Process Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols

Shengbin He, Zhenshou Wang, Yang Zou, Shaofang Chen, Xiaoping Xu*

Research Institute of Photocatalysis, College of Chemistry, Fuzhou University, Fuzhou 350108, China

ARTICLE INFO

Article history:
Received 24 December 2013
Received in revised form 18 March 2014
Accepted 19 March 2014
Available online xxx

Keywords:
Aromatic β-amino ketone
Oxidoreduction
NADH
Purification

ABSTRACT

Aromatic β-amino ketones/alcohols such as adrenalone play an important role in some stereoselective synthesis of pharmaceuticals. Unfortunately, the transformation of aromatic β-amino ketones to their chiral alcohols has been carried out chemically as no corresponding biocatalyst has been available. Here, a novel carbonyl reductase responsible for the reduction of adrenalone to (*R*)-(−)-epinephrine was identified and characterized from *Kocuria rhizophila*. This enzyme was purified to homogeneity by ammonium sulfate precipitation followed by ion-exchange column chromatography, hydrophobic chromatography and gel chromatography. The purified enzyme yielded pure (*R*)-enantiomer product with high activity and utilized NADH as the cofactor. The enzyme had special significance by showing selectivity for many aromatic β-amino ketones/alcohols such as 2-amino-acetophenone, 2-amino-4′-hydroxyacetophenone, isoproterenol and ephedrine. The maximum reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_m) for adrenalone and NADH were 14.62 μmol/(min mg) protein and 0.189 mM, 11.66 μmol/(min mg) protein and 0.204 mM respectively. These properties ensure the enzyme a promising future for industrial application as a replacement of chemical synthesis of aromatic β-amino chiral alcohols.

1. Introduction

The therapeutically non-active isomer in a racemate should be regarded as an impurity (50% or more) which lead to a waste of resources and may have side effect on patients and additional metabolic burden to viscera [1]. Therefore, the problem of stereoselective synthesis of optically active compounds is an extremely important and pressing one. As a result of the ongoing progress in pharmacy industry, an increasing number of novel methods for synthesis of pure chiral compounds are now accessible, among which biocatalytic transformation using isolated enzymes or whole cell systems has gained popularity due to its advantages such as high stereoselectivity, few by-products, mild reaction conditions, no residual metals in the products and avoidance of protection and deprotection of functional groups during the bioreaction [2,3].

Chiral alcohols with β-amino functional group, called 1,2-amino alcohol, often participate in some important stereoselective synthesis and reactions [4]. Among the numerous 1,2-amino alcohols, aryl amino alcohols, such as ephedrine, pseudoephedrine and norephedrine are of particular interest due to their versatile application in pharmaceuticals [5]. For instance, adrenomimetic drugs are widely used in injectable solutions for acute treatment of hypotension, anaphylactic shocks, and as vasoconstrictive additives in local anesthetic formulations to prolong the analgesic effect [6]. Unfortunately, most of alcohol dehydrogenases reported showed catalytic activity to substrates limited to aromatic ketones, long chain fatty ketones and some more simple ketones, but showed no catalytic activity to aryl amino ketones/alcohols. For instance, yeast dehydrogenase showed catalytic activity to aldehydes and methyl ketone [7]; reductase from *Bacillus* showed activity to acetophenone [2]; *Thermoanaerobacter brockii* alcohol dehydrogenase catalyzed aliphatic methyl ketone and ethyl ketone to their alcohols [8]; dehydrogenase from horse liver showed activity to monocyclic/bicyclic ketones, and lactic dehydrogenase was used for biological asymmetric transformation of alpha keto acid [9]. However, reported methods for the reduction of aromatic β-amino ketones are limited in contrast to the ubiquitous biocatalytic reduction of keto esters, though amides and esters are similar groups [10].

Previously, our research group had isolated a stain named *Kocuria rhizophila* (16S rDNA accession number EF138624) capable of catalyzing adrenalone to (*R*)-(−)-epinephrine, and the method has been patent protected [11–14]. In the current study, we report the purification and characterization of a carbonyl reductase from

* Corresponding author. Tel.: +86 13067237982.
E-mail addresses: comhsb@163.com, xu@fzu.edu.cn (X. Xu).

http://dx.doi.org/10.1016/j.procbio.2014.03.023
1359-5113/

K. rhizophila, which could catalyze the asymmetric reduction of adrenalone to (*R*)-(−)-epinephrine with an enantiomeric excess (e.e value) of more than 99%. It was named (*R*)-(−)-epinephrine dehydrogenase (EPID). Moreover, this enzyme shows great selectivity for aromatic β-amino ketones/alcohols, and very poor or almost no affinity for aliphatic ketones or amino acids.

2. Materials and methods

2.1. Chemicals

(*R*)-epinephrine and adrenalone were purchased from Sigma (USA). Coenzymes (NADH, NADPH) were purchased from ROCHE (Germany). DEAE Sepharose F. F, Butyl Sephrose™ 4 Fast Flow and Sephacryl™ S-200 High Resolution were purchased from Amersham Biosciences (GE, USA). Standard proteins for gel filtration and SDS-gel electrophoresis were purchased from Sangon (ShangHai, PRC). All other reagents were of analytical grade.

2.2. Production of (R)-epinephrine dehydrogenase

K. rhizophila was cultured as described previously to obtain mycelia [11].

2.2.1. Freeze/thaw

Air-dried mycelia (1 g) were suspended in buffer A (5 ml). The mixture were frozen at −196 °C for 1 min and then thawed at 37 °C for 1 min. The freeze/thaw processes were repeated for three cycles. Finally, the mixture was centrifuged at 12,000 × *g* for 20 min and the supernatants were recovered as crude enzymatic extracts.

2.2.2. Ammonium sulfate fractionation precipitation

The ammonium sulphate was added to the samples (20 ml) to give a saturation concentration of 40%, and the resulting precipitate was removed by centrifugation (12,000 × *g* for 20 min at 4 °C). Then, the ammonium sulphate concentration was increased stepwise to 60% saturation, and precipitate was collected by centrifugation.

2.2.3. DEAE-sepharose fast flow (ion-exchange column chromatography)

The DEAE-sepharose FF column (1.6 cm × 50 cm) was equilibrated with 20 mM Tris–HCl buffer at pH 7.2 and eluted with a linear gradient of sodium chloride (0–0.5 M, pH 7.0) in the equilibrating buffer. The fractions showing activity were collected and used for the next step.

2.2.4. Butyl-sepharose 4 fast flow (hydrophobic chromatography)

The butyl-sepharose 4 fast flow column (1.6 cm × 50 cm) was equilibrated and eluted by 50 mM potassium phosphate buffer (pH 7.0).

2.2.5. Sephacryl S-200 HR (gel chromatography)

Sephacryl S-200 HR column (1.6 cm × 50 cm) was equilibrated and eluted with 50 mM potassium phosphate buffer (pH 7.0), containing 0.15 M NaCl. The active fractions were pooled and dialyzed against buffer E. The enzyme purity was assessed by SDS–PAGE.

2.3. Determination of enzymatic activities

The determination of enzyme activity was described in Vallee and Hoch method [15]: coenzymes NAD(P)H absorbs at 340 nm while oxidized form $NAD(P)^+$ does not. Thus, coenzymes' oxidation or reduction would cause absorbance rise or fall at 340 nm respectively. Enzyme activity, therefore, could be measured through the determination of absorbance changes at 340 nm in the reaction process.

2.4. Protein assay and electrophoresis

Protein concentration was determined by the Lowry method using bovine serum albumin as the standard [16]. Protein concentration in fractions derived by column chromatography was estimated by measurements of absorbance at 280 nm.

The purity and molecular mass of the separated proteins were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [17].

2.5. Native molecular weight determination of the purified enzyme

The native molecular weight of the purified enzyme was determined using Sephadex G-100 column pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The elution rate was 0.3 ml/min with the same buffer. Gel filtration chromatography was performed at room temperature and a gelfiltration protein marker comprising myoglobin (17.8 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and ovalbumin-dimer (90 kDa) was used.

2.6. Enzyme characterization

2.6.1. Cofactor specificity and enzymatic enantioselectivity

Cofactor specificity was detected on the basis of the standard enzyme activity determination. When the dehydrogenase catalyzed a reduction reaction, NADH or NADPH was selected as the cofactor and adrenalone was the substrate in the reaction system. Analogously, NAD^+ or $NADP^+$ was the cofactor and (*R*)-(−)-epinephrine was the corresponding substrate when it was a oxidation reaction. The reactions were carried out at 40 °C for 2 min, and then each system's enzymatic activity was examined.

Separation of enantiomers was performed on an automated P/ACE™ MDQ CE system (Beckman Instruments, Fullerton, CA, USA) equipped with fused-silica capillaries (I.D. 75 μm, efficient length 50 cm). Separation solutions were prepared by dissolving methylol-β-cyclodextrin (20 mM) as the chiral selector in the background electrolyte (55 mM Tris–H_3PO_4, pH 3.0). The capillaries were conditioned according to the following procedure before every run: UP (ultra pure) water for 5 min; 0.1 M NaOH for 5 min; UP water for 5 min. Separations were then started thermostated at 25 °C using 10 kV, and UV absorbance was monitored at 214 nm.

The reaction system, which was used to determine enzymatic enantioselectivity, comprising 2.5 mM substrates (adrenalone), 1 mM coenzyme NADH (or NADPH), and 0.5 ml pure enzyme solution. 50 mM phosphate buffer was then added to the system above to obtain a final volume of 1 ml. The enzymatic reaction was carried out at 40 °C for 30 min, and then reaction products were detected with high performance capillary electrophoresis (HPCE).

2.6.2. Effects of pH and temperature on enzyme activity

The effect of pH on activity of (*R*)-epinephrine dehydrogenase was estimated on the basis of standard activity assays carried out at 45 °C and over pH range between 3.0 and 10.0 buffers of Different pH values were used. The pH stability of the purified enzymes was determined by measuring residual activity after protein pre-incubation (for 4 h at 45 °C) in same buffers with various pH values. The optimum temperature of (*R*)-epinephrine dehydrogenase was determined by measuring relative activity in 100 mM phosphate buffer (pH 6.0) over a temperature range between 20 and 70 °C. Thermostability of purified enzymes was evaluated by their incubation for 60 min at temperature varying from 20 to 60 °C in 100 mM phosphate buffer (pH 6.0) followed by residual activity assays under standard conditions.

2.6.3. Effects of various compounds on enzyme activity

The effect of various metals ions (Ag^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, NH_4^+) and EDTA on the activity of (*R*)-epinephrine dehydrogenase was investigated by their pre-incubation with these compounds for 30 min at 37 °C followed by measuring residual activity under standard conditions.

2.6.4. Substrate specificity and enzyme kinetics

For determination of substrate specificity of the purified enzyme, various substrates such as norepinephrine, isoproterenol, ephedrine, phenylephrine, acetaldehyde, acetone, 2-amino-acetophenone, L-tyrosine, ethanol, acetanilide and other chemicals were used. Kinetic constants (K_m and V_{max}) for (*R*)-epinephrine, adrenalone, NADH and NAD^+ were determined by measurement of initial velocities at various concentrations of one substrate at fixed concentrations of other substrates under the standard assay condition. The data was plotted on a Lineweaver–Burk double-reciprocal plot. Reactions were then performed at the optimal pH and temperature of the dehydrogenase. All experiments were done in triplicates and the average data were used to calculate the KM and V_{max}.

3. Results and discussion

3.1. Purification of enzymes

As shown in Fig. 1C, only one protein peak (fractions from 29 to 36) containing (*R*)-epinephrine dehydrogenase activities was eluted from DEAE-sepharose column, while butyl-sepharose 4 fast flow gave one main peak of proteins that displayed (*R*)-epinephrine dehydrogenase activities (Fig. 1A). The third step of purification gave one main peak of proteins that displayed (*R*)-epinephrine dehydrogenase activity (Fig. 1B). Results of the three-step purification procedure are summarized in Table 1. (*R*)-epinephrine dehydrogenase was purified 226-fold with 32% recovery of its activity. The specific activity of (*R*)-epinephrine dehydrogenase was 190 U/mg.

The purified enzyme showed a single peak in Sephadex G-100 gel filtration chromatography, and the molecular mass was estimated to be 67 kDa in comparison with the elution volume of standard proteins. The purified enzyme migrated as a single band with a size of about 33 kDa on SDS–PAGE (Fig. 2). The results of gel

Please cite this article in press as: He S, et al. Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.03.023

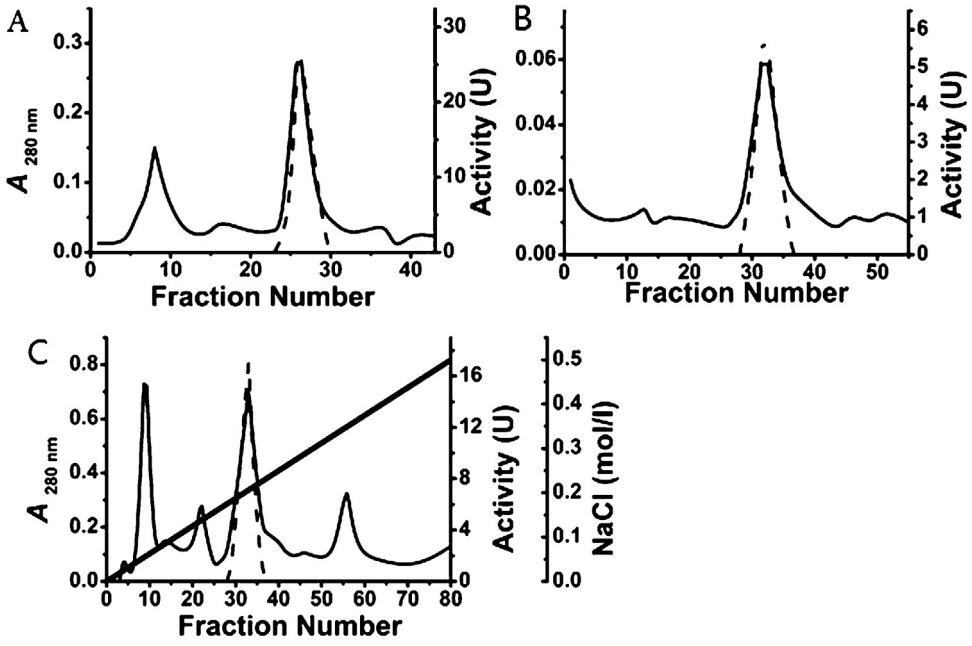

Fig. 1. Purification of (R)-(−)-epinephrine dehydrogenase. (C) Chromatogram of DEAE-sepharose fast flow. The bolder line (▬) indicates NaCl concentration of the eluting buffer. (A) Chromatogram of butyl-sepharose 4 fast flow. (B) Chromatogram of sephacryl S-200 HR. In each step of chromatography, elution of proteins was followed at 280 nm (—) and (*R*)-(−)-epinephrine dehydrogenase activity assay was followed at 340 nm (-----).

filtration chromatography together with SDS–PAGE suggested that this enzyme is a homodimeric enzyme. Dehydrogenases or reductases generally have low molecular weight and homomeric subunit. Many reported carbonyl reductases or dehydrogenases were low molecular and monomeric enzyme [18,19]. However, the result of this purified enzyme was homodimeric, which was coincident with alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 [20].

3.2. Properties of (R)-epinephrine dehydrogenase

3.2.1. Cofactor specificity and enzymatic enantioselectivity

The results showed that (R)-epinephrine dehydrogenase displayed activity with NADH (1.151 U/mg) or NAD^+ (0.368 U/mg), but no activity for the NADPH or $NADP^+$. It illustrated that the enzyme was a NAD (H)-dependent dehydrogenase.

The analysis of HPCE found that this NAD(H)-dependent dehydrogenase could catalyze the asymmetric reduction of adrenalone (corticosterone) to (*R*)-epinephrine, with an enantiomeric excess (e.e value) of more than 99% (Fig. 3).

3.2.2. Effects of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was studied in different buffer systems (100 mM) in the pH range of 3.0–9.0 (Fig. 4). The higher enzyme activity was observed in phosphate buffer at the same pH values (data not shown), compared to activity in acetate and glycine–NaOH. The results indicated that (*R*)-epinephrine dehydrogenase was optimally active at pH 6.0 and more than 80% of the peak activity was displayed in the pH range of 5.5–6.5. Below pH 4.5 (or above pH 7.0) the activity decreased rapidly. The enzyme retained 80% of its original activity over a broad range of pH 4.5–7.0 at 45 °C for 24 h, showing that it was relative stable at acidic pH.

The effect of temperature on the enzyme activity revealed that (*R*)-epinephrine dehydrogenase was optimally active at 45 °C and exhibited more than 80% of the maximum activity between 40 and 55 °C. However, when exposed to a temperature of over 60 °C, the enzyme activity decreased sharply. The enzyme was stable over a temperature range of 20–40 °C. Purified (*R*)-epinephrine dehydrogenase could retain almost 40% of its peak activity when incubated at 55 °C for 60 min. However, it lost almost all of its initial activity after incubation at 60 °C. Details are shown in Fig. 5.

3.2.3. Effects of various compounds on enzyme activity

The effects of metal ions and EDTA on the enzyme activity were summarized in Table 2. The enzyme activities were found to be enhanced by the addition of Ca^{2+}, Mg^{2+}, Mn^{2+}, and NH_4^+, but to be inhibited notably by the addition of Fe^{2+}, Al^{3+}, Co^{2+}, and Pb^{2+}, and to be inactive by the addition of Ag^+, Hg^{2+} and Cu^{2+} ions even at much lower concentrations. Simultaneously, the enzyme could also lose activity with EDTA at relative high concentration. Carbonyl reductases are generally known to be thiol enzymes and therefore activity was found to be severely inhibited by thiol specific metal ions such as Cu^{2+}, Ag^+ [21]. It was in good accordance with the results above. In addition, it was a well known fact that Zn^{2+} was essential to maintain activities of most dehydrogenases or carbonyl reductases as cofactors, but in this study Zn^{2+} displayed no effect on

Table 1
Purification of (*R*)-epinephrine dehydrogenase.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification degree [fold]	Yield (%)
Crude extract	36.400	43.300	0.841	1.000	100.000
40–60% ammonium sulfate precipitation	30.100	8.690	3.464	4.119	82.692
DEAE-sepharose F.F	25.200	2.180	11.560	13.745	69.231
Butyl-sepharose 4 F.F	19.900	0.706	28.187	33.516	54.670
Sephacryl S-200 HR	11.600	0.061	190.164	226.116	31.868

Please cite this article in press as: He S, et al. Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.03.023

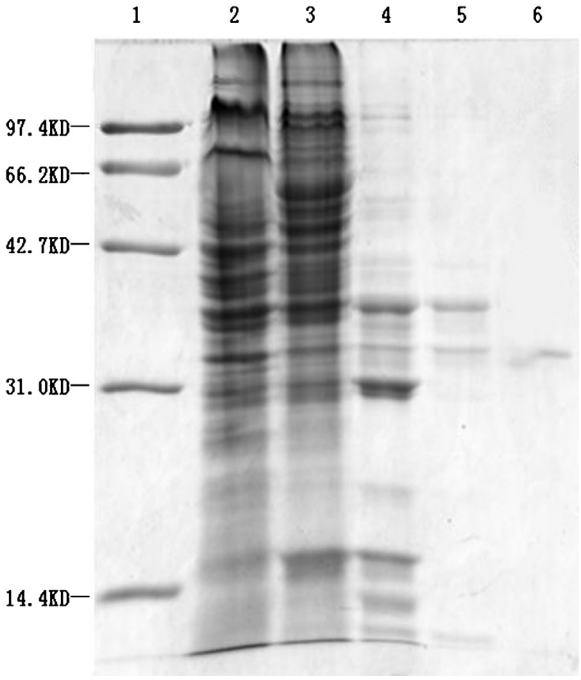

Fig. 2. SDS-polyacrylamide gel (12%) electrophoresis of purified (*R*)-(−)-epinephrine dehydrogenase. Lane 1: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14.4 kDa); lane 2: cell extract by freeze/thaw; lane 3: ammonium sulfate fraction precipitation; lane 4: DEAE sepharose F.F; lane 5: butyl sepharose 4 fast flow; lane 6: sephacryl S-200 HR. Gel was stained with 0.1% Coomassie Blue R-250.

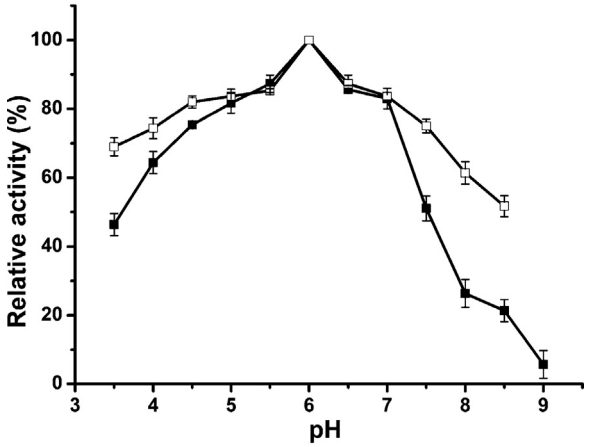

Fig. 4. Effect of pH on the activity (■) and stability (□) of (*R*)-epinephrine dehydrogenase. Enzyme assay was performed using standard assay procedure: 20 mM phosphate buffer, 4 mM NADH, 0.1 M corticosterone, and appropriate quantity of enzyme in different buffers (100 mM) from 3.5 to 9.0 in total 1 ml at 45 °C. Buffers used: 100 mM sodium acetate; 100 mM potassium phosphate; 100 mM glycine–NaOH. The maximum activity in tested buffers was taken as 100% and used as control. Relative activity of other pHs was expressed as a percentage of control. Results shown are means ± SD of triplicate samples.

the native dehydrogenase at 0.1 mM, and even inhibition at 1 mM. This demonstrated that (*R*)-epinephrine dehydrogenase was not a zinc metalloenzyme.

3.2.4. Substrate specificity and enzyme kinetics

To determine the substrate specificity of the enzyme, various substrates were used. As shown in Table 3, the enzyme could scarcely catalyze the reduction of aliphatic ketones, aldehydes

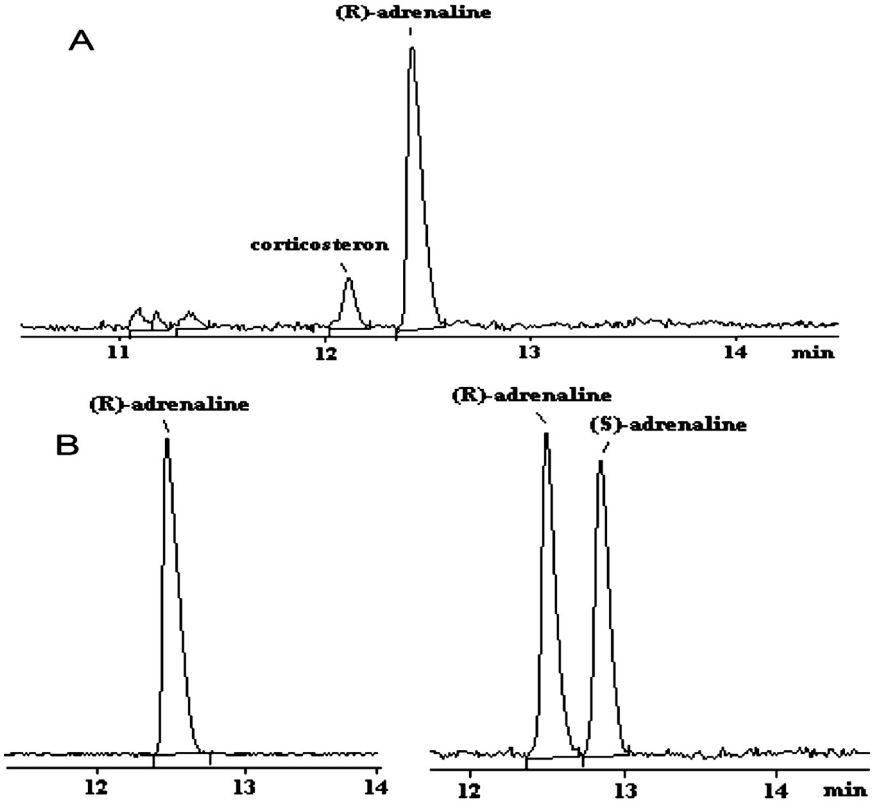

Fig. 3. (A) Capillary electropherogram of enzymatic reaction samples. The reaction system (1 ml), which was used to determinate enzymatic enantioselectivity, comprising 2.5 mM substrates (adrenalone), 1 mM coenzyme NADH, 0.5 ml pure enzyme solution and 50 mM phosphate buffer. (B) Electropherogram of standard (*R*)-adrenaline and racemic adrenaline.

Please cite this article in press as: He S, et al. Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.03.023

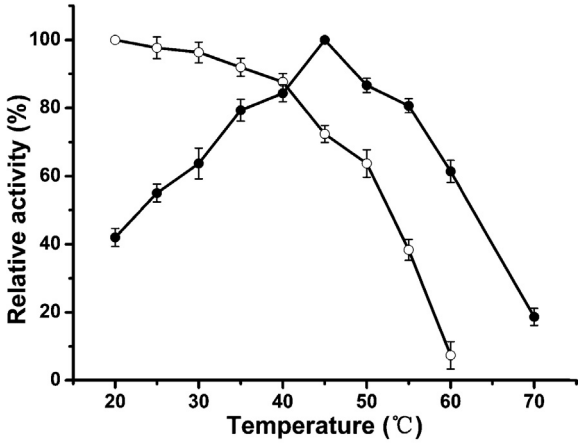

Fig. 5. Effect of temperature on the activity (●) and stability (○) of (*R*)-epinephrine dehydrogenase. Enzyme assay was performed using standard assay procedure: 20 mM phosphate buffer, 4 mM NADH, 0.1 M corticosterone, and appropriate quantity of enzyme at various temperatures from 20 to 70 °C in phosphate buffer (100 mM, pH 6.0) in total 1 ml. The maximum activity in tested buffers was taken as 100% and used as control, and relative activity of other pHs was expressed as a percentage of control. Results shown are means ± SD of triplicate samples.

and alcohols. In contrast, it presented different degrees of activity toward many aromatic ketones, aldehydes and alcohols. (*R*)-(−)-epinephrine dehydrogenase showed low activity for aromatic ketones, but high activity when aromatic ketones contained exposed amino groups. For the oxidation of alcohols compounds, the optimum substrates for this enzyme were some adrenomimetic agents such as norepinephrine, isoproterenol, ephedrine and phenylephrine, which had the same core structure of phenyl β-amino alcohol. In addition, this enzyme rarely participated in the catalytic reduction of amino acids except for L-phenylalanine.

It is of great significance that such enzyme catalyzing compounds with core structure of phenyl β-amino ketones/alcohols was obtained. It was supposed that (*R*)-(−)-epinephrine dehydrogenase contain a negatively charged active center composed of negatively charged amino acids under physiological pH condition, which is consistent with the results that electrophilic substituents such as $-NO^2$, –Cl and –Br promoted the asymmetric transformation of aromatic ketone by alcohol dehydrogenase from *Rhodotorula* sp., and vice versa [22]. If this is the case, we may further analyze the active center of (*R*)-epinephrine dehydrogenase to obtain conserved amino acid sequences responsible for catalysis of phenyl β-amino ketones/alcohols. Such sequence may be developed into gene probes for isolation of relevant enzymes with more merits as it's a rare event to screen a microorganism strain fit for such catalytic reactions. Additionally, the purified enzyme may be further applied in organic solvent reaction system and ionic liquid reaction system to improve its catalytic characteristics such as increasing the substrate solubility, increasing the conversion rate, improve the stereoselectivity, enhance the thermostability, increase the rate of reaction and improve the enzyme utilization.

The kinetic constants of the purified enzyme were calculated by fitting data by linear regression to a Lineweaver–Burk double-reciprocal plot (plot not shown). All the kinetic constants for (*R*)-epinephrine, adrenalone, NADH and NAD^+ were presented in Table 4. As can be seen, the K_m value for (*R*)-epinephrine was 0.455 mM and the corresponding V_{max} was 9.38 μmol mg^{-1} min^{-1}. In contrast, the K_m value for adrenalone was 0.189 mM and corresponding higher V_{max} 14.62 μmol mg^{-1} min^{-1}. Assume that the K_m can be approximately considered as a measure of binding affinity between the enzyme and its substrate, and the smaller K_m meant the stronger affinity. Adrenalone was therefore more preferred than (*R*)-epinephrine for the dehydrogenase, which illustrated the

Table 2
Effects of various compounds on enzyme activity.

Chemicals	Concentration (mmol/l)	Relative activity (%)
–	0.0	100.0
EDTA	1.0	0.0
EDTA	0.1	99.3
$AlCl_3$	1.0	73.1
$CaCl_2$	1.0	109.6
$CuSO_4$	1.0	0.0
$MgSO_4$	1.0	118.6
$MnSO_4$	1.0	117.6
$FeSO_4$	1.0	17.7
$ZnSO_4$	1.0	88.5
$ZnSO_4$	0.1	100.0
$CoCl_2$	1.0	70.1
$AgNO_3$	1.0	0.0
$Pb(CH_3COO)_2$	1.0	54.9
$HgNO_3$	1.0	0.0
$(NH_4)_2SO_4$	1.0	106.5

Table 4
Kinetic parameters of different substrates for (*R*)-adrenaline dehydrogenase.

Substrates	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Adrenalone	0.189	14.62
(*R*)-Adrenaline	0.455	9.38
NADH	0.204	11.66
NAD^+	0.284	8.54

Table 3
Substrate specificity of (*R*)-epinephrine dehydrogenase.

Substrates	Relative activity (%)	Substrates	Relative activity (%)
Aldehydes and ketones		Alcohols	
Acetaldehyde	1.00	Phenylephrine	41.00
Formaldehyde	1.00	Ephedrine	35.00
Acetone	1.40	Isoproterenol	33.00
Acetophenone	4.10	Norepinephrine	46.00
Benzoylformic acid	7.00	Epinephrine	90.00
Adenosterone	100.00	2-Phenethyl alcohol	3.30
2-Amino-acetophenone	67.00	Phenylmethanol	4.00
2-Amino-4′-hydroxyacetophenone	81.00	Ethanol	<1.00
2-Amino-4′-aminoacetophenone	21.00	Methanol	<1.00
2-Amino-3′,4′-dihydroxycetophenone	48.00	Others	
α-Bromoacetophenone	<1.00	Acetanilide	0.00
Amino acids		Ethylacetoacetate	0.00
L-Tyrosine	0.00		
Tryptophane	0.00		
L-Phenylalanine	3.20		

Please cite this article in press as: He S, et al. Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.03.023

catalytic transformation of corticosterone to (*R*)-epinephrine in practical by (*R*)-epinephrine dehydrogenase was achievable and had potential industrial application value.

4. Conclusions

A novel carbonyl reductase from *K. rhizophila* capable of catalyzing adrenalone to (*R*)-(−)-epinephrine was purified and characterized. The purified enzyme preferred the inexpensive cofactor NADH as specific electron donor. The results of gel filtration chromatography together with SDS–PAGE suggested that this enzyme is a homodimeric enzyme with 67 kD by gel filtration and 33 kD by SDS–polyacrylamide gel electrophoresis, and it exhibited broad substrate specificity for many adrenomimetic agents. The enzyme activity was perfect under pH 6.0 and 45 °C, and was enhanced by Ca^{2+}, Mg^{2+}, Mn^{2+} and NH_4^+, while inhibited by Fe^{2+}, Al^{3+}, Co^{2+} and Pb^{2+}. The maximum reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_m) for adrenalone and NADH were 14.62 μmol min^{-1} mg^{-1} protein and 0.189 mM, 11.66 μmol min^{-1} mg^{-1} protein and 0.204 mM, respectively. These properties ensure the enzyme a promising future for industrial application as a replacement of chemical synthesis of aromatic β-amino chiral alcohols. Further molecular analysis of the enzyme is needed to pave the road of isolating more relevant versatile enzymes by DNA probe detection methods.

Acknowledgements

The authors gratefully acknowledge financial assistance by the Specialized Research Fund for the Doctoral Program of Higher Education of China (20103514110002).

References

[1] Murakami H. From racemates to single enantiomers-chiral synthetic drugs over the last 20 years. Top Curr Chem 2007;269:273–99.
[2] Xie Y, Xu JH, Xu Y. Isolation of a Bacillus strain producing ketone reductase with high substrate tolerance. Bioresour Technol 2010;101:1054–9.
[3] Wang P, Cai JB, Ouyang Q, He JY, Su HZ. Asymmetric biocatalytic reduction of 3,5-bis(trifluoromethyl) acetophenone to (1R)-[3,5-bis (trifluoromethyl) phenyl] ethanol using whole cells of newly isolated Leifsonia xyli HS0904. Appl Microbiol Biotechnol 2011;90:1897–904.
[4] Juaristi E, Soloshonok V. Enantioselective synthesis of beta-amino acids. Wiley.com; 2005.
[5] Testa ML, Zaballos E, Zaragozá RJ. Reactivity of β-amino alcohols against dialkyl oxalate: synthesis and mechanism study in the formation of substituted oxalamide and/or morpholine-2,3-dione derivatives. Tetrahedron 2012;68:9583–91.
[6] Hoellein L, Holzgrabe U. Ficts and facts of epinephrine and norepinephrine stability in injectable solutions. Int J Pharm 2012;434:468–80.
[7] Zhang ZJ, Pan J, Liu JF, Xu JH, He YC, Liu YY. Significant enhancement of (*R*)-mandelic acid production by relieving substrate inhibition of recombinant nitrilase in toluene–water biphasic system. J Biotechnol 2011;152:24–9.
[8] Keinen E, Sinha SC. Thermostable enzymes in organic synthesis. 5. Total synthesis of S-(+)-Z-dodec-3-en-11-olide (ferrulactone) using a TBADH-generated bifunctional chiron. Tetrahedron 1991;47:4631–8.
[9] Zhang YB. Chiral synthesis by biological catalysis, 1. Beijing: Chemical Industry Press; 2002. p. 170–99 (Chinese).
[10] Stella S, Chadha A. Biocatalytic reduction of α-keto amides to (*R*)-α-hydroxy amides using *Candida parapsilosis* ATCC 7330. Catal Today 2012;198:345–52.
[11] Chen B, Yang K, Chen P, Ruan W, Xu X. Optimization of (*R*)-adrenaline high produing strain fermentation medium. Comput Appl Chem 2009;26:935–9.
[12] Lin M, Chen B, Li Z, Xu X. Determination of the optical purity of biotransfortated R-adrenaline. J Anal Sci 2010;26:395–9.
[13] Xu X, Li Z, Yang K. Application of *Kocuria rhizophila* in asymmetric synthesis of R-adrenaline. CN Patent CN101067121A.10.12.05.
[14] Xu X, Li Z, Chen B. A biological asymmetric transformation method for preparation of R-adrenaline. CN Patent CN201010245540.8.10.12.08.
[15] Vallee BL, Hoch FL. Zinc, a component of yeast alcohol dehydrogenase. Proc Natl Acad Sci U S A 1955;41:327.
[16] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[18] Cao H, Mi L, Ye Q, Zang G, Yan M, Wang Y, Zhang Y, Li X, Xu L, Xiong J. Purification and characterization of a novel NADH-dependent carbonyl reductase from *Pichia stipitis* involved in biosynthesis of optically pure ethyl (S)-4-chloro-3-hydroxybutanoate. Bioresour Technol 2011;102:1733–9.
[19] Richter N, Hummel W. Biochemical characterisation of a NADPH-dependent carbonyl reductase from *Neurospora crassa* reducing α- and β-keto esters. Enzyme Microb Technol 2011;48:472–9.
[20] Kosjek B, Stampfer W, Pogorevc M, Goessler W, Faber K, Kroutil W. Purification and characterization of a chemotolerant alcohol dehydrogenase applicable to coupled redox reactions. Biotechnol Bioeng 2004;86:55–62.
[21] Li N, Ni Y, Sun Z. Purification and characterization of carbonyl reductase from *Candida krusei* SW 2026 involved in enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate. J Mol Catal B: Enzym 2010;66:190–7.
[22] Yang W, Xu JH, Xie Y, Xu Y, Zhao G, Lin GQ. Asymmetric reduction of ketones by employing *Rhodotorula* sp. AS2.2241 and synthesis of the β-blocker R-nifenalol. Tetrahedron: Asymmetry 2006;17:1769–74.

Please cite this article in press as: He S, et al. Purification and characterization of a novel carbonyl reductase involved in oxidoreduction of aromatic β-amino ketones/alcohols. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.03.023