrifugation at 9,000 rpm for 20 min. Saturated $(\text{NH}_4)_2\text{SO}_4$ was again added to the supernatant solution to 0.5 saturation. The precipitate was collected by centrifugation at 9,000 rpm for 20 min. The precipitate, after being dialyzed against MCLVAINE buffer (pH 5.1), was dissolved in an aliquot of the buffer. This solution was used as a preparation of the crude enzyme. The specific activity of this preparation was 5.5 times higher than that of the cell free extract.

Reaction system. The reaction mixture consisted of 0.5 ml of the cell free extract, 0.1 ml of the ^{35}S -DMPT (50,000 cpm) and 5.0 ml of MCLVAINE buffer containing 0.45 M NaCl (final pH 6.2), unless otherwise described.

Determination of DMS, DMPT and ACA. The reaction mixture was incubated with aeration of argon gas at 27°C for 1 hour, and DMS evolved was trapped by 3% HgCl_2^{11} . Radioactivity of DMS trapped was determined by use of a Packard Tri-Carb liquid scintillation counter. To stop the reaction PCA was added to the mixture so as to be 5% in the final concentration.

Determination of DMPT was made by use of a thetin-reineckate method based on that of CANTONI *et al.*⁶⁾. ACA formed in the reaction system was extracted with ether and determined by a SHIMADZU model GC-1C gas chromatograph, equipped with brass tube containing Polyester FA²³⁾.

Results

Evolution of DMS by the cell free extract. In a preliminary experiment evolution of a small amount of DMS from the resting cells was observed, but the amount of DMS produced was comparable to that from the cells inactivated by heating. It was also found that when the cells were incubated with ^{35}S -DMPT, the radioactivity did not incorporate into the cells. It was suggested that this negative result was due to the impermeability of the cell membranes to certain substrates, as was reported with S-adenosylmethionine in yeast cells^{9,10)}. In this experiment, therefore, the cell free extract was used instead of the resting cells.

Table 1. Production of DMS from ^{35}S -DMPT by cell free extract of *G. cohnii* treated with or without heat.

Preliminary treatment of cell free extract	DMS evolved (cpm)
none	27,600
heated at 100°C for 10 min.	70

To ascertain whether or not the evolution of DMS is enzymatical the evolution of DMS from ^{35}S -DMPT by the extract was compared with that by the heated extract (Table 1). These data suggest that DMS is enzymatically evolved in this organism.

Effect of pH and temperature on enzymatic evolution of DMS. The effect of pH value on the rate of evolution of DMS by the extract was examined, using ^{35}S -DMPT as the substrate. The pH value of the reaction mixture was adjusted by use of 1/10 MCLVAINE buffer (pH 4.5–7.7). The data obtained are shown in Fig. 1. Approximately the same results were obtained with the phosphate buffer. The pH optimum for DMS evolution was 6.0 to 6.5. Although DMS was non-enzymatically produced from DMPT in alkaline solution, such a reaction did not take place in the mixture, of which the pH value was lower than 8.0.

To determine the effect of pH value on stability of the DMS producing enzyme, the extract, after being treated at different pH values for 2 hours at 0°C , was examined with respect to the DMS producing activity. As illustrated in Fig. 2, this enzyme was most stable at pH 5.1. The stability of this enzyme was not influenced by the concentration of NaCl.

Fig. 3. Effect of temperature on production of DMS from DMPT by *G. cohnii*.

Fig. 1. Effect of pH value on the production of DMS from ^{35}S -DMPT by cell-free extract.

●—●, in 1/10 McIlvaine buffer prepared with 0.45 M NaCl solution; ▲---▲, in Tris buffer prepared with 0.45 M NaCl solution.

Fig. 2. Effect of pH value on the stability of the DMS-producing enzyme of *G. cohnii*.

The optimum temperature for the reaction was around 27°C (Fig. 3).

Activation of DMS evolution by salts.

Effect of NaCl concentration on the evolution of DMS was observed by use of the cell free extract. Fig. 4 shows that the activity was at its maximum in the presence of more than 0.4 M NaCl. The other inorganic salts were also examined in this respect, for comparison's sake. As will be seen from Figs. 4 and 5, the enzymatic evolution of DMS was activated by addition of various inorganic and organic salts at high concentration in an order of increasing effectiveness; $\text{NaCl} \geq$

Fig. 4. Effect of NaCl concentration on the enzymatic production of DMS from ^{35}S -DMPT.

Fig. 5. Effect of salts on the enzymatic production of DMS from ^{35}S -DMPT.

○—○, KCl; ●—●, MgCl₂; △—△, CaCl₂; ▲—▲, LiCl;
 □—□, NaBr; ■—■, LiBr; ▽—▽, NaNO₃; ▼—▼, Na₂SO₄;
 ●—●, NaI; ○—○, CH₃COONa; ▽—▽, CH₂CHCOONa.

$\text{KCl} > \text{MgCl}_2 = \text{CaCl}_2 > \text{LiCl} > \text{NaBr} \approx \text{LiBr} > \text{NaNO}_3 \gg \text{NaI} \approx \text{KI} > \text{Na}_2\text{SO}_4 > \text{Li}_2\text{SO}_4 > \text{CH}_3\text{COONa} > \text{CH}_2\text{CHCOONa}$. This may suggest that the effect of the salt resides primarily in the anion rather than the cation. The anions seemed to activate the reaction in the following order; $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- \gg \text{I}^- > \text{SO}_4^{2-} > \text{CH}_3\text{COO}^- > \text{CH}_2\text{CHCOO}^-$.

Other anions such as PO_4^{3-} , SCN^- , ClO_3^- and citrate, and urea did not activate the enzyme.

The extract was stable under prolonged exposures to salt free environments.

Inhibition of DMS evolution.

Inhibition of the enzymatic activity by some reagents and its release were examined (Table 2). As will be seen in Table 2, the enzyme was inhibited completely by *p*CMB, IAA and KCN, and partly by EDTA. The inhibitions by the sulfhydryl reagents and KCN were more or less released by Hcys or ETG.

It is suggested from these results that the DMS-producing enzyme is a SH-enzyme which requires some metals for its activity⁽⁸⁾.

Kinetics of DMS evolution. Time course of the evolution of DMS by the crude enzyme was observed to consider the kinetics of the reaction. As shown in Fig. 6, the evolution of DMS stoichiometrically increased in inverse proportion to the decrease of DMPT. This reaction reached a maximum in approximately an hour. It was found by an experiment using gas chromatograph that ACA as well as DMS was produced from DMPT by this enzyme. The K_m value for DMPT-Cl as a substrate was 1.5×10^{-3} M.

Requirements of cofactors by the crude enzyme.

Effects on the enzyme reaction of some substances which were expected to be cofactors were examined with the crude enzyme, as shown in Table 3. It was found that Hcys, ascorbic acid, betaine and trace metals such as Mn, Zn, Co and Fe had no influence on the reaction.

Table 2. Effect of inhibitors and sulfhydryl compounds on the DMS evolution.

Inhibitors	(M) Concn.	SH- compounds	(M) Conc.	DMS produced (cpm)
<i>p</i> CMB	1×10^{-4}	none		3,000
	5×10^{-4}	"		30
	1×10^{-3}	"		20
	"	Hcys	2×10^{-3}	2,100
	"	"	5×10^{-3}	4,900
	5×10^{-4}	ETG	2×10^{-3}	2,800
IAA	5×10^{-4}	none		90
KCN	5×10^{-4}	none		2,800
	2×10^{-3}	"		560
	2×10^{-4}	Hcys	5×10^{-4}	17,750
	2×10^{-3}	"	5×10^{-3}	9,900
EDTA	1×10^{-2}	none		3,600
	1×10^{-1}	"		4,300
None		none		15,900

Fig. 6. Time course of the evolution of ^{35}S -DMS from ^{35}S -DMPT by the crude enzyme.

Discussion

It was reported that the activity of cytochrome oxidase from a halotolerant *Micrococcus* was increased several fold by NaCl in concentration of 1–2M, but the activity in extract from heart muscle preparation showed a clear decrease at NaCl concentration above 0.1M⁽¹¹⁾. In their early experiments, BAXTER *et al.*^(12,13,14), HOLMES *et al.*⁽¹⁵⁾ and LARSEN⁽¹⁶⁾ found that some enzymes extracted from *Halobacterium sali-*

Table 3. Requirements of cofactors by the crude enzyme.

System	DMS evolved (%)
Control*	100
+ Hcys (2×10^{-3} M)	95
+ ascorbic acid (2×10^{-4} M)	103
+ betaine (1×10^{-2} M)	100
+ 0.03 M MgCl_2 + 0.008 M KCl + 0.003 M CaCl_2	102
+ 0.1 mM Mn^{2+} + 0.01 mM Zn^{2+} + 0.1 mM Fe^{2+} + 0.01 mM Co^{2+} + 0.01 mM Mo^{6+}	100
without NaCl (0.45 M)	0

* Consisted of 0.5 ml of the crude enzyme, 0.1 ml of DMPT solution (final concn.: 20 mM) and 5 ml of 1/10 McIlvaine buffer containing 0.45 M NaCl (final pH 6.2).

narium were activated by salts in high concentration. These reports, together with our findings that evolution of DMS by the enzyme of *G. cohnii* was activated by addition of NaCl and other salts in high concentration, support our speculation that the ability to evolve DMS of unicellular algae may be related to the high salinity of their habitats.

Concerning the effects of inorganic anions on the enzyme activity, COLE¹⁷⁾ showed that the activity of animal α -amylases was so greatly affected that Cl^- ion and other monovalent ions were regarded as the natural activators of these enzymes. MANN *et al.*¹⁸⁾ and MASSEY¹⁹⁾ reported, on the other hand, that fumarate hydratase was activated only by divalent and trivalent anions. Recently TONOMURA *et al.*²⁰⁾ and WARREN *et al.*^{21, 22)} demonstrated that salts in high concentrations tended to inhibit the activity of some enzymes such as myosin ATPase^{20, 21)}, fumarase¹⁹⁾ and other several enzymes²²⁾, and that the inhibition in this case might be caused by some structural changes in the enzyme proteins. These observations provide evidence that salts alter enzyme activity by altering the organized structure of the protein macromolecule.

Based on our findings that the DMS producing enzyme of *G. cohnii* is a SH enzyme, that this enzyme is activated by anions at high concentration in the following order; $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- \gg \text{I}^- > \text{SO}_4^{2-}$, and that urea and SCN^- inactivate this enzyme, it is considered that the activation of the enzyme by salts results from the exposure by the anions of some buried groups such as SH-group in the enzyme proteins.

Summary

It was found that the evolution of DMS from *G. cohnii*, a heterotrophic alga of marine origin, resulted from an enzymatical cleavage of DMPT in the cells. This reaction was activated by various inorganic salts at high concentration in an order of increasing effectiveness for anions: $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- \gg \text{I}^- > \text{SO}_4^{2-}$. The maximum

rate of the reaction was obtained at pH 6.2. The enzyme catalyzing this reaction was most stable at pH 5.1. Activity of the enzyme was inhibited by *p*CMB, IAA and KCN. The inhibition by these compounds was released in part by the addition of Hcys.

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