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The Properties of the Multistage Bioreactor Constructed by the LB Technique

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A multistage bioreactor consisting of organic ultrathin layers was successfully realized by immobilizing glucose oxidase and glucoamylase by the Langmuir–Blodgett technique (LB technique). Starch as the substrate was quickly converted into gluconic acid and hydrogen peroxide as the products in this bioreactor via the intermediate, glucose. The rate of the sequential reactions was electrochemically measured. This rate increased proportionally with increasing the concentration of starch, and also increased with increasing the amount of glucose oxidase in the LB film. This bioreactor, however, decayed soon. The reason of this decay was considered to be the structural conversion of the LB layers with glucoamylase. The multistage bioreactor constructed by the LB technique will be used not only as a super small-sized, rapid bioreactor or biosensor but also as an investigating means of the reconstitution of membrane enzyme and biomembrane.

The biomembrane is commonly a complex of lipid and protein constituting sequential enzymatic reaction systems. The same reactions may be realized in an artificially reconstructed system. A system using the microcapsule technique[1] as previously been demonstrated. Glucoamylase was adsorbed to the outside of a microcapsule within which glucose oxidase was contained. The construct functioned as an extremely small-sized bioreactor which converted starch into gluconic acid via glucose. Glucose as the intermediate, however, moved slowly into the inside of the microcapsule, and the rate of the overall reaction was not rapid.

If the enzymes are immobilized in ultrathin films the intermediates of sequential reactions will be able to move rapidly from one enzyme to the next. It is easy to immobilize protein in an organic ultrathin film if the Langmuir–Blodgett technique (LB technique) is used.[2]

The LB technique provides the following advantages: i) It is expected that the denaturation of enzymes is minimized as the technique is a wet process at normal temperature and pressure. ii) The devices constructed by this technique are so small and their that rapid diffusion of the substrate molecules of the enzymatic reactions are allowed. iii) The construction of multilayer structures is easy since the LB film is made by consecutively depositing monolayers. Onoue and Moriizumi constructed a glucose sensor by immobilizing glucose oxidase by the LB technique.[3,4] i) and ii) have already been demonstrated by these previous workers. We realized a two-step sequential reaction by constructing an LB film incorporating different enzymes in successive layers, by the introduction of the viewpoint of iii). This multistage bioreactor is a composite system combining the reaction of glucoamylase which hydrolyzes starch to glucose, and that of glucose oxidase which oxidizes glucose to gluconic acid and hydrogen peroxide.

Experimental

Materials. Enzymes: Glucose oxidase (EC 1. 1. 3. 4) from *Aspergillus niger* (Sigma Chemical) and glucoamylase (EC 3. 2. 1. 3) from *Aspergillus oryzae* (Sigma Chemical) were used. Each enzyme was dissolved in ultra pure water at a concentration of 500 mg dm^{-3}. The electric conductivity of ultra pure water produced by using Milli Q Organex-Q type (Millipore) was more than 17.5 MΩ cm.

Amphiphilic Materials: As a result of considering the surface charges of the enzymes, icosanoic acid (Applied Science) was used to adsorb glucose oxidase and a mixture of trimethyloctadecylammonium chloride (Tokyo Kasei Kogyo) and methyl icosanoate (Applied Science), weight ratio 1 : 4, was used to adsorb glucoamylase.[4,5] The trimethyloctadecylammonium chloride was recrystallized from hot ethanol–ether. The spreading solvents of the amphiphilic materials were prepared in chloroform (Spectrosol series of Dojindo Laboratories), at a concentration of 1 mg cm^{-3}.

Substrates: Glass substrates with two gold-evaporated electrodes were used. The area of each electrode was 10 mm^2. The surface of the substrate was treated with trichlorooctadecylsilane (Shin-etsu Chemical) and made hydrophobic.[6]

Preparations. Fromherz Method:[2] A Fromherz-type trough (Mayer–Feintechnik) was used. The trough has eight trough compartments, and two barriers can move from one trough compartment to another ad libitum. This Fromherz method was used to carry out the following process. The subphase solution is placed in several trough compartments. Then, a monolayer of amphiphilic molecules is organized on the surface of the subphase. Next, a protein solution is placed in the adjoining trough compartments. The monolayer is then moved over onto the surface of the protein solution to allow the protein to adsorb to the monolayer. This composite monolayer with adsorbed protein is returned onto the pure subphase. The composite monolayer is then deposited onto a glass substrate by the LB technique, and in this way the LB film incorporating protein can be constructed.

Conditions for Constructing the Enzyme LB Film: The Fromherz method was carried out. Ultra pure water was used as the subphase (pH approximately 6, 20 °C). The surface pressure of the monolayer of icosanoic acid was 30 dyne cm^{-1} (1 dyne=10^{-5} N) through the adsorption step, and that of the mixed monolayer described above was 20 dyne cm^{-1}. The time allowed for adsorption was 1 h. The surface pressure of the composite monolayer on the subphase was increased to 35 dyne cm^{-1} for deposition. The dipping speed

was 5 mm min^{-1}. A Y-type deposition was obtained for each composite monolayer. The transfer ratio was between 0.9 and 1.0.

The Measurement of the Enzymatic Reaction. Hydrogen peroxide generated by the enzymatic reaction was measured electrochemically.[3] The substrate was placed in a 1 cm×1 cm cell for spectroscopy containing 1 ml of 0.05 M (1 M=1 mol dm^{-3}) acetate buffer (pH 5, 20 °C).[7] The electrodes on the substrate were connected to a potentiostat with the applied potential set at 0.8 volt. The electrode current was recorded by an X-T recorder connected to the potentiostat. Glucose or soluble starch of reagent grade was used as the substrate of the enzymatic reactions. The substrate solution was prepared in acetate buffer, and 200 μl of a concentrated solution was injected into the buffer in the cell to produce the necessary concentrations.

Results and Discussion

The enzymatic reactions is following:

Glucoamylase

$$\text{starch} + (n-1)\cdot H_2O \longrightarrow n\cdot \text{D-glucose}$$

Glucose oxidase

$$\text{D-glucose} + O_2 \longrightarrow \text{D-gluconic acid} + H_2O_2$$

First, the properties of a bioreactor constructed by the deposition of an LB film incorporating glucose oxidase was studied. The response current at 1 min after injecting glucose, two LB layers incorporating glucose oxidase was directly proportional to the concentration of glucose in the range less than 3 g dm^{-3} (Fig. 1). When this bioreactor was kept at 5 °C for several months, the response current changed very little, indicating high stability. These results agreed with the results by Onoue and Moriizumi.[3]

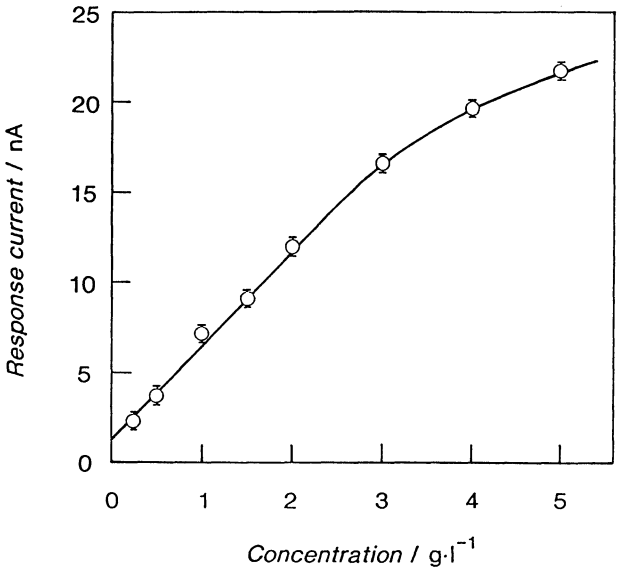

Fig. 1. Response current vs. concentration of glucose. Two LB layers incorporating glucose oxidase was measured. The value (average ±standard error) was observed at 1 min after the addition of glucose solution.

Next, the properties of a multistage bioreactor constructed by depositing two LB layers incorporating glucoamylase onto eight LB layers incorporating glucose oxidase was studied. The time-dependences of the response currents for a series of concentrations of starch are shown in Fig. 2. Rapid response was observed at each concentration of starch solution. These response currents attained steady states in a few seconds. The microcapsule-type bioreactor showed a lag time of sev-

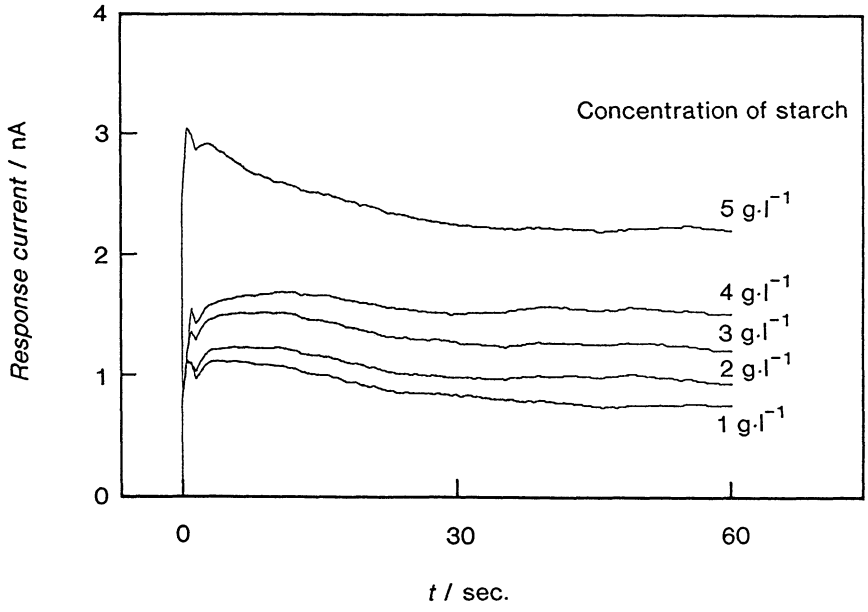

Fig. 2. Response current vs. time after addition of starch solution (t). Two LB layers incorporating glucoamylase on eight LB layers incorporating glucose oxidase were measured.

eral minutes in the reactions.[1] The very short lag time indicates that the diffusion of the intermediate species, glucose, was very rapid in the ultrathin LB film.

Greater concentrations of starch increased the response current. Figure 3 shows the dependence of the response current of the bioreactor, at 1 min after injection, on the concentration of starch. The response current was linearly related to the concentration of starch from 2 to 5 g dm^{-3}, suggesting the possibility of its use as a starch sensor. The reason the fitted line does not go through the origin is probably due to the fact that the solution is not stirred, resulting in some nonuniformity of concentration.

Figure 4 shows the response currents at 1 min after the injection of starch solution plotted against the number of the LB layers incorporating glucose oxidase. In each sample, two LB layers incorporating glucoamylase were deposited on to the LB layers incorporating glucose oxidase. The concentration of starch was 5 g dm^{-3}. The response current was proportional to the number of the glucose oxidase LB layers. As the amount of adsorbed glucose oxidase per one LB layer is constant, this indicated that the activity of the reaction was proportional to the total amount of enzyme incorporated.

The storage stability of the bioreactor constructed was investigated. The bioreactor constructed with two LB layers of glucoamylase deposited on eight LB layers of glucose oxidase was kept at 5 °C. The response current at 1 min after injection of a 5 g dm^{-3} starch solution is plotted in Fig. 5. The response current decreased significantly in five days after construction. The decrease in response current for a bioreactor incorporating only glucose oxidase was found to be very small. Thus, it is suspected that the decay was caused

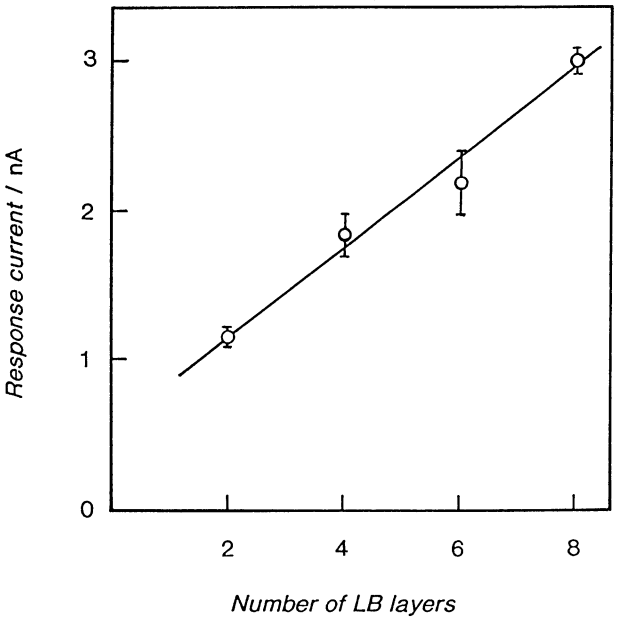

Fig. 4. Response current vs. number of LB layers incorporated with glucose oxidase.
Two LB layers incorporated with glucoamylase on LB layers incorporated with glucose oxidase were measured. The value (average ±standard error) was observed at 1 min after the addition of starch solution. The concentration of starch was 5 g dm^{-3}.

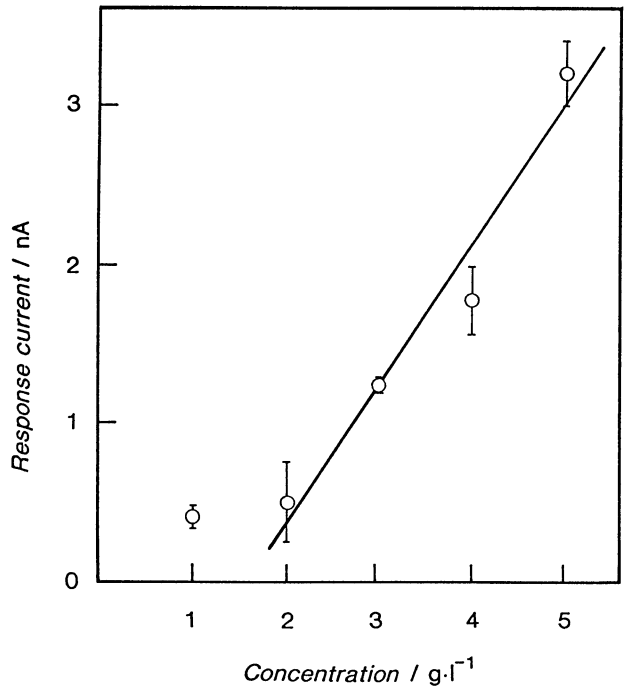

Fig. 3. Response current vs. concentration of starch.
The value (average ±standard error) was observed at 1 min after the addition of starch solution.

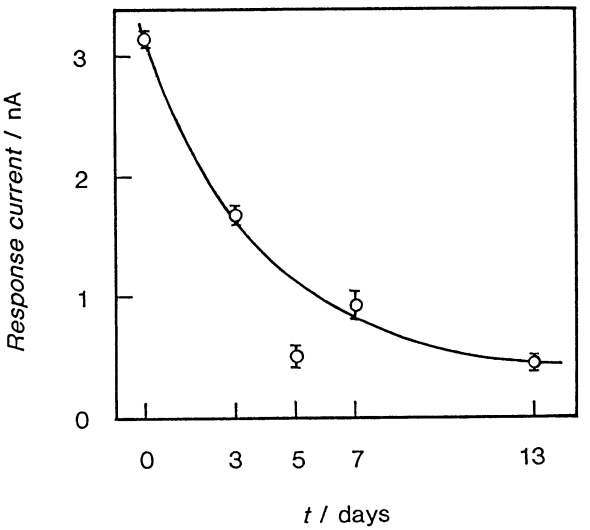

Fig. 5. Storage stability of the bioreactor. Days after preparation (t) vs. response current.
Storage temperature was 5 °C. The bioreactor was constructed with two LB layers incorporated with glucoamylase on eight LB layers incorporated with glucose oxidase. The value (average ±standard error) was observed at 1 min after the addition of starch solution. The concentration of starch was 5 g dm^{-3}.

by the LB layers incorporated with glucoamylase.

The structure of the multistage bioreactor constructed by the LB technique is similar to a biomembrane. Therefore, it will be possible to apply this approach to the study of the property of reconstructed membrane enzyme systems as well as to the construction of ultramicro or ultrarapid bioreactors or biosensors.[8,9] This approach would allow the control of the adsorbed site and the amount of enzyme.[4,10,11]

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