Biochimica et Biophysica Acta 1814 (2011) 622–629

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Characterization of the aldol condensation activity of the *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) cloned from *Pseudomonas fluorescens* N3

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ARTICLE INFO

Article history:
Received 19 July 2010
Received in revised form 1 March 2011
Accepted 21 March 2011
Available online 7 April 2011

Keywords:
Recombinant production
*t*HBP-HA
Aldol condensation
Pseudomonas

ABSTRACT

The gene encoding *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) was isolated from *Pseudomonas fluorescens* N3, an environmental strain able to degrade naphthalene. This enzyme is an aldolase of class I that reversibly catalyzes the transformation of the *trans*-*o*-hydroxybenzylidenepyruvate (*t*-HBP), releasing pyruvate and salicylaldehyde. The enzyme was expressed in *Escherichia coli* as a recombinant protein of 38 kDa with a His6-Tag at its N-terminus. The recombinant protein His-*t*HBP-HA was purified by affinity chromatography and we present here the biochemical characterization of its activity in the aldol condensation reaction. The aldol condensation reaction parameters were determined using as acceptors both salicylaldehyde, which is the natural substrate taking part to the naphthalene degradative pathway, and benzaldehyde. In both cases, His-*t*HBP-HA shows similar apparent K_m and apparent V_{max} values. Further analyses showed that the optimal pH and temperature of His-*t*HBP-HA activity are 7.0 and 30 °C, respectively. The *t*HBP-HA catalytic rates and the availability of an efficient system to produce large amounts of purified protein are relevant from a biotechnological point of view.

1. Introduction

Aldolases are a specific group of lyases that catalyze the reversible stereoselective addition of a donor compound (nucleophile) to an acceptor compound (electrophile). Aldolases are known to participate to both the anabolism and the catabolism of highly oxygenated metabolites, essential for many biosynthetic pathways of carbohydrates, keto acids, and amino acids [1–3]. On the basis of their enzymatic mechanism, aldolases were grouped into two classes, I and II [2,3]. Class I aldolases activate the donor substrate by forming a Schiff base as an intermediate, thanks to a well-conserved lysine in the active site. This activated donor is then stereoselectively added to the acceptor aldehyde. In class II aldolases, a divalent metal ion (mostly Zn^{2+} but also Co^{2+} or Fe^{2+}) is coordinated by three histidine residues to the enzyme active site. The metal cation acts as a Lewis acid and activates the carbonyl donor substrate. Moreover, aldolases show strict selectivity for donor compounds, and therefore, they can also be functionally classified according to the donor used [2,3]. The only exception reported in literature is the novel D-fructose-6-phosphate aldolase isoenzyme (FSA) from *Escherichia coli* as reported by Clapés et al. [3]. This enzyme which accepts unphosphorylated dihydroxyacetone (DHA) as donor also readily accepts monohydroxylated donors in place of DHA and glycolaldehyde (GA) as an alternative donor substrates.

The making and breaking of carbon—carbon (C—C) bonds is a significant component of synthetic organic chemistry in the formation of larger and more complex compounds from small and simple starting materials [4,5]. In addition, C—C bond formation by aldolases can generate up to two new stereocenters in the resulting aldol products. Along with the ability to use a wide range of aldehydes as acceptors, this capacity indicates that most aldolases are interesting tools in the asymmetric syntheses of rare, deoxy and fluoro sugars, or sugar-derived compounds as iminocyclitols, statins, epothilones, D- and L-sialic acids and other analogs [1–3].

The involvement of aldolases in the metabolism of sugars, keto acids, and amino acids has been extensively described. Examples are dihydroxyacetone phosphate-dependent aldolases, such as the well-known fructose 1,6-bisphosphate aldolase [3,4]; aldolases depending on pyruvate/phosphoenolpyruvate or acetaldehyde as donor substrates; and glycine-dependent aldolases, for the formation of hydroxylated amino acids such as D- and L-threonine and serine [1–3,6].

To a lesser extent, aldolase activities were also shown to participate with their retro-aldol activity in the aromatic hydrocarbon degrading

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doi:10.1016/j.bbapap.2011.03.013

pathways in bacteria [7–11]. In particular, Eaton et al. cloned the gene encoding the *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) from the naphthalene catabolic plasmid NAH7 of *Pseudomonas putida* and showed that this enzyme transforms *t*-HBP to salicylaldehyde and pyruvate (Fig. 1) [7]. In addition, the capability of *t*HBP-HA to condense aromatic and non-aromatic aldehydes with pyruvate was assessed using crude extracts of recombinant *E. coli* strains [8]. Kuhm et al. purified from *Pseudomonas vesicularis* DSM 6383 (strain BN6) a *trans-o*-hydroxybenzylidenepyruvate aldolase [9] which catalyzes the cleavage of *trans-o*-hydroxybenzylidenepyruvate to salicylaldehyde and pyruvate. This reaction is part of the degradative pathways for naphthalene and naphthalene sulfonate metabolism by *P. vesicularis*. In this paper, the purification by conventional methods and the characterization in the retro-aldol activity is reported. Ohmoto described the presence and the purification of two *t*HBP-HAs in *Sphingomonas paucimobilis* TA-2 [10] involved in naphthalene sulfonate metabolism, comparing the different characteristics of the *t*HBP-HA A and the *t*HBP-HA B after purification from a cell-free extract.

However, the application of bacterial *t*HBP-HAs as aldol condensation tools has not been extensively explored yet; in fact, no previous studies resulted in the detailed biochemical characterization of the aldol condensation activity. To address this issue, we aimed at the cloning and the expression in *E. coli* of the *t*HBP-HA from *Pseudomonas fluorescens* N3 able to degrade naphthalene [12]. The *t*HBP-HA as a recombinant His-Tag protein was prepared, purified by affinity chromatography, and we present here for the first time the biochemical characterization of its aldol condensation activity using as acceptors both salicylaldehyde, which is the natural substrate taking part to the naphthalene degradative pathway, and benzaldehyde.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this paper are listed in Table 1. *P. fluorescens* N3 was isolated from activated sludges from a waste–water treatment plant because of its ability to use naphthalene as the only carbon and energy source for its growth [12]. *E. coli* JM109 [13] and *E. coli* M15 (pREP4) (Qiagen, Hilden, Germany) were chosen as the host strains: the former was used for gene cloning and expression, and the latter was well suited for protein purification. Recombinant plasmids (see below for details) were constructed by standard procedures [14].

2.2. Identification, PCR-amplification, and cloning of gene encoding *t*HBP-HA

Isolation of the complete coding region of *t*HBP-HA from *P. fluorescens* N3 was obtained from purified total DNA of this strain by PCR-amplification. The reaction was carried out in 20 μl of a mixture containing 100 ng of total DNA, 1 μM of each primer, 0.2 mM of dNTPs, 1 U of *Pfu* DNA polymerase, and PCR buffer (Fermentas, M-Medical, Milano, Italy). The PCR-amplification was conducted at 95 °C for 30 s, 61.2 °C for 45 s, and 72 °C for 2 min for a total of 35 cycles. Forward primer F1 (5′-ATCGGACGCCAATTCTGATG-3′) and reverse primer R1 (5′-GGCGCAAGAGATGAGCTTTA-3′) were used to amplify a fragment

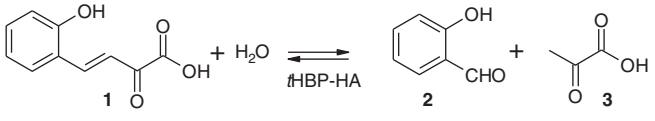


Fig. 1. Reaction catalyzed by the *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) in naphthalene degradative pathway in bacteria belonging to the *Pseudomonas* genus. The *trans-o*-hydroxybenzylidenepyruvate (1) is converted to salicylaldehyde (2) and pyruvate (3). Salicylaldehyde is a key intermediate that is transformed to catechol, cleaved by a 2,3-catechol dioxygenase, and then enters in the TCA cycle.

Table 1
Strains and plasmids.

	Relevant genotype and phenotype	Reference
Strains		
Pseudomonas fluorescens N3	Nah^+; Sal^+	Di Gennaro et al., 1997
Escherichia coli JM 109	[*recA1 endA1 gyrA96 thi hsdR17* (r^+_k M^+_k) *supE44 relA1* λ$^-$ Δ(*lac-proAB*)(F′ *traD36 proAB* + *lacI*^{q} *lacZ*Δ*M15*)]	Yanish-Perron et al., 1985
E. coli M15 [pREP4]	(mal, str^r, rif^r, thi$^-$, lac$^-$, ara$^+$, gal$^+$, mtl$^-$, F$^-$, recA$^+$, uvr$^+$, lon$^+$)	Qiagen
Plasmids		
pVLT33	Km^r, RSF1010-*lacI*^{q}/P*tac* expression vector, containing MSC of pUC18	de Lorenzo et al., 1990
pVLT33-ALD	Km^r; a pVLT33 derivative containing the *t*HBP-HA gene from *P. fluorescens* N3 cloned with *Eco*RI/*Xba*I ends	This study
pQE30	Amp^r,T5 promoter, lac operator, ribosome-binding site, start codon, 6×His tag sequence, multiple cloning site, stop codons in all three reading frames, Col E_1 origin of replication, *lacI*^{q} repressor gene.	Qiagen
pQE30-ALD	Amp^r, a pQE30 derivative for the expression of *t*HBP-HA gene from *P. fluorescens* N3 as His-tagged protein cloned with *Bam*HI/*Hind*III	This study

that included the gene encoding *t*HBP-HA (*nahE*) with its flanking regions. Forward primer F2 (5′-CAATCGAATTCGGGTGTTTCCATGTC-GAATAA-3′, *Eco*RI site underlined) and reverse primer R2 (5′-CCAACTCTAGAGGAGGTGAACTACTTCAATTCATTA-3′, *Xba*I site underlined) were used to generate an *Eco*RI/*Xba*I 1.0 kb DNA fragment harboring only the *nahE* gene to be cloned into the vector pVLT33 [15], giving the pVLT33-ALD construct. The construct was transferred in *E. coli* JM109 by electroporation. pQE30-ALD was generated for *t*HBP-HA expression as an N-terminal His6-tagged protein. Direct primer F3 (5′-CAATCGGATCCATGTCGAATAAAATTATGAA-3′, *Bam*HI site underlined) and reverse primer R3 (5′-CCAACAAGCTTGGTGAACTACTTCAATTCAT-TACTG-3′, *Hind*III site underlined) were used for amplification from pVLT33-ALD. The pQE30 plasmid (Qiagen, Hilden, Germany) was used as the expression vector and *E. coli* M15 (pREP4) as the host strain. All cloned inserts were verified before use by automated sequencing (Eurofins MWG, Ebersberg, Germany). The nucleotide sequence of *nahE* gene of *P. fluorescens* N3 is reported in the GenBank DataBase (Accession number GU319975).

2.3. Media, cultivation, and induction conditions

P. fluorescens N3 was grown in M9 mineral medium [16] under a saturated atmosphere of naphthalene. *E. coli* recombinant strains were grown in Luria–Bertani medium or M9 medium [16] supplemented with 10 mM glucose at 37 °C. Ampicillin and kanamycin were used in selective media at concentrations of 200 and 100 μg/ml, respectively. IPTG was added when the culture reached OD_{600} equal to 0.6, using a final concentration of 1 mM, and the cultivation temperature was decreased to 30 °C to minimize the potential formation of inclusion bodies.

2.4. SDS–PAGE analysis of cell extracts

Protein cell extracts from 2 ml of induced cultures were prepared according to standard procedures [14] and analyzed on NuPAGE Novex 10% Bis–Tris Gel (Invitrogen, San Diego, CA, USA) using NuPAGE MOPS SDS as running buffer (Invitrogen, San Diego, CA, USA) and stained with Coomassie Brilliant Blue R-250. The extracts were centrifuged to separate the soluble fraction from the inclusion bodies. The inclusion bodies were analyzed as follows: the pellet containing

inclusion bodies was resuspended in BC300 buffer (KCl, 300 mM; Tris, 20 mM pH 7.8; glycerol, 10%) and sonicated 4 times in ice in a Soniprep 150-MSE (Sanyo MSE, London, UK) with a pulse of 30 s and 2-min intervals between each pulse. The samples were centrifuged for 1 h at 24,000*g* at 4 °C and the pellets were resuspended in 100 μl 7 M urea. The samples were sonicated again 4 times with pulses of 10 s and intervals of 50 s in ice.

2.5. Western blotting experiments

Protein cell extracts were loaded on NuPAGE Novex 10% Bis–Tris Gel (Invitrogen, San Diego, CA, USA), and after electrophoresis, the proteins were transferred onto a nitrocellulose membrane (pore size, 0.2 μm; Amersham Biosciences, Uppsala, Sweden) at 30 V for 1 h in NuPAGE Novex Tris–Glycine transfer buffer (Invitrogen, San Diego, CA, USA) using an XCell-Blot Module (Invitrogen, San Diego, CA, USA) for Western blot analysis. The membrane was blocked with BSA (25 mg/ml) in TBS-T (TBS with 0.1% Tween 20) at room temperature for 1 h; it was washed 3 times for 10 min with TBS-T and incubated with HisProbe-HRP (Thermo Scientific Pierce, MA, USA) diluted 1:5000 in TBS-T for 1 h. The membrane was then washed 3 times for 10 min with TBS-T, incubated with ECL Western Blotting Substrate (Thermo Scientific Pierce, MA, USA) according to the manufacturer's instructions and visualized by chemiluminescence.

2.6. Preparation of cell extracts for aldol condensation assays

Cells from 500 ml of induced cultures were collected by centrifugation at 4 °C, washed twice, and resuspended in 50 mM potassium–sodium phosphate buffer (pH 7); the suspension was sonicated 4 times in ice in a Soniprep 150-MSE with a pulse of 30 s and 2-min intervals between each pulse. The lysate was cleared for 1 h at 24,000*g*.

2.7. Enzymatic assays for aldol condensation reaction

Aldol condensation assays using crude extracts were performed at 30 °C in 50 mM potassium–sodium phosphate buffer (pH 7) with 0.2–1.6 mg of total proteins, 10 mM of pyruvate (donor), and 0.1 mM of salicylaldehyde or benzaldehyde (acceptor), or 1 M of pyruvate (donor) and 10 mM of salicylaldehyde or benzaldehyde (acceptor). The reactions were performed in a final volume of 2 ml and followed for 3 h.

Aldol condensation assays for kinetics parameter determination using purified His-*t*HBP-HA were performed using 0.001–0.01 mg/ml of purified protein, 0.08–2 M of donor compound, and 0.8–20 mM of acceptor compound in a final volume of 2 ml in the same conditions utilized for the crude extracts.

Preparative reactions with crude extract were carried out at 30 °C in a final volume of 100 ml using 100 mg of total proteins dissolved in 50 mM potassium–sodium phosphate buffer (pH 7), and 20 mM of donor and 1 mM of acceptor substrates. The reactions were stopped after 7 h.

In case of His-*t*HBP-HA, preparative reactions were carried out at 30 °C in a final volume of 10 ml using 0.1–1 mg/ml of purified protein dissolved in 50 mM potassium–sodium phosphate buffer (pH 7), and 20 mM of donor and 1 mM of acceptor substrates. The reactions were stopped after 3 h.

Temperature activity assays were performed in 50 mM potassium–sodium phosphate buffer (pH 7) with 0.01 mg/ml of purified protein, 10 mM benzaldehyde, and 1 M pyruvate in a final volume of 2 ml, at *T* from 25 to 40 °C.

pH activity assays were performed at 30 °C using 0.01 mg/ml of purified protein, 10 mM benzaldehyde, and 1 M pyruvate in a final volume of 2 ml. The following buffers were used: 50 mM sodium acetate (pH 5.5–6.5); 50 mM potassium–sodium phosphate (pH 6.5–7.5); 50 mM Tris–HCl (pH 7.5–8.5).

The activity was calculated based on the production of the aldol condensation products and the aldehyde consumption. The specific activity was defined as the amount of enzyme required to catalyze the release of 1 μmol of the product per minute under the above mentioned conditions.

The kinetic parameters of apparent K_m and apparent V_{max} were calculated from Lineweaver–Burk plots.

Three independent data sets from the aldol condensation assays performed with different substrate concentrations were analyzed.

2.8. His-tagged protein purification

The purification procedure of His-*t*HBP-HA protein was performed using pellet from 500 ml of induced culture broths treated as described previously. Forty-eight milligrams of crude cell extract of *E. coli* M15 (pREP4) (pQE30-ALD) were applied to 2 ml of Co^{2+} TALON Metal Affinity Resin (Clontech, Euroclone, Milano, Italy) bed volume. Washing procedures were carried out according to the manufacturer's instructions. Proteins were eluted with a linear gradient of imidazole, from 50 to 250 mM. Eighteen fractions, each one of 1 ml, were collected and analyzed for their protein content by Bradford assay (Sigma, St. Louis, MA, USA), SDS–PAGE, and Western blot (50 μg of sample were loaded per lane) [14]. Imidazole was removed by membrane dialysis tubes with a cutoff of 12,000 Da against 50 mM potassium–sodium phosphate buffer (pH 7) overnight at 4 °C. Fractions were collected and frozen at −20 °C or lyophilized, using a Bench Lyophilizator LIO-5P (Cinquepascal S.r.l., Milano, Italy). The lyophilized protein was stored at −20 °C.

2.9. Characterization of transformation products

The aldol condensation reactions were followed by HPLC analyses using a Waters 515 HPLC instrument (Waters, Milford, MA, USA) coupled with a Waters 2487 Dual λ Absorbance Detector and a Millennium32 Waters data analysis program. The column was an Alltech Adsorbosphere XL C18 5U (length, 250 mm; ID, 46 mm) (Alltech, Bologna, Italy). Flow rate was set at 0.8 ml/min using water:acetonitrile 1:1 as the mobile phase. The double-beam detector allowed the visualization of substrate consumption salicylaldehyde or benzaldehyde at 256 nm and product formation *trans*-*o*-hydroxybenzylidenepyruvate or *trans*-benzylidenepyruvate at 296 nm.

The isolation and identification of the condensation products was performed as follows. For benzaldehyde, the reaction mixture (10–100 ml) was acidified to pH 3.0 and the products were extracted 3 times with an equal volume of ethyl acetate; the collected organic phases were dried over Na_2SO_4 and the solvent was removed in vacuum; the residue was dissolved in $CDCl_3$. For salicylaldehyde, the reaction mixture was frozen at −40 °C, lyophilized for 24 h, and the residue directly extracted with DMSO-d6. ^{1}H-NMR and ^{13}C-NMR analyses were performed using a Brucker AC-200 NMR.

2.10. Chemicals

Acetic acid, benzaldehyde, ethylacetate, naphthalene, isopropyl-ß-D-thiogalactopyranoside (IPTG), sodium pyruvate, salicylaldehyde, and sodium acetate were supplied by Sigma-Aldrich.

3. Results

3.1. Identification and cloning of the gene encoding tHBP-HA from P. fluorescens N3

In order to isolate a gene encoding a *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) activity from *P. fluorescens* N3, putative flanking sequences needed to be initially identified. To this end, the amino acid sequence (Swiss-Prot entry Q51947) of *t*HBP-

HA encoded by the *nahE* gene carried by the NAH7 plasmid of *P. putida* G7 was used as bait in reiterated BLASTp [17] searches. This analysis resulted in a set of proteins with a specific degree of similarity to the NAH7 *t*HBP-HA.

The DNA sequences upstream and downstream of the genes encoding this set of proteins were aligned with ClustalW [18] to detect conserved anchor sequences (data not shown). Several forward and reverse PCR primers were designed within conserved flanking sequences. Different pairs of these primers were used in PCR reactions with the *P. fluorescens* N3 total DNA as template. A primer pair composed of oligos F1 and R1, annealing to the upstream and the downstream regions, amplified a 1.1 kb fragment that was then sequenced and found to include a 1.0 kb ORF sharing 94% of sequence identity with the NAH7 *nahE* gene of the G7 strain. In order to amplify the N3 *nahE* gene alone with no adjoining intergenic sequences, primers F2 and R2 were designed. Finally, a construct was generated by cloning the 1.0 kb PCR fragment amplified from *P. fluorescens* N3 into the broad host range *lacI*^{q}/*Ptac*-based vector pVLT33 [15], giving rise to the construct pVLT33-ALD. *E. coli* JM109 was chosen as host strain.

3.2. Expression of the tHBP-HA in E. coli and aldol condensation assay in crude extracts

The *t*HBP-HA from *P. fluorescens* N3 was expressed in *E. coli* JM109 (pVLT33-ALD) strain and enzyme capability to perform aldol condensation of salicylaldehyde and pyruvate was tested using crude cell lysates.

The aldol condensation reaction experiment was followed by HPLC analysis monitoring the substrate consumption (salicylaldehyde) and product formation (*trans*-*o*-hydroxybenzylidenepyruvate) (Fig. 2A).

In order to ensure that the product of the aldol condensation was the *trans*-*o*-hydroxybenzylidenepyruvate, a preparative reaction was performed. This reaction was stopped after 7 h, when the peak corresponding to the concentration of salicylaldehyde appeared to be almost halved with respect to the initial concentration. The reaction product was recovered and identified as *trans*-*o*-hydroxybenzylidenepyruvate by ^{1}H-NMR and ^{13}C-NMR analysis (Table 2).

In an attempt to influence the equilibrium of the reaction in favor of the aldol condensation product and further characterize the enzyme behavior, benzaldehyde was used as an acceptor. In fact, in the literature [8], it is reported that the *trans*-benzylidenepyruvate retro-aldol reaction is 200 times slower than that of *trans*-*o*-hydroxybenzylidenepyruvate. The reaction was followed by HPLC analysis and a decreasing peak corresponding to benzaldehyde consumption was detected at 256 nm together with a growing peak at 296 nm that can be assigned to product formation (Fig. 2B). The product was extracted for ^{1}H-NMR and ^{13}C-NMR analysis after a preparative reaction, resulting in the *trans*-benzylidenepyruvate (*t*-BP) (Table 2).

3.3. Expression and purification of the His-tHBP-HA protein

In order to characterize the biochemical and kinetic parameters of the aldol condensation reaction, the *t*HBP-HA protein was engineered at its N-terminus with a His6-Tag and expressed in *E. coli* M15 (pREP4) carrying pQE30-ALD.

To assess the capability of *E. coli* M15 (pREP4) host strain to efficiently express the His-tagged *t*HBP-HA of *P. fluorescens* N3, samples of induced culture were taken after 4, 6, and 24 h from the inducer addition, and the protein content of the soluble fraction and inclusion bodies was analyzed by Bradford assay and SDS–PAGE (data not shown). In the soluble fraction, a band corresponding to the expected molecular weight (38 kDa) increased and a maximum level of expression was observed 6 h after induction (Fig. 3, lane 2). Western blot analysis with HisProbe-HRP performed on these same samples revealed a unique band of 38 kDa corresponding to the one visualized by SDS–PAGE; this further indicated that the His-tagged aldolase had been expressed.

The aldol condensation activity of crude extract (0.1 mg/ml) of *E. coli* M15 (pREP4) carrying pQE30-ALD was tested using pyruvate (1 M) and benzaldehyde (10 mM) as substrates. HPLC analysis

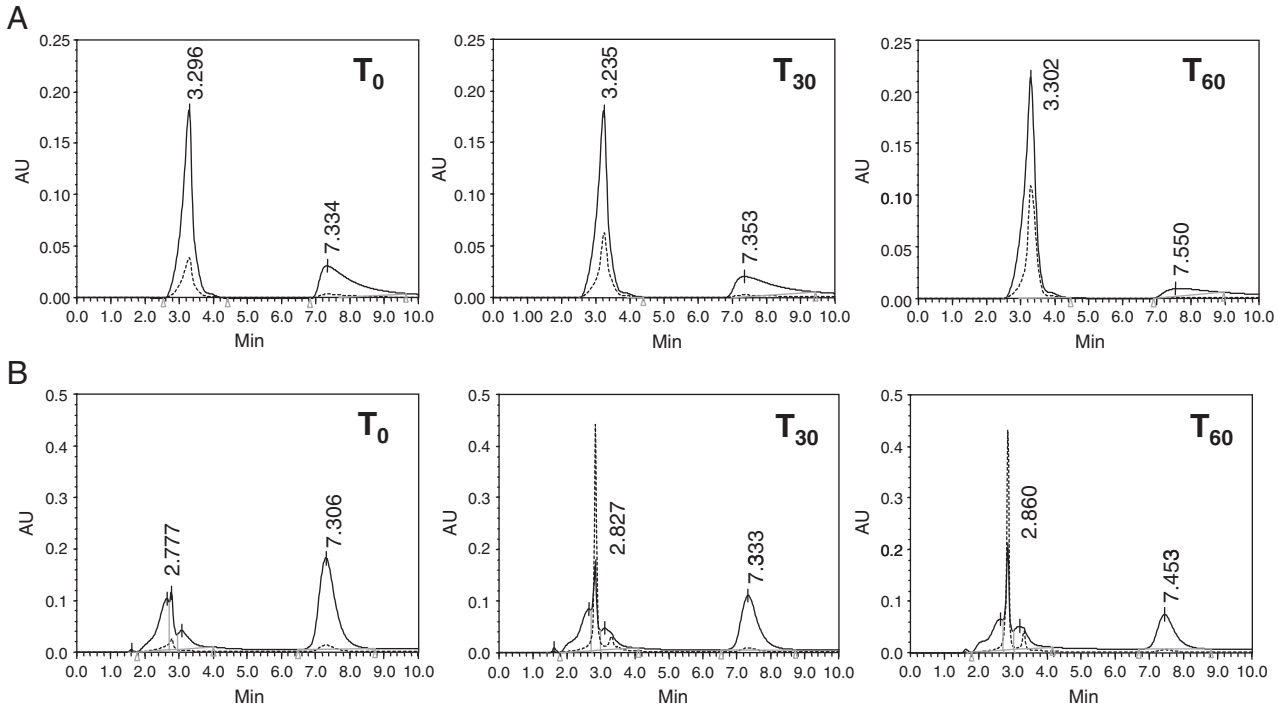
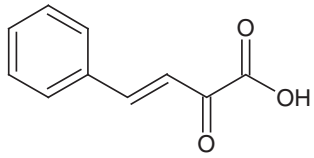
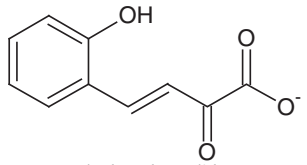


Fig. 2. HPLC analysis of aldol condensation of benzaldehyde/salicylaldehyde and pyruvate catalyzed by cell extracts of *Escherichia coli* JM109 (pVLT33-ALD) strain as described in Materials and methods. The solid line is the absorbance at 256 nm; the dotted line is the absorbance at 296 nm. The retention time around 3 min is product condensation signal; the retention time around 7 min is aldehyde signal. Part A, salicylaldehyde reaction; part B: benzaldehyde reaction.

Table 2
^{1}H-NMR and ^{13}C-NMR data of the aldol condensation reaction by *t*HBP-HA of *P. fluorescens* N3.

Compound	^{1}H-NMR and ^{13}C-NMR
 *Trans*-benzylidenepyruvic acid	$CDCl_3$, δ: 7.53, 1H, d, J = 16.0 Hz; 7.6–7.64, 3H, m; 7.86, 2H, dd, J = 2.3 Hz, 9 Hz; 8.0, 1H, d, J = 16.0 Hz CDCl3, δ: 196.80 1C (s); 167.5 1C (s); 151.574, 1C (d); 128.721–142.773, 5C (d) + 1C (s); 117.840 1C (d)
 *Trans-o*-hydroxybenzylidenepyruvate	DMSO-d6, δ: 6.72, 1H, d, J = 16.2 Hz, 6.8–6.9, 2H, m; 7.2, 1H, d, J = 8.1 Hz; 7.52, 1H, dd, J = 8.1, 1.0 Hz; 7.6, 1H, d, J = 16.2 Hz DMSO-d6, δ: 198.4 1C (s); 168.6 1C (s); 158.2 1C (s); 140.5 1C (d); 132.4 1C (d); 129.1 1C (d); 125.2 1C (d); 121.0 1C (s); 120.2 1C (d); 117.4 1C (d)

showed that initial amount of benzaldehyde had been completely consumed in 120 min. These data led to the quantification of the specific activity of the crude extract corresponding to 4.59 μmol mg^{-1} min^{-1}.

His-*t*HBP-HA was prepared in a soluble form by a metal-affinity purification procedure. The whole soluble fraction, containing 6.85 mg of total proteins in a final volume of 7 ml, was loaded onto the Co^{2+} metal-affinity column. Fractions were pooled and analyzed to determine their protein content by Bradford assay, SDS–PAGE (Fig. 3, lanes 3–9), and Western blot analysis. Successively, imidazole was removed by dialysis and the fractions were stored at −20 °C.

The enzymatic activity of the fractions containing the recombinant His-*t*HBP-HA was tested by condensation assays using 1 M pyruvate and 10 mM benzaldehyde as substrates in 2-ml reactions at 30 °C. The initial amount of benzaldehyde was halved in 30 min, while a peak corresponding to the *trans*-benzylidenepyruvate increased as the reaction progressed. From the analysis of the kinetics, the specific activity of the His-*t*HBP-HA was established to be 9.56 μmol mg^{-1} min^{-1}.

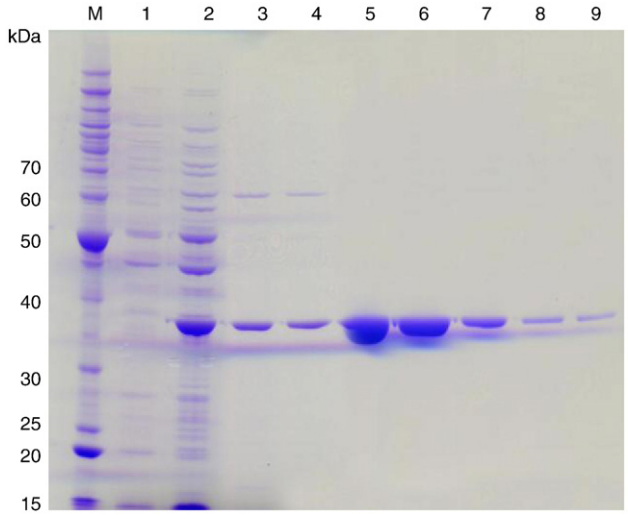


Fig. 3. Purification of His-*t*HBP-HA performed by a metal-affinity chromatography from crude extract of *Escherichia coli* M15 (pREP4) (pQE30-ALD) strain induced with IPTG 1 mM in LB medium at 30 °C for 6 h. SDS–PAGE analysis of some eluted fractions: lane M, molecular mass markers; lane 1, *E. coli* JM109 (pVLT33) extract, the negative control; lane 2, *E. coli* M15 (pREP4) (pQE30-ALD) extract; lane 3, fraction 3 (imidazole, 50 mM); lane 4, fraction 4 (imidazole, 50 mM); lane 5, fraction 6 (imidazole, 150 mM); lane 6, fraction 7 (imidazole, 150 mM); lane 7, fraction 8 (imidazole, 150 mM); lane 8, fraction 11 (imidazole, 250 mM); lane 9, fraction 15 (imidazole, 250 mM).

3.4. Biochemical characterization and kinetic parameter determination

The kinetic characterization of the condensation reaction of the purified His-*t*HBP-HA was performed in the presence of pyruvate as donor and using as acceptors either salicylaldehyde or benzaldehyde, with 0.001 mg/ml or 0.01 mg/ml of purified protein, respectively. Pyruvate was added at concentration ranging from 0.08 M to 2 M (100 times the concentration of acceptors), while the salicylaldehyde or benzaldehyde concentrations ranged from 0.8 mM to 20 mM. The reported values are the average of three independent experiments. Kinetic parameters of the recombinant His-*t*HBP-HA were determined; the results are shown in Fig. 4. The values of apparent K_m and apparent V_{max} were calculated from Lineweaver–Burk plots. The apparent K_m and apparent V_{max} values for benzaldehyde and salicylaldehyde were 8.98 and 3.58 mM, and 17.24 and 35.46 μmol mg^{-1} min^{-1}, respectively.

For further characterization of the recombinant His-*t*HBP-HA, the effects of temperature and pH on enzyme activity with 1 M donor and 10 mM acceptor were studied. At a fixed pH of 7.0, maximum His-*t*HBP-HA activity was observed at 40 °C (Fig. 5A). Further temperature increase caused a decrease of the specific activity. A control of the enzyme stability at selected temperatures (40 °C, 35 °C, and 30 °C) was then performed; the enzyme was kept at these temperatures for 30 min and then an enzymatic assay was run. The decreases of the activity were 64% at 40 °C and 24% at 35 °C, while at 30 °C, the activity remained unchanged. However, at 30 °C, the decreases of the activity were 24% after 5 h and 54% after 24 h. Hence, 30 °C was the selected temperature of the successive enzymatic activity measurements.

The effect of pH on the enzyme activity was studied. Fig. 5B shows the initial reaction rate measured by the aldol condensation assay method using benzaldehyde (10 mM) and pyruvate (1 M) as substrates with the following buffers: acetate (pH 5.5–6.5), potassium phosphate (pH 6.5–7.5), and Tris–HCl (pH 7.5–8.5). The maximum activity of His-*t*HBP-HA was at pH 7.0. These data confirm that the conditions of 30 °C and pH 7.0 are the best choice for running the enzyme assays.

3.5. Enzyme stability of lyophilized His-tHBP-HA

We also tested the level of protein enzymatic activity after lyophilization. We compared the performance of the purified component before and after lyophilization. Approximately 0.02 mg of lyophilized protein was rehydrated in 50 mM sodium–phosphate buffer, pH 7.0; then, the aldol condensation activity was assayed vs. the same amount of protein not lyophilized, using 1 M pyruvate and 10 mM benzaldehyde. No significant difference was detected between the two reactions.

4. Discussion

It is known that the catabolic pathway of naphthalene metabolism in *Pseudomonas* includes a retro-aldol reaction that transforms *trans-o*-hydroxybenzylidenepyruvate into pyruvate and salicylaldehyde (Fig. 1). The reaction is described as being reversible and not needing any cofactors [7,9,19]. Several aldolases belonging to the pathways of sugar metabolism have been described in detail [1–3]; in contrast, limited data are available concerning the aldol activity of aldolases belonging to other pathways [7,9,11]. In order to contribute some more data, in this work, we report the cloning of the gene and the isolation and characterization of the corresponding *t*HBP-HA enzyme from *P. fluorescens* N3.

The amino acidic sequence of the 37 kDa protein from *P. fluorescens* N3 shares 95% of similarity with the *t*HBP-HA of *P. putida* G7 strain. Besides, the N3 *t*HBP-HA enzyme probably belongs to the group that forms Schiff bases as is the case for the *t*HBP-HA of *P. putida* G7 [7–9]. This is supported by the presence in the sequence of the motif GxxGE involved in the binding of the alpha-keto acid moiety and of the highly

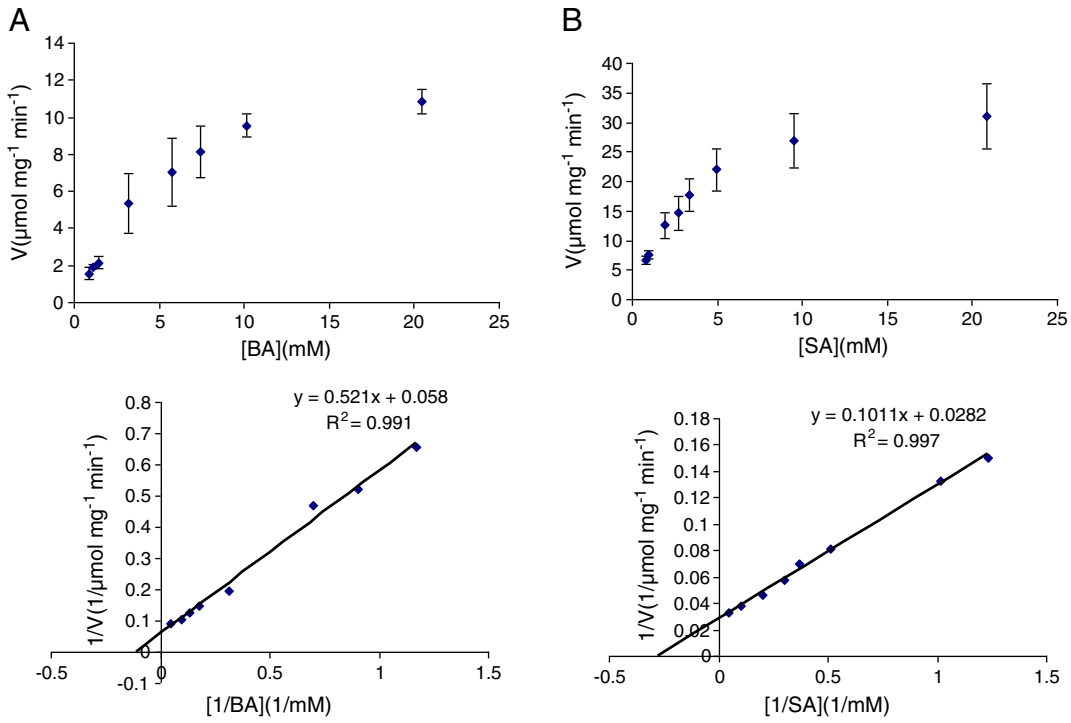


Fig. 4. Michaelis–Menten curves and Lineweaver–Burk plots of the His-*t*HBP-HA aldol condensation reaction with various concentrations of benzaldehyde (A) and salicylaldehyde (B). Reactions were performed under the aldol condensation assay conditions described in Materials and methods; concentrations of salicylaldehyde and benzaldehyde are indicated in the figure.

conserved lysine (Lys183) involved in the Schiff base formation [8,9,20,21].

Other genes encoding the *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (*t*HBP-HA) from the naphthalene catabolic pathway that can transform *t*-HBP to salicylaldehyde and pyruvate have been reported [7–9].

In particular, Eaton et al. reported the capability of NAH7 *t*HBP-HA to transform different substrates into aromatic and non-aromatic aldehydes and pyruvate using crude extracts of recombinant *E. coli* strains [8], but no kinetic parameter with the purified enzyme was determined. Nevertheless, the authors reported the *t*-HBP rates which varied from 1.6 to 3.6 µmol mg^{-1} min^{-1} measured using the crude extract; in addition, a K_m value of 4 µM for *t*-HBP was reported by Kuhm et al. [9].

Ohmoto et al. reported the purification of two *t*HBP-HAs, A and B, from *S. paucimobilis* TA-2; their K_m values for *t*-HBP were 9 and 3 µM, respectively [10].

Finally, Kuhm et al. purified from *P. vesicularis* DSM 6383 (strain BN6) a *trans-o*-hydroxybenzylidenepyruvate aldolase [9]. In this paper, the purification by conventional methods and the characterization of the retro-aldol activity is reported. The K_m value of 17 µM for *t*-HBP in contrast with the value of 0.4 mM for *t*-BP was determined.

We present here for the first time the characterization of the aldol condensation activity of a *t*HBP-HA. This extends and complements the literature data [8–10] that describe the characterization of the retro-aldol activity.

In our case, the successful determination of the kinetic parameters in the condensation reaction was made possible by the availability of an efficient integrated production process based on the use of an expression system yielding intracellular level of 50% of the total soluble protein that allowed the study and characterization of the His-*t*HBP-HA enzyme.

Since the equilibrium in the reaction of salicylaldehyde favors cleavage while retro-aldol reaction of benzylidenepyruvate is very slow

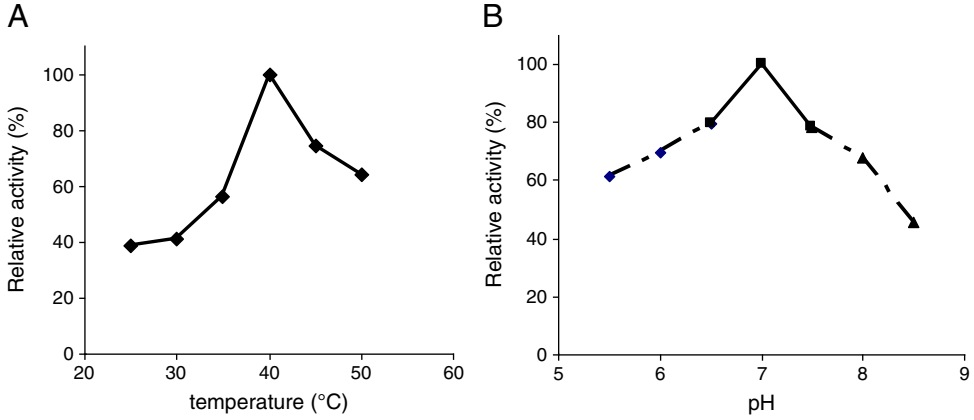


Fig. 5. Temperature (A) and pH (B) effects on the activity of His-*t*HBP-HA. Reactions were performed under the aldol condensation assay conditions described in Materials and methods; temperature range was 25–50 °C, pH range was 5.5–8.5 (pH 5.5–6.5 acetate buffer, dotted line; pH 6.5–7.5 phosphate buffer, solid line; pH 7.5–8.5 Tris–HCl buffer, dotted line).

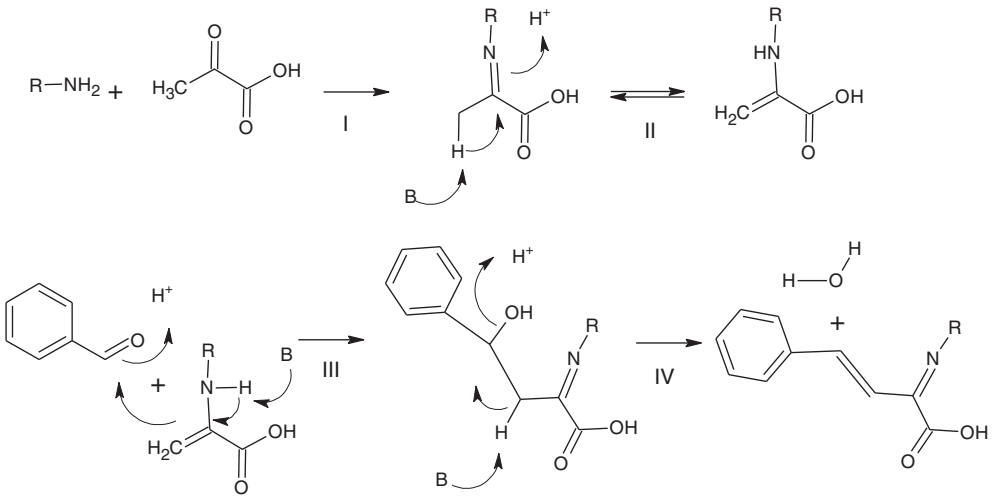


Fig. 6. Hypothetical mechanism of the aldol condensation reaction catalyzed by *t*HBP-HA. Schiff base formation (I); imine–enamine equilibrium (II); aldol condensation reaction (III); aldol dehydratation (IV).

[8], and because it is known that the *t*HBP-HA catalyzes the condensation reaction of several aromatic aldehydes including benzaldehyde [8], we used also this molecule as the acceptor for aldol condensations.

The apparent K_m values show that His-*t*HBP-HA has a similar affinity for benzaldehyde (8.98 mM) and salicylaldehyde (3.58 mM) that is the natural substrate taking part to the naphthalene degradative pathway. The apparent V_{max} values are also similar: the salicylaldehyde apparent V_{max} (35.46 μmol mg^{-1} min^{-1}) is two-fold the benzaldehyde apparent V_{max} (17.24 μmol mg^{-1} min^{-1}). However, considering that cleavage reaction rate of *t*-HBP is reported to be 200 times greater [8] than *t*-BP rate, it is evident that the measured salicylaldehyde condensation rate is lower than the actual rate. This result could be explained by the participation of the acidic phenol proton to the several acid–base reactions present in the hypothetical mechanism [8] (Fig. 6).

The comparison of the enzyme affinity for substrates and products and of enzymatic rates is not easy; in fact, all the available literature data refer to the reaction performed from the unsaturated acids to the corresponding aldehydes and pyruvate. Nevertheless, we can attempt a qualitative comparison. Concerning substrate affinity, it appears that the values reported by Kuhm et al. [9] showed a higher affinity with *t*-HBP (4–20 μM) than that we determined for salicylaldehyde (3.58 mM); in contrast, the value that we obtained with benzaldehyde (8.98 mM) is more similar to the value obtained by Kuhm et al. for the transformation of the *t*-BP into benzaldehyde and pyruvate (0.4 mM). Concerning rates, Eaton et al. [7,8] report rates measured using crude extracts; these were in the range of 1.6 to 3.6 μmol min^{-1} mg^{-1}. On the other hand, Kuhm et al. report a specific rate of 23.7 μmol min^{-1} mg^{-1} for the purified enzyme. Both these values are similar to our calculated rate of 35.5 μmol min^{-1} mg^{-1}, and all refer to the reaction of *t*-HBP and salicylaldehyde.

Our results also show that the optimum pH and temperature of the enzyme activity are 7.0 and 30 °C, respectively. The aldolase activity rapidly decreases even at pH 7.5 or 6.5 (−20%); in contrast, it increases at temperatures between 30 °C and 40 °C (+60%). However, experiments performed to test the enzyme stability show an activity decrease of 64% at 40 °C and 24% at 35 °C, after 30 min. In addition, at 30 °C, the activity decrease was 24% after 5 h and 54% after 24 h.

The N3 His-*t*HBP-HA catalytic rates and the availability of an efficient system to produce a good amount of purified protein can be also relevant from a biotechnological point of view. Indeed, the data obtained in this work have extended previous data [8–10] and could allow the use of N3 His-*t*HBP-HA as biocatalyst in aldol condensations for the production of α,β-unsaturated carboxylic acids conjugated to aromatic rings.

Acknowledgments

The authors are particularly grateful to G. Bertoni and P. Parenti for helpful discussion and critical reading of the manuscript, and E. Lanfranchi for technical support. This work was supported by MIUR-PRIN 2007 (grant 20077MY8M9 004) and was the recipient of a Sovvenzione Globale Ingenio fellowship granted to Silvia Ferrara from the Lombardia Regional Government.

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