catalysts

MDPI

Supplementary Materials

Efficient Amino Donor Recycling in Amination Reactions: Development of a New Alanine Dehydrogenase in Continuous Flow and Dialysis Membrane Reactors

David Roura Padrosa [1,2], Zoya Nissar [2] and Francesca Paradisi [1,2,*]

[1] Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland; david.roura@dcb.unibe.ch

[2] School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK; zoyanisar@outlook.com

* Correspondence: francesca.paradisi@dcb.unibe.ch; Tel.: +41-(0)-316-314-002

Supplementary information:

Materials and methods:

Size exclusion chromatography

The molecular mass of the native proteins was determined by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column with a total bed column of 24 mL from GE Healthcare. The column was equilibrated with 20 mM sodium phosphate buffer pH 8 with 150 mM NaCl. The purified enzymes (0.5-2 mg) were then injected and the experiment run at a flow of 0.8 mL/min. Fractions were collected and those corresponding to the eluted peak were assayed for concentration and activity to confirm the protein was still active and therefore, correctly folded. A calibration curve was prepared using the weight markers from Sigma-Aldrich: β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and the void volume indicated by blue dextran (2000 kDa).

Kinetic characterisation

To measure the kinetic properties of HeAlaDH, the concentration of the corresponding substrate was varied, and the enzymatic activity measured using the same method as in the standard activity assay. Data was then plotted and fitted to the standard Michaelis-Menten curve using GraphPad™.

Stability assays

For the stability assays, typically, 0.1 mg/mL of the enzyme were incubated at the desired conditions. At regular times, a sample was taken, and the activity of the enzyme assessed using the standard activity assay. All stability assays were performed at 4ºC to minimise the temperature effect.

Supplementary tables and figures:

HeAlaDH characterisation:

- ***Protein expression:***

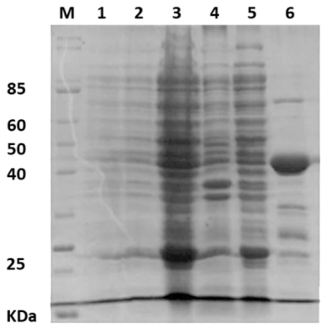


Figure S1. SDS-page analysis of HeAlaDH expression. M: molecular marker 1: Cell extract after expression; 2: Soluble fraction; 3: Insoluble fraction; 4: Flow through in IMAC; 5: Washing step; 6. Pure protein.

- ***Michaelis-Menten kinetics:***

In the deamination direction, HeAlaDH exhibited a K_M for alanine of 10.3 ± 2.4 mM and of 0.20 ± 0.04 mM for NAD+, with specific activity of 18 ± 2 U/mg at pH 8. In the reverse direction, the enzyme was much more active, with a specific activity of 124 ± 13 U/mg at pH 10. Moreover, the affinities for the substrates in the reductive reaction were lower when compared to alanine (0.60 ± 0.11 mM for pyruvate, 0.05 ± 0.01 mM for NADH) except for ammonia, for which the K_M was 77.8 ± 12.6 mM

Oxidative deamination:

Reductive amination:

Figure S2. Kinetic characterisation of HeAlaDH in both the oxidative deamination and the reductive amination direction. Reactions in the oxidative direction were performed at pH 8 while the reductive deamination was performed at pH 10.

- ***Resistance to organic co-solvents:***

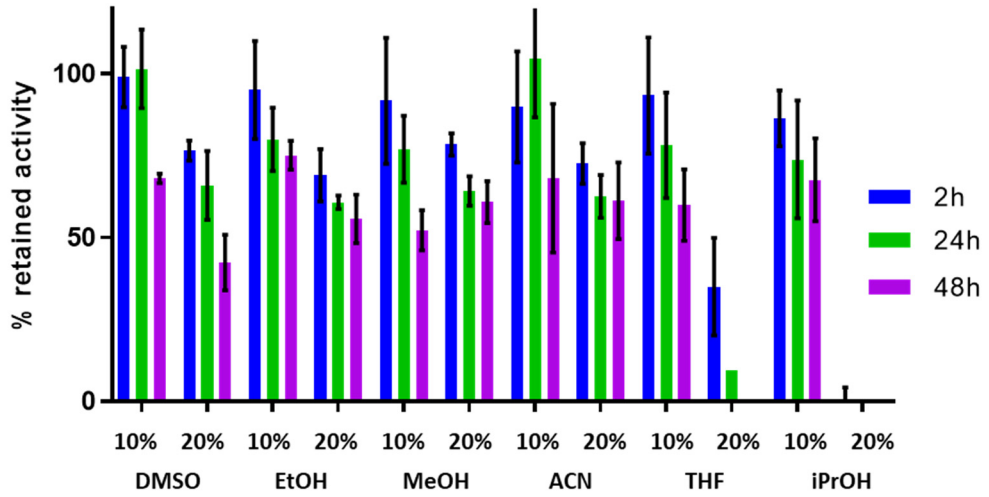


Figure S3. Cosolvent effect on the stability of HeAlaDH. 0.1 mg/mL of protein was incubated at 4ºC for 48h in the presence of 10% or 20% (v/v) of the desired cosolvent. Activity was tested at different time points using the standard activity assay. The values correspond to the average of 3 replicates.

- ***pH stability:***

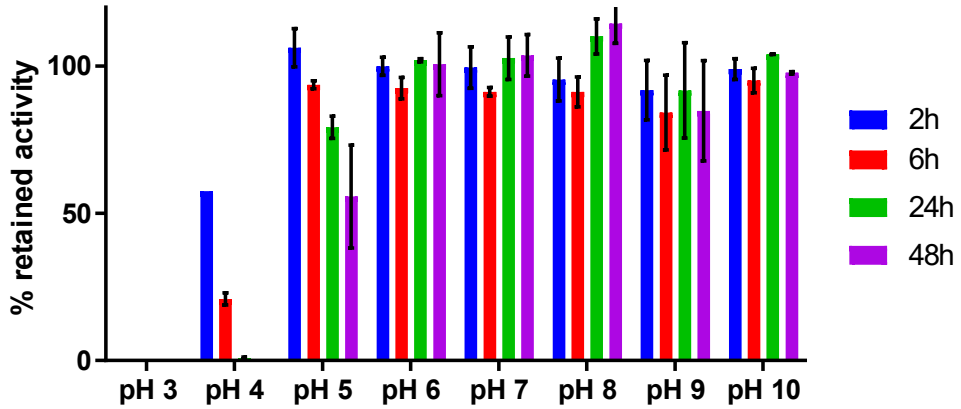


Figure S4. pH effect on the stability of HeAlaDH. The enzyme was incubated at 4ºC for 48h and the activity determined at different time points using the standard activity assay. The values correspond to the average of 3 replicates.

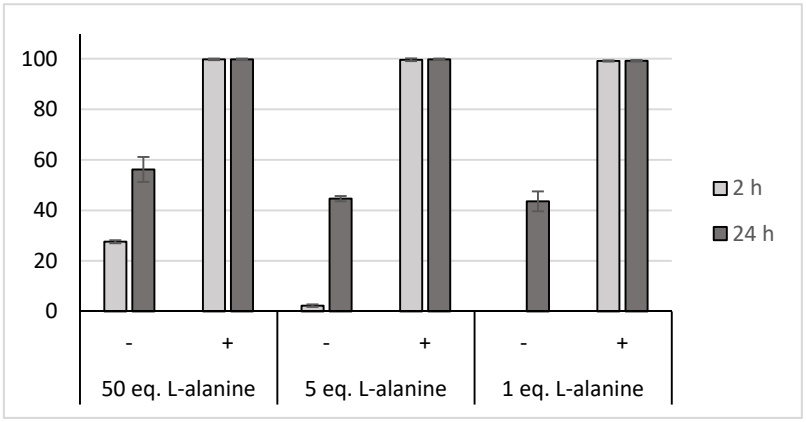


Figure S5. Amination of vanillin at the 10 mM scale with different equivalents of alanine and the presence (+) or absence (-) of the cascade for the amino donor recycling.. Reactions were performed in a final volume of 1 mL, with 10 mM aldehyde, the desired amount of alanine, 250 mM ammonium formate, 1 mM NAD+ in 100 mM phosphate buffer pH8 with 1 mg/mL HeWT, 1 mg/mL HeAlaDH and 2 mg/mL CbFDH. Samples were taken at the specified times and the results analysed with HPLC by the consumption of substrate and product formation compared to a standard curve.

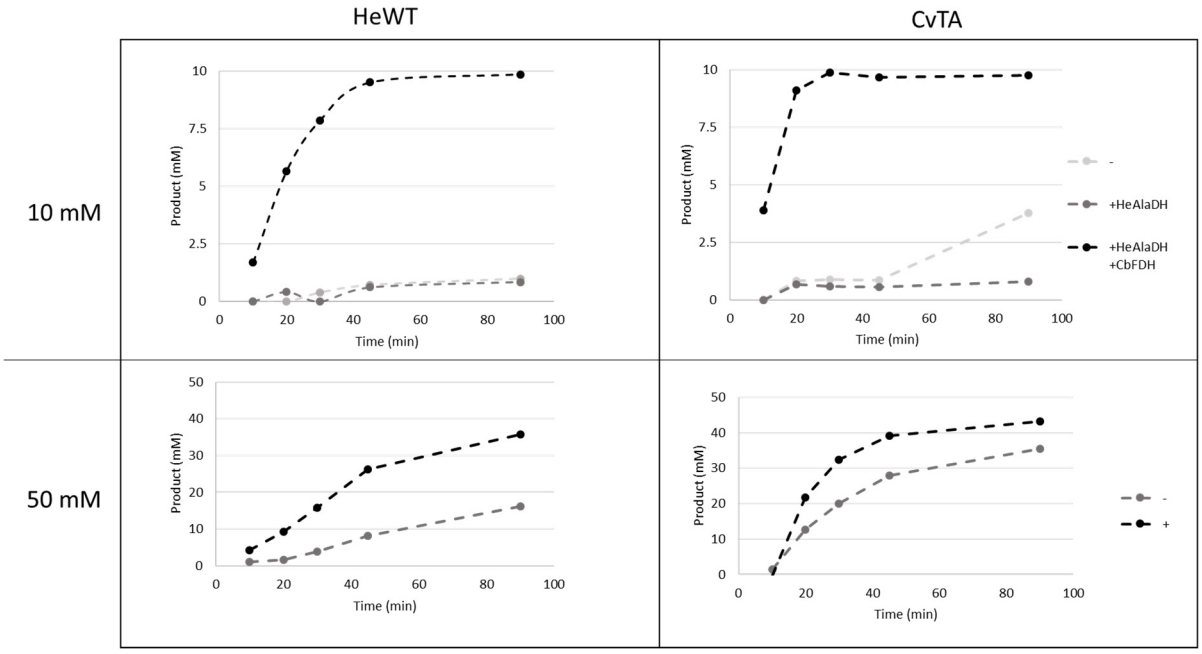


Figure S6. Amination of vanillin at the 10- and 50-mM scale with 1 equivalent of alanine. Reactions were performed in a final volume of 1 mL, with 10 mM aldehyde, 1 equivalent of alanine, 250 mM formate dehydrogenase, 1 mM NAD+ in 100 mM phosphate buffer pH8. Samples were taken at the specified times and the results analysed with HPLC by the consumption of substrate and product formation compared to a standard curve.

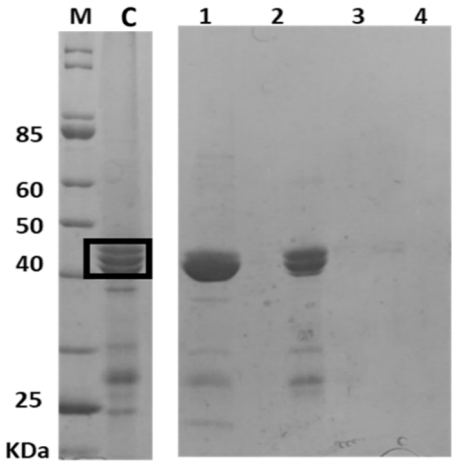
- ***HeAlaDH immobilisation:***

Table S1: Results of the immobilisation of HeAlaDH in various supports. Both the functional group and the pore size of each matrix is indicated. Enzyme loading was fixed at 0.5 mg/g and the recovered activity is expressed as percentage of the deamination capacity of HeAlaDH in phosphate buffer pH8 (3.4 U/mg).

Support	Functional group	Pore size	Enzyme loading (mg/g)	Recovered activity (%)	Expressed activity (U/g)
EC/EP-S	Epoxy	10-20 nm	0.5	17 ± 2	0.25 ± 0.03
EC403-S	Epoxy	40-60 nm		19 ± 1	0.28 ± 0.02
EC/HFA-S	Amino Epoxy	10-20 nm		20 ± 5	0.43 ± 0.10
HFA403-S	Amino Epoxy	40-60 nm		32 ± 2	0.60 ± 0.02
Ag-Ep	Epoxy	24-70 nm		42 ± 1	0.67 ± 0.02

- ***Post-immobilisation coating effect:***

A.



B.

Support + coating	Recovered activity (%)
HFA403-S	36 ± 7
HFA403-S+GA	21 ± 3
HFA403-S+PEI	27 ± 2
HFA403-S+dexCHO	31 ± 1

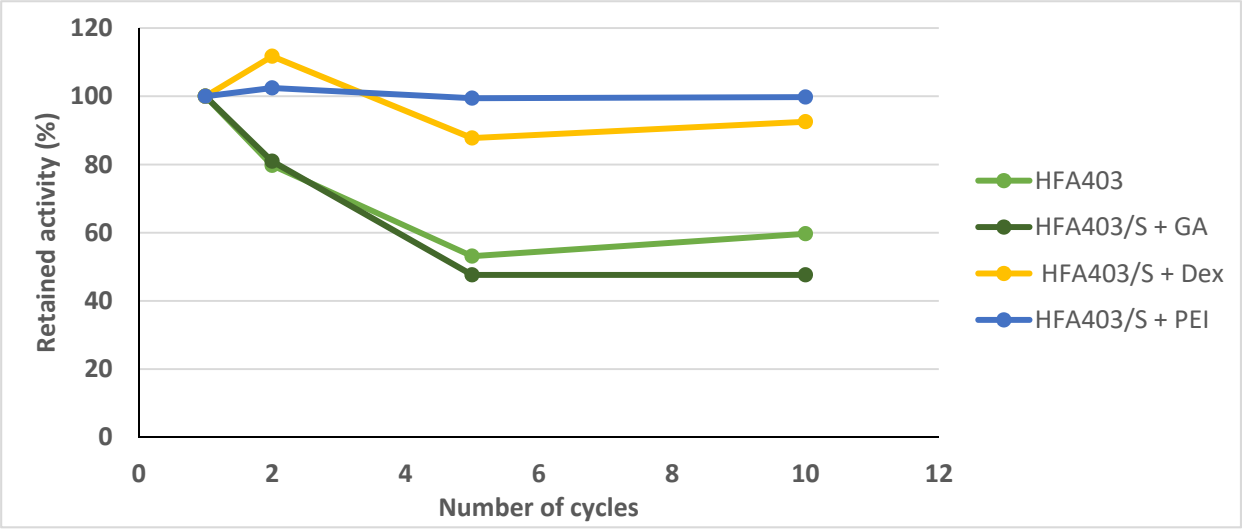


Figure 7. A. SDS-PAGE analysis of the immobilised biocatalysts. The supernatant of a boiled sample (10 minutes at 95ºC) of 20 mg of the sample in a final volume of 200 µL was loaded. M: molecular markers; C: HFA403-S-HeAlaDH with no post-immobilisation treatment 1: Non-treated; 2: PEI; 3: dexCHO; 4: GA. **B.** The table shows the recovered activity for the different post-immobilisation techniques used with the HFA403-S resin with a loading of 0.5 mg enzyme/g of resin. The results correspond to the mean of two measurements. The graph below, corresponds to the operational stability expressed as the retained activity after 10 cycles of reaction of HeAlaDH immobilised in HFA403-S with the different post-immobilisation treatments. 100% is defined as the initial activity of each resin in the 1st cycle.

Immobilised biocatalyst recovered activities:

Table S3: Co-immobilization of HeAlaDH (1 mg/g) and CbFDH (5 mg/g) in the three different tested resins. The initial activity of both enzymes was 3.5 U/mg. Reactions were performed in 50 mM phosphate buffer pH8 with 40 mM alanine, 1 mM NAD^+ to test HeAlaDH and 100 mM ammonium formate, 1 mM NAD^+ to test CbFDH. Immobilization was performed either simultaneously immobilization (S), HeAlaDH followed by CbFDH (AF) or vice versa (FA).

		HeAlaDH			CbFDH		
		Imm. yield (%)	**Rec. activity (%)**	**Expressed activity (U/g)**	**Imm. yield (%)**	**Rec. activity (%)**	**Expressed activity (U/g)**
HFA403-S	**S**	>99 %	33 ± 5%	1.22 ± 0.02	44 ± 1 %	16 ± 1%	1.20 ± 0.05
	AF	>99 %	52 ± 3%	1.78 ± 0.11	64 ± 18%	24 ± 7%	0.72 ± 0.06
	FA	>99 %	43 ± 1%	1.44 ± 0.04	76 ±1 %	17 ± 3%	0.80 ± 0.28
EC/HFA-S	**S**	>99 %	29 ± 2%	0.99 ± 0.09	26 ± 1%	23 ± 1%	1.04 ± 0.09
	AF	>99 %	27 ± 1%	0.90 ± 0.03	34 ± 2%	21 ± 5%	1.20 ± 0.18
	FA	>99 %	31 ± 9%	1.08 ± 0.30	25 ± 1%	30 ± 5%	1.31 ± 0.16
Ep-Ag	**S**	>99 %	35 ± 1%	1.22 ± 0.01	>99 %	< 5%	0.27 ± 0.12
	AF	>99 %	40 ± 1%	1.21 ± 0.27	>99 %	< 5%	0.39 ± 0.19
	FA	>99 %	30 ± 2%	1.20 ± 0.21	>99 %	< 5%	0.54 ± 0.03

Table S4. Recovered activities and specific activity of the biocatalysts of the three different enzymes.

Resin		**Loading**	**Recovered activity (%)**	**Expressed activity (U/g)**
HFA403-S	HeWT	5	42 %	4.2
	HeAlaDH	1	28 %	1.5
	CbFDH	5	5 %	1.4
Ag-Ep	HeWT	5	24 %	2.5
	HeAlaDH	1	47 %	2.4
	CbFDH	5	3 %	0.9

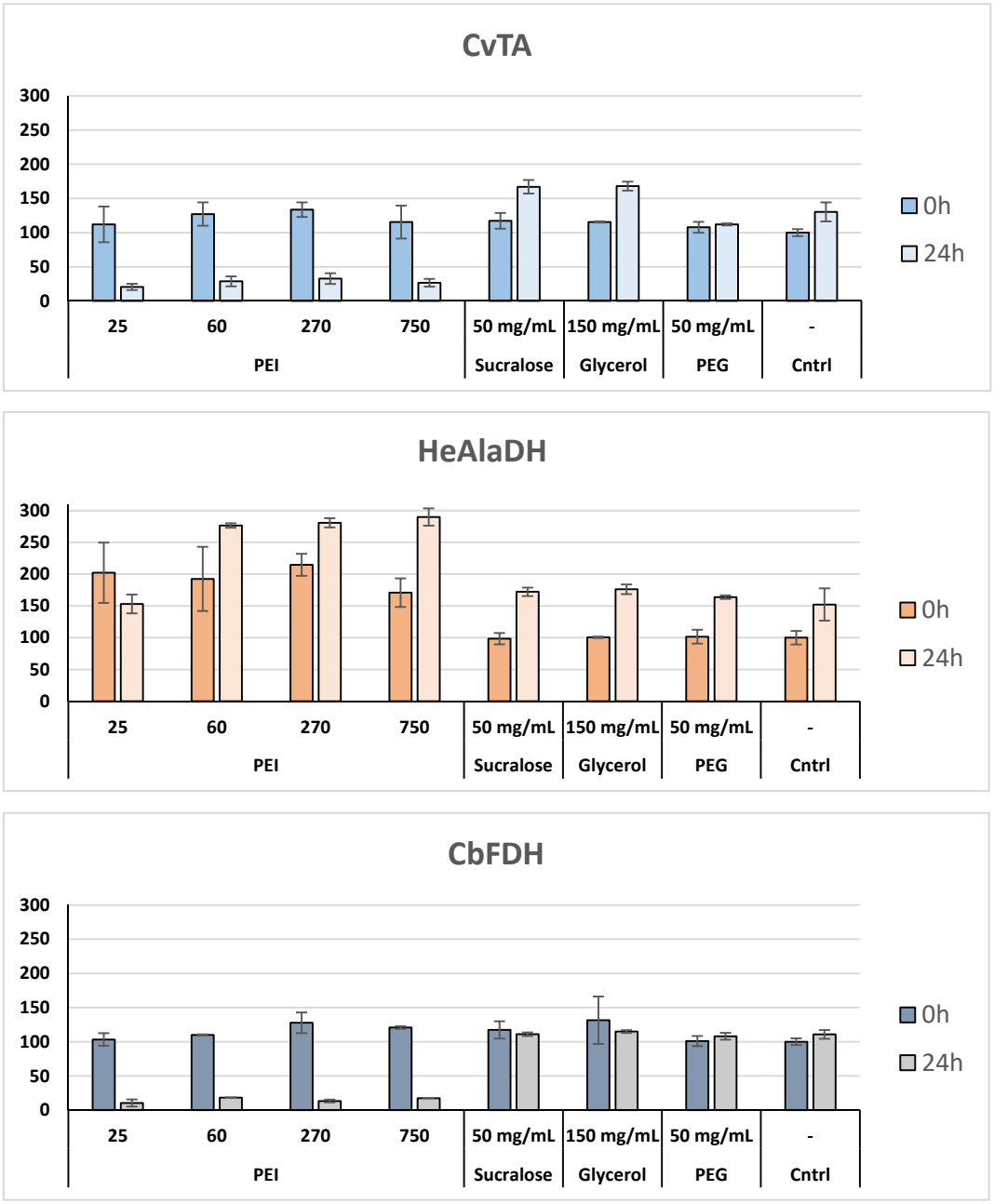


Figure S8. Stability of the different biocatalysts in the presence of the different additives. All biocatalysts were mixed at the same concentration as in the reaction (1 mg/mL of CvTA, 1 mg/mL of HeAlaDH and 2 mg/mL of CbFDH) and the activity measured after 30 minutes incubation at 37ºC and after 24h. Activities were measured using the standard assay for each enzyme. For alanine dehydrogenase, the deamination of alanine was monitored. All results are the shown as the average of 3 replicates and their standard deviation.