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Toshihiro Suzuki & Noboru Takizawa

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NOTE

Purification and enzymatic characterization of *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase from *Rhodococcus opacus* and enzymatic formation of α, β-unsaturated ketones

Toshihiro Suzuki[a] and Noboru Takizawa[b]

[a]Department of Fermentation Sciences, Faculty of Applied Biosciences, Tokyo University of Agriculture, Tokyo, Japan; [b]Department of Applied Chemistry and Biotechnology, Faculty of Engineering, Okayama University of Science, Okayama, Japan

ABSTRACT

Trans-o-hydroxybenzylidenepyruvate (*t*HBPA) hydratase-aldolase (RnoE) catalyzes the conversion of *t*HBPA to 2-hydroxybenzaldehyde and pyruvate. We purified RnoE from *Rhodococcus opacus* and characterized its enzymatic properties. It exhibited maximum enzyme activity at 60°C and catalyzed the reverse reaction, converting various aromatic benzaldehydes and pyruvate to benzylidenepyruvate, indicating that this enzyme can be adapted for the enzymatic synthesis of α, β-unsaturated ketones.

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Microorganisms degrade polycyclic aromatic hydrocarbons (PAHs), for example, naphthalene, phenanthrene, and anthracene, via 4-substituted 2-ketobut-3-enoate (α, β-unsaturated ketones) intermediates [1]. Among α, β-unsaturated ketones, *trans-o*-hydroxybenzylidenepyruvate (*t*HBPA) is produced as an intermediate in the upper pathway of microbial PAH-degradation and is converted to hydroxyl-aromatic aldehyde and pyruvate by hydratase-aldolase (via a retro-aldol reaction) [2,3]. Hydratase-aldolase is widely distributed in PAH-degrading bacteria and exhibits a unique activity that catalyzes α, β-unsaturated ketone hydroxylation and aldol cleavage [4].

*t*HBPA hydratase-aldolase (RnoE) converts *t*HBPA to 2-hydroxybenzaldehyde and pyruvate (Figure 1(a)). In addition, RnoE has been known to catalyze the reverse aldol reaction and produce various α, β-unsaturated ketones from several aldehydes and pyruvate [3]. Thus, this enzyme is not only important for microbial degradation of PAHs but also suited to being a biocatalyst for the synthesis of various useful compounds. However, only a few reports on its enzymatic characterization and function have been published [3,5–7]. Here we purified native RnoE from the naphthalene-degrading soil bacterium *Rhodococcus opacus* CIR2 and investigated its enzymatic properties and aldol reaction activity using several aromatic aldehydes and pyruvate.

Owing to its commercial unavailability, *t*HBPA and its derivative were synthesized by an aldol condensation reaction from 2-hydroxybenzaldehyde and pyruvate under alkaline conditions. *Trans-m*-hydroxybenzylidenepyruvate (*m*HBPA) and *trans-p*-hydroxybenzylidenepyruvate (*p*HBPA) were similarly synthesized using the same reaction.

To obtain native RnoE, the *R. opacus* strain CIR2 was cultured for 45 h at 30°C with shaking in W minimal salt medium [8] containing 0.3% solid naphthalene (Nacalai Tesque, Kyoto, Japan) as the sole carbon and energy source. Cells were collected and washed twice with 50 mM P Buffer (50 mM Na_2HPO_4 and 50 mM KH_2PO_4, pH 7.5) and then re-suspended in the same buffer (5×the cell pellet wet-weight). Re-suspended cells were disrupted using a Multi-Beads-Shocker (Yasui Kikai, Osaka, Japan), and the lysate was harvested by ultracentrifugation at 92,300 × g for 60 min at 4°C.

Enzyme activity was measured by monitoring the decrease in absorbance at 340 nm, the λ_{max} of *t*HBPA [4], at 30°C. Enzyme activity was calculated using a molar extinction coefficient of 12.8 mM^{-1} for *t*HBPA [7], and one unit of enzyme was defined as the amount of enzyme that produces one μmole of 2-hydroxybenzaldehyde per min at 30°C. Protein concentration was determined using the method of Bradford [9], and bovine serum albumin was used as standard.

The cell-free extract reduced the intensity of the absorbance peaks of 2-hydroxybenzaldehyde at 296 nm and 340 nm and increased absorbance at 255 nm (Figure 1(b)) [4]. In addition, a retro-aldol reaction with the extract using *t*HBPA as a substrate was analyzed by HPLC (LC-10A system; Shimadzu, Japan) with a Luna 5μ C-18 column (Ø4.6 mm × 250 mm; Shimadzu). The analysis

CONTACT Toshihiro Suzuki ts206188@nodai.ac.jp

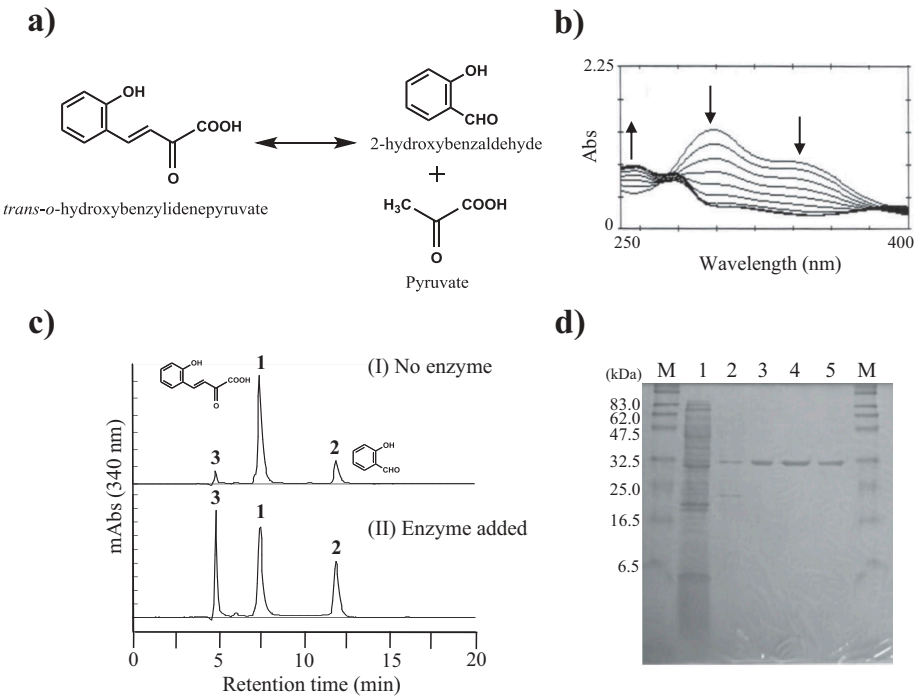

Figure 1. Enzymatic reactions and purification of RnoE.

(a) Catalytic activity of RnoE. (b) Spectrophotometric analysis of the enzyme reaction using RnoE and *t*HBPA with a Shimadzu UV-Vis UV-1200 spectrophotometer. The upward arrow indicates the 255 nm peak, whereas the downward arrows indicate the 296 nm and 340 nm peaks. (c) HPLC analysis of *t*HBPA hydrolysis in the presence (I) or absence (II) of RnoE. Peak 1 = *t*HBPA, peak 2 = 2-hydroxybenzaldehyde and peak 3 = pyruvate. (d) SDS-PAGE of each step in the purification of RnoE. Lane M, molecular weight markers; lane 1 cell-free extract; lane 2, active fraction from DEAE-Toyoperl 650 M; lane 3, active fraction from HiLoad 16/10 Phenyl Sepharose; lanes 4 and 5, active fractions from MonoQ HR 5/5. Marker sizes (kDa) are indicated on the left. All proteins were stained with Coomassie brilliant blue R-250.

revealed an increase in 2-hydroxybenzaldehyde and a decrease in *t*HBPA levels (Figure 1(c)), indicating that the strain CIR2 possesses a soluble form of RnoE.

Purification of native RnoE was performed using DEAE Toyopearl 650 M (Tosoh, Tokyo, Japan), HiLoad 16/10 Phenyl Sepharose (Amersham), and MonoQ HR 5/5 (Amersham) connected to an FPLC system (Amersham, Sweden). Purity at each purification step was assessed by SDS-PAGE. The final material produced a single band of 32 kDa on SDS-PAGE (Figure 1(d)) and was purified 23.9-fold with a yield of 0.6% from the lysate. Gel filtration on Sephadex 200 HR 10/300 (Amersham) gave a native molecular mass of 64.5 kDa, suggesting that the enzyme is a homodimeric complex. It has been reported that the native molecular mass of *Pseudomonas vesicularis* DAM6383 is 120 kDa and that it is consists of a homotrimeric complex [7]. In addition, to compare native molecular mass and enzymatic properties, we also purified the native *t*HBPA hydratase-aldolase (PahE) from another naphthalene degrader, *Pseudomonas aeruginosa* PaK1 [10], and determined it to be a homotetrameric complex.

A summary of the enzymatic characterization of purified RnoE and a comparison to other *t*HBPA hydratase-aldolases is shown in Table 1. The optimum pH and temperature of RnoE were 7.5–8.0 and 60°C, respectively. PahE also exhibited the same optimum temperature. To date, there have been few reports on the enzymatic characterization of hydratase-aldolases using *t*HBPA as substrate; those that exist used enzymes from *P. vesicularis* DSM6383 [7], *P. fluorescens* N3 [2], and *Sphingomonas paucimobilis* TA-2 [6]. The optimum pH of RnoE was similar with that of enzymes from these other known species, although the optimum reaction temperature of hydratase-aldolases/RnoE has not been reported for other species except for *P. fluorescens*.

The substrate specificity of RnoE was investigated using *trans-m*-hydroxybenzylidenepyruvate (*m*HBPA) and *trans-p*-hydroxybenzylidenepyruvate (*p*HBPA), which were synthesized by chemical aldol condensation reactions using 3- or 4-hydroxybenzaldehyde and pyruvate. RnoE was equally active using *p*HBPA as a substrate compared with *t*HBPA but only 32% as active when using *m*HBPA. Conversely, the activity of PahE with *p*HBPA

Table 1. Comparison of enzymatic characteristics of various purified *t*HBPA hydratase-aldolases.

Bacterial strains	*R. opacus* CIR2	*P. aeruginosa* Pak1	*P. vesicularis* DSM6383	*S. paucimobilis* TA-2	*P. fluorescens* N3
Growth substrate	Naphthalene	Naphthalene	Naphthalenesulfonate	Naphthalenesulfonate	Naphthalene
Enzyme substrate	*t*HBPA	*t*HBPA	*t*HBPA	*t*HBPA	*t*HBPA
Native enzyme	Homodimer	Homotetramer	Homotrimer	Homotrimer (HA A, HA B)	Unknown
Enzyme subunit	32 kDa	37 kDa	38 kDa	37.6 kDa (HA A), 37.2 kDa (HA B)	38 kDa (His_6-Tagged)
Optimum pH	7.5–8.0	8.0	8.0	9.0 (HA A, HA B)	7.0
Optimum temperature	60°C	60°C	Unknown	Unknown	30°C
Km value	20μM	5 μM	17 μM	9 μM (HA A), 3 μM (HA B)	3.58 mM (for 2-hydroxybenzaldehyde)
Vmax value	0.82 μmol/min/mg	15.9 μmol/min/mg	Unknown	Unknown	35.46 μmol/min/mg
kcat	0.244 s^{-1}	0.03 s^{-1}	Unknown	Unknown	Unknown
kcat/Km (s^{-1} mM^{-1})	12.2 s^{-1} mM^{-1}	6.0 s^{-1} mM^{-1}	Unknown	Unknown	Unknown

and *m*HBPA relative to *t*HBPA was 26% and 68%, respectively.

The K_m and V_{max} of RnoE for *t*HBPA were determined by linear regression using Lineweaver–Burk plots. The K_m value was 20 μM, as was for the enzyme from *P. vesicularis* DSM6383, and the V_{max} was 0.82 μmol/min/mg. The K_m and V_{max} values of PahE of *P. aeruginosa* Pak1 were determined to be 5 μM and 15.9 μmol/min/mg, respectively. These enzymatic parameters differed between enzymes from different species (Table 1). The K_m and V_{max} values of *t*HBPA hydratase-aldolase from *P. fluorescens* N3 for 2-hydroxybenzaldehyde were recently reported as 3.58 mM and 35.46 μmol/min/mg, respectively [2]; however, these values were for the 2-hydroxybenzaldehyde and not *t*HBPA. In addition, Ohmoto et al. determined the K_m values of two purified *t*HBPA hydratase-aldolases (HA A and HA B) from *S. pucimobilis* TA-2, which were found to be 9 (HA A) and 3 (HA B) μM, respectively [6]. Subsequently, the specificity constants k_{cat} and k_{cat}/K_m of RnoE were determined using *t*HBPA as a substrate. We determined the k_{cat} and k_{cat}/K_m values of RnoE as being 0.24 s^{-1} and 12.2 s^{-1} mM^{-1}, respectively. These values differed from those of *P. aeruginosa* Pak1 ($k_{cat,}$ 0.03 s^{-1} and $k_{cat}/K_{m,}$ 6.0 s^{-1} mM^{-1}). Although the K_m, k_{cat} and k_{cat}/K_m values of RnoE is higher than that of PahE, the specificity constants of other hydratase-aldolases have not been reported.

Although RnoE catalyzes the aldol reaction of *t*HBPA to 2-hydroxybenzaldehyde and pyruvate (Figure 2(a)), its activity was investigated using 2-chlorobanzaldehyde, 3-chlorobanzaldehyde, 4-chlorobanzaldehyde, and 2-fluoro-3-(Trifluoromethyl)benzladehyde as substrates using HPLC. The substrate peak areas decreased, and condensation products were produced with all these halogenated substrates (Figure 2(b-e)), indicating that RnoE can catalyze the formation of various α, β-unsaturated ketones from aldehyde and pyruvate. The conversion from fluorinated benzaldehyde and pyruvate to α, β-unsaturated ketones by RnoE in this study is a remarkable catalytic reaction by hydratase-aldolase because there have been no reports of fluorinated benzaldehyde being a substrate of aldol reaction by hydratase-aldolase.

In conclusion, RnoE can catalyze both aldol and retro-aldol reactions using benzaldehydes and pyruvate as substrates. We believe that this is the first report to show that fluorinated benzaldehyde can be a substrate for aldol reaction by hydratase-aldolase. Aldol condensation is generally performed under acidic or alkaline conditions; however, RnoE could catalyze the reactions at neutral pH and relatively high temperatures. Therefore, various chemical conversions might be possible using this enzyme, although further characterization is needed to achieve this goal.

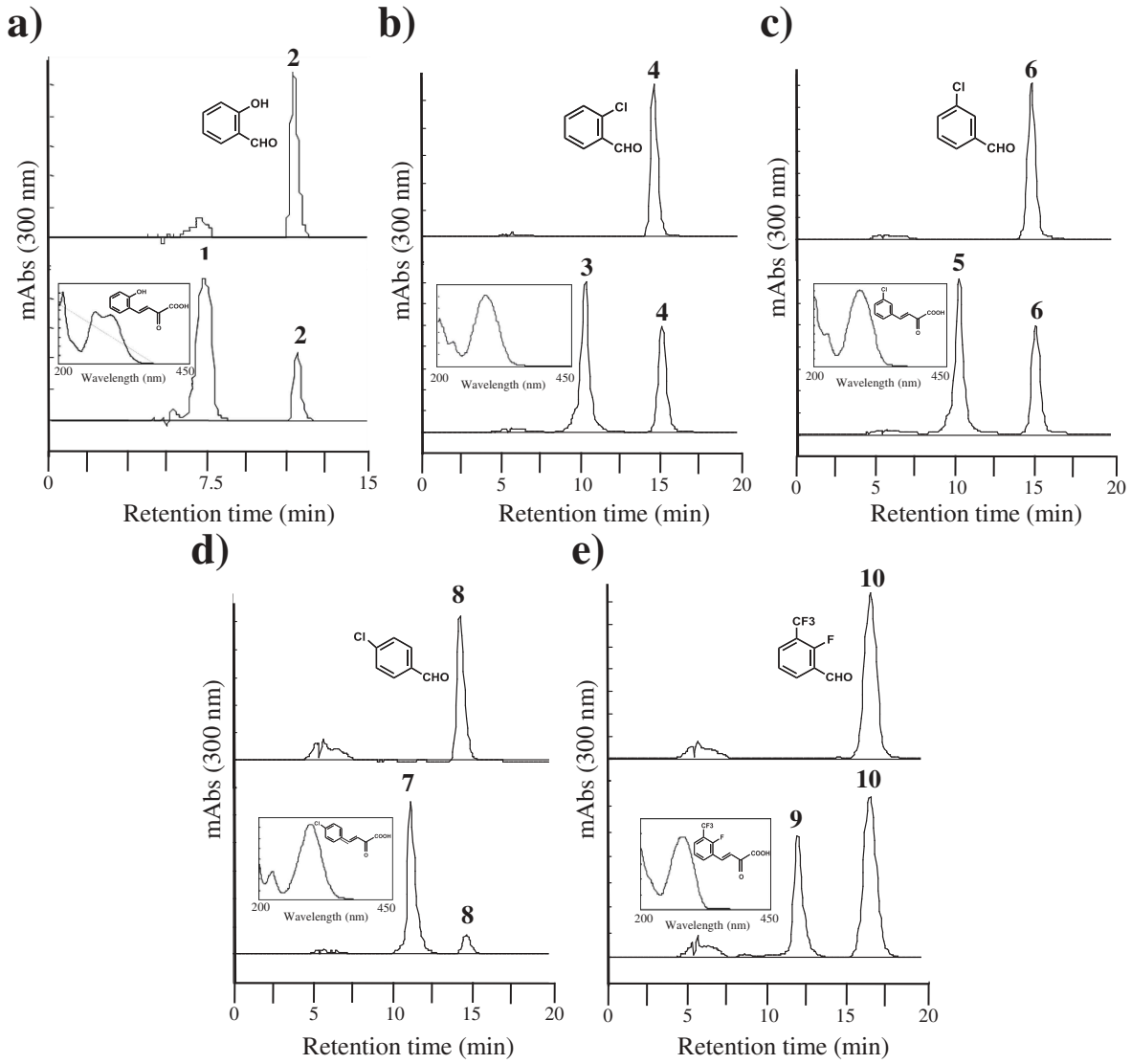

Figure 2. HPLC chromatograms from *in vitro* aldol reactions with various-halogenated banzaldehydes and pyruvate catalyzed by RnoE.

(a) Production of *trans-o*-hydroxybenzylidenepyruvate (peak 1) from 2-hydroxybenzaldehyde (peak 2) and pyruvate. Retention time in this reaction is short because this reaction was separately performed from reactions b–e. (b) Production of *o*-chlorobenzilidenepyruvate (peak 3) from 2-chlorobanzaldehyde (peak 4) and pyruvate. (c) Production of *m*-chlorobenzilidenepyruvate (peak 5) from 3-chlorobanzaldehyde (peak 6) and pyruvate. (d) Production of *p*-chlorobenzilidenepyruvate (peak 7) from 4-chlorobanzaldehyde (peak 8) and pyruvate. (e) Production of 2-fluoro-3-(Trifluoromethyl) benzilidenepyruvate (peak 9) from 2-fluoro-3-(Trifluoromethyl) benzladehyde (peak 10) and pyruvate. In all chromatograms, upper panels represent reactions without RnoE and lower panels represent reactions with RnoE. Each reaction mixture (Total volume 3.0 ml) contains 100 mM sodium pyruvate, 10 mM benzaldehydes, and 0.2 mg RnoE. All enzyme reactions were performed at 60°C for 60 min.

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Author Contribution

T.S. and N.T. conceived the study and planned the experiments; T.S. performed the experiments and wrote the manuscript; N.T. participated in redaction of the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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