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Reversible Denaturation Behavior of Immobilized Glucose Oxidase

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Abstract

Glucose oxidase (GOD) was immobilized by using glutaraldehyde crosslinking and various stabilizing agents such as BSA, gelatin, lysozyme, and polyethylenimine (PEI). Studies on the denaturation of the soluble as well as immobilized GOD were carried out for 1 h at various concentrations of guanidine hydrochloride (GdmCl) in 50 m*M* phosphate buffer, pH 6.0 at 25 ± 1°C. The soluble enzyme required a GdmCl concentration of 5 *M* for total activity loss, whereas for GOD immobilized with BSA, gelatin, lysozyme, and heat-inactivated lysozyme, the corresponding GdmCl concentration required was 8 *M*. GOD immobilized with PEI, however, was more stable and retained 25% activity when denatured for 1 h using 8 *M* GdmCl. However, after undergoing denaturation for 1 h, GOD immobilized with lysozyme regained 72% original activity within 20 min of renaturation, while GOD immobilized with BSA, PEI, gelatin, and heat-inactivated lysozyme regained only 39, 21, 20, and 25% of activity, respectively. After five cycles of repeated denaturation and renaturation with 8 *M* GdmCl, GOD immobilized with lysozyme retained 70% of the original activity. Refolding ability of lysozyme, glutaraldehyde crosslinkages between lysozyme and GOD, together with ionic interactions between them, appear to play an important role in the denaturation–renaturation behavior of the immobilized enzyme.

Index Entries: Glucose oxidase; guanidine hydrochloride; reversible denaturation; renaturation; stabilizing agents; immobilization.

Introduction

Denaturation of immobilized enzymes is a critical problem in industrial applications. A reversal of the denaturation to bring back the original activity is important from an application point of view. The ability to regain the functional state of the immobilized enzyme after undergoing denaturation has been known in some cases *(1–3)*. In most of the cases a combination

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of intermolecular and intramolecular interactions responsible for the protein folding adversely affect the renaturation of immobilized enzymes *(4–6)*. Most of the reported renaturation studies have been carried out on single protein immobilized on solid support. Trivedi et al. *(7)* demonstrated the importance of heteromolecular interaction between the acidic and basic proteins on the renaturation of lysozyme in solution. They claimed that the renaturation of lysozyme (basic protein) was drastically affected due to the electrostatic interactions and aggregation by the addition of acidic proteins like BSA and alcohol dehydrogenatase (ADH) to the renaturation buffer. In another study, the importance of electrostatic interaction between the barnase (basic protein) and chaperoning growl (acidic protein) during denaturation of barnase has been reported by Gray and Fershat *(8)*. Furthermore, aggregation of proteins during renaturation can be avoided by immobilization *(3)*. While it is known that the electrostatic interactions between proteins that play a crucial role in the renaturation and aggregation of proteins can be avoided by immobilization, information on the renaturation of immobilized multiple proteins is not available. Also, while it is known that proteins like lysozyme can by themselves exhibit reversible denaturation with denaturants like GdmCl and urea *(9,10)*, that their incorporation during the enzyme immobilization may help the process of renaturation of the desired enzymes has not been known. We had earlier observed that incorporation of lysozyme during the immobilization step increases the thermal stability of GOD, which may be attributed to the ionic interactions between lysozyme (basic protein) and GOD (acidic protein) *(20)*. In this context, it would be interesting to study the denaturation–renaturation behavior of immobilized multiproteins, where the role of ionic interactions and influence of refolding ability of the stabilizing agent on the desired enzyme can be investigated.

Glucose oxidase (GOD) is an important enzyme for biotechnological applications *(11,12)*. GOD (β-D-glucose, oxygen-oxidoreductase) from *Aspergillus niger* is a dimer and flavine adenine dinucleotide (FAD) containing glycoprotein catalyzes the oxidation of (β-D-glucose by molecular oxygen to D-gluconolactone and hydrogen peroxide. The carbohydrate content accounts for 24% of the molecular size 157 kDa *(13)*. Recently X-ray crystallography has been carried out for the GOD from *A. niger* at 1.9 Å resolution *(14)*. Although molecular properties of GOD have been studied extensively, information on its denaturation–renaturation behavior is not available.

Denaturation–renaturation behavior of proteins can be studied by using CD or NMR measurement. However, these methods have several constraints with respect to their application to immobilized enzymes. As an alternative approach the activity of enzymes like GOD in an immobilized form can be easily followed with the help of a dissolved oxygen electrode wherein the depletion of oxygen in the vicinity of the electrode membrane results in a change in current due to the enzymatic reaction that bears a definite relationship with the activity of the immobilized enzyme. In the present work, GOD immobilized with various stabilizing agents like BSA,

gelatin, lysozyme, and PEI using glutaraldehyde crosslinking has been investigated for the denaturation–renaturation behavior with respect to enzyme activity measured by a dissolved oxygen electrode.

Materials and Methods

Materials

Glucose oxidase (E.C. 1.1.3.4.) from *A. niger*, lysozyme, BSA, gelatin, and glutaraldehyde were from Sigma. Guanidine hydrochloride (GdmCl) was from SRL, India. The cellophane membrane of molecular cutoff 6000–8000 was from Spectra/Por; oxygen permeable Teflon membrane was from WTW, Germany. Immobilized enzyme activity was measured by using dissolved oxygen meter (EDT, UK) containing Clark electrode. A 8 *M* GdmCl solution was prepared by dissolving the required quantity of GdmCl in a small volume of 50 m*M* phosphate buffer pH 6.0. The solution was adjusted to pH 6.0 by the addition of 2 *M* NaOH. The final volume was adjusted by adding the required amount of phosphate buffer solution pH 6.0.

Heat Inactivation of Lysozyme

In order to avoid the influence of refolding ability of lysozyme on the immobilized GOD, lysozyme was heat inactivated as reported by Fischer et al. *(15)*. Briefly, 0.2 mg/mL of lysozyme in distilled water was heated for 2 h at 90°C. The heat-inactivated protein solution was cooled to room temperature. The residual activity was observed by lysozyme assay *(16)* for 48 h. After confirming that there is no reactivation of the enzyme, the denatured enzyme solution was freeze dried and used as the heat-inactivated lysozyme.

Preparation of Immobilized Glucose Oxidase

GOD was immobilized by glutaraldehyde crosslinking method reported by Cass *(17)*. One milligram of GOD was dissolved in 180 µL of 50 m*M* phosphate buffer pH 6.0; 200 mg each of BSA, gelatin, and lysozyme were dissolved in 1 mL of 0.05 *M* sodium phosphate buffer. A 2.5% (w/v) of glutaraldehyde solution was prepared by appropriate dilution of 70% (w/v) glutaraldehyde. On a 2 cm × 2 cm cellophane membrane, 10 µL (10 IU) of GOD and 30 µL (6 mg) of the stabilizing agent were placed and mixed using a glass rod. Fifty microliters of 2.5% glutaraldehyde was then added and mixed so that the enzyme and the stabilizing agent were distributed uniformly throughout the enzyme membrane. This mixture was allowed to remain for 1 h and then the enzyme membrane washed repeatedly with 50 m*M* phosphate buffer pH 6.0 to remove the excess glutaraldehyde.

Activity Measurement of Immobilized GOD

In order to measure the immobilized enzyme activity in the presence and absence of various concentrations of GdmCl, an amperometric prin-

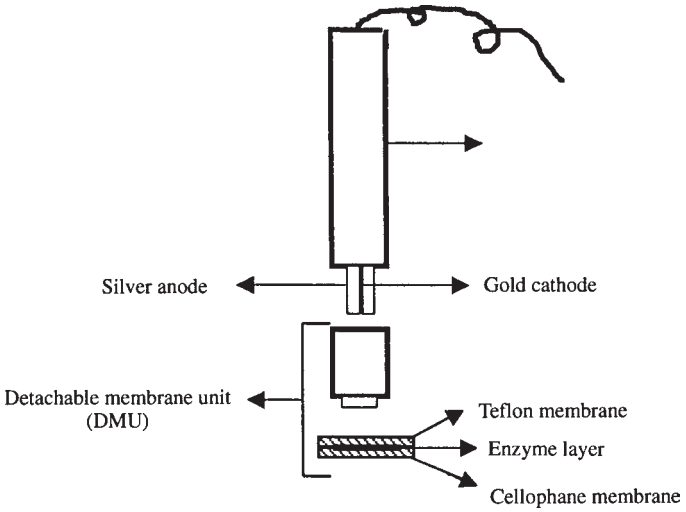

Fig. 1. A schematic diagram of the dissolved oxygen electrode set up with the sandwich enzyme membrane system.

ciple based Clark-type dissolved oxygen electrode along with a dissolved oxygen meter (EDT, UK) was used. A composite membrane system comprising the enzyme layer was held in a sandwiched form between a Teflon membrane and a cellophane membrane, secured tightly with an O ring on the electrode surface (*see* Fig. 1). The activity of the immobilized enzyme was measured by immersing the enzyme-sensing element in a glass sample cell of 25 mL containing 5 mL buffer, kept agitated continuously with air bubbled through a portable air pump in order to eliminate dissolved oxygen limitations. After initial bubbling of air for saturation, the dissolved oxygen meter was set at 100%. A known, relatively high concentration of glucose solution (chosen here as 10%) was now injected and the decrease in percentage dissolved oxygen at the end of 3 min (time taken to reach steady state) was monitored. This value is proportional to the enzyme activity.

Denaturation Studies of Immobilized GOD

Denaturation studies on GOD immobilized with various stabilizing agents and PEI were carried out at different GdmCl concentrations using freshly immobilized GOD membrane on the electrode surface, the initial activity a of the enzyme membrane was measured by injecting 40 µL of 10% glucose at 25 ± 1°C. The enzyme electrode was then immersed in a known concentration of GdmCl solution in phosphate buffer (50 m*M*) pH 6.0 for 1 h with constant stirring. The residual activity b of the immobilized enzyme membrane was observed by again injecting 40 µL of 10% glucose solution. Percentage residual activity was calculated as $(b/a) \times 100$. In order to measure the soluble enzyme activity, a known concentration of enzyme (10 IU, in our studies) was injected first. Then 40 µL of 10% glucose

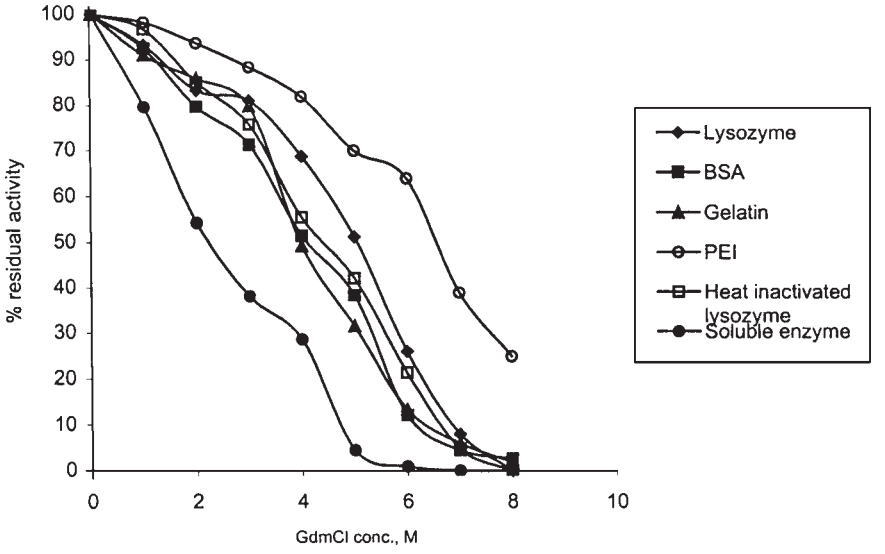

Fig. 2. Denaturation behavior of the soluble and immobilized GOD (with different stabilizing agents) at various GdmCl concentrations. (◆) Lysozyme; (■) BSA; (▲) gelatin; (○) PEI; (□) heat-inactivated lysozyme; (●) soluble GOD.

solution was injected and activity a was measured. Denaturation of the soluble enzyme was carried out as follows. The oxygen electrode sensor alone (without the enzyme membrane) was immersed in the desired GdmCl solution in phosphate buffer pH 6.0; 10 IU of the enzyme was now injected and incubated for 1 h, after which the residual activity b of the soluble GOD was measured by injecting 40 µL of 10% glucose solution.

Renaturation Studies

Renaturation of the immobilized GOD denatured in 8 *M* GdmCl was carried out in 50 m*M* phosphate buffer pH 6.0 at 25 ± 1°C for 1 h. The dissolved oxygen electrode containing the denatured enzyme membrane was repeatedly washed by immersing the enzyme electrode in 50 m*M* phosphate buffer pH 6.0. At desired intervals the regained enzyme activity c was measured by injecting 40 µL of 10% glucose solution. Then the percentage renaturation was calculated as $[(c - b)/(a - b)] \times 100$, where a is the initial activity of immobilized GOD and b is the residual activity of immobilized GOD after undergoing denaturation in 8 *M* GdmCl for 1 h. In the case of soluble enzyme renaturation was carried out by diluting the mixture of enzyme and denaturant with 40 volumes of phosphate buffer pH 6.0 and then activity was measured by using the oxygen electrode.

Results and Discussion

Figure 2 demonstrates the denaturation behavior of immobilized GOD with various stabilizing agents in different GdmCl concentrations at pH 6.0

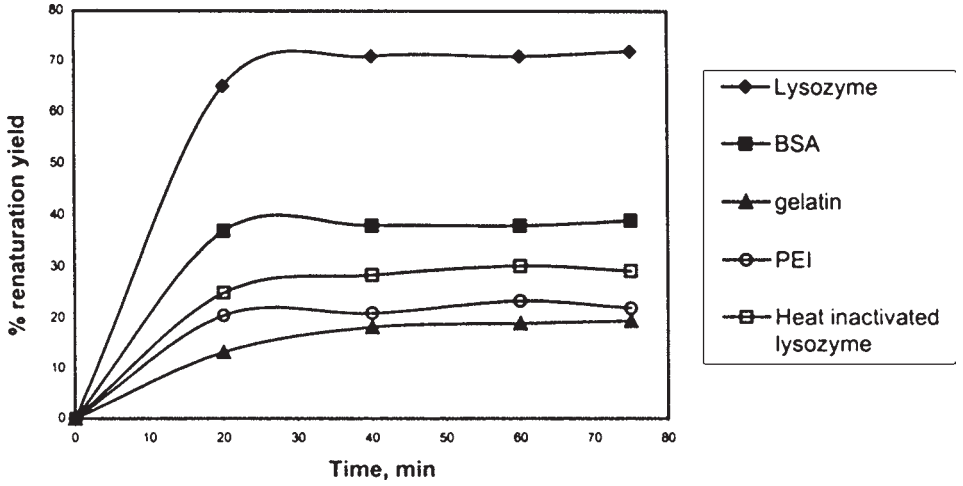

Fig. 3. Renaturation behavior of GOD immobilized with various stabilizing agents. (◆) Lysozyme; (■) BSA; (▲) gelatin; (○) PEI; and (□) heat-inactivated lysozyme.

for 1 h at 25 ± 1°C. The soluble enzyme loses its activity completely beyond 6 *M* GdmCl concentration. Similarly, GOD immobilized with BSA, gelatin, lysozyme, and heat-inactivated lysozyme lost their activity completely beyond 8 *M* GdmCl, whereas GOD immobilized with PEI retained 37% activity after undergoing denaturation in 8 *M* GdmCl for 1 h. In fact, GOD immobilized with PEI as stabilizing agent, even after 10 h of 8 *M* GdmCl treatment retained 25% initial activity (data not shown). Increase in the stability of the immobilized GOD compared to that of the soluble enzyme against GdmCl concentrations can be attributed to the crosslinkages between the enzyme and stabilizing agents, which results in stearic hindrance of active site of the enzyme in the three-dimensional lattice. Variation in the stability of GOD immobilized with various stabilizing agents after undergoing denaturation with different GdmCl concentrations at pH 6.0 for 1 h at 25 ± 1°C can be ascribed to the physicochemical properties of the stabilizing agent. If the carrier itself is unstable to the denaturants compared to the enzyme, then the stability of enzyme preparation is adversely affected. As shown in Fig. 2, a remarkable stability of GOD immobilized with PEI against GdmCl concentrations was observed, probably owing to the inertness of PEI to GdmCl. Correspondingly, as the stability of stabilizing agent itself decreased as reported for lysozyme *(9,10)*, BSA *(18)*, and gelatin *(19)*, stability of GOD immobilized with these stabilizing agents decreased against GdmCl.

Figure 3 demonstrates renaturation behavior of GOD immobilized with various stabilizing agents after undergoing denaturation in 8 *M* GdmCl for 1 h. The renaturation studies were carried out in 50 m*M* phosphate buffer pH 6.0 at 25 ± 1°C. As shown in the figure, GOD immobilized with lysozyme showed a remarkably higher renaturation yield compared to other stabilizing agents. GOD immobilized with lysozyme regained 72%

Table 1
Effect of NaCl on the Reactivation
of Glucose Oxidase Immobilized with Different Stabilizing Agents

	Percentage renaturation yield in presence of	
Stabilizing agent	None	1 *M* NaCl
PEI	20.41	20.26
BSA	38.36	39.21
Gelatin	20.41	21.26
Lysozyme	72.56	37.95
Heat-inactivated lysozyme	25.13	18.59

of original activity, whereas GOD immobilized with PEI, BSA, heat-inactivated lysozyme, and gelatin regained 21, 39, 25, and 19% activity, respectively, within 20 min of renaturation. Another observation from Fig. 3 is that the renaturation is almost complete in 20 min in all the cases. The significantly beneficial renaturation ability of GOD immobilized with lysozyme compared to other stabilizing agents may be attributed to the influence of refolding ability of the lysozyme on GOD through crosslinkages after undergoing denaturation in 8 *M* GdmCl. Glutaraldehyde crosslinkages between GOD and lysozyme thus appear to play an important role in the renaturation behavior of the immobilized GOD. A significant evidence for the influence of refolding ability of the stabilizing agent on GOD after undergoing denaturation can perhaps be seen from the sharply decreased percentage renaturation of the GOD immobilized with heat-inactivated lysozyme (Fig. 3). After heat inactivation, lysozyme loses its refolding ability and, correspondingly, the renaturation influence on the immobilized GOD. Thus, the refolding ability of the stabilizing agent appears to play a crucial role in the renaturation behavior of the immobilized enzyme.

In a further support of the above hypothesis, Table 1 shows the effect of 1 *M* sodium chloride on the renaturation of the immobilized GOD after undergoing denaturation in 8 *M* GdmCl for 1 h. The presence of 1 *M* sodium chloride in the renaturation buffer leads to a significant decrease in percentage renaturation only in the case of GOD immobilized with lysozyme as stabilizing agent and not in the case of other stabilizing agents, namely, BSA, gelatin, PEI, and heat-inactivated lysozyme. Gouda et al. *(20)* have reported that the increased thermal stability of soluble as well as immobilized GOD in the presence of lysozyme is attributable to the ionic interactions between them. The considerably decreased renaturation yield in the presence of 1 *M* sodium chloride for GOD immobilized with lysozyme supports this hypothesis. Addition of sodium chloride to the renaturation buffer may lead to a modification of the surface charges of denatured GOD

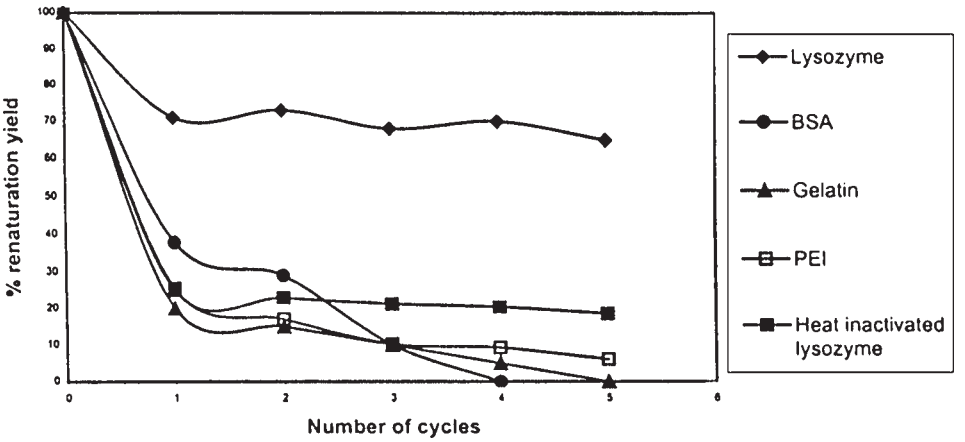

Fig. 4. Repeated denaturation–renaturation of GOD immobilized with various stabilizing agents after undergoing denaturation in 8 *M* GdmCl for 1 h. (◆) Lysozyme; (●) BSA; (▲) gelatin; (□) PEI; and (■) heat-inactivated lysozyme.

and lysozyme by a shielding effect and thus adversely affect the renaturation process. Trivedi et al. *(7)* reported that the heterochain interaction between acidic protein (BSA) and basic protein (lysozyme) resulted in a decrease in the renaturation of the lysozyme due to aggregation. This aggregation occurs by specific conformation and interaction of certain folding intermediates. The influence of electrostatic interactions between the folding protein, barnase, and the chaperonin, GroEL, during renaturation of barnase has been demonstrated by Gray and Fershat *(8)*. Our results indicate that the glutaraldehyde crosslinkages between lysozyme and GOD, together with ionic interactions between them may play an important role in the renaturation behavior of the immobilized enzyme.

Figure 4 demonstrates the repeated denaturation (in 8 *M* GdmCl) for 1 h and renaturation (in 50 m*M* phosphate buffer) of GOD immobilized with various stabilizing agents at pH 6.0 and at 25 ± 1°C. In all the cases, there is a steep fall in the renaturation in the first cycle followed by gradual fall. However, as shown in Fig. 4, BSA and gelatin as stabilizing agents negligible extent of the renaturation at the end of fifth cycle, whereas GOD immobilized with PEI and heat inactivated lysozyme resulted in a slower decrease in renaturation yields, reaching saturation values of 32 and 20%, respectively, by the fifth cycle. Remarkably, lysozyme as the stabilizing agent, after a steep fall (72%) in the immobilized GOD activity in the first cycle, shows stable and almost constant renaturation yields in the subsequent cycles, retaining 70% renaturation yield at the end of fourth cycle. The decrease in activity in the first cycle to 72% for GOD immobilized with lysozyme may be due to the irreversible denaturation of loosely bound enzyme molecules on the surface of immobilized enzyme membrane during exposure to 8 *M* GdmCl. This hypothesis of the irreversible denaturation of surface-bound enzyme molecule is supported by the gradual

decrease of the renaturation yield in the subsequent cycles (Fig. 4). These results indicate that the GOD immobilized with lysozyme can show almost quantitative reversible denaturation behavior after undergoing denaturation by GdmCl.

In conclusion, the refolding ability of the lysozyme, glutaraldehyde crosslinkages between lysozyme and GOD, together with ionic interactions between them appear to play an important role in the reversible denaturation behavior of the immobilized enzyme. Our results thus suggest that the refolding ability of stabilizing agents can modulate the reversible denaturation behavior of the immobilized enzyme. These results should provide useful information in understanding the role of the refolding ability of the stabilizing agent on the immobilized enzyme. Incorporation of lysozyme during immobilization of enzymes can be employed as a useful tool for an intrinsic evaluation of the various refolding reagents by avoiding the aggregation which is a common problem in the denaturation study of soluble protein. By selecting a suitable stabilizing agent which has a refolding ability, the desired enzyme can be efficiently renatured.

Acknowledgments

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