Journal of Molecular Catalysis B: Enzymatic 61 (2009) 30–35

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Comparative characterisation of thiamin diphosphate-dependent decarboxylases

Dörte Gocke[a], Thorsten Graf[a], Helen Brosi[a], Ilona Frindi-Wosch[a], Lydia Walter[b], Michael Müller[b], Martina Pohl[a,*]

[a] Institute of Molecular Enzyme Technology, Heinrich-Heine University Düsseldorf, Research Centre Jülich, D-52426 Jülich, Germany
[b] Institute of Pharmaceutical Sciences, Albert-Ludwigs-University Freiburg, 79104 Freiburg, Germany

ARTICLE INFO

Article history:
Available online 5 April 2009

Keywords:
Enzymatic decarboxylation
Enzymatic carboligation
2-Hydroxy ketones
Phenylacetylcarbinol
Acetoin

ABSTRACT

Several 2-keto acid decarboxylases catalyse an acyloin condensation-like carboligase reaction beside their physiological decarboxylase activity. Although many data concerning stability and catalytic potential of these enzymes are available, a standard evaluation under similar reaction conditions is lacking. In this comprehensive survey we assemble already published data combined with new studies of three bacterial pyruvate decarboxylases, yeast pyruvate decarboxylase, benzoylformate decarboxylase from *Pseudomonas putida* (BFD) and the branched-chain 2-keto acid decarboxylase from *Lactococcus lactis* (KdcA). The obtained results proof that the optima for activity and stability are rather similar if comparable reaction conditions are used. Although the substrate ranges of the decarboxylase reaction of the various pyruvate decarboxylases are similar as well, they differ remarkably from those of BFD and KdcA. We further show that the range of acceptable donor aldehydes for the carboligase reaction of a respective enzyme can be reliably predicted from the substrate range of decarboxylase reaction.

1. Introduction

Pyruvate decarboxylases (PDC, E.C. 4.1.1.1) catalyse the non-oxidative decarboxylation of pyruvate to acetaldehyde requiring the cofactors thiamin diphosphate (ThDP) and divalent cations like Mg^{2+}. They are the key enzymes in homo-fermentative pathways cleaving the central intermediate pyruvate into acetaldehyde and CO_2. PDCs are commonly found in plants, yeasts and fungi, but absent in mammals [1]. Beside several PDCs from yeasts only four enzymes from prokaryotes have been cloned and characterised so far, encompassing three enzymes from the gram-negative bacteria *Zymomonas mobilis* (*Zm*PDC) [2], *Zymobacter palmae* (*Zp*PDC) [3,4] and *Acetobacter pasteurianus* (*Ap*PDC) [5], as well as one from the gram-positive bacterium *Sarcina ventriculi* (*Sv*PDC) [6]. Among these *Ap*PDC is an exception as it is a central enzyme of the oxidative metabolism [5]. PDCs are key enzymes in the production of biofuels from bulk plant materials like sugars or cellulose. Currently bacterial strains with engineered ethanol pathways, e.g. *Escherichia coli* strains with an inserted *Zm*PDC, alcohol dehydrogenase (ADH) and acyltransferase are investigated for industrial applications [7].

Beside their decarboxylase activity it is known that several decarboxylases are able to catalyse an acyloin condensation-like carboligation of aldehydes. Since the 1930s this activity of PDC from yeast is used in an industrial fermentative process for the production of (*R*)-phenylacetylcarbinol (**17**), a pre-step in the synthesis of ephedrine [8,9]. A similar carboligase activity yielding chiral 2-hydroxy ketones was detected and intensively studied with *Zm*PDC and further ThDP-dependent enzymes like benzoylformate decarboxylase from *Pseudomonas putida* (BFD, E.C. 4.1.1.7), branched-chain 2-keto acid decarboxylase from *Lactococcus lactis* (KdcA, E.C. 4.1.1.72) and benzaldehyde lyase from *Pseudomonas fluorescens* (BAL, E.C. 4.1.2.38) [10–18]. Different substrate-, regio- and stereoselectivities of these enzymes open the way to a broad range of 2-hydroxy ketones.

As decarboxylation and carboligation are catalysed at the same active site and are influenced by similar steric and electronic factors, the investigation of the substrate range of the decarboxylase reaction yields important information about the putative acyl donor spectrum for the carboligase activity. If a respective 2-keto acid is a substrate for decarboxylation, the binding of the corresponding aldehyde to the C2-atom of ThDP located in the active centre is most likely, meaning that this aldehyde may be a possible donor aldehyde in enzyme-catalysed carboligation reactions.

Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; BFD, benzoylformate decarboxylase; DMSO, dimethylsufoxide; *ee*, enantiomeric excess; KdcA, branched-chain 2-keto acid decarboxylase; ThDP, thiamin diphosphate.

* Corresponding author. Tel.: +49 (0)2461 613704; fax: +49 (0)2461 612490.
E-mail address: ma.pohl@fz-juelich.de (M. Pohl).

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doi:10.1016/j.molcatb.2009.03.019

Although various data concerning the decarboxylase activity of ThDP-dependent enzymes are reported in literature, a thorough characterisation of the substrate range of these enzymes under comparable reaction conditions is still missing. Here we report the characterisation of the decarboxylase activity of various 2-keto acid decarboxylases under similar reaction conditions with regard to pH- and temperature-dependent optima, stability, kinetic parameters and substrate ranges. These data provide important information not only for the determination of the acyldonor spectrum but also for the identification of suitable reaction conditions for the carboligase activity. Still optimal reaction conditions for the carboligase reaction might differ slightly from optimal conditions for the decarboxylase reaction.

2. Experimental

2.1. Cloning

The *pdc* gene (1674 bp) from *Acetobacter pasteurianus* (DSMZ 2347) was cloned into a pET22b-vector (Novagen) carrying a C-terminal His_6-tag (see supplementary material). The *pdc* gene of *Zymobacter palmae* (DSMZ 10491) was cloned into the pET28a(+)-vector (Novagen), also carrying a C-terminal His_6-tag. *E. coli* BL21(DE3) (Novagen) was transformed with the respective vector by electroporation. The sequences were verified by sequencing (Sequiserve). KdcA (with N-terminal His_6-tag) [14] and BFD (with C-terminal His_6-tag) [18] were cloned according to the published procedures. The *pdc* gene of *Saccharomyces cerevisiae* [19] was kindly provided by Stephan König from the Martin-Luther University Halle-Wittenberg and recloned as a C-terminal hexahistidine-fusion protein by Dr. Marion Wendorff in our lab.

2.2. Enzyme preparation

High cell density cultivation according to the method of Korz et al. [20] was performed in a 40 L Techfors reactor (Infors AG, CH) at 30 °C, pH 7.0. Cells were harvested by a separator (Westfalia).

Harvested *E. coli* cells were suspended by addition of 1:10 (w/v) buffer (50 mM potassium phosphate buffer, pH 6.5, containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP). After addition of 0.33 mg/mL lysozyme and incubation for 30 min on ice, cells were further disrupted by sonification before centrifuged. Enzyme purification to homogeneity >95% was performed by immobilised nickel chelate chromatography and additional size exclusion chromatography, using a purification protocol previously developed for *Zm*PDC [21] using the following buffers: Ni-NTA-chromatography: disintegration buffer (50 mM potassium phosphate buffer, pH 6.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP), washing buffer (50 mM potassium phosphate buffer, pH 6.5, 50 mM imidazole), elution buffer (50 mM potassium phosphate buffer, pH 6.5, 250 mM imidazole); G25-chromatography (20 mM potassium phosphate buffer, pH 7.0, 2.5 mM $MgSO_4$, 0.1 mM ThDP). The enzyme was either freeze-dried or diluted with 50% (v/v) glycerol and stored at −20 °C.

2.3. Decarboxylase activity

One unit of decarboxylase activity is defined as the amount of enzyme which catalyses the decarboxylation of 1 μmol pyruvate (PDCs), benzoylformate (BFD) or 3-methyl-2-oxo-butanoate (KdcA) per minute under standard conditions. Standard conditions are defined in Table 1, bottom line. Protein determination was performed according to Bradford [22] using BSA as a standard.

Two continuous decarboxylase assays were used. In the *coupled decarboxylase assay* an alcohol dehydrogenase reduces the aldehyde obtained by enzymatic decarboxylation of the respective 2-keto acid. The simultaneous consumption of nicotinamide adenine dinucleotide (NADH) was followed spectrophotometrically for 90 s at 340 nm (molar extinction coefficient ε of NADH = 6.22 L mmol^{-1} cm^{-1}). Typical assay composition (1 mL): 700 μL buffer (for buffer composition see Table 1, bottom line), 100 μL sodium pyruvate or another 2-keto acid (final concentration 20–40 mM, depending on enzyme kinetic), 100 μL NADH in buffer (final concentration 0.25 mM), 50 μL ADH. Yeast ADH (Roche, E.C. 1.1.1.1, final concentration 0.1 U) was used to monitor indirectly the decarboxylation of pyruvate to acetaldehyde. For all other 2-keto-acids horse liver ADH (Sigma, final concentration 0.25 U) was added. The assay was started by addition of 50 μL enzyme. The enzyme concentration was chosen such that a linear decay of NADH was observed over 90 s. ADH concentration was chosen in a way that the reduction of the aldehyde formed in the decarboxylase reaction was not rate limiting.

Further, a *direct decarboxylase assay* following the direct decay of pyruvate was used to measure decarboxylase activity under NADH-degrading or ADH-inactivating conditions. Assay composition: a solution of sodium pyruvate or 2-keto acid (final concentration 20–40 mM) in 950 μL buffer. The reaction was started by addition of 50 μL enzyme solution. The decay of pyruvate was followed at 320 nm ($\varepsilon_{pyruvate}$ = 0.022 L mmol^{-1} cm^{-1}) in quartz cuvettes.

Both decarboxylase assays show a standard deviation of about 10%. All decarboxylase activities in this paper are given as the average of minimum 3–6 measurements. Kinetic parameters were calculated by non-linear regression using the Michaelis-Menten equation and Origin 7G SR4 (OriginLab Coop.) except for PDC from *Saccharomyces cerevisiae* (*Sc*PDC), where the Hill equation including substrate inhibition was used ($v = v_{max} S^h/(S^h_{0.5} + S^h(1 + S/K_i))$). Activation energies were calculated for the decarboxylase reaction from an $\ln v_{max}/[1/T]$-plot in the linear range of 20–50 °C.

Substrate range of decarboxylase activity. Activity towards different 2-keto acids was measured with the coupled decarboxylase assay using horse liver ADH as coupling enzyme. It was verified that the respective aldehydes, which are obtained by decarboxylation, are substrates for the horse liver ADH. All substrates were applied in a concentration of 30 mM, except indole-3-pyruvate (**15**), which was applied in a concentration of 1 mM due to low solubility and strong UV absorbance.

Optima and stability investigations. pH and temperature optima were measured under initial rate conditions within 90 s using the direct decarboxylase assay. To investigate the stability towards pH, temperature and organic solvents, enzymes were incubated under the conditions given in Table 1, bottom line. Samples, withdrawn at appropriate time intervals, were subjected to residual activity determination using the coupled decarboxylase assay. It was ensured, that the pH in the cuvettes was stable during the time of detection.

2.4. Carboligation

Acetoin (**16**) *formation* (conditions for *Ap*PDC, *Zp*PDC, KdcA, BFD). Acetaldehyde (8.8 mg, 40 mM) was dissolved in 3 mL potassium phosphate buffer (50 mM, pH 7.5, containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP) and 1 mL dimethylsulfoxide (DMSO). After addition of 1 mL purified enzyme dissolved in the same buffer (~1.5 mg/mL protein content, final assay concentration 0.3 mg/mL) the reaction was stirred with 100 rpm at 30 °C. To avoid evaporation of the aldehydes the reaction batch was divided into 200 μL tubes with iced tips. Incubation for 1 min at 90 °C stopped the reaction. After vortexing and centrifugation the precipitate was removed.

The conversion was determined by GC (6890 N Agilent Technologies, column 50 m × 320 μm Cyclodex b-1/P, flow 3.4 mL/min H_2, pressure 0.8 bar, 1 μL injection volume, split 5:1, FID-detector, time program (oven): 5 min 50 °C, 40 °C min^{-1} to 190 °C), R_t

Table 1
Optima and stabilities of various 2-ketoacid decarboxylases. n.d. = not determined, h = Hill coefficient, K_i = inhibition constant.

	*Ap*PDC	*Zp*PDC	*Zm*PDC [48]	*Sc*PDC	KdcA [14]	BFD [18]
pH-optimum	pH 3.5–6.5	pH 4.5–8 maximum at pH 7	pH 5.5–8.0 maximum pH 6–6.5	pH 5–7	pH 6–7	pH 5.5–7.0 maximum at 6.2
pH-dependent stability half-life time	pH 5–7: no activity loss within 60 h pH 4: 2.3 h	pH 6.5/8: stable for several days pH 4/9: complete activity loss within 2 h	n.d. in potassium phosphate buffer	pH 5: 80 h pH 7: 53 h pH 8: 13 h	pH 5–7: no activity loss within 60 h pH 8: 40 h pH 4: < 2 h	pH 5.5: deactivation 0.3% min^{-1} pH 10: deactivation 0.1% min^{-1}
Temperature optimum	65 °C	55 °C	60 °C	43 °C	50 °C	68 °C
Temperature dependent stability half-life time	30 °C: 144 h 40 °C: 34 h 50 °C: 12 h 60 °C: 2 h 70 °C: 0.4 h	30 °C: 150 h 40 °C: 40 h 50 °C: 10 h 60 °C: 0.4 h	50 °C: 24 h	20 °C: 235 h 30 °C: 78 h 35 °C: 62 h	40 °C: 80 h 50 °C: 9 h 55 °C: 4 h	60 °C: 2 h 80 °C: 0.3 h
Natural substrate	pyruvate	pyruvate	pyruvate	pyruvate	3-methyl-2-oxobutanoic acid	benzoylformate
v_{max} [U/mg]	110 ± 1.9	116 ± 2.0	121	112.0 ± 35.6	181.6 ± 1.7	400 ± 7
K_M ($S_{0.5}$) [mM]	2.8 ± 0.2	2.5 ± 0.2	1.3	$S_{0.5}$: 21.6 ± 7.4	5.02 ± 0.2	0.37 ± 0.03
Shape of v/[S]-curve	Michaelis-Menten kinetic	Michaelis-Menten kinetic	Michaelis-Menten kinetic	sigmoidal (h = 1.6 ± 0.2) & substrate surplus inhibition (K_i = 35.1 ± 16.5)	Michaelis-Menten kinetic	Michaelis-Menten kinetic
Activation energy	27.1 kJ mol^{-1}	41 kJ mol^{-1}	43 kJ mol^{-1}	n.d.	8.5 kJ mol^{-1}	38 kJ mol^{-1}
Buffer used for the characterisation of decarboxylase activity and stability	50 mM potassium phosphate buffer pH 6.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP	50 mM potassium phosphate buffer pH 6.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP	100 mM potassium phosphate buffer pH 6.5, 5 mM $MgSO_4$, 0.1 mM ThDP	50 mM potassium phosphate buffer pH 6.5, 2.5 mM $MgSO_4$, 0.5 mM ThDP	50 mM potassium phosphate buffer pH 6.8, 2.5 mM $MgSO_4$, 0.1 mM ThDP	50 mM potassium phosphate buffer pH 6.0[a], 2.5 mM $MgSO_4$, 0.1 mM ThDP

[a] Substrate range was determined at pH 6.5.

[(*R*)-acetoin] = 6.98 min, R_t [(*S*)-acetoin] = 7.12 min. Absolute configuration was assigned according to the method of Crout et al. [12,23].

Mixed ligations of aromatic and aliphatic aldehyde. The conversion was followed by GCMS, employing a HP 6890 series GC-system fitted with a HP 5973 mass selective detector (Hewlett Packard; column HP-5MS, 30 m × 250 μm; T_{GC} (injector) = 250 °C, T_{MS} (ion source) = 200 °C, time program (oven): T_{0min} = 60 °C, T_{3min} = 60 °C, T_{14min} = 280 °C (heating rate 20 °C min^{-1}), T_{19min} = 280 °C). The enantiomeric excess (*ee*) was determined by chiral phase HPLC employing a HP 1100 HPLC system (Agilent) fitted with a diode-array detector. NMR spectra were recorded on a Bruker DPX-400. Chemical shifts are reported in ppm relative to $CHCl_3$ (^{1}H NMR: δ = 7.27) and $CDCl_3$ (^{13}C NMR: δ = 77.0) as internal standards. CD-spectra were recorded on a JASCO J-810 spectropolarimeter using acetonitrile as solvent.

Example: *(R)-phenylacetylcarbinol* (**17**) (conditions shown for *Ap*PDC; *Zp*PDC done similarly):

Reactions conditions: Benzaldehyde (159 mg, 30 mM) and acetaldehyde (110 mg, 50 mM) were dissolved in 40 mL buffer (pH 7.0) and 10 mL DMSO. After addition of purified *Ap*PDC dissolved in the same buffer (63 U/mL decarboxylase activity) the reaction was stirred slowly at 30 °C. After 24 h additional acetaldehyde (50 mM) and *Ap*PDC (42 U/mL) were added. After 72 h the reaction mixture was extracted three times with ethyl acetate (25 mL) and the organic layer was dried over Na_2SO_4. The solvent was evaporated and the crude product was dissolved in diethylether (5 mL) in order to remove traces of DMSO. The solution was washed with brine and dried over Na_2SO_4 followed by evaporation of the solvent. Purification of the crude product by flash column chromatography (cyclohexane/ethyl acetate 10:1) revealed the designated product. Isolated yield: 30%. HPLC: Chiralcel OD-H, *n*-hexane/2-propanol 90:10, 0.75 mL min^{-1}, 25 °C, R_t [(*S*)-**17**] = 11.25 min, R_t [(*R*)-**17**] = 12.56 min; ^{1}H NMR (400 MHz, $CDCl_3$, 300 K, ppm) δ = 2.10 (s, 3H, CH_3), 4.40 (bs, 1H, OH), 5.10 (s, 1H, *CH*OH), 7.30–7.41 (m, 5H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$, 300 K, ppm) δ = 25.3 (CH_3), 80.2 (CHOH), 127.4 (CH), 128.8 (CH), 129.1 (CH), 138.1 (Cq), 207.2 (C=O); GCMS R_t = 7.83 min; MS (70 eV, EI): *m*/*z* (%): 150 (3%) [M^+], 107 (100%), 79 (68%).

3. Results and discussion

3.1. Optimal reaction conditions

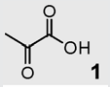
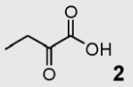
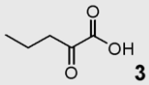
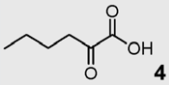
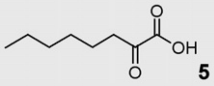
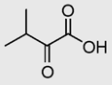
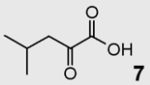
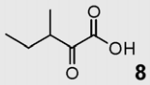
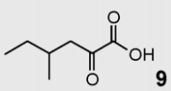
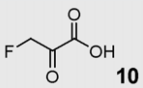
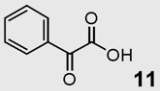
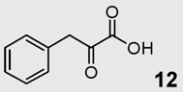
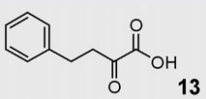
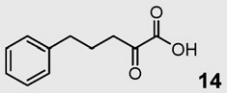
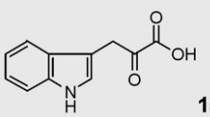
Optimal cofactor concentration. In all known ThDP-dependent decarboxylases the cofactors are bound non-covalently to the active site [24]. For the stability of the holoenzyme most ThDP-dependent enzymes require the addition of cofactors to the buffer. Under the applied enzyme concentrations the addition of 2.5 mM $MgSO_4$ and 0.1 mM ThDP is sufficient to keep the enzymes stable and active in potassium phosphate buffer for several days.

pH-dependent activity and stability. All tested 2-keto acid decarboxylases are most stable and most active in the slightly acidic to neutral pH range of pH 5–7 (Table 1). Although *Ap*PDC shows a maximal initial rate activity in a lower pH-range (pH 3.5–6.5), which was also observed by Raj et al. in sodium citrate buffer [4], the stability in this acidic range is drastically reduced. For all other decarboxylases optimal initial rate activities overlap very well with the stability optima. At alkaline pH values >8 the activity and stability of 2-keto acid decarboxylases is impaired.

Temperature-dependent activity and stability. Under initial rate conditions temperature optima for all bacterial enzymes range between 50 and 68 °C, whereas *Sc*PDC shows maximal activity already at 43 °C (Table 1). Carboligase reactions carried out for 2–3 days are performed at 30 °C with a half-life ($t_{1/2}$) of all enzymes >3 days. Most activation energies for the decarboxylase reaction of physiological substrates are in the range of 27–43 kJ mol^{-1}, except for KdcA showing exceptionally low activation energy of 8.5 kJ mol^{-1}.

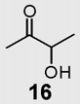
Stability towards dimethylsulfoxide (DMSO). The biotransformation of aromatic aldehydes is often hampered by their low solubility in aqueous systems. To enhance solubility the water-miscible organic solvent DMSO has been already successfully implemented

Table 2
Substrate range of the decarboxylation. The natural substrates (bold face) resulted in highest specific activity and were used for further kinetic studies (Table 1).

2-Keto acid	Specific activity [U/mg]					
	*Ap*PDC	*Zm*PDC [17]	*Zp*PDC	*Sc*PDC	KdcA [14]	BFD [44]
 **1**	**89.3**	**120**	**147.0**	43.4	1.9	0.4
 **2**	60	79	85.1	16.9	14.1	21.4
 **3**	12.9	13	20.7	18.8	22.7	12.5
 **4**	4.2	0.2	6.2	5.3	20.1	9.2
 **5**	1.1	n.d.	0.6	0.0	0.0	7.2
 **6**	n.d.	0.0	10.4	6.9	**152**	0.0
 **7**	1.1	0.3	2.8	0.3	40	3.3
 **8**	2.2	0.0	2.8	0.0	57.9	3.6
 **9**	0.6	0.0	0.4	0.0	28.9	6.9
 **10**	0.3	n.d.	0.6	0.0	0.0	0.0
 **11**	1.1	0.0	0.3	0.0	12.8	**420.1**
 **12**	0.8	0.0	1.8	0.0	13.1	0.0
 **13**	0.3	0.0	0.3	1.7	2.4	4.4
 **14**	0.0	0.0	0.2	0.2	1.7	0.0
 **15**	0.0	n.d.	0.5	0.0	1.3	1.5

n.d. = not determined; 0.0 = activity < 0.05 U/mg.

Table 3A
Specific activities, enantioselectivities and absolute configuration for acetoin obtained by carboligation of two molecules acetaldehyde catalysed by various ThDP-dependent decarboxylases.

Enzyme	Acetoin *ee*  **16**	Specific activity [U/mg]
*Ap*PDC	31% (*S*)	59
*Zp*PDC	58% (*S*)	2
*Zm*PDC [17]	20% (*S*)	n.d.
*Sc*PDC [34]	44% (*R*)	n.d.
KdcA [14]	46% (*R*)	4.4
BFD [12]	35% (*R*)	0.7

n.d. = not determined under similar reaction conditions.

in conversions with BAL [25] and BFD [26]. While *Sc*PDC and KdcA [14] show the same stability in the presence or absence of 20% (v/v) DMSO, *Ap*PDC exhibits a considerable stabilisation in the presence of 20% and even 30% (v/v) DMSO (half-life: ~430 h, data not shown). Therefore DMSO is generally implemented in biotransformations containing aromatic aldehydes.

3.2. Substrate range and kinetic parameters of decarboxylation

Kinetic constants were determined for the physiological substrates. With pyruvate (**1**) all bacterial PDCs (*Zm*PDC, *Zp*PDC, *Ap*PDC) exhibit hyperbolic v/[S]-plots with highly similar kinetic parameters (v_{max} 110–121 U/mg, K_M 1.3–2.8 mM, Table 1). Significantly higher $S_{0.5}$-values have been observed for *Sc*PDC where substrate activation gives rise to a sigmoidal v/[*S*]-plots. Although the maximal velocity of pyruvate decarboxylation could be calculated to 112 U/mg, which is in a similar range compared to the bacterial PDCs, this values cannot be reached due to a decrease of activity at concentrations >30 mM pyruvate. The latter might be a result of substrate surplus inhibition or increased ionic strength. The sigmoidal v/[*S*]-plot of *Sc*PDC [19,27,28] has already been described in detail and similar results have been obtained with PDCs from various plants [29] as well as the prokaryotic PDC from *Sarcina ventriculi* (*Sv*PDC) [30]. Most recently the mechanism for the allosteric activation of *Sc*PDC could be elucidated [31].

The substrate range of the investigated PDCs is mainly limited to short-chain aliphatic 2-keto acids (Table 2), whereas branched-chain and aromatic 2-keto acids are only poorly converted. In contrast, KdcA shows an exceptionally broad substrate range encompassing linear, branched-chain aliphatic and aromatic 2-keto acids (Table 2) [32,33]. Here maximal velocity was observed with 3-methyl-2-oxobutanoate (**6**) while the lowest K_M-values were detected with phenylpyruvate (**12**) [14]. BFD shows by far the highest specific activity for the decarboxylation of benzoylformate (**11**, Table 2), with a maximal velocity three times higher than those observed with other 2-keto acid decarboxylases and their physiological substrates (Table 1).

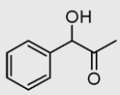
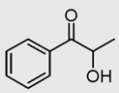
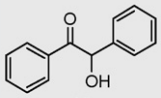
It should be mentioned that the substrate range was investigated with a concentration of 30 mM for each substrate and it cannot be ruled out that the maximum rate for each reaction could not be detected correctly due to high K_M values or substrate surplus effects.

3.3. Carboligase activity

Based on the preference of PDCs for pyruvate as the substrate, the corresponding acetaldehyde is preferred as a donor substrate in PDC-catalysed carboligation reactions. Formation of acetoin (**16**) is known for yeast PDCs [34–37], *Zm*PDC [17,23,34,38], *Zp*PDC [39] and BFD Acetoin can be obtained starting either from acetaldehyde and/or pyruvate. Our recent studies on *Ap*PDC carboligase ability demonstrate a considerably higher acetoin forming activity compared to the one of other decarboxylases (Table 3A). For all 2-keto acid decarboxylases the enantioselectivity of the acetoin forming reaction is with 20–50% *ee* comparatively low and probably a consequence of insufficient stabilisation of small aldehydes in the active site. Bacterial PDCs catalyse the synthesis of predominantly (*S*)-acetoin, whereas (*R*)-acetoin is formed in excess by *Sc*PDC, BFD and KdcA. A molecular model explains the opposite selectivities of *Zm*PDC and *Sc*PDC by size effects in the active site [40,41].

Determination of the product range obtained by the mixed carboligation of benzaldehyde and acetaldehyde was used as a preliminary test reaction for all enzymes to obtain valuable information about their chemo- and enantioselectivity. For all investigated PDCs (*R*)-phenyl-acetylcarbinol (PAC) (Table 3B, **17**) was identified as the predominant product, with traces of acetoin (**16**) for all decarboxylases except for *Sc*PDC. Even with benzaldehyde as the only substrate no benzoin (**19**) could be detected for *Zp*PDC and just very low benzoin-forming activity was observed with *Ap*PDC (2.4×10^{-3} U/mg), which is typical for pyruvate decarboxylases [17]. Consequently the investigated PDCs almost exclusively accept benzaldehyde as the acceptor aldehyde. In contrast BFD, which decarboxylates benzoylformate with very high activity (Table 2), prefers predominantly benzaldehyde as a donor and acetaldehyde as an acceptor yielding (*S*)-2-hydroxypropiophenone (HPP, **18**). The correlation between the substrate range of the decarboxylation reaction and the donor spectrum for carboligation can be demonstrated best with KdcA, which decarboxylates a broad range of 2-keto acids. Consequently,

Table 3B
Product range, enantioselectivities and absolute configuration for 2-hydroxy ketones obtained by mixed carboligations of acetaldehyde and benzaldehyde with various ThDP-dependent decarboxylases.

Enzyme	Acetoin *ee*  **16**	PAC-product *ee*  **17**	HPP-product *ee*  **18**	Benzoin *ee*  **19**
*Ap*PDC	n.d.	93% (*R*)	–	n.d.
*Zp*PDC	n.d.	89% (*R*)	–	–
*Zm*PDC [17]	20% (*S*)	98% (*R*)	–	–
*Sc*PDC [43]	–	90% (*R*)[a]	–	–
KdcA [14]	n.d.	92% (*R*) (40%)	93% (*R*) (60%)	n.d.
BFD [44]	–	–	92% (*S*)	99% (*R*)

– = no product formation; n.d. = very low amount of product was formed, not sufficient for *ee* determination.
[a] Data for *Sc*PDC (without His_6-tag) were obtained with crude cell extracts only.

the corresponding aldehydes might act as donors and acceptors in KdcA-catalysed carboligation reactions. Chemoselectivity can be controlled by the side chains of the respective aldehydes in mixed carboligations transformed by KdcA [14]. Thus, in case of benzaldehyde and acetaldehyde as substrates, both aldehydes may act either as donor or acceptors yielding almost equal amounts of **17** and **18**, which is a consequence of the sterical properties in the active site [42]. With larger aliphatic aldehydes, such as propanal, butanal and pentanal, the chemoselectivity can be shifted exclusively to the (*R*)-PAC-analogue product, with the aliphatic aldehyde acting as donor. On the other hand, the (*R*)-HPP-analogue product can be obtained exclusively if 3,5-dichlorobenzaldehyde and acetaldehyde are employed in mixed carboligations [14].

Like KdcA the PDCs are *R*-selective in mixed carboligations. Thus, (*R*)-PAC is formed enantioselectively by *Zm*PDC (*ee* 98%), whereas *Zp*PDC and *Ap*PDC show lower selectivity of 89–91% *ee* (*R*) (Table 3B). The same holds for *Sc*PDC were an *ee* of 90% (*R*) was reported for PAC [43]; however in this case data refer to biotransfomations with crude cell extracts. Currently, no wild type 2-keto acid decarboxylase is able to form (*S*)-PAC or derivatives thereof. Still, HPP is formed (*S*)-selectively with an *ee* of 92% by BFD. Recently the molecular reasons for this selectivity could be explained by steric demands in the active site of the enzyme [44,45].

4. Conclusions

By employing similar reaction conditions it becomes obvious that all investigated 2-keto acid decarboxylases show similar optima and stabilities regarding pH and temperature. In contrast the substrate spectra for decarboxylase activity vary considerably. While the substrate ranges of all pyruvate decarboxylases are restricted to small aliphatic 2-keto acids, BFD and KdcA additionally and predominantly decarboxylate branched-chain, respectively aromatic 2-keto acids. Most importantly, the substrate range of the decarboxylase reaction gives clear indication concerning the suitability of the corresponding aldehydes as the donor substrates for the carboligation. If a 2-keto acid is decarboxylated with high activity, the corresponding aldehyde might also act as a putative donor substrate for carboligase reactions. On the other hand, if a 2-keto acid is not decarboxylated by a particular decarboxylase it can be supposed that the corresponding aldehyde cannot serve as a donor in carboligation reactions as well, although it can still be an acceptor substrate for this enzyme in C–C coupling reactions.

Therefore an investigation of the decarboxylase potential of 2-keto acid decarboxylases using a fast photometric decarboxylase assay is part of our basic screening procedure to identify the donor spectrum for carboligation. Additionally a simple and reliable colorimetric tetrazolium chloride assay is used, which is based on the reduction of formazane in the presence of formed 2-hydroxy ketones [46,47]. The combination of both assays allows a reliable prediction concerning the substrate range of donor- as well as acceptor aldehydes accepted by the respective 2-keto acid decarboxylases in carboligase reactions. Subsequently, interesting enzyme candidates are further investigated using complete instrumental analysis in order to analyse chemo- and enantioselectivity of the respective mixed carboligation reaction.

Acknowledgements

The skilful technical assistance of Katharina Range is gratefully acknowledged. The *Sc*PDC gene was kindly provided by PD Dr. Stephan König from the Martin-Luther University Halle-Wittenberg. The authors thank Evonik Industries (formerly Degussa AG) for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.03.019.

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