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The kinetic behavior of dehydrogenase enzymes in solution and immobilized onto nanostructured carbon platforms

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ABSTRACT

This paper describes the kinetic behavior of alcohol (ADH) and aldehyde (AldDH) dehydrogenases in solution and immobilized onto carbon platform via polyamidoamine (PAMAM) dendrimers. All the kinetic constants achieved for soluble ADH and AldDH are in agreement with literature data. The influence of pH and temperature was evaluated. Results showed that physiological conditions and ambient temperature can satisfactorily be applied to systems containing dehydrogenase enzymes, so as to ensure an environment where both ADH and AldDH display good activity. It is noteworthy that the affinity between both ADH and AldDH and their substrates and coenzyme is retained after the immobilization process. Investigation of the influence of the storage time demonstrated that there was no appreciable reduction in enzymatic activity for 50 days. Results showed that the PAMAM dendrimers provide a good environment for immobilization of dehydrogenase enzymes and that the affinity observed between the enzymes and their substrates and coenzymes seems to be retained, despite the considerable loss of enzymatic activity after immobilization. Furthermore, the anchoring methodology employed herein, namely layer-by-layer (LbL), required very low catalyst consumption.

1. Introduction

More than two hundred enzymes are known to catalyze reactions in which nicotinamide adenine dinucleotide hydrate (NAD^+) receives the hydride ion from a reduced substrate. The general nomenclature used for this kind of enzyme is oxidoreductase or dehydrogenase. Alcohol dehydrogenase (ADH) obtained from baker's yeast *Saccharomyces cerevisiae* was one of the first enzymes to be isolated and purified. It is classified as 1.1.1.1 and is part of a large family of dehydrogenase enzymes containing the element zinc in their structure. The primary structure of ADH consists of a tetramer formed by four identical subunits with a molecular weight of 36 KDa each [1]. ADH specificity is restricted to primary alcohols with linear aliphatic chains, and ethanol is by far the best substrate for this enzyme [2]. Several studies on the ADH steady-state kinetic mechanism have definitely shown that this enzyme follows a random mechanism and have also indicated that the crucial stage of the reaction is the dissociation of the NADH species from the formed complex [3–6].

The enzyme responsible for the second step of ethanol oxidation, aldehyde dehydrogenase (AldDH), is part of a set of three isoenzymes known as AldDH A, B, and C, also obtained from baker's yeast. AldDH has two identical subunits with a molecular weight of approximately 200 kDa, and it is classified as 1.2.1.5 [7]. AldDH is responsible for catalyzing the oxidation of aldehydes to carboxylic acids, and it displays activity for a wide variety of aliphatic, aromatic, and heterocyclic aldehydes [8]. The mechanism proposed for aldehyde oxidation involves a sequence of two substrates/two products, with formation of binary and ternary complexes, similar to the kinetic mechanism described for ADH.

Recently, there has been growing biotechnological interest in the use of immobilized enzymes, such as dehydrogenases, for many kinds of purposes; e.g., bioremediation, sensors, and biofuel cells. The presence of several functional groups on the protein structure allows one to employ different procedures for enzyme anchoring onto solid supports. Enzyme immobilization is generally accomplished by chemical or physical means. In the former case, covalent linkage and also the cross-linking process are utilized for binding the enzyme molecules. Sometimes, covalent binding is not necessary, so enzyme immobilization can be achieved by using membranes, adsorption processes, or entrapment into polymer gels and microcapsules. In most cases, the simplicity of the physical methodologies makes such processes advantageous

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over chemical methods [9]. One of the most important points to consider for enzyme anchoring onto solid platforms is enzyme stability, so it is very important to ensure that the enzyme is placed in a friendly environment, so that it can resist sudden changes in temperature, pH, and solution composition, which could inactivate the anchored enzymes. In this context, the choice of a suitable immobilization process for enzyme anchoring onto solid platforms is of great importance, because it directly affects the lifetime of the immobilized enzyme.

The PAMAM dendrimer represents a class of branched and monodisperse polymers. Unlike classical polymers, dendrimers exhibit larger uniformity, narrow molecular weight distribution, and highly functionalized terminal surface. Due to their organized structure and adsorption characteristics, dendrimers have been extensively exploited for production of film layers that can be used as sensors for detection of many different compounds [10]. Recent papers have described the viability of anchoring enzymes onto PAMAM dendrimers using the layer-by-layer technique. Perinotto et al. [11] have shown that ADH can be anchored with PAMAM onto Au electrodes, and that the resulting electrodes can be applied for ethanol detection with a detection limit of 1 ppm.

In this context, the study of the kinetic behavior of enzymes is very helpful for comparison of the effectiveness of the different methodologies employed for enzyme immobilization. Our group has evaluated two immobilization processes, namely LbL and passive adsorption technique, and has observed that the methodology employed for enzyme immobilization directly influences the enzymatic activity [12,13]. In fact, our previous work on enzymatic biofuel cells has demonstrated that the combination of the LbL technique with PAMAM dendrimers seems to be a better and more feasible way of anchoring enzymes onto carbon platforms, since good control of enzyme disposition onto the surface of the bioanode is obtained with very low enzyme consumption [13].

Despite the several literature studies on the structure and kinetic mechanism of dehydrogenase enzymes, there are few comparative studies of the kinetic behavior of immobilized enzymes. In this paper, the kinetic behavior of both ADH and AldDH dehydrogenase enzymes in solution and of the corresponding enzymes anchored onto carbon platforms using PAMAM dendrimers is investigated.

2. Materials and methods

2.1. Chemicals

All the reagents were analytical reagent grade and were used without further purification. The enzymes ADH (E.C. 1.1.1.1, initial activity of 331 U mg^{-1}) and AldDH (E.C. 1.2.1.5, initial activity of 1.02 U mg^{-1}), both obtained from *Saccharomyces cerevisiae* lyophilized powder, were purchased from Sigma–Aldrich and used as received. The coenzyme NAD^+ and the polyelectrolyte PAMAM generation 4 were also purchased from Sigma–Aldrich and used as received. All solutions were prepared with high-purity water from a Millipore Milli-Q system, and pH measurements were carried out with a pH electrode coupled to a Qualxtron model 8010 pH meter. All enzyme and coenzyme solutions were freshly prepared and rapidly used.

2.2. Enzyme immobilization

The enzymes were anchored onto a 1 cm^2 carbon platform (carbon fiber paper, TGP-H-060, Fuel Cell Earth, Stoneham, MA) with a homemade gas diffusion layer specifically designed to have low Teflon® content [13]. The choice of support was made so that two goals would be achieved, namely a support with the hydrophilicity required for the LbL process and increase in the diffusional limits on the bioanode surface. In fact, the presence of a gas diffusion layer tends to increase the total surface area through formation of a more disperse three-dimensional structure, thus providing sufficient uniformity and enough porosity that culminate in enhanced kinetics of the substrates and co-enzymes in terms of reaching the active center of the enzyme.

There are several studies employing NAD^+-dependent enzymes, such as amperometric sensors and biofuel cells [12–19]. In these devices, the regeneration of the coenzyme from its reduced form is quite important, and this process requires the use of an electrocatalyst because of the high overpotential of the reaction. Hence, in order to keep the same architecture of the generally employed bioanodes, a stable methylene green film was electropolymerized at the carbon support before the immobilization step [18].

Fig. 1. Mechanism for the two-step oxidation of ethanol to acetate catalyzed by ADH and AldDH, with concomitant NAD^+ consumption.

After formation of poly(methylene green), enzyme immobilization was performed by anchoring the dehydrogenase enzymes onto both sides of a 1 cm^2 Toray® paper (pretreated with nitric acid, in order to enhance the hydrophilicity of the surface) using the LbL technique [13]. Briefly, sample preparation was carried out by immersing the substrate into the PAMAM solution (2 mg mL^{-1}) for 5 min, followed by immersion onto the enzyme solutions (1 mg mL^{-1}) for 15 min. The substrates were rinsed with the buffer solution after each deposition, followed by drying [13].

2.3. Determination of enzymatic activity by the continuous method

The two-step oxidation of ethanol to acetate catalyzed by ADH and AldDH occurs with concomitant NAD^+ consumption (Fig. 1). So, the substrate hydrolysis activity of dehydrogenase enzymes was investigated at 25 °C, by folowing the reduction of NAD^+ to NADH at 340 nm ($\varepsilon_{340\,nm,\,pH\,7.5}$ = 6.220 L mol^{-1} cm^{-1}) in a UV/vis spectrophotometer Ultrospec 5300 pro from Amersham Biosciences, using thermostatic quartz cells of 1 cm path length. The assays were accomplished in phosphate buffer, pH 7.2, to a final volume of 1 mL. The reaction was initiated by addition of the soluble enzymes or the substrate containing the immobilized proteins, depending on the study that was being performed. Enzymatic activity was determined by quantification of NADH formation, as measured by the increase in absorbance at 340 nm. The absorbances were recorded for 5 min (with an interval of 2 s between each measurement), and the initial velocity was calculated by linear regression during the first 2–3 min of reaction. Assays were conducted in triplicate, and controls without added enzyme were included in each experiment, to quantify the non-enzymatic hydrolysis of the substrate. One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1.0 μmol of substrate per minute at 25 °C.

K_m (Michaelis–Menten constant) and V_{max} (maximum velocity) for substrate and coenzyme were obtained from substrate hydrolysis and were calculated using the Lineweaver–Burk plot [20]. Data are reported as the mean ± S.D. of triplicate measurements, which were considered to be statistically significant at $P \leq 0.05$.

2.4. Effect of pH and temperature on enzymatic activity

The influence of both pH and temperature on the kinetics of the enzymes was determined by assaying enzymatic activity from 15 to 55 °C at various pH levels between 4 and 10. To this end, the following 0.1 mol L^{-1} buffer solutions were used: acetate buffer (NaAc/HAc) for pH 4–5; phosphate buffer (NaH_2PO_4/Na_2HPO_4) for pH 6–7; tris-hydroxymethyl amino methane–HCl ($Tris^+$) buffer for pH 8–10.

3. Results and discussion

Once most studies aim at obtaining high enzymatic activity and enhanced lifetime for anchored enzymes, evaluating and understanding all the parameters influencing the kinetic behavior of dehydrogenase enzymes is very important. Therefore, evaluation of how the amount of enzyme, substrate, and coenzyme influences the enzymatic activity was carried out by always having the kinetic parameters of the enzymes in solution as reference values.

The initial kinetic results obtained after the immobilization process curiously showed that the anchored enzymes apparently displayed enzymatic activity during only one cycle; i.e., the immobilized enzyme had no significant activity after the first kinetic assay. This is because the presence of dendrimers combined with enzymes and NAD^+ species on the carbon platforms probably imposed some diffusional limits, thereby hindering flow of the reduced form of the coenzyme from the dendrimers to the bulk solution. Due to this diffusional obstruction, it seems that the enzymatic activity of the anchored enzymes is lost after the first assay; however, if the NADH species formed during the catalytic reaction are forced to leave the active site, the system can be regenerated. For this reason, it was necessary to design a reproducible method and a reliable system, to ensure NADH species removal from the anchored

Table 1
Substrate kinetic parameters for both dehydrogenase enzymes in solution and immobilized onto a carbon platform.

Enzyme		Parameters			
		K_m (mmol L^{-1})	V_{max} (μmol NADH min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
ADH	Soluble	18.2 ± 0.1	69.4 ± 0.1	172.6 ± 0.1	9500 ± 10
	Immobilized	17.9 ± 0.1	0.45 ± 0.01	1.07 ± 0.02	59 ± 1
AldDH	Soluble	$17 \times 10^{-3} \pm 1$	24.1 ± 0.2	80.3 ± 0.2	$4.7 \times 10^{6} \pm 2$
	Immobilized	$16 \times 10^{-3} \pm 1$	0.13 ± 0.001	0.43 ± 0.001	$26.8 \times 10^{3} \pm 0.1$

enzymes in all the assays. So, the supported enzymes were regenerated by applying a potential of 0.3 V (close to the NADH oxidation potential) in a Potentiostat/Galvanostat Model 273A – PAR for a few minutes after each assay, which forced the NADH species to leave the PAMAM dendrimers.

3.1. ADH kinetic behavior

Before performing experiments as a function of the substrate and coenzyme concentration, the influence of the amount of ADH on the enzymatic kinetics in solution was evaluated in the 0.01–0.8 U range. The results evidenced that there is a linear influence of the ADH load on the initial reaction rate up to 0.2 U, followed by formation of a plateau; thereafter, this behavior remained constant up to 0.8 U ADH (data not shown). In the case of the anchored enzymes, the influence of the amount of ADH on the enzymatic kinetics was evaluated in the range 1–36 ADH bilayers. The mass of enzyme deposited per bilayer was about 95 ng cm^{-2}, which represented approximately 1.13 U ADH [13]. The results revealed that the ADH load directly influences the reaction kinetics with loss of linearity above ca. 12 bilayers and subsequent formation of a plateau [13]. This result indicates that a significant enzymatic activity on the carbon support is obtained only when a high amount of enzyme is added, (above 1 U ADH). Although above 12 bilayers the amount of enzyme does not significantly influence the kinetics, the value of 36 bilayers was chosen for subsequent experiments due to the greater stability of this sample.

For investigation of the influence of ethanol concentration on the activity of ADH in solution, the quantity of substrate was varied from 0.5 to 500 mmol L^{-1}, while the enzyme load and the co-enzyme concentration were kept at 0.2 U and 1.9 mmol L^{-1}, respectively. At low substrate concentrations, there was a linear increase in the rate of NADH formation; thereafter, the NADH conversion rate became constant, leading to a typical equilateral hyperbola curve. From the obtained double reciprocal graph, both K_m and V_{max} were determined (18.2 ± 0.1 mmol L^{-1} and 69.4 ± 0.1 μmol NADH min^{-1} mg^{-1}, respectively). In addition, the K_{cat} constant was 172.6 ± 0.1 s^{-1}, and the K_{cat}/K_m ratio was 9500 ± 10 mol L^{-1} s^{-1} (see Table 1).

To evaluate the effect of the coenzyme concentration on the kinetics of ADH in solution, experiments were performed by varying the amount of NAD^+ from 0.12 to 7.6×10^{-3} mol L^{-1}. The final concentration of reagents was kept at 0.2 U ADH, 0.1 mol L^{-1} EtOH, pH 7.2. Fig. 2 shows that the expected equilateral hyperbola curve occurred only up to 2.6×10^{-3} mol L^{-1} NAD^+. After this point, there was a decrease in enzymatic activity. The obtained profile suggests possible inhibition due to excess substrate. This behavior is supported by the ADH kinetic mechanism, which indicates that the crucial stage of the process is dissociation of the NADH species from the formed complex. In fact, at high NAD^+ concentration, there might be a competition between the formed NADH and the NAD^+ species in solution, since both compete for the active site of the enzyme. This is an important result that must be considered for maximization of the performance of the enzymatic system both in solution and anchored onto solid platforms. The kinetic constant values K_m and V_{max} were determined from the double reciprocal graph plotted in the region in which there was no inhibition (inset of Fig. 2), and values of 0.14 ± 0.01 mmol L^{-1} and 70.7 ± 0.2 μmol NADH min^{-1} mg^{-1} were obtained, respectively. K_{cat} was 176.7 ± 0.2 s^{-1} and K_{cat}/K_m was $1.3 \times 10^{6} \pm 200$ mol L^{-1} s^{-1} (see Table 1).

Comparing the results obtained for the kinetic parameters of both substrate and coenzyme in this paper with literature data, it can be inferred that all the kinetic constants for soluble ADH presented here are in agreement with previously published works [3,4,21,22]. Also, the huge difference in the K_m data for ethanol and NAD^+ corroborated with the ADH structure, in which the NAD^+ binding site is easily available to the solution while the substrate binding site is quite narrow and almost inaccessible to the solution, thus hindering the access of ethanol to the ADH active site [1].

In order to obtain both K_m and V_{max} for the anchored ADH, assays as a function of ethanol and NAD^+ concentration were performed by employing the same conditions used in the experiments with the enzyme ADH in solution. Considering the substrate variation, the values determined for K_m and V_{max} were 17.9 ± 0.1 mmol L^{-1} and 0.45 ± 0.01 μmol NADH min^{-1} mg^{-1}, respectively. Results as a function of coenzyme concentration (Fig. 2) furnish values of 0.15 ± 0.01 mmol L^{-1} and 0.46 ± 0.01 μmol NADH min^{-1} mg^{-1}, for K_m and V_{max}, respectively. Comparison between the kinetic data obtained for ADH in solution and results achieved with the anchored enzymes, demonstrate that, although there is considerable loss of enzymatic activity after immobilization, the affinity between the ADH molecules and the substrate and coenzyme is retained.

In order to evaluate the stability of the anchored enzymes as a function of time, kinetic assays were performed for 90 days, using the same sample with 36 ADH bilayers. The results evidenced that there was no appreciable enzymatic activity reduction for 30 days (Fig. 3).

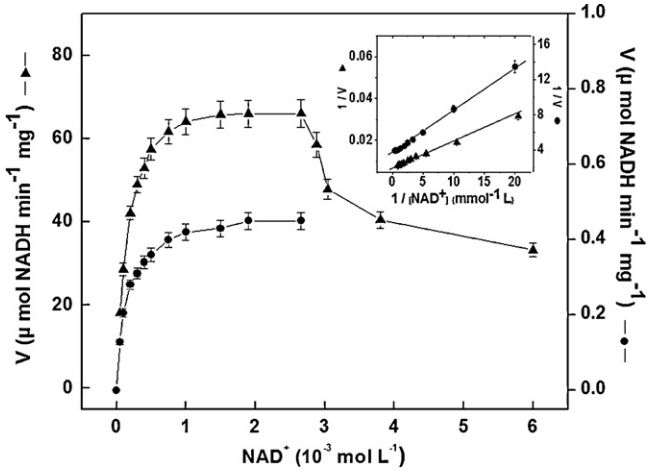

Fig. 2. NADH conversion rate as a function of the coenzyme concentration. (▲) ADH in solution, 0.2 U; (●) immobilized ADH, 36 bilayers, 0.1 mol L^{-1} EtOH, phosphate buffer, pH 7.2. Inset: double reciprocal graph obtained for (▲) soluble ADH and (●) immobilized enzyme.

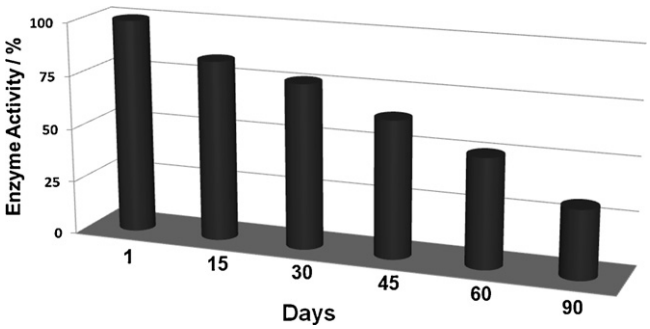

Fig. 3. Enzymatic activity of anchored ADH as a function of time. 36 bilayers of immobilized ADH, 0.1 mol L^{-1} EtOH, phosphate buffer, pH 7.2.

3.2. AldDH kinetic behavior

Prior to the assays as a function of substrate and coenzyme concentrations, the influence of the presence of potassium ions and thiols was evaluated. This is important to evaluate a wide range of parameters that could influence the final performance of the immobilized enzymes. Our results gave evidence that the yeast enzyme is highly dependent on both species (Fig. 4). The assays were carried out as a function of the potassium concentration from 10 to 100 mmol L^{-1}, and as a function of 2-mercaptoethanol concentration from 1 to 10 mmol L^{-1}. The best results were achieved in the assays employing 100 and 10 mmol L^{-1} potassium ions and 2-mercaptoethanol, respectively. These data reveal that, besides anchoring the enzyme properly, it is important to control other experimental parameters, so that a good final performance is achieved.

Once the assay conditions were established, the influence of the amount of AldDH on the enzymatic kinetics in solution was evaluated in the 0.025–0.1 U range. In a final volume of 1 mL, the following conditions were maintained: acetaldehyde 0.5×10^{-3} mol L^{-1}, 1.67×10^{-3} mol L^{-1} NAD^+, 0.1 mol $\times L^{-1}$ KCl, and 2-mercaptoethanol 0.01×10^{-3} mol L^{-1}. There was a linear increase in the initial rate, which was followed by formation of a plateau up to 0.05 U AldDH (data not shown). Similarly to the results obtained with ADH, the assays as a function of the load of AldDH anchored by the LbL technique showed that considerable enzymatic activity was achieved up to 20 bilayers only. The same architecture employed in the study on ADH (carbon supports containing 36 AldDH bilayers) was utilized in all the subsequent assays.

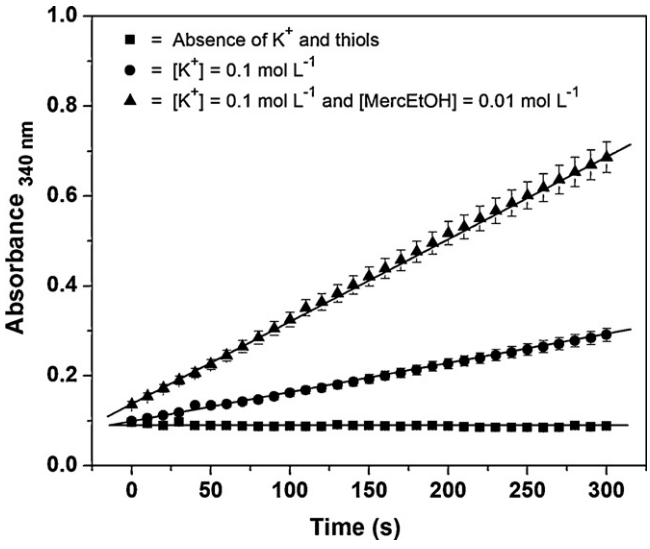

Fig. 4. Influence of the presence of K^+ ions (0.1 mol L^{-1}) and thiols (0.01 mol L^{-1}) on AldDH activity in solution. 0.05 U AldDH, 0.5×10^{-3} mol L^{-1} acetaldehyde, 1.67×10^{-3} mol L^{-1} NAD^+ in phosphate buffer, pH 7.2.

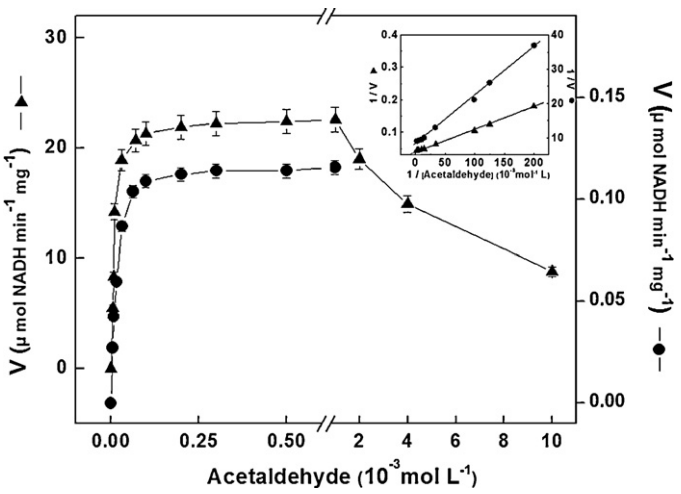

Fig. 5. NADH conversion rate as a function of the acetaldehyde concentration. (▲) AldDH in solution, 0.05 U; (●) immobilized AldDH, 0.8 U. 1.67×10^{-3} mol L^{-1} NAD^+, KCl 0.1 mol L^{-1}, 2-mercaptoethanol 0.01 mol L^{-1}, pH 7.2. Inset: double reciprocal graph obtained for (▲) soluble AldDH and (●) immobilized enzyme.

To evaluate the effect of coenzyme concentration on AldDH kinetics in solution, experiments were performed by varying the amount of NAD^+ from 0.1 to 5×10^{-3} mol L^{-1}. The final concentration of reagents was kept at 0.05 U AldDH, 0.5×10^{-3} mol L^{-1} acetaldehyde, 0.1 mol L^{-1} KCl, and 0.01 mol L^{-1} 2-mercaptoethanol, pH 7.2. Contrary to the results obtained with ADH, data as a function of the NAD^+ concentration demonstrated that no inhibition process occurred in the evaluated concentration range. Indeed, the expected kinetic behavior was verified; i.e., the initial rate increased linearly at low NAD^+ concentrations, followed by formation of a plateau, which resulted in a typical equilateral hyperbola curve. From the obtained double reciprocal graph, K_m and V_{max} were determined as 0.41 ± 0.01 mmol L^{-1} and 23.2 ± 0.1 μmol NADH min^{-1} mg^{-1}, respectively. The K_{cat} constant was 77.3 ± 0.1 s^{-1} and the K_{cat}/K_m ratio was $1.9 \times 10^5 \pm 10$ mol L^{-1} s^{-1} (see Table 1).

For the AldDH in solution, the assays as a function of substrate concentration were performed by varying the amount of acetaldehyde from 0.005 to 10×10^{-3} mol L^{-1}. The final concentration of reagents was kept at 0.05 U AldDH, 1.67×10^{-3} mol L^{-1} NAD^+, 0.1 mol L^{-1} KCl and 0.01 mol L^{-1} 2-mercaptoethanol, pH 7.2. The results from Fig. 5 show that at relatively low acetaldehyde concentrations, the rate of NADH formation follows the typical equilateral hyperbola curve. Thereafter (above 1×10^{-3} mol L^{-1}), there is a sharp decrease in the initial NADH conversion rate, suggesting another possible inhibition due to excess substrate. It is noteworthy that acetaldehyde molecules can also bind to the formed ternary complex, thus diminishing the enzymatic activity [23]. By tracing the double reciprocal graph (inset of Fig. 5) in the region in which there was no inhibition, the kinetic constants K_m and V_{max}, were determined as 16.9 ± 0.2 μmol L^{-1} and 24.1 ± 0.2 μmol NADH min^{-1} mg^{-1}, respectively. The K_{cat} constant was 80.3 ± 0.2 s^{-1} and the K_{cat}/K_m ratio was $4.7 \times 10^6 \pm 200$ mol L^{-1} s^{-1} (see Table 1).

The kinetic parameters obtained in this work for AldDH in solution are in agreement with literature reports, for both substrate and coenzyme [8,24,25]. In addition, the results clearly show the great affinity between enzyme/substrate as well as the very easy access of aldehyde molecules to the active site, as compared to the interaction AldDH/NAD^+.

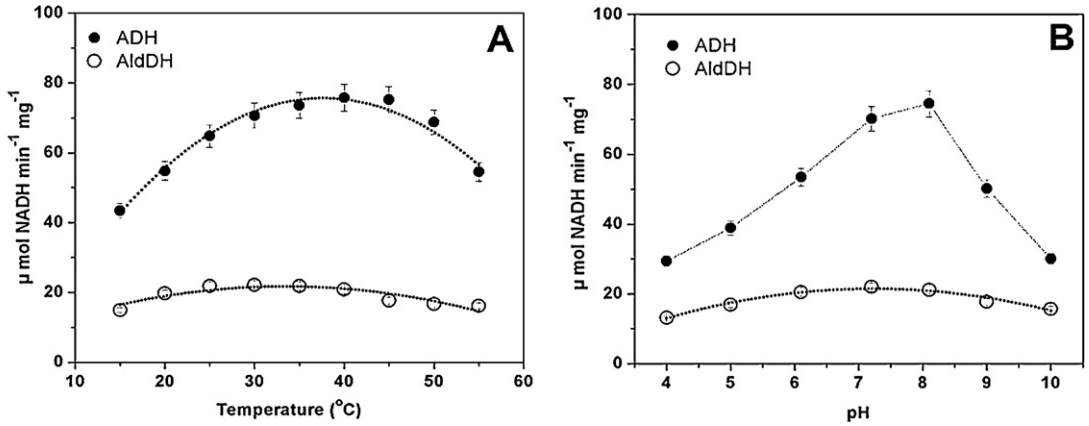

Fig. 6. Influence of temperature (A) and pH (B) on the enzymatic activity of ADH and AldDH in solution. (●) ADH 0.2 U, 0.1 mol L^{-1} EtOH, 1.67×10^{-3} mol L^{-1} NAD^+, phosphate buffer, pH 7.2. (○) AldDH 0.05 U, 1.67×10^{-3} mol L^{-1} NAD^+, KCl 0.1 mol L^{-1}, 2-mercaptoethanol 0.01 mol L^{-1}.

By keeping the same assay conditions described in the study using soluble AldDH, both K_m and V_{max} were determined for the anchored enzyme as a function of acetaldehyde (Fig. 5) and NAD^+ concentrations. Once again, the kinetic values indicated that, despite the large decrease in enzymatic activity after the immobilization process, the affinity between the anchored enzyme and its substrate and coenzyme was preserved. The values determined for K_m and V_{max} from the substrate variation assay were 16.3 ± 0.1 μmol L^{-1} and 0.13 ± 0.01 μmol NADH min^{-1} mg^{-1}, respectively. As for the coenzyme assays, K_m and V_{max} were 0.53 ± 0.01 mmol L^{-1} and 0.12 ± 0.01 μmol NADH min^{-1} mg^{-1}.

Table 1 summarizes all the kinetic parameters determined for both dehydrogenase enzymes in solution and anchored onto carbon platform.

3.3. Influence of temperature and pH on the enzymatic activity

Fig. 6 depicts the kinetic behavior of both dehydrogenase enzymes in solution as a function of pH and temperature. The obtained curves allowed determination of the optimum temperature (Fig. 6A) and pH (Fig. 6B) for ADH and AldDH. The highest activity was achieved in the 7.0–8.0 pH range for ADH, and around 7.0 for AldDH. The optimum temperature for ADH lay between 35 and 40 °C and at about 35 °C for AldDH.

The results in terms of pH and temperature coincide with data reported in the literature for both enzymes in solution [24,26,27]. Moreover, the results also show that, in order to maintain an environment in which both enzymes display good activity and to provide conditions for future technological applications, physiological conditions and ambient temperature can satisfactorily be applied to an enzymatic system involving dehydrogenase enzymes.

In order to verify product formation after the kinetic tests, product yields were followed by high performance liquid chromatography (HPLC) employing the best conditions obtained in all the assays utilizing the anchored ADH and AldDH. A highly specific behavior was verified for both dehydrogenase enzymes. Considering the ADH kinetic behavior, an average of acetaldehyde recovery of 92% was obtained after the assay (aldehyde leakage may have occurred during the experiment), but acetic acid was not detected, probably due to the low activity of ADH for acetaldehyde oxidation, $K_{cat} = 2.3\ s^{-1}$ at pH 8.8 [28]. In the case of AldDH, an acetic acid recovery of about 90% was achieved.

3.4. Double enzymatic system

Aiming at the complete oxidation of fuels, multiple immobilized dehydrogenase enzymes are generally used by anchoring the enzymes onto a solid platform in cascade [29,30]. Despite the lower kinetic values obtained with AldDH compared with ADH, the large affinity between AldDH and acetaldehyde enables construction of an efficient integrated system employing both enzymes; i.e., as soon as ADH catalyzes the first step of ethanol oxidation, AldDH is able to rapidly catalyze the oxidation in a second step.

So, in order to simulate these conditions in the kinetic assays, the LbL technique was employed for immobilization of both ADH and AldDH onto carbon platforms. To this end, a sample with 36 bilayers containing both dehydrogenase enzymes was prepared by anchoring the enzymes onto separate layers, which furnished a final architecture sequence of ADH/PAMAM/AldDH. The kinetic results obtained with both enzymes anchored onto the carbon platform by using the self-assembly methodology, evidence enhanced enzymatic activity behavior ($V_{max} = 0.62$ μmol NADH min^{-1} mg^{-1}) as compared to the individual systems (Table 1). By comparing the results between the double enzymatic system with those obtained in the case in which only ADH was anchored, it is clear that the use of multiple dehydrogenase enzymes enhances the kinetic performance of the whole system, without causing damage to the first oxidation step. In addition, these results evidence that a very good arrangement of the anchored enzymes on the carbon support is provided by the layered structure, which facilitates the diffusional processes during the catalysis is and contributes to the overall performance of the system.

Although all the kinetic results indicate that the use of the LbL technique provides good control of enzyme disposition on the carbon platform, thus providing good kinetic rates for the anchored enzymes, a large decrease in enzymatic activity is still observed when one compares the results from the tridimensional condition in solution and the two-dimensional situation on the carbon support (Table 1). In this context, the question that arises is related to the effect of immobilization employing dendrimers on the enzymatic activity. In fact, a few factors must be considered in order to understand the kinetic behavior of dehydrogenase enzymes anchored with dendrimers onto carbon platforms. The first factor involves the possibility of enzyme inactivation after the immobilization step. Normally, this risk is pronounced when chemical bonds are formed during the immobilization step, and also when sudden changes in temperature occur during the anchoring process. These two situations have not been reported for dendrimers yet. In fact, no enzymatic denaturation was observed in a previous investigation using PAMAM dendrimers for the immobilization of Cl-cathecol 1,2 deoxygenase [31]. However, denaturation may occur after protein immobilization and, considering such reduction in activity after enzyme anchoring, this possibility cannot be disregarded.

Considering the enzymes in solution, diffusional limitations can normally be neglected; however, in the case of enzymes anchored with PAMAM dendrimers, the effects of mass transfer should be much relevant. In this way, the better the diffusion rate, the higher the enzymatic activity of the anchored enzymes. So the better kinetic results obtained with the double enzymatic system as compared to the individual cases is a clear indication that good mass transfer is provided by the self-assembly methodology.

Finally, the present work clearly showed that various kinetic parameters (mainly the concentration of substrates and coenzyme, which act as inhibitors of the enzymes) should be considered, for achievement of maximum enzymatic activity for the anchored enzymes. Additionally, the immobilization process seems to be crucial for preparation of a viable anchored system, making a careful choice of immobilization process for each type of anchored system very important.

4. Conclusions

All the kinetic constants for soluble enzymes presented in this paper are in agreement with literature data. It is important to control the concentration of both substrate and coenzyme because of the possible inhibition due to substrate excess. The kinetic rates obtained for the anchored enzymes showed that the choice of a proper immobilization method is very important, since activity reduction after the anchoring process may be pronounced. PAMAM dendrimers provide a good environment for the immobilization of dehydrogenase enzymes and, despite the considerable loss of enzymatic activity observed after immobilization, the affinity between the enzymes and their substrates and coenzymes seems to be retained. Also, the employed anchoring methodology (LbL) required very low catalyst consumption, and the anchored enzymes were quite stable over a period of approximately 30 days.

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