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Stabilization and Reutilization of *Bacillus megaterium* Glucose Dehydrogenase by Immobilization

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ABSTRACT

Glucose dehydrogenase (GDH) from *Bacillus megaterium* was immobilized using aminopropyl controlled-pore silica (CPS, average pore sizes of 170 and 500 Å) as a support and glutaraldehyde as a bifunctional crosslinking agent. The CPS-immobilized enzyme could be reused 12 times and the best results were obtained using aminopropyl CPS-500 and bovine serum albumin as a feeder for stabilizing the protein layer on the support. DEAE-Sephadex (A-25 and A-50) was also used as a support for immobilizing GDH, with yields of around 42% for A-25 and 25–30% for A-50. The effect of pH on the immobilization procedure showed pH 6.5 to be better than pH 7.5 with respect to the recovery of enzyme activity. Both preparations of DEAE-Sephadex immobilized GDH could be reused several times and were thermostable at 40°C for 7 h. The kinetic parameters as Michaelis constant and maximum rate were determined for the immobilized enzyme and compared with those for the freeform.

Index Entries: *Bacillus megaterium*; glucose dehydrogenase; aminopropyl controlled-pore silica; DEAE-Sephadex; immobilization.

INTRODUCTION

Glucose dehydrogenase (GDH) from *Bacillus megaterium* catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone using NAD^+ or $NADP^+$ as the coenzyme *(1)*. Coupling GDH and hydrogenase from *Pyrococcus*

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furiosus, which uses NADPH as an electron donor, may provide a convenient method for the biological production of hydrogen from glucose that in turn may derive from renewable sources as cellulose, starch, and lactose *(2)*. When adsorbed on metal hydrides, hydrogen may be used in refrigerators and air conditioners, as a coolant in place of freon *(3)*. The other product obtained in the GDH-catalyzed reaction, gluconic acid from the hydrolysis of D-glucono-1,5-lactone, is also a high value chemical for a variety of industries including alkaline detergents for metal cleaning, pulp and paper, textile, glass, and pharmaceuticals *(4)*. GDH is a tetrameric protein displaying molecular weight of 116,000 by gel permeation chromatography. In the presence of 0.1% sodium dodecylsulfate and 8*M* urea, the enzyme dissociates into four identical subunits, each one containing 262 amino acid residues *(5)*, with molecular weight around 30,000 as determined by dodecylsulfate gel electrophoresis. Unfolding of the enzyme in 8*M* urea is strongly inhibited by high concentrations of NaCl *(6)*. At 67 m*M* phosphate buffer, pH 6.5, in the presence of 3*M* NaCl, GDH shows optimal stability against thermal inactivation, and solutions of the enzyme may be stored for months at room temperature without loss of activity. The addition of polyethyleneimine (PEI), a water-soluble cationic polymer, to solutions of GDH at a molar ratio of PEI to GDH >10 also increases the thermal stability of GDH *(7)*. In presence of PEI, the rate for the GDH-catalyzed oxidation of β-D-glucose increases in a low concentration range of NAD^+ and $NADP^+$ suggesting that negatively charged GDH interacts with cationic polymers by electrostatic attraction and the negatively charged coenzymes are adsorbed by the polymers, resulting in enrichment of the coenzymes in the vicinity of GDH.

Enzymatic stability may sometimes be enhanced by immobilization of the enzyme onto a solid support, where it can be recovered and continuously reused. The easiest method for immobilizing proteins is by adsorption involving noncovalent interactions as ionic, metal bridge, and hydrophobic binding *(8)*. In this case, immobilization is normally achieved by simply incubating the support with a given amount of enzyme at a specific temperature, pH, and ionic strength. Alternatively, the proteins can be covalently attached to various support surfaces with a wide range of choices in selecting support materials and binding methods.

In this study, two methods for immobilizing GDH from *B. megaterium* were examined. Firstly, aminopropyl controlled-pore silica with average pore sizes of 170 and 500 Å was tested as an inorganic support, and glutaraldehyde was used as a bifunctional cross-linking agent in the covalent binding. Secondly, the anion exchanger DEAE-Sephadex was selected, that binds to the enzyme by electrostatic interactions. We compared the immobilization efficiencies for the covalent and non-covalent methods. Some enzymatic properties of the preparations and the possibility for reusing the immobilized biocatalyst are described and discussed.

MATERIALS AND METHODS

Materials

Glucose dehydrogenase (GDH, EC 1.1.1.47) from *Bacillus megaterium* (G-7653) with 250 U/mg protein (Biuret), β-nicotinamide adenine dinucleotide (β-NAD^+, 260-150, grade III), β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$, N-3886), bovine serum albumin (BSA, A-7638, essentially globulin free), glutaraldehyde (G-5882, grade I), aminopropyl controlled-pore silica (CPS, G-4643: average pore size = 500 Å, 200–400 mesh, amine content = 70 μmol/g silica and G-4518: average pore size = 170 Å, 200–400 mesh, amine content = 165 μmol/g silica) were purchased from Sigma Chemical Company, St. Louis, MO. DEAE-Sephadex A-50 (17-0180-01, useful MW range = 30,000–200,000) and A-25 (17-0170-01, useful MW range ≤30,000) were products from Pharmacia, Uppsala, Sweden. β-D-Glucose (34635) was obtained from Calbiochem, San Diego, CA and dye reagent concentrate (Coomassie Brilliant Blue G-250) for protein assay was from Bio-Rad Laboratories, Hercules, CA. All other reagents were of analytical grade.

Enzyme Assay

The enzyme activity was performed using β-D-glucose as substrate and NAD^+ or $NADP^+$ as coenzyme. Each assay contained 10 m*M* glucose and 1 m*M* $NADP^+$ or 2 m*M* NAD^+ prepared in 50 m*M* sodium phosphate buffer, pH 7.5. The reaction was initiated by the addition of enzyme (free or immobilized as indicated in each assay) and proceeded for 2 min or other indicated time, at room temperature (23°C). The production of NADH or NADPH was measured spectrophotometrically at 340 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that oxidizes 1 μmol of β-D-glucose to D-glucono-δ lactone per minute at pH 7.5 at 23°C.

Immobilization of GDH Using Controlled-Pore Silica (CPS)

Two types of aminopropyl-CPS with average pore sizes of 170 and 500 Å (50–100 mg) were activated by addition of 2.5% (v/v) glutaraldehyde (0.25–0.50 mL) in 0.1*M* phosphate buffer, pH 7.5, with stirring for 1 h at 23°C, and the excess glutaraldehyde was washed off with deionized water. Enzyme solutions with 0.54 g% BSA (49.045 U/100 mg support) or without BSA (5.586 U/50 mg support) and the activated supports were shaken at 4°C for 41 h or at 22°C for 1 h, respectively. The immobilized enzyme was then washed with 50 m*M* phosphate buffer, pH 7.5, containing 1*M* NaCl until no enzymatic activity could be detected in the washings. The preparations were tested for enzyme activity as above, and after each assay, the immobilized enzyme was centrifuged and washed with phosphate-NaCl buffer in order to remove the residual products and reassayed to determine enzyme stability and reusability.

Immobilization of GDH Using DEAE-Sephadex

Effect of pH in the immobilization procedure: Two samples of DEAE-Sephadex A-50 (10 mg) were equilibrated with 50 m*M* phosphate buffer, pH 6.5 and pH 7.5, before enzyme immobilization. Enzyme solutions (0.419 U) prepared in phosphate buffer, pH 6.5 and pH 7.5, and the support in a total volume of 1 mL were stirred at 4°C for 15 h and the excess enzyme was washed off with the respective buffer solutions. The preparations were assayed as described in the Enyzme Assay Section, and after each assay recovered, washed and reassayed.

Effect of the type of DEAE-Sephadex in the immobilization procedure: Two types of DEAE-Sephadex A-25 (100 mg) and A-50 (100 mg) were equilibrated in 50 m*M* phosphate buffer, pH 6.5, and enzyme solutions (5.226 U), prepared in the same buffer, were added to the supports. The mixtures were shaken at 4°C for 1 h (DEAE-Sephadex A-50) or 5 h (DEAE-Sephadex A-25), and the excess enzyme was washed off with 50 m*M* phosphate buffer, pH 6.5. The preparations, after lyophilization, were assayed, as described in Enzyme Assay Section, recovered, and reassayed.

Determination of Kinetic Parameters for Free and DEAE-Sephadex Immobilized GDH

The kinetic parameters, Michaelis constant and maximum rate, for free GDH were determined by measuring the reaction rate at substrate concentrations between 1–30 m*M* whereas for immobilized GDH, concentrations of β-D-glucose between 2–160 m*M* were used.

Stability at 40° for Free and DEAE-Sephadex Immobilized GDH

Free GDH solutions (10.36 μg/mL) prepared in 50 m*M* phosphate buffer, pH 6.5 with NaCl (0.0857*M*), and without NaCl (after rapid desalting using Sephadex G-25 M), were heated at 40°C for 30 min, 1, 3, 5, and 7 h prior to the enzymatic activity measurement at 23°C. For the thermal stability of DEAE-Sephadex A-25 and A-50 immobilized GDH, samples of each preparation (2 mg) were heated at 40°C for the aforementioned periods of time and then assayed for enzymatic activity.

RESULTS AND DISCUSSION

Table 1 summarizes the results for covalent immobilization of GDH using aminopropyl CPS. In this method, glutaraldehyde was used to activate the amino groups on CPS and then to cross-link the enzyme to the support. In all cases, the covalent attachment of GDH resulted in most of the activity being lost (≥98.8%). Two types of aminopropyl-CPS with average pore sizes of 170 and 500 Å were used for immobilizing GDH, and the best results were obtained using CPS-500 with respect to the recovery of total activity after immobilization. Concerning the conditions for

Table 1
Covalent Immobilization of GDH Using CPS[a]

Immobilized GDH	Activity added (U)	Activity in washings (U)	Immobilized activity (U)		Immobilization yield (%) [(B/A) x 100]
			Theoretical (A)	Actual (B)	
CPS-170 (100 mg) With BSA (0.54g%)	49.05	2.32 (4.73)	46.72 (95.27)	0.03 (0.06)	0.06
CPS-500 (100 mg) With BSA (0.54g%)	49.04	8.22 (16.76)	40.82 (83.24)	0.31 (0.62)	0.75
CPS-170 (50 mg) Without BSA	5.59	0.07 (1.32)	5.51 (98.68)	0.04 (0.72)	0.73
CPS-500 (50 mg) Without BSA	5.59	0.11 (1.93)	5.48 (98.07)	0.06 (1.15)	1.18

[a]CPS = Controlled-pore silica; BSA = Bovine serum albumin; The values in parentheses refer to the activity retention (%). The conditions for immobilization were: 50 m*M* phosphate buffer, pH 7.5, containing 1*M* NaCl, shaking at 4°C for 41 h (with BSA) or shaking at 22°C for 1 h (without BSA).

immobilization, a shorter time (1 h instead of 41 h) and smaller enzyme: support ratio (0.11 U/mg instead 0.49 U/mg) were preferable for obtaining best recovery. BSA was used as a feeder *(9)* for stabilizing the enzymatic protein layer on the support and, after repeated assays with CPS-immobilized GDH, it was observed that the enzyme could be reused twelve times without total loss of activity (Fig. 1). Although, GDH immobilized on CPS could be reused several times, the immobilization yield was very low possibly because of exposure of the enzyme to harsh environments or toxic reagents such as glutaraldehyde. This crosslinking agent could act on amino groups located at or close to the active site [e.g., His-148 and Lys-201 are involved in catalysis or binding of ligands *(5)*] and could hinder the conformational adaptation of the enzyme to the substrate resulting in an inactivation of the enzyme molecules *(9)*. The effect of pH on the immobilization of GDH using DEAE-Sephadex A-50 was examined in phosphate buffer solutions, pH 6.5 and pH 7.5. The reason for this is because pH 7.5 represents the optimum pH for determination of the GDH activity whereas pH 6.5 is more suitable for long-term storage of GDH (in presence of 3*M* NaCl) for months, without loss of activity *(6)*. When the immobilized preparations made at pH 6.5 and pH 7.5 were subjected to repeated assays, it could be noted that pH 6.5 was better than pH 7.5 with respect to the recovery of enzyme activity (Fig. 2). Thus, pH 6.5 was chosen

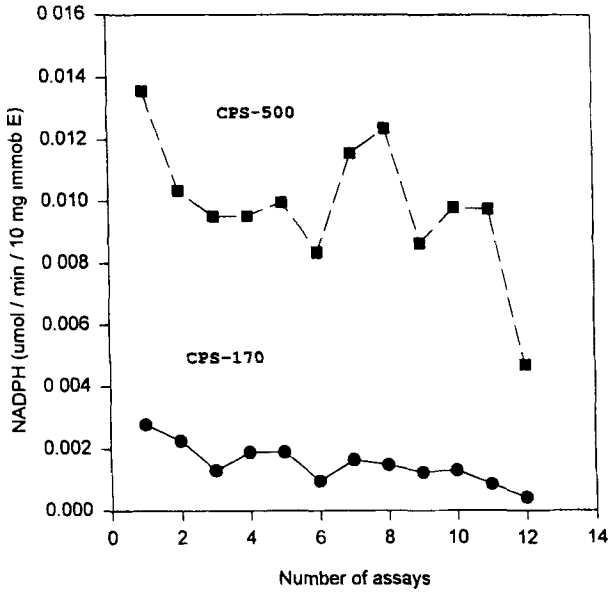

Fig. 1. Effect of repeated assays on the activity of CPS-170 and CPS-500 immobilized GDH/with BSA. The conditions for immobilization are in the footnote of Table 1. Incubation system: [glucose] = 10 μmol/mL, [NADP+] = 1 μmol/mL, [immobilized GDH] = 10 mg/mL, pH 7.5, room temperature, for 15 min.

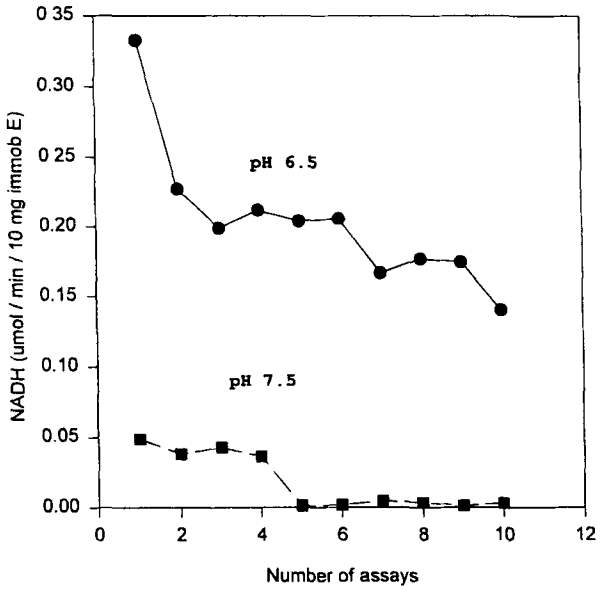

Fig. 2. Effect of repeated assays on the activity of DEAE-Sephadex A-50 immobilized GDH/pH 6.5 and pH 7.5. The conditions for immobilization were: 50 m*M* phosphate buffer, pH 6.5 or pH 7.5, shaking at 4°C for 15 h. Incubation system: [glucose] = 10 μmol/mL, [NAD+] = 2 μmol/mL, [GDH immobilized at pH 6.5] = 1.65 mg/mL, [GDH immobilized at pH 7.5] = 1.35 mg/mL, pH 7.5, room temperature for 2 min.

Table 2
Immobilization of GDH by Ionic Adsorption Using DEAE-Sephadex[a]

Immobilized GDH	Activity added (U)	Activity in washings (U)	Immobilized activity (U)		Immobilization yield (%) [(B/A) x 100]
			Theoretical (A)	Actual (B)	
DEAE-Sephadex A-25 (100 mg):					
Duplicate 1	5.23	0.00 (0)	5.23 (100)	2.23 (42.67)	42.67
Duplicate 2	5.23	0.00 (0)	5.23 (100)	2.22 (42.43)	42.43
DEAE-Sephadex A-50 (100 mg):					
Duplicate 1	5.23	1.49 (28.47)	3.74 (71.53)	0.96 (18.33)	25.63
Duplicate 2	5.23	1.50 (28.75)	3.72 (71.25)	1.14 (21.85)	30.67

[a]Actual activity was obtained after second reuse assay (*see* Figs. 3 and 4). The values in parentheses refer to the activity retention (%). The conditions for immobilization were: 50 m*M* phosphate buffer, pH 6.5, shaking at 4°C for 1 h (DEAE-Sephadex A-50) or 5 h (DEAE-Sephadex A-25).

for immobilizing GDH on DEAE-Sephadex. Two types of DEAE-Sephadex, namely A-25 and A-50, were tested as support in order to compare the immobilization efficiencies. The support A-25 presents useful MW range ≤30,000 and, in this case, GDH (MW = 116,000) is totally excluded from the gel while the support A-50 has useful MW range equal to 30,000–200,000 and there is some possibility for the inclusion of GDH inside the gel beads. Table 2 shows the results for immobilizing GDH on DEAE-Sephadex and the support A-25 was more efficient for obtaining superior activity recovery (42%) when compared to the support A-50 in which the recovery was around 25–30%. Both immobilized enzymes could be reused, as shown in Fig. 3 for support A-50 and Fig. 4 for support A-25, and both enzymes maintained their activities after several recycles. It is important to observe that the maximum activity for DEAE-Sephadex immobilized GDH, using $NADP^+$ as coenzyme (Figs. 3 and 4), was only obtained after the second reutilization assay. In comparison to the result shown in Fig. 2 using NAD^+ as cofactor, it is possible that the additional negative charge from phosphate group in the $NADP^+$ molecule plays an important role in its adsorption to the support. Indeed, NADPH was demonstrated to adsorb to DEAE-Sephadex (results not shown). It is possible, therefore, that the concentration of $NADP^+$ in the first use was effectively reduced in the assay by this nonspecific adsorption. In the second and subsequent assays, most if not all the $NADP^+$ would be available to the enzyme and not subject to further adsorption.

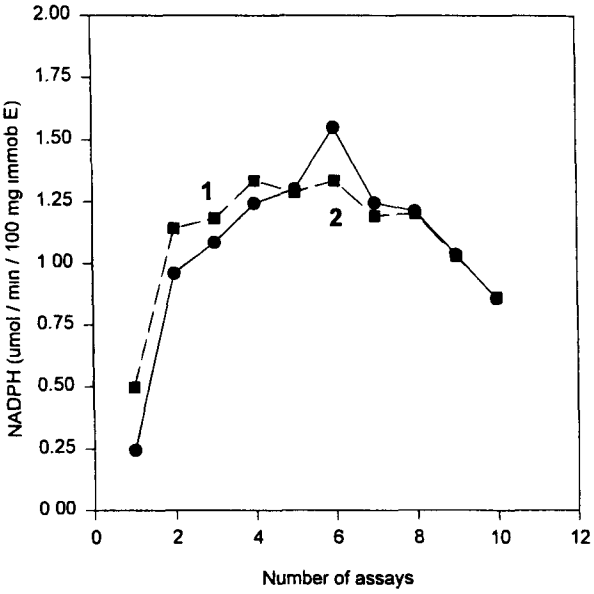

Fig. 3. Effect of the repeated use on the activity of DEAE-Sephadex A-50 immobilized GDH/pH 6.5. The conditions for immobilization were in the footnote of the Table 2. Incubation system: [glucose] = 10 μmol/mL, [NADP+] = 1 μmol/mL, [immobilized GDH] = 5 mg/mL, pH 7.5, room temperature for 2 min.

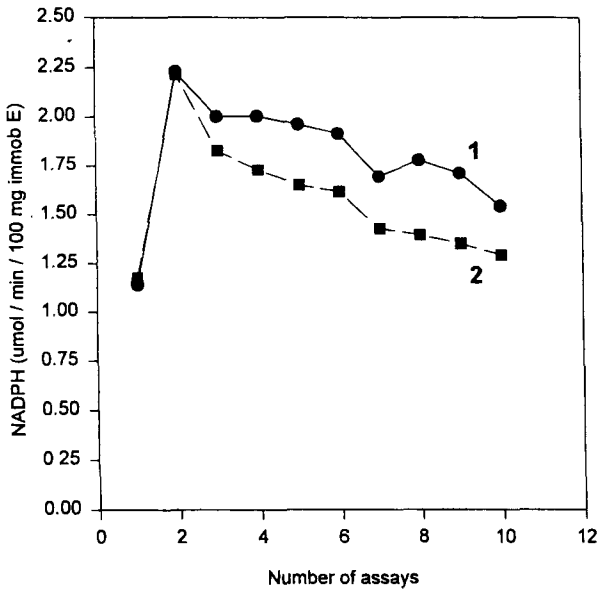

Fig. 4. Effect of the repeated use on the activity of DEAE-Sephadex A-25 immobilized GDH/pH 6.5. The conditions for immobilization were in the footnote of the Table 2. Incubation system = [glucose] = 10 μmol/mL, [NADP+] = 1 μmol/mL, [immobilized GDH] = 3.33 mg/mL, pH 7.5, room temperature for 2 min.

Table 3
Kinetic Parameters for Free and DEAE-Sephadex Immobilized GDH

Kinetic parameter	Free GDH	Immobilized GDH	
		DEAE-Sephadex A-25	DEAE-Sephadex A-50
Km(a)	3.83	3.84	12.11
Vmax	5.77(b)	2.60(c)	1.50(c)
r^2(d)	0.99	0.85	0.98

(a) Michaelis constant in m*M*.

(b) Maximum rate in μmol NADPH/min/0.2 mL (volume of free enzyme used in the immobilization procedure).

(c) Maximum rate in μmol NADPH/min/100 mg immobilized enzyme.

(d) Regression coefficient for Lineweaver–Burk plot.

The incubation conditions for assay with free enzyme were: [GDH]free = 0.36 μg/mL, [glucose] = 1–30 m*M*, [NADP+] = 1 m*M*, 50 m*M* phosphate buffer, pH 7.5, room temperature for 2 min whereas for immobilized enzyme were: [GDH]immobilized A-25 = 2 mg/mL or [GDH]immobilized A-50 = 1 mg/mL, [glucose] = 2–160 m*M*, [$NADP^+$] = 1 m*M*, in the same conditions of pH, temperature, and time.

The kinetic parameters for the preparations of DEAE-Sephadex immobilized GDH are shown in the Table 3 and the apparent *Km* for DEAE-Sephadex A-50 immobilized GDH was calculated as being 12.11 m*M*, which is higher than the *Km* value for free GDH. According to the literature *(7)*, *Km* values for free GDH at pH 7.0 and pH 7.8, using $NADP^+$ as cofactor, were respectively 2 m*M* and 4.55 m*M* and, in our procedure, the *Km* value at pH 7.5 was found as 3.83 m*M*. The apparent *Km* for DEAE-Sephadex A-25 immobilized GDH was 3.84 m*M* and it is similar to that for free GDH. The stability for GDH is an important study because it is a stable tetramer at pH 6.5 but is readily dissociated into four inactive protomers by shifting the pH to 8.5 *(6)*. Rapid reactivation can be achieved by readjustment to the original pH value. Adding NaCl to the GDH solutions and the presence of the coenzyme stabilize the quaternary structure of the enzyme. For thermal stability of free GDH at 40°C, the effect of NaCl can be observed in the Fig. 5. When the free enzyme was preheated at 40°C, without presence of NaCl, the enzyme activity dropped very quickly after heating of 30 min. If the enzyme solution contained NaCl (0.086*M*), it maintained its activity after heating at 40°C for 7 h. In the Fig. 6, the thermostability at 40°C for DEAE-Sephadex immobilized GDH was analyzed and after preheating for 7 h, the immobilized enzyme was thermostable without loss of activity. In this case, the presence of NaCl was not required for stability of GDH. It can be

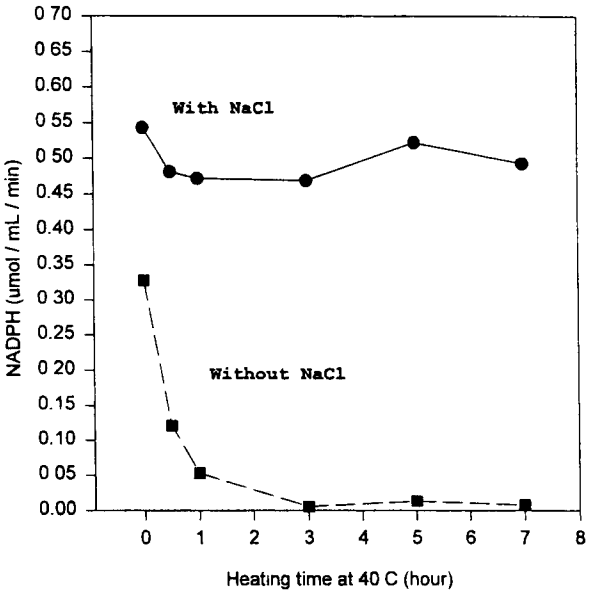

Fig. 5. Effect of sodium chloride for thermal stability of free GDH at 40°C. Incubation system: [glucose] = 10 μmol/mL, [NADP+] = 1 μmol/mL, [GDH solution, previously heated at 40°C, with NaCl (0.086*M*)] = 10.36 μg/mL, [GDH solution, previously heated at 40°C, without NaCl] = amount above after rapid desalting using Sephadex G-25 M, pH 7.5, room temperature for 2 min.

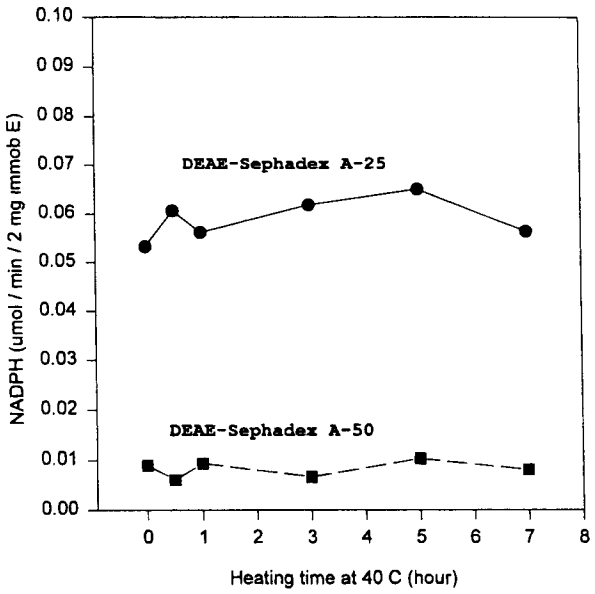

Fig. 6. Stability at 40°C for DEAE-Sephadex A-25 and A-50 immobilized GDH. Incubation system: [glucose] = 10 μmol/mL, [NADP+] = 1 μmol/mL, [immobilized GDH] = 2 mg/mL, pH 7.5, room temperature, for 2 min.

concluded that the immobilization of GDH using DEAE-Sephadex is a method to obtain stability of GDH that could be then reused several times with maintenance of its enzymatic activity. The stability of GDH is also necessary for coupling it with other enzymes as hydrogenase from *Pyrococcus furiosus (2)* since GDH is an useful enzyme for generating reduced coenzyme as NADPH, which in turn can be used as an electron donor for the hydrogenase activity in the production of hydrogen. Moreover, the coimmobilization of hydrogenase and GDH will also deserve attention in ongoing work in order to obtain an enzymatic system more stable than that formed with the free enzymes. The enzymatic pathway for the conversion of biomass-derived glucose to hydrogen is advantageous by requiring relatively mild conditions without the formation of waste gases as carbon dioxide and carbon monoxide when compared with other routes to hydrogen production as gasification, pyrolysis, and fermentation of biomass.

CONCLUSIONS

GDH from *Bacillus megaterium* was immobilized using aminopropyl controlled-pore silica as support and glutaraldehyde as bifunctional crosslinking agent. This method resulted in most of the activity being lost even so the immobilized enzyme activity was reusable. GDH immobilized on CPS-500 was reused twelve times without total loss of activity; BSA, as a feeder in the covalent interaction, stabilized the enzyme protein layer on the support. DEAE-Sephadex was also used as support for immobilizing GDH, through electrostatic binding, resulting in recovered activities around 42% (A-25) and 25–30% (A-50); pH 6.5 in the immobilization procedure was better than pH 7.5 with respect to the enzymatic activity obtained. The preparations of DEAE-Sephadex immobilized GDH could be reused several times with a good maintenance of activity and were thermostable at 40°C for 7 h.

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