BBA 66087

PREPARATION AND CHARACTERIZATION OF GLUCOSE OXIDASE COVALENTLY LINKED TO NICKEL OXIDE

HOWARD H. WEETALL AND LEROY S. HERSH
Research and Development Laboratories, Corning Glass Works, Corning, N.Y. 14830 (U.S.A.)

(Received November 18th, 1969)

SUMMARY

The enzyme glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) has been covalently coupled to NiO on Ni screening. The product is extremely stable over a long time and shows greater thermal stability than soluble enzyme. Kinetic values are similar to the soluble enzyme.

INTRODUCTION

Enzymes have been insolubilized by covalent attachment to various organic polymers[1–6], and cellulose derivatives[7–9]. Immobilization has also been accomplished by entrapment in starch[10], and acrylamide gel[11,12]. These types of derivatives have been studied in detail and were recently reviewed by SILMAN AND KATCHALSKI[13].

The insolubilized enzyme derivative described in this report was prepared by covalently bonding glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) to an inorganic carrier through a silane coupling agent.

Enzymes can be covalently coupled to many inorganic materials with the aid of an intermediate coupling agent[14,15]. Inorganic carriers in general, are not subject to microbial attack; they do not change configuration over an extensive pH range or under various solvent conditions, and with their greater rigidity, they immobilize enzymes to a greater degree than do organic polymers.

MATERIALS AND METHODS

Preparation of Ni–NiO screens

Ni screen 150 mesh, 0.1 mm was cut into strips of 1 inch $\times$ 5 inch each. The screens weighed approx. 1 g each. These strips were placed in a furnace at 700° for 2 h with O_2 flowing to oxidize the Ni surface. The strips were then rolled into cylinders of 0.5 inch internal diameter and soldered to prevent unravelling. The oxidized screens had a surface area as determined by N_2 adsorption[16] of 3.1 m^2/g.

Preparation of the insolubilized enzyme derivative

The oxidized screens were refluxed overnight in a toluene solution of aminopropyltriethoxysilane. The product was washed with acetone and dried. Several samples of the Ni–NiO screens were not reacted with aminopropyltriethoxysilane. Both silanized and untreated Ni–NiO screens were later employed as controls.

The Ni–NiO–aminopropyltriethoxysilane screens were refluxed overnight in 10% thiophosgene in chloroform. The product was washed with chloroform, air-dried and immediately coupled to glucose oxidase. The insolubilized enzyme derivatives were stored at 5° in distilled water.

Several Ni–NiO screens, untreated and treated with aminopropyltriethoxysilane, were added to a 1.0% solution of glucose oxidase dissolved in 0.1 M $NaHCO_3$ (pH 9.0) and allowed to stand overnight at 5°. The products were washed in distilled water and assayed for activity.

Determination of enzyme activity

The enzyme activity on the screens was determined in terms of μg of enzyme activity based on the activity of known quantities of soluble enzyme. The substrate for all experiments was anhydrous D-glucose (dextrose) in the concentration range of 0.55–55 mM, dissolved in 0.01 M phosphate buffer (pH 6.0) unless otherwise stated.

Assays

The soluble enzyme was assayed as follows. A 0.5-ml aliquot containing 250 μg of enzyme was added to 50 ml substrate containing 50 μg of horseradish peroxidase (Nutritional Biochemical Corp., Cleveland, Ohio) and 0.0005% of *o*-dianisidine. The reactants were stirred and a 2-ml sample was immediately taken as a control. Over a 5-min period, 2.0-ml samples were withdrawn at 1-min intervals and placed in tubes containing one drop 4 M HCl in 0.5 ml distilled water. The solutions were examined spectrophotometrically at 460 mμ. Unless otherwise stated, all experiments were carried out at 23°. The molar absorbency index for oxidized *o*-dianisidine was taken as $1.13 \cdot 10^4$ cm^{-1}.

The bound enzyme was assayed by a similar procedure. In this case, however, the substrate solution did not contain the horseradish peroxidase and *o*-dianisidine as these materials tended to absorb on the NiO screens. The peroxidase was added to aliquots of substrate removed during the reaction. The quantity of active enzyme coupled to the screen was determined by making the following assumptions: (a) the rate of product formation for a given concentration of soluble enzyme is equal to that for an equivalent concentration of bound enzyme, (b) the total weight of active enzyme bound may be determined by multiplying the experimental value for the enzyme concentration, as determined by assay, by the volume of substrate employed for the assay of the bound enzyme.

Determination of K_m and V

Values for the Michaelis constant, K_m, and maximum reaction velocity, V, were determined by assaying the soluble and bound enzymes at various substrate concentrations ranging from 0.55 to 55 mM. The temperature was held constant at 23°, as previously described.

Determination of temperature coefficient and apparent activation energy

The soluble and bound enzymes were assayed as previously described. Temperatures were maintained at 10 ± 0.01° for the first series of assays and at 20.0 ± 0.01° for the second series of assays. The substrate concentration was 1.0% which would give maximum velocity.

Determination of enzyme stability

An insolubilized enzyme screen was assayed at four weekly intervals to determine losses in enzyme activity with time. The screen was stored at 5°. Assays were carried out at 23°. The screen was then stored for 12 months at 5° and reassayed to determine long-term stability.

Determination of thermal stability

Several samples of the bound and soluble enzymes were exposed to increasing temperatures for 20-min periods and assayed as previously described at these temperatures to determine comparative thermal stability. The samples were exposed at 23, 33, and 43°. The exposures were cumulative.

Determination of optimal pH range

Assays as previously described were carried out in phosphate buffer adjusted to the appropriate pH. The maximum activity observed was taken as the pH optimum.

RESULTS

Quantity of enzyme adsorbed on Ni–NiO screens

The glucose oxidase adsorbed on the Ni–NiO and Ni–NiO–aminopropyltriethoxysilane screens was assayed immediately after preparation. The silanized screens showed a maximum activity equivalent to 175 μg of active enzyme. A second assay immediately following the first revealed a loss of 80% of the original activity. When assayed a third time, no activity was detected. The Ni–NiO untreated screens showed no enzyme activity.

Quantity of enzyme covalently coupled to Ni–NiO–aminopropyltriethoxysilane screens

Five screens were assayed for activity. The active enzyme coupled ranged from a minimum of 390 μg/screen to a maximum of 500 μg/screen. Assays were repeated several times with no observable losses of activity. The total nitrogen determined by Kjeldahl, present on each screen was always 20% less than the values determined by assay after subtraction of the nitrogen contributed by the silane.

K_m and V

The values for K_m and V were obtained by plotting the reciprocal of substrate concentration, $[S]$, against the reciprocal of reaction velocity, V, at that substrate concentration, $[S]$. These velocities were reproducible to ± 10%. Similar data were collected for the soluble enzyme preparation and both are plotted in Fig. 1. The value for V was calculated, and K_m was determined using the relationship: $1/v = K_m/V \cdot [S] + 1/V$, which assumes Michaelis–Menten kinetics.

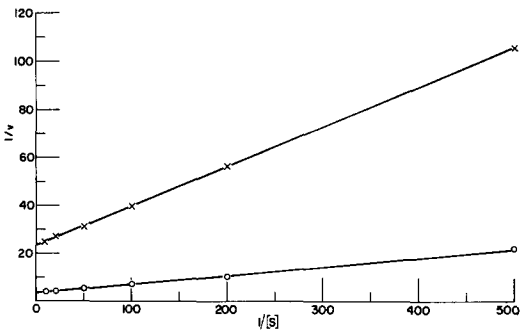

Fig. 1. Determination of V and K_m. Reciprocal of velocity, $1/v \times 10^6$, in units of $(\text{moles} \cdot \text{sec}^{-1})^{-1}$ *vs.* the reciprocal of concentration of D-glucose, $1/[S]$, in units of $1 \cdot \text{mole}^{-1} \times 10^2$. ×, experimental data for bound enzymes; ○, experimental data for the soluble enzyme. The curves for both free and bound enzyme were used for determining the values of V and K_m.

Temperature coefficient and apparent activation energy

The soluble and bound enzymes were assayed at 10 and 20°. The activation energy E_a and temperature coefficient Q_{10} were determined for each. The results are given in Table I. The bound enzyme was found to have a higher Q_{10} and E_a than the free enzyme.

Stability of the insolubilized enzyme

One screen was assayed for four consecutive weeks. The results (Table II)

TABLE I

CHARACTERISTICS OF THE CHEMICALLY-COUPLED AND SOLUBLE GLUCOSE OXIDASE

	Soluble enzyme	*Bound enzyme*
K_m (mM)	9.2	7.1
V (μmole·sec^{-1})	0.025	0.043
Q_{10} (10–20°)	1.48	1.72
E_a (cal)	6590	9050
Enzyme activity (mg)	0.50	0.38

TABLE II

ENZYMATIC ACTIVITY OF CHEMICALLY-COUPLED GLUCOSE OXIDASE WITH TIME

Time (days)	*Enzyme activity (μg)*	*Percent original activity remaining*
0	375	100
7	375	100
14	300	80
21	225	60
28	225	60
178	200	53
365	200	53

TABLE III

INCREASED THERMAL STABILITY OF BOUND GLUCOSE OXIDASE OVER THE SOLUBLE FORM

Enzyme was maintained at prescribed temperature for 20 min and assayed at that temperature before increasing temperature. The samples were subjected to the cumulative effect of increasing temperatures.

	Activity (μg)		
Temp.:	*23°*	*33°*	*43°*
Soluble glucose oxidase	480	400	386
Insoluble glucose oxidase	480	500	420

indicate that no losses occurred for at least 7 days. By 21 days the activity had leveled off at 60% of the original activity. After storage for 178 days, only an additional 7% loss was observed. After 365-day storage, the activity had not diminished further.

Thermal stability

Exposure of soluble and bound enzyme to increasing temperatures caused more rapid inactivation of the soluble enzyme (Table III). The activity increase between 23 and 33° for the bound enzyme is not large. However, in the case of the soluble enzyme a loss in activity was observed over the same temperature range.

Optimal pH

Maximum enzyme activity for the bound enzyme was observed at pH 5.6 (Fig. 2), and decreased rapidly on both the acid and base side of the optimum. The soluble enzyme gave maximal activity also at pH 5.6.

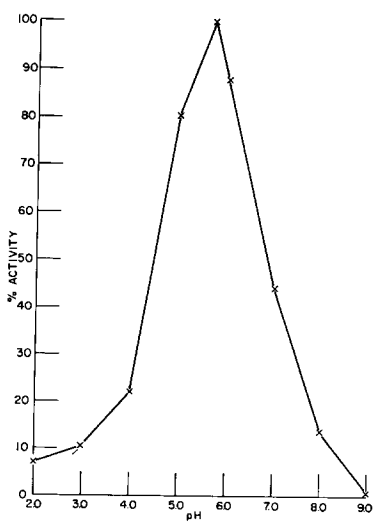

Fig. 2. Optimal pH of bound glucose oxidase. Maximum activity arbitrarily set at 100%. See text for detailed explanation.

DISCUSSION

The NiO was prepared in the hope that the ethoxy-silane groups would be covalently bound to the surface, leaving the primary amines available for coupling to the enzymes. There is a strong possibility that the silane amine groups are covalently linked to the NiO rather than the ethoxy groups[17]. However, because the silane polymerizes, a great number of amine groups could still be available for conversion to the isothiocyanate.

The surface area of the oxidized screens was $2.1 \cdot 10^{20}$ Å^2/g. Assuming an average size of 14 000 Å^2 per glucose oxidase molecule, one should expect a complete monolayer to contain over 13 mg of enzyme per g of screen. However, only 0.4–0.5 mg of enzyme per g of screen was found. Although the enzyme activity and protein nitrogen values were within reasonable agreement, comparisons may be misleading. At present we can only assume that the activity of the bound enzyme is equivalent to the unbound and make our comparisons on this basis. The 20% discrepency between activity based on assay and total protein based on nitrogen may be inactivated protein, or increased activity of the bound protein.

Additional evidence for presence of a covalent bond between the glucose oxidase and the Ni–NiO screens was obtained by the observation that physically adsorbed glucose oxidase is quickly desorbed from the silanized Ni–NiO screens.

The stability of the chemically bonded enzyme appears to have been increased over the soluble enzyme. This is easily visible in many aspects of the enzymatic reaction. Thermal stability is greater over the range of 10–40°.

The soluble enzyme appears to denature even at very low temperatures, as indicated by the Q_{10} (10–20°) values. Between 23 and 33° denaturation is so great that the soluble enzyme activity actually decreases.

The results presented in this communication are consistent with several general observations on insolubilized enzymes, such as those reported in a recent colloquium on *Properties of Enzymes Attached to Solid Matrices*[18].

Most observed factors can be understood by considering the microenvironment of the enzyme. Stability toward heat and extremes of pH can usually be explained in this manner.

The Michaelis constant is particularly sensitive to the nature of the microenvironment. If the substrate is uncharged, there is little change in K_m. On the other hand, K_m may change as much as an order of magnitude when both substrate and support are charged, increasing with like charge and decreasing when the charges are unlike[18].

No change in pH optimum and only a slight change in K_m were observed with the insolubilized glucose oxidase. This would indicate that little or no charge–charge interaction is involved in this system. The value for pH optimum in the literature is given as pH 5.6 (ref. 19).

Enzymes bound to metallic oxides may have advantages over enzymes entrapped in gels, or bound to porous glass or organic polymers, for several reasons: (a) they are not as diffusion-controlled as shown by linearity of reaction kinetics; (b) greater flow rates are achievable with less chance of clogging during continual usage since the enzymes are attached to a very porous screen; (c) greater ease of handling; and (d) no apparent charge (–charge) interaction with the support material.

ACKNOWLEDGMENTS

We thank Dr. G. S. Eadie of Duke University for his discussions concerning the kinetics of enzyme reactions. We also thank Mr. R. A. Messing, Dr. M. L. Hair and Dr. J. R. Hutchins, III for their aid and criticism in the preparation of this manuscript.

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