Immobilization of Enzymes on Electrostatic Medium of Positively Charged Dimethylamino Nylon Gel

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SYNOPSIS

Urease and glucose oxidase were immobilized by positively charged nylon gel matrix made from dimethylaminated nylon. The stability of the immobilized enzyme after preheating and incubation in organic solvent were measured and compared with that of native enzymes. Also, pH dependency of the immobilized enzymes was examined. The pH activity curves of the immobilized enzyme shift toward a low pH compared with that of the native enzyme. Using the pH difference, the electrostatic potential of the positively charged nylon gel was estimated. The estimated values of the positively charged gel were +90 and +102 mV for the immobilized urease and immobilized glucose oxidase, respectively. In the presence of buffer salt, they decreased to +30 and +24 mV, respectively. © 1992 John Wiley & Sons, Inc.

INTRODUCTION

Charged gel is useful material for the ion-exchange[1,2] and immobilization of enzymes.[3-5] The fixed charges in the matrix have electrostatic interaction between the matrix and the charged molecule.[6,7] No experimental examination of the electrostatic potential was previously performed for hydrophilic and swelled gel matrices having fixed charges. In micelles,[8] vesicles,[9] and polyelectrolytes,[10] however, the electrostatic potential was measured using probe molecules such as pH-indicator dyes. In these cases, it is necessary to bind the probe molecules to the potential environment of the charged assemblies.

Using charged matrix for the immobilized enzyme, the immobilized enzyme prepared has different pH dependence of the activity compared with the native enzyme[11-15]; that is, for positively charged matrix, the optimum pH of the immobilized enzyme activity shifts toward a low pH region relative to that of native one. This is due to the covalent binding of the enzyme to the high potential portion of the charged matrix. The phenomena were quantitatively studied by Goldstein et al.[11,12] The electrostatic potential of the ethylene–maleic acid copolymer of the immobilized matrix was calculated using the activity of the immobilized enzyme.

In this report, positively charged gels made from aqueous soluble dimethylamino nylon (Scheme 1) were used for the immobilization of urease and glucose oxidase. The stability, pH dependence, and electrostatic potential of the positively charged gel were measured.

MATERIALS

Various dimethylamino nylons having a 2, 11, 33, 55, and 100% degree of dimethylamination were produced by Toray Industries. The preparation methods were reported previously.[16,17] The nylons of 2, 11, 33, and 55% degree of dimethylamination are not soluble in water, but that of 100% degree is soluble. In this work, the aqueous soluble nylon of 100% degree of dimethylamination was used without further purification. The molecular weight (M_w = 8–9 × 10^3) of the aqueous soluble nylon was measured in 0.2 M phosphate buffer using HPLC (Toso; pump, CCPD; column, TSKgel 6000PW_{XL}, 30 cm). The dextran samples were used as standards to calculate molecular weight.

Cross-linked reagents (Scheme 2) as synthesized in previous studies[5] were used for the experiments. Urease (EC 3.5.1.5) and glucose oxidase (EC 1.1.3.4)

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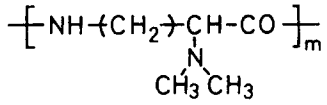
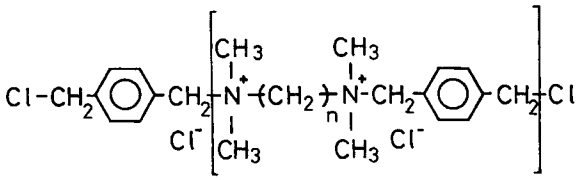


Scheme 1



Scheme 2

were from Merck Co. and Toyobo Co., respectively. Other reagents were commercial types of special grade. Water was purified by using ion-exchange resin and then distilled.

PREPARATION OF IMMOBILIZED ENZYME AND MEASUREMENT OF ACTIVITY

Details of the preparation of charged gel-entrapping enzymes have been described in previous reports.[4,5] Table I shows the composition of positively charged matrices of dimethylamino nylon gel for the immobilization of enzymes. Immobilization of urease and glucose oxidase was carried out at 4°C in a refrigerator for 20–24 h. The prepared gel having 0.01 mg enzyme per gram gel was used for the following experiments: For measuring the activity of the immobilized enzyme, a 45 mL reaction solution of 0.1 *M* phosphate buffer containing 2 g immobilized enzyme gel and 0.005 *M* substrate was incubated at 30°C with stirring at 80 rpm. Substrates of urease and glucose oxidase are urea and β-D-glucose, respectively. The enzyme activity was measured according to the previous method[3–5]; that is, the produced NH_3 for urease and the consumed O_2 for glucose oxidase were monitored by using absorption spectroscopy through the indophenol method[18] and the oxygen detector (Shibata Kagakukikai Co. Ltd., type DO-2), respectively.

Absorbances were measured by using a Shimadzu UV-190 double-beam spectrophotometer. Titrations of dimethylamino nylon were carried out in water using pH readings obtained on a Horiba F-11 pH meter equipped with a Horiba combination electrode no. 6366.

RESULTS AND DISCUSSION

Gelation was carried out by quaternization of dimethylamino nylon with the chloromethyl group of cross-linked reagents. The cross-linked nylon gel in Table I is of hydrophilic nature. As previously reported,[4] water contents are about 96% and positively charged contents are 21.3–21.6 mol/g-dry gel. The densities of the swelled gel for n = 2, 6, and 11 of the cross-linked reagents are 0.996, 0.992, 0.981, respectively. The partition coefficient (K_p) previously calculated[4] for urea and β-D-glucose did not change with the n of the cross-linked reagent. This shows little difference in the substrate concentration for the gels cross-linked by the reagents.

Table I contains values of relative activity of immobilized enzymes. The obtained activity of the immobilized enzyme is lower than that of the native one. The decrease may arise from the low diffusion of the substrate into the enzyme entrapped in the gel.[4]

Produced NH_3 for the immobilized urease and consumed O_2 for the immobilized glucose oxidase are plotted in Figures 1 and 2, respectively, as function of reaction time. The amounts of the produced NH_3 and the consumed O_2 increase with increasing

Table I Composition of Positively Charged Dimethylamino Nylon Gel for Immobilization of Enzyme[a]

No.	n of Cross-linked Reagents	K_p		Relative Activity[b] (%)	
		Urea	β-D-Glucose	Urease	Glucose Oxidase
1	2	3.3	0.7	2	30
2	6	3.1	0.6	10	36
3	11	3.7	0.6	23	42

[a] Mol ratio of cross-linked reagent to the dimethylamino group of the nylon is 0.065.

[b] Relative activity is of relative values to 0.02 mg native enzyme in 0.1 *M* phosphate buffer at 30°C, using 2 g gel containing 0.02 mg enzyme.

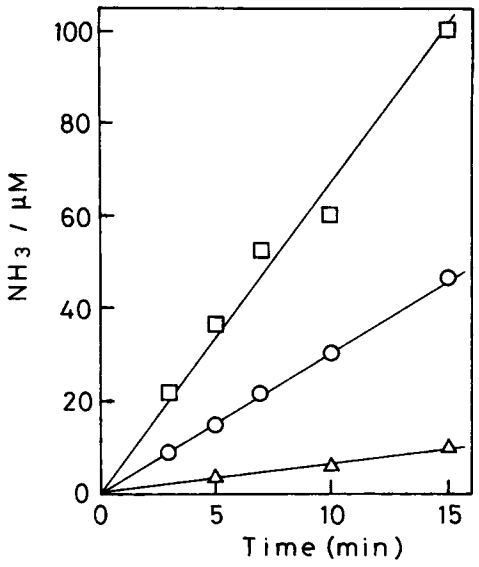


Figure 1 Changes of produced NH_3 for immobilized urease with reaction time at 30°C. Mol ratio of cross-linked reagent to dimethylamino group of the nylon is 0.065. Reactions were carried out in 0.1 *M* phosphate buffer at pH 7.0: (□) $n = 11$; (○) $n = 6$; (△) $n = 2$.

reaction time and depend on the number of n of the cross-linked reagent used; that is, the decrease of the number, n, leads to the decrease of the immobilized enzyme activity. This may be due to the decrease of network distance of the gel with decreasing the number.

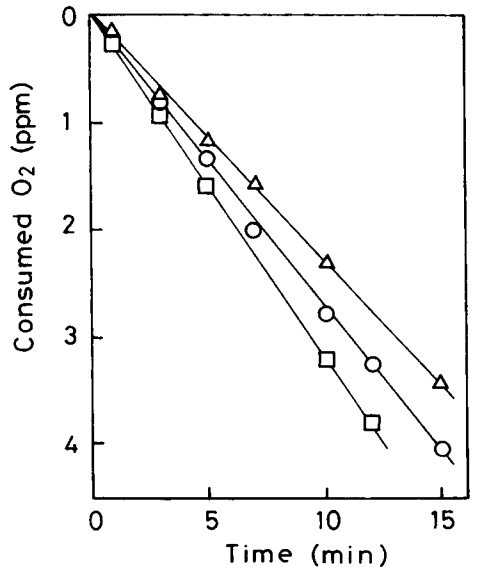


Figure 2 Changes of consumed O_2 for immobilized glucose oxidase with reaction time at 30°C. The mol ratio is 0.065. Reactions were carried out in 0.1 *M* phosphate buffer at pH 4.6: (□) $n = 11$; (○) $n = 6$; (△) $n = 2$.

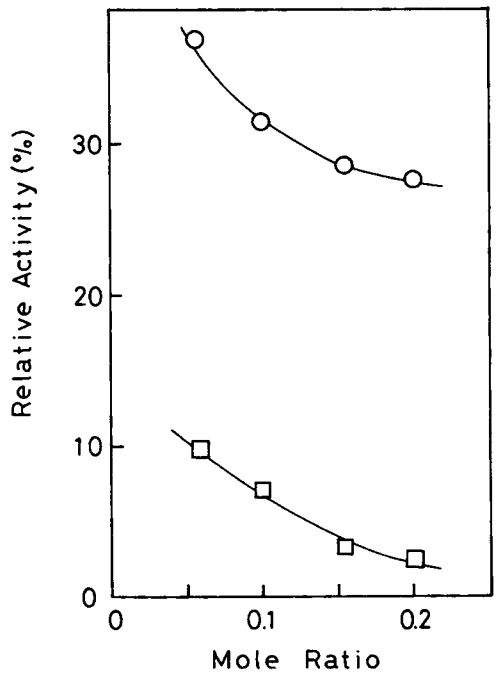


Figure 3 Relationship between relative activity of the immobilized enzymes and mol ratio of the cross-linked reagent ($n = 6$) to the dimethylamino group of the nylon: (○) immobilized glucose oxidase (pH 4.6); (□) immobilized urease (pH 7). The activities were compared with those of native enzymes.

In Figure 3, relative activities of the immobilized enzymes to those of native enzymes are plotted vs. mol ratios of the cross-linked reagents to the dimethylamino groups of the nylon in gels presented as

Mol ratio = (crosslinked reagent)/
(dimethylamino group of nylon)

The plots indicate that the increases of the cross-linked reagent in the gel result in the decrease of the activity. In the presence of the cross-linked reagent ($n = 6$), the activity of native urease and glucose oxidase was measured. The activity losses of urease and glucose oxidase are 10 and 7%, respectively. Here, the values refer to that of the native enzyme without the reagent. These show that the cross-linked reagents act as the inhibitor. In the figure, the decreases of the activity with increase of the mol ratio arise from the increased degree of cross-linked density of gels and the inhibition of the enzyme activity.

Figure 4 shows changes of remaining activity for immobilized glucose oxidase with preheating for 1 h in water at various temperatures. This shows that

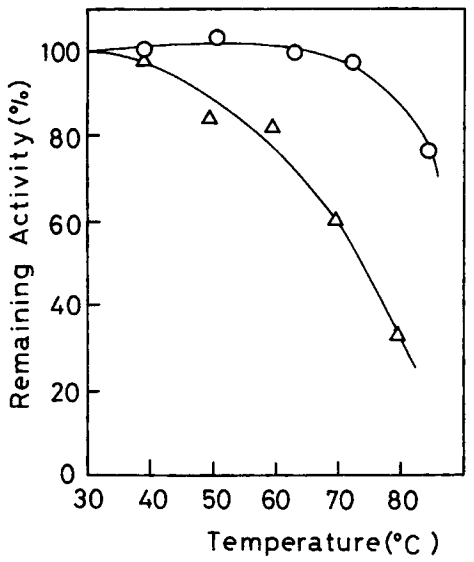


Figure 4 Preheat stabilities of immobilized (○) and native (△) glucose oxidase at the optimum pH 4.6 in 0.1*M* phosphate buffer. The mol ratio of the cross-linked reagent (n = 6) used is 0.065. Remaining activity is of relative values before and after the preheat.

the remaining activity of the immobilized enzyme is higher than that of native glucose oxidase. Here, the remaining activity is of relative values before and after the preheat. Figure 5 shows changes of remaining activity with incubation times in ethanol.

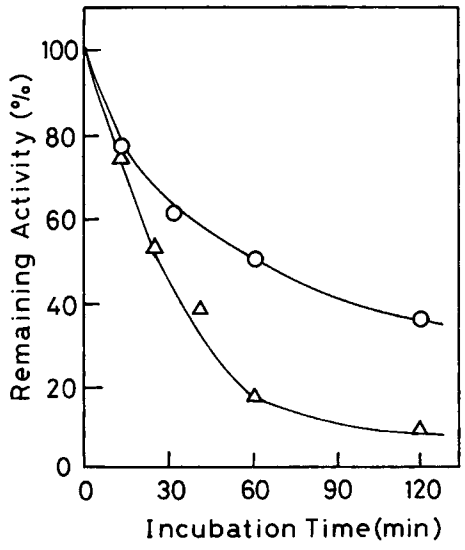


Figure 5 Stability of incubation in methanol for immobilized (○) and native (△) glucose oxidase. The mol ratio of the cross-linked reagent (n = 6) used is 0.065. Remaining activity is of relative values before and after the incubation.

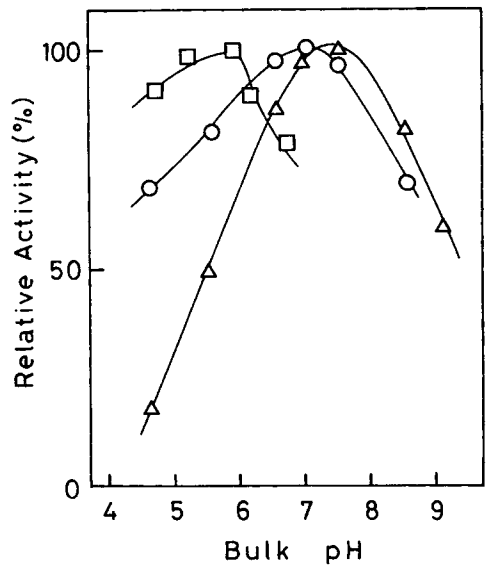


Figure 6 pH activity curves for immobilized urease in the absence and the presence of phosphate buffer. Relative activity is of relative values to that of the optimum pH: (△) native urease; (○) immobilized urease in the presence of 0.1*M* phosphate buffer; (□) immobilized urease in the absence of the buffer. The mol ratio of the cross-linked reagent (n = 6) used is 0.065.

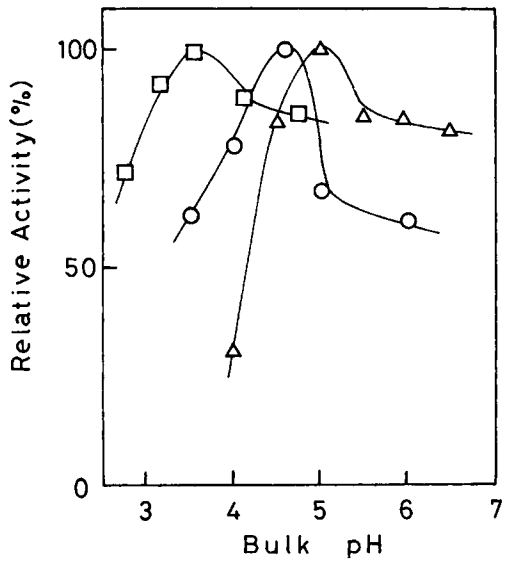


Figure 7 pH activity curves for immobilized glucose oxidase in the absence and the presence of phosphate buffer: (△) native glucose oxidase; (○) immobilized glucose oxidase in the presence of 0.1*M* phosphate buffer; (□) immobilized glucose oxidase in the absence of the buffer. The mole ratio of the cross-linked reagent (n = 6) used is 0.065.

Table II Optimum pH and Electrostatic Potential of Positively Charged Gel for Immobilized Enzyme

No.	Added Buffer (M)	Optimum pH		ψ (mV)	
		Urease	Glucose Oxidase	Urease	Glucose Oxidase
Native	0	7.5	5.0	—	—
2	0	6.0	3.3	+90	+102
2	0.6	7.0	4.6	+30	+24
3	0.6	7.0	4.6	+30	+24

After the incubation, the decreased degree of the activity for immobilized glucose oxidase is lower than that for the native one. The data in both figures indicate that the immobilization protects the decrease of the enzyme activity, as is well known.[19]

Relationships of relative activity of the immobilized enzyme with bulk pH are shown in Figure 6 for the immobilized urease and in Figure 7 for the immobilized glucose oxidase. Apparently, the optimum pH of immobilized enzymes shifts toward a low pH region relative to that of the native enzyme. The shift in the absence of phosphate buffer is larger than that in the presence of 0.1 M phosphate buffer. It is well known[11,12] that, in the cases of immobilization of enzymes linked to charged matrix, the shift of the optimum pH is due to low H^+ concentration in the positively charged matrix relative to the external bulk solution of the gel; electrostatic repulsion between the matrix and H^+ result in the low concentration. Assuming the distribution of H^+ between the gel phase and the external solution according to the Boltzmann's law, the pH difference (Δ pH) between the immobilized and the native enzyme relates to the electrostatic potential (ψ) as follows[8,11,12]:

$$\Delta \text{pH} = -F\psi/2.3RT$$

where F is the faraday constant; R, the gas constant; and T, the absolute temperature.

Table II presents optimum pH of the immobilized enzymes and the calculated ψ. In the presence of 0.1 M phosphate buffer, the difference of the optimum pH between the native and the immobilized enzymes is about 0.4–0.5 for both systems. The calculated ψ is +30 and +24 for the immobilized urease and glucose oxidase, respectively. In the absence of the buffer salt, the values of optimum pH further shift toward a low pH region relative to that in the presence of the salt, as shown in Figures 6 and 7.

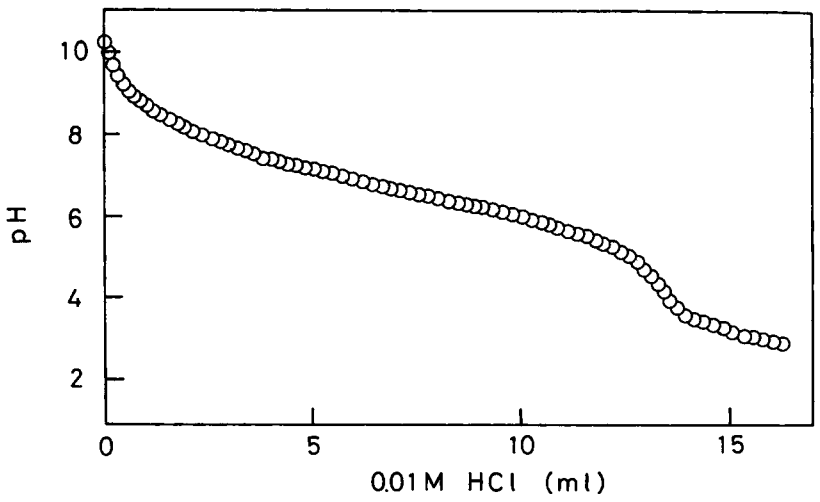


Figure 8 Titration curve of dimethylamino nylon in water at 30°C. For the titration with 0.01 M HCl, a 0.02 g dimethylamino nylon was used.

The obtained ψ of the both immobilized enzymes for urease and glucose oxidase is +90 and +102 mV, respectively. With decrease of the buffer salt concentration, the ψ values increased due to the effect of the electrostatic shield of the salts.

The value of the immobilized glucose oxidase is a little larger than that of the immobilized urease. To explain the different ψ in the systems, the titration curve of dimethylamino nylon in water is measured and shown in Figure 8. This indicates that, at pH = 3 of the optimum condition for the immobilized glucose oxidase, most of the dimethylamino group in the nylon has the protonated form $[-N^+-(CH_3)_2(H)]$. At pH 6 for the immobilized urease, in the titration curve, the volumes of the added 0.01 *M* HCl is 10 mL. The degree of the positively charged dimethylamino group by the protonation is about 70% at the pH and is lower than that for the immobilized glucose oxidase, resulting the large ψ of the immobilized glucose oxidase.

In addition, the values obtained in this work are lower than those of positively charged assemblies such as micelles, vesicles, and polyelectrolytes reported as about +150 mV. The assemblies can form high charge density through the aggregation of the molecules in water. On the other hand, the hydrophilic charged gels may not form the high charge density corresponding to the assemblies because of the swelled properties. The enzyme and water molecules are entrapped with the expanded segments of the positively charged nylon. Consequently, the surrounding enzyme may indicate H^+ concentration of the gel compartment.

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