Expanding the catalytic activity of amine dehydrogenases

Rational enzyme engineering via computational analysis and application in organic synthesis

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Chapter 6

Development of a screening methodology for idenification of (*S*)-selective amine dehydrogenases

The project described in this chapter is in progress

6.1 Abstract

Amine dehydrogenases (AmDHs) represent one of the most rapidly developed enzyme family for the synthesis of a-chiral amines. However, most of the AmDHs developed up to date display (*R*)-selectivity. Engineering of new (*S*)-selective AmDHs is one of the main challenges. In this study, we have developed a screening assay for the identification of (*S*)-configured amines in solution that can be applied for screening novel (*S*)-selective AmDHs. After expression and purification of the potential AmDHs in the 96-deep well blocks, the variants can be screened for the desirable reductive amination direction. The high enantioselectivity of this assay relies on a monoamine oxidase (MAO) capable of oxidizing exclusively (*S*)-configured amines to their corresponding imines producing H_2O_2. The H_2O_2 formed is subsequently used by a horseradish peroxidase (HRP) to form a colored product that can be identify both by eye and spectrophotometrically. Amines with (*R*)-configuration or samples with no amines did not result in any coloration of the sample significantly reducing the possibility of false positive results. It is expected that the assay will be apply for identification of novel (*S*)-selective AmDHs in the future.

6.2 Introduction

Amine dehydrogenases (AmDHs) catalyze the reductive amination of carbonyl compounds at the sole expense of ammonia and a hydride source yielding a-chiral amines with excellent enantioselectivity. When combined with cofactor recycling enzymes (e.g., FDH, GDH), AmDHs represent an enzyme family that displays increased atom economy for the synthesis of chiral amines. Therefore, AmDHs has been the subject of extensive investigation during the last years, In fact, since 2012, fourteen AmDHs are now available, using genome mining or semi-rational approaches as discussed in the first chapter. Apart from the recently discovered (*S*)-selective native AmDHs (nat-AmDHs),[1] all other members have been created starting from L-amino acid dehydrogenases (L-AADHs) by targeting residues that interact with the a-carboxyl-moiety of the substrate. For instance, *Bs*-AmDH,[2] *Bb*-AmDH,[3] *Rs*-AmDH,[4] *cal*-AmDH,[5] *Es*-AmDH,[6] *Lf*-AmDH[7] and *Bsp*-AmDH (displaying *R*-selectivity) were all engineered from L-PheDH or L-LeuDH by substituting the two highly conserved residues that interact with the carboxyl group of the substrate (i.e., lysine and aspartate). Moreover, we have recently created (*R*)-selective AmDHs (LE-AmDHs) starting from L-lysine-(ε-deaminating)dehydrogenase (LysEDH) that does not catalyze any apparent asymmetric transformation on its natural reaction/substrate but displayed (*S*)-selectivity when interrogated for the amination of 6-oxo-hexanoic acid, which is structurally related to the natural substrate (*L*-Lysine).[8] As a result, until now nine out of fourteen available AmDHs display (*R*)-selectivity. These (*R*)-selective AmDHs are capable of transforming a wide range of carbonyl compounds into the corresponding amines. In contrast, the (*S*)-selective nat-AmDHs are all active towards a similar substrates, practically limited to small aliphatic ketones and cycloalkanones.[1]

One of the major limitations of the AmDHs is the limited availably of (*S*)-selective members. This is due to the similar scaffolds and approach that have been used for engineering of new AmDHs as well as due to the lack of a screening assay for the detection of (*S*)-configured amines. So far, the identification of AADH variants as potential AmDHs have been performed using an NAD^+ auto fluorescence

assay. Fluorescence-relative quantification of NAD^+ produced after reductive amination could be monitored at high pH (pH >13) and ranked based upon the differential increase in fluorescence over a background reaction. The long reaction times and the low signal to noise ratio are two major limitations of this assay.[2] A formazan-based assay have been also used for screening of AmDHs.[2,4] This assay is based on the methodology developed by Chen *et al*.[9] The enzymatic deamination activity is related to the conversion of NAD^+ to NADH, which is reoxidized to NAD^+ by phenazine ethosulfate (PES). The resulting reduced PES subsequently reduces 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride hydrate (INT) to create formazan. Formazan creates a deep red color, which can be monitored at 495 nm. However, it is expected that not all AmDHs that are able to perform the reductive amination are sufficiently active in the opposite direction (or even are not active at all) as known for alcohol dehydrogenases. As a results, active variants for the reductive amination may never be detected using this assay and, very regretfully, discarded as false negatives. Another limitation reported is the increased number of false positive samples. To eliminate these false positive readings another spectrophotometric assay has been developed by comparing the samples' absorbance in two different wavelengths (340 nm and 600 nm) over a background plate at the same wavelengths.[2,5] The increased absorbance at 340 nm corresponds to the production of NADH in the deamination direction, while absorbance at 600 nm estimates the amount of biomass present. This simple assay can only be applied for the deamination reaction. It should also mentioned that the amine substrate or the ketone product may interfere with the detection if absorbance occurs in wavelengths close to NADH absorbance.

Herein, we reported the development of a screening methodology for the detection of (*S*)-configured amines. Both expression and purification can be performed in 96-deep well blocks using Ni-NTA coated magnetic beads. Purification with Ni-NTA coated magnetic beads is optional. The amines can be detected in aqueous solutions without the need for extraction. Moreover, the potential AmDHs can be screened in the desirable, reductive amination,

direction. One of the main advantages of this colorimetric assay is its high enantioselectivity, since in the presence of (*R*)-configured amines the samples did not result in any coloration. Moreover, in the absence of the amine product, the reaction did not occur, thus eliminating the possibility to obtain false positive results. This assay will be applied for the screening of D-AADH variants as potential (*S*)-selective AmDHs.

6.3 Results and discussion

6.3.1 Electrocompetent cells and electroporation

The electrocompetent cells were prepared based on BioRad MicroPulser application guide as describe in methods (section 6.5.1). The electrocompetent BL21(DE3) cells were used for high efficiency electrotransformation of the plasmid encoding for D-AADH[10] using MicroPulser electroporation apparatus (BioRad) and following manufacturer's instructions (see methods 6.5.1). Aliquots of 100 μL of the 1 mL transformed cells were spread in large petri dishes (approx. 100 mL), and grown at 37 °C overnight. Next day more than 1000 colonies were observed in each petri dish indicating that the method is suitable for the preparation of large number of colonies (~ 30.000) for screening purposes.

6.3.2 Development of an expression and purification assay in 96-deep well blocks

In this assay the Ch1-AmDH[11] was chosen as tested enzyme because of its high expression yield in order to exclude any limitation of the methodology due to enzyme's poor expression. Cultures of Ch1-AmDH were performed in 1 or 2 mL 96-deep well blocks, in order to find the best cultivation volume. In both conditions the enzyme expressed well using the reported methodology (see methods 6.5.2). Higher expression yield was observed in 2 mL cultures in relation to the 1 mL cultures. The lysis of the cells with the reported procedure resulted in enough amount of protein in the soluble fraction for downstream applications.

Purification of the 96-deep well blocks were performed after optimization of the procedure reported by manufacturer using Ni-NTA coated magnetic beads. Optimization was performed by purification trials of known concentration of Ch1-AmDH. The aim was to find the best relationship between the amount of the enzyme that the beads can bind and that can be eluted in one fraction. In order to achieve this, five parameters were tested and adjusted: the quantity of protein that can be bound to a specific volume of beads, the induction time of the sample with beads, the imidazole concentration in the elution buffer as well as the number and volume of elution fractions. After a series of experiments, we

obtained approximately 300 µg of the Ch1-AmDH in the first elution fraction when eluted with 500 mM imidazole (50 µL, 6 mg/mL). Incubation time of the sample with the beads was 20 min and elution time was 5 min. Using these conditions, the supernatants obtained from the *E. coli* cultures of the 2 mL and 1 mL 96-deep well blocks were purified and analyzed by SDS-PAGE. In both cases the enzyme was obtained in high purity at the first elution fraction. Moreover, the purification yield was consistent among the different wells of the same plate.

In order to investigate a possible variation of the conversion levels for the amination catalyzed by the AmDH enzyme obtained from different positions within the same plate, we performed biocatalytic reactions using the eluted enzymes. The substrate *para*-fluoro-phenylacetone was chosen as it is known to be fully converted by Ch1-AmDH to the corresponding amine after 24 h using 90 µM of enzyme.[12] More specifically, the reaction shown in **Figure 1** was performed for 3 h and for 24 h. After 3 h, almost 40% conversion was observed in all different samples tested; after 24 h, all samples tested gave full conversion (**Figure 1**), thus confirming the reproducibility of the assay.

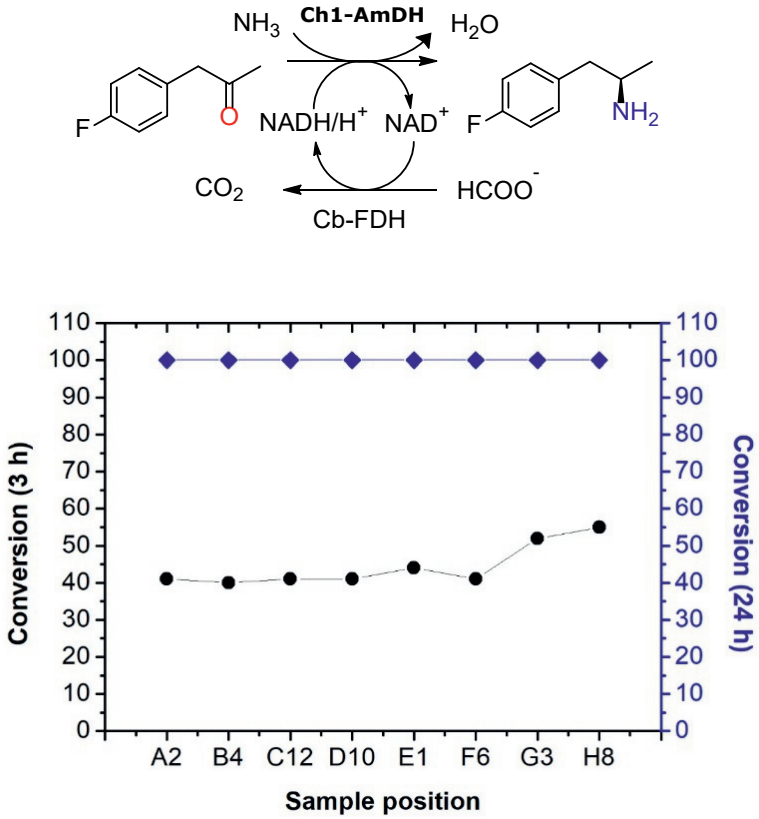


Figure 1. Biocatalytic reactions performed with Ch1-AmDH obtained after expression and purification in 96- deep well blocks.

6.3.3 Small scale expression and purification of D-AADH

The D-AADH has been reported to catalyze the reductive amination of D-amino acids.[10] However the enzyme showed no activity for the reductive amination of ketones or aldehydes tested (2-heptanone, 2-octanone, *para*-fluoro-phenylacetone, phenylacetaldehyde, hexanal, heptanal) but it was found to be active with phenyl-pyruvate. Therefore, the D-AADH was used as a starting template for the construction of library of potential (*S*)-selective AmDHs. This library was constructed by saturation mutagenesis (using NBT degenerated codons) of the enzyme's amino acid residues that are involved in the interaction with the substrate's carboxyl group (Asn244, Asn270, Ser149). For 95% coverage of the library, a screening of 5175 colonies was calculated to be required (i.e.,

including codon usage bias). Prior to the screening of the colonies, the applicability of the assay was tested using the parental enzyme.

Expression and purification were performed with 0.5 mM IPTG, overnight at 25 °C in 2 mL 96-deep well blocks (see methods 6.5.3) as in the case of Ch1-AmDH. These conditions resulted in high expression of the enzyme (**Figure 2**, line 2), while no enzyme was observed before induction (**Figure 2**, line 1). Cell disruption was performed with 900 µL B-PER bacterial protein extraction reagent supplemented with lysozyme (9.6 mg mL^{-1}) and DNAse (0.04 mg mL^{-1}) for 90 min at 25 °C. This lysis protocol resulted in high levels of enzyme in the soluble fraction, thus indicating efficient cell lysis (**Figure 2**, line 3), while only a minor part of the enzyme was observed in the insoluble fraction (**Figure 2**, line 4). The flow-through sample (after incubation of the Ni-NTA beads with the lysate) showed that almost all of the enzyme was bound into the beads (**Figure 2,** line 5). Remarkably, almost all the enzyme was eluted under the reported conditions in the first elution fraction (**Figure 2, line 6**).

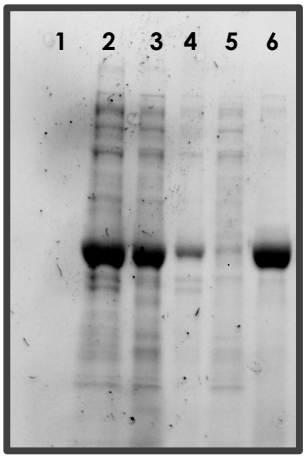


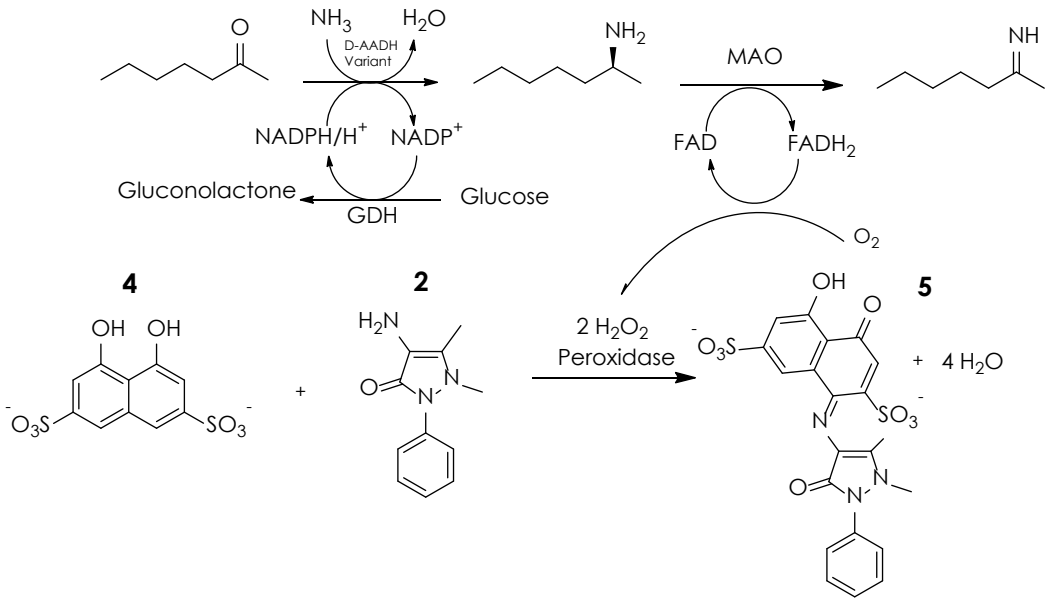
Figure 2. SDS page analysis of the expression, solubility and purification of D-AADH with magnetic beads. (1) Sample before induction; (2) sample after induction); (3) soluble fraction; (4) insoluble fraction; (5) sample after incubation of the enzyme with magnetic beads; (6) elution fraction.

Since the purified enzyme was in imidazole-containing buffer that absorbs at 280 nm, the concentration of the enzyme could not be determined

spectrophotometrically and therefore was calculated using the Bradford method. The calibration curve for the Bradford solution was calculated using BSA (see methods 6.5.3). On average 0.5 mg mL^{-1} of enzyme was present in each elution sample (50 µL, 25 µg in total).

6.3.4 Assay for the detection of (*S*)-configured amines using chromotropic acid

After purification of D-AADH variants (50 µL), they will be tested for the reductive amination of 2-hexanone. The 2-hexanone was chosen as substrate because of the reported high activity of D-AADH with 2-oxo-heptanoic acid. We hypothesize that the more hydrophobic active site of the engineered D-AADH variants will favor the acceptance of 2-hexanone. Furthermore, 2-hexylamine is accepted by the MAO-D5 in the next step of the screening procedure, which is graphically summarized in **scheme 1**.



Scheme 1. Propose screening assay for D-AADH variants using chromotropic acid.

This assay is based on the peroxidase assay reported by Wong *et al*.[13] After the reductive amination of 2-hexanone by D-AADH variants, the MAO-D5[14]—which accepts only (*S*)-configured amines—will be used for oxidizing (*S*)-2-hexylamine to its corresponding imine.[15] The hydrogen peroxide formed from this reaction will

be used by the horseradish peroxidase (HRP) to conjugate compounds **4** and **2** forming compound **5** (**Scheme 1**). This compound (**5**) can be easily identified by eye (deep blue) or spectrophotometrically at 590 nm.

Initially the assay was tested with the reported conditions using 100 mM sodium phosphate buffer pH 6.5, 3 mM 4-aminoantipyrine (AAP, compound **2**) and 15 mM chromotropic acid (CTA, compound **4**) as well as 10 mM H_2O_2. In this assay, 5 mM of (*S*)-2-heptylamine were added and the reaction started by addition of HRP (10 µg mL^{-1}). **Figure 3** shows an intensive blue color that is formed after 5 min in the reaction samples (A2-C2). In contrast, the non-protein-containing control (NPC without MAO) did not result in any coloration of the samples (A1-C1).

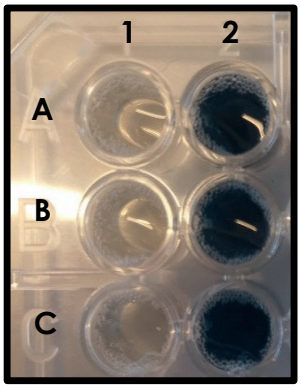


Figure 3. Initial test of the colorimetric assay using chromotropic acid.

Another set of reactions were prepared using 100 mM KPi 7.6, which is the preferred buffer by MAO-D5.[15] Indeed, after 5 min the reactions performed in KPi buffer also became blue. All reactions (both in sodium phosphate and in KPi) were measured spectrophotometrically at 590 nm. Results are shown in **Table 1**. In general, the reactions showed higher absorption values in KPi pH 7.6 compared with sodium phosphate after 5 min. Moreover, in KPi the absorption values at 590 nm for the negative controls remained low (i.e., <0.25) even after 30 min.

Table 1. Absorption values at 590 nm of the colorimetric assay in different buffers.

100 mM Sodium Phosphate pH 6.5 Abs 590 nm				**100 mM KPi pH 7.6** Abs 590 nm			
5 min		30 min		5 min		30 min	
NPC	Reaction	NPC	Reaction	NPC	Reaction	NPC	Reaction
0.17	0.84	0.50	3.6	0.08	1.03	0.2	3.4
0.13	0.86	0.51	3.6	0.12	0.83	0.22	3.3
0.13	0.90	0.56	3.5	0.11	0.90	0.19	3.3

NPC: Non protein control

In the next step, the sensitivity of the assay at low concentrations of H_2O_2 was investigated. The amount of H_2O_2 is related with amount of (*S*)-configured amine produced from the first step of the screening process (reductive amination of ketone). To investigate the lowest amount of H_2O_2 that will still result to measurable amounts of compound **5**, the reactions were performed with 0.1-5 mM of H_2O_2. **Table 2** summarizes the absorption values obtained from the reactions performed after 5 min reaction time. It is evident that the assay can be used even at concentrations of H_2O_2 as low as 0.1 mM. At this concentration the values obtained are still significantly higher than the negative control (0 mM H_2O_2) thereby confirming the sensitivity of the assay. The absorption values above 2 mM of H_2O_2 were observed to be 3.4 because of the saturation of the reaction above this concentration.

Table 2. Absorption values at 590 nm of the colorimetric assay at different concentrations of H_2O_2.

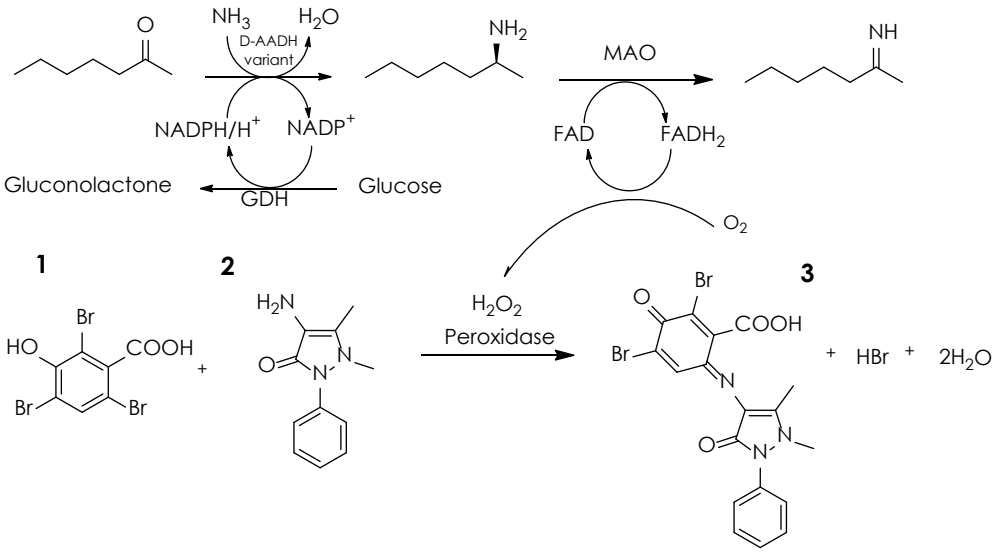
[H_2O_2] mM	Reaction Time (min)	Abs 590 nm
0.0	5	0.15
0.1	5	0.35
0.2	5	0.86
0.3	5	1.30
0.4	5	1.70
0.5	5	2.13
1	5	3.35
2	5	3.4
3	5	3.4
4	5	3.4
5	5	3.4

All results using the CTA assay were obtained with external addition of H_2O_2. However, the activity of MAO may be influenced by CTA. Therefore, in another set of reactions, the assay was performed without any external addition of H_2O_2. The amount of H_2O_2 required was *in situ* produced by the oxidation of (*S*)-2-heptylamine to its corresponding imine by the action of MAO (see methods part 6.5.4 for expression of MAO-D5). The reactions were performed with 15 µL of MAO-D5 (cell free extract) and using different concentrations of (*S*)-2-heptylamine. Unfortunately, no color was observed in any of these reactions, indicating that MAO is possibly inhibited by CTA. A positive control using the 2,4,6-tribromo-3-hydroxybenzoic acid was also included and it was ensured that MAO was active. Therefore, this assay was not investigated any further.

6.3.5 Assay for the detection of (*S*)-configured amines using 2,4,6-tribromo-3-hydroxybenzoic acid

An alternative assay for the detection of (*S*)-configured amines, which is schematically shown in **Scheme 2**, has been developed. The

2,4,6-tribromo-3-hydroxybenzoic acid (**Scheme 2**, compound **1**) was used instead of chromotropic acid (CTA). This compound is commonly used to evaluate the activity of the MAOs for screening both enzyme and substrate libraries.[15] The conjugated product (**Scheme 2**, compound **3**) produces an intensive magenta color and can be determined spectrophotometrically at 510 nm.



Scheme 2. Colorimetric assay with 2,4,6-tribromo-3-hydroxybenzoic acid for the detection of (*S*)-configured amines.

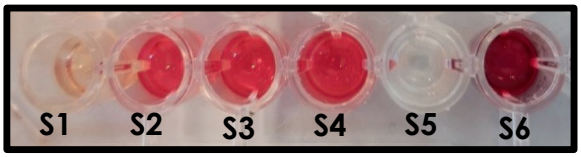
In the first set of reactions, it was ensured that MAO-D5 is active in the presence of 2,4,6-tribromo-3-hydroxybenzoic acid using (*S*)-configure a-methylbenzylamine. The a-methylbenzylamine has been reported to be accepted by MAO-D5.[14,15] Reactions were performed with 5 mM (*S*)-configured a-methylbenzylamine with different amounts of MAO-D5 (5-15 µL cell free extract). In all cases the reaction mixture became colored (magenta) after the first 10 min. The same assay was also performed using the (*R*)-configured a-methylbenzylamine. These reactions remained uncolored indicating no activity of MAO-D5 at all, even after 24h. In all cases, a non-protein-containing control (NPC), which did not contain MAO-D5, was also included; thus, it was

ensured that the reaction does not occur in the absence of MAO-D5 due to the required H_2O_2 for the HRP. Next, the acceptance of *rac*-2-hexylamine was tested using the same assay since this is the target compound for screening of the library. Reactions were performed at different concentrations of *rac*-2-hexylamine from 0.1-5 mM. Results showed that after 20 min a magenta color was visible in the reactions samples containing the substrate, the exception being the reaction performed with 0.1 mM substrate; this latter sample required approximately 3 h for the color to be visible by eye.

For the screening of the library of variants of D-ADDH, two separated reaction steps are performed after purification of the biocatalyst. The first step is the addition of the reaction mixture (**RM**, 50 µL) into the purified enzyme (50 µL). The reaction mixture contains: ketone (10 mM), GDH (2 mg/mL), D-glucose (100 mM), and $NADP^+$ (2 mM) in 400 mM NH_4Cl buffer pH 9 supplemented with 100 mM K_2CO_3 as previously reported for this enzyme.[10,16] The second step is the addition of 200 µL of assay solution (**AS**) containing 0.54 mM of compound **1**, 0.75 mM of compound **2** (**Scheme 2**), 15 µL MAO-D5 (15 µL) and HRP (10 µg/ mL) in 500 mM KPi pH 7.6. To simulate the above mentioned conditions in a hypothetical scenario in which D-AADH converted 50% of 2-hexanone substrate at initial 10 mM concentration, 5 mM of 2-hexanone and 5 mM of 2-hexylamine were added into the reaction mixture; next, 50 µL of this mixture were added into 50 µL of elution buffer containing 1 mg/mL DAADH. After that, 200 µL of the assay solution was added. This reaction was performed in triplicate (**Figure 4**, S2-S4). Another reaction was prepared without addition of amine and used as a negative control (**Figure 4**, S1). Finally, two positive controls (**Figure 4** S5 and S6) were prepared containing 5 mM of 2-hexylamine in the assay solution (**AS**). These controls were prepared without any addition of D-AADH and without addition of the reaction mixture (**RM**). Additionally, in the sample (S5) 2-hexanone (5 mM) was added in order to investigate any effect of the unreacted starting compound in the reaction. Results (**Figure 4**) show that after 24h all reactions became colored (S2-S4) while the negative control remained uncolored (S1). The positive control without ketone also gave the expected magenta color (**S6**).

Unexpectently, the positive control containing both ketone and amine did not result in any coloration (**S5**) after this time which colored after longer reaction time (~48h).

Figure 4. Assay for the detection of amines under screening conditions.



6.4 Conclusions

The scarce availability of (*S*)-selective AmDHs is one of the major limitations of this class of enzymes. This can be partly overcome using highly enantioselective assays for screening of new (*S*)-selective AmDH variants. In most of the reported cases, engineering new AmDHs have been achieved by screening libraries in the oxidative deamination direction leading to a decreased possibility to identify active variants in the reductive amination reaction. Moreover, the variants were used as cell free extracts and therefore consumption of the cofactor by other enzymes produced by the host organism may lead to false positive results. Herein, we have developed an assay for the screening of (*S*)-selective AmDHs. Expression and purification can be performed in 96-deep well blocks using NI-NTA coated magnetic beads that bind to the His-tagged variants. After optimization of the purification procedure, we were able to obtain most of the enzyme in the first elution fraction. The enzymes were screened for the desirable reductive amination direction. The formation of (*S*)-configured amines can be detected by eye using two enzymes: a monoamine oxidase (MAO) and a peroxidase (HRP). The first enzymes oxidizes exclusively (*S*)-configured amines into the corresponding imines producing H_2O_2, which can be subsequently used by the second enzyme that produces a colored product. The HRP was tested for the conjugation reaction using both chromotropic acid (CTA) and 2,4,6-tribromo-3-hydroxybenzoic with 4-aminoantipyrine (AAP). Although the HRP was found to be active in all reactions tested, the presence of MAO negatively affected the reaction between CTA and AAP. In contrast the reactions with 2,4,6-tribromo-3-hydroxybenzoic and AAP resulted in coloration of the samples in the presence of MAO without any external addition of H_2O_2. Moreover, in the presence of (*S*)- but not (*R*)-configured amines the reaction samples were colored, indicating that the enzymes producing the desirable (*S*)-configured product (with or without perfect enantioselectivity) can be identified using this assay.

6.5 Methods

6.5.1 Electrocompetent cells and electroporation

For the preparation of electrocompetent cells, a glycerol stock of BL21(DE3) cells containing no plasmid, was used for streaking an antibiotic free LB-agar plate, grown overnight at 37 °C. Next day, a single colony was used to inoculate an overnight culture (ONC) containing LB medium (5 mL). This ONC was used to inoculate the main culture (500 mL). When the OD was ~ 0.7-1, the main culture was chilled on ice for 20 min. After this time, the main culture was transferred under flame to 10 sterile falcon tubes (10 x 50 mL) on ice. The falcon tubes were centrifuged for 15 min at 4500 rpm (4000g). The supernatants were removed and it was ensured that no supernatant remained into the falcon tubes that could affect the transformation efficiency. The harvested cells were gently resuspended under flame in 50 mL ice cold 10% glycerol (sterile) and the samples were centrifuged for another 15 min at 4500 rpm. The supernatants were removed and the pellets were resuspended in another 25 mL of 10% ice cold glycerol. After another round of centrifugation (4500 rpm) and removal of the supernatants, the cells were finally resuspended in 2 mL 10% ice cold glycerol under sterile conditions. Aliquots of 40 µL were prepared and the electrocompetent cells were store at –80 °C. For the electroporation, an aliquot of 40 µL of cells was used and gently mixed with 1.5 µL of the plasmid encoding for the D-AADH (pET28b). The mixture was cooled on ice for 1 min and transferred to a pre-cooled cuvette (sterile, 0.2 cm) under flame. The cuvette was inserted into the slide of the shocking chamber and a single pulse was delivered with the optimal voltage of 2.5 kV. The cuvette was removed from the chamber and 1 mL of SOC medium was immediately added to the cuvette. The cells were gently resuspended and transferred to a 17 x 100 mm polypropylene tube which was incubated for 1h at 37 °C.

6.5.2 Development of an expression and purification assay in 96-deep well blocks

For the development of the high throughput screening method in 96-deep well blocks, modifications of the protocol reported by Bougioukou *et al.*[17] were made in order to optimize the process. The Ch1-AmDH was used as the testing enzyme because of its favorable properties. Aliquots of 100 µL ONC were used to inoculate new 96-deep well block containing 400 µL of LB medium (in the case of 1 mL 96-well plate) or 900 µL of LB medium (in the case of 2 mL 96-well plate), supplemented with 50 µg mL^{-1} kanamycin. After 3 h incubation at 37 °C with shaking at 390 rpm another 500 µL (for the 1 mL blocks) or 1 mL (for the 2 mL blocks) of LB medium supplemented with 50 µg mL^{-1} kanamycin and 1 mM isopropyl-thiol –β-D-galactoside (IPTG) were added (final concentration of IPTG is 0.5 mM) and the plate were incubated at 25 °C overnight. The expression levels were observed by SDS-PAGE. The next day, the plate was centrifuged and the supernatants were discarded. The pellets were frozen at -20 °C for at least one hour following by thawing and resuspension in 150 µL washing buffer (phosphate buffer pH 7.0, NaCl, Tween 20, 0.04% NaN_3, IMAC purification kit). The plate was then shock frozen with liquid nitrogen and immediately thawed with warm water for five times. The lysis of the cells was finally completed by the addition of lysozyme (2 mg mL^{-1}) for 2 h at 4 °C and shaking in an orbital shaker (390 rpm). After incubation, the lysate was centrifuged at 5600 rpm for 45 minutes and the supernatants were collected for purification.

Purification was performed with QuickPick IMAC kit protocol using PickPen 8-M, after modifications of manufacturer's instructions: 100 µL of magnetic beads transferred to 150 µL regeneration buffer (Aqueous $NiSO_4$ solution, Tween 20, 0.02% NaN_3) and were washed with washing 1 buffer (phosphate buffer pH 7.0, NaCl, Tween 20, 0.04% NaN_3). After that, the beads were transferred to the sample (150 µL) and incubated at RT for 20 min with shaking at 700 rpm using an orbital shaker. After one washing step with washing 2 buffer (100 mM phosphate buffer pH 7.0, 250 mM NaCl, Tween 20, 0.04% NaN_3, 20 mM imidazole) the protein was eluted with 100 µL elution buffer (50mM sodium phosphate buffer, 300 mM

imidazole, 500 mM NaCl, pH 8.0). The final imidazole concentration of 500 mM was chosen after purification trials of known quantity Ch1-AmDH by varying its final concentration (300 mM and 500 mM) in the elution buffer and the elution volume (50 µL, 70 µL, 140 µL) in order to obtain as much protein possible in one elution fraction. The incubation time of the sample with the magnetic beads (2-20 min.) as well as the time the beads were been incubated into the elution buffer (1- 10 min) also was adjusted after a series of experiments with the aim to find the optimum incubation times.

For the high-throughput biocatalytic reactions, the enzymes were immediately used after purification. First a mixture was prepared containing $HCOONH_4/NH_3$ buffer (2M, pH 8.5), 2 mM NAD^+, FDH (3mg 10 mL^{-1}) and 10 mM of substrate (*fluoro*-phenylacetone) diluted in DMSO. The mixture was then splited in each well, until the volume of the reaction was 200 µL (100 µL of the mixture and 100 µL of the purified enzyme). The reactions were performed at 30 °C, overnight. The reactions were quenched after 24 h by the addition of aqueous 10M KOH (100 µL) and the extraction was performed with dichloromethane (CH_2Cl_2, 1 x 250 µL) followed by centrifugation. The organic phases were dried with magnesium sulfate and the conversions were measured by GC and determined on an Agilent GC system equipped with an Agilent J&W DB-1701 column (30 m, 320 µm, 0.25 µm); GC program parameters: injector 250 °C; constant pressure 6.9 psi; temperature program: 60 °C/hold 6.5 min; 100 °C/rate 20 °C min^{-1}/hold 5 min; 280 °C/rate 20 °C min^{-1}/hold 1 min.

6.5.3 Small scale expression and purification of D-AADH

For the expression of D-AADH in 96-deep well blocks (2 mL) an ONC was prepared (10 mL LB supplemented with 50 µg mL^{-1} kanamycin). For each well of the plate, 100 µL of this ONC were used to inoculate 900 µL of LB supplemented with 50 µg mL^{-1} kanamycin. This main culture was grown at 37 °C for 3 h. After this time, a 1 mL LB supplemented with 50 µg mL^{-1} kanamycin and 1 mM IPTG was added and the culture was grown at 25 °C overnight. The next day, the plate was centrifuged at 5600 rpm for 1h. The supernatants were removed and the

pellets were resuspended in 150 µL lysis buffer buffer (pH 8.0, 50 mM KH_2PO_4, 300 mM NaCl). In total 50 µL from a home-made cell lysis reagent (900 µL B-PER bacterial protein extraction reagent, 96 µL lysozyme of 100 mg/mL (concentration of stock solution) and 4 µL DNAse of 10 mg mL^{-1} (concentration of stock solution)) were added to each sample and incubated at 25 °C for 90 min to allow for enough time for cell disruption. The lysates were centrifuged for 90 min at 5700 rpm and the supernatants were transferred in a flat-bottom 96-well plate for downstream purification.

Purification was performed with 50 µL of beads using the following procedure. For each sample (well) to be purified, 50 µL of beads were transferred to a 96-well plate. This plate was put on top of a home-made magnetic plate to keep the beads on the bottom of the plate and the liquid phase (storage solution) could be easily removed. 250 µL of lysis buffer was added to the well containing the beads. The beads were incubated in lysis buffer for 1 min. After that the lysis buffer was removed and 100 µL of regeneration buffer were added to the beads and incubated for another minute. After the incubation time, the regeneration buffer was removed and another 250 µL of lysis buffer were added to the beads. After another minute, the lysis buffer was removed following by the addition of the 200 µL lysate. The lysate was incubated with the Ni-charged magnetic beads for 10 min at 4 °C. After that time the 96-well plate containing the beads was placed on the top of the magnetic plate and the liquid phase was removed. 250 µL of wash buffer (pH 8.0, 50 mM KH_2PO_4, 300 mM NaCl, 30 mM imidazole) were added and incubated for 2 min. Finally the enzyme was eluted with 50 µL elution buffer (pH 8.0, 50 mM KH_2PO_4, 300 mM NaCl, 500 mM imidazole) after incubation of another 5 min. After this step, the elution buffer containing the enzyme was stored and the beads were incubated into the 200 µL elution buffer to remove any traces of bound enzyme.

The enzyme concentration was determined using the Bradford assay. A BSA stock (10 mg/mL) was prepared. Using this stock, samples with different concentrations of BSA (0.15-0.5 mg/ mL) were prepared. 20 µL of each sample were added in 980 µL of Bradford solution and the absorbance at 595 nm was

determined. Based on the obtained values, a calibration curve was determined with slope 0.791 (R^2: 0.993). A diluted sample (1:2) of the purified D-AADH was made and 20 μL of this sample were added into 980 μL Bradford solution and the absorbance at 595 nm was measured. In order to calculate the concentration of the enzyme (mg/mL), the obtained value was divided with the slope and multiply by the dilution factor (2).

6.5.4 Expression of MAO-D5

E. coli cells (glycerol stocks) harboring pET-28a plasmids encoding for the MAO-D5 and MAO-D9 were used to prepare ONCs (5 mL LB supplemented with ampicillin 100 μg mL^{-1}). Next day, 100 μL of the ONCs were used to inoculate 10 mL of LB supplemented with ampicillin 100 μg mL^{-1} . This small main culture was grown in 30 °C until the OD_{600} ~ 0.7-1. After the required time, 8 mL of this culture were used to inoculate the main large culture (800 mL LB supplemented with ampicillin 100 μg/mL) which was grown at 30 °C overnight. The next day, the cells were harvested by centrifugation (15 min 4.500 rpm at 4 °C) and the pellet was resuspended in 50 mM KPi buffer, pH 7.6. Lysis of the cells was performed with ultrasonication (total sonication time 10 min). After centrifugation the soluble part was stored in -80 °C supplemented with FAD^+ and used as cell free extract in all reactions performed.

6.5.5 Colorimetric assays for the detection of (*S*)-configured amines.

2,4,6-tribromo-3-hydroxybenzoic acid based assay

In this assay, 15 μL of the supernatant (cell free extract) containing MAO-D5 supplemented with FAD was used. Using 0.75 mM of 4-aminoantipyrine (AAP, 1 M stock in DMSO) and 0.54 mM of 2,4,6-tribromo-3-hydroxybenzoic acid (stock 2% w v^{-1} in DMSO) an assay solution was prepared in 100 mM KPi pH 7.6. The reaction consisted of 180 μL of assay solution,15 μL of MAO-D5 cell free extract and 5 μL of horseradish peroxidase (HRP) solution (HRP stock 1 mg mL^{-1}). The reaction was started by the addition of amine (stock 1 M) at the desirable concentration.

Chromotropic acid based assay

1M of 4-aminoantipyrine (AAP, compound 2) and 500 mM of chromotropic acid (CTA, compound **4**) were prepared as stock solution in DMSO. In addition, 1M H_2O_2 stock solution in dH_2O was freshly prepared. The assay solution 2 (1 mL) was prepared by adding 3 mM AAP, 15 mM CTA and 10 mM H_2O_2 in either 100 mM sodium phosphate buffer pH 6.5 or 100 mM KPi buffer pH 7.6. The substrate (5 mM) was added from a stock solution 1 M in DMSO. Reaction was started by the addition of 10 µg mL^{-1} HRP. Reactions were measured spectrophotometrically using a fluorometer with excitation filter A-590 and no emission filter.

6.6 References

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