3 Kinetic Characterization of Enzymes

3.1 Methodological Background

The pre-screening of enzymes by the industrial partner exhibited two ADH which are suitable for the enantioselective reduction of prochiral ketones, the NADP-dependent LB-ADH and the NAD-dependent SADH-X2. Of the LB-ADH two different preparations will be used: a standardized lyophilized preparation, and a liquid preparation which was specially purified by column chromatography. The SADH-X2 is also applied as standard lyophilized preparation. Furthermore, for enzyme dependent cofactor regeneration a lyophilized NAD-dependent and a lyophilized NADP-dependent MDH were pre-screened.

For the characterization of enzymes in general and ADH in particular kinetic parameters like maximum activity v_{max} and the Michaelis-Menten constant K_M are significant values. Furthermore, in case of substrate-excess inhibition the constant K_S is of importance. To perform kinetic measurements UV/Vis spectroscopy is applied. In case of an ADH characterization the absorbing species are the reduced cofactors NADH and NADPH. The change in absorption is direct proportional to the reaction rate. The change in absorbance is monitored at different substrate concentrations, i.e. ketone or alcohol, the initial reaction rate v_0 is calculated from it using the slope m (Figure 3-1, for more details see Chapter 7.5) and is plotted against the initial substrate concentration (Figure 3-2). This is the so-called Michaelis-Menten plot using Equation 3-1 providing two kinetic parameters, v_{max} (Equation 3-2) and K_M (Equation 3-3) based on the standard reaction scheme (Scheme 3-1). v_{max} is the maximum reachable reaction rate, K_M is the Michaelis-Menten constant describing the affinity of the enzyme towards the substrate at the given reaction conditions. A high value for the concentration at which ½ v_{max} is reached means a low affinity of the enzyme towards the substrate.

For the UV/Vis spectroscopy a multiplate reader by Biotek is used. The measurements are done using 96-well plates with a maximum volume of 220 µL. A maximum of 8 measurements is done in parallel. Reactions are usually started by addition of enzyme; measurements are started immediately after enzyme addition.

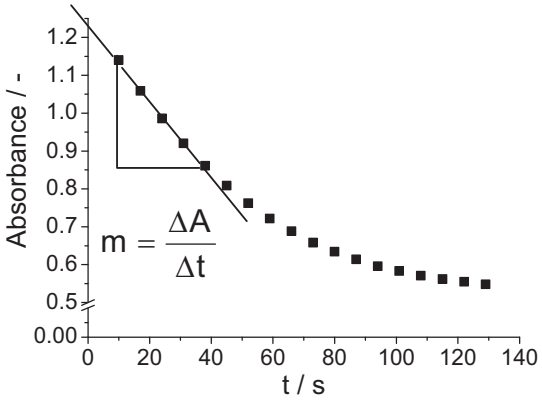

Figure 3-1 Illustration of typical absorption/time plot

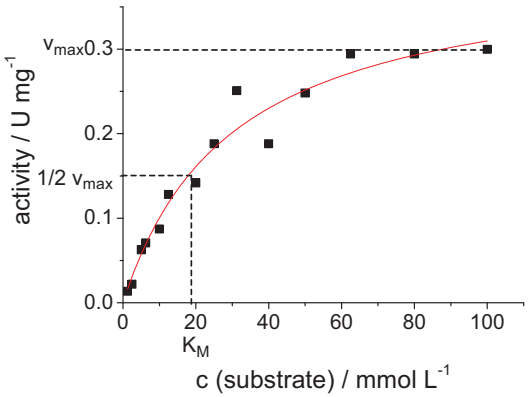

Figure 3-2 Illustration of typical Michaelis-Menten plot

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E+P$$

Scheme 3-1 General reaction scheme of an enzyme catalyzed reaction

$$v = \frac{v_{max} \cdot c_S}{K_M + c_S}$$

Equation 3-1 Michaelis-Menten equation

$$v_{max} = v \frac{K_M + c_S}{c_S}$$

Equation 3-2 Definition of v_{max} resulting from Michaelis-Menten equation

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

Equation 3-3 Definition of K_M

The pre-screening of reaction conditions by the industrial partner showed the following standard conditions for the enzymatic assays for all three ADH preparations. Enzyme assays and the resulting activity data are the only way to characterize enzymes properly. As they are produced by means of molecular biology the protein content may vary which does not ensure continuous quality of activity.

c (cofactor) = 0.5 mmol L^{-1}
c (buffer) = 50 mmol L^{-1} potassium phosphate buffer
pH = 6.5
T = 30 °C

A pool of prochiral ketone substrates and acetone were selected for the screening as well as the corresponding alcohols (Figure 3-3). Substrate concentrations were chosen depending on the water-solubility of the respective substrate. Based on the standard assay the substrate spectrum of the three ADH will be tested. Furthermore, the dependence of the enzymes on the following parameters will be tested:

- buffer concentration
- cofactor concentration
- substrate concentration
- addition of salts ($MgCl_2$, $MnCl_2$)
- pH value
- saturation of the buffer with MTBE

The tolerance towards MTBE is important for the application of the enzymes in biphasic reaction systems. From the recorded data enzyme activity is calculated and plotted against the respective substrate concentration. If possible, a Michaelis-Menten fit is obtained using Origin. At the beginning of each sub-chapter all performed measurements including v_{max} and K_M are summarized to give an overview of the obtained results. The results show that every system reacts different to a change of a certain parameter. Optimum reaction conditions cannot be generalized.

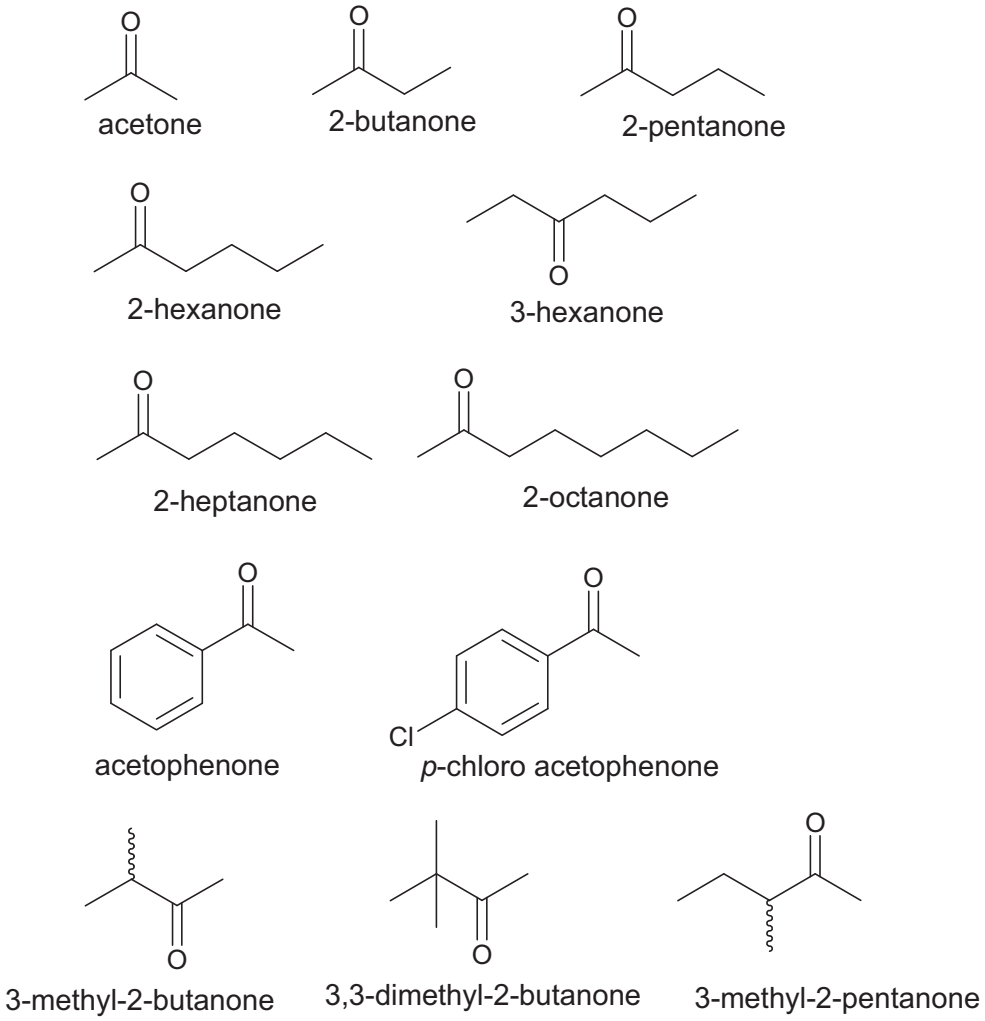

Figure 3-3 Prochiral ketone substrates

3.2 Substrate Screening

The solid SADH-X2, the solid and the liquid LB-ADH preparation are tested for their activity towards different substrates. All show good activities in presence of the selected ketones.

The SADH-X2 shows high activities of 12 – 14 U mg^{-1} for the reduction of 2-hexanone, 2-heptanone, and 2-octanone (Figure 3-4). The shorter the chain of the ketone is the lower is the activity. It increases from 0.5 U mg^{-1} for 2-butanone and 2.8 U mg^{-1} for 2-pentanone to 11.7 U mg^{-1} for 2-hexanone. The activity towards 3-hexanone is 7-times lower than towards 2-hexanone. Obviously, the ethyl group is too large to enter the active site of the enzyme. Acetone as a non-prochiral ketone is also accepted as a substrate, and the SADH-X2 has a larger activity in presence of this ketone than for the reduction of 2-butanone. The orientation of 2-butanone is not well defined although it has two different R-groups. The methyl and the ethyl group are very similar, but the ethyl group cannot enter the active site. Therefore,

activity towards 2-butanone is lower than towards acetone. Activity shows a certain dependence on the size of the non-methyl group of the secondary ketone. With increasing chain length the hydrophobic interactions with the enzyme increase leading to higher reaction rates.

The SADH-X2 is able to reduce aromatic and branched prochiral ketones as well (Figure 3-5). For acetophenone (4.7 U mg^{-1}) activity is lower than for *p*-chloro acetophenone (9.3 U mg^{-1}) due to electronic effects. The values are settled between those for 2-pentanone and 2-hexanone. For the branched ketones activities are lower than for aromatic ketones. Regarding the size of the R-groups the same trends as for the linear ketones can be found. The activity towards 3-methyl-2-butanone is 2.4 U mg^{-1} and, therefore, lower than towards 3-methyl-2-pentanone which provides a larger size difference in the R-groups. The sterically more demanding 3,3-dimethyl-2-butanone is too bulky to enter the active site although it contains a methyl group. Activity is only 1.5 U mg^{-1} which is 1.6-times lower than towards 3-methyl-2-butanone.

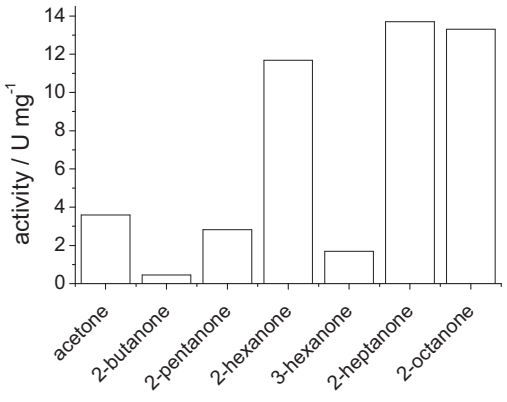

Figure 3-4 Activity of SADH-X2 towards linear ketones

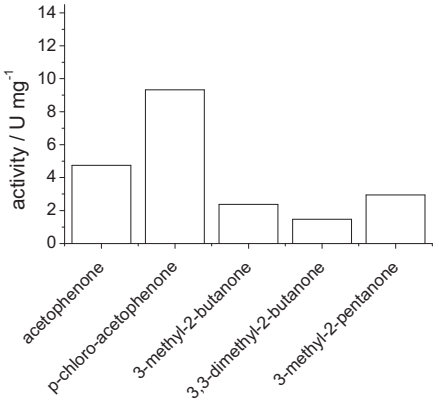

Figure 3-5 Activity of SADH-X2 towards bulky aliphatic and aromatic ketones

The solid LB-ADH preparation is tested for its activity towards linear, aromatic, and branched ketones. The activity towards 2-butanone is higher than towards acetone (Figure 3-6). The gap between the activity towards 2-pentanone (1.8 U mg^{-1}) and 2-hexanone (2.2 U mg^{-1}) is only small. For 2-heptanone a maximum in activity of 6.1 U mg^{-1} is reached. For 2-octanone activity is 4.6 U mg^{-1}.

As well as the SADH-X2 the solid LB-ADH shows activity for aromatic and branched ketones (Figure 3-7). Activity towards acetophenone is 4.7 U mg^{-1} and lower than towards *p*-chloro acetophenone with 8.2 U mg^{-1}. There is only a small difference of 0.3 U mg^{-1} obtained in activity for the reduction of 3-methyl-2-butanone (2.4 U mg^{-1}) and

3-methyl-2-pentanone (2.7 U mg^{-1}). In contrast to the SADH-X2 activity for 3,3-dimethyl-2-butanone is with 3.2 U mg^{-1} higher than towards the less bulky 3-methyl-2-butanone. The reason might be a larger active site of the LB-ADH compared to the SADH-X2.[105]

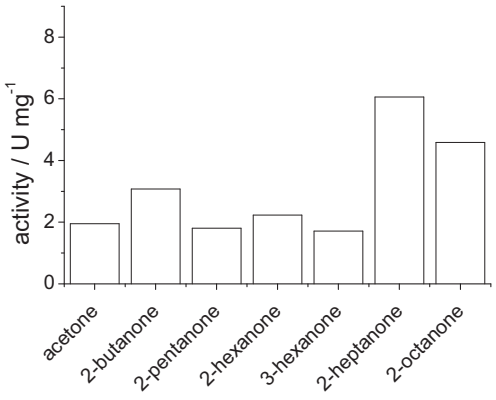

Figure 3-6 Activity of solid LB-ADH towards linear ketones

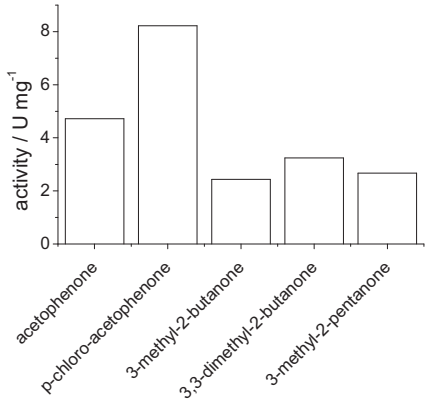

Figure 3-7 Activity of solid LB-ADH towards bulky aliphatic and aromatic ketones

The results obtained from measurements using the liquid LB-ADH preparation show that the preparation of the enzyme has a huge impact on activity. The liquid LB-ADH has high activity towards all tested ketones. The highest activity is observed for acetone with 606 U mL^{-1} (Figure 3-8). For this LB-ADH preparation the tendency of activity cannot be correlated to the chain length of the linear ketones. Surprisingly, the activity towards 3-hexanone is higher than towards 2-hexanone. This was neither found for the SADH-X2 nor for the solid LB-ADH preparation.

The activity measured towards acetophenone is 515 U mL^{-1} and lower than towards *p*-chloro acetophenone with 620.5 U mL^{-1} (Figure 3-9). For the bulky aliphatic ketones no tendency regarding the chain length is observed. Activity towards 3-methyl-2-butanone and 3,3-dimethyl-2-butanone is 369.3 U mL^{-1} and 385.1 U mL^{-1}, respectively. In contrast to the SADH-X2 and the solid LB-ADH the activity of the liquid LB-ADH towards 3-methyl-2-pentanone is lower than for the other two bulky ketones.