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Purification and characterization of a *cis*-epoxysuccinic acid hydrolase from *Bordetella* sp. strain 1–3

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ABSTRACT

Purification of a *cis*-epoxysuccinic acid hydrolase was achieved by ammonium sulfate precipitation, ionic exchange chromatography, hydrophobic interaction chromatography followed by size-exclusion chromatography. The enzyme was purified 177-fold with a yield of 14.4%. The apparent molecular mass of the enzyme was determined to be 33 kDa under denaturing conditions. The optimum pH for enzyme activity was 7.0, and the enzyme exhibited maximum activity at about 45 °C in 50 mM sodium phosphate buffer (pH 7.5). EDTA and *o*-phenanthrolin inhibited the enzyme activity remarkably, suggesting that the enzyme needs some metal cation to maintain its activity. Results of inductively coupled plasma mass spectrometry analysis indicated that the *cis*-epoxysuccinic acid hydrolase needs Zn^{2+} as a cofactor. Eight amino acids sequenced from the N-terminal region of the *cis*-epoxysuccinic acid hydrolase showed the same sequence as the N-terminal region of the beta subunit of the *cis*-epoxysuccinic acid hydrolase obtained from *Alcaligenes* sp.

Introduction

Optically pure vicinal diol compounds, which are highly versatile chiral synthons and often used in the synthesis of biologically active molecules, are becoming increasingly important to the chemical and pharmaceutical industries [1]. One of the practical processes to obtain such building blocks is enantioselective hydrolysis of the corresponding epoxides catalyzed by epoxide hydrolases (EHs, EC 3.3.2.3).[1] Because this enzymatic process is usually conducted at mild conditions with a high transformation rate and enantioselectivity with a great variety of EH sources, the application of EHs in chemical synthesis has become a focus of the organic chemical industry.

EHs can catalyze the conversion of epoxides to the corresponding diols by addition of a water molecule. They are found ubiquitously in mammals, insects, plants and microorganisms [2]. Recently, microbial EHs have been received considerable attention as they can be produced on an almost unlimited scale [3]. A number of microbial EHs have been purified and characterized to determine their applicability to enantioselective transformation [4–9].

One of the potential applications of EHs in biotransformation is producing L(+)- or D(−)-tartaric acid. These enantiomeric tartaric acids are well-known organic chiral auxiliaries and building blocks [10,11] that have a broad application in the pharmaceutical and food industries. At present, L(+)-tartaric acid is produced as a byproduct of the wine industry from lees and argols or by biotransformation with immobilized microorganisms on an industrial scale [12]. However, D(−)-tartaric acid rarely exists in natural resources [13] and the cost of production by chemical resolution is high. If some EHs able to produce D(−)-tartaric acid were available and could be applied in the manufacturing process, it would make the production of D(−)-tartaric acid much simpler and more economical.

The EHs that catalyze *cis*-epoxysuccinic acid hydrolysis to form D(−)-tartaric acid as well as L(+)-tartaric acid were also named *cis*-epoxysuccinic acid hydrolases (Fig. 1). At present most of the *cis*-epoxysuccinic acid hydrolases produced by microorganisms can only produce L(+)-tartaric acid [12] and only three microorganisms, *Pseudomonas putida*, *Alcaligenes* sp. and *Bordetella* sp., were reported to possess enzymes with the ability to catalyze the hydrolysis of *cis*-epoxysuccinic acid to form D(−)-tartaric acid [14–16]. Currently there have been few studies on the characteristics of these D(−)-tartaric acid-transforming EHs. Although the *cis*-epoxysuccinic acid hydrolase from *Alcaligenes* sp. was overexpressed in *Escherichia coli* for D(−)-tartaric acid production by Asai et al. [14], and it was found that the enzyme is a heterodimer with a

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[1] *Abbreviations used:* EHs, epoxide hydrolases; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ICP-MS, inductively coupled plasma mass spectrometry.

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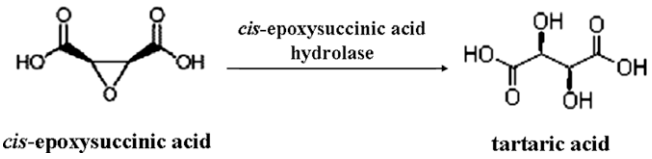

Fig. 1. Hydrolysis of *cis*-epoxysuccinic acid by *cis*-epoxysuccinic acid hydrolase to form tartaric acid.

molecular mass of 80 kDa, the detailed physiological properties of this enzyme were not determined.

In previous work, a *Bordetella* sp. strain 1–3 was isolated in our laboratory and found to have the ability to produce D(−)-tartaric acid from *cis*-epoxysuccinic acid [15]. In this paper, the *cis*-epoxysuccinic acid hydrolase from that strain was purified to homogeneity and the properties of the enzyme were studied.

Materials and methods

Organism and culture conditions

A *Bordetella* sp. strain 1–3 producing the *cis*-epoxysuccinic acid hydrolase was isolated from soil, which was grown on the basal medium slant at 30 °C for 36 h and then stored at 4 °C. The basal medium contained 10 g/L *cis*-epoxysuccinic acid, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 2 g/L $K_2HPO_4{\cdot}3H_2O$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 10 ml/L trace element solution at pH 7.0. The trace element solution consisted of 1.5 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L NaCl, 0.5 g/L $FeSO_4{\cdot}7H_2O$, 0.5 g/L $ZnSO_4{\cdot}7H_2O$, 0.5 g/L $MnSO_4{\cdot}H_2O$, 0.05 g/L $CuSO_4{\cdot}5H_2O$ and 0.25 ml/L 4 M HCl. For enzyme production, bacteria were cultivated at 30 °C for 36 h in a 10-L bioreactor (WKT-2, Jiangsu Weikete Bioengineering Equipment Co., Ltd.) in basal medium. The aeration rate and stirring speed of flat impeller were 10 L min^{-1} and 250 rpm, respectively.

Extraction of enzyme from Bordetella sp. strain 1–3

Unless stated, all purification steps were performed at 4 °C and buffers A and B were 50 and 10 mM sodium phosphate buffer (pH 7.5), respectively.

Cells were harvested from culture medium by centrifugation at 8000 rpm for 30 min, re-suspended in two volumes of buffer A and disrupted by a high pressure homogenizer, followed by centrifugation at 8000 rpm for 30 min to remove the cell wall debris. Ammonium sulfate was then added to the supernatant stepwise with stirring to give 35%, 40%, 45%, 50%, 55%, 60%, 65% and 70% ammonium sulfate. At each concentration, the precipitates were obtained by centrifugation at 8000 rpm for 30 min and the fractions containing *cis*-epoxysuccinic acid hydrolase were collected and re-dissolved in buffer A.

Enzyme purification with chromatography

The crude enzyme obtained by ammonium sulfate precipitation was dialyzed overnight against buffer B and then applied to a DEAE-cellulose column (5.5 × 50 cm) that had been previously equilibrated with the same buffer. The *cis*-epoxysuccinic acid hydrolase was eluted with a linear gradient of KCl from 0 to 1.0 M at a flow rate of 1 ml min^{-1}. The fractions containing *cis*-epoxysuccinic acid hydrolase activity were pooled and dialyzed against buffer B. After ammonium sulfate had been added to 40% saturation, the enzyme solution was then loaded onto a Toyopearl HW-65 column (5.5 × 50 cm) equilibrated with the same buffer containing 40% ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate from 40% to 0% at 1 ml min^{-1} flow rate. After the fractions containing the enzyme activity had been collected, ammonium sulfate was added to 70% saturation. The precipitate of the enzyme was then collected by centrifugation at 8000 rpm for 30 min, dissolved in 15 ml buffer A and applied onto a Sephadex G-75 column (5.5 × 100 cm) equilibrated with buffer B containing 0.1 M KCl. The enzyme was then eluted with the same buffer and the fractions containing pure *cis*-epoxysuccinic acid hydrolase were combined for subsequent study.

Enzyme assay and protein measurement

To measure enzyme activity, 0.1 ml supernatant of enzyme solution was added to 0.9 ml phosphate buffer (50 mM, pH 7.5) containing 60 mM *cis*-epoxysuccinic acid and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 400 μL 1.0 M H_2SO_4. The tartaric acid generated in the reaction was measured by the Ammonium Meta Vanadate Method [17]. One unit of enzyme was defined as the amount of enzyme that generates 1 μmol of D(−)-tartaric acid per minute under the above conditions. The protein was measured by the Folin-phenol method [18].

Electrophoresis and mass spectrometry

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli [19] with a 4% stacking and 12% separating gel containing 0.1% SDS. The proteins were stained with Coomassie Brilliant Blue-250 and destained with a methanol (30%) and acetic acid (7%) solution. The protein standards used for estimation of molecular masses were rabbit phosphorylase b (97 kDa), bovine serum albumin (66 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20 kDa) and hen egg white lysozyme (14 kDa).

To determine the molecular mass of the native *cis*-epoxysuccinic acid hydrolase, a matrix-assisted laser desorption ionization (MALDI) experiment was carried out on a delayed extraction time-of-flight (TOF) mass spectrometer Voyager-DE PRO (Applied Biosystems, USA), equipped with a 337 nm nitrogen laser, operating in the positive ion mode. Measurement was performed in liner mode with +25 kV total acceleration voltage, 90% applied to the grid, 0.15% applied to the guide wire and 300 ns extraction delay. Expected mass was calculated using Protein Prospector.

Effect of pH and temperature on the activity and stability of cis-epoxysuccinic acid hydrolase

The optimum pH for *cis*-epoxysuccinic acid hydrolase activity was determined by incubation of the enzyme solution with the pH range from 4.5 to 9.5 at 37 °C for 30 min. In order to eliminate the buffer effects on enzyme activity, a mix of 50 mM zwitterionic buffers (MES, CAPS, HEPES and AMPS) covering the desired range was used. The optimum temperature for the enzymatic reaction was determined in 50 mM sodium phosphate buffer (pH 7.5) at temperatures in range from 25 to 65 °C for 30 min.

The thermostability of the purified enzyme was determined in microcentrifuge tubes (0.5 ml) containing about 0.1 U of *cis*-epoxysuccinic acid hydrolase in buffer A. Samples were incubated from 25 to 65 °C with 5 °C intervals for 30 min, immediately cooled to 4 °C and the remaining enzyme activity was then measured. To determine the effect of pH on the stability of the *cis*-epoxysuccinic acid hydrolase, the enzyme solution was incubated for 24 h at 25 °C in the buffers described above and the residual enzyme activity was measured with the standard method as described above.