Biphasic Enzyme-Catalyzed Reactions

Is log *P* a Convenient Criterion to Guide the Choice of Solvents for Biphasic Enzymatic Reactions?**

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The high chemo- and enantioselectivity of enzyme-catalyzed hydrogen transfer are well documented in the literature.[1] Recent articles compared the biocatalytic methods for the reduction of ketones with classical chemical reactions.[2] The advantages of the former were summarized by Faber and co-workers.[3] Although a vast variety of ketones are reduced enantioselectively by commercially available enzymes under mild conditions, isolated alcohol dehydrogenases (ADH) are in comparison seldom used as catalysts in daily chemical laboratory work. The major reason for this discrepancy is the incompatibility of the required reaction conditions for the enzyme with most substrates.[4] Enzymes usually require aqueous environments in which the organic substrates are poorly soluble and in some cases even unstable. A straightforward solution to this problem is the application of biphasic systems.[5] In these systems, the enzyme and the cofactor are dissolved in the aqueous phase, while the hydrophobic substrate is present in a high concentration in the organic phase. As the occurrence of a liquid–liquid interface and presence of residual amounts of organic solvent in water can lead to deactivation of the biocatalyst, methods to guide the choice of the proper solvent have been proposed, such as that reported by Brink and Tramper.[6] Laane et al. suggested using the polarity of solvents, expressed by the log *P* value,[7] as the main criterion for optimizing organic solvents in multi-liquid-phase biocatalysis.[8] This concept has since had a widespread influence on the choice of organic solvents used as the second phase in enzymatic transformations and was assumed to be valid for cofactor-requiring enzymes.[1c,9] Attempts to use ADH under liquid–liquid biphasic conditions employing solvents with a high log *P* afforded unsatisfactory results,[10] which led to the conclusion that alcohol dehydrogenases were not stable in biphasic systems.[11]

Herein we present results guiding the choice of organic solvents suitable for use as the second phase in ADH-catalyzed reactions and their application in the reduction of poorly soluble ketones.

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Because there are some examples of enzymatic reactions for which no correlation between activity retention and log *P* were found,[12] we assumed that a new method of searching for a water-immiscible organic solvent for use as the second phase in ADH-catalyzed reductions should be developed. Mozhaev et al. noticed that log *P* should only be used to compare solvents of the same functionality (e.g. alcohols) and that it is not a convenient parameter for the comparison of solvents of different functionality.[13] Therefore, we decided to test the stability of three different commercially available ADH (from horse liver, HLADH, from *Thermoanaerobium brockii*, TBADH, and from *Lactobacillus brevis*, LBADH) in the presence of water-immiscible organic solvents of different functionality, regardless of their log *P*.[14] Thus, nonane was used as a representative example of unbranched alkanes, cyclohexane of cyclic alkanes, toluene of aromatic compounds, dichloromethane of halogenated solvents, *tert*-butyl methyl ether (MTBE) of ethers, and ethyl acetate of esters. Though soluble in water, *tert*-butanol was also tested as a model to study the effect of tertiary alcohols on the enzymes activity, as these alcohols cannot be accepted as substrates. The corresponding log *P* values were calculated according to the fragmentation method of Crippen (Figure 1).[15]

Samples of the enzymes were dissolved in phosphate buffer and an equal amount of an organic solvent was added.[16] The system was stirred continuously to maintain an emulsion during the whole experiment. To ensure that the observed loss of activity can be mainly attributed to the presence of the organic solvent and is not caused by thermal deactivation, the emulsion was kept at 4 °C. Samples were taken periodically and the activity was measured under standard assay conditions.[17]

For all three enzymes the measured residual activity (A_t) can be described by considering the contributions of instantaneous inhibition (I) and time-dependent deactivation ($k'_{dea}t$) [Eq. (1)].

$$A_t = A_0\, I\, e^{-k'_{dea}t} \quad (1)$$

I was determined by comparing the enzyme activity in the water phase before and five minutes after the addition of the organic solvent. Its magnitude depends on the specific interaction between organic solvent and enzyme and no general tendency can be observed.[18] Surprisingly, an increase in activity after the addition of the solvent was observed in some cases.

The observed time-dependent deactivation of all three enzymes can be described as a first-order exponential decay of the activity.

This model allows the calculation of the apparent rate constant of deactivation (k'_{dea}) and the half-life for every combination of enzyme and solvent.[19] To evaluate the importance of the hydrophobicity of the solvent for the retention of enzyme stability in multiliquid systems, histograms showing the calculated half-life were ordered according to increasing log *P*-values (Figure 1). The enzyme stability shows no dependence on the solvent hydrophobicity.

A comparison of the three graphs reveals great similarity concerning the stability pattern of the three enzymes in the presence of organic solvents. While unsatisfactory results were obtained with aromatic, aliphatic hydrocarbons, or halogenated solvents, all three enzymes have the highest stability in the presence of MTBE. This finding is consistent with the assumption that the solvent functionality would be of greater significance for enzyme deactivation than the frequently mentioned log *P*. To test this hypothesis, the stability of the enzymes in the presence of another three short-chained ethers (diethyl ether, diisopropyl ether and di-*n*-butyl ether) was studied. In the case of diethyl ether and diisopropyl ether the stabilities observed were again higher than those in the presence of aromatic and aliphatic hydrocarbons, and halogenated solvents, thus strengthening our assumption. For di-*n*-butyl ether the results obtained were less satisfactory and comparable with those of other solvents. Thus, it was established that log *P* was not an adequate parameter to describe the trend of enzyme tolerance to organic solvents, even while comparing ethers. Rather, a trend of higher stability of the enzymes in the presence of ethers with short-chained aliphatic residues was observed.

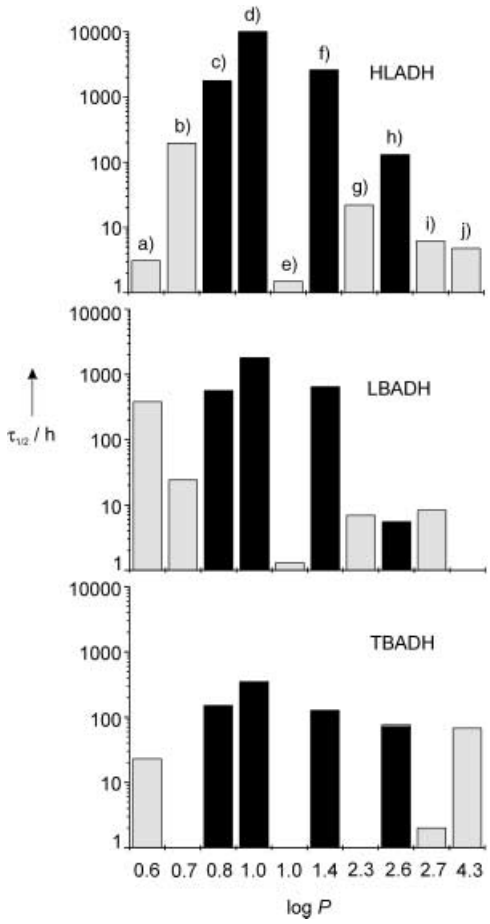

Figure 1. Logarithmic plot of the half-lives of HLADH, LBADH and TBADH at 4 °C in biphasic systems (aqueous/organic solvent 50:50, v/v): a) *tert*-butanol (log *P* = 0.6), b) ethyl acetate (log *P* = 0.7), c) diethyl ether (log *P* = 0.8), d) *tert*-butyl methyl ether (log *P* = 1.0), e) dichloromethane (log *P* = 1.0), f) di*iso*propyl ether (log *P* = 1.4), g) toluene (log *P* = 2.3), h) di-*n*-butyl ether (log *P* = 2.6), i) cyclohexane (log *P* = 2.7), j) nonane (log *P* = 4.3).

Further studies were carried out with MTBE because of the good results obtained and the absence of peroxide formation. LBADH was arbitrarily chosen as the enzyme to be used in the biphasic biotransformation studies.

The feasibility of the biphasic biotransformation was confirmed by reducing acetophenone to (*R*)-phenylethanol. A solution containing LBADH and the reduced cofactor NADPH was mixed with a solution of 1.0 mM acetophenone in MTBE in a repetitive batch mode (Figure 2).[20] In this way,

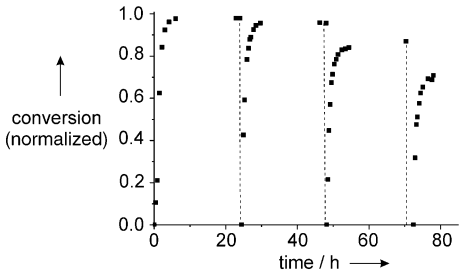

Figure 2. First four repetitive batches of the LBADH-catalyzed reduction of acetophenone in MTBE/water 50:50, v/v biphasic system at 20 °C (enzyme concentration = 0.4 U mL^{-1}).

the productivity of enzyme and cofactor was enhanced. The simple distillation of the solvent allows a facile workup. This procedure was repeated for 1400 h since the half-life of LBADH at room temperature under these conditions is 480 h. The obtained (*R*)-phenylethanol is optically pure (*ee* > 99 %, GC-analysis), thus confirming that the enantioselectivity of the enzyme is not altered by the presence of an organic phase.[21] NADPH is regenerated in situ by the addition of 2-propanol to the emulsion in a concentration of 1.5 M.[22] The acetone accumulation caused a progressive limitation of the achievable conversion.[23]

The utility of the biphasic approach was demonstrated by its application in the reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate (**1**) to *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate (**2**) by LBADH.[24] This reaction is of practical interest because the product is an advanced building block in the synthesis of HMG-CoA reductase inhibitors.[25] The substrate is poorly soluble and also unstable in water. Through the elimination of HCl the corresponding furanone **3** is formed (Scheme 1). In an MTBE/water biphasic system ten times the amount of **1** could be converted in one single batch than would have been possible in pure water. A 210 mM solution of **1** in the organic solvent was used, thus increasing the productivity of enzyme and cofactor. Moreover, the undesired degradation of the substrate could be discriminated; high conversion (98 %) and selectivity (70 %) were achieved. Once again, the enantioselectivity of the enzymatic reduction was not affected (*ee* > 99.5 %). For the regeneration of the cofactor 2-propanol was used as a reducing agent.[26]

In conclusion, we have shown that the log *P* concept is not satisfactory to guide the choice of an organic solvent serving as the second phase in ADH-catalyzed reactions. The biphasic enzymatic reduction of ketones is possible if short-chained aliphatic ethers are employed. This suggests that a single physicochemical parameter is incapable of satisfactorily predicting the biocompatibility of organic solvents, given the complexity of the enzyme deactivation in biphasic systems.[27] Rather a set of properties is necessary to guide the solvent choice. Based on our results, we propose that the set of properties inherent in the chemical functionality (e.g. ether) give rise to the compatibility of solvents with alcohol dehydrogenases.[28] Therefore, we recommend that instead of the hydrophobicity of the solvent (log *P*) its functionality should be considered when screening for a solvent. The identification of the same pattern of stability in the presence of solvents for benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I,[29] and the report that an *S*-hydroxynitrile lyase from *Hevea brasiliensis* is stable in the presence of MTBE encourages the application of this idea for other enzymes which are active in the bulk of the aqueous phase.[30]

The tolerance of ADH towards organic solvents described herein increases the compatibility of the enantio- and regioselectivity of the enzymes with hydrophobic nonnatural substrates. Moreover, the presence of a water-immiscible solvent facilitates the use of ADH in continuous operations.

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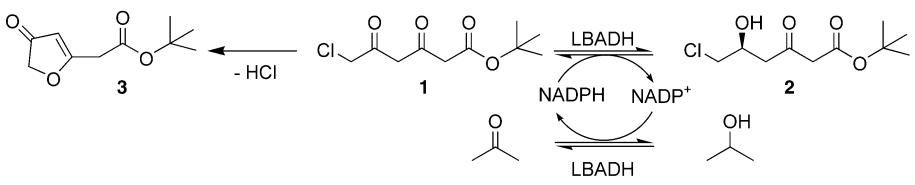

Scheme 1. Enzymatic reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate with substrate-coupled cofactor regeneration and the undesired formation of furanone **3**.

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[16] To minimize changes in the phase volumes, the organic solvent was previously saturated with water. The reaction mixture was shaken vigorously for one minute resulting in the saturation of the aqueous phase with the organic solvent.
[17] Protocol for the standard assays are available as Supporting Information.
[18] Neither the nature (competitive, uncompetitive or noncompetitive) nor the cause of this instantaneous inhibition was further investigated; the values are available as supporting information.
[19] Half-lives are calculated as $\tau_{1/2} = \ln 2/k'_{dea}$.
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