Immobilization of β-Glucosidase in Fibroin Membrane

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Fibroin membrane was used as a support for immobilized β-glucosidase. The immobilized enzyme was prepared by drying fibroin-enzyme solution on a horizontal plate, followed by ethyl alcohol treatment which was an essential process for immobilization. The immobilized β-glucosidase was characterized enzymatically compared with soluble enzyme, using p-nitrophenyl-β-D-glucopyranoside as a substrate. The immobilized enzyme showed 47% of the activity of soluble enzyme and little decrease of the activity was observed both on re-use and storage. There were no significant differences in pH dependency between immobilized and soluble enzyme activity. Activation energy was slightly larger with immobilized enzyme than soluble enzyme. The enzyme was considerably enhanced by immobilization in stabilities against heating, electrodialysis and protease treatment. Apparent affinity for the substrate decreased as membrane thickness increased. Enzyme affinity for substrate in the membrane was discussed.

Various attempts have recently been made to immobilize enzymes in membrane,1-3) in order to produce a material with both reaction and separation capabilities. As support for membraneous immobilized enzyme, collagen has been successfully adapted.4-6)

In our laboratory, fibroin membrane has been studied with permeability for sugars.7) Fibroin membrane shows rather high permeability. To develop this fibroin membrane as a biochemically reactive membrane, we immobilized β-glucosidase as a test enzyme in membrane.

In this report, fundamental studies are described on the properties of immobilized β-glucosidase and of fibroin membrane as support.

MATERIALS AND METHODS

Preparation of immobilized enzyme. Pure fibroin was obtained from Bombyx mori cocoon, born in spring 1975 (species, Taihei × Choon), by the established method including successive treatment with ethyl ether and boiling water.8) Purified fibroin (3 g, dry weight) was completely dissolved in 100 ml of 9.3 M of LiBr solution at 40°C,8) and the solution was placed in a cellophane bag (cellulose tubing, Union Carbide Co.), followed by dialysis against 60 liters of distilled water for 48 hr at 20°C. After dialysis, the solution was centrifuged for 10 min at 104 x g to remove aggregates. The clear supernatant (130 ml) was mixed with 20 ml of β-glucosidase solution (5.4 mg/ml) from sweet almond (Tokyo Kasei Kogyo Co.), cast on a horizontal polyacrylate plate (24 x 20 cm), and dried in air with an electric fan for 24 hr at 20°C, and a translucent membrane of about 67-μ thick (at wet state) was obtained. The membrane was suspended in 50 vol% ethyl alcohol for 30 min at 20°C. The membrane was removed and kept in vacuo for 30 min to remove ethyl alcohol, followed by suspending in distilled water for 1 hr and drying in vacuo. The membrane-immobilized enzyme was stored at 4°C (relative humidity, 60%), and enzyme activity was measured in need. A small amount of enzyme was released from the membrane by washing with water. The content of enzyme in the membrane was equivalent to 4% of membrane weight. When ethyl alcohol treatment was omitted from the preparation process described, about two-thirds of the activity was solubilized from the membrane during 1 hr incubation in 0.05 M sodium acetate buffer (pH 5.7) at 25°C.

Measurement of enzyme activity

Activity of enzyme in soluble form. Enzyme reaction was carried out at 25°C in a reaction mixture of 1 ml containing 0.05 M p-nitrophenyl-β-D-glucopyranoside (p-NPG; Koch-Light Laboratories), 0.05 M sodium acetate buffer (pH 5.7) and 100 μg of β-glucosidase. After 10-min reaction, 0.5 ml of reaction mixture was
sampled and added to 2.5 ml of 0.6 M sodium carbonate, and the amount of p-nitrophenol (p-NP) formed was measured by an increase in optical density at 410 nm. One unit of enzyme is defined as the amount that forms 1 μmol of p-NP per min under the conditions used.

Activity of immobilized enzyme. Preparation of immobilized enzyme of about 67 μ (thickness) × 1 cm² (square) at wet state was previously buffered by being kept in 0.05 M buffer for 4 hr at 25°C. The wet membrane was transferred into 5 ml of solution containing 0.05 M p-NPG and 0.05 M acetate buffer (pH 5.7), and the reaction was conducted at 25°C for 30 min with gentle shaking. p-NP formed was measured. Activity was determined from the data at steady state.

Electrodialysis. Five ml water solution containing 10 mg of the soluble or immobilized enzyme was placed in a cellophane bag and put into a vessel containing 800 ml of deionized and distilled water, in which electrodialysis was conducted at 25°C for 20 hr. The cross section of the vessel was about 50 cm², and the distance between two electrode plates was 12 cm. Current density was kept at about 8 μA/cm² and voltage at about 450 V by a Toyo direct current generating system.

Protease treatment. Proteases employed were trypsin (2000 U/g, E. Merck), α-chymotrypsin (45 U/g, E. Merck), papain (20,000 U/g, E. Merck) and pronase E (70,000 U/g, Kaken Kagaku Kogyo Co.). Protease treatment was performed in the following mixture. Five milliliter of reaction mixture contained: 2.5 mg protease; 0.05 M K, Na-phosphate buffer (at indicated pH) and 10 mg of soluble enzyme or 67 μ × 1.9 cm² of membrane (equivalent to 0.4 mg enzyme); pH 7.5 for trypsin, α-chymotrypsin and papain treatments, and pH 7.0 for pronase E. The treatment mixture was allowed to stand for 150 min at 30°C. Before and after treatment, activity of 0.05 ml soluble enzyme was assayed directly. Protease-treated and non-treated immobilized enzyme membranes were washed with distilled water, immersed in acetate buffer, and then β-glucosidase activity was measured as described above.

Determination of diffusion coefficient. Diffusion coefficients of p-NPG and p-NP in fibroin membrane were determined with the apparatus reported previously. Fibroin membrane without enzyme (72 μ thick), previously immersed in 0.05 M buffer (acetate buffer, pH 4.2 and 5.7; phosphate buffer, pH 7.0) for 4 hr at 25°C, was attached to the end of a glass tube with an inner diameter of 3 cm, and fixed by being pressed with a gum ring. This was termed an inner cell. A 500-ml beaker used as an outer cell and containing 400 ml of 0.05 M buffer was regulated at 25°C. The experiment was started by placing 20-ml solution composed of the substrate and 0.05 M buffer in the inner cell put in the outer cell. The inner cell was so settled as surfaces of the solutions in both cells coincided with each other to eliminate the effect of hydrostatic pressure. The solutions in both cells were continuously stirred throughout the experiment. At appropriate intervals, 1-ml samples from the outer cell and 0.1 ml from the inner cell were withdrawn. The amount of p-NPG was estimated from the absorbance of the sample at 300 nm in the presence of 0.5 M sodium carbonate. With a membrane less than 1 mm thick, a steady state was essentially established within a few min for a low-molecule-weight substrate, such as sucrose. A diffusion coefficient D: cm²/sec was obtained from the data at 60-min by using formula (I)

\[
d = \frac{(AD)}{(V_o)} (S_i - S_o)
\]

where, \(d\) is diffusion rate (m/sec), \(A\) the membrane area (cm²), \(l\) the membrane thickness (cm), \(V_o\) the volume of the solution in the outer cell (cm³), \(S_i\) and \(S_o\) the concentration of the substrate in the inner and outer cells (m), respectively.

Estimation of partition coefficient. Partition coefficients of p-NPG and p-NP in fibroin membrane were measured by placing a membrane of 0.5 cm³ in 5 ml solution composed of 0.01 M substrate and 0.05 M buffer (acetate buffer, pH 4.2 and 5.7; phosphate buffer, pH 7.0). The system was left for 10 hr at 25°C to attain equilibrium and the change in concentration of the substrate in solution was determined. No further change in the concentration was observed after 10 hr.

RESULTS

Time course of enzyme reaction

The time course was followed in both the soluble and immobilized enzyme systems. As shown in Fig. 1, soluble enzyme activity decreased slightly with time, while immobilized enzyme hydrolyzed the substrate with an ap-

![Fig. 1. Time Course of Enzyme Reaction.](image-url)
proximately constant rate, after a lag time of about 10 min. The immobilized enzyme showed 47% of the activity of soluble enzyme in 60-min incubation.

**pH dependency**

No significant differences were observed in pH dependency between immobilized and soluble enzyme activity (Fig. 2). The pH-activity curve of immobilized enzyme shifted a little to the alkaline side. The optimum pH was 5.6 for soluble enzyme, and 5.7 for immobilized enzyme.

**Temperature dependency**

Figure 3 shows the temperature dependency in a range of 10°C to 40°C by Arrhenius plots. The plots for soluble enzyme were two straight lines intersecting at 27°C and those for immobilized enzyme were two lines intersecting at 25°C. Activation energies of the soluble enzyme were calculated to be 11.1 and 7.3 kcal/mol at temperatures below 27°C and above 27°C, respectively. With immobilized enzyme, the values were 14.8 and 7.9 kcal/mol at the temperatures below and above 25°C. Thus, activation energy was larger with immobilized enzyme than with soluble enzyme at this temperature range.

**Heat stability**

Heat stability was investigated by heat treatment for 30 min in 0.05 M sodium acetate buffer (pH 5.7) at the indicated temperatures. After treatment at 55°C, soluble enzyme lost about one-half of the activity, while immobilized enzyme lost only one-tenth (Fig. 4).
Furthermore, 65°C treatment reduced the activity of soluble enzyme to about one-seventh, while that of immobilized enzyme was reduced to about half. Thus, immobilization enhanced heat stability. Amounts of protein released from immobilized enzyme during heat treatment were found to be less than 1% in measurements with the Lowry-Folin method.

**Michaelis constant and maximum velocity**

Figure 5 shows Lineweaver-Burk's plots on immobilized and soluble enzymes. The plots were linear with immobilized enzyme. Apparent Michaelis constants at pH 5.7 were calculated to be 5.6, 7.8 and 10.7 mM with immobilized enzyme in membranes of 36 μ, 67 μ and 98 μ thickness, respectively, and so apparent affinity decreased as membrane thickness increased. The Michaelis constant of free enzyme was 2.3 mM. Maximum velocities were calculated to be 146, 126 and 110 units per gram of enzyme with membranes 36 μ, 67 μ and 98 μ thick, respectively. The maximum velocity of free enzyme was 256 units per gram of enzyme. The affinity of the enzyme for the substrate in membrane will be discussed later.

![Figure 5](image)

**Storage stability**

Stability of activity during storage was tested (Table I). When stored in a storage chamber (4°C, 10°C) at a relative humidity of 60%, the soluble enzyme was a little more stable than immobilized enzyme, while storage in such solutions, as buffer solution containing the substrate, buffer solution or water, gave reverse results. Under the conditions used here, the immobilized enzyme showed an approximate 10% decrease in activity and no release of protein during storage.

![Figure 6](image)

**TABLE I. STABILITY DURING STORAGE**

<table>
<thead>
<tr>
<th>Condition of storage</th>
<th>Period (days)</th>
<th>Residual activity</th>
<th>Released protein from the membrane (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>In air at 4°C</td>
<td>180</td>
<td>89</td>
<td>94 b</td>
</tr>
<tr>
<td>In air at 10°C</td>
<td>30</td>
<td>92</td>
<td>97 b</td>
</tr>
<tr>
<td>In water at 4°C</td>
<td>30</td>
<td>86</td>
<td>79</td>
</tr>
<tr>
<td>In buffer at 4°C</td>
<td>30</td>
<td>88</td>
<td>84</td>
</tr>
<tr>
<td>In buffer containing substrate at 4°C</td>
<td>30</td>
<td>89</td>
<td>82</td>
</tr>
</tbody>
</table>

Data points include:
- Released protein was measured by the Lowry-Folin method.
- Enzyme preparation was powder.
- 0.05 M sodium acetate buffer.
- 0.05 M sodium acetate buffer with 0.02 M p-NPG.
**β-Glucosidase-fibroin Membrane**

*Stability in repeat usages*

Retention of activity in membrane during repeat usage was measured every day as described below. After each measurement, the membrane was stored under different conditions. As shown in Fig. 6, retention of enzyme activity in the membrane tended to decrease in the order of storing in water, air, buffer solution without substrate (0.05 M acetate buffer, pH 5.7) and buffer solution with substrate, at 4°C. When stored in air, the decrease in membrane weight was within 5%, and it was 3% in other storing conditions. Released enzyme activity was negligible, while, the remaining activity in the membrane was 87%, when stored in the buffer-substrate solution.

*Effect of electrodialysis*

The enzyme-immobilized membrane might contain trace amounts of salts, such as LiBr from fibroin solution and others from the original enzyme. Addition of LiBr to the reaction mixture at a concentration of 0.01 M did not at all affect the activity of soluble enzyme. However, these salts in membrane might be undesirable for some applications. Electrodialysis should usefully remove salts from the membrane, though its effect on enzyme activity is not known. Therefore, electrodialysis was conducted and its effects were examined. The table shows the residual activity and solubilized membrane of various proteases.

**Table II. Effect of Electrodialysis**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Residual activity (%)</th>
<th>Solubilized membrane (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>104</td>
<td>93</td>
</tr>
<tr>
<td>Papain</td>
<td>95</td>
<td>76</td>
</tr>
<tr>
<td>Pronase E</td>
<td>88</td>
<td>61</td>
</tr>
</tbody>
</table>

*Protease treatment*

The proteolytic stability of soluble and immobilized enzymes was compared. Solubilization of membrane was not observed during protease treatment, as seen in Table III. Although soluble enzyme decreased in activity by treatment with pronase E or papain, the immobilized enzyme was little affected, except with pronase E.

**Table III. Effect of Protease Treatment**

For details on experiments, see MATERIALS AND METHODS.

Diffusion and partition coefficients in fibroin membrane

Diffusion coefficients (D) in fibroin membrane at pH 4.2, 5.7 and 7.0 were calculated respectively to be 8.2, 8.3 and 9.5×10⁻⁹ cm²/sec for 10 mM p-NPG (from data in Fig. 7), and 4.2, 3.9 and 2.4×10⁻⁷ cm²/sec for 10 mM p-NP (data not shown). As D values of p-NP are considerably larger than those of p-NPG, p-NP diffusion can not be a rate limiting factor in enzymatic reaction. Also, D values for p-NPG at pH 5.7, at initial concentrations of p-NPG 1.9, 4.8, 19 and 48 mM, were calculated from data in Fig. 7 to be 9.6, 8.9, 7.7 and...
FIG. 7. Time Course of Diffusion of p-NPG through Fibroin Membrane.

Solid lines represent external substrate concentrations and dashed lines, the internal concentrations. Initial internal concentrations were as follows, at pH 5.7: ○, 1.9 mM; △, 4.8 mM; □, 10 mM; ●, 19 mM; ▲, 48 mM.

The initial concentration at 10 mm and pH 7.0, with phosphate buffer.

The time course at pH 4.2 (acetate buffer) and 10 mm initial internal concentration was not illustrated, because it was quite similar to that at pH 5.7 and 10 mm.

$7.0 \times 10^{-8}$ cm$^2$/sec, respectively. This result shows that the diffusion coefficient depends somewhat on internal substrate concentration.

Considering that the typical value of $D$ of a low-molecular-weight substrate (such as sucrose) is about $5 \times 10^{-6}$ cm$^2$/sec at 25°C in free water, the diffusion rate of p-NPG in fibroin membrane may have some effect on the reaction of immobilized $\beta$-glucosidase.

According to the method described in the text, partition coefficients ($P$) were calculated to be 0.56 for p-NPG and 0.68 for p-NP. These values were not affected by pH.

DISCUSSION

Laidler et al.\textsuperscript{10,11} presented four main reasons why the kinetics of immobilized enzyme are different from those of soluble formed one. (a) The enzyme structure may be conformationally altered when immobilized in the membrane.

(b) The different interaction between the enzyme and substrate may take place in the membrane from that in free solution. Rate constant and Michaelis constant, as modified by effects (a) and (b), are denoted as $kc'$ and $Km'$. (c) Partition of the substrate between phases of the membrane and medium may affect the substrate concentration in the environment the enzyme is located. (d) The reaction in the membrane may be to some extent diffusion-controlled.

By supposing that diffusion of the substrate in membrane obeys Fick's second law of diffusion and enzyme reaction in membrane obeys Michaelis-Menten kinetics, they proposed equations (II)\textendash(VI) to express enzyme reaction in membrane to a good approximation.\textsuperscript{10,11} The rate ($v'$) of a reaction catalyzed by the immobilized enzyme is represented by

$$v' = kc'(app)(E)m(S)/[Km(app) + (S)]$$  \hspace{1cm} (II)

where $(E)m$ is the molar concentration of enzyme in the membrane, $(S)$ the concentration of substrate in solution. $kc(app)(E)m$ (M/sec) is equivalent to maximum reaction rate by 1 liter of enzyme-immobilized membrane, calculated from Lineweaver-Burk's plots. $kc(app)$ and $Km(app)$, signifying apparent rate constant and apparent Michaelis constant, are given by

$$kc(app) = kc'$$  \hspace{1cm} (III)

$$Km(app) = Km'/PF$$  \hspace{1cm} (IV)

The function $F$ relates to the extent to which the reaction in membrane is diffusion-controlled, and is expressed as follows

$$F = (\tanh\gamma l)/\gamma l$$  \hspace{1cm} (V)

where $l$ (cm) is the thickness of the membrane and $\gamma$ (cm$^{-1}$) is given by

$$\gamma = [kc'(E)m/4DKm']^{1/2}$$  \hspace{1cm} (VI)

By applying equations (II)\textendash(VI), values of $Km$ for p-NPG of $\beta$-glucosidase in fibroin membrane were calculated on the basis of the data of $l$, $D$, $P$, $Km(app)$ and $kc(app)(E)m$, obtained in RESULTS. As shown in Table IV, $Km'$ varies with each membrane and with the $D$ value employed, but the difference between
TABLE IV. CALCULATED VALUES OF MICHAELIS
CONSTANTS OF ENZYME IN MEMBRANE

<table>
<thead>
<tr>
<th>Membrane thickness (µ)</th>
<th>Maximum velocitya (µmol/sec/liter of membraneb)</th>
<th>Km(app)a (mm) at D values employed</th>
<th>Km’ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>78</td>
<td>5.6</td>
<td>2.3 2.2 2.0</td>
</tr>
<tr>
<td>67</td>
<td>67</td>
<td>7.8</td>
<td>2.2 2.0 1.7</td>
</tr>
<tr>
<td>98</td>
<td>60</td>
<td>10.7</td>
<td>2.3 2.0 1.7</td>
</tr>
</tbody>
</table>

a Obtained from Fig. 5.
b Equivalent to 32 g of β-glucosidase.
c, d, e Obtained at initial internal substrate concentrations of 1.9, 10 and 48 mm, respectively.

membranes was not so large. It could be said that Km’, in any case, is close to Km (2.3 mm) of the soluble enzyme. Thus, it might be concluded that the affinity of the enzyme for the substrate was not affected by immobilization, and apparent Michaelis constant was affected chiefly by partition and diffusion effects of membrane.

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REFERENCES