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Oscillatory reaction of catalase wrapped by liposome

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Abstract

To date we have been studying the enzymatic oscillatory reaction caused by gradual entry of substrate via semi-permeable membrane. It has been found that many enzymes cause oscillatory reaction. Here we present an oscillatory reaction of enzyme wrapped by liposome. We used catalase as an enzyme, since its oscillatory reaction has been already investigated in detail in the absence of liposome. Distinct oscillation with shorter period than without liposome was obtained. On the other hand, it was shown that the presence of liposome facilitated the permeation rate of hydrogen peroxide through semi-permeable membrane. This is thought to be factor of shortening the oscillation period compared with that in the absence of liposome. Oscillation was also temperature dependent. Our finding may provide an important insight into the study of enzyme reaction taking part in rhythms in living systems.

Keywords: Oscillatory reaction; Enzyme; Catalase; Liposome

1. Introduction

Living systems are nonlinear and in far-from-equilibrium situation. Thus, oscillatory reaction and other kinds of self-organization phenomena are found in all levels of biological organization, from unicellular to multicellular organisms, with periods ranging from a second to days or even years. It is considered that the network of molecular species controlled by positive or negative feedback loops is required for causing oscillation. A well-known example is glycolytic oscillation that occurs in yeast and muscle cells. Oscillation of cyclic AMP that governs in *Dictyostelium amoebae* is also well known. Allosteric effects are thought to be responsible for these oscillations [1]. Furthermore, the other oscillations have been observed: (1) oscillatory reaction by horse radish peroxidase [2], (2) intercellular Ca^{2+} oscillation in many cell types either spontaneously or after simulation by a hormone or a neurotransmitter [1], (3) the mitotic oscillator that drives the cell division cycle, (4) the biochemical oscillator that governs the circadian rhythms and (5) ultradian rhythms that are observed in yeast system [3].

Besides these oscillatory reactions, we discovered the oscillatory reaction of enzyme induced by gradual entry of substrate into enzyme solution. As a means of gradual entry of substrate, we used permeation to substrate through dialysis membrane. So far we have observed oscillatory reactions for many kinds of enzymes by using this method [4,5]. When catalase was used as an enzyme, for example, the oscillation lasted for more than 2 days, although the period of oscillation gradually increased with time [5]. In this system, the period did not change in the temperature range from 25 to 37 0 and within a pH range of 6.0–7.0. It was found from numerical analysis that both the rates of the permeation of substrate into enzyme solution and of the outflow of product from enzyme solution are essential for causing the oscillation. Since, in varying temperature or pH, the effect of change of rate constants is cancelled by the change of permeation rate of substrate, the period remains constant.

In a previous paper [6], oscillatory reaction by catalase wrapped by liposome made of synthetic phospholipids, dimyristoyl lecithin (DMPC) was investigated. Sustained periodic oscillation of electric potential with long period and downward peaks was observed after aperiodic oscillation. The result was quietly different from that in the absence of liposome. Here we present oscillatory reaction of catalase wrapped by liposome made of naturally occurring phospholipids, egg

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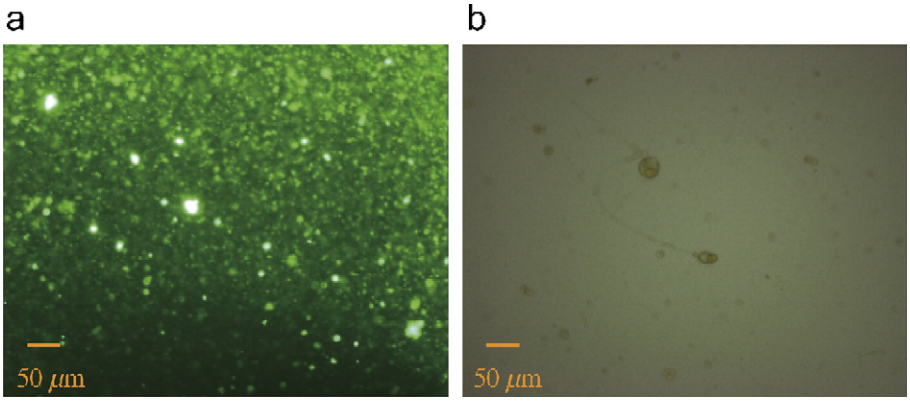

Fig. 1. Microscopic image of liposome (a) fluorescence image (b) image by phase-difference microscope.

lecithin, to put our system closer to the reaction in living systems.

2. Methods

Egg lecithin and hydrogen peroxide was purchased from Wako Pure Chemical Industry and catalase from bovine liver (EC.1.11.1.6), from Sigma. The pH of aqueous solution was adjusted at 7 with Tris–HCl buffer.

Liposome solution was prepared as follows: at first, toluene including egg lecithin was layered on aqueous solution of catalase of equal volume. After 24 h, only solution containing enzyme was taken out and used for oscillatory reaction. In addition, to confirm the formation of liposome, using the 1:6 mixture of NBD–lecithin (from Avanti polar-lipids, inc) and egg lecithin, the same procedure was performed. The fluorescence image of liposome was taken on Leica DM LB with DC300F Chilled CCD camera.

The concentration of catalase, which was not including within liposome, was measured by Bradford method using Bio-Rad protein assay. This method can detect only the protein out of liposome, since the pigment (Coomassie Blue) cannot pass through lipid membrane.

For measuring the oscillatory reaction, the dialysis membrane (Seamless Cellulose Tubing, Viscose Sales Corp.) was sandwiched between two cells made of glass. In one cell, solution of catalase enwrapped by liposome was put in the other cell with substrate (H_2O_2) solution. The oscillatory reaction induced by the gradual entry of substrate into the enzyme solution was measured electrically by oxygen electrode and oxidation–reduction potential (ORP) electrode. The method of measuring was quietly the same as that reported already [5]. Instead of catalase solution, we used liposome solution involving catalase in the present study. The volume of enzyme solution and hydrogen peroxide solution were 25 ml and 20 ml, respectively, when measurement was performed using Horiba DO meter OM-51 that the range is limited from 0–20 mg/l, whereas each volume was 10 ml in the oxidation–reduction potential (ORP) measurement. The measurement cells were immersed in thermally controlled water bath and all the experiments were carried out at 37 °C.

3. Results

The transfer of lipid into aqueous phase was confirmed with the following method. One day after laying the toluene solution containing egg lecithin on the aqueous solution of catalase, the toluene solution was evaporated to dryness and the mass of egg lecithin remained in toluene was weighted. The result indicated that ~50% of egg lecithin transferred into the aqueous solution and that liposome could be formed. The fluorescence image of liposome of 10–20 μm in diameter was observed under the microscope (Fig. 1a). An analogous image was also observed with phase-difference microscope (Fig. 1b). In addition, the concentration of catalase existing outside lipid membrane in varying egg lecithin concentration was measured with Bio-Rad protein assay. The result showed that no protein exists outside the lipid membrane at >0.3 mg/ml of egg lecithin (Fig. 2).

In the current research, hydrogen peroxide transfers through dialysis membrane to an aqueous solution of liposome-enwrapped catalase and further passes through liposome membrane to react with catalase which exists within liposome. Produced oxygen is extruded from liposome and evaporates into the air in part. Time course of dissolved oxygen concentration (DO) in the bulk of liposome solution containing catalase was measured with DO meter.

At first, the effect of the amount of egg lecithin, which was used for forming liposome, was investigated. A large amount of egg lecithin indicated distinct oscillation of dissolved oxygen concentration with a period of ~15 min (Fig. 3a and b). Fig. 3d shows the time course of oscillatory reaction in the absence of

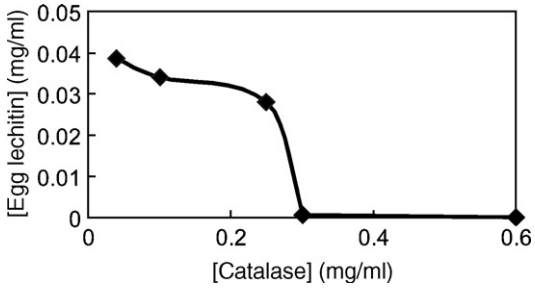

Fig. 2. Measurement of protein concentration existing outside the liposome membrane. The concentration of catalase is 0.125 mg/ml.

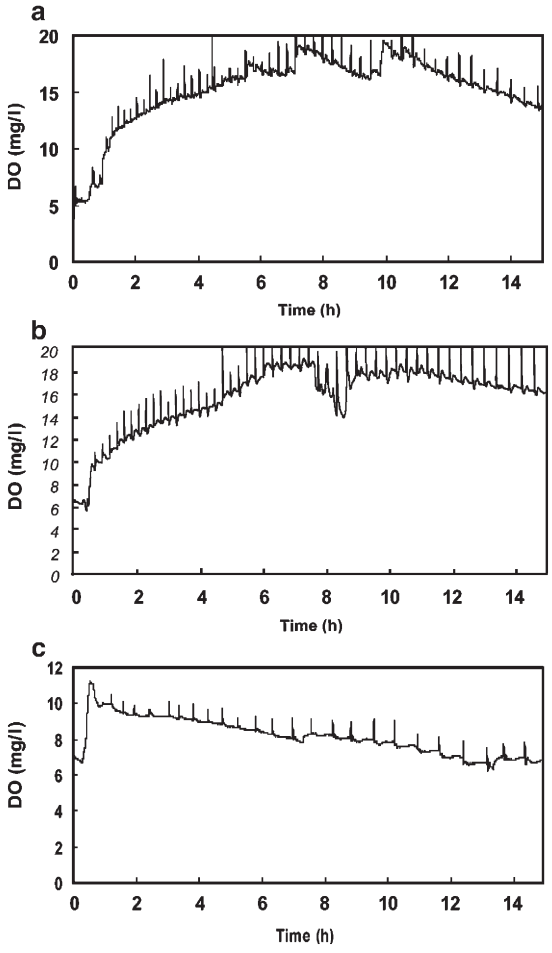

Fig. 3. Time course of oscillatory reaction by liposome-enwrapped catalase. The concentrations of egg lecithin 1 mg/ml (a), 0.3 mg/ml (b) and 0 mg/ml (c), respectively. The concentrations of catalase and hydrogen peroxide are 0.125 mg/ml and 0.256 mol/l, respectively. Abscissa denotes dissolved oxygen concentration (DO).

liposome as a reference. The period was ~25 min in the first stage. Compared with these results, it was concluded that the period of oscillatory reaction of liposome-enwrapped catalase was short compared with that in the absence of liposome. In addition to the short period oscillation, we also observed long period oscillation as shown in Fig. 3a.

Figs. 4 and 5 show the hydrogen peroxide concentration dependency on oscillatory reaction at 37 °C. With the increase in hydrogen peroxide concentration, the period of oscillation became shorter. However, time interval between peaks of oscillation became longer with time. These tendencies were the same as those in the absence of liposome. When the catalase concentration was 0.125 mg/ml, the amplitude of oscillation became larger with increase in concentration of H_2O_2 (Fig. 4). When the catalase concentration was lowered to 1/10 (0.0125 mg/ml), the period similarly became shorter with the increase in H_2O_2 concentration. However, the period was short as compared with 0.125 mg/ml catalase in the same concentration of H_2O_2 (Fig. 5).

The effect of temperature was also investigated. As shown in Fig. 6, the period became longer and the amplitude became smaller as the temperature decreased. Temperature compensation, which was observed in the absence of liposome, was not observed in this case.

On the other hand, the time course of oxidation–reduction potential in liposome solution was measured with ORP electrode instead of measuring dissolved oxygen concentration with DO meter in higher concentration of hydrogen peroxide. In a previous paper [6], the oscillation was observed in higher concentration of hydrogen than that in the absence of liposome and the corresponding oxygen concentration to its value was beyond the region (0–20 mg/l) that can be measured by DO meter. It was already confirmed that oscillation could be also purchased by the measurement of potential as well as dissolved oxygen concentration [5]. Fig. 7 shows the results of oscillatory reaction in varying the concentration of hydrogen peroxide at the fixed concentration of catalase (0.125 mg/ml). Like the previous paper, the induction time appeared before regular oscillation. The time interval between peaks during regular oscillation was gradually increased with time. The average time interval between peaks was, however, much smaller than that of

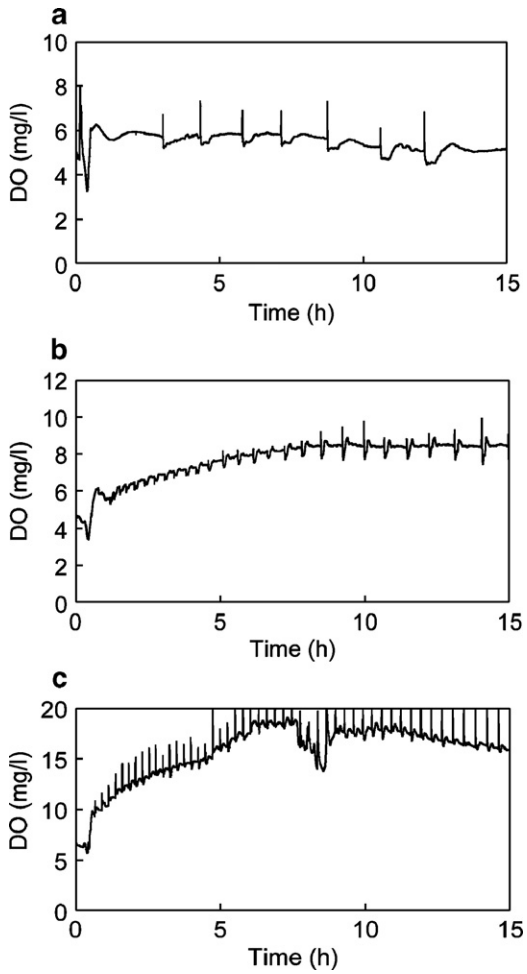

Fig. 4. Time course of oscillatory reaction by liposome-enwrapped catalase with higher concentration. The concentration of catalase is 0.125 mg/ml and liposome was prepared with 0.6 mg/ml egg lecithin–toluene solution. The initial concentrations of H_2O_2 are 0.0853 mol/l (a), 0.128 mol/l (b) and 0.256 mol/l (c), respectively.

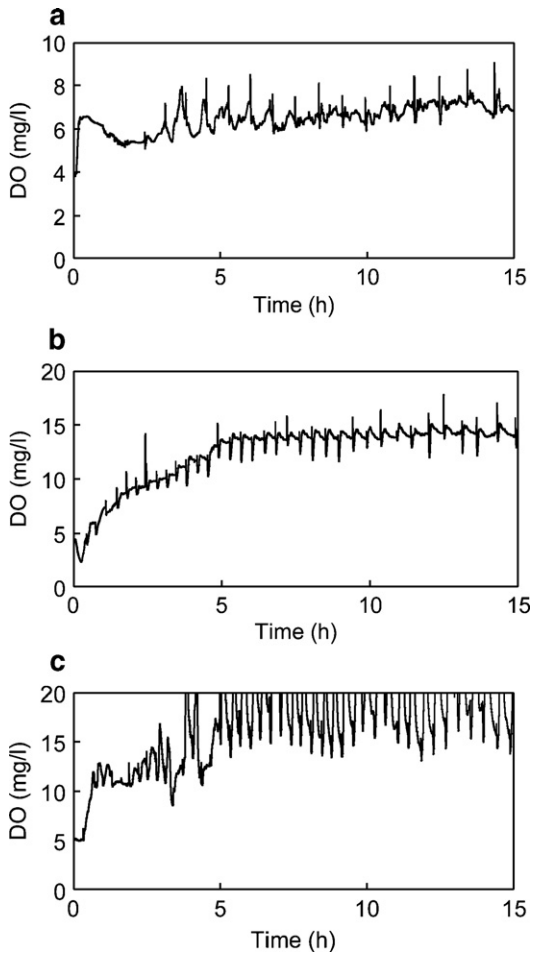

Fig. 5. Time course of oscillatory reaction by liposome-enwrapped catalase with lower concentration. The concentration of catalase is 0.0125 mg/ml and liposome was prepared with 0.6 mg/ml egg lecithin–toluene solution. The initial concentrations of H_2O_2 are 0.0853 mol/l (a), 0.128 mol/l (b) and 0.256 mol/l (c), respectively.

DMPC and the oscillation was observed in low concentration of hydrogen peroxide compared with that of DMPC. As the concentration of hydrogen peroxide increased, the period of oscillation became longer and oscillation disappeared gradually with further increase in concentration of H_2O_2.

4. Discussion

With respect to the liposome formed from egg lecithin, Mason et al. already studied the structure in detail [7]. As described above, it was found that catalase was included within liposome in current condition. It is well known that the permeation rate of nonelectrolyte such as hydrogen peroxide through bilayer membrane of phospholipid is related to the partition ratio between lipid and aqueous phase and can be described by solubility-diffusion model [8]. Hydrogen peroxide is thought to permeate easily through lipid membrane. The permeation rate of H_2O_2 through dialysis membrane was measured in the presence of liposome without catalase by the similar method as previous paper [5] (Fig. 8a). The time course of absorbance at 215 nm was measured. As shown in Fig. 8b, the

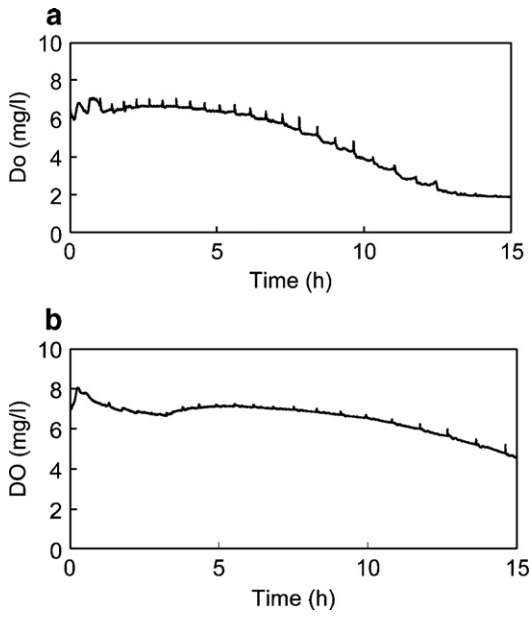

Fig. 6. Effect of temperature on oscillatory reaction. The concentrations of catalase and hydrogen peroxide are 0.125 mg/ml and 0.256 mol/l, respectively. 30 °C (b) 25 °C.

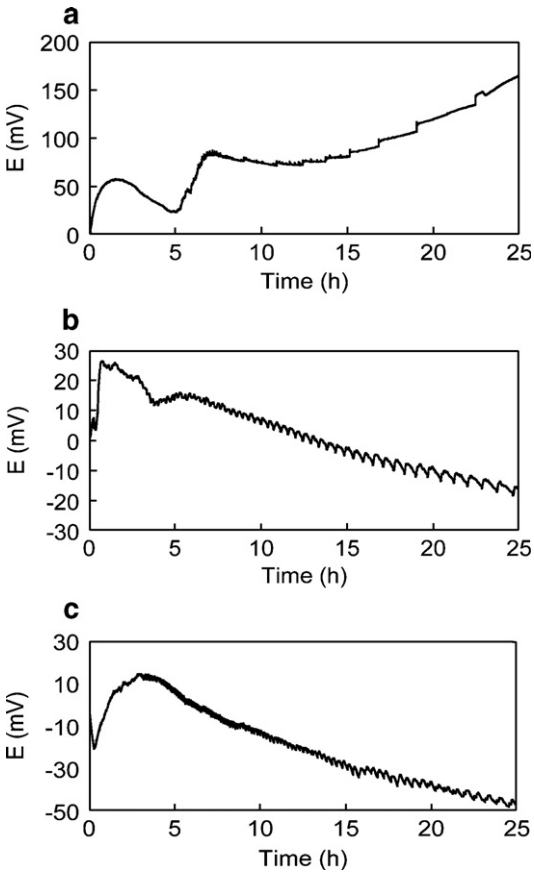

Fig. 7. Time course of electric potential measured by ORP electrode in the presence of liposome. The concentration of catalase is 0.125 mg/ml and liposome was prepared with 0.6 mg/ml egg lecithin–toluene solution. The initial concentrations of H_2O_2 are 0.128 mol/l (a), 0.426 mol/l (b) and 4.26 mol/l (c), respectively. The initial value of potential was taken as a criterion in each experiment.

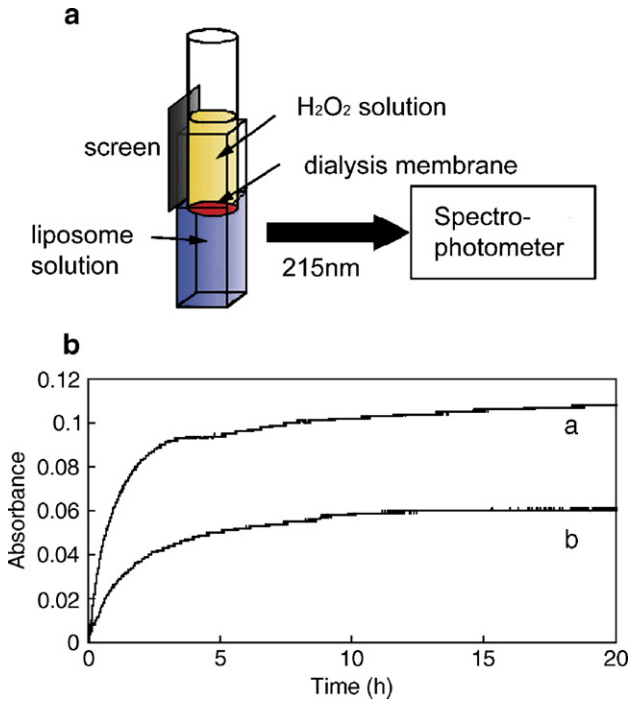

Fig. 8. (a) Apparatus for measuring the permeation rate of H_2O_2. (b)Time course of absorption at 215 nm in the permeation of H_2O_2 through semi-permeable membrane. Initial concentration of is 0.256 mol/l. Symbolic a and b in figure refer to the result in the presence of liposome and in the absence of liposome, respectively.

permeation rate in the presence of liposome was found to be larger than that in the absence of liposome. Also the partition coefficient of H_2O_2 from H_2O_2 solution to liposome solution at equilibrium condition in the absence of catalase was found to be larger than unity (1.27) from oxidation–reduction titration using $KMnO_4$ solution. This means that H_2O_2 was taken into liposome. These facts indicated that the presence of liposome facilitated the permeation rate of H_2O_2, although the cause of shortening the oscillation period remains to be cleared. It is also well known that O_2 can permeate easily through lipid membrane.

Our new data also showed oscillation with short period compared to that of DMPC. This might be thought that substrate and product easily permeate through membrane of egg lecithin, which is actually a mixture of phosphatidylcholine molecules with different acyl chain constituents [7].

As shown in Figs. 4 and 5, we obtained the oscillation with shorter period with an increase in hydrogen peroxide concentration. It has already been reported that the oscillation period became shorter with increasing in the concentration of hydrogen peroxide when liposome was absent [5]. The tendency of our new data is the same as that in the absence of liposome and shows that the increase in hydrogen peroxide, which causes the increase in permeation rate, leads to decreasing the oscillation period. On the other hand, the effect of temperature was observed, although temperature compensation of oscillation was observed in the absence of liposome. This is thought to be due to the fact that the permeation rate of substrate depends on the membrane structure of liposome.

Judging from the above discussion, the reaction of catalase and hydrogen peroxide should occur within individual liposome. The underlying causes of oscillatory reaction of enzyme under the condition via membrane have been thought to be suitable rates of the permeation of substrate into enzyme solution and of the outflow of oxygen [5]. Rate equations for each species in individual liposome are considered as follows like a previous paper [5], provided that substrate permeates through membrane, and the product is eliminated in proportional to its concentration.

$$\frac{d[E]}{dt} = -k_1[E][S] + (k_2 + k_3)[ES] - k_4[E][P]^{1/2}[P_2]$$

$$\frac{d[S]}{dt} = k_s - k_1[E][S] + k_2[ES]$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES] + k_4[E][P_1]^{1/2}[P_2]$$

$$\frac{d[P_1]}{dt} = k_3[ES] - k_4[E][P_1]^{1/2}[P_2] - k_p[P_1]$$

where [E], [S], [ES], $[P_1]$ and $[P_2]$ are the concentration of enzyme, substrate (H_2O_2), intermediate, product (O_2) and product (H_2O), respectively; k_1, k_2, k_3 and k_4 are rate constants for each reaction, respectively , k_s is permeation rate of H_2O_2 and k_p is the rate constant of outflow of O_2. In fact, we measured the concentration of O_2 that exude from liposome and was accumulated outside the liposome, being evaporated into air in part. According to linear stability analysis for this mechanism, the condition of instability of the steady state holds if $k_s - [E]_i$ $k_2 < 0$, where $[E]_i$ is the initial concentration of catalase [4].

As shown in Fig. 8, although the permeation rate of H_2O_2 in the presence of liposome was larger than that in the absence of liposome. As suggested from computer simulation, increase in permeation rate shortens the oscillation period. Consequently, the increase in permeation rate is thought to lead to the decrease in oscillation period. In the absence of liposome, the permeation rate was expressed by $k_s([S]_e - [S])$, where $[S]_e$ is equilibrium concentration. However, in the presence of liposome, the flow of H_2O_2 through semi-permeable membrane was nonlinear against the concentration difference from equilibrium. This nonlinearity seems to cause the short period oscillation and the long period oscillation found in Fig. 3a. Detailed analysis will be carried out in the future study.

On the other hand, as in the absence of liposome, the oscillation period became longer with the time. Oscillation with fixed period was obtained when glucose oxidase or lactic oxidase with its substrate was added to the system of catalase and hydrogen peroxide in the absence of liposome [9,10]. These are cyclic systems in which hydrogen peroxide and oxygen alternately become substrate and product. It was confirmed that the same occurred in the system with liposome containing glucose oxidase and glucose (data not shown) .

So far, we have been studying the oscillatory reaction of enzyme in bulk phase via semi-permeable membrane. Together

with a previous paper [6], we could obtain the oscillatory reaction that occurred in small compartment. The important role of liposome as model systems for protocell formation has been discussed by Szostak et al. [11]. Stange et al. provided experimental models for testing oscillatory behaviors of allosteric enzyme in a small confined volume [12]. Oscillatory reaction of liposome entrapped horseradish peroxide was reported [13]. On the other hand, it is known that liposome-entrapped catalase prevents lethal O_2 toxicity [14]. Catalase is needed during period of starvation to protect the organization from oxidative damage.

Oscillatory reactions of our system are different from these enzymatic oscillatory reactions in the mechanism. The underlying causes for inducing oscillatory reaction of enzyme are gradual entry of substrate and proper outflow of product as described above. Consequently, it is suggested that many kind of enzymes have a possibility to cause oscillatory reaction ubiquitously in living system. Our finding may provide an important insight into the study of enzyme reaction in organelles of living system.

Circadian rhythms are widespread among eukaryotes and prokaryotes. An ultradian-time keeping requirement is also essential for the maintenance of the ordered complexity of life [15]. Lloyd et al. showed the ultradian time keeper with a period of ~40 min and discussed a relation between ultradian and circadian time keeping [16]. Our method also may provide an important clue for elucidating these rhythms.

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