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ENZYMATIC REDUCTION BY ALCOHOL DEHYDROGENASE TA1316 FROM
Thermoplasma acidophilum

REDUCCIÓN ENZIMÁTICA POR ALCOHOL DESHIDROGENASA TA1316
PROVENIENTE DE *Thermoplasma acidophilum*

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Abstract

In this work we present the NADH-dependent Ta1316 alcohol dehydrogenase (ADH) characterization for its reducing reaction in the presence of aldehydes, ketones and keto-esters. In the presence of 2 mM acetaldehyde, Ta1316 ADH showed a remarkable thermal activity, displaying activity at temperatures up to 90°C and low pH with a requirement of Zn^{+2} to reach its maximum activity. In contrast to other characterized ADHs, Ta1316 ADH reduces methyl pyruvate, an alpha-ketoester as its preferred substrate. We conclude that Ta1316 ADH's principal function is the reduction of substrates and is potentially an enzyme that can be used in biotechnology, as well as in industrial applications.

Keywords: alcohol dehydrogenase, Ta1316, *Thermoplasma acidophilum*, reduction reaction, characterization.

Resumen

En este trabajo presentamos la caracterización de la actividad enzimática reductora de la enzima alcohol deshidrogenasa (ADH) Ta1316 dependiente de NADH en presencia de aldehídos, cetonas y cetoésteres. La ADH Ta1316 mostró estabilidad térmica notable con actividad máxima a temperatura de 90°C y pH ácido en presencia del cofactor Zn^{+2} y acetaldehído 2 mM. En contraste con otras ADHs caracterizadas, Ta1316 es capaz de reducir metil-piruvato, un alfa-cetoéster como su sustrato preferido. Concluimos que la función principal de la Ta1316 ADH es la reducción de sustratos y es una enzima que podría ser utilizada potencialmente en aplicaciones biotecnológicas e industriales.

Palabras clave: alcohol deshidrogenasa, Ta1316, *Thermoplasma acidophilum*, reacción reductora, caracterización.

1 Introduction

Alcohol dehydrogenases (ADHs) are part of the ubiquitous oxidoreductase family within the three domains of life. This cofactor-dependent enzyme catalyzes the oxidation of alcohols in the presence of NAD^+ or $NADP^+$ (Reid & Fewson, 1994). ADHs are involved in the interconversion between alcohols, aldehydes and ketones that are key in biotechnological processes such as the production of optical active alcohols for fragrances, pharmaceuticals and agrochemicals. These enzymes are also useful in bioelectrocatalysis for the production of biosensors, biofuels (Eichler, 2001) (Kroutil *et al.*, 2004) (Yakushi & Matsushita, 2010) (Reyes-Valadez, 2016),

bio-reduction (Yang *et al.*, 2012), optically active compounds (Nie *et al.*, 2007; Nie *et al.*, 2007) or fermented spirits (De los Rios-Deras *et al.*, 2015).

Hence, the demand in industry for active and stable ADHs; able to tolerate elevated temperatures, organic solvents or distinct pHs has increased. Extremophile organisms contain enzymes that withstand hard conditions to survive (Tsigos *et al.*, 1998). In this context, *Thermoplasma acidophilum* (Darland *et al.*, 1970) (Ruepp *et al.*, 2000) is an archaea with a source of enzymes with robust features to survive in extreme conditions, as well as having the potential for biotechnological applications.

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Table 1. Closest structural homologues displayed by the protein data bank analyses

PDB Hit	Description	Oligomeric state	Ion dependence	TM-score	Reference
4JBG	<i>Pyrobaculum aerophilum</i> ADH	Tetramer	Zn ⁺²	0.964	(Vitale et al. 2013)
2EIH	<i>Thermus thermophilus</i> HB8 ADH	Tetramer	Zn ⁺²	0.959	To be published
4A0S	<i>Streptomyces</i> sp JS360 Octenoyl-CoA reductase/carboxylase	Tetramer	-	0.936	(Quade et al. 2012)
1RJW	<i>Bacillus stearothermophilus</i> strain LLD-R htADH	Tetramer	Zn ⁺²	0.931	(Ceccarelli et al. 2004)
3KRT	<i>Streptomyces coelicolor</i> A3(2) Crotonyl CoA reductase	Tetramer	Cl ⁻	0.930	To be published
3MEQ	<i>Brucella melitensis</i> ADH	Tetramer	Zn ⁺² , Cl ⁻ , Na ⁺	0.930	To be published
1R37	<i>Sulfolobus solfataricus</i> ADH	Tetramer	Zn ⁺²	0.929	(Esposito et al. 2003)
4WGZ	<i>Saccharomyces cerevisiae</i> ADH	Tetramer	Zn ⁺²	0.928	(Raj et al. 2014)
3S1L	<i>Cupriavidus necator</i> JMP134 ADH	Tetramer	Zn ⁺²	0.927	(Kang et al. 2012)
2EER	<i>Sulfolobus tokodaii</i> strain7 ADH	Tetramer	Zn ⁺²	0.925	To be published

We have previously reported the analysis of the oxidative reaction of *T. acidophilum* NAD⁺ dependent Ta1316 ADH throughout the biocatalysis of ethanol to acetaldehyde (Marino-Marmolejo *et al.*, 2009). This work describes the enzymatic characterization towards the reductive reaction in the presence of ketones, aldehydes and keto-esters.

2 Materials and methods

2.1 Chemicals

All chemicals were analytical grade; aldehydes, ketones and keto-esters were purchased from Sigma-Aldrich.

2.2 Sequence analysis

Homologues of Ta1316 ADH (NCBI reference sequence: NP_394771.1) sequenced open reading frames were identified using the BLAST tool server at NCBI (www.blast.ncbi.nlm.nih.gov/). The 3D structure prediction was performed by using the I-TASSER server (Zhang, 2008; Roy *et al.*, 2010; Roy *et al.*, 2012) and the UCSF-Chimera program for interactive analyses of the structures (Meng *et al.*, 2006).

2.3 Expression and purification of the recombinant Ta1316 ADH

Ta1316 ADH cloned into the vector pET28a(+) was expressed in *E. coli* BL21 (DE3) pLysS from Novagen (Madison, WI) and purified as previously described (Marino-Marmolejo *et al.*, 2009).

2.4 Mass spectrometry

Recombinant Ta1316 ADH was identified by mass spectrometry analysis (LC-ESI-MS/MS) in the LINAN laboratory facility at the IPICYT. Data identification was carried out on the MS/MS spectra datasets using the MASCOT search algorithm (Matrix Science, London, UK). Searches were performed using the NCBI database.

2.5 Reduction assay

Consumption of NADH to NAD⁺ (oxidation) was measured at 340 nm (Kagi & Vallee, 1960) using a Variant Cary 50 spectrophotometer with a peltier temperature-controlled cuvette-holder and the Kinetics v3.0 software (Variant). The reaction mixture was completed in a total volume of 1 ml containing 50 mM phosphate buffer (pH 5.0), 1 mM NADH, 0.5 mM ZnSO₄, 10 µg of pure recombinant protein, 2 mM pure aldehyde, or 1 mM aldehydes, ketones or

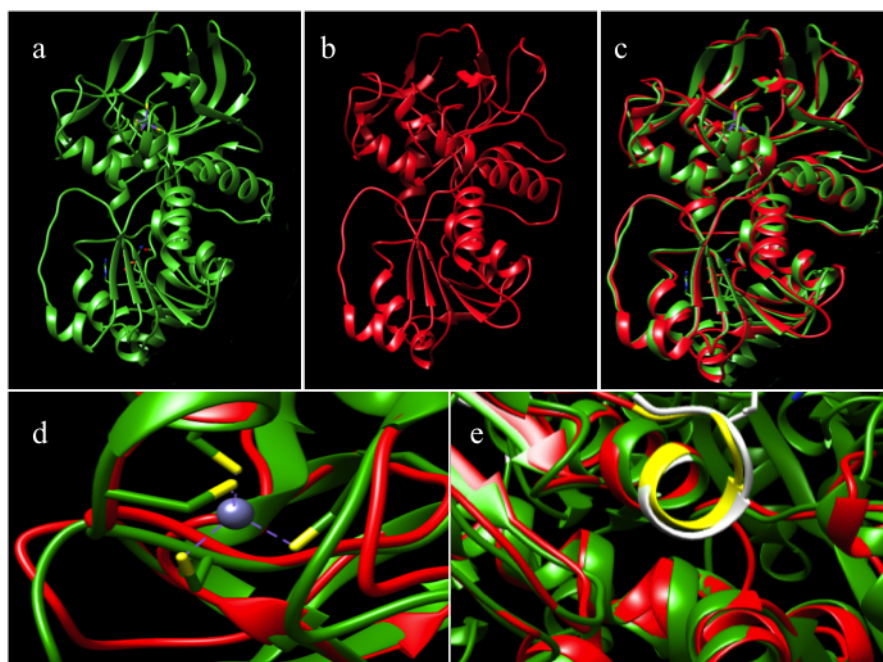


Fig. 1: In silico modeling of Ta1316 ADH against its closest homologue. a) Crystal structure of *P. aerophilum* str. IM2 ADH, PyAeADHIII (green), and b) 3D model of Ta1316 ADH (red). c) Superposition of PyAeADHIII (red) and Ta1316 ADH (red). d) The active ligand site is zoomed in where the Zn^{2+} ion is represented as a sphere. e) Sequences of the NAD-binding site in Ta1316 ADH and PyAeADHII are represented in yellow and white respectively.

keto-esters (Table 2). All pH, temperature, substrate specificity, kinetics and effects of compounds studies were conducted in triplicate. Maximum activity was determined with a temperature range of 25 to 90°C and pH range of 2 to 8 at 75°C. K_m and V_{max} values were estimated using the method of least squares.

3 Results

Recombinant Ta1316 ADH was identified by electrospray ionization mass spectrometry and MASCOT dataset. The average nominal mass reported of 36278 Da was equivalent to the calculated theoretical value of the sequence. 57% sequence coverage of matched peptides was confirmed,

Table 2. Effect of chemicals on the Ta1316 ADH activity

Chemicals	Concentration (mM)	Relative activity (%) ^a
None	-	100.00
ZnSO ₄	1	194.78
MnCl ₂	1	130.96
CoSO ₄	1	117.52
MgCl ₂	1	95.55
FeCl ₃	1	73.71
CuCl ₂	1	0.001
EDTA 2Na	10	68.63
2-Mercaptoethanol	1	46.41
N-Ethylmaleimide	1	52.89

^aThe activity was measured at 75°C as described in Materials and Methods section in the presence of 2 mM acetaldehyde

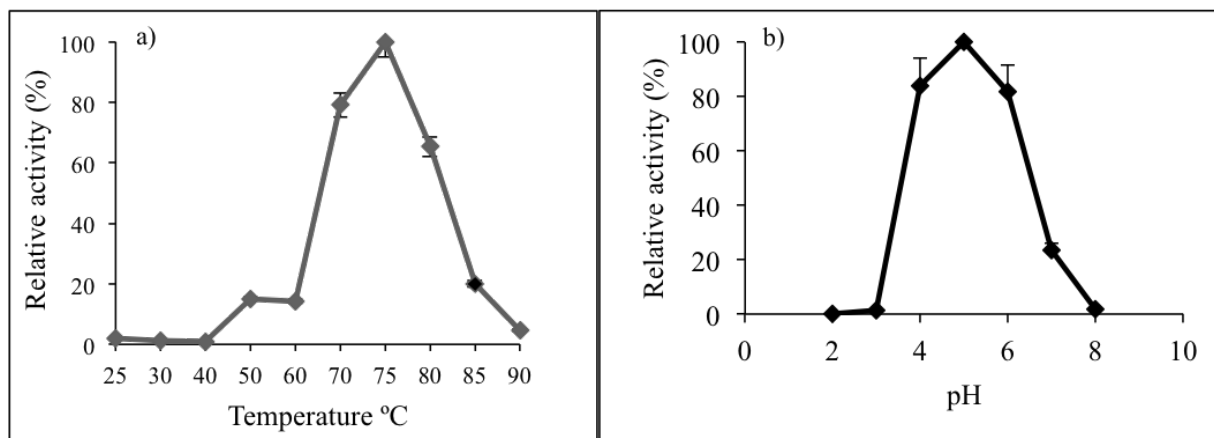


Fig. 2: a) Effect of temperature on Ta1316 ADH activity. b) Effect of pH on Ta1316 ADH activity.

and the NCBI Entrez gi 10640659 or 16082305 reports indicated an alcohol dehydrogenase from *Thermoplasma acidophilum* DSM 1728.

Structural analyses displayed by the PDB platform showed the crystal structure of *Pyrobaculum aerophilum* ADH (Vitale *et al.*, 2013) as the closest structural conformation against Ta1316 ADH (Figure 1, Table 1). Unexpectedly, *P. aerophilum* does not possess any apparent ADH activity for comparison against Ta1316 ADH. The second best TM-score found, *Thermus thermophilus* ADH (Pennacchio *et al.*, 2008), was similar and presents a comparable substrate preference.

The enzymatic oxidation reaction of NADH to NAD⁺ produced a reaction rate that increases up to 75°C as shown in Figure 2a, then activity decays gradually until inactivation at 90°C. The maximum temperature found is comparable to *Sulfolobus acidocaldarius* ADH (Pennacchio *et al.*, 2010).

Maximum activity displayed by Ta1316 ADH was at pH 5.0 in the presence of 2 mM acetaldehyde (Figure 2b). This pH value is closely related to the intracellular pH of *T. acidophilum* (pH 5.5) (Searcy, 1976) and those found for the reduction of NAD⁺ (Marino-Marmolejo *et al.*, 2009) and *S. acidocaldarius* (Pennacchio *et al.*, 2010).

Table 3. Substrate specificity of Ta1316 ADH in the reduction reaction

Substrate (1 mM)	Relative activity (%)
Ketones	
3'-Chloroacetophenone	67.98
3-Octanone	61.57
4-Acetylpyridine	53.68
2'-Nitroacetophenone	14.91
Acetylacetone	0
4-Fluoroacetophenone	0
Aldehydes	
2-Chlorobenzaldehyde	60.06
3-Nitrobenzaldehyde	59.10
Benzaldehyde	58.07
2-Nitrobenzaldehyde	42.61
Keto-esters	
Methyl pyruvate*	100

*The activity towards methyl pyruvate (1 mM) was taken as 100%.

Table 4. Biochemical features of several ADH members in the reduction reaction

Source	Specific activity (U/mg)	optimum pH	optimum T(°C)	K _m (mM)	K _{cat} /K _m (s ⁻¹ mM ⁻¹)	Reference		
Ta1316 ADH ^{a,b}	ND	5.0	75	3.97	46	0.56	11.6	This work
<i>Geobacillus thermodenitrificans</i> NG80-2 ADH1 ^a	ND	8.0	60	4.71	404.7	568.7	85.94	(Liu et al. 2009)
<i>Desulfovibrio gigas</i> ^a	31.8	7.5	40	1.8	ND	ND	ND	(Hensgens et al. 1993)
<i>Thermococcus</i> strain ES1 ^b	ND	7.0	ND	0.84	22.9	30.5	27	(Ying et al. 2009)
<i>Mus musculus</i> ^c A2	0.012	9.5	30	0.041	ND	17.83	ND	(Algar et al. 1983)
<i>Rattus norvegicus</i> ^c ADH3	ND	7.5	25	0.17	170	ND	1000	(Julia et al. 1987)

ND, Not determined; ^aArchaea; ^bBacteria; ^cEukaryote; ^d2mM acetaldehyde as substrate.

The effect of various divalent cations was also examined (Table 2). Apparently, no metal cofactor is required for the enzymatic activity however, CoSO₄, MnCl₂ and ZnSO₄ produced increasing activities of 117, 130 and 194% respectively against the blank reaction (no metal ion added). It has been reported that ZnCl₂ is able to inhibit *Oenococcus oeni* or *Yokenella* sp. ADH activities (Elleuche *et al.*, 2013) (Ying *et al.*, 2014). Surprisingly, in the presence of MgCl₂, Ta1316 ADH conserved 95.5% activity. FeCl₃ retained virtually 75% activity. Moreover, evident obstruction of activity was observed in the presence of the Cu⁺² ion as reported for *Moraxella* (Tsigos *et al.*, 1998). The metal chelating agent EDTA was used at increased concentrations and arrest of activity was only observed at 10 mM producing less than 50% inhibition (63% relative activity). This behavior has been reported for the yeast ADH (Magonet *et al.*, 1992). It is expected that Zn⁺² ions confer high stability to the reduction reaction as shown for other ADHs (Kawano *et al.*, 2011; Rong Chen, 2011) (Pennacchio *et al.*, 2010) (Kizaki *et al.*, 2008). Lastly, inhibition of Ta1316 ADH activity by the thiol group 2-mercaptoethanol (Cheng & Lek, 1992) (Langeland *et al.*, 1999) and N-ethylmaleimide reduced 50% the activity.

Ta1316 ADH substrate specificity was standardized reaching a maximum activity of 100% at 1 mM methyl pyruvate (Table 3). We observed that in the presence of side chains ketones, such as 3'-chloroacetophenone, 3-octanone, 4-acetylpyridine and 2'-nitroacetophenone the enzyme showed decreasing relative activities of 68 to 15%. Surprisingly, the enzyme did not show activity with ketones such as acetylacetone or 4'-fluoroacetophenone in contrast to *Lactobacillus kefir* ADH (Chen *et al.*, 2010).

Aldehyde substrates (benzaldehydes) were evaluated for conversion to their corresponding aromatic alcohols. We found minor differences in the relative activities: 2-chlorobenzaldehyde > 3-nitrobenzaldehyde > benzaldehyde > 2-nitrobenzaldehyde (60-42%) as shown in Table

3. Ta1316 ADH showed similar activities when comparison was performed against *Devosia riboflavina* ADH (Kizaki *et al.*, 2008), and higher activity than *Thermococcus guaymasensis* and *Moraxella* sp. ADHs (Ying & Ma, 2011) (Tsigos *et al.*, 1998).

Lastly, we found Ta1316 ADH was able to reduce keto-ester methyl pyruvate producing its highest activity (adjusted to 100%) and preferred substrate of reaction (table 3). This substrate has been used with low efficiency by *Candida maris* ADH (Kawano *et al.*, 2011), however it is capable of processing acetylacetone in contrast to Ta1316 ADH.

Kinetic parameters were measured in the presence of acetaldehyde. Ta1316 ADH displayed higher affinity for acetaldehyde (K_m 3.97 mM and V_{max} 0.56 mM/s) rather than ethanol (Marino-Marmolejo *et al.*, 2009), suggesting that Ta1316 ADH's main function is the reduction of aldehydes and ketones. A comparative analysis of several ADHs is shown in Table 4 to support biochemical and kinetic data.

Discussion

An enzymatic reduction of prochiral substrates was performed to characterize the reducing activity of Ta1316 ADH. In general, ADHs show some advantages for asymmetric catalysis against chemical catalysis; especially for challenging reactions where the purpose is the production of enantiopure substances.

Biotransformation is therefore key in industry; highly valuable products such as short chain alcohols (fine chemicals) are used as intermediates, e.g. (*R*)-2-butanol, which is 120 times more expensive than 2-butanone. In this context, Ta1316 ADH displayed broad substrate specificity both in oxidation and reduction reactions with special preference towards the reductive activity. Characterization of the enzymatic properties showed that Ta1316 ADH prefers NAD⁺ as cofactor and ZnSO₄ enhances 200% the relative activity. Moreover, Ta1316 ADH has

the ability to reduce methyl pyruvate as its principal substrate. The recombinant form of Ta1316 ADH can be produced in high quantities in *E. coli*, it is stable in a broad range of temperature and pH and has a wide substrate specificity in the reduction reaction. The properties reported here makes Ta1316 ADH an attractive catalyst in several industry processes, principally for the production of chiral compounds under extreme conditions.

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