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Preparation, Characterization, and Potential Application of an Immobilized Glucose Oxidase

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ABSTRACT

A simple, one-step process, using 0.25*M* *p*-benzoquinone dissolved in 20% dioxane at 50°C for 24 h was applied to the activation of polyacrylamide beads. The activated beads were reacted with glucose oxidase isolated from *Aspergillus niger*. The coupling reaction was performed in 0.1*M* potassium phosphate at pH 8.5 and 0–4°C for 24 h. The protein concentration was 50 mg/mL. In such conditions, the highest activity achieved was about 100 U/g solid. The optimum pH for the catalytic activity was shifted by about 1 pH unit in the acidic direction to pH 5.5. Between 35 and 50°C, the activity of the immobilized form depends on the temperature to a smaller extent than that of the soluble form. Above 50°C, the activity of immobilized glucose oxidase shows a sharper heat dependence. The enzyme–substrate interaction was not profoundly altered by the immobilization of the enzyme. The heat resistance of the immobilized enzyme was enhanced. The immobilized glucose oxidase is most stable at pH 5.5. The practical use of the immobilized glucose oxidase was tested in preliminary experiments for determination of the glucose concentration in blood sera.

Index Entries: Glucose oxidase, immobilized; polyacrylamide-type support, prepared by *p*-benzoquinon activation; support, poly-

acrylamide type; catalytic properties, immobilized glucose oxidase; glucose determination, blood sera.

INTRODUCTION

Polyacrylamide has several advantageous properties, such as a hydrophilic character, resistance against microbial attacks, controlled pore size, and the like. Therefore, several methods are known to produce polyacrylamide-type supports having functional groups [cf. ref. (*1*)]. However, in most cases, the production of activated polyacrylamide supports is relatively tedious and requires special reaction conditions or expensive reagents. We have found (*2*) that polyacrylamide gels might be activated by *p*-benzoquinone in a very simple way, and the activated polymer is able to bind various nucleophylic compounds. Because of its great practical importance in the determination of glucose concentration in blood sera glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4), it was chosen to test the applicability of *p*-benzoquinone-activated polyacrylamide beads as enzyme support.

Both from a theoretical and practical point of view, it is interesting to study the effects of immobilization on the catalytic properties and stabilities of the enzymes (*3*). Therefore, the catalytic properties and stabilities of immobilized glucose oxidase were investigated and compared with the corresponding properties of the soluble enzyme.

MATERIALS AND METHODS

Akrilex P-100, a polyacrylamide-type bead polymer (particle size 40–120 μm; pore size, max. 12 nm) was produced by Reanal Factory of Laboratory Chemicals, Budapest, Hungary. Glucose oxidase isolated from *Aspergillus niger* was purchased from Sigma Chemical Company, St. Louis, MO, USA. Its specific activity on a protein basis was 150–200 U/mg protein. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol β-D-glucose to D-gluconic acid/min at pH 6.5 and 25°C. All other chemicals were reagent grade commercial products of Reanal.

General Method of Immobilization

Glucose oxidase might be immobilized on Akrilex P type supports as follows: 100 mg of dry beads were suspended and swollen in 4 mL of 0.1*M* potassium phosphate (pH 8.0) and 1 mL of 0.25*M* *p*-benzoquinone dissolved in 20% dioxane was added. During a 24-h incubation at 50°C, the originally colorless beads became brownish, indicating the activated state. After activation, the suspension was filtered by suction and washed 10 times with 7 mL of 20% dioxane and 10 times with water.

Then, an appropriate quantity (5–100 mg) of glucose oxidase dissolved in 2 mL of 0.1*M* potassium phosphate solution was added to the activated support. The mixture was incubated at 0–4°C during 24 h, with an 8-h period of agitation.

The gel was filtered by suction and successively washed three times with 2 mL of 0.1*M* potassium phosphate buffer (pH 7.5), three times with 2 mL of the same buffer containing 1.0*M* sodium chloride, three times again with 2 mL of buffer to remove the unbound proteins, and, finally, with distilled water to remove the phosphate ions. The product was then lyophilized.

In control experiments, 100 mg of support were treated with glucose oxidase in identical circumstances, but without previous activation by *p*-benzoquinone.

Measurement of Protein

Protein determinations were performed according to the method of Lowry et al. (*4*), as modified by Schacterle and Pollack (*5*). The amount of immobilized protein was calculated as the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Glucose Oxidase Activity

For the determination of glucose oxidase activity, a coupled-enzyme reaction, using horse radish peroxidase and *o*-dianisidine, was applied (*6*).

At the activity test of the soluble enzyme, the reaction mixture (3.03 mL) contained 140 μg *o*-dianisidine, 270 μmol glucose, 20 μg peroxidase, and 0.02 mL enzyme (10–20 μg/mL) in 0.1*M* potassium phosphate buffer (pH 6.5). The increase in absorbance at 436 nm was recorded. The activity was calculated from the initial linear portion of the curve. In the case of immobilized glucose oxidase, 10 mg of the immobilized enzyme was stirred in 2 mL of 0.1*M* potassium phosphate buffer (pH 5.5), containing 0.5*M* glucose, for appropriate periods of time (1–5 min). Then the enzyme was filtered off quickly (a few seconds), and an aliquot (0.1-mL) of filtrate was treated with a mixture of *o*-dianisidine and peroxidase. The absorbance was measured spectrophotometrically at 436 nm.

Heat Stability Tests on Soluble and Immobilized Glucose Oxidase

The experiments were performed in 0.1*M* potassium phosphate, with reaction mixtures of 1.5 mL vol at 50°C. After appropriate times of incubation, the samples were rapidly cooled in an ice bath and the residual activities were assayed at 25°C by the standard method.

RESULTS

Effect of pH and Protein Concentration of Coupling Reaction Mixture on the Activity of Immobilized Glucose Oxidase

The most favorable conditions for the immobilization of glucose oxidase were studied. The optimal pH for the coupling was found to be pH 8.5 (Fig. 1). The support could be saturated with protein (Fig. 2). In optimal conditions, i.e., pH 8.5 and 50 mg/mL protein concentration, the highest activity achieved was about 100 U/g dry support. The loading was 190 mg protein/g dry support.

In control experiments performed without previous activation by *p*-benzoquinone, the total quantity of glucose oxidase added could be washed out of the support.

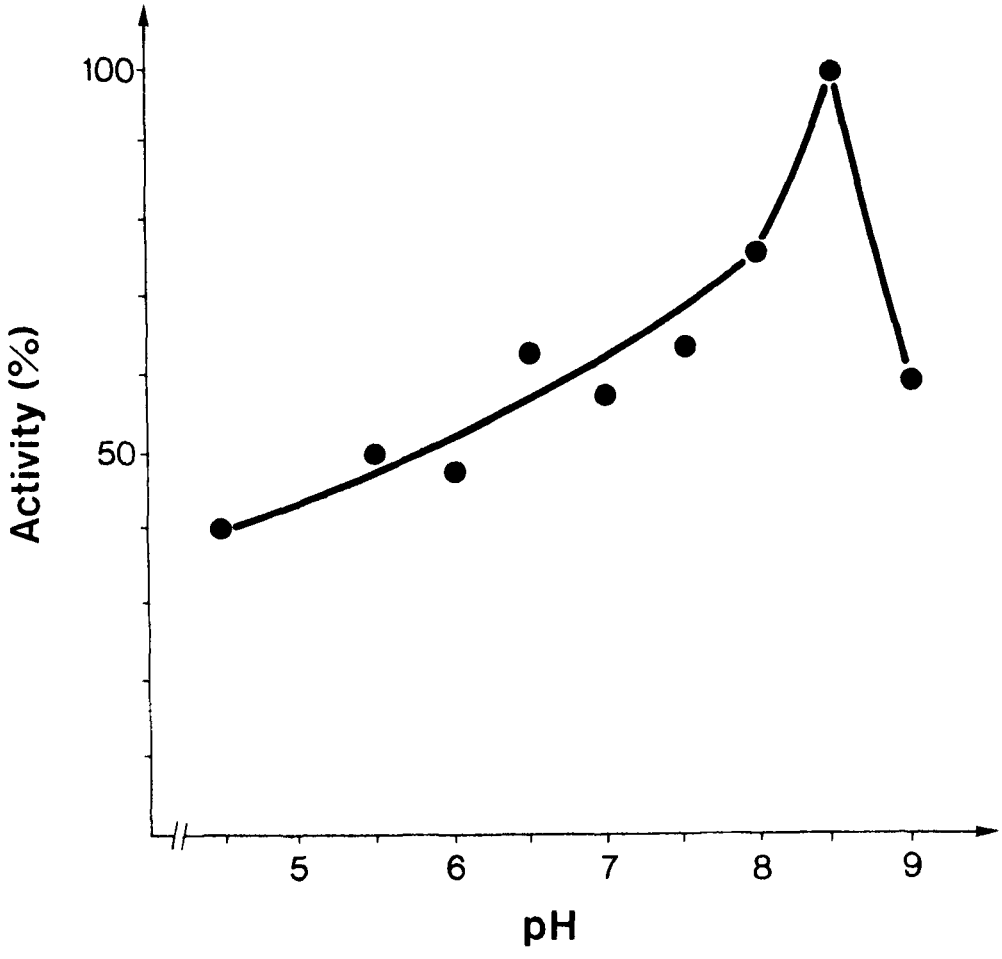

Fig. 1. Effect of pH of coupling reaction mixture on the activity of immobilized glucose oxidase. Experiments were performed in 0.1*M* potassium phosphate at 0–4°C. The Akrilex P-100 support was activated by *p*-benzoquinone at pH 8.0.

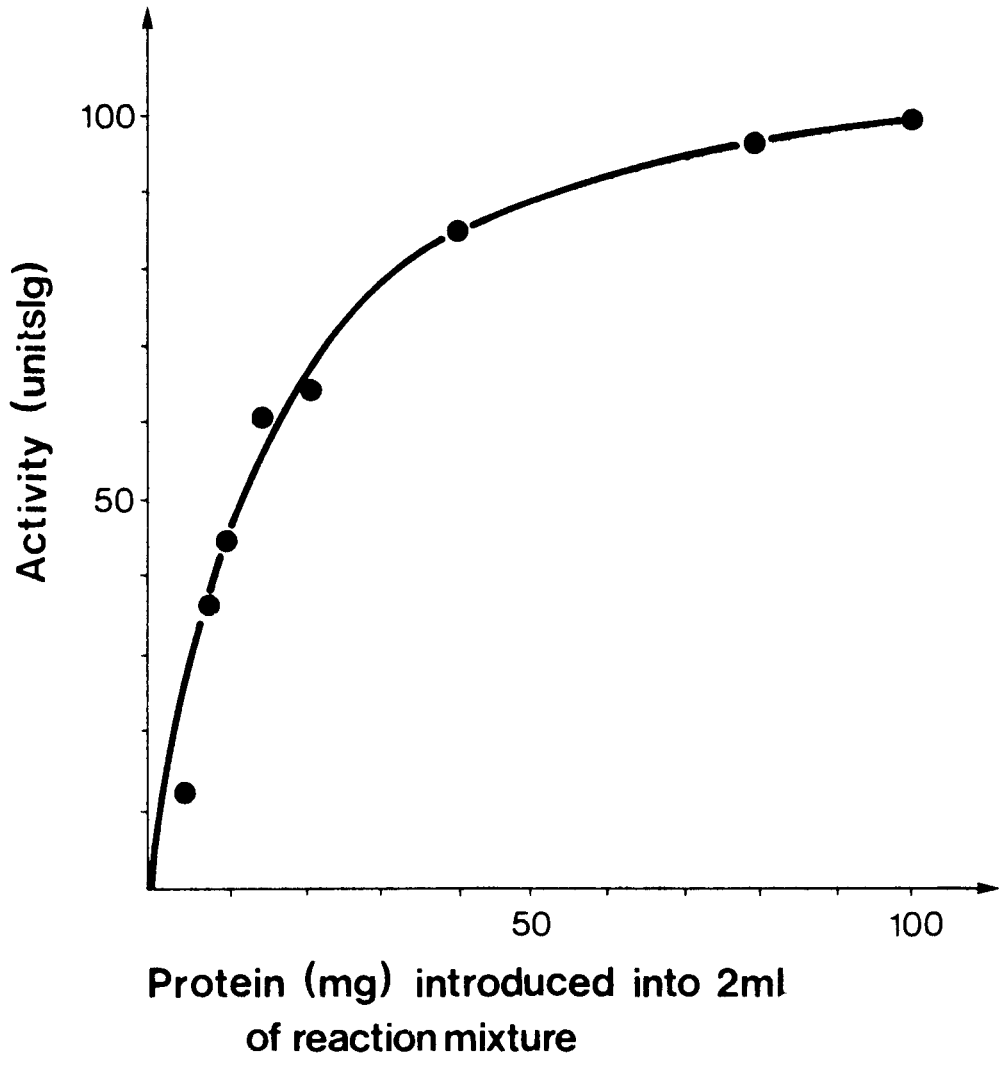

Fig. 2. Effect of protein concentration in the coupling reaction mixture on the activity of immobilized glucose oxidase. Experiments were performed in 0.1*M* potassium phosphate at pH 8.5 and 0–4°C. The Akrilex P-100 support was activated by *p*-benzoquinone.

pH Dependence of Catalytic Activity

The pH dependence of the initial rates of glucose oxidation was studied at the same ionic strength in the pH range of 4.5–9.0. The optimum pH for the catalytic activity of immobilized glucose oxidase was shifted by about 1 pH unit in the acidic direction to pH 5.5 by the effect of immobilization.

Dependence of Catalytic Activity on Temperature

The temperature dependence of the activity of soluble and immobilized glucose oxidase was studied in 0.1*M* potassium phosphate at the

optimum pH for the catalytic activity (soluble enzyme, pH 6.5; immobilized enzyme, pH 5.5) in the temperature range of 15–60°C.

Initial velocities were derived by measuring the activity after a 5-min incubation at selected temperatures (Fig. 3). The apparent optimum temperature for the catalytic activity of immobilized glucose oxidase was about 40°C, i.e., nearly identical to that of the soluble enzyme.

Effect of Substrate Concentration on the Catalytic Reaction

The effect of glucose concentration on the initial rates of catalyzed reaction was investigated in the concentration range of 0.02–0.5*M* at the

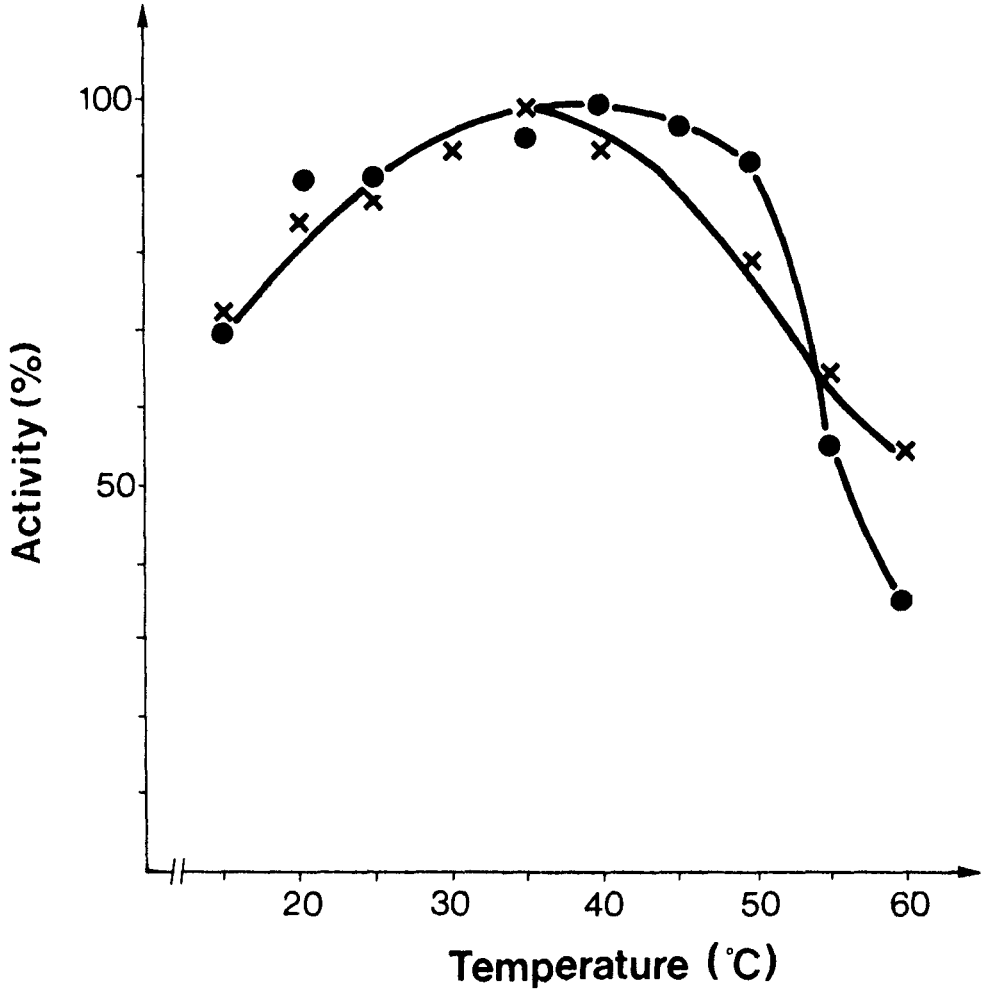

Fig. 3. Effect of temperature on the activity of soluble and immobilized glucose oxidase. Experiments were performed in 0.1*M* potassium phosphate buffer at the optimum pH for the catalytic activity (soluble enzyme, pH 6.5; immobilized enzyme, pH 5.5) with glucose as substrate: ×, Soluble enzyme; •, immobilized enzyme. With both soluble and immobilized enzyme the maximum activity was taken as 100%.

optimum pH for catalytic activity. Experiments were carried out in 0.1*M* potassium phosphate. The temperature was 25°C. For calculation of K_m values, kinetic plots according to Hanes (*7*) were used. From the plots, K_m of soluble glucose oxidase was calculated to be 33 m*M* and $K_{m\ app}$ of immobilized enzyme to be 44 m*M*, i.e., the enzyme–substrate interaction was not profoundly altered by immobilization of the enzyme.

Heat Stability

The rates of heat inactivation of soluble and immobilized glucose oxidase were investigated at 50°C and the optimal pH for the catalytic activity (soluble enzyme, pH 6.5; immobilized enzyme, pH 5.5) in 0.1*M* potassium phosphate. The time curves of the heat inactivation are presented in Fig. 4. The graph demonstrates the enhanced heat resistance of the immobilized enzyme.

The pH Dependence of Heat Inactivation ("pH Stability")

The pH stability of soluble and immobilized glucose oxidase was compared at selected pH values in 0.1*M* potassium phosphate at 50°C. The results are presented in Fig. 5. The soluble glucose oxidase was most stable at pH 6.0, whereas the immobilized form was most stable at pH 5.5, which is identical with the optimal pH for the catalytic activity. The apparent half life values derived from the time curves at different pH values are compared in Table 1. As can be seen from Table 1, the heat stability of immobilized glucose oxidase at every pH exceeded that of the soluble enzyme.

Storage Stability

The shelf life of the immobilized glucose oxidase was very good. It lost only 9% of its original activity during a 1-yr storage at 4°C in lyophilized form under exclusion of air.

Practical Use of the Immobilized Glucose Oxidase

The practical use of the immobilized glucose oxidase was tested in preliminary experiments. Ten milligrams of immobilized enzyme was stirred with 2 mL of sample containing glucose at 25°C for 15 min. Then the enzyme was filtered off, the filtrate treated with a peroxidase–*o*-dianisidine mixture (*6*), and the absorption measured at 436 nm. The glucose content was calculated using a calibration curve. In one of these experiments, the glucose content of a corn-starch syrup produced by total enzymatic hydrolysis was determined. With the same enzyme aliquot, seven successive determinations could be carried out. The average glucose concentration (*M*) was found to be 1.36 ± 0.15 (SD), the iodometrically determined concentration of the same sample was 1.62*M*. The immobilized glucose oxidase was used in a similar way for the determi-

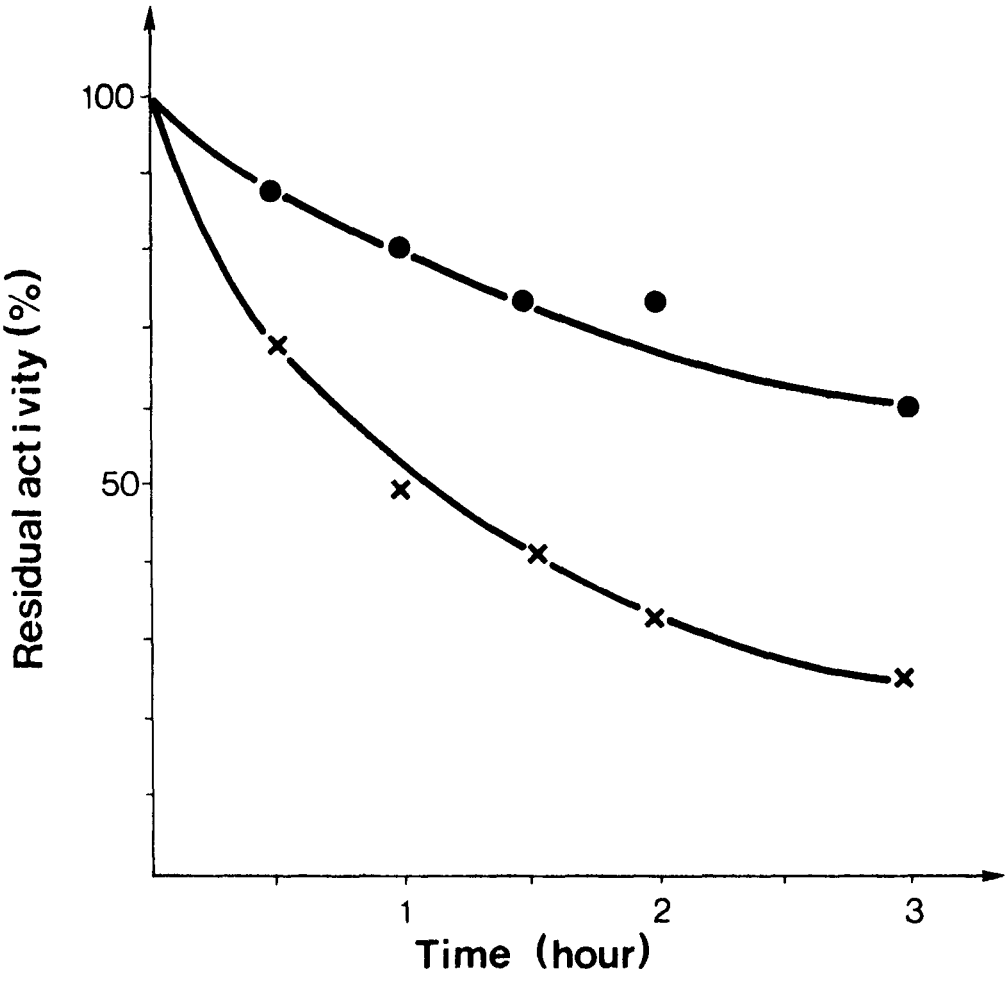

Fig. 4. Heat inactivation of soluble and immobilized glucose oxidase at 50°C in absence of glucose. Experiments were carried out in 0.1*M* potassium phosphate buffer at the optimum pH for the catalytic activity (soluble enzyme, pH 6.5; immobilized enzyme, pH 5.5). Enzyme concentrations used were soluble enzyme, 1.0 mg protein/mL; immobilized enzyme, 8.5 mg solid/mL: ×, Soluble enzyme; •, immobilized enzyme. In the case of both soluble and immobilized enzyme the starting activity was assigned the value of 100%.

nation of the glucose content in two different, individual human blood sera (sample 1 and 2). The average values of glucose concentrations (m*M*) were 3.12 ± 0.14 (SD) for sample 1 and 7.74 ± 1.04 (SD) for sample 2, respectively.

DISCUSSION

Polyacrylamide gels have great practical importance as enzyme supports because of their advantageous chemical and mechanical properties.

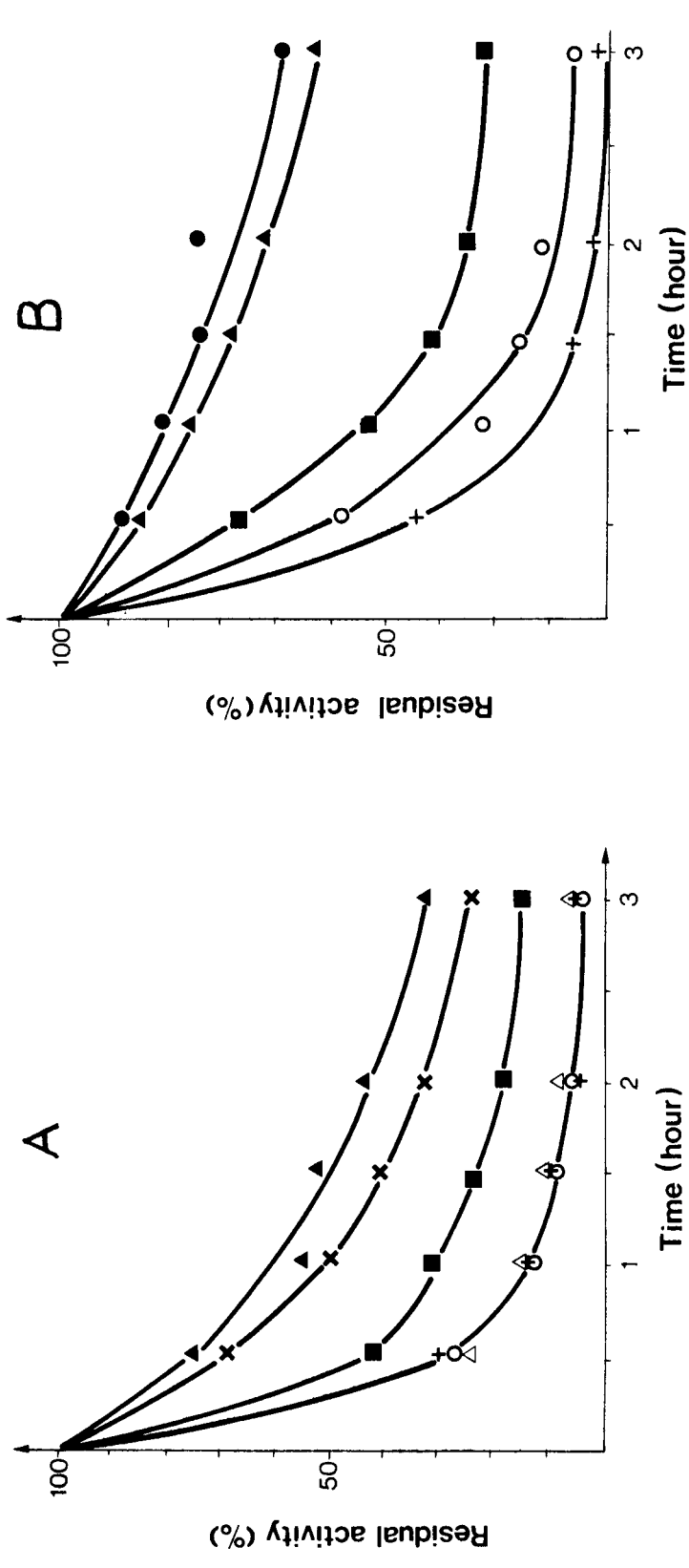

Fig. 5. The pH dependence of heat stability ("pH stability") of soluble (A) and immobilized (B) glucose oxidase at 50°C. Experiments were carried out in 0.1*M* potassium phosphate. Enzyme concentrations used were soluble enzyme, 1.0 mg protein/mL; immobilized enzyme, 8.5 mg solid/mL. o, pH 4.0; Δ, pH 5.0; ●, pH 5.5; ▲, pH 6.0; ×, pH 6.5; ■, pH 7.0; +, pH 8.0. In the case of both soluble and immobilized enzyme the starting activity was assigned the value of 100%.

TABLE 1
pH Stability of Soluble and Immobilized Glucose Oxidase[a]

	$t_{1/2,app}$, min	
pH	Soluble enzyme	Immobilized enzyme
4.0	15.6	28.6
6.0	90	180
7.0	23.9	51.7
8.0	15.6	20.3

[a]Experiments were carried out in 0.1*M* potassium phosphate at 50°C. Enzyme concentrations used were soluble enzyme, 1.0 mg protein/mL; immobilized enzyme, 8.5 mg solid/mL.

Several derivatization techniques are known to convert the amide groups into chemically more reactive groups (*8,9*). Formerly, *p*-benzoquinone was used only for the activation of polymers having hydroxylic groups (*10,11*). However, we have found (*2*) that at an elevated temperature (50°C), using longer incubation time (24 h), polyacrylamide gels could be activated by *p*-benzoquinone. The activated polymers react with various nucleophylic compounds, including enzymes. The mechanism of the activation and the coupling reaction has not been explained yet. Glucose oxidase was used to study the effects of immobilization on *p*-benzoquinone-activated polyacrylamide support.

The catalytic properties of the immobilized glucose oxidase showed characteristic changes in comparison with those of the soluble enzyme. The possible cause of the acidic shift in the optimum pH for the catalytic activity is an alteration in the number and/or character of the ionic amino acid side chains localized on the surface of enzyme molecules. Since the support is nonionic, the microenvironmental effects are considered to be less essential. The heat dependence of the activity is also changed in the case of the immobilized enzyme. Probably, the alteration is a complex function of the changes in the heat stability of enzyme and the activation energy of the catalytic reaction. The enzyme–substrate interaction was not profoundly altered by the immobilization.

The predicted stabilizing effect of polyacrylamide support, as a strongly hyrophylic microenvironment, was experienced in the case of the immobilized glucose oxidase, too.

The preliminary experiments testing the practical applicability of immobilized glucose oxidase indicated that it would be a useful tool for glucose determination in fluids of different origin. Considering the experimental errors, the performance of the glucose determination with immobilized glucose oxidase has to be improved. Such experiments are in progress.

REFERENCES

1. Inman, J. K. (1974), in *Methods in Enzymology*, 34, Jakoby, W. B., and Wilchek, M., eds., Academic, New York, San Francisco, London, pp. 30–58.
2. Kálmán, M., Szajáni, B., and Boross, L. (1983), *Appl. Biochem. Biotechnol.* **8,** 515.
3. Goldstein, L., and Katchalski—Katzir, E. (1976), in *Applied Biochemistry and Bioengineering*, **1,** Wingard, L. B., Katchalski-Katzir, E., and Goldstein, L., eds., Academic, New York, pp. 1–22.
4. Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
5. Schacterle, G. R., and Pollack, R. L. (1973), *Anal. Biochem.* **51,** 654.
6. Biochemica Information, Boehringer Mannheim GmbH (1973), **1** p. 97.
7. Hanes, C. S. (1932), *Biochem. J.* **26,** 1406.
8. Weston, P. D., and Avramess, S. (1971), *Biochem. Biophys. Res. Commun.* **45,** 1574.
9. Epton, R., Hibbert, B. L., and Thomas, T. H. (1976), in *Methods in Enzymology*, **44,** Jakoby, W. B., and Wilchek, M., eds., Academic, New York, San Francisco, London, pp. 84–107.
10. Brandt, J., Anderson, L.-O., and Porath, J. (1975), *Biochim. Biophys. Acta* **386,** 196.
11. Stambolieva, N., and Turková, J. (1980), *Collection Czechoslov. Chem. Commun.* **45,** 1137.