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Factors influencing the operational stability of NADPH-dependent alcohol dehydrogenase and an NADH-dependent variant thereof in gas/solid reactors

Liliya Kulishova[a,1], Kerasina Dimoula[b,1], Max Jordan[b], Astrid Wirtz[a], Diana Hofmann[c], Beatrix Santiago-Schübel[c], Jörg Fitter[d], Martina Pohl[a,*], Antje C. Spiess[b,**]

[a] Institute of Molecular Enzyme Technology, University of Düsseldorf, D-52426 Jülich, Germany
[b] AVT-Biochemical Engineering, RWTH Aachen University, Worringerweg 1, D-52074 Aachen, Germany
[c] ZCH/BioSpec, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany
[d] Institute of Structural Biology and Biophysics 2, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany

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ABSTRACT

The continuous enzymatic gas/solid bio-reactor serves both for the production of volatile fine chemicals and flavors on an industrial scale and for thermodynamically controlled investigation of substrate and water effects on enzyme preparations for research purposes. Here, we comparatively investigated the molecular effects on the operational stability of NADPH-dependent *Lactobacillus brevis* alcohol dehydrogenase and an NADH-dependent variant thereof, *Lb*ADH G37D, in the gas/solid bioreactor. The reference reaction is the reduction of acetophenone to (*R*)-1-phenylethanol with concomitant oxidation of 2-propanol to acetone for the purpose of regeneration of the redox cofactor.

It could be clearly shown that not the thermostability of the cofactor, but the thermostability of the proteins in the solid dry state govern the order of magnitude of the operational stability of both purified enzymes in the gas/solid reactor at low thermodynamic activity of water and substrate. However, at higher thermodynamic activity the operational stability in the gas/solid reactor is overlaid by stabilizing and destabilizing effects of the substrates that require further investigation. We demonstrated first evidence that the substrate affinity of the two variants in the gas/solid reactor is similar to the affinity in aqueous medium. We could also show that partial unfolding of the proteins with subsequent aggregation are the factors governing protein thermo-in-stability both in the dissolved and in the dry state. Thus, stability investigations of enzymes in the dry state are suggested to predict their basal level of operational stability in gas/solid reactions.

1. Introduction

Gas/solid biocatalysis represents an alternative to common liquid biocatalytic reaction systems, where the solid dry enzyme catalyzes conversion of gaseous substrates to gaseous products. The technology is used for production of volatile compounds like esters and alcohols [1]. It exhibits significant advantages, such as high productivity, pronounced stability of the immobilized biocatalyst [2] and simplified downstream processing [3]. The possibility to control thermodynamic parameters in a gas/solid system additionally allows adjusting the enzyme micro-environment, which is important for scientific purposes, such as studies of enzyme hydration or solvent influences [4].

It is generally accepted that dry enzymes are significantly more stable than the dissolved ones. However, progressive inactivation of alcohol dehydrogenases is observed under the gas/solid reactor conditions [5–8]. Yang and Russell described the conversion of 3-methyl-2-buten-1-ol, catalyzed by immobilized yeast alcohol dehydrogenase in a continuous gas/solid reactor at 22–50 °C at different water activities and observed steady-state periods of 4–16 days prior to progressive inactivation [8]. Reduction of acetophenone with the solid immobilized wild type ADH from *Lactobacillus brevis* (*Lb*ADHwt) performed in a continuous gas/solid reactor at 40 °C and a water activity (a_w) of 0.5 revealed a half-life between 1 and more than 40 days for the enzymatic activity depending on the immobilization conditions [7]. Up to now the mecha-

Abbreviations: AcPh, acetophenone; a_w, water activity; BSA, bovine serum albumin; HPLC/MS, high pressure liquid chromatography/mass spectroscopy; *Lb*ADH, *Lactobacillus brevis* alcohol dehydrogenase; RT, room temperature; TEA, triethanolamine; wt, wild type.

* Corresponding author. Present address: Institute of Biotechnology 2, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany.
Tel.: +49 0246 1614388.
** Corresponding author. Tel.: +49 241 8028111; fax: +49 241 8022570.
E-mail addresses: ma.pohl@fz-juelich.de (M. Pohl), antje.spiess@avt.rwth-aachen.de (A.C. Spiess).

[1] Both authors contributed equally to this work.

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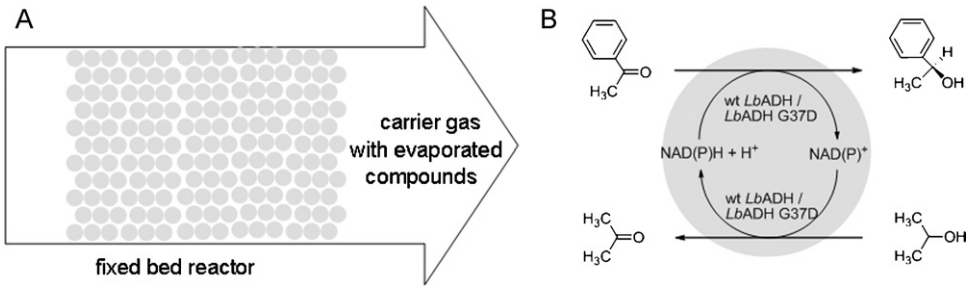

Fig. 1. Biocatalytic reduction of acetophenone in a gas solid reactor. (A) Reaction principle of passing gas mixture across a fixed bed of immobilized enzyme preparation. (B) The cofactor NAD(P)H is regenerated by a substrate coupled approach using 2-propanol as a second substrate.

nisms leading to enzyme inactivation in the solid state remain unclear.

To elucidate the effects of temperature on activity and stability, previous studies focused on the comparison of operational stabilities of ADHs derived from different thermophilic and mesophilic sources [9]. It was shown that high thermal stability in aqueous media was not necessarily correlated with higher stability under gas/solid reactor conditions [9]. However, the enzymes used in that study were from different organisms, showing significant sequence and structural differences. Engineered enzymes, differing only in few sequence positions were never comparatively studied in the gas/solid system.

Here we present a comparative study of the NADPH-dependent wild type ADH from *L. brevis* (*Lb*ADHwt) and the NADH-dependent variant thereof, *Lb*ADH G37D. Both are able to reduce ketones to the corresponding (*R*)-alcohols with high stereoselectivity [10,11]. As can be deduced from structural data, both enzyme variants are homotetramers with a molecular weight of 107 kDa, they contain two Mg^{2+} binding sites and four active centres (one per subunit) [11,12]. The variant was created to accept cheaper and more stable cofactor NADH [13,14] with higher affinity than NADPH [11].

Activity and stability of the *Lb*ADHwt and the *Lb*ADH G37D were studied in aqueous solution, in the dry solid state and with respect to their operational stability in a gas/solid reactor (see principle in Fig. 1A). To identify the main factors causing enzyme inactivation in the gas/solid system, the reduction of acetophenone to (*R*)-1-phenylethanol with concomitant oxidation of 2-propanol to acetone for the cofactor regeneration was studied (Fig. 1B), while varying thermodynamic parameters, such as water activity, and acetophenone activity. Additionally, the thermostability of both enzyme variants and the cofactors was investigated in the solid state and in solution using tryptophan fluorescence spectroscopy and HPLC/MS, respectively.

2. Results and discussion

In case of substrate-coupled cofactor regeneration the cofactors for the biocatalytic reduction step are required in equimolar amounts with respect to the enzyme's active sites, here four per *Lb*ADH molecule. Therefore, the cofactor stability in the gas/solid reactor is as important as the stability of the respective enzyme. In aqueous solution the oxidized and the reduced cofactor molecules are able to freely diffuse between the binding site in the enzyme and the solution, enabling substrate coupled and enzyme coupled cofactor regeneration [15]. The situation is different in gas/solid biocatalysis, as the cofactor remains in the enzyme's active site and must be regenerated by a substrate coupled approach. As a consequence, inactivated cofactor molecules remain in the active sites and render the respective site inactive. There are currently no data available concerning the stability of NAD(P)H in the dry state. Thus, our studies started with a detailed investigation of the stability of the reduced cofactors in the liquid and dry state.

2.1. Thermostability of NAD(P)H

Up to now, the stability of the redox cofactors has exclusively been studied in aqueous solution, demonstrating that NADH is significantly more stable than NADPH under various conditions, such as alkaline or acidic pH or elevated temperature [13,16–19]. Additionally, these studies showed that factors such as buffer salt, ionic strength, and pH increase the speed of NAD(P)H degradation even at moderate temperatures [13,18,19]. In order to obtain the respective data for the solid phase, we studied the thermal stability of NAD(P)H under the same conditions (buffer, ionic strength and pH) as used for the immobilization of the enzyme and compared their stability in the solid and the dissolved state.

The thermal stability of the dissolved cofactor at pH 7.2 was analyzed in a temperature range of 30–70 °C by measuring the decay of absorbance at 340 nm, which is proportional to the concentration of reduced nicotinamide moieties. Further, the residual reducing activity of the heat-treated cofactor samples was studied by the standard activity assay using the *Lb*ADHwt for NADPH and the variant *Lb*ADH G37D for NADH (Fig. 2). In separate experiments the thermal degradation products derived after thermal treatment of the solid cofactor probes for 16 h at 95 °C were analyzed by HPLC/MS analysis (see below).

The absorption at 340 nm and the residual activity decreased over time following a linear decay at lower temperatures and a single-order exponential decay at higher temperatures (Fig. S1, supplementary material). The estimated half-life significantly decreased with increasing temperature for both NADH and NADPH. As expected, the dissolved NADH was 3.5–5.5 times more thermostable than the phosphorylated cofactor. In a range of 30–60 °C, half-life values calculated from residual activity measurements were 20–50% lower than the ones estimated from $absorbance_{340}$ decay curves. The tendency was more pronounced for NADPH. This fact indirectly suggests the presence of enzymatically inactive degradation products containing a reduced nicotinamide ring. At 70 °C half-life values measured by both methods were statistically the same. Thermal stabilities of the solid cofactor samples were examined by the same techniques, $absorbance_{340}$ and reducing activity (Fig. 3).

Surprisingly, the solid cofactors were stable for several weeks upon incubation at 50 °C, as was indicated by a constant absorbance at 340 nm (Fig. 3A). However, the corresponding reducing activity of NADPH gradually decreased to approximately 60% of the initial activity (Fig. 3B), whereas both the $absorbance_{340}$ and the reducing activity of the solid NADH remained almost unchanged (Fig. 3). In comparison to the half-life of > 24 days at 50 °C for both solid cofactors, half-life of dissolved NADPH and NADH at 50 °C equaled to 60 ± 1 and 240 ± 20 min, respectively.

Due to the slow degradation process, the incubation temperature of the solid samples was increased to 95 °C to study the degradation products after 16 h by HPLC/MS. Interestingly, even under these harsher conditions, a significant part of the solid