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Improved Stability of Formate Dehydrogenase by Coating with Didodecyldimethylammonium Bromide

Matthias Reichert[a], Marco Haumann[b]*, Rainer Buchholz[a,c], Christoph Lindenberger[a]*,

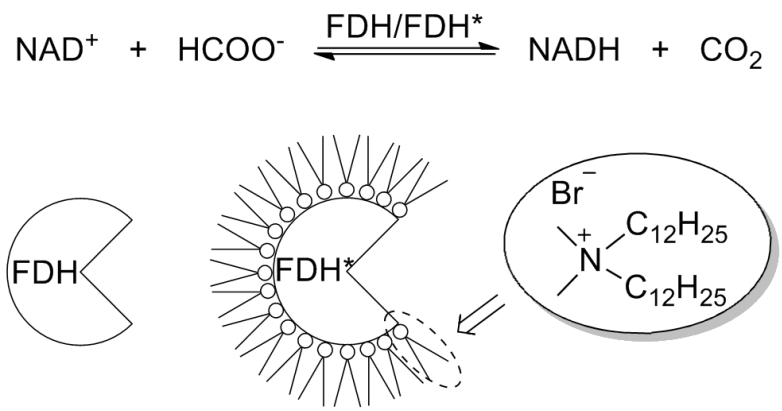
[a]*Friedrich-Alexander Universität Erlangen-Nürnberg, Busan Campus, Bioprocess Engineering, 1276 Jisa-Dong, Gangseo-Gu, Busan 618-230, Republic of Korea*
[b]*Friedrich-Alexander Universität Erlangen-Nürnberg, Chemical Reaction Engineering, Egerlandstr. 3, 91058 Erlangen, Germany*
[c]*Friedrich-Alexander Universität Erlangen-Nürnberg, Bioprocess Engineering, Paul-Gordan-Straße 3, 91052 Erlangen, Germany*

Abstract: *Hydrophilic formate dehydrogenase (FDH) from candida boidinii was chemically modified by coating it with didodecyldimethylammonium bromide (DDAB). This coating changed the phase behavior of the enzyme, making it highly soluble in hydrophobic solvents and thereby offering the chance for biphasic enzyme recycling from hydrophilic substrates and products. Different coating procedures of FDH with DDAB were investigated and all proved suitable for efficient coating of the enzyme's outer surface. A 50 mM Tris-(hydroxymethyl)-amminomethan (tris) buffer at pH 8 was chosen to make DDAB soluble and avoid aggregation. The reaction of NAD^+ with uncoated and coated FDH to NADH and CO_2 was monitored by UV-vis spectroscopy and kinetic parameters (r_{max}, K_m, K_I, E_A) for the the FDH were determined. The coated enzyme resulted in a lower relative initial activity between 40-60% compared to the uncoated one. The stability of the coated enzyme (FDH*) was improved significantly and remained stable in long-term experiments, resulting in a deactivation rate k_D smaller than 3% per day and a half-life time $t_{1/2}$largerthan 23 days, while the deactivation rate of the uncoated enzyme was 260% per daywitha $t_{1/2}$of 0.3 days. Both activation energies were similar, with 42 kJ mol^{-1} for the coated and 48 kJ mol^{-1} for the uncoated enzyme.This result suggests that there is not significant transport resistance originating from the DDAB coating layer. The reason for the significantly lower activity of the coated FDH probably stems from accumulation of formed CO_2 in the coating layer, thereby preventing high equilibrium conversions.*

I. Introduction

The highly selective production of chemicals is one of the major challenges for modern chemical engineering since most feedstock for chemical transformations is based on crude oil or gas (Cornils and Herrmann, 2002).Catalysis is the key technology to achieve high selectivity and enzymes are superior in this regard compared to homogeneous and heterogeneous catalysts. Especially for stereoselective reaction many enzymes produce the target enantiomer in high yields. Introducing enzymatic reactions into chemical production processes lowered the production complexity and costs significantly in the past, e.g. in the synthesis of L-Ephedrine or Pregabalin(Mahmoud, El-Sayed and Coughlin, 1990). Due to high enzyme prices and product purity requirements the separation after reaction and consecutive use of the precious catalyst can help to improve the enzyme productivity significantly. Biphasic catalysis with enzymes is a promising technique to achieve this goal. For such recycling to be effective, the enzyme solubility must be low in the product phase and high in the catalyst solvent. Biphasic catalysis with hydrophilic enzymes is possible if the products are hydrophobic and the substrates have a good miscibility in the water phase, the most common solvent for enzymes. If hydrophobic substrates should be converted, the poor miscibility in the enzyme containing water phase will result in a low conversion, making such processes unfavorable. This is even more critical, if the products are hydrophilic and need to be separated from the water phase.

One possibility to separate hydrophilic products from the enzyme catalyst would be the modification of the enzyme's phase behavior, namely its solubility in water. This could be achieved by coating the enzyme with a surfactant, so that the polar head group of the surfactant will coordinate to the hydrophilic enzyme surface, while the nonpolar tail of the surfactant will point away from the enzyme. Such coated enzymes will be soluble in nonpolar media now and could be easily separated from an aqueous product phase.



Scheme1. Top: reaction of NAD^+ and formate to yield NADH and CO_2, catalyzed by FDH or lipid-coated FDH (FDH*). Bottom: Schematic presentation of the FDH* coated with DDAB.

The coating of enzyme surfaces with polymers has been described as an efficient method to prevent negative interaction of enzyme interfaces (e.g. gas bubbles) that may produce enzyme inactivation (Betancor, Lopez-Gallego, Hidalgo, Alonso-Morales, Fuentes, Fernandez-Lafuente, and Guisan, 2004). For enzyme stabilization and solubility in organic media, some chemical modification techniques are promising and known. The dissociation of multimeric enzymes can be avoided by coating their surface with a large cationic polymer e.g. polyethylene imine (PEI) (Bolivar, Rocha-Martin, Mateo, Cava, Berenguer, Fernandez-Lafuente, and Guisan, 2009), polyethylene glycol (PEG) (Turner, Spear, Huddleston, Holbrey, and Rogers, 2003; Laszlo and Compton, 2001; Maruyama, Nagasawa and Goto, 2001) or with amphiphiles (lipids/surfactants) (Okahata, Fujimoto and Ijiro, 1995). However, since most of the glycolipids are not available on the market and have to be newly synthesized, makes them expensive. One of the most extensively investigated double chained cationic surfactant (Marques, Regev, Khan, and Lindman, 2003), is didodecyldimethylammonium bromide (DDAB). It has market value between 7 and 15Euro per gram and it is forming micelles in aqueous solution (Lichtenberg, Opatowski, and Kozlov, 2000).

In this work we present different coating procedures for FDH by the surfactant didodecyldimethylammonium bromide (DDAB), which is expected to form a two phase emulsion consistent of bilayer structures (Marques, Regev, Khan and Lindman 2003; Lichtenberg, Opatowski, and Kozlov, 2000). The coating quality and the performance of the lipid-coated FDH (FDH*) will be investigated for the test reaction of formate with NAD^+ to yield NADH and CO_2 as shown in Scheme 1.To the best of our knowledge, this system in tris-(hydroxymethyl)-amminomethane (tris) buffer has not been described for the particular enzyme in literature so far.

II. Experimental

Chemicals and materials

The 50 mM Tris-(hydroxymethyl)-amminomethane (tris) buffer (DAE Jung, Korea) was prepared, adjusted with HCL to a pH of 8, autoclaved and stored at room temperature. For the FDH stock solution the lyophilized formate dehydrogenase (FDH) from *candida boidinii* (Sigma-Aldrich, Germany) was weighed and added to ice cold 50 mM tris buffer of pH 8. The stock solution wasstored at 4 °C for further experiments. For the NADH stock solution the lyophilizedβ-nicotinamide adenine dinucleotide hydrate (NAD^+) from yeast (Sigma-Aldrich, Germany) and β-nicotinamide adenine dinucleotide(NADH) reduced dipotassium salt (Sigma-Aldrich, Germany) solution were weighed, dissolved in 50 mM tris buffer of pH 8, stored at -20 °C and defrosted for experiment. The DDAB stock solutions containing32 mg of didodecyldimethylammonium bromide (DDAB) (Steinbeis GmbH, Germany) were dissolved in 1 ml of ethanol and stored at 4 °C. Additionally, 4 mg of DDAB were dissolved in 1 ml of 50 mM tris buffer of pH 8 and stored at 4 °C.The formate solution (Dae Jung, Korea) was weighed, dissolved in 50 mM tris buffer of pH 8 and stored at room temperature.

FDH coating procedures

Three different coating methods were used to examine the influence on the relative activity (a/a_0) of coated-FDH (FHD*). In method 1, the enzyme solution was first added to the vial, followed by the prepared DDAB-ethanol solution. Method 2 reversed the steps, with the enzyme solution being added to the vial after the DDAB-ethanol solution. In Method 3, a DDAB tris buffer was used instead of the DDAB-ethanol solution. The buffer was then added to the enzyme solution, similar to method 1. Both, an unstirred and a stirred enzyme solution were taken as reference.

The FDH*reaction solutions were obtained by simply mixing prepared FDH* enzyme and DDAB solutions. 8 mg of lyophilized FDH was mixed in a glass sample vial (Wheaton, USA) with 5 ml of 50 mM tris buffer pH 8 at 4 °C. Four other vials with magnet stirrers were prepared, one with 2600 µl, twowith 2950 µl and one with 3000 µl of tris buffer (4 °C, 50 mM and pH 8). 1 ml of the enzyme stock solution was transferred in different steps, to each of the other fourstirred vials. Under stirring, 50 µl of 32 $mgml^{-1}$DDAB ethanol stock solution was added, in different steps, to two of the vials. 400 µl of 4 $mgml^{-1}$ DDAB tris buffer stock solution was added to the last vial. 3 ml of tris buffer (4 °C, 50mM, and pH 8) was given to the unstirred enzyme reference vial in which the enzyme concentration was determined with Bradford's method (Bradford, 1976). After the preparation, all vials had a total volume of 4 ml and were stirred at least 20 hours (with 300 min^{-1} or 500 min^{-1}at 4 °C) before measurement. The sequence of the different coating steps andthe end concentrations areitemized in Table 1.

Table 1. Sequence of different coating steps and final concentrations.

	FDH		FDH*		
Step	Unstirred FDH	Stirred FDH	Method 1	Method 2	Method 3
1	5000 µl FDH	3000 µl tris	2950 µl tris	2950 µl tris	2600 µl tris
2	4 x 1000 µl FDH	1000 µl FDH	1000 µl FDH	50 µl DDAB/EtOH	1000 µl FDH
3	3000 µl tris		50 µl DDAB/EtOH	1000 µl FDH	400 µl DDAB/tris
V_{total}	4 ml	4 ml	4 ml	4 ml	4 ml
[FDH]	0.4 $mgml^{-1}$	0.4 mg ml^{-1}	0.4 mg ml^{-1}	0.4 mg ml^{-1}	0.4 mg ml^{-1}
[DDAB]	0 mg ml^{-1}	0 mg ml^{-1}	0.4 mg ml^{-1}	0.4 mg ml^{-1}	0.4 mg ml^{-1}

Kinetic experiments

FDH concentration

To determine the exact enzyme concentrationthe absorption was measured in a spectrophotometer UV-1800 (Shimadzu, Japan) at 595 nm. The formate dehydrogenase (FDH) from *candida boidinii* concentration was determined and compared with a BSA standard (Sigma, Germany) using Bradford's method.

NAD^+/ NADH activity

Prior to all measurements exact concentration of the active $NAD^+$and NADH were determined. The activities of the FDH preparations were evaluated by their UV absorption, recording the increase of the absorption at 340 nm (ε=6220 $M^{-1}cm^{-1}$ at 340 nm)which resulted from the formation of NADH during the oxidation of formic acid.The prepared NAD^+ stock solution was unfrozen and setwithin the experiment on flocked ice. 100 µl of 1.67 mM NAD^+ stock solution, 400 µl of 1000 mM formate stock solution and 350 µl of 50 mM tris buffer of pH 8 were prepared in a tube. The tube was placed in a thermomixer (compact, Eppendorf, Germany) and mixed for 10 minutes at 400 min^{-1} to adjust the 25 °C. As well the precision cells 105.201-QS (Hella GmbH, Germany)weretempered in an oven (T20, Thermo Scientific, Germany) with the required temperature for at least 10 minutes. After tempering, the reactive solution was converted into the cell. The cell was placed in the spectrophotometer. By adding 150 µl of ice cold (4 °C) 0.4 mg ml^{-1}FDH stock solution, the reaction was started. The total volume, of 1 ml inside the cell, was gently mixed twice with a pipette (VWR, Korea). After the reaction was completed (30 minutes) the concentration was calculated from the difference of the start and end absorption values according to Equation 1.For the determination of the NADH concentration the stock was unfrozen and diluted by afactor of 10 (100 µl of 1mM NADH stock solution, 900 µl 50 mM tris buffer of pH 8). A cell filled with tris buffer served as zero value. The NADH concentration was calculated directly with Lambert Beer's law usingEquation 1. The procedure of allother experiments was handled as described above.

$$\frac{[NAD^+]}{[NADH]} = \frac{[Absorption]}{6220} \qquad (1)$$

Long term stability of FDH in tris buffer

The stability of FDH in a 50 mM tris buffer of pH 8 was tested under following reaction conditions: 100 µl of 1.67 mM NAD^+ stock solution, 400 µl of 1000 mM formate stock solution, 350 µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

Kinetic parametersof FDH catalyzed reaction

The kinetic parameters of enzymatic reactions were determined in different reaction media set upswith varying concentrations of substrate (NAD^+, $HCOO^-$), inhibitor (NADH, EtOH) and DDAB concentrations.Reactions condition for the determination of the kinetic parameters were as follows:

K_{m,NAD^+}:10-200 µl of 1.67 mM NAD^+ stock solution, 200 µl of 1000 mM formate stock solution, 550-740 µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

$K_{m,HCOO^-}$:600 µl of 1.67 mM NAD^+ stock solution, 5-200 µl of 1000 mM formate stock solution, 150-345µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

$K_{I,NADH}$: 600 µl of 1.67 mM NAD^+ stock solution, 10-200 µl of 1000 mM formate stock solution, 0-150 µl of 1mM NADH stock solution, 0-320µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

$K_{I,EtOH}$: 400 µl of 1.67 mM NAD^+ stock solution, 10-200 µl of 1000 mM formate stock solution, 0-350 µl of EtOH, 0-450 µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

Influence of DDAB: 100 µl of 1.67 mM NAD^+ stock solution, 400 µl of 1000 mM formate stock solution, 0-100µl of 4mg ml^{-1}DDAB in 50 mM tris buffer of pH 8, 490-540µl of 50 mM tris buffer of pH 8, 50µl of 0.4 mg ml^{-1}FDHstock solution.

Activation energy FDH/FDH*

Activation energies were determinedexperimentally measuring at different temperatures T of 10 °C, 15 °C and 25 °C.The experiments at a temperature of 10 °C and15 °Cwere carried out with the complete setup being placed in a cooling chamber (Coldtop Co., Korea). Reactions conditions for the determination of activation energies were as follows:

$E_{a,FDH}/E_{a,FDH*}$: 100 µl of 1.67 mM NAD^+ stock solution, 25-750 µl of 8000 mM formate stock solution, 0-725 µl of 50 mM tris buffer of pH 8, 150µl of 0.4 mg ml^{-1}FDH/FDH* stock solution. FDH* was covered with 0.4 mg ml^{-1}DDAB using coating method 1.

Influence of coating methodson stability of FDH/FDH*

The FDH was coated with 0.4 mg ml^{-1}DDAB as described at coating procedures. Either the non-coated enzymes (FDH) or the coated enzymes (FDH*) were stirred at speed of 300 min^{-1} or 500 min^{-1}, and the activity *a*was determined over 90 hours in order to the initial activity $a_0$of FDH at 0 hours.Reactions conditions for the determination of the kinetic parameters were as follows: 300 min^{-1}: 100 µl of 1.67 mM NAD^+ stock solution, 400 µl of 1000 mM formate stock solution, 350 µl of 50 mM tris buffer of pH 8, 150 µl of 0.4 mg ml^{-1}FDH/FDH*. 500 min^{-1}: 100 µl of 1.67 mM NAD^+ stock solution, 750 µl of 8000 mM formate stock solution, 150 µl of 0.4 mgml^{-1} FDH/FDH*.

Evaluation of kinetic data

Initial velocity $v_{initial}$

The spectrophotometer UV-1800 measured the reaction solution's UV absorption of formed NADH at 340 nm every two seconds over a time period of 200 seconds. The initial velocity was taken by the change in absorption *A* over time through linear regression usingEquation (2). In case of FDH the interval was60 seconds over at least 20 data points and in case of FDH^* within the first 34 seconds over the first ten measured data points.

$$A = v_{initial} \cdot t + A_0 \qquad (2)$$

With A = absorption, $v_{initial}$ = dA/dt = initial velocity in s^{-1}, t = time in s, A_0 = absorptionat t_0.

Kinetic parameters v_{max}, K_M and K_I

The activity was calculated with Equation 1 and 2 referred to the FDH concentration (see Equation 3). The kinetic parameters and inhibition models of enzymatic reactions were determined and evaluated using SigmaPlot's Enzyme Kinetic Wizard (SigmaPlot 12, Systat Software Inc., USA)plotting activity against concentration.

$$activity = \frac{v_{initial}}{[FDH]} = \frac{\frac{d[NADH]}{dt}}{[FDH]} \qquad (3)$$

Activation energy

Activation energies were determined by plotting the logarithm of k_{eff}against T^{-1} and determining the slope of the straight line with Equation 4.

$$\ln k_{eff} = \ln k_0 - \frac{[E_a]}{[R]} \cdot \frac{1}{T} \qquad (4)$$

With k_{eff} = effective rate constant, k_0 = frequency factor, E_a = activation energy. The effective rate constant $k_{eff}$was calculated from plots of the logarithm of activity against the logarithm of formate concentration at different temperaturesT.

Deactivation constant k_D

In the stirring experiment the deactivation constants were calculated by measuring the activity *a* of either FDH or FDH* over the stirring time. This activity was correlated to the initial activity a_0 of the non-coated FDH at time t=0. The decay in activity in % was exponentially fitted and k_D determined with Equation 5.

$$activity = \frac{a}{a_0} = y(t) = A_0 \cdot e^{-k_D \cdot t} \qquad (5)$$

With t = stirring time in days, *a* = activity of FDH/FDH* at t in μMmin^{-1}mg^{-1}, a_0 = activity of FDH at t = 0 in μM min^{-1} mg^{-1}, A_0 =initial activity of FDH/FDH* at t = 0 in %, k_D = deactivation constant of FDH/FDH*in day^{-1}.

FDH/FDH* half-life time

The FDH/FDH* half-life time $t_{1/2}$was defined as the time when the enzyme (FDH or FDH*)has lost half of its initial activity A_0, and was calculated according to Equation 6.

$$t_{1/2} = \frac{\ln 2}{k_D} \qquad [day] \qquad (6)$$

FDH/FDH* productivity

FDH and FDH* productivity was calculated by integrating Equation 5 within a determined interval of time (Equation7).

$$P_{FDH/FDH^*} = y(t) = \int_{t=0}^{t} A_0 \times e^{-k \times t} dt \qquad (7)$$

With P_{FDH/FDH^*} = *productivity*, t = stirring time in days, A_0 =initial activity of FDH or FDH* at t = 0 in %, k_D = deactivation constant of FDH or FDH* in day^{-1}.

III. Results

In a preliminary set of experiments the solubility of the DDAB coating agent in different buffer solutions has been investigated (Figure 1). DDAB was nearly insoluble in 5mM and 50mM potassium-phosphate buffer at pH 8, yielding white swollen aggregates that could not be resuspended upon prolonged stirring for 48 h (Figure 1 A and B). However, DDAB readily dissolved in large quantities in ethanol. For that reason, two ternary systems consisting of either ethanol, DDAB and potassium-phoasphate buffer or ethanol, DDAB and tris buffer, both at pH 8 and 25 °C were investigated. Only the system using the tris buffer gave an emulsified and stable system (Figure 1 C and D), while the other systems tended to agglomerate. Hence, all further experiments were conducted with the tris buffer.

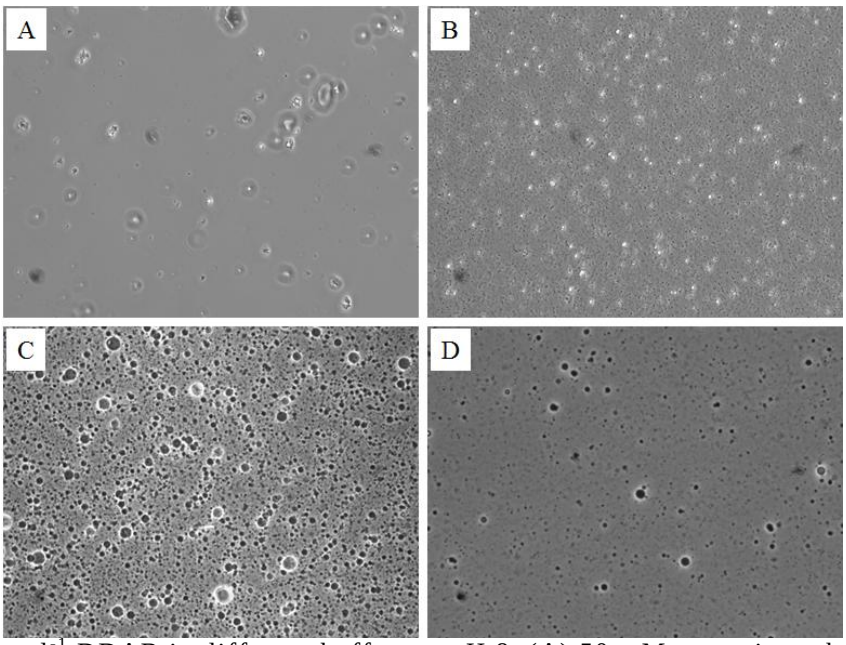


Figure 1. 4 mg ml^{-1} DDAB in different buffers at pH 8. (A) 50 mM potassium phosphate, (B) 5 mM potassium phosphate, (C) 50 mM tris-(hydroxymethyl)-amminomethan (tris), (D) 50 mM tris-(hydroxymethyl)-amminomethan (tris) and 1 Vol% EtOH.

Non-coated enzyme

In a first set of experiments the non-coated FDH stability in a 50 mM tris buffer of pH 8 at 25 °C has been investigated. 400 mg ml^{-1} FDH was stored in this buffer at 4 °C for 30 days. Kinetic measurements with 0.167 mM NAD^+ and 400 mM formate at 25 °Cindicated that the relative enzyme activity remained stable over three days and that even after 30 days storage time still 94 of the initial activity could be preserved (see Table 2).

Table 2. Stability of 0.4 $mgml^{-1}$ FDH stored at 4 °C in a tris buffer of pH 8.

Storage time / h	Activity (a/a_0) / %	RSD / %
0	100	± 2.7
24	102	± 5.6
48	103	± 0.4
720	94	± 2.4

Reaction conditions: 25 °C, 0.167 mM NAD^+, 400 mM formate, 2 µg ml^{-1}FDH.

Enzymes interact often with substances that affect the reaction rate. These substances (effectors) are usually low-molecular compounds. Activators increase the reaction rate, whereas inhibitors decrease it. In general irreversible inhibitors are covalent bound to the enzyme. It is separated between unspecific inhibitions, where the interaction takes place beyond the active site and specific inhibitions, where the inhibitor has an affinity to catalytic center. Reversible changes in activity caused by inhibitors are essentially separated in four types and are derived by the Michaelis-Menten kinetics in Equation (8):

- competitive inhibition
- non-competitive inhibition
- partial-competitive inhibition
- uncompetitive inhibition

Competitive inhibition appears if substrate and inhibitor compete against the active site. Mostly they are of similar structure. There are some possibilities this inhibition can arise:

1. S and I compete against the same binding site
2. Only S or I can bind, cause of their steric interference
3. S and I have an additional common binding site

At our reaction formate serves as substrate and NAD^+ is applied as the coenzyme. Since the CO_2-concentration is negligible, the reaction can be seen as irreversible. The byproduct NADH has an inhibitory character (product inhibition). To simplify the calculations a competitive inhibition model was used.

The kinetic parameter estimation was based on an inhibition model. For this case of competitive inhibition the classical Michaelis-Menten kinetics in Equation (8) has to be expanded into Equation (9).

$$\nu = \nu_{\max} \cdot \frac{[S]}{K_m + [S]} \qquad \text{with } K_m = \frac{k_1 + k_2}{k_{-1}} \tag{8}$$

$$\nu = \nu_{\max} \cdot \frac{[S]}{K_m \cdot \left(1 + \frac{[I]}{K_I}\right) + [S]} \text{ with } K_I = \frac{k_I}{k_{-I}} = \frac{[E] \cdot [I]}{[EI]} \tag{9}$$

Linearization of Equation (9) yields the Lineweaver-Burk Equation (10).

$$\frac{1}{\nu} = \frac{K_m}{\nu_{\max}} \cdot \left(1 + \frac{[I]}{K_I}\right) \cdot \frac{1}{[S]} + \frac{1}{\nu_{\max}} \tag{10}$$

The uncoated FDH activity was investigated in a 50 mM tris buffer at a pH value of 8 and a constant NAD^+ concentration of 1 mM with varying formate concentrations from 5 mM to 200 mM. Figure 2 depicts the corresponding Michaelis-Menten and Lineweaver-Burk plots, from which the kinetic parameters of the uncoated FDH were determined. The Michaelis-Mentenplot reveals a typical progression of an enzymatic reaction with $K_{m,HCOO^-}$ of 6.18 mM and $\nu_{\max}$ of 5.30 µM mg^{-1} min^{-1}. Variation of the NAD^+ concentration between 16.7 µM and 334µM at constant FDH of 200 mM yielded a $K_{m,NAD+}$ of 23.1µM and a $\nu_{\max}$ of 5.07 µM mg^{-1} min^{-1}.

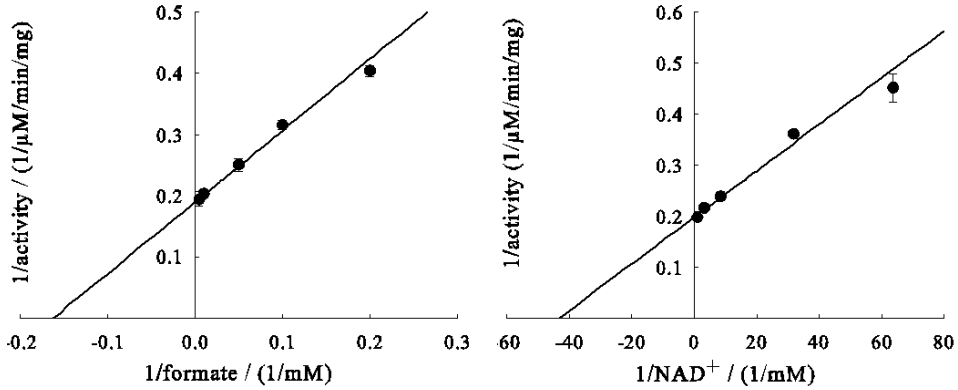


Figure 2. Michaelis-Menten and Lineweaver-Burk plots for FDH kinetics at varying formate (left) and NAD^+ (right) concentrations. Reaction conditions: (left) 25 °C,1 mM NAD^+, 5-200 mM formate, 2 µg ml^{-1}FDH and (right) 25 °C, 16.7-334 µM NAD^+, 200 mM formate, 2 µg ml^{-1}FDH.

In good agreement with the proposed competitive product inhibition model, the concentration of the substrate NADH had a negative influence on the enzyme activity indicated by a value for $K_{I,NADH}$ of 14.6 µM, determined from the Lineweaver-Burk plot shown in Figure 3.

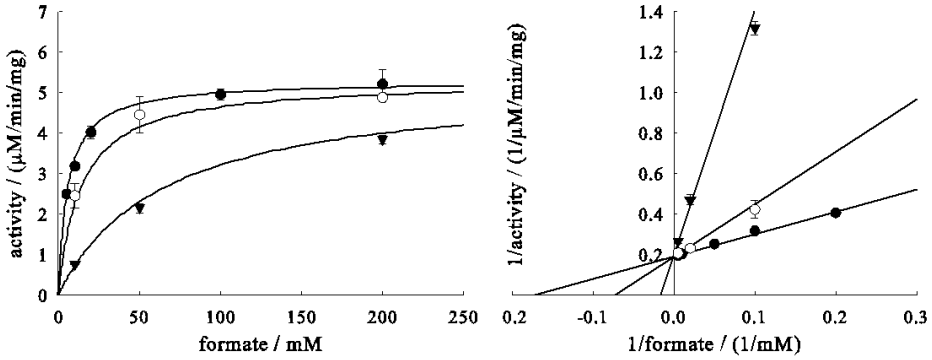


Figure 3. Michaelis-Menten (left) and Lineweaver-Burk (right) plots for FDH kinetics at varying NADH concentrations. Reaction conditions: 25 °C, 1 mM NAD^+, 10-200 mM formate, 2 µg ml^{-1}FDH, [NADH] = 0 (●), 20 (○) and 150 (▼) µM.

The influence of the coating agent DDAB and its solvent ethanol on the enzyme activity was also tested. While ethanol had no significant influence on the activity in the range of 171 µM to 1.71 M, addition of the coating agent DDAB led to a distinct decrease in activity as shown in Table 3 while Table 4 lists obtained kinetic data.

Table3. Influence of the DDAB concentration on the activity of FDH

[DAAB] / mg mL^{-1}	Activity (a/a_0) / %
0	100
0.04	63.9
0.10	53.6
0.20	50.2
0.40	58.0

Reaction conditions: 25 °C, 167 µM NAD^+, 400 mM formate, 0-0.4 mg/ml DDAB, 2 µg ml^{-1}FDH.

Table 4. Kinetic parameters determined for various inhibitors.

Substrate	v_{max} / µM mg^{-1} min^{-1}	$K_{m,NAD+}$ / µM	$K_{m,HCOO-}$ / mM	$K_{I,NADH}$ / µM	$K_{I,EtOH}$ / mM
$HCOO^-$	5.30		6.18		
NAD^+	5.07	23.1			
NADH	5.27		5.83	14.6	
EtOH	4.69		7.98		11.97

Reaction conditions: [$HCOO^-$]: 25 °C, 1 mM NAD+, 5-200 mM formate, 2 µg/ml FDH; [NAD^+]; 25 °C, 16.7-334 µM NAD+, 200 mM formate, 2 µgml^{-1}FDH; [NADH]: 25 °C, 1 mM NAD+, 10-200 mM formate, 0-150 µM NADH, 2 µgml^{-1}FDH: [EtOH]: 668 µM NAD+, 10-200 mM formate, 0-6 M EtOH, 2 µgml^{-1} FDH.

Lipid coated enzyme

Three coating methods were tested that differed according to whether the enzyme was coated directly with DDAB (method 1 and method 3) or method 2. Furthermore, no ethanol was present in the coating solution when using method 3. All three coating methods resulted in similar initial activity of the FDH*, yielding 38.8 % (method 1), 40.8 % (method 2) and 39.0 % (method 3) of the non-coated FDH activity, respectively. Interestingly, the non-coated FDH gradually lost activity over time.After 20 hours a relative FDH activity of 60.3 % was obtained which decreased to 12.3 %, after 90 hours. In contrast, all coated FDH* resulted in stable, yet lower activity as shown in Figure 4.

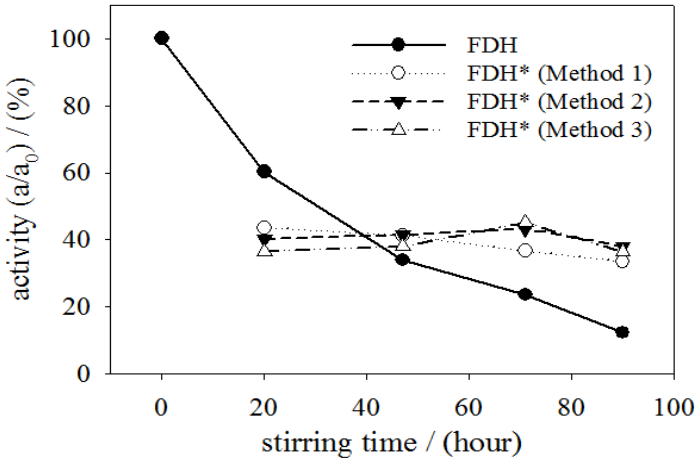


Figure 4. Stability studies of non-coated (●) FDH and coated FDH* using different coating methods.Coating conditions: 4 °C, stirring rate 300 min^{-1},0.4 mg ml^{-1}FDH coated with 0.4 mg mL^{-1} DDAB according to methods 1 (○), 2 (▼) and 3 (Δ).Reaction conditions: 25°C, 167 µM NAD+, 400 mM formate,6µgml^{-1}FDH/FDH*.

To further elucidate the influence of coating on overall kinetics, the reaction temperature was varied in the concentration regime between 200 and 800 mM of formate. For the non-coated FDH an effective activation energy of 41.7 kJ mol^{-1} was determined. Applying the coated FDH* under identical reaction conditions resulted in a similar activation energy of 47.3 kJ mol^{-1}. With both activation energies being similar, limitation of the overall kinetics by transport of substrate through the coating layer to the active site of the enzyme can be excluded.

The half-life time$t_{1/2}$of the enzyme (FDH) was strongly decreased from 1.3 days to 0.3 days when the stirring rate was increased from 300 min^{-1} to 500 min^{-1} as shown in Figure 5. In contrast, coated FDH* was stable even under conditions of more sheer stress and showed a $t_{1/2}$ of more than 23 days when stirring at 300 min^{-1} and a $t_{1/2}$ of more than 43 days at a stirring rate of 500 min^{-1}. The results indicate that the coating layer somehow protectedthe FDH* against mechanical stress from stirring and cavitationand didnot hamper the transport of substrate from the organic phase to the active site of the FDH*.

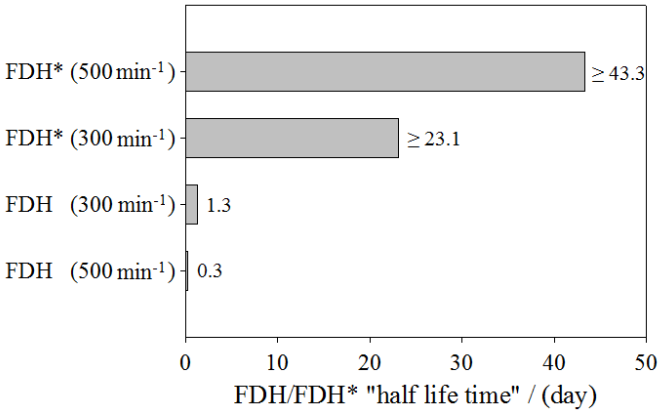


Figure 5. Half-life time of non-coated FDH and coated FDH*. Reaction conditions: 4 °C, stirring rate 300-500 min^{-1}, 0.4 mg ml^{-1} FDH coated with 0.4 mg mL^{-1} DDAB using method 1. Reaction conditions: 300 min^{-1}:25°C, 167 µM NAD^+, 400 mM formate,6µgml^{-1} FDH/FDH*; 500 min^{-1}:25°C, 167 µM NAD^+, 6 M formate,6µgml^{-1}FDH/FDH*.

According to Equation 5 an exponential fit was used to describe the decay in activity of un-coated FDH and coated FDH* with stirring time. The deactivation rate $k_D$of non-coated FDH increased from 53.5% per day to 261% per day when increasing the stirring speed from 300 to 500 min^{-1}. In contrast, the coated FDH* remained stable under these conditions with a k_D of 3% per day (300 min^{-1}) and a k_D of 1.6% per day (500 min^{-1}). However, the initial activity of FDH* dropped from 100% to values around 50 %. The productivity P_{FDH*} of DDAB coated enzyme (method 1) was enhanced by a factor of 3.8 (at 300 min^{-1}) and even 31.7 (at 500 min^{-1})with respect to un-coated FDH.

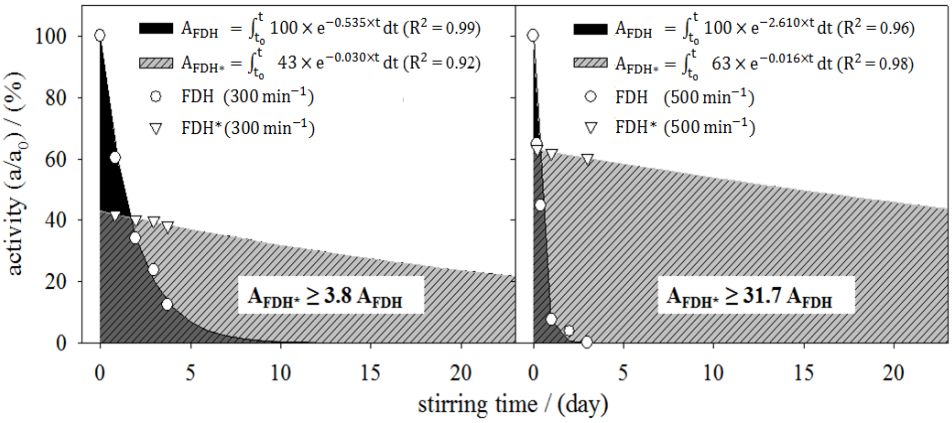


Figure 6. Stability and productivity$P_{FDH/FDH*}$of non-coated FDH and coated FDH* and exponential fits with R^2.Coating conditions: 4 °C, stirring rate 300-500 min^{-1}, 0.4 mg ml^{-1} FDH coated with 0.4 mg mL^{-1} DDAB using method 1. Reaction conditions: 300 min^{-1}:25°C, 167 µM NAD^+, 400 mM formate,6µgml^{-1} FDH/FDH*; 500 min^{-1}:25°C, 167 µM NAD^+, 6 M formate,6µgml^{-1}FDH/FDH*.

IV. Discussion

Kinetic data

The first step to be able developing a new coating method of a hydrophilic enzyme was using a tris buffer. FDH remained stable over three days and that even after 30 days storage. The obtained kinetic data are in good agreement with literature data using the same enzyme system (see Table 5).

Table 5. Comparison ofobtained kinetic datawith literature.

ν_{max} / µM mg^{-1} min^{-1}	$K_{m,NAD+}$ / µM	$K_{m,HCOO-}$ / mM	$K_{I,NADH}$ / µM	pH	T / °C	Ref.
5.30	23.1	6.18	14.6	8	25	This work
3.42	39.0	8.70	30.0	8	25	[1]
n.a.	40.0	2.42	n.a.	7.6	25	[2]
6.00	45.0	5.60	n.a.	7.5	30	[3]

[1] Kragl, Vasic-Racki, and Wandrey, 1996; [2] Labrou and Ridgen, 2001; [3] Slusaarczyk, Felber, Kula, and Pohl, 2000.

Coating procedure and stability

To simplify the coating method used in this work DDAB was dissolved in EtOH. Converting the concentration, used in the developed coating method, to wt% shows that the experiment produces a two-phase emulsion of isotropic liquid L1 in the water corner (x_{buffer}=98.98%;x_{DDAB}=0.04%; x_{EtOH}=0.98%).However results indicate that adding a DDAB/ethanol solution to a tris buffer produces a trinary phase system DDAB/EtOH/tris buffer. This system is meta stable and strongly dependent on phase composition. Moreover, adding ethanol in a certain amount seems to stabilize this system, shown by the fact that a gel is formed in the DDAB/tris buffer stock solution without adding ethanol after two weeks. These observations are also established by the work of Friberg, Hasinovic, Yin, Zhang and Patel. The authors have shown that the addition of the ethanol to the surfactant-water lamellar liquid crystal caused a disordering of it to an isotropic liquid, a micro emulsion, which can be separated easily in a process.

A comparison of the method developed by Okahata, Fujimoto and Ijiro with the method developed furtherin this thesis, shows some remarkable improvements. Okahata et al. mixed anaqueous solution (25 ml, 0.01 M acetate buffer, pH 5.6) containing lipase (50 mg)with an aqueous solution dispersion (25 ml) of dioctadecyldimethylammoniumbromide (50 mg). The preparation was stirred for 20 hours at 4°C. Precipitates were gathered by centrifugation at 4°C (5000 rpm, 5min) and lyophilized. The lyophilized powder was taken for kinetic measurements. However precipitates indicate that no emulsion has been formed which is similar to the

observation when using a potassium phosphate buffer. This shows that, if at all, only a very low enzyme amount was coated and that an adsorption of the lipase on the surfactant surface is more likely. Results have been shown that the yield was 34.7mg containing a protein content of 10 wt% (obtained from UV absorption). In other words 93.06 wt% of the used lipase could not be used. In contrast we have coated all FDH present in the stock solution. This assumption seems justified by a lower conversion of FDH* since free uncoated-FDH present in the reaction media always lead to total conversion. However, this is more time consuming and total conversion could be achieved adding FDH.

Application

1000 units (U) of FDH are necessary to form 1 mmole of NADH. If we want to produce NADH in a stirred tank reactor (STR) with a stirring rate between 300-500 min^{-1} and assume the FDH hold a deactivation rate k of 50% a day, with an initial activity of 100%,then, a one year repeated fed batch process would consume 181.000 U of FDH. The current market price of 1000 U of FDH is EUR 2000. In the described process the annual cost of enzyme refilling is EUR 362.000 Euro. Coating the enzyme with DDAB using our method, would reduce the deactivation rate k from 50% to 2% a day with respect to un-coated FDH, however with a reduced initial activity of 50%. To produce 1 mmole of NADH would now require 2000 units (U) of FDH* in the STR to achieve 100% initial activity but only 32.000 U of FDH* would have been consumed per year. This leads to an around 11 fold annual cost reduction from EUR 362.000 Euro to EUR 32.800. Figure 7 shows described example process of NADH production in a STR.

However it is known that the deactivation rate of uncoated enzyme can be reduced to 3% a day by pressurizing the reactor system to avoid cavitation and maintain the protective and catalytic required hydrate shell. Assuming the latter system would reduce the deactivation rate of FDH* from 1.6% to 1% a day, enzyme consumption and costs still could be reduced by a factor 1.3 per year. Neglecting the fact that hydrophobic coated FDH* easily could be recycled with a settler.

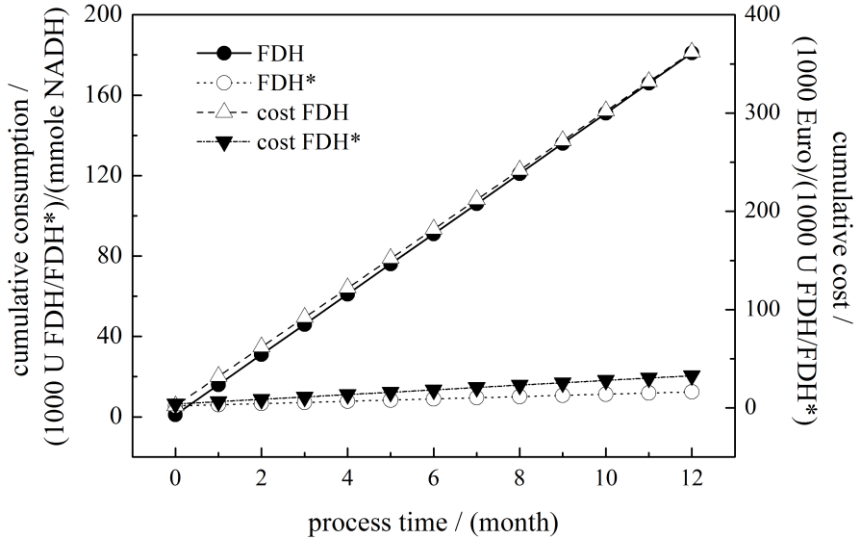


Figure 7. Example process of NADH production in a stirred tank reactor (STR) in comparison with FDH and coated FDH* shown as annual cumulative enzyme consumption per mmole NADH and cumulative cost in Euro per consumed enzyme.

V. Conclusion

A coating method was developed which enables hydrophilic enzymes being solvable in hydrophobic solvents and improved long-term stability due to protection against mechanical stress. There is not a significant transport resistance originating from the coating layer, however FDH* showed a lower initial activity between 40-60%. The deactivation rate of hydrophobic coated enzyme was reduced to less 1.6% a day which makes developed method economic attractive when running a stirred process in which the enzyme-emulsion could be easily recycled. It would also be interesting to test this coating technology with an alcohol dehydrogenase (ADH) and a glutamate dehydrogenase (GDH). Their products, aldehyde andα-Ketoglutarate, are interesting as precursor for chemical industry.

Acknowledgements

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