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Studies on Dissolved Metalloenzymes in Lake Water. I. Identification of Alkaline Phosphatase

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Metalloenzyme (alkaline phosphatase) dissolved in lake water has been identified by means of high performance liquid chromatography (HPLC). Water samples were collected from Lake Kasumigaura in Ibaraki, and filtered with a 0.2—0.45 μm membrane filter immediately after sampling. Filtered water was concentrated to 100—2000 fold by ultrafiltration membrane which could concentrate the components of molecular weight larger than 10000. The enzymatic activity of alkaline phosphatase was measured using *p*-nitrophenyl dihydrogenphosphate as a substrate. Chelating agents added to the concentrated lake water inhibited the appearance of enzymatic activity, while the enzymatic activity was recovered with addition of zinc ion. The HPLC chromatograms of the concentrated lake water were examined, where an aqueous porous gel column was used for separation. The effluent at molecular weight of *ca.* 90000 showed specific activity of alkaline phosphatase. Zinc was also found in the same fraction. These facts suggest that alkaline phosphatase with the nature similar to procaryotes was present in the lake water.

Recently, the present authors reported that alkaline phosphatase existed as a dissolved form in sea water.[1,2] In these studies the identification of alkaline phosphatase was made by measuring the high performance liquid chromatogram as well as enzymatic activity and zinc content, and also biological significances of dissolved metalloenzymes in natural water were discussed along with the natural cycles of nutrient and trace elements. As is well known, alkaline phosphatase is one of the typical metalloenzymes containing four zinc atoms in one molecule,[3] and concerns with the phosphorus cycle in animals and microorganisms. Thus, further study on dissolved metalloenzymes in natural water may help to elucidate not only biological activities, but also elemental cycles. In addition, the identification of the chemical forms of trace metals in water has also been receiving great attention in the fields of bioinorganic and analytical chemistry.[4] In this sense, the dissolved metalloenzymes can be considered as one of the chemical forms of trace metals in water.

As was suggested in the previous papers,[1,2] alkaline phosphatase is also one of the most significant enzymes in limnology, because it may concern with the phosphorus cycles in natural water. Free dissolved phosphatase activity in pond water was first reported by Overbeck and Babenzien in 1963.[5] Reichardt *et al.* did consider the ecological importance of free phosphatase in lake water.[6] Since then, a number of investigations on phosphatase activity and its role in freshwater have been done. Furthermore, several reports on other types of enzymatic activities have been also given in terms of natural water. For example, Morrison *et al.* determined cellulase activity,[7] and Little *et al.* determined proteolytic enzymes in lake water.[8]

In this paper, the study on dissolved alkaline phosphatase has been extended to lake water. Lake Kasumigaura was chosen as the target, since it is one of the typical eutrophic lakes in Japan. The dissolved alkaline phosphatase activity was measured and identification of the enzyme was discussed along with the enzymatic properties.

Experimental

Chemicals. *E. coli* alkaline phosphatase (Boehringer-Mannheim, No. 15429) whose specific activity was 20 U mg^{-1} was used as a standard enzyme. *p*-Nitrophenyl dihydrogenphosphate was dissolved in 0.6 M tris(hydroxymethyl)-methanamine hydrochloride (pH 8.0; abbreviated as Tris buffer) (1 M=1 mol dm^{-3}). Chloride salts were used as metal(II) ion standards. Calibration proteins for the molecular weight (M. W.) standard of HPLC are as follows: Urease (Jack bean; M. W.: 480000);[9] aldolase (rabbit muscle; M. W.: 161000);[10] alkaline phosphatase (*E. coli*; M. W.: 89000);[11] carbonic anhydrase (Bovine erythrocytes; M. W.: 31000);[12] and ribonuclease (Bovine pancreas; M. W.: 13700).[13]

Instruments. A Shimadzu UV-210A spectrophotometer with a SFU-6 semi-auto flow cell unit was used for the measurements of enzymatic activity. The optical path of the absorption cell was 10 mm. A Shimadzu LC-3A high performance liquid chromatography with a SPD-2A spectrophotometric detector was used for HPLC-separation. Zinc concentration was determined by a Jarrell-Ash Model 975 Plasma Atom Comp. Ultrafiltration and incubation for activity measurements were all performed under the controlled temperature by a thermostatic bath circulator (Model TRL-111SP; Thomas Scientific Co., Ltd.).

Sampling and Filtration of Lake Water. The sampling stations in Lake Kasumigaura are located as shown in Fig. 1. In Stations 4 and 9, water samples were collected from depths of 0.5, 1.5, and 5.0 m; in the other stations, only from a depth of 0.5 m. Samples were taken once a month starting in April, 1980.

Water samples were also collected from the Shinobazu pond in Tokyo.

The water was filtered through a glass-fiber membrane filter (pore size: 1.0 μm) and then a Toyo Kagaku UK-4 membrane filter (pore size: 0.2 μm) as soon as possible after collection, in order to remove microorganisms. Enzymes in thus filtered water are hereafter called *dissolved enzymes.*

Concentration of Lake Water Samples. As enzyme levels in lake water are too low to be analyzed by the chromatographic technique, the filtered water samples were concentrated using the ultrafiltration technique. An Amicon YM-10 or UM-10 type membrane, or a Toyo Kagaku UK-10 type membrane, through which only molecules with a mo-

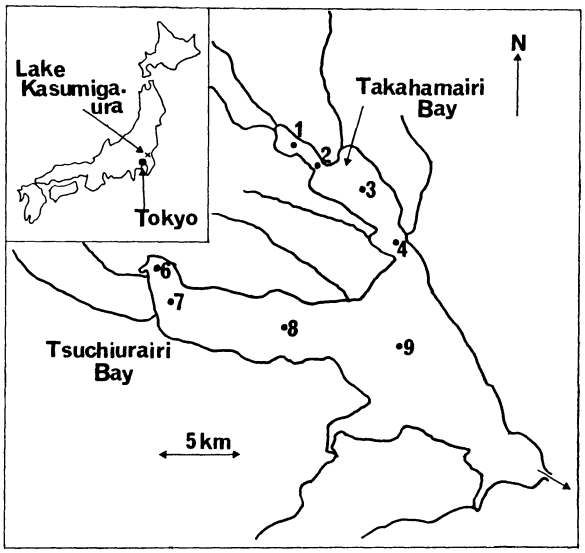

Fig. 1. Sampling stations in Lake Kasumigaura.

lecular weight over 10000 can be concentrated, was attached to an Amicon ultrafiltration cell (Model 202), in which samples were concentrated to 1/100—1/2000 of their original volumes. Samples were kept at about 4 °C throughout the operation.

Measurements of Phosphatase Activity. The enzymatic reaction of alkaline phosphatase for the substrate *p*-nitrophenyl dihydrogenphosphate proceeds as follows:

$$O_2N\text{-}C_6H_4\text{-}OPO_3H_2 + H_2O \longrightarrow O_2N\text{-}C_6H_4\text{-}OH + H_3PO_4. \quad (1)$$

The absorbance of the product, *p*-nitrophenol, was measured at 410 nm.[14] Assay procedures were as follows: The substrate solution (volume: V_1) and either a water sample or distilled water (blank) (volume: V_2) were mixed and then kept at 25 °C. After an interval of time, t, the absorbance of the mixture at 410 nm was measured using spectrophotometer equipped with a 10-mm long light path cuvette. When the absorbance of the sample-mixture and the blank-mixture, and the absorption coefficients of the substrate (S) and the product (P) at 410 nm are A, A_0, ε_s, ε_p, respectively, the enzymatic activity of the water sample, α, is given in Eq. 2:

$$\alpha = \frac{(A-A_0)(V_1+V_2)}{(\varepsilon_p-\varepsilon_S)V_2 t}. \quad (2)$$

When V_1, V_2, and t were taken as 4 cm^3, 1 cm^3, and 1440 min, respectively, in measuring unconcentrated samples, and $(\varepsilon_p-\varepsilon_s)$ is determined as 16200 mol^{-1} dm^3, the value of α is shown in Eq. 2′:

$$\alpha = 2.1 \times 10^{-7} (A-A_0) \text{ mol dm}^{-3} \text{ min}^{-1}. \quad (2')$$

Separation and Detection Using High Performance Liquid Chromatography (HPLC). The HPLC separation was performed using a Shimadzu W-71 aqueous porous gel column (7.9 mm i.d. × 50 cm), with which molecules can be separated according to their molecular weight (range 10000—500000). Two types of the carriers were used, *i.e.*, a) 0.2% KH_2PO_4-KOH buffer (pH 7.3) for general separation, and b) 0.01 M Tris·HCl buffer (pH 8.0) for the distribution measurement of phosphatase activity, since phosphate inhibits phosphatase activity. The flow rate of the carrier was 1.0 cm^3 min^{-1}, and the solutes in the effluent were detected with the absorbance at 210 nm (general) and 280 nm (for protein). Chromatography was carried out at room temperature. Chromatographic effluents were collected by a fraction collector every 0.3—0.5 min.

Results and Discussion

Phosphatase Activity in Lake Waters. Table 1 shows the phosphatase activity of the water in Lake Kasumigaura. All the activity measurements were performed for the untreated samples. Activity values varied with sampling seasons and sampling points from less than 0.4 to 9.0 nmol dm^{-3} min^{-1}. The average value of activity, 1.4 nmol dm^{-3} min^{-1}, is comparable to the value in Lake Kinneret reported by Berman[15] and the value in the Shinobazu pond, Tokyo, measured by the present authors, and higher than the level in sea water samples.[16]

Characterization of Phosphatase Activity in Lake Water. Phosphatase activity in the lake water was inhibited

Table 1. Dissolved phosphatase activity in lake water

Station	Depth / m	Dissolved phosphatase activity/nmol dm^{-3} min^{-1}								
		Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Lake Kasumigaura										
1	0.5	1.3	1.9	0.9	3.9	7.4	1.8	1.7	2.5	<0.4
2	0.5	2.6	2.1	1.1	9.0	2.9	2.4	0.9	1.2	0.4
3	0.5	2.4	0.9	0.9	4.9	1.2	2.0	<0.4	0.7	0.4
4	0.5	1.7	1.7	1.5	1.1	<0.4	1.9	<0.4	0.8	0.6
	1.5	1.5	0.9	0.4	1.3	0.6	1.5	<0.4	1.2	0.6
	5.0	1.3	0.9	2.8	1.1	0.6	1.7	1.5	1.6	0.6
6	0.5	1.1	2.1	<0.4	2.6	3.6	2.9	1.7	4.6	0.4
7	0.5	1.7	1.7	<0.4	1.9	1.8	1.0	0.6	3.9	0.7
8	0.5	1.7	2.1	1.5	0.9	1.0	0.5	<0.4	1.6	0.6
9	0.5	1.3	1.3	1.3	0.4	<0.4	0.9	<0.4	1.3	0.4
	1.5	1.5	0.9	1.5	1.5	1.5	1.2	<0.4	1.2	0.5
	5.0	1.5	0.9	0.5	1.1	<0.4	0.8	<0.4	1.0	0.5
The Shinobazu Pond										
H	0.1	—	1.0	—	—	—	—	0.8	—	—

by treating with chelating agents such as ethylendiaminetetraacetic acid (EDTA) or 2,6-pyridinedicarboxylic acid (PDA). However, it is well known that such inhibited activity may be recovered by adding metal ions, such as zinc(II) and cobalt(II). The recovery effect of metal ions added to *apo-lake water* (the sample treated with chelating agents) is summarized in Table 2. Phosphatase activity was completely recovered when zinc(II) was added, and partly when cobalt(II) or manganese(II) was added. When heated over 80 °C, the sample completely lost activity. These results suggest that phosphatase activity in lake water originates from the zinc-metalloenzyme *alkaline phosphatase*.

In Fig. 2, the pH dependence of dissolved alkaline phosphatase activity in Lake Kasumigaura is shown. The maximum activity of *lake water* alkaline phosphatase was obtained at pH 8.9, which was a little higher than that of *E. coli* alkaline phosphatase (8.0).

The variations of phosphatase activity of *lake water* alkaline phosphatase, and of *E. coli*, with temperature and substrate concentration were also examined. The apparent activation energy (E_a) and the Michaelis constant (K_m) were estimated from the Arrhenius and the Michaelis plots.[6] The results are summarized in Table 3. Both the E_a and K_m of *lake water* alkaline phosphatase were different from those of *E. coli* alkaline phosphatase. This may indicate that *lake water* alkaline phosphatase is similar to that of *E. coli*, but exactly not the same.

Separation by HPLC. The HPLC chromatogram of concentrated lake water using 0.2% phosphate buffer

Table 2. Recovery ratio of phosphatase activity in lake water

Apo-sample(mm^3)[a]	Metal ion[b]	Relative activity %
50	None	0
50	Manganese(II)	41
50	Cobalt(II)	32
50	Nickel(II)	4
50	Copper(II)	9
50	Zinc(II)	100
50	Cadmium(II)	4

a) Concentrated (×400) lake water dialyzed against 0.01 M 2,6-pyridinedicarboxylic acid (pH 7.0). Lake water was sampled in Lake Kasumigaura in August, 1980. b) 50 μg of each metal ion was added in the form of chloride salt.

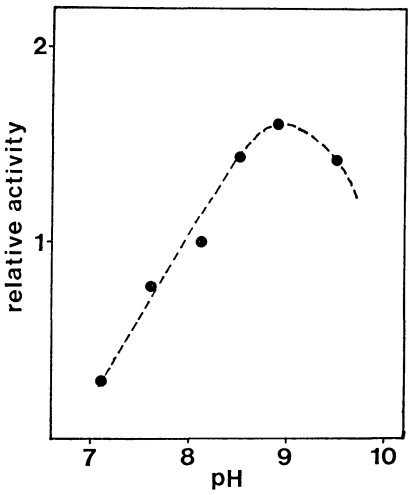

Fig. 2. pH dependence of dissolved alkaline phosphatase activity in lake water.
Activity was measured using 1 mM *p*-nitrophenyl dihydrogenphosphate in 0.1 M Tris–HCl buffer at various pH, at 30 °C. The sample was collected in Lake Kasumigaura (Sta. 2) on November 20, 1980.

Table 3. Apparent activation energies and Michaelis constants of alkaline phosphatases

Source of alkaline phosphatase	Apparent activation energy[b] E_a/kcal mol^{-1}	Michaelis constant[c] K_m/μM
Lake water[a]	6.93	1.0
E. coli	8.73	1.5

a) Concentrated lake water collected in Lake Kasumigaura (Sta. 2) on November 20, 1980. b) Obtained with the Arrhenius plot. Activities were measured using 1 mM *p*-nitrophenyl phosphate in 0.1 M Tris·HCl buffer (pH 8.1), at various temperatures (0—30 °C). c) Obtained with the Michaelis plot. Activities were measured using *p*-nitrophenyl dihydrogenphosphate of various concentrations (1 μM—1 mM) in 0.1 M Tris·HCl buffer (pH 8.1) at 30 °C.

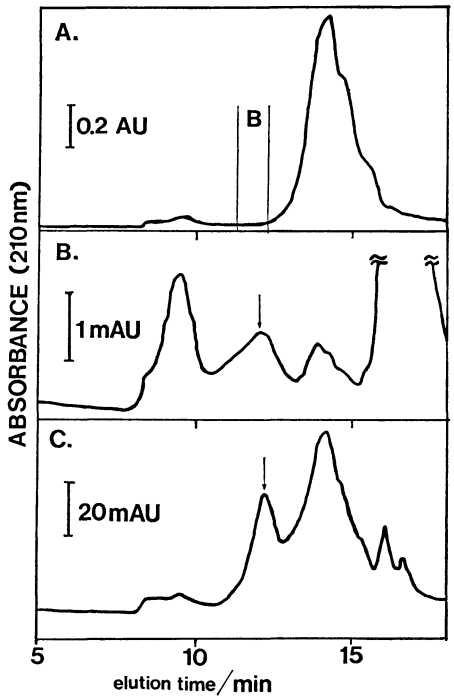

Fig. 3. Chromatograms of lake water.
Column: Shimadzu W-71 (7.9 mm i.d.×50 cm), flow rate: 1.0 mL min^{-1}, carrier: 0.2% KH_2PO_4–KOH (pH 7.3), sample: concentrated (×500) lake water from Lake Kasumigaura sampled in February, 1980, temperature: at room temperature. (A): Lake water only, 100 μL; (B): fraction of (A) between 11.3 and 12.3 min; (C): (A) +20 μg of *E. coli* alkaline phosphatase.

(pH 7.3) as a carrier is shown in Fig. 3(A). As has been reported in the previous papers,[1,2] the chromatogram peak of alkaline phosphatase is located near 12 min. However, no marked peak could be noticed near the elution time of alkaline phosphatase in the chromatogram (A). Then, the effluent between 11.3 and 12.3 min of A was collected and concentrated. The chromatograph of the fractionated effluent was again examined under the same conditions, and the chromatogram B was obtained. In the chromatogram B, some apparent peak can be observed at the elution time at *ca.* 12 min. The chromatogram C is that of concentrated lake water spiked with *E. coli* alkaline phosphatase, which provided the elution peak at 12 min. From the comparison between the chromatograms B and C, the peak near 12 min in the chromatogram B corresponds to the distinct peak at the elution time of *E. coli* alkaline phosphatase. Fig. 4 shows the chromatograms of concentrated lake water detected both by absorbance and phosphatase activity, using 0.01 M Tris buffer (pH 8.0) as the carrier. There is a sharp peak of activity, and its elution time is the same as that of *E. coli* alkaline phosphatase. Zinc could also be found in this fraction. These results suggest that phosphatase activity in lake water originates from the alkaline phosphatase, whose molecular weight is close to that of *E. coli* alkaline phosphatase.

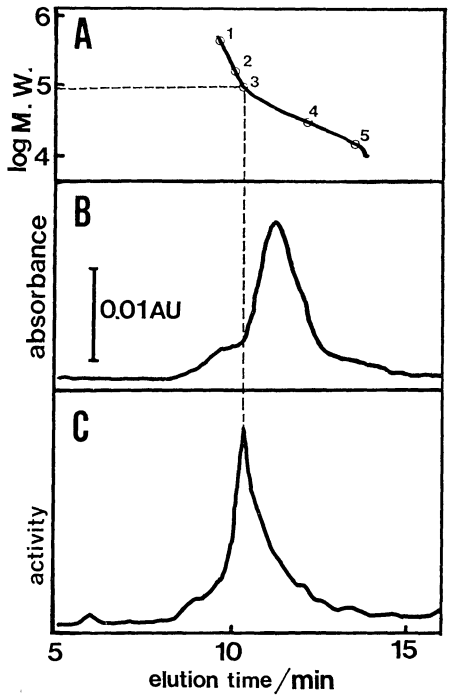

Fig. 4. Distribution of phosphatase activity of lake water.
Column: Shimadzu W-71 (7.9 mm i.d. × 50 cm), flow rate: 1.0 mL min^{-1}, carrier: 10 mM Tris buffer (pH 8.0), sample: concentrated (×2,300) lake water from Lake Kasumigaura sampled in August, 1980, 100 μL, temperature: 25 °C. (A) Calibration curve by standard enzymes [1, Urease (Jack bean; M. W.: 480000); 2, aldolase (rabbit muscle; M. W.: 161000); 3, alkaline phosphatase (*E. coli*; M. W.: 89000); 4, carbonic anhydrase (Bovine erythrocytes; M. W.: 31000); 5, ribonuclease (Bovine pancreas; M. W.: 13700)]; (B) chromatogram detected in absorbance at 280 nm; (C) distribution of phosphatase activity.

Conclusion

The results described above lead us to the conclusion that dissolved zinc-metalloenzyme alkaline phosphatase, which may be provided by procaryotes, is present in Lake Kasumigaura. It has been known that alkaline phosphatase of eucaryotes (molecular weight around 150000) is usually larger than the alkaline phosphatase of procaryotes (molecular weight around 90000).[17] As Lake Kasumigaura is known to be an algae-rich lake, a large portion of the alkaline phosphatase in the lake would be provided by algae, such as *Microcystis*. Further work to identify the isozymes using electrophoresis, *etc.* is in progress.

In Lake Kasumigaura, the concentration of alkaline phosphatase-formed zinc has been calculated as about 0.2 ng dm^{-3} (0.2 ppt), using the value of standard *E. coli* alkaline phosphatase, that is, almost one ten-thousandth of the total zinc in the lake water.

In consequence, the existence of active dissolved alkaline phosphatase may be related to the phosphorus cycles in the lake water, as has been discussed in other lakes.[15,18–20] The study on the correlation between the dissolved alkaline phosphatase and phosphorus concentration/cycle in the eutrophic lake including Lake Kasumigaura should be biochemically and ecologically important and interesting.

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References

1) H. Haraguchi, K. Kobayashi, M. Matsui, and K. Fuwa, *Chem. Lett.*, **1982**, 175.
2) K. Kobayashi, M. Matsui, H. Haraguchi, and K. Fuwa, *J. Inorg. Biochem.*, in press.
3) J. E. Coleman and J. F. Chlebowski, "Advances in Inorganic Biochemistry," ed by G. L. Eichhorn and L. G. Marzilli, Elsevier, New York (1979), Vol. 1, Chap. 1.
4) T. M. Florence and G. E. Batley, *Talanta*, **24**, 151 (1977).
5) J. Overbeck and H. D. Babenzien, *Naturwissenschaften*, **50**, 571 (1963).
6) W. Reichardt, J. Overbeck, and L. Steubing, *Nature*, **216**, 1345 (1967).
7) S. J. Morrison, J. D. King, R. J. Bobbie, R. E. Bechtold, and D. C. White, *Mar. Biol. (Berlin)*, **41**, 229 (1977).
8) J. E. Little, R. E. Sjogren, and G. R. Carson, *Appl. Environ. Microbiol.*, **37**, 900 (1979).
9) W. N. Fishwein and P. P. Carbone, *Proc. Soc. Exp.*

Biol. Med., **134**, 1083 (1970).
10) C. L. Siam and B. L. Horecker, *Arch. Biochim. Biophys.*, **123**, 186 (1968).
11) R. T. Simpson, B. L. Vallee, and G. H. Tait, *Biochemistry*, **7**, 4336 (1968).
12) J. E. Coleman, "Inorganic Biochemistry," ed by G. L. Eichhorn, Elsevier, Amsterdam (1973), Vol. 1, Chap. 16.
13) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).
14) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, **38**, 470 (1960).
15) T. Berman and G. Moses, *Hydrobiologia*, **40**, 487 (1972).
16) H. Kobori and N. Taga, *J. Exp. Mar. Biol. Ecol.*, **36**, 23 (1979).
17) J. F. Chlebowski and J. E. Coleman, "Metal Ions in Biological Systems," ed by H. Sigel, Marcel Dekker, New York (1976), Vol. 6, Chap. 1.
18) T. Berman, *Limnol. Oceanogr.*, **5**, 663 (1970).
19) R. J. Stevens and M. P. Parr, *Freshwater Biol.*, **7**, 351 (1977).
20) S. Niewolak, *Rocz. Nauk. Roln., Ser. H*, **98**, 147 (1978).