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EXPLANATION OF ANOMALOUS BINDING KINETICS WITH A HIGH YIELD IMMOBILIZED ENZYME SYSTEM

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Summary

The activities of glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) and catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) from commercial preparations do not give typical adsorption curves upon immobilization on non-porous polyethylenimine-coated glass microbeads. The cause of this effect with glucose oxidase was investigated. Protein binding exhibited a rectangular hyperbolic adsorption isotherm, approaching saturation at high concentrations, however, enzyme activities did not. The isotherm for activities exhibited a maxima which corresponded to less than 50% saturation with regard to total protein adsorption. The enzyme preparation was found to contain small quantities of several low molecular weight impurities as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. These impurities apparently compete with glucose oxidase for binding. When large excesses of protein are added to beads, the binding of impurities becomes significant and the amount of enzyme activity per unit of bead is reduced.

Introduction

A number of methods have been developed for the immobilization of enzymes on inorganic carriers such as glass beads [1]. However, until recently little data has been reported citing yields, either with respect to the expression of total original soluble activity or the percent protein immobilized. We have recently developed a procedure for immobilizing enzymes on non-porous glass microbeads coated with polyethylenimine, which is rapid and combines the

characteristics of high activities and yields [2].

One of the parameters shown to be important in achieving maximal enzyme activities per unit wt. of bead, was the protein concentration of the immobilizing solution. Immobilized *Aspergillus niger* glucose oxidase and catalase, prepared from solutions containing high concentrations of protein, exhibited enzyme activities several times lower than samples prepared with more dilute solutions. Both total and specific activity significantly declined as a function of protein loading. Herein we present evidence that the decline is due to the differential binding of enzyme and minor contaminants in the enzyme preparation.

Methods

Glucose oxidase from *A. niger* was purchased from Worthington Biochemical Corp., Freehold, NJ. Rough polyethylenimine-coated glass microbeads (13—44 μm) were prepared as reported previously [2]. Immobilizations were performed at 25°C in 50 mM citrate-phosphate buffer, pH 7.5. The enzyme and beads were mixed and after 15—20 min, excess protein was removed by repeated washing. Activities of soluble and immobilized glucose oxidase and catalase were assayed using an oxygen polarograph [2]. Soluble protein was assayed by the method of Lowry et al. [3], using glucose oxidase as the standard. Protein adsorbed on beads was determined by a modification of the Lowry procedure after extraction in SDS [4]. The ability of SDS to quantitatively extract adsorbed bovine serum albumin from polyethylenimine-coated glass beads, was confirmed by comparing the results obtained by the above method with results obtained by the direct counting of adsorbed ^{14}C-labeled bovine serum albumin. A series of beads was prepared with increasing concentrations (0—10 mg/ml) of either ^{14}C-labeled bovine serum albumin (prepared by reductive formylation) or non-labeled BSA. The binding isotherm of the labeled bovine serum albumin was obtained by direct counting. Unmodified bovine serum albumin was measured by SDS extraction. Values for adsorbed protein by the two methods were in general agreement. Saturation binding was observed. The binding capacity of the beads for bovine serum albumin was 200 μg/g bead. SDS-polyacrylamide gel electrophoresis was carried out using the buffer systems of Laemmli [5].

Results

With glucose oxidase and catalase, simple adsorption kinetics were not observed when bound activity was compared with protein concentrations initially present. In the presence of glutaraldehyde, glucose oxidase activities expressed per unit wt. of bead were 8-times higher when the initial enzyme concentration was 0.67 mg/ml compared to 10 mg/ml (data not shown). When glutaraldehyde was omitted from the immobilizing solution, as in the experiments reported here, a 4-fold difference in activity between samples prepared at the low and high protein concentrations was observed. To further investigate the cause of this effect, resolubilization of bound protein was necessary. Since glucose oxidase immobilized by adsorption exhibits a similar activity binding pattern to

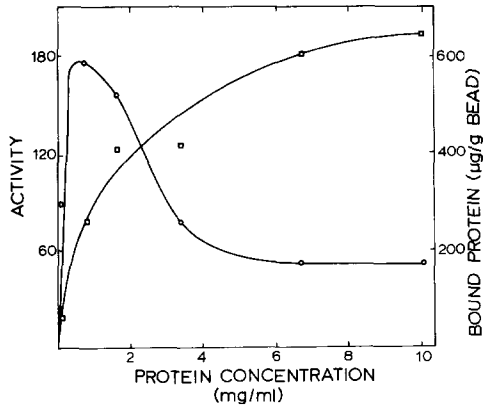

Fig. 1. Correlation of immobilized activity (○———○) (pmol O_2/mg bead per s) with protein binding (□———□) (mg protein/g bead). The specific activities (µmol O_2/mg protein per min) of the samples prepared at 0.67 and 10 mg/ml were calculated to be 39.5 and 4.5, respectively. Immobilizing solutions contained 400 mg of beads and 600 µl enzyme solution. After 15 to 20 min, beads were washed to remove unadsorbed enzyme and assayed for activity. Bound protein was extracted twice with 400 µl of 3% SDS at 65°C for 5 min and assayed by a modified procedure of Lowry et al. [4]. A sample containing beads without protein served as control.

samples prepared in the presence of glutaraldehyde and in addition can be desorbed by extraction in either SDS at 60°C or acidic buffer [2] all experiments were performed on samples prepared by adsorption.

Following adsorption and the removal of excess protein, a series of beads prepared by incubation with glucose oxidase solutions, ranging in concentration between 0.067 and 10 mg/ml were analyzed for activity and bound protein (Fig. 1). Activity binding was non-hyperbolic. However, protein exhibited saturation binding. The maximal expression of enzyme activity did not correlate with the protein binding capacity of the beads (628 µg/g bead), but occurred when the beads were only one-third saturated with respect to protein. The sharp decline in activity began to occur when the quantity of bound protein exceeded approx. one-third saturation. To rule out the possibility that the high activities obtained in samples prepared using immobilizing solutions, containing protein concentration of less than 10.0 mg/ml, were due to activation of the bound enzyme, the specific activity of the bound enzyme was compared

TABLE I

SPECIFIC ACTIVITY OF DESORBED GLUCOSE OXIDASE

The specific activity of native glucose oxidase was 53.6 µmol O_2/mg protein per min. Protein was extracted twice from 400 mg of beads in 0.5 ml 50 mM citrate phosphate buffer, pH 5.5. The supernatants were combined and adjusted to 1.0 ml. A 10-µl aliquot was assayed for enzyme activity and the remainder for protein [4]. Data are represented as the average values for 3 determinations ± one S.D.

Concentration of glucose oxidase in immobilizing solution (mg/ml)	Immobilized enzyme activity before extraction (pmol O_2/mg bead per s)	Specific activity of resolubilized enzyme (µmol O_2/mg protein per min)
0.67	423.3 ± 9.2	65.2 ± 1.3
10.0	270.8 ± 17.1	24.6 ± 2.0

with the specific activity of soluble glucose oxidase. The specific activity of soluble glucose oxidase was greater than any of the values calculated for the immobilized samples, thus indicating that activation was not occurring.

To determine whether diffusional or steric effects were responsible for the diminished enzyme activities observed concomitant with the saturation binding of protein, the enzyme was desorbed from bead samples prepared at high and low protein concentrations and assayed for specific activity. Results from this experiment are shown in Table I. The specific activity of the extracted enzyme immobilized at 0.67 mg/ml was close to the value obtained for native enzyme, whereas the specific activity of extracted glucose oxidase immobilized at 10.0 mg/ml was 3-times lower. If diffusional or steric effects alone were responsible for the lowered activity, the specific activity of the desorbed enzyme from the sample prepared at the high concentration should have equaled the specific activity of the enzyme prepared at 0.67 mg/ml. It is highly unlikely that diffusion effects exert a significant effect on this system. This is because the beads are non-porous and relatively small (13—44 μm).

The K_m values of soluble glucose oxidase and samples prepared by immobilization at protein concentrations of 10.0 and 0.67 mg/ml were identical, 29.4 mM. However, V decreased from 1667 to 285.9 pmol O_2/mg bead per s. Since at high protein concentrations, the K_m of glucose oxidase does not change, but V is reduced, these results are consistent with either inactivation or the presence of impurities. To discern between the two possibilities, the homogeneity of the enzyme preparation before and after binding to the beads was investigated.

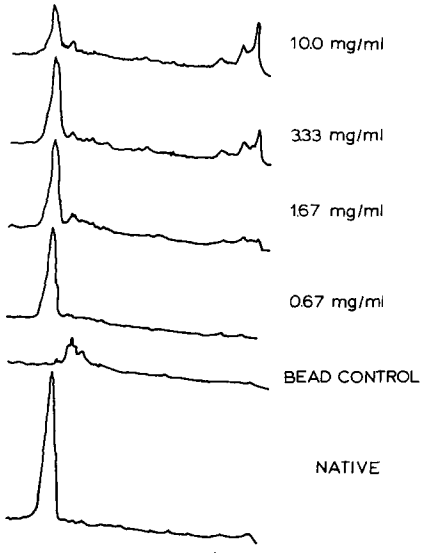

Fig. 2. Densitometer tracings of SDS-polyacrylamide gels of native (30 μg) and glucose oxidase extracted from beads. Immobilizations were performed as in Fig. 1 using the protein concentrations indicated to the right of each scan. An SDS-polyacrylamide gradient slab gel system (8—18% acrylamide) with a 4.6% acrylamide stacking gel was used. Protein was extracted from beads as described [4]. Scanning and computation of the relative areas under each peak were performed with an ACD-18 automatic computing densitometer (Gelman Instrument Co.).

The native enzyme preparation was analyzed for purity by SDS-polyacrylamide gel electrophoresis. Native glucose oxidase (30 μg) was essentially homogeneous except for a relatively faint band co-migrating with the dye front. On overloading with 50 μg or more of protein, three bands could be detected in this region. The possibility, that these low molecular weight trace impurities competed with glucose oxidase for binding at high protein concentrations was investigated. A series of beads was prepared with solutions of protein concentration ranging between 0.67 and 10.0 mg/ml. The protein adsorbed to the beads was electrophoresed. Results in the form of densitometer tracings are shown in Fig. 2. This experiment shows that a progressively larger proportion of the trace impurities become bound compared with enzyme at the high protein concentrations. At 0.67 mg/ml the trace impurities accounted for only about 6% of the resolubilized protein, whereas at 10 mg/ml over 50% of the desorbed material were low molecular weight proteins.

Since the production of low molecular weight peptides during the extraction procedure was shown not to be time dependent, the possibility of their formation by proteolysis during extraction seems unlikely. A small amount of material migrating in the 60 000—70 000 molecular weight range was extracted from both the control beads and beads containing glucose oxidase. The molecular weight of this material corresponded closely to the molecular weight of the polyethylenimine used to derivatize the beads.

The data suggest that the protein impurities bind preferentially to polyethylenimine-coated glass beads. These impurities had a low molecular weight on protein denaturing gels. With the amount of enzyme solution employed, at the low protein concentration, these impurities are not present in sufficient amounts to compete with the enzyme for bead binding sites. However at higher concentrations, the quantity of impurities relative to the number of enzyme binding sites becomes quite substantial, and bound protein becomes enriched with the lower molecular weight polypeptides. The identity of these polypeptides is unknown.

Discussion

Many immobilization procedures are designed to favor the saturation of surfaces with protein. With heterogeneous enzyme preparations such as the one used in this study, by simply varying the protein concentration of the immobilizing solution it is possible to select for conditions which favor the binding of one group of proteins over another. These results demonstrate that maximal ratios of enzyme to impurity binding can be achieved by utilizing low protein concentrations or by avoiding the addition of excess quantities of protein to the immobilizing medium.

In studies directed at obtaining maximal reaction rates, two different methods of describing immobilized enzyme activity have been used. One common method of quantitation is to report reaction rates in terms of immobilized specific activity which is the ratio between bound enzyme activity and the activity of an identical amount of soluble enzyme. The other form of expressing immobilized activities has been termed total activity and is the reaction velocity per unit wt. of support material. In several systems [6,7] declines in

immobilized specific activity have been observed as a function of increasing enzyme loading. Although changes in total activities were not explicitly stated, one may calculate that total activities did not significantly decrease as a function of enzyme loading. These systems exhibited significant diffusional effects.

Maximal enzyme activities per unit wt. of support are not necessarily achieved by optimizing conditions for protein binding. It cannot be predicted whether major or even trace impurities present in an enzyme preparation will compete with an enzyme for binding. If the goal is to immobilize most, if not all, of the total enzyme in the immobilization mixture, a comparison of protein and activity binding curves will give the necessary information for determining the optimum concentration of protein to be used.

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