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STABILIZATION OF GLUCOSE OXIDASE FROM *Penicillium vitale* ENTRAPPED IN HIGH-CONCENTRATION GELS

J. J. KULYS, B. S. KURTINAITIENĖ, and V. F. AKULOVA
Institute of Biochemistry, Academy of Sciences Lithuanian SSR, Vilnius, USSR

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Glucose oxidase from *Penicillium vitale* was immobilized in a 2-hydroxyethyl methacrylate (HEMA) gel containing 0.3 to 2% of methacrylic acid (MAA) or MAA neutralized by allylamine (AA). Depending on the MAA quantity of MAA in the gel, the thermal irreversible inactivation (k_{in}) constants of the immobilized enzymes sharply decrease at gel concentrations higher than 29 to 50% at pH 5.8. A 220- to 250-fold decrease of k_{in} was observed in 60 to 80% gel. The inactivation rate of enzyme in HEMA gel also decreases considerably under the action of urea. Over the range of pH 5.0 to 9.0 the k_{in} of the native enzyme depends on pH by a degree of 2.1, and of the immobilized enzyme, 0.3 to 0.55. Over the pH range of 5.2 to 5.7, k_{in} of the native and immobilized enzymes are approximate, whereas at pH 8 and over the difference between k_{in} values exceeds four orders of magnitude. The activity dependence of the immobilized enzyme on pH is shifted two units to the alkaline region. This shift depends on the ionic strength of the solution. This dependence is best reflected in the high gel concentrations. A mechanism of enzymes stabilization in the concentrated HEMA gel is discussed.

INTRODUCTION

The preparation of thermally stable enzymes appears to be one of the essential problems in the enzymology. This problem is related to the elaboration of methods that permit the suppression or retardation of the molecular processes of biocatalyzer inactivation.

It is believed that a multisided fixation of an immobilized protein molecule on a matrix is one of the possible ways to stabilize an enzyme. It was shown that a multisided attachment of trypsin molecules to rigid polysaccharidic carriers by covalent bonds considerably increased the stability of the immobilized enzyme to the action of denaturing agents (1). Multisided fixation of enzyme molecules can also occur through electrostatic interaction between the charges of protein molecules and a matrix (2). Stabilization of enzymes by modification of the protein molecules with unsaturated compounds and subsequent entrapment of the attached monomeric links in the growing polymeric chains of a matrix has also been

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demonstrated (3,4). In the present work a method of subunit-consisting enzyme stabilization, by entrapment in high concentration gels, is proposed. The enzyme used in this study was glucose oxidase (EC 1.1.3.4) from *P. vitale.*

METHODS

The glucose oxidase of commercial manufacture (USSR) was purified as previously described (5). An electrophoretically homogeneous enzyme (activity 180–190 units per mg of protein) was used in this investigation.

2-Hydroxyethyl methacrylate (HEMA) was prepared as previously described (6). A monomer was distilled *in vacuo* at 60°C/12 mm of HEMA under nitrogen. Ethyleneglycol dimethacrylate (EDMA) was prepared by the etherification of methyl methacrylate with anhydrous ethylene glycol (4). After double distillation a monomer, boiling at 120 to 126°C/15 mm of EDMA was obtained. Allylamine (AA) was distilled at 53.5°C. Acryl amide, ammonium persulfate, tetramethyl ethylene diamine (TEMED), tris(oxymethyl) amino methane (Tris) were manufactured by "Reanal" (Hungary). Chemically pure acetic acid, natrium acetate, ethylene diamino tetraacetic acid (EDTA), azotic acid, glucose and other reagents used were manufactured in the USSR.

The immobilization of glucose oxidase was carried out by copolymerizing 20, 30, 40, 50, and 60% HEMA solutions with the cross-linking agent EDMA in 50 mM Tris-HNO_3 buffer, pH 7.2, containing 1 mM EDTA, 2.5% acryl amide, and 0 to 5 mg of enzyme per 1 ml of a gel. Each gel contained varying amounts (0.3–20%) of MAA or MAA neutralized by AA. Polymerization was carried out in a 6-ml volume activated by TEMED (0.6 ml of 4.3% solution) and 1.8 mg of ammonium persulfate under nitrogen at 4°C for 20 h. The gels obtained were cut to 1-mm^3 size blocks, washed on a glass filter with running water for 24 h and kept for a day in a 100-fold volume of 0.2 M acetate buffer, pH 5.8. If the measurements were carried out at other pH values, the latter procedure was omitted and the gels were dried in a vacuum dessicator for 3 days. Dried gel was ground with a mortar and pestle and sieved in order to obtain a definite range of particle sizes (60–100 μm). Before the experiment the dried gel granules were placed in a buffered solution for no less than 4 h. The high concentration gels were prepared by partial drying of gels equilibrated with acetate buffer at pH 5.8.

Enzymatic activity was monitored by measuring the rate of oxygen consumption in the glucose oxidation reaction at 25°C using a membraneous oxygen electrode (5) placed in a thermostated cell (10 ml). The reaction was carried out in 0.2 M acetate buffer containing 0.17 M D-glucose and 1 mM

EDTA. The solution of glucose was used after storing for 24 h to mutarotate to equilibrium.

The thermal inactivation of the immobilized enzyme was carried out by incubating at 65°C with 0.1 g of preliminarily swollen gel in 3 ml of 0.2 M acetate buffer, pH 5.8, with the addition of 0.1 mM EDTA, or in 0.005 M Tris-HNO_3 buffer or phosphate buffer over pH ranges from 5.8 to 8.9. Eight to ten samples were used for each inactivation curve. The enzyme inactivation in the case of concentrated gels was carried out at 65°C by incubating tightly shut test tubes (5 ml) each of which contained 0.1 g of ground gel. The inactivation curves were plotted on the basis of six to nine measurements determining a residual activity for each sample. Inactivation of the native enzyme was carried out at 56° in 0.1 M phosphate buffered solution containing 1 mM EDTA (pH 5.3 to 8.0) or in 0.2 M acetate buffered solution containing 1 mM EDTA at 65°C. The enzyme concentration used in these studies was equivalent to 6.5 nM.

The inactivation constant was calculated using a formula for the first-order reaction: $k_{in} = 2.303\ \mathrm{tg}\alpha$, where $tg\alpha$ is tangent of the inclination angle of the inactivation straight line in the coordinates; the logarithm of residual activity vs time.

The denaturing effect of urea on the native glucose oxidase was examined by placing the latter ($c = 10$ nM) in the thermostated cell at 25°C in 2 ml of 0.05 M Tris-HCl buffer, pH 7.2, containing different quantities of urea. The activity of the glucose oxidase was measured after incubating for 20 min and adding 7 ml of Tris-HCl buffer and 1 ml of 1.7 M glucose solution to the cell. The denaturation of the immobilized glucose oxidase was carried out under identical conditions by placing 100 mg of preliminarily swollen gel in the solution of urea.

The dependence of the native and immobilized glucose oxidase activity on pH was determined over a pH range of from 5.8 to 9.6 in 0.1 M phosphate and citrate buffered solutions as well as in 0.005 M Tris-HNO_3 buffer with the addition of 1 mM EDTA.

RESULTS

The immobilization of glucose oxidase was carried out in HEMA gel adding 2.5% of acrylamide and a given amount of methacrylic acid or methacrylic acid and an equimolar amount of allyl amine. As a cross-linking agent EDMA was used.

The investigation of enzyme retention by the gels of different concentrations has shown that glucose oxidase most firmly is retained in the gels obtained from 50% solutions of monomers. Less than 0.06% activity was

eluted from such a gel in 1 h at pH 7.2 in 0.1 M Tris-HCl at 25°C. From the gels prepared at lower concentrations of monomers—20, 30, and 40%—the enzyme was more easily eluted. The rate of enzyme elimination from these gels increased with decreasing monomer concentrations and accounted, respectively, for 96, 24, and 6% of the original activity lost in 1 h. The gels based on 60% or more of the monomers were brittle and, consequently, the enzyme retained was weaker (the rate of elution is 0.6% in 1 h) than that in the 50% gel.

Increasing the amount of the cross-linking agent by more than 1% resulted in the formation of brittle gels. With this in mind the gels prepared from 50% solutions of monomer with 1% of the cross-linking agent were used in most of the experiments. These gels cannot be swollen. The addition of MAA or MAA and AA as the starting monomers for the gels enabled swelling in the buffered solutions to increase to 2.5 times their original weight. Increases in the amount of the cross-linking agent did not prevent swelling.

The immobilized enzyme in the gels retained 12 to 20% of its original activity. The dependence of the gels' specific activity on the concentration of the entrapped enzyme achieved a limit at the enzyme concentrations of higher than 1.3 μM. For that reason all investigations of immobilized enzyme stability were carried out at concentrations that were lower than this value.

The thermal inactivation of the immobilized glucose oxidase proceeds by the first order until 60 to 80% of the activity is lost (Fig. 1A). The glucose oxidase inactivation constant in 50% gel with 0.3% of MAA is one-half that of the native enzyme. However the partial removal of water from these gels and, accordingly, the increase in enzyme concentration, results in a sharp decrease in the inactivation constant (Fig. 1B). It is seen that in 80% gel approximately a 250-fold decrease in the inactivation constant occurs. As MAA is immersed in the gel there is a sharp decrease of the inactivation constant observed at lower gel concentrations. However, this decrease does not exceed the 250-fold difference previously observed.

The entrapment of the enzyme in gels containing equimolar amounts of MAA and AA does not significantly change the rate of immobilized enzyme inactivation (Table 1). With the gels negatively charged by carboxylic groups the increase of the ionogenic group concentration results in intensification of the gel swelling and in a small decrease in the inactivation constant. The addition of greater amounts of the cross-linking agent in these mixed gels decreases swelling. The inactivation rate of enzymes entrapped in such gels containing 1% and 5% of MAA also decreases with an increase in EDMA. In the presence of 7.6 of MAA with 5% of EDMA, the inactivation constant was found to be 0.014 min^{-1} which is 12.7% of the constant value obtained

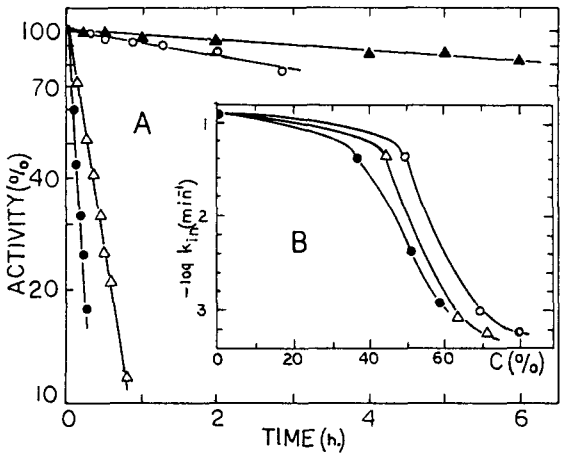

FIG. 1. (A) Thermal inactivation of native (●) and entrapped in 50% (△), 70% (○), and 80% (▲) respectively, of HEMA gels of glucose oxidase *Penicillium vitale*. The amount of MAA in gels is 0.3%; the conditions of inactivation are 65°C, pH 5.8, 0.2 M acetate buffer, and 1 mM EDTA. (B) Dependence of thermal inactivation constants of immobilized glucose oxidase on the concentration of gels containing 0.3 (○), 0.6 (△), and 4.6%(●) MAA (conditions of inactivation are the same).

for the native enzyme. The immobilized enzyme is about 10 times as stable as the native enzyme. A 1.6-fold increase in the inactivation constant of the same enzyme was observed with an increase in the cross-linking agent that added up to 15% (Table 1).

TABLE 1. **Dependence of the Thermal Inactivation Constants (k_{in}, min^{-1}) of Glucose Oxidase *Penicillium vitale* Entrapped in HEMA Gels Containing Different Amounts of MAA and Equimolar Amounts of AA**[a]

Weight ratio of HEMA/EDMA	Amount of methacrylic acid (%)							
	1.04		5.0		7.6		20.0	
	k_{in}	C^b	k_{in}	C^b	k_{in}	C^b	k_{in}	C^b
100:1	0.083	50.7	0.043	44.3	0.046	40.7	—	—
100:3	0.020	51.3	0.044	40.3	—	—	0.057	20.6
100:5	—	—	—	—	0.014	46.5	—	—
100:6	—	—	0.035	51.8	—	—	—	—
100:10	—	—	0.032	52.2	0.017	48.0	—	—
100:15	—	—	—	—	0.023	53.2	—	—

[a] Conditions: pH 5.8, 0.2 M acetate buffer, 1 mM EDTA, 65°C.
[b] C = the concentration of swollen gels (%).

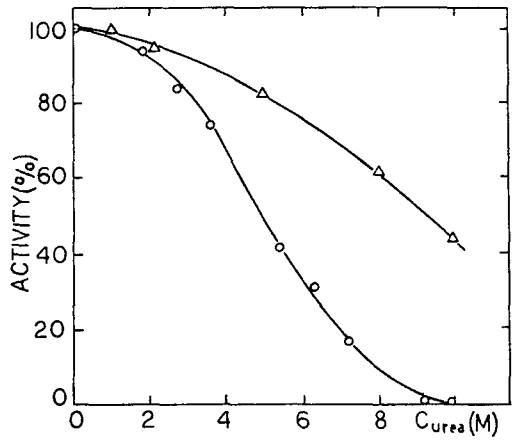

FIG. 2. Inactivation of native (○) and immobilized glucose oxidase (△) under the action of urea with a gel concentration of 29%; amount of MAA, 4.6%. Conditions of inactivation: 25°C, pH 7.2, 0.05 M Tris-HCl, incubation time 20 min.

The inactivation of immobilized glucose oxidase in the equilibrium swollen 29% gel containing 4.6% of MAA under the action of urea is shown in Figure 2. Also see Figure 2 for comparison with a curve of the native enzyme inactivation. It is seen, that even in a 10 M urea solution, the immobilized enzyme retains more than 40% of its original activity. The native enzyme is completely inactivated under these conditions.

The dependence of the inactivation constant vs pH for native glucose oxidase as well as for enzyme entrapped in gels is given in Figure 3. Over the

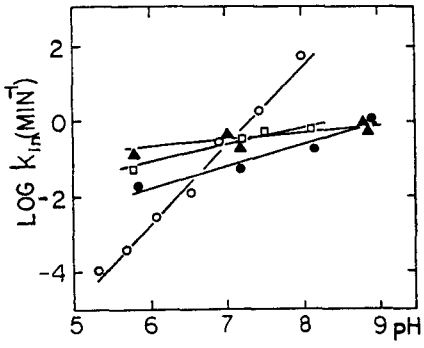

FIG. 3. Dependence of inactivation constants on pH of native glucose oxidase (○) and immobilized in HEMA gels containing 1.04 (▲), 5% (□), and 20% (●), respectively, of MAA. Conditions of experiment for native enzyme: 56°C, 0.1 M phosphate buffer, 1 mM EDTA; for immobilized enzyme: 65°C, 5 mM Tris-HNO_3 or phosphate buffer; 51.9, 23, and 20.6% concentrations, respectively, of gels containing 1.04, 5, and 20% of MAA, respectively.

range of pH from 5.8 to 8.0 the change in the native enzyme inactivation constant depends on the hydrogen ions concentration. This dependence may be described as

$$\log k_{in} = (2.1 \pm 0.2)\,\mathrm{pH} + (-15.3 \pm 1.4)$$

The k_{in} of the immobilized enzymes depends far less on pH. For the gel containing 20% of MAA this equation may be written

$$\log k_{in} = 0.55\,\mathrm{pH} - 5.06$$

for the gel containing 5% of MAA:

$$\log k_{in} = 0.46\,\mathrm{pH} - 3.90$$

and for the gel containing 1.04% of MAA:

$$\log k_{in} = 0.30\,\mathrm{pH} - 2.85$$

A tendency for the decreasing dependency of the inactivation constant on pH is observed with the decrease of the MAA concentration for the immobilized enzyme.

The pH optimum for the enzyme entrapped in gels containing different amounts of MAA is shifted to an alkaline region (Fig. 4). Thus, for glucose oxidase immobilized on the 51.9% gel containing 1.04% of MAA and with an ionic strength of 5 mM, the pH optimum is shifted to the alkaline region by two pH units. The effect is much less at the ionic strength of 0.1 M. In the

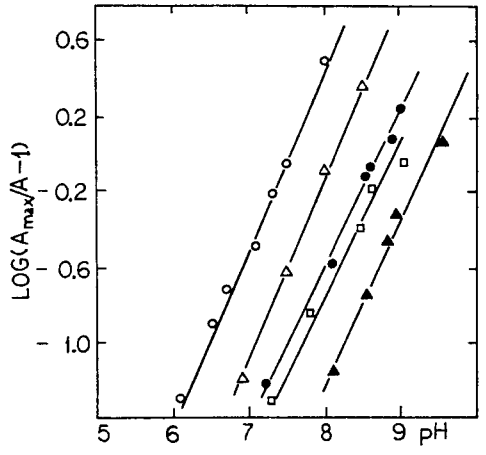

FIG. 4. Dependence of enzymatic activity on the pH of native glucose oxidase (○) and entrapped in gels containing 1.04 (△, ▲), 5 (□), and 20% (●) of MAA, respectively, at ionic strength of solutions of 0.1 M (○, △) and 5 mM (●, □, ▲), respectively.

latter case the pH optimum shifts only to 0.65 pH units. In the 20.6% gel containing 20% of MAA and at a low ionic strength, the shift into the alkaline region was found to be 1.25 pH units. For a 44.3% gel containing 5% of MAA this shift is close to that of the 20.6% gel. It is seen that the most significant shifts of the maximal activity into the alkaline region are observed in gels containing the smallest amounts of MAA, that is, in gels that have poor swelling capacity.

DISCUSSION

An immobilized enzyme which retained 10 to 20% of its original activity was prepared by the entrapment of glucose oxidase *P. vitale* in the HEMA gel. Thermal stability of glucose oxidase from *Aspergillus niger* immobilized on HEMA gel containing 20% polyvinyl pyrrolidone was also investigated at 35, 45, and 50% of HEMA (7). The stability was shown to be close to that of the native enzyme. Some new effects were observed while investigating the stability of the immobilized glucose oxidase *P. vitale*. At pH of 5.8 the thermal stability of the immobilized enzyme entrapped in 20 to 50% gel was 2.7 times as high as that of the soluble enzyme (Fig. 1). However, on the removal of water (57 to 64%) from these gels, preparations were obtained with inactivation constants which were over two orders of magnitude lower than that of the native enzyme (Fig. 1B). Further increase in the gel concentration decreased the inactivation constant until a limit was reached. The maximal decreased inactivation constant observed was 220- to 250-fold lower than that of the native enzyme. Adding MAA into the gels caused a sharp increase in the stability at lower gel concentrations.

Over the pH range of from 5.0 to 9.0, thermal inactivation of the native enzyme depends on the pH of the solution (Fig. 3) to a significant degree. The pH dependence of the immobilized glucose is far less and falls down as MAA concentrations decrease. Extrapolation of the dependence of the native enzyme inactivation constant on pH up to 65°C[1] and the comparison of these with that for the immobilized enzyme at 65°C, shows that the immobilized enzyme is much more stable over the pH range of from 5.2 to 5.7. At pH 8.0 the difference between the inactivation constants reaches at least four orders of magnitude.

The immobilized glucose oxidase pH optimum is shifted to the alkaline region (Fig. 4). The dependence of the shift value on the ionic strength of a solution indicates that the nature of the shift is due to the influence of the microenvironment (8). The ionized carboxylic groups of MAA serve to form

[1] $k_{in} = 0.11\ min^{-1}$ at 65°C, pH 5.8. A thermal inactivation of the native enzyme at 65°C was not determined because the process was so fastly occurring especially in the region of high pH.

a surface charge. Nevertheless, the pH shift value is greater using a small amount of methacrylic acid. This is due to the slight swelling of gels containing from 0.3 to 1% of MAA. The addition of higher MAA concentrations into the gels results in gel swelling. As a consequence the surface density of carboxylic groups decreases and leads to a decrease in the local charge density within the cells of the gel.

Such a significant stabilizing effect on the matrix, however, cannot be attributed to a local acidification of the medium within the cells since, the dependence of the immobilized enzymes inactivation constants on the pH is rather insignificant. In the case of a gel containing 1.04% of MAA, a local change of pH per unit changes the inactivation constant no more than twice. Besides, the addition of an equimolar amount of allyl amine into the gels with MAA creates an electroneutral gel in which a significant stabilizing effect on the matrix close to that in the gels without AA is observed. In the case of partially dried gels (Fig. 1) the thermal inactivation was carried out at high ionic strengths where the effect of the microenvironment is suppressed (8). A small alteration of pH after the equilibration in 0.2 *M* acetate buffer gels should not exceed 0.1 to 0.2 pH units at most (9). Consequently, the observed increase in the inactivation constant of the immobilized enzyme cannot be due to this alteration.

To explain the observed stabilizing effect of the matrix, data on the structure of native glucose oxidase must be taken into account. It has been shown (10–12) that glucose oxidase from *P. vitale* readily dissociates into two subunits in 6 M guanidine chloride at neutral pH and in maleic anhydride in the alkaline medium. Under more drastic conditions these subunits disentegrate into two peptide chains. These data show the enzyme to possess a quarternary structure in which the subunits are bound by noncovalent bonds. On the basis of the studies on the thermal and ultrasonic inactivation of this enzyme it was concluded that the subunits in the native molecule are bound through two salt bridges and that enzyme inactivation was due to its decomposition into subunits (5).

If the inactivation of the immobilized glucose oxidase proceeds according to a scheme:

$$D \rightleftharpoons 2\mathrm{M} \cdots K$$

$$\mathrm{M} \rightarrow P \cdots k_{\mathrm{in}}$$

where D, M, P = concentrations of native enzyme, subunits, and inactivated products, respectively; K and k_{in} = enzyme dissociation and inactivation constants the factors affecting the process of the dissociation and (or) inactivation must change the rate of the enzyme inactivation.

In the case of a high enzyme concentration, the relative concentration of subunits is decreased which in turn reduces the enzyme inactivation.

Indeed a decrease in the inactivation constant with increasing enzyme concentration is observed in the case of a native enzyme (5). However, the alteration of enzyme concentration is negligible for the immobilized enzyme, especially in the case of partially dried gels. Consequently, the stabilizing effects of a matrix cannot be explained in terms of concentration.

Evidently, the entrapment of enzymes in high concentration gels containing hydroxyethyl groups capable of forming hydrogen bonds allows a multisided interaction between the protein molecules and the matrix. The presence of charged groups in the gel increases the number of the interaction points. The motion of the individual subunits is significantly retarded by these interactions. This decreases the enzyme dissociation rate. This explains the weak pH dependence of the inactivation rate of the immobilized enzymes since the ionic interaction between subunits is not essential for supporting the native structure of the enzyme. In such enzymes the native structure is supported not only by ionic interactions between the subunits but by the matrix too. On the other hand, the decrease in the glucose oxidase dissociation rate in the gel results in the reduction of the inactivation rate and the increase in thermal stability of these biocatalyzers. A multisided interaction must also decelerate the process of the enzyme inactivation due to denaturating agents. It is especially noteworthy that a minimum concentration of gel is essential for effective interaction between the protein molecule and the matrix. The stabilizing effects are reflected in the high concentration gels where a multisided interaction is the most effective.

A multisided interaction between a matrix and protein molecule must also retard the unfolding of the polypeptide chain (1, 2), the dissociation of cofactors, and other processes. However the contribution of these processes to the common mechanism of the stabilization of glucose oxidase immobilized in high concentration gels seems to be rather small.

The stabilization of glucose oxidase by a matrix at pH 5.8 achieves a limiting value even at high gel concentrations. Apparently this is due to the involvement of other mechanisms of enzyme inactivation since the rate is 220 to 250 times smaller than that of native glucose oxidase inactivation.

The stabilization of glucose oxidase at high gel concentrations investigated in the present work has a more common character and may be applied for other subunit-consisting enzymes.

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