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Development and characterization of an immobilized enzyme reactor based on glyceraldehyde-3-phosphate dehydrogenase for on-line enzymatic studies

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Abstract

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been extensively studied as a target for new drugs to be used in the treatment of various parasitic diseases. The standard approach to the determination of GAPDH activity utilizes solubilized free enzyme and is limited by the enzyme's low stability. In the current study the stability of GAPDH was significantly increased through the covalent immobilization of the enzyme on a wide-pore silica support containing glutaraldehyde (Glut-P). The optimal conditions for the immobilization were: 100 mg Glut-P stationary phase, ~150 μg of enzyme dissolved in pyrophosphate buffer (15 m*M*, pH 8.5). The mixture was gently agitated for 6 h at 4 °C. Under these conditions 91.3% of protein was immobilized on 100 mg of Glut-P support with retention of 2.97% of the initial enzymatic activity. The activity of the immobilized GAPDH was stable for over 30 days. The GAPDH–Glut-P stationary phase was packed into a glass column to produce a GAPDH immobilized enzyme reactor (GAPDH-IMER). The activity and kinetic parameters of the GAPDH-IMER were investigated and the results demonstrated that the enzyme retained its activity and sensitivity to the competitive inhibitor agaric acid.

Keywords: Immobilized enzymes; Kinetic studies; Enzymes; Glyceraldehyde-3-phosphate dehydrogenase

1. Introduction

The homotetramer D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a key enzyme involved in the glycolysis and gluconeogenesis pathways in bacteria and eukaryotes. The enzyme catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (D-GA3P) to produce 1,3-diphosphoglycerate (1,3-DPGA) in presence of NAD^+ and inorganic phosphate (Fig. 1) [1]. The currently accepted mechanism of reaction involves an initial oxidative acylation followed by phosphorylation with the concomitant production of NADH. A direct consequence of the enzymatic conversion is the production of ATP.

GAPDH plays a central role in controlling ATP production in pathogenic parasites such as *Trypanosoma cruzi*, the causative agent of Chagas' disease, *Trypanosoma brucei*, the causative agent of sleeping sickness, and *Leishmania mexicana* [2–4]. Based upon the enzyme's role in the life cycle of these parasites, GAPDH has become a key target for drug discovery. Although crystallographic studies

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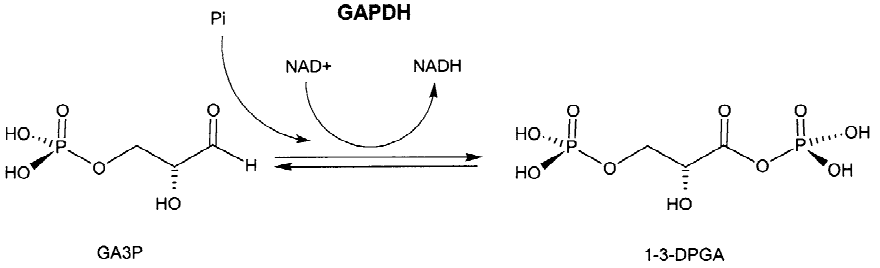

Fig. 1. Scheme of the reversible oxidative phosphorylation of D-glyceraldehyde-3-phosphate (D-GA3P) catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of NAD^+ and inorganic phosphate.

have indicated that the enzyme's structure has been highly conserved in bacterial and eukaryotic organisms [5], there are significant inter-species differences in GAPDH activity and sensitivity. For example, the active sites of GAPDH in *Trypanosoma cruzi* and humans differ by a substitution of Asp^{210} (*Trypanosoma cruzi)* by Leu^{194} (human) [6]. This difference creates the possibility that a *Trypanosoma cruzi*-specific GAPDH inhibitor could be developed to treat Chagas' disease [6].

GAPDH activity is usually determined by monitoring NADH formation at λ=340 nm [7,8]. However the utility of the standard methods are limited by the low stability of the enzyme in solution as previously reported by Ferdinand [9] and confirmed in our preliminary studies on free enzyme. When a cloned parasitic GAPDH is the target for drug discovery, the instability of the solubilized enzyme is a critical problem.

Enzymatic immobilization on a solid support is a technique that has been demonstrated to increase the stability of an enzyme. For example, the immobilization of brain glutamine synthetase on a liquid chromatographic stationary phase increased the stability of the enzyme from hours to weeks [10]. A widely used support for the immobilization of proteins and enzymes is a silica-based liquid chromatographic stationary phase containing glutaraldehyde (Glut-P) [11]. Glut-P is a wide-pore silica that has been covalently clad with a hydrophilic polymer (polyethylenimine) and then activated with glutaraldehyde. A Schiff base reaction is involved in the coupling of the ligand to the affinity medium. This support has been successfully used by Markoglou and Wainer to develop on-line immobilized enzyme reactors (IMERs) containing dopamine β-hydroxylase [12] and phenylethanolamine *N*-methyltransferase [13].

In the present study, GAPDH obtained from rabbit muscle was covalently immobilized on the Glut-P stationary phase. The immobilized GAPDH–Glut-P stationary phase was initially characterized using batchwise techniques and the immobilization procedure was optimized. The GAPDH–Glut-P was then packed into a chromatographic column to create a GAPDH-IMER. The GAPDH-IMER was placed in a liquid chromatographic system and on-line chromatographic studies were performed. The results of the study demonstrate that GAPDH was immobilized with retention of enzymatic activity and increased stability. The immobilized enzyme can be placed in an on-line system for the rapid screening of compounds for inhibitory activity.

2. Materials and methods

2.1. Materials

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) crystalline suspension derived from rabbit muscle, D,L-glyceraldehyde-3-phosphate free acid (GA3P), β-nicotinamide adenine dinucleotide (NAD^+), β-nicotinamide adenine dinucleotide reduced form (NADH) and agaric acid were purchased from Sigma (St. Louis, MO, USA). Buffer components and other chemical used during the immobilization procedure were of analytical grade and supplied by Sigma. Glutaraldehyde-P 40 μm affinity packing matrix was obtained from J.T. Baker (Phillipsburg, NJ, USA). The buffer solutions were filtered through cellulose nitrate membrane filters

(0.45 μm) purchased from Whatman International (Maidstone, UK) before their use for HPLC.

2.2. Apparatus

Spectrophotometric determinations were performed using a Shimadzu (Columbia, MD, USA) UV 160U spectrophotometer. The chromatographic experiments were carried out using a Thermo Separation Products P2000 pump, a Thermo Separation Products AS3000 autosampler equipped with a 100-μl loop, a Thermo Separation Products SpectraSystem UV 6000LP interfaced with a computer equipped with CHROMQUEST software version 2.51 for data collection (ThermoQuest, San Jose, CA, USA).

The chromatographic separations were performed connecting in series the GAPDH-IMER to a Chromolith SpeedROD RP 18e column (50×4.6 mm I.D.) supplied by Merck (Darmstadt, Germany).

2.3. Activity assay and kinetic studies on free enzyme

The activity of GAPDH was determined by measuring the formation of NADH [7,8]. One unit of enzymatic activity is defined as the amount catalysing the reduction of 1 μmol of NAD^+/min at 25 °C. The buffer used to measure the enzymatic activity (buffer A) contained triethanolamine (100 m*M*, pH 7.6), 1 m*M* dithiothreitol, 1 m*M* sodium azide, 5 m*M* magnesium sulfate, 1 m*M* EDTA, and 10 m*M* K_2HPO_4. The 1 ml reaction mixture contained: 780 μl of buffer A, 100 μl NAD^+ (2.0 m*M*, final concentration) and 100 μl GA3P (0.8 m*M*, final concentration). The reaction was initiated with the addition of the enzyme. The extent of the enzymatic conversion was monitored by following the increase in absorbance at λ=340 nm. Enzymatic activity was calculated from the initial slope of the curve obtained during the first minute of reaction. The reference sample contained only buffer A.

The dependence of enzymatic activity on NAD^+, GA3P and inorganic phosphate was also investigated. NAD^+ concentrations varied between 2.0 and 0.05 m*M* and the GA3P concentrations between 1.2 and 0.06 m*M* (corresponding to (D-GA3P) between 0.6 and 0.03 m*M*). The parameters for NAD^+ were determined under saturating conditions of GA3P (D-GA3P, 0.4 m*M*) and the parameters for GA3P were determined using saturating concentrations of NAD^+ (2.0 m*M*). Phosphate concentration was kept constant at 10 m*M*.

Data were plotted using Michaelis–Menten and Lineweaver–Burk plots to evaluate K_m and V_{max} [14].

2.4. Immobilization of GAPDH onto Glut-P

GAPDH was immobilized onto the Glut-P stationary phase using a modification of the procedure initially reported by Narayanam et al. [11]. Briefly, Glut-P stationary phase (50 or 100 mg) was placed in a 1.5-ml eppendorf tube and washed three times with 1 ml of the same buffer used for the solubilization of the enzyme (as described below, several buffers were tested during the optimization of the immobilization procedure). The washings were carried out by the addition of 1 ml of buffer to the packing material, the resulting mixture was vortex-mixed, centrifuged for 30 s at 7500 *g* and the supernatant was discarded. A GAPDH solution was prepared by dissolving 10 μl of crystalline suspension in 1 ml of buffer in order to obtain a solution containing 300 μg/ml of protein. A 0.5-ml aliquot (~150 μg of enzyme) of the solution was added to the washed matrix and gently agitated overnight at 4 °C. The mixture was then centrifuged and the supernatant was removed. The remaining solid support was washed three times with 1 ml aliquots of sodium pyrophosphate buffer (15 m*M*, pH 8.5). The amount of protein immobilized on Glut-P was determined by measuring the residual enzyme present in all supernatants using the Bio-Rad protein assay (Bio-Rad Labs., Hercules, CA, USA).

The Schiff bases formed during the immobilization procedure were reduced using 1.0 ml of sodium cyanoborohydride [100 m*M* in sodium pyrophosphate buffer (15 m*M*, pH 6.9)] [11]. The cyanoborohydride solution was added to the matrix and allowed to react for 1.5 h at 4 °C. The matrix was then washed with 1 ml pyrophosphate buffer (15 m*M*, pH 8.5) three times and then allowed to react at 4 °C for 1.5 h with 1.0 ml of monoethanolamine [200 m*M* in sodium pyrophosphate buffer (15 m*M*, pH 8.0)]. Finally, the matrix was washed three times with 1 ml of sodium pyrophosphate buffer (15 m*M*,

pH 8.0) and then washed twice with 1.5 ml of buffer A. The reacted matrix was kept at 4 °C in buffer A.

2.5. Optimization of immobilization procedure and conditions of storage

The relationship between reaction time and the amount of protein immobilized was determined by reacting 50 mg of Glut-P with 125–150 μg of enzyme at 4 °C for 18 h (overnight), 6 h and 3 h.

The effect of pH on the amount of GAPDH immobilized on the Glut-P was evaluated using 15 m*M* pyrophosphate buffers adjusted to pH 6.5, 7.0, 7.5, 8.0 and 8.5. The enzyme–matrix mixtures were gently agitated overnight at 4 °C. As a control, an immobilization was carried out using bidistilled water as a solvent for the GAPDH.

In order to determine the optimal conditions for storage of the GAPDH–Glut-P, the enzymatic stability of GAPDH was investigated after storage in several buffers. The buffers were: (a) sodium pyrophosphate buffer (15 m*M*, pH 7.5) containing sodium azide (1 m*M*); (b) pyrophosphate buffer (15 m*M*, pH 7.5) containing ascorbic acid (20 m*M*) and EDTA (1 m*M*); (c) buffer A (d) ammonium sulfate buffer (130 m*M*, pH 7.5) containing EDTA (1 m*M*) and 2-mercaptoethanol (20 m*M*). The enzymatic activity of GAPDH–Glut-P was evaluated each day in order to estimate the decrease of specific activity.

2.6. Activity assay and kinetic studies for GAPDH–Glut-P in batchwise

The enzymatic activity of GAPDH immobilized on Glut-P was evaluated adding NAD^+ and GA3P to GAPDH–Glut-P in buffer A (final volume 1.0 ml) in saturating concentrations of substrate and cofactor (final concentration 5.34 m*M* NAD^+, 4.8 m*M* GA3P). The mixture was then vortex-mixed for 1 min, centrifuged for 5 s at 7500 *g* and the supernatant was rapidly transferred into a 1-ml plastic cuvette. The UV spectra between $\lambda = 450$ and 300 nm were recorded and the absorbance at $\lambda = 340$ nm, corresponding to NADH formation, was used to evaluate the activity. The GAPDH–Glut-P was washed three times with 1 ml of buffer A between each experiment. The kinetic studies were carried out using NAD^+ concentrations ranging between 5.34 and 0.165 m*M* and GA3P concentrations ranging between 4.79 and 0.15 m*M*. The Michaelis–Menten constants K_m and V_{max} were calculated from the data using Lineweaver–Burk reciprocal plots [14].

2.7. Preparation of the GAPDH-IMER

A HR 5/2 glass column (5×25 mm) purchased from Pharmacia Biotech (Uppsala, Sweden) was used to prepare a GAPDH-IMER. The column was washed with bidistilled water followed by sodium pyrophosphate buffer (15 m*M*, pH 8.5) and then 10–20 ml of buffer A. A suspension containing 100 mg of GAPDH–Glut-P in buffer A was then added to the column. The top of the column was fastened and 20 ml of buffer A were pumped through the packed column at a flow-rate of 0.4 ml/min. The resulting GAPDH-IMER had a 10 mm×5 mm (I.D.) chromatographic bed and a volume of 0.785 ml.

2.8. Chromatographic procedures

The GAPDH-IMER was connected to a Thermo Separations HPLC system and covered with aluminum foil to reduce light-induced oxidative processes. In order to preserve enzymatic activity, the GAPDH-IMER was kept at 4 °C when not in use.

A mobile phase consisting of buffer A was utilized to perform the kinetic studies. In order to obtain the desired chromatographic separation of the products from the substrates a Cromolith SpeedROD RP 18e column was connected in series to the GAPDH-IMER. The NADH formation was quantified at $\lambda =$ 340 nm. The influence of flow-rate on enzyme activity was evaluated by injecting duplicate 20-μl aliquots of a solution containing NAD^+ (53.5 m*M*) and GA3P (48 m*M*) at flow-rates of 0.1, 0.2, 0.4, 0.6 and 0.8 ml/min. Kinetic studies were performed in order to determine saturating conditions for NAD^+, GA3P and inorganic phosphate. Solutions with NAD^+ concentrations ranging from 53.5 to 0.835 m*M* and GA3P concentrations between 96 and 1.5 m*M* were injected in duplicate. The parameters for NAD^+ were determined under saturating conditions of GA3P (96 m*M*) and the parameters for GA3P were determined at saturating concentrations of NAD^+ (53.5 m*M*). Since the concentration of phosphate ions also has a critical effect on the activity,

the mobile phase concentration of the dipotassium hydrogenphosphate varied between 2.5 and 100 m*M*.

2.9. Inhibitory assay for agaric acid

The effect of agaric acid on the enzymatic activity of the not-immobilized enzyme and the GAPDH–Glut-P was examined using off-line techniques. The reaction mixtures contained Buffer A, NAD^+ (2.0 m*M*) and GA3P (0.8 m*M*) (final concentrations) and agaric acid concentrations ranging from 360 to 7.2 μ*M*. Samples were incubated at room temperature for 5 min before adding GA3P and NAD^+ and starting the enzymatic reaction. The percent of remaining activity was calculated using the initial velocities measured from 0 to 1 min and by comparison with an inhibitor-free control experiment. IC_{50} values were extrapolated from the curve obtained by plotting percent of inhibition versus log (final concentration of the inhibitor).

The agaric acid inhibition of enzymatic activity in the GAPDH-IMER was investigated using a solution containing NAD^+ (53.5 m*M*), GA3P (96 m*M*) and agaric acid concentrations ranging from 2.06 to 4.13 m*M* and the optimized chromatographic parameters determined from the experiments described in Section 2.8. Each concentration was analyzed in duplicate. The percent of inhibition was calculated comparing the peak areas of NADH obtained in the absence and presence of inhibitor and the IC_{50} was extrapolated.

3. Results and discussion

3.1. Activity and kinetic studies of the soluble enzyme

The determination of the activity and kinetic parameters of free GAPDH was carried out in buffer A. NAD^+ and GA3P concentrations varied independently in order to ascertain the saturating conditions for substrate and cofactor. Under the experimental conditions, the saturating concentrations for NAD^+ and D,L-GA3P were 2.0 and 0.8 m*M*, respectively. The K_m and V_{max} values for NAD^+ and D-GA3P were determined plotting 1/activity versus 1/(substrate or cofactor) according to the Lineweaver–Burk approach [14]. Since only D-GA3P is the substrate for GAPDH, D,L-GA3P concentration was divided by 2 before being used in the calculations. Halved values were used in all calculations presented in this manuscript. The calculated values for NAD^+ and D-GA3P are presented in Table 1.

3.2. Optimization of the immobilization of GAPDH

Effect of the pH on the total amount of protein immobilized on the Glut-P and the residual activity of the GAPDH was investigated using 150±15 μg of GAPDH, 50 mg of Glut-P and 15 m*M* sodium pyrophosphate buffer with the pH adjusted to 6.5, 7.0, 7.5, 8.0, 8.5. The amount of protein immobilized on the Glut-P phase ranged from a low 85% (at pH 6.5) to 95% (at pH 8.0 and 8.5), Table 2 ($n=2$). The pH of the immobilization buffer did not appear to significantly affect the amount of protein immobilized.

In order to evaluate the effect of the pH of the immobilization buffer on the activity of the immobilized GAPDH, the enzymatic activities of the enzyme/buffer solutions were determined immediately before addition of the Glut-P stationary phase. Then, after completion of the immobilization process, the enzymatic activity of the GAPDH–Glut-P was determined. Both sets of experiments were carried out under saturating concentrations of substrate and cofactor. Under these conditions, the retained

Table 1
K_m and V_{max} calculated for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in solubilized (free GAPDH), immobilized (GAPDH–GLUT-P) and immobilized enzyme reactor (GAPDH-IMER) formats

	NAD^+	D-GA3P
Free GAPDH		
K_m (μmol/U)	9.84±1.18	9.06±0.52
V_{max} (μmol/min)	0.01942±0.0022	0.02408±0.0091
GAPDH–GLUT-P		
K_m (μmol/U)	5.79±0.37	5.55±0.83
V_{max} (μmol/min)	0.0912±0.0112	0.1039±0.0132
GAPDH-IMER		
K_m (μmol/U)	1.23±0.08	1.85±0.14
V_{max} (μmol/min)	0.0534±0.0021	0.0577±0.0029

Data reported are the mean of two independent measurements, each performed in duplicate.

Table 2
Effect of pH on the amount of protein immobilized and activity retained onto Glut-P; all the experiments were carried out in pyrophosphate buffer 15 m*M*

pH	% Protein immobilized	% Activity retained
6.5	85.2±0.5	0.69±0.06
7.0	91.3±0.8	0.78±0.01
7.5	93.3±1.2	0.83±0.01
8.0	95.1±0.4	0.87±0.04
8.5	95.1±0.1	0.90±0.06

Results represent the mean of duplicate experiments for each pH value.

GAPDH activity varied between 0.69±0.06% of the initial activity at pH 6.5 and 0.90±0.06% at pH 8.5, Table 2 ($n=2$). A comparison of the % activity retained/% protein immobilized versus the pH indicated that the residual activity retained onto the Glut-P stationary phase linearly increases with pH within the used buffer range. The optimum pH for the immobilization buffer was 8.5. Although <1% of the enzymatic activity was retained after immobilization, the GAPDH–Glut P stationary phase could be readily used to determined enzymatic activity and calculate the Michaelis–Menten kinetic parameters.

In the initial studies of the effect of pH on the immobilization of GAPDH, the enzyme solution and Glut-P stationary phase were gently agitated at 4 °C for 18 h. However, solubilized GAPDH rapidly loses activity and, therefore, the effect of time on the yield of the coupling reaction and the retained enzymatic activity was also investigated using the optimized buffer at pH 8.5. The immobilizations were carried out for 3, 6 and 18 h. The amount of initial protein immobilized was 72% after 3 h and 90% at 6 h and 18 h. On the basis of the results, the optimal conditions for the immobilization were set at buffer pH 8.5 and 6 h. Under these conditions 91.3% of protein was immobilized on 100 mg of Glut-P support and 2.97% of the initial enzymatic activity was retained. The low rate of activity retained onto the Glut-P is probably related to the the high enzyme instability in aqueous solutions [9] and the time needed for the binding reaction. Moreover, it should be considered that the biopolymer immobilization could modify the substrate access to the binding sites. However, the GAPDH–Glut-P obtained was used to pack a GAPDH-IMER as reported in Section 2.7, which, despite the low enzymatic activity, allowed several kinetic and inhibition studies to be performed for many weeks.

3.3. Enzymatic activity of the GAPDH–Glut-P

The Michaelis–Menten constants K_m and V_{max} were determined for the GAPDH–Glut-P using a batchwise approach. The results are presented in Table 1. Although the affinity (K_m) and kinetics (V_{max}) of the GAPDH–Glut-P stationary phase were higher for both NAD^+ and D-GA3P relative to the values determined for the free enzyme, there were no significant differences between the two forms of the enzyme.

3.4. On-line GAPDH-IMER chromatographic system

The enzymatic activity of GAPDH is monitored by following the conversion of NAD^+ to NADH. Thus, the ability to separate NAD^+ and NADH is a crucial element in the GAPDH-IMER chromatographic system. The chromatographic efficiency of the GAPDH-IMER was not sufficient to separate NAD^+ from NADH and the activity of the GAPDH-IMER could not be directly determined. In order to separate NAD^+ from NADH, a Chromolith SpeedROD RP 18e column was placed in-line after the GAPDH-IMER. This column was chosen because it had enough efficiency and selectivity to separate the analytes and a low backpressure, insuring that the immobilized enzyme was not unduly stressed. At a flow-rate of 0.6 ml/min, the retention factors (k') of NAD^+ and NADH were 0.67 and 2.27, respectively with a separation factor (α) of 3.4 (Fig. 2). The identity of the NADH peak was confirmed by injecting a NADH standard solution in the same chromatographic conditions and comparing retention times and spectra.

The effect of flow-rate on the production of NADH was determined using flow-rates from 0.1 to 0.8 ml/min, reflecting substrate–enzyme contact times ranging from 8 min to about 1 min, respectively. While the flow-rates between 0.1 and 0.4 ml/min produced the greatest amounts of NADH (Fig. 3)

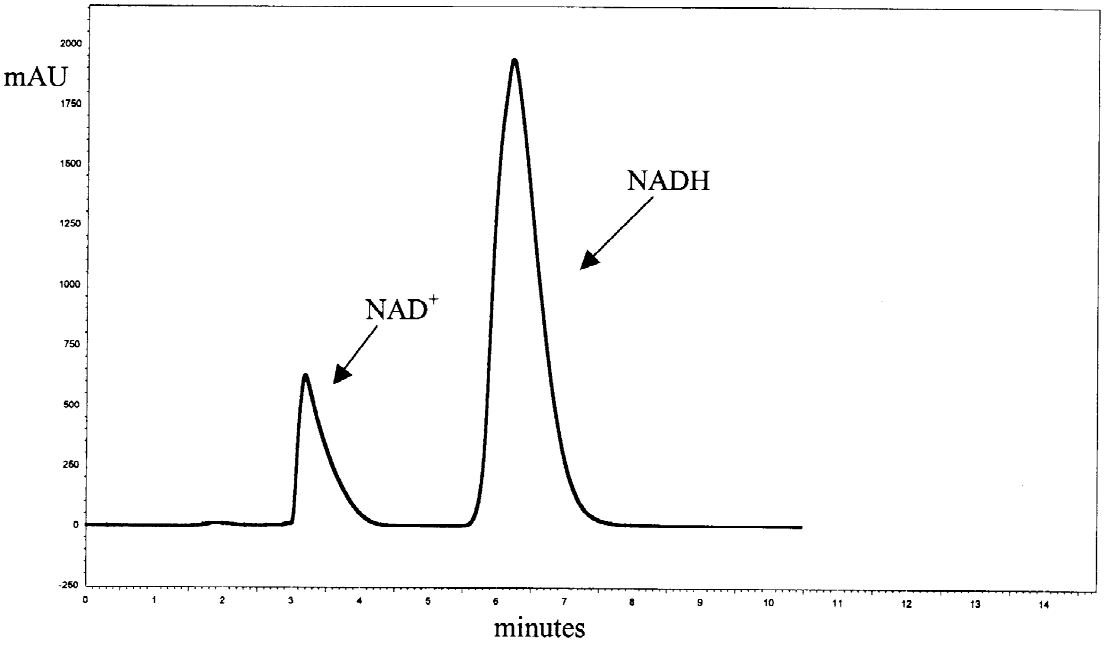

Fig. 2. Separation of NAD^+ from NADH by a Chromolith SpeedRod RP 18e column.in-line coupled to the GAPHD-IMER. Experimental conditions as described in the text. Mobile phase: buffer A; flow-rate was 0.4 ml/min. Detection was fixed at 340 nm.

they also produced the greatest variation in the areas of the NADH peaks; for example, an increase in flow-rate from 0.1 to 0.2 ml/min produced an over 200% decrease in the area of the NADH peak. Thus, slight fluctuations in pump speed would produce significant variations and error in kinetic studies. The relationship between the pump speed and the extent of NADH production was not as great for the 0.6 and 0.8 ml/min flow-rates (Fig. 3) and these flow-rates were easier for the pumping system to maintain. However, at the 0.8 ml/min flow-rate, NAD^+ and NADH peaks partially overlapped. Therefore, a flow-rate of 0.6 ml/min was used for all of the further studies on GAPDH-IMER system.

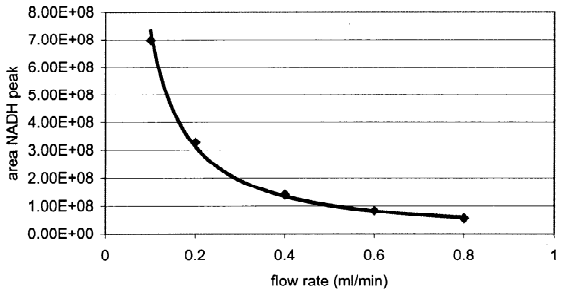

Fig. 3. Effect of flow-rate on the observed enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GADPH) determined as the peak area of the NADH produced during the oxidative phosphorylation of D-glyceraldehyde-3-phosphate.

GAPDH catalyzes the incorporation of inorganic phosphate into the GA3P molecule (Fig. 1). Being a substrate, the concentration of phosphate ions in the reaction buffer affects the observed enzymatic activity. The effect of phosphate ion concentration on NADH production was investigated by varying the phosphate ion concentration in the mobile phase from 2.5 to 100 m*M*. Saturation in the Michaelis–Menten plot of μmol NADH produced versus m*M* concentration of phosphate ions was reached at a 50 m*M* phosphate concentration (Fig. 4). This concentration was used in the following activity assays and kinetic studies.

3.5. Enzymatic activity of the GAPDH-IMER

The enzymatic activity of the GAPDH-IMER was determined by following the production of NADH. In order to accurately quantify the amount of NADH

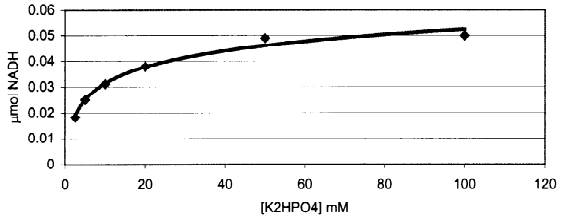

Fig. 4. Michaelis–Menten plot for the immobilized GAPDH as a function of (phosphate) in the mobile phase. Experimental conditions as described in the text.

produced by the on-line system, a calibration curve was obtained by injecting standard solutions of NADH onto the chromatographic system. The concentration of the NADH solutions ranged from 0.19 to 3.80 m*M* and the on-column concentrations (following 20-µl injections) ranged from 3.8 to 76.0 n*M*. The correlation between NADH peak areas and injected concentrations was linear ($y = 2^{e9}x + 2^{e6}$; $R^2 = 0.9979$).

The kinetic parameters of the GAPDH-IMER were determined by varying the substrate and cofactor concentrations. NADH production was correlated to the concentration of substrate or cofactor injected to obtain Michaelis–Menten plots for NAD^+ and D-GA3P, Fig. 5A and B respectively. Lineweaver–Burk reciprocal plots of 1/activity and 1/(substrate

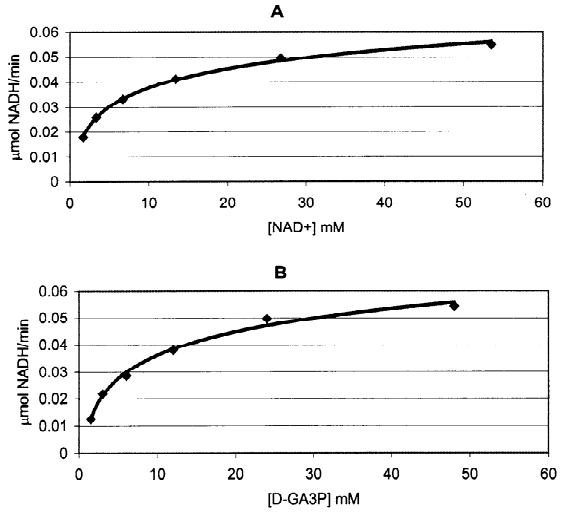

Fig. 5. Michaelis–Menten plots for immobilized GAPDH in the reactor format (GAPDH-IMER) as a function of (A) (NAD^+) and (B) (D-GA3P). Experimental conditions as described in the text.

or cofactor) allowed the estimation of the value of K_m and V_{max} for D-GA3P ($y = 63.435x + 18.717$; $R^2 = 0.9907$) and NAD^+ ($y = 92.35x + 17.316$; $R^2 = 0.9948$). The apparent affinities (K_m) of NAD^+ for GAPDH as free enzyme, in the Glut-P stationary phase and in the IMER format, respectively, were (from highest to lowest affinity): IMER > Glut-P > free enzyme. However, there was less than a 10-fold difference between the K_m value for the free enzyme (9.84 µmol/U) and the IMER (1.23 µmol/U). A similar trend was observed for the K_m values determined for the substrate D-GA3P (Table 1). It appears that placing the GAPDH in the IMER format does not significantly alter the enzyme's affinities for NAD^+ or D-GA3P.

When enzymatic conversion of NAD^+ to NADH, expressed as V_{max} (µmol/min), were compared the order from fastest to slowest was Glut-P > IMER > free (Table 1). As observed with the K_m values, there was less than a 10-fold difference between the V_{max} value for the free enzyme (0.0194 µmol/min) and the Glut-P (0.0912 µmol/min). The same trend was observed for the V_{max} values determined for the substrate D-GA3P (Table 1). It appears that placing the GAPDH in the IMER format does not significantly alter the enzyme activity.

3.6. Stability of immobilized and free enzyme

The stability of GAPDH was also examined for the free enzyme, GAPDH–Glut-P and for the GAPDH-IMER. The activity was evaluated in saturating concentrations of substrate and cofactor daily for GAPDH–Glut-P and every hour for the free enzyme.

The studies with the free enzyme confirmed the rapid inactivation of GAPDH upon diluition in water. During the first 4 h, the solution lost 30% of its activity and more than 50% after 1 day (data not shown). Ferdinand [9] demonstrated that only traces of heavy metals were needed to cause inactivation. The presence of a chelating agent can minimize the inactivation effect of heavy metals ions and the presence of a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol can protect the sulfhydryl groups involved in the enzymatic activity [15]. Buffer A contains 1 m*M* DTT and 1 m*M* EDTA and when used, it caused no significant decrease in GAPDH activity for at least 6 h.

The optimal conditions of storage for the immobilized GAPDH were investigated using GAPDH–Glut-P and several buffers. When the GAPDH–Glut-P was stored in sodium pyrophosphate buffer (pH 7.5) containing 1 m*M* sodium azide, GAPDH lost almost 90% of activity overnight (Fig. 6). With the addition of ascorbic acid as an antioxidant agent and EDTA as a chelating agent to the same buffer, up to 20% of the initial GAPDH activity was preserved for 5 days and there was detectable activity after 3 weeks. Additional studies were carried out using buffer A and a buffer composed of ammonium sulfate (130 m*M*, pH 7.5) containing EDTA (1 m*M*) and 2-mercaptoethanol (20 m*M*). The buffers were freshly prepared every day. Over 20% of the initial activity was retained up to 8–11 days and there was detectable enzymatic activity up to 1 month.

Based on the results, buffer A was chosen to store the GAPDH-IMER. Additional steps were also taken to preserve the enzymatic activity of the GAPDH-IMER. Since previous studies demonstrated that the specific activities of GAPDH measured in an oxygen-free atmosphere were the same as those measured in air [9], freshly deaerated solutions were used to perform HPLC experiments in order to minimize any on-line oxidative processes. The column was also kept covered with aluminum foil to avoid light-catalyzed reactions. Under these conditions, the stability of the GAPDH-IMER was significantly increased (Fig. 7). After an initial lost

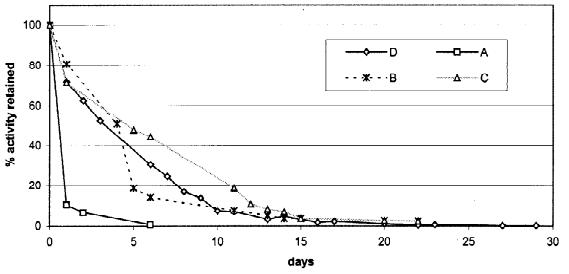

Fig. 6. Effect of various storage buffers on the stability of immobilized GAPDH. Buffers were: (A) 0.015 *M* sodium pyrophosphate buffer, pH 7.5, containing 1 m*M* sodium azide; (B) 0.015 *M* pyrophosphate buffer, pH 7.5, containing 0.02 *M* ascorbic acid and 1 m*M* EDTA; (C) 0.13 *M* ammonium sulfate buffer, pH 7.5, containing 1 m*M* EDTA and 0.02 *M* 2-mercaptoethanol; (D) 0.1 *M* triethanolamine–HCl, 1 m*M* sodium azide, 5 m*M* magnesium sulfate, 1 m*M* DTT, 1 m*M* EDTA.

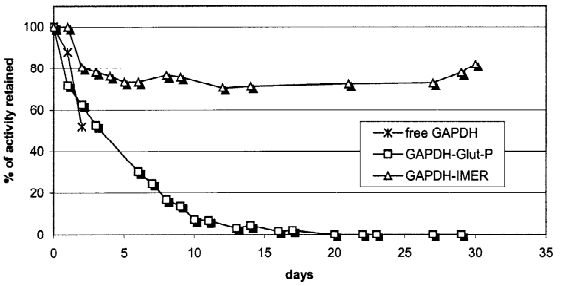

Fig. 7. Stability of free and immobilized glyceraldehyde-3-phosphate dehydrogenase during storage in 0.1 *M* triethanolamine–HCl, 1 m*M* sodium azide, 5 m*M* magnesium sulphate, 1 m*M* DTT, 1 m*M* EDTA and 10 m*M* K_2HPO_4.

of 20% of the enzymatic activity, GAPDH-IMER enzymatic activity remained almost unchanged for over 30 days and decreased 15% after 60 days. The inclusion of GAPDH in a chromatographic column, the addition of an antioxidant and chelating agent to the buffer and low temperature for the overnight storage were the necessary conditions to preserve the activity of immobilized GAPDH.

3.7. Effect of agaric acid on GAPDH activity

The sensitivity of the immobilized GAPDH in the GAPDH-IMER format to GAPDH inhibitors was examined using agaric acid. Agaric acid, a toxin in mushrooms poisoning is a well-known GAPDH inhibitor [16]. The IC_{50} value obtained on the GAPDH-IMER (103 μM) was compared with the value obtained for the free enzyme and was found to be consistent with it. The results indicate that the GAPDH-IMER could be used as an on-line screen for new GAPDH inhibitors.

4. Conclusions

The proposed immobilization procedure was found appropriate to covalently bind GAPDH to a modified silica matrix maintaining enzyme activity. Moreover, the inclusion of GAPDH–Glut-P in a chromatographic column preserved the enzymatic activity from inactivating processes, widely increasing the stability of the enzyme with advantages in term of accuracy and reproducibility. The same immobilization procedure can be potentially applied to prepare

IMERs containing GAPDHs from different sources such as human and parasite enzymes. Usual enzymatic assays do not allow the recovery of the enzyme for re-use. Considering the high cost and difficulty in overexpression, isolation and purification of GAPDHs from a parasitic source, this analytical technique represents an extremely useful approach to preserve the activity of the small amount of enzyme available, to perform kinetic studies and to rapidly screen for potential drugs candidates.

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