Hyperthermophilic aldolases as biocatalyst for C-C bond formation: Rhamnulose 1-phosphate aldolase from *Thermotoga maritima*

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Abstract

The TM1072 gene from *Thermotoga maritima* codifies for a putative form of a rhamnulose-1-phosphate aldolase (Rha-1PA Tm). To investigate this enzyme further, its gene was cloned and expressed in *Escherichia coli*. The purified enzyme was activated by Co^{2+} as a divalent metal ion cofactor, instead of Zn^{2+} as its *E. coli* homologue, and exhibited a maximum of activity at 95 °C. Furthermore, the enzyme displayed a high stability against extreme reaction conditions, retaining 90% of its activity in the presence of 40% of acetonitrile and showing a half-life greater than three hours at 115 °C. The kinetic parameters at R/T were also studied; the K_M was calculated to be 3.6 ± 0.33 mM, while K_{cat}/K_M was found to be $0.7 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$. Given these characteristics, Rha-1PA Tm is an attractive enzyme for use as a biocatalyst for industrial applications, offering intriguing possibilities for practical biocatalysis.

Keywords

Aldolases

Biocatalysis

Enzyme catalysis

Hyperthermophilic enzymes

Thermostability

Introduction

Carbon-Carbon bond formation reactions can be considered as being the essence of organic synthesis (Corey and Cheng 1989). The aldol addition reaction has long been recognized as one of the most useful tools that the synthetic chemist possesses for the construction of new C–C bonds since the formation of one or two new stereocenters, which allow the chemist to deal with a broad range of both natural and novel compounds, is concomitant with the bond forming process (Li 2005; Palomo et al. 2004). Nature builds up carbohydrate, aminoacids, α -hydroxy acids and other molecules by use of aldol reactions catalyzed by aldolase enzymes. Aldolases, mainly dihydroxyacetone phosphate (DHAP) dependent aldolases, have been extensively used in chemoenzymatic syntheses and many of these applications have been compiled in several superb reviews over the last years (Clapés and Joglar 2013; Fesko and Gruber-Khadjawi 2013; Müller 2012; Brovetto et al. 2011; Clapés and Fessner 2011; Clapés et al. 2010; Iturrate and García-Junceda 2008). Despite this well proven synthetic utility of aldolases, there are still few processes that have been developed at the industrial scale and none of them —to the best of our knowledge— have used DHAP-dependent aldolases (Patel 2011; Baik and Yoshioka 2009; Wolberg et al. 2008; Castillo et al. 2006; Müller 2005; Greenberg et al. 2004). Therefore, the search for new enzymes with improved properties from a biocatalysis point of view it is still necessary.

Enzymes as catalysts offer many advantages, such as having high efficiency, chemo-, regio- and stereoselectivity, avoiding the need for functional group activation and protection/deprotection steps, being environmentally friendly since they are biodegradable, using mostly water as a solvent system, and working under mild reaction conditions (Anastas and Eghbali 2010). Despite these advantages, enzymes present some limitations, of which perhaps the most important —especially from the point of view of industrial application — is their intrinsic instability since they have evolved to work in aqueous media and in a narrow range of pH and temperature. One approximation for overcoming the instability of enzymes under adverse reaction conditions is to use enzymes from extremophile microorganisms (Canganella and Wiegel 2011; Champdoré et al. 2007; Unsworth et al. 2007). These organisms have adapted to live in extreme environments offering useful enzymes that should enable us to expand the range of reaction conditions suitable for biocatalysis. In particular, thermophiles (an optimal growth temperature of between 50 and 80 °C) and hyperthermophiles (an optimal growth temperature of between 80 and 110 °C) have attracted attention from a biocatalytical point of view since these microorganisms are a source of thermostable enzymes (thermozymes) that display an outstanding stability against high temperatures and, furthermore, it has also been reported that these enzymes show enhanced tolerance against other denaturalization conditions such as the presence of organic solvents, detergents or extreme pHs (Atomi et al. 2011; Bertoldo and Antranikian 2008; Atomi 2005; Vieille and Zeikus 2001). Moreover, carrying out reactions at high temperatures brings other advantages, such as that it allows for the use of higher concentrations of substrates, to decrease the viscosity of the reaction medium, to prevent against possible microbial contamination and frequently leads to higher reaction rates.

Despite these advantages, DHAP-dependent aldolases from hyperthermophilic organisms have been barely used in synthesis (Falcicchio et al. 2014). Recent example are those described by

Wever group, in which rhamnulose-1-phosphate aldolase (Rha-1PA) from *Thermotoga maritima* —a hyperthermophilic eubacterium with an optimal growth temperature of 80 °C— was used soluble in a one-pot four-enzyme catalytic cascade for the simple and inexpensive synthesis of a variety of non-natural heterocycles or immobilized on epoxy beads in a continuous flow reactor for the synthesis of various aldol products (Babich et al. 2011; Babich et al. 2012).

In this paper we describe the heterologous expression of rhamnulose-1-phosphate aldolase from *Thermotoga maritima*, the functional characterization of the recombinant enzyme and a study of its applicability as a biocatalyst.

Materials and Methods

Materials and General Procedures

Thermotoga maritima MSB8 was provided from the Leibniz Institute DMSZ (German Collection of Microorganisms and Cell cultures, DSM nº 13995). *E. coli* BL21 (DE3) competent cells were purchased from Promega (Madison, WI, USA). Luria Bertani (LB) medium and agarose D-1 Medium EEO for DNA gels were obtained from Pronadisa. Restriction enzymes *Nde*I and *Hind*III, Taq polymerase, 1kb DNA ladder and T4-DNA ligase were purchased from MBI Fermentas AB (Lithuania). Triosephosphate isomerase (TIM), α -glycerophosphate dehydrogenase (α GDH), proteinase K, RNA-ase and acetate kinase (AK) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lisozyme was acquired from USB Corporation and DNAase from Roche. PCR primers were purchased from Isogen Life Science (Spain) and the pET-28b(+) expression vector was purchased from Novagen (Beeston, Nottingham, UK). Isopropyl- β -D-thiogalactopyranoside (IPTG) and imidazole were purchased from Acros Organics. Antibiotics, ATP, NADH and other reagents were obtained from Sigma-Aldrich. Plasmids and PCR purification kits were from Promega (Madison, WI, USA) and the DNA purification kit from agarose gels was from Eppendorf (Hamburg, Germany). Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using 13% and 5% acrylamide in the resolving and stacking gels, respectively. Gels were stained with Coomassie brilliant blue R-250 (Applichem GmbH, Germany). Electrophoresis was always run under reducing conditions in the presence of 5% β -mercaptoethanol. Protein and DNA gels were quantified by densitometry using GeneGenius Gel Documentation and Analysis System (Syngene, U.K.). Nickel-iminodiacetic acid (Ni^{2+} -IDA) agarose was supplied by Agarose Bead Technologies (Spain). Dialysis was performed in SPECTRA/Por® membranes (Spectrum) with 12,000-14,000 Da cut-off. HPLC analyses were carried out on a chromatograph JASCO, Dual Gradient Pump, with UV/VIS detector. L-Lactaldehyde (**2a**) was prepared from D-threonine as previously described (Zagalak et al. 1966). L-Rhamnulose-1-phosphate was synthesized in our lab by aldol condensation of DHAP and L-lactaldehyde catalyzed by Rha-1PA from *E. coli*, according to the procedure previously reported (Liu et al. 1991). Rha-1PA was obtained from the recombinant *E. coli* strain ATCC # 86983 and purified as previously described (García-Junceda et al. 1995). Benzyloxyacetaldehyde (**2b**), phenylacetaldehyde (**2c**) and ethyl-3-methyl-4-oxocrotonate (**2d**) were purchased from Sigma–Aldrich. ^1H and ^{13}C NMR spectra, using D_2O as solvent, were recorded on a Varian System 500 spectrometer equipped with a 5 mm HCN cold probe with field z-gradient, operating at 500.13 and 125.76 MHz for ^1H and ^{13}C , respectively. The sample

temperature was maintained constant at 298 K. One dimensional NMR experiments were performed using standard Varian pulse sequences. Two-dimensional [^1H , ^1H] NMR experiments (gCOSY and TOCSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 3000 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 256 time increments, and linear prediction to 512. The data were zero-filled to 4096 x 4096 real points. Two-dimensional [^1H - ^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^1H spectral window, a ^{13}C spectral windows of 15,000 Hz, 1 s of relaxation delay, 1024 data points, and 256 time increments, with a linear prediction to 512. The data were zero-filled to 4096 x 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively.

Cloning, over-expression and purification of Rha-1PA from *Thermotoga maritima*

DNA manipulation was carried out according to standard procedures (Sambrook et al. 1989). Leftward primer was 5'- TATAACATATGATGAGAGAGACGATA-3' (*NdeI* restriction site underlined) and rightward primer was 5'- TATAAAAGCTTTCACAGCCATCCCTC-3' (*HindIII* restriction site underlined). To extract the chromosomal DNA, *T. maritima* cells were suspended in 40 μL lysis buffer containing Tris (50 mM), EDTA (50 mM), Tween 20 (0.5%), Triton X (0.5%), lysozyme (40 μg), RNA-ase (16 μg) and proteinase K from *Tritirachium album* (32 μg). The mixture was incubated 1 h at 37 $^{\circ}\text{C}$ (Kim et al. 1993). PCR amplification was performed in a 10 μL reaction mixture and subjected to 30 cycles of amplification. The cycle conditions were set as follows: denaturation at 94 $^{\circ}\text{C}$ for 1 min, annealing at 50 $^{\circ}\text{C}$ for 1.5 min and elongation at 72 $^{\circ}\text{C}$ for 2 min. The purified PCR product was digested with *NdeI* and *HindIII* and ligated into the doubled digested vector pET-28b(+) to yield the plasmid pET28b(+)-*r1patm*. This plasmid was transformed into *E. coli* BL21 (DE3) competent cells. A colony containing the plasmid pET28b(+)-*r1patm* was grown in LB broth containing kanamycin (26 $\mu\text{g}\cdot\text{mL}^{-1}$) at 37 $^{\circ}\text{C}$ with shaking. When the culture reached an OD_{600} of 0.5-0.6, protein expression was induced with IPTG (0.4 mM) and the temperature was decreased to 30 $^{\circ}\text{C}$. The culture was maintained overnight and then centrifuged (3800 x g) for 30 min at 4 $^{\circ}\text{C}$. The resulting pellet was treated with lysozyme and DNA-ase for protein extraction (Bastida et al. 2001). The solution was centrifuged for 40 min (8000 x g, 4 $^{\circ}\text{C}$) and collected for purification. The recombinant protein was firstly purified by a heat treatment at 60 $^{\circ}\text{C}$ during 20 min. Heat-denatured proteins were removed by centrifugation for 30 min (15,600 x g, 4 $^{\circ}\text{C}$). The recovered supernatant was further purified by IMAC on a Ni^{+2} -IDA agarose column pre-equilibrated with sodium phosphate buffer (20 mM, pH 7.5). Rha-1PA Tm was eluted with the same buffer containing imidazole (0.5 M). All the fractions containing protein were pooled together dialyzed to remove the imidazole and lyophilized. SDS- PAGE showed a single band matching the expected molecular weight of the recombinant Rha-1PA Tm (29 kDa).

Protein analysis

Amino acid analysis of purified recombinant protein was performed at the Protein Chemistry Service of the Centre of Biological Research (CIB-CSIC, Spain) to determine the protein concentration. The absorption spectrum of different quantified samples allowed the determination of the molar extinction coefficient at $\lambda=280\text{ nm}$ for recombinant Rha-1PA Tm ($\epsilon_{280}= 32,161\text{ M}^{-1}\text{cm}^{-1}$). Peptide-mass fingerprint analyses from the SDS-PAGE band that

correspond to the putative Rha-1PA Tm were performed at the Proteomic Unit of the Spanish National Center of Biotechnology (CNB-CSIC). The samples were digested with sequencing-grade trypsin overnight at 37 °C. The analysis by MALDI-TOF mass-spectrometric analysis produces peptide-mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. Data were collected in the range m/z 800–3600.

Quaternary structure of Rha-1PA Tm was determined by size-exclusion chromatography with no-denaturing conditions. Recombinant protein was loaded into a HiLoad 26/60 Superdex 200 PG column controlled by the AKTA-FPLC system (GE Healthcare Life Science). The column was developed in phosphate buffer (50 mM, pH 7.2) containing NaCl (0.15 M) at a constant flow rate of 1.0 mL·min⁻¹. A calibration curve was made with the retention times of well-known proteins with molecular weight (MW) between 100 kDa and 190 kDa: fucose-1-phosphate aldolase from *E. coli* (104.8 kDa), dihydroxyacetone kinase from *Citrobacter freundii* (127.2 kDa), Rha-1PA from *E. coli* (137.2 kDa), fructose-1,6-bisphosphate aldolase from *Oryctolagus cuniculus* (RAMA) (160 kDa) and the fusion protein DLF (Iturrate et al. 2009; Iturrate et al. 2010) (186.8 kDa).

Enzyme activity assays and steady-state kinetic analysis

Rha-1PA Tm activity was spectrophotometrically measured by retro-aldol reaction using Rha-1P as substrate. The activity assays were run at 25 °C or 45 °C (as indicated) by following the decrease in absorbance at $\lambda=340$ nm ($\epsilon_{NADH} = 6220 \text{ M}^{-1}\cdot\text{cm}^{-1}$) for 10 min in 1 mL reaction mixture containing Tris-HCl buffer (40 mM, pH 8.0), NADH (0.2 M), α GDH/TIM (2 U), Rha-1P (10 mM), Rha-1PA Tm and Co^{2+} 187.5 μM where indicated. Steady-state kinetic assays were measured at 25 °C in 96 well plates in a total volume of 0.2 mL. Enzyme kinetic parameters were determined employing the standard coupled assay with α -GDH with purified Rha-1PA Tm (4 μg) and concentrations of Rha-1P ranging from 0.005 to 18.75 mM. Initial velocities (V_i) were fitted to the Michaelis-Menten equation and kinetic constants were obtained using the built-in nonlinear regression tools in SigmaPlot 12.0. Values presented are the mean of three replicates measurements.

Characterization of purified recombinant Rha-1PA Tm

Optimal temperature of Rha-1PA Tm was determined by measuring its activity at temperatures ranging from 25 °C to 95 °C. Reactions at 25 °C and 45 °C were carried out in phosphate buffer (20 mM, pH 7.5) containing Rha-1PA Tm (0.08 mg), DHAP (15 mM) and L-lactaldehyde (15 mM) in a final volume of 2 mL. For reactions at other temperatures the volume of the mixture was increased to 10 mL keeping constant the concentrations of DHAP and L-lactaldehyde. In all cases reactions were incubated in a sand bath. Aldol product formation was quantified by retro-aldol reaction catalysed by the Rha-1PA from *E. coli* (0.05 U). Thermostability of Rha-1PA Tm was evaluated by measuring the remaining activity at 45 °C after incubation of the enzyme at different temperatures for a maximum of 166 h. The temperatures assayed were 80 °C, 95 °C and 115 °C. The same experiment was carried out at 4 °C and R/T to know the storage stability of the enzyme.

The activity of the purified Rha-1PA Tm was measured at different pH values. The pH was adjusted using the following buffers of 0.1 M ionic strength: Bis/Tris (pH 5.5; pH 6.0; pH 6.5; pH 7.0), sodium phosphate (pH 7.0; pH 7.5; pH 8.0) and Tris/HCl (pH 8.0; pH 8.5; pH 9.0).

Reactions were performed at 45 °C in 1 mL mixtures containing L-lactaldehyde (8.3 mM), DHAP (16.7 mM) and Rha-1PA Tm (18.2 µg; 0.01 U measured at 45 °C with the standard activity assay). Formation of aldol product was monitored by retro-aldol reaction as described above.

Activity of the Rha-1PA Tm in organic solvents was analyzed in different mixtures of organic co-solvent, aqueous buffer. Reactions were carried out in the same conditions described above.

The assayed co-solvents were acetonitrile, isopropanol, THF, DMF and DMSO in concentrations ranging from 0% to 40% of the total reaction volume.

Effect of metal ions on Rha-1PA Tm activity

Rha-1PA Tm activity was measured with several divalent cations (Zn^{2+} , Co^{2+} , Fe^{2+} , Cu^{2+} , Ni^{2+} , Mg^{2+}). Rha-1PA Tm was previously incubated with EDTA 0.1 M during 1 h at 80 °C, dialyzed at 4 °C overnight and lyophilized. Retro-aldol reactions were carried out at 25 °C in 96 well plates in a final volume of 0.2 mL. Reaction mixtures contained Tris/HCl buffer (40 mM pH 8.0), α GDH/TIM (0.4 U), NADH (0.2 mM), Rha-1PA Tm (28 µg), Rha-1P (10 mM) and different divalent cations in concentrations ranging from 0 to 675 µM. Decrease of the absorbance at 340 nm was monitored for 10 min.

Aldol reactions catalyzed by Rha-1PA Tm

Rha-1PA Tm was assayed in a multi-enzyme system using a one-pot/two-steps protocol. In the first step, DHA was phosphorylated to DHAP (**1**) by the enzyme dihydroxyacetone kinase (DHAK) in reaction mixtures containing HEPES buffer (90 mL, 60 mM, pH 7.5) DHA (55 mM), acetyl phosphate (80 mM), MgSO_4 (14 mM), DHAK (431 U) and AK (225 U). The reactions were initiated upon addition of ATP (1.8 mM). When DHAP accumulation was higher than 95%, the pH was adjusted to 5 to avoid DHAP degradation during the storage of the mixture. The reaction progress was followed spectrophotometrically, measuring the amount of DHAP formed, which was quantified by an enzymatic assay, based on the reduction of DHAP, catalyzed by α -GDH with concomitant oxidation of NADH to NAD^+ monitoring the decrease of the absorbance at 340 nm. The assays were run at room temperature during 15 min, containing a 1 mL aliquot of reaction mixture, Tris-HCl (40 mM, pH 8.0), NADH (0.2 M) and a mixture of α -GDH (2 U) and TIM (25 U) from rabbit muscle. For the measurement of the DHA consumption, DHAK (0.375 U), ATP (5 mM) and MgSO_4 (3.75 mM) were added to the reaction mixture (Oroz-Guinea et al. 2012). In the second step, aldol reaction was performed. Reactions (6 mL final volume) containing DHAP (33.3 mM, pH 6.9) and the corresponding aldehyde (16.7 mM) were started by addition of Rha-1PA Tm (0.37 U measured at 45 °C). To dissolve aldehydes **2b** and **2c** was necessary to add 10% of DMSO in the reaction media. Reaction progress was followed spectrophotometrically, measuring the amount of remaining DHAP and aldol formation. Both were quantified by an enzymatic assay, based on the reduction of DHAP, catalyzed by α -GDH with concomitant oxidation of NADH to NAD^+ monitoring the absorbance decreasing at 340 nm ($\epsilon_{\text{NADH}}=6220 \text{ cm}^{-1} \text{ M}^{-1}$). Reaction mixture (1 mL) contained Tris-HCl buffer (40 mM, pH 8.0), NADH (0.2 M), ZnCl_2 (15.0 mM) and the aldol reaction aliquot. Rha-1PA Tm was eliminated from the reaction aliquot by acidification followed by centrifugation. After that,

the pH of the aliquot was neutralized to 6.8-7.0. With these conditions, remaining DHAP was measured by adding α -GDH/TIM (2U), being the absorbance change proportional to DHAP content. After that, Rha-1PA Ec (0.05 U) was added to promote the retro-aldolization of the formed aldol and quantify the amount of newly DHAP produced which is proportional to the aldol formed. The aldol concentration can be calculated from the decrease in absorbance due to the NADH oxidation by applying the law of Lambert-Beer.

DHAP is labile at high temperatures, thus its concentration varies with time in the reaction medium at a given temperature. Therefore, we realized a study of the DHAP degradation at 25, 45, 65, 80, 85, 90 and 95 °C. Degradation assay was started adding DHAP (20 μ mol; 33.3 mM) to 0.6 mL of phosphate buffer (20 mM, pH 7.5), under magnetic stirring in a sand bath at the desired temperature. DHAP present in the medium was measured spectrophotometrically as described above.

Purification and characterization of reaction products

Reactions with aldehydes **2a-c**, were stopped and passed through activated carbon. The eluted was freeze-dried for NMR characterization.

Aldol **3d** was purified by HPLC. The reaction mixture was passed through an active carbon and filter (0.45 μ m, Milipore) before injection in HPLC. The separation was carried out using a *Mediterranea Sea*₁₈ (25 cm x 1.0 cm, particle $\varnothing$ =5 μ m) and the mobile phase gradient was modified as follow:

Channel A: H₂O + TFA 0.1%; Channel B: CH₃CN. Time 0 min: 83% of A; flow 2.3 mL.min⁻¹; time 8 min: 83% of A; flow 2.3 mL.min⁻¹; time 10 min: 5% of A; flow 3.0 mL.min⁻¹; time 14 min: 5% of A; flow 3.0 mL.min⁻¹; time 16 min: 83% of A; flow 3.0 mL.min⁻¹; time 19 min: 83% of A; flow 3.0 mL.min⁻¹; time 25 min: 83% of A; flow 2.3 mL.min⁻¹; time 26 min: 83% of A; flow 2.3 mL.min⁻¹.

The purified aldol was characterized by ¹H-NMR. In all cases the NMR spectra were coincident with those previously reported (Sánchez-Moreno et al., 2009)

Results

Heterologous expression and purification of Rha-1PA from *Thermotoga maritima*

Some years ago, in a databank search for predicted aldolase enzymes we found that the TM1072 gene from *Thermotoga maritima* codifies for a putative rhamnulose-1-phosphate aldolase (Rha-1PA Tm) (Oroz-Guinea et al. 2009). Although since then this enzyme has been used by others authors (Babich et al. 2011; Babich et al. 2012), to the best of our knowledge, there has been no reported any study describing its biochemical characterization. To investigate this enzyme further, the TM1072 gene was cloned and expressed in *Escherichia coli*. The gene was amplified by PCR from the genomic DNA of the *T. maritima* strain MSB8. Specific primers were designed, including restriction sites for the enzymes *Nde*I and *Hind*III. The resulting amplified DNA fragment was of the expected size (711 bp) and was digested and cloned in the expression vector pET-28b(+), which introduces a 6xHis tag in the N-terminal of

the recombinant protein. A sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Rha-1PA Tm expression showed a band of the expected molecular mass (29 kDa) which was consistent with the 28.9 kDa calculated for a protein of 256 amino acid residues, including the fused 6xHis tag. This band in the soluble fraction represented more than the 50% of the total protein (see SI, Figure S1). After growing and disrupting the *E. coli* cells, the recombinant enzyme was easily purified by heat treatment. The time and temperature of the heat treatment were optimized, resulting in a purification degree greater than 90% (Figure 1).

Figure 1

In cases where a higher degree of purity was required, the enzyme was purified by heat treatment at 60 °C for 20 min followed by a step of IMAC chromatography obtaining degrees of purification higher than 98% (Figure 1). To unambiguously identify the overexpressed protein as the TM1072 gene product, the amino acid composition and peptide-mass fingerprinting of the recombinant protein were obtained. An amino acid analysis showed a good correlation with the theoretical values (see SI, Table S1). The Molar extinction coefficient at $\lambda=280$ nm experimentally determined was $\epsilon_{280} = 32,161 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The identity of the recombinant protein was further confirmed by peptide-mass fingerprinting that showed fifteen peptides covering the protein sequence (Figure 2).

Figure 2.

The quaternary structure of the enzyme was analyzed by size-exclusion chromatography. The recombinant protein showed a retention time of 187 min with a constant flow rate of $1.0 \text{ mL}\cdot\text{min}^{-1}$. When this value was interpolated into the calibration curve prepared with proteins of known mass, a weight of about 114 kDa corresponding to a tetramer was found (see SI, Figure S2). This result agreed with the high sequence homology usually displayed by hyperthermophilic and mesophilic proteins (in the case of the Rha-1PA, the sequence identity between the enzyme from *T. maritima* and *E. coli* is 46.3%), which normally entail superposable 3D structures and similar catalytic mechanisms (Davies et al. 1993; Bauer and Kelly 1998; Maes et al. 1999).

Functional characterization of recombinant Rha-1PA Tm

Temperature and optimum pH determination

To determine the optimal temperature of Rha-1PA Tm, the specific activity of the enzyme was measured in a temperature range of between 20 and 95 °C. Rha-1PA Tm was active in the tested range of temperatures and the specific activity increased with temperature, up to a maximum of 95 °C (Figure 3). Nevertheless, the optimum temperature of 95 °C could not be confirmed because of the impossibility of performing reactions at higher temperatures. Interestingly, the calculated maximum activity temperature is higher than the optimum growth temperature of *T. maritima*, which is 80 °C. Usually, most enzymes characterized from hyperthermophiles have a maximum or optimal temperature near the microorganism's optimum growth temperature. However, *T. maritima* seems to behave differently because, besides the aldolase described in this work, many of the enzymes isolated from this

organism exhibit a similar performance with optimum temperatures around 95 °C (Bock et al. 1999; Song et al. 2008; Drzewiecki et al. 2010; Park et al. 2010; Huang et al. 2012). This fact could explain the capacity of this eubacteria to grow at temperatures above 90 °C (Huber et al. 1986).

Figure 3.

The Rha-1PA Tm optimum pH was determined by measuring the rate of aldol formation at different pHs (Figure 4). The maximum reaction rate was observed at pH 8.0. This value is similar to that described for the Rha-1PA from *E. coli* and other class II aldolases (Chiu and Feingold 1969). The range of the pH where the Rha-1PA Tm kept at least 50% of its maximum activity was between pH 5.7 and pH 9.0. This result is especially attractive because it allows the enzyme to be used in slightly acidic media where DHAP is more stable.

Figure 4.

Enzyme stability

The thermal stability of the Rha-1PA Tm was evaluated by incubating the enzyme at 80, 95 and 115 °C (for more details see the Experimental Section). In this study the irreversible denaturation of the enzyme was evaluated since the remaining activity was measured at 45 °C. As can be observed in Figure 5, the half-life of the Rha-1PA Tm did not greatly change between 80 and 95 °C (about 44 h and 33 h respectively). However, at 115 °C the half-life dropped to 3.5 h. These results indicate that the Rha-1PA Tm is not only thermophile but also thermo resistant and the most stable DHAP-dependent aldolase described until now.

Figure 5.

The thermal stability was also assayed at 25 and 4 °C to determine possible storage conditions. After 28 days at 4 °C, the remaining activity was greater than 80% of the initial activity. At 25 °C, the stability was slightly lower but over 70% of the initial activity was still detected after 28 days (see SI, Figure S3).

As previously discussed, thermozymes are usually extremely resistant to the action of organic solvents; therefore, a study of *T. maritima* enzyme activity in the presence of different co-solvent concentrations was carried out. Solvents displayed partition coefficients ($\log P$) between -1.378 and 0.526 to cover the range of $\log P$ for which the water-solvent mixture forms a single phase (Figure 6). Rha-1PA Tm showed a quite remarkable stability in all cases, although stability increased as the $\log P$ of the solvent decreased. The enzyme's stability in acetonitrile was remarkably high with a rate of 90% of the initial activity in H₂O/AcN 60:40 conditions. The high stability exhibited by the enzyme in DMSO, which keeps nearly 100% of its activity in the presence of a 20% concentration of co-solvent, and DMF, where 50% of the activity was found at percentages of organic solvent close to a 25% concentration, was also remarkable. The stability of the enzyme was lower in isopropanol and THF but, even in these cases, 40% of the activity was retained at a 10% concentration of co-solvent.

Figure 6.

Catalytic mechanism

Rha-1PA Tm has been described as a putative class II aldolase by nucleotide sequence homology (Osipiuk et al., 2003). In order to confirm this assignation, we incubated the recombinant aldolase in EDTA 0.1 M during 1 h at R/T. After that, the mixture was dialyzed to eliminate the quelation complex and, subsequently, to remove any divalent cation present. The activity of the resulting enzyme was measured with increasing concentrations of Zn^{2+} at R/T. The activity of Rha-1PA from *E. coli*, which, as is well known, belongs to class II aldolases, was used as a reference in the same experiment (Chiu and Feingold 1969). Meanwhile, the specific activity of the Rha-1PA from *E. coli* was clearly dependent on the Zn^{2+} concentration, and the activity of the Rha-1PA Tm remained unalterable in the absence or presence of different concentrations of Zn^{2+} (see SI, Figure S4). This behavior may be explained by the fact that the enzyme active site retained, even after treatment with EDTA, the divalent cation responsible for the residual activity. Incubation with EDTA at 80 °C was required to completely remove the metal from the enzyme active site and, thus, inactivate the aldolase. High temperature was needed to reduce the rigidity and compactness of the enzyme, allowing the exit of the divalent cation from the catalytic site. The enzyme prepared in this manner showed no activity in the absence of divalent cation and it was not possible to recover the activity by increasing the concentration of Zn^{2+} in the medium (Figure 7). Consequently, the metal ion-dependent activation of the Rha-1PA Tm was assayed with several divalent cations (see Materials and Methods Section). As can be observed in Figure 7, Co^{2+} was found to be the most potent activator, reaching a maximum activity at a concentration of Co^{2+} of 187.5 μ M. The specific activity of the enzyme increased 5.7 folds from 0.15 U per mg of protein before the treatment with EDTA and Co^{2+} to 0.85 U per mg of protein with the optimal concentration of Co^{2+} .

Figure 7.

Basic kinetic parameters for Rha-1PA Tm

Kinetic parameters for Rha-1PA Tm were calculated for the cleavage reaction of rhamnulose-1-phosphate. The kinetic behavior of two different preparations of the recombinant enzyme was analyzed. In the first preparation (Rha-1PA Tm1), the recombinant enzyme was purified by a heat shock followed by IMAC purification (for details see Materials and Methods Section). In the second case (Rha-1PA Tm2), after purification, the enzyme was incubated with EDTA at 80 °C, as described above, to remove the metal and the activity recovered with Co^{2+} 200 μ M. In both cases, Rha-1PA Tm showed Michaelis-Menten behavior and interestingly, inhibition by substrate excess was not detected (see SI, Figure S5). The calculated kinetic parameters are summarized in Table 1. The different treatment of the enzyme did not substantially modify the K_M of the aldolase but k_{cat} was 7-fold higher in Rha-1PA Tm2 demonstrating the importance of all the active sites being occupied by Co^{2+} .

Table 1.

Preliminary studies of Rha-1PA Tm as a biocatalyst

Four different aldehydes were used as a donor to test the synthetic utility of the Rha-1PA Tm: the natural acceptor L-lactaldehyde (**2a**), benzyloxyacetaldehyde (**2b**), phenylacetaldehyde (**2c**) and ethyl-3-methyl-4-oxocrotonate (**2d**). Reactions were carried out using 100 μ mol of the aldehyde (aldehyde/DHAP 1:2 ratio) at two different temperatures: 45 and 80 °C (Figure 8).

Figure 8.

Rha-1PA Tm was able to use the four aldehydes tested at both temperatures. Results are summarized in Table 2. At 45 °C more than 90% (percentage represents the non-isolated molar yield based on the aldehyde input) of aldol **3a** was formed after a 20 min reaction. In the same way, for aldol **3d**, the maximum formation was achieved after a 20 min reaction. For aldols **3b** and **c** the formation rate was lower and 2.5 and 3 h were necessary to raise the maximum accumulation (68 and 53%, respectively). As expected, when the reactions were carried out at 80 °C the reaction time decreased dramatically. However, aldol formation percentages varied only slightly. In general, the percentages of aldol formation decreased probably due to the much faster rate of DHAP degradation at this temperature (see SI, Figure S6). However, the end result in aldol yield is a compromise between the rate of the aldol addition reaction and the rate of degradation of DHAP. Thus, the formation of aldol **3a** dropped to 84% and aldol **3c** formation decreased to 48%. On the other hand, in the case of aldol **3b**, the percentage of formation obtained was identical at 45 and 80 °C and for aldol **3d** an increase in the formation rate to 98% was observed.

Table 2

Finally, a preliminary study of the stereochemistry of the reaction catalyzed by the Rha-1PA Tm was carried out. A reaction with **2d** was repeated in a 0.5 mmol of aldehyde scale and aldol **3d** was purified and characterized by NMR in order to evaluate if the *threo:erythro* ratio was modified with the reaction temperature. The NMR spectra of aldol **3d** were coincident with those previously described for this compound (Sanchez-Moreno et al. 2009a), showing a 80:20 *threo:erythro* ratio both at 45 and 80 °C. These results indicate that the temperature does not affect the stereochemical course of the reaction and were consistent with the stereochemistry expected for this enzyme and with the results previously reported for the Rha-1PA from *E. coli* (Sanchez-Moreno et al. 2009a).

Discussion

In this work, the gene TM1072 from *Thermotoga maritima* encoding a putative rhamnulose-1-phosphate aldolase was cloned and expressed in *E. coli*, and the encoded protein (Rha-1PA Tm) purified and characterized. Our results proved that Rha-1PA from *T. maritima* is a hyperthermophile enzyme with an optimum temperature (95 °C) even higher than the optimum microorganism growth temperature (80 °C). Another interesting feature of this enzyme, especially from the perspective of its application in synthesis, is that it maintains a significant percentage of activity at temperatures far below 95 °C (e.g., about 20% of its activity at 45 °C) allowing for it to be used in a broad range of temperatures. This is a very interesting and distinctive property since most hyperthermophile enzymes are often inactive at low temperatures due to an excessive structural rigidity (Vieille and Zeikus 2001). It is possible to hypothesize that Rha-1PA Tm combines local flexibility in its active site (which must

be responsible for the activity at low temperatures) with a high overall rigidity (which must be responsible for the thermostability) (Dellus-Gur et al. 2013; Wintrode and Arnold 2001; Merz et al. 1999; Ichikawa and Clarke 1998; Sterner et al. 1996). The Rha-1PA Tm displayed a great stability to extreme reaction conditions, such as high temperatures (a half-life of more than 3 h at 115 °C) and the presence of different organic solvents. Besides this, Rha-1PA Tm showed activity in a wide range of pH with an optimum activity at pH 8.0.

Mechanistically, two classes of aldolases can be recognized: (i) class I aldolases form a Schiff-base intermediate between the donor substrate and a highly conserved lysine residue in the active site of the enzyme; (ii) class II aldolases depend on a metal cation, mainly Zn^{2+} , as a cofactor, which acts as a Lewis acid in the activation of the donor substrate (Rutter 1964; Horecker et al. 1972). This hyperthermophile aldolase belongs to class II and uses Co^{2+} as a divalent metal ion cofactor instead of Zn^{2+} . Besides Co^{2+} , only Ni^{2+} showed a slight recovery capacity at high concentrations above 400 μM . In the literature, this preference has been reported also for other meso- or thermophile DHAP-dependent aldolases (La et al. 2009; Labbé et al. 2011). The kinetic parameters previously reported in the literature for the Rha-1PA from *E. coli* (Chiu et al. 1975) differ significantly from the values obtained for the *Thermotoga* enzyme. Especially remarkable is the k_{cat} value, which is more than one order of magnitude higher in the *E. coli* enzyme (Table 1). However, it should be taken into consideration that the kinetic parameters for Rha-1PA Tm were calculated at 25 °C where the specific activity of the enzyme is more than 20-fold lower than at 95 °C, therefore at higher temperature both K_{M} and k_{cat} may change substantially. Considering that the Q_{10} values —an average fold-increase in reaction rate per 10 °C increase in temperature— of the majority of thermophilic enzymes are the same as for mesophilic enzymes, that is $Q_{10} = 2$, when Rha-1PA Tm work at temperatures closer to the optimal, the turnover number (k_{cat}) must increase significantly (Elias et al. 2014). Although in the case of the *E. coli* enzyme can be also expect an increase in the turnover number, this parameter can only grow until the denaturation temperature of the enzyme, which can be assume will not be as high as the one of Rha-1PA Tm.

Reaction with aldehyde **2d** was performed at two different concentrations, 16.7 and 83.5 mM. In both cases the yield of aldol formation was practically the same (97.3 vs 98%) when the reactions were carried out at 80 °C. However, when the reactions were performed at 45 °C, the yield was slightly higher (95.7 vs 87%) when the concentration of aldehyde substrate was higher. Besides, the time required to reach the maximum yield was practically the same at the two concentrations of aldehyde assayed and both at 45 °C and 80 °C (25 min and 7 min respectively). Although in the figure S5 can be observed that the enzyme was saturated at relatively low concentrations of substrate, it is necessary to notice that the kinetic study was carried out in the retro-aldol reaction direction (monosubstrate reaction) and therefore their behavior can be different than the one displayed when the enzyme is used in synthetic sense (bisubstrate reaction). These data suggest that, likewise has been described for other hyperthermophilic enzymes (Li et al. 2005), the Rha-1PA Tm keep unaltered its activity at relatively high concentrations of substrate.

The utility of Rha-1PA Tm as a biocatalyst for C-C bond formation was proved at the analytical scale in the context of the multienzyme system previously developed in our group (Sánchez-Moreno et al. 2004; Sanchez-Moreno et al. 2009a; Oroz-Guinea et al. 2012). This system is

based on the use of the recombinant ATP-dependent dihydroxyacetone kinase (DHAK) from *C. freundii* CECT 4626 for the *in situ* phosphorylation of DHA (Sánchez-Moreno et al. 2009b). One advantage of this method is that it can be used as a one-pot/one-step or one-pot/two-steps system depending on the reaction conditions required. Rha-1PA Tm was assayed using the one-pot/two-steps protocol. In this way, DHAP is easily produced and thus may be used in excess with respect to the aldehyde, reducing the problem of its greater degradation at high temperatures. When comparing the results described in this work (Table 2) with those previously obtained in our laboratory using Rha-1PA from *E. coli* as biocatalyst (Iturrate, 2008), can be observed that in all cases the formation of aldol is higher, or at least equal, when the Rha-1PA Tm was used as biocatalyst (Table 2). Particularly noteworthy were the results obtained with aldehydes **2b** and **2d**, since the yield of aldol products **3b** and **3d** increased from 32% and 62% to 67% and 98% respectively when the reactions were catalyzed by Rha-1PA Tm at 80 °C. Furthermore, our preliminary studies on the stereochemistry of the reaction catalyzed by the enzyme from *T. maritima* indicate that it is similar to that shown by its homologous from *E. coli* (Sanchez-Moreno et al. 2009a).

In conclusion, the rhamnulose-1-phosphate aldolase from *Thermotoga maritima* has a very interesting number of features as biocatalyst and may be the alternative of choice when extreme reaction conditions are necessary.

Acknowledgements

We thank the Spanish Ministerio de Economía y Competitividad (Grant PI11/01436) and Comunidad de Madrid (Grant S2009/PPQ-1752) for financial support. I. O.-G. is a JAEPredoc fellow from CSIC.

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Figure captions

Figure 1. SDS-PAGE gel of recombinant Rha-1PA Tm purification. Lane 1, low molecular weight markers; lane 2, cell free extract of transformed cells; lanes 3 and 4 heat treatment at 60 °C; lanes 5 and 6 heat treatment at 70 °C; lanes 7 and 8 heat treatment at 80 °C; lane 9 IMAC purification; lane 10 heat treatment at 60 °C during 20 min followed by IMAC purification.

Figure 2. Peptide-mass fingerprint of Rha-1PA Tm. The shaded sequence indicates the region covered by the peptides identified after tryptic digestion. Each identified peptide is over- or underlined and its experimental mass is indicated in Da.

Figure 3. Temperature–activity profile for Rha-1PA Tm. Maximum activity was reached at 95 °C indicating that Rha-1PA Tm is a hyperthermophile enzyme.

Figure 4. pH profile for recombinant Rha-1PA Tm. Reactions were performed at different pHs in appropriate buffers with overlapping pH ranges: Bis/Tris (●); sodium phosphate (○); Tris/HCl (▼).

Figure 5. Thermal stability of recombinant Rha-1PA Tm. Enzyme was incubated at 80 °C (●), 90 °C (▼) and 115 °C (■).

Figure 6. Activity of Rha-1PA Tm in the presence of different organic solvents: acetonitrile (▼); DMF (○); DMSO (●); isopropanol (△); THF (■).

Figure 7. Rha-1PA Tm re-activation by different divalent metals at concentrations between 0 and 675 μM: Co²⁺ (●); Zn²⁺ (○); Fe²⁺ (▼); Cu²⁺ (△); Ni²⁺ (■) and Mg²⁺ (□).

Figure 8. Aldol addition reaction between DHAP (**1**) —*in situ* obtained by enzymatic phosphorylation of DHA— and aldehydes **2a-d**, catalyzed by Rham-1PA at (A) 45 °C and (B) 80 °C

Tables

Table 1

Table 1. Basic kinetic parameters for Rha-1PA Tm.

	K_M (M) $\pm$ (SD)	k_{cat} (s^{-1}) $\pm$ (SD)	k_{cat} / K_M ($s^{-1} M^{-1}$)
Rha-1PA Tm1 ^a	$2.9 \cdot 10^{-3} \pm 0.6$	0.36 ± 0.01	$0.12 \cdot 10^3$
Rha-1PA Tm2 ^a	$3.6 \cdot 10^{-3} \pm 0.3$	2.52 ± 0.04	$0.7 \cdot 10^3$
Rha-1PA <i>E. coli</i> ^b	$0.2 \cdot 10^{-3}$	38.3	$1.9 \cdot 10^5$

^a Kinetic parameters calculated at 25 °C. Values presented are the mean of three replicates measurements. ^b data taken from reference (Chiu et al., 1975).

Table 2

Table 2. Percentage^a of aldol formation using Rha-1PA Tm or Rha-1PA from *E. coli*

Aldehyde (2)	Aldol 3a-d (%)		
	Rha-1PA Tm (45 °C)	Rha-1PA Tm (80 °C)	Rha-1PA Ec (R/T)
a	>90	84	83
b	68	67	32
c	53	48	49
d	87	98	62

^a Percentage represents the non-isolated molar yield based on the aldehyde input.

Figures

Figure 1. SDS-PAGE gel of recombinant Rha-1PA Tm purification.

Figure 2. Peptide-mass fingerprint of Rha-1PA Tm.

Figure 3. Temperature–activity profile for Rha-1PA Tm.

Figure 4. pH profile for recombinant Rha-1PA Tm.

Figure 5. Thermal stability of recombinant Rha-1PA Tm.

Figure 6. Activity of Rha-1PA Tm in the presence of different organic solvents

Figure 7. Rha-1PA Tm re-activation by different divalent metals

Figure 8. Aldol addition reaction between DHAP (**1**) —*in situ* obtained by enzymatic phosphorylation of DHA— and aldehydes **2a-d**