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Purification and characterization of a novel (–) gamma-lactamase from *Microbacterium hydrocarbonoxydans*

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Abstract – A (–) gamma-lactamase from *Microbacterium hydrocarbonoxydans* was purified to homogeneity by chromatography methods. SDS-PAGE showed the molecular weight of the enzyme was about 31 kDa. The purified enzyme had a specific activity of 61.3 ± 2.5 U mg^{-1} for 2-azabicyclo [2.2.1] hept-5-en-3-one [(–) gamma-lactam]. The enantioselectivity factor (E) of the purified enzyme was 9.5 ± 0.8 for unreacted (+) gamma-lactam. The K_m and V_{max} value were 2.3 ± 0.2 mM and 80.0 ± 15.4 U mg^{-1} respectively. The highest activity was found at 30 °C and pH 8.0. ESI-MS mass spectrometry analysis results and N-terminal sequence indicated the (–) gamma-lactamase might be a new enzyme.

Key words: gamma-lactamase; *Microbacterium hydrocarbonoxydans*; gamma-lactam.

INTRODUCTION

Current chemistry allows for the multi-ton scale production of asymmetric compounds in commercial applications, however, advantages such as mild reaction conditions, stereospecificity, high specificity, low energetic consumption, reuse capability and high-quality bioconversion make biocatalysis an alternative method to chemical synthesis for certain products (Fessner and Jones, 2001; Hauerand Roberts, 2004). Biotransformation with whole cells or partially purified enzymes has been used in some instances, particularly when co-factor recycling is required or the enzyme is unstable. However, low rates of production due to poor substrate transport and expensive downstream processing costs limit their use. The use of purified enzymes would solve these problems by enzyme immobilization and also allow the use of flow-through reactors. They would be especially useful in the production of fine chemicals and pharmaceuticals due to their chemo-, regio-, and more particularly, their enantioselectivity (Schulze and Wubbolts, 1999).

The bicyclic lactam, 2-azabicyclo [2.2.1] hept-5-en-3-one (gamma-lactam) has been shown to be a versatile synthon for the preparation of carbocyclic nucleosides such as abacavir and carbovir (Daluge and Vince, 1978). Method of asymmetric synthesis of gamma-lactam has been reported before (Velazquez and Olivo, 2002) and approach of resolution of racemic gamma-lactam by enzymatic methods had also been applied (Nakano *et al.,* 1996; Mahmoudian *et al.,* 1999).

Gamma-lactamase from species such as *Sulfolobus solfataricus* and *Pseudomonas fluorescens* that have the ability to catalyze the enantio-selective hydrolysis of gamma-lactam have been reported before (Brabban *et al.,* 1996; Toogood *et al.,* 2004). In a previous study, we screened a strain of *Microbacterium hydrocarbonoxydans* that produced high levels of both (+) and (–) gamma-lactamase (Li *et al.,* 2006; Zheng *et al.,* 2007). *Microbacterium hydrocarbonoxydans* was a newly found species in 2005 (Schippers *et al.,* 2005). To our knowledge it is the first time that gamma-lactamase had been purified and characterized from the strain.

MATERIALS AND METHODS

Materials. 2-Azabicyclo [2.2.1] hept-5-en-3-one was purchased from Sigma (Germany). Methanol, acetonitrile and isopropanol of HPLC grade were purchased from Acros (USA). Unless otherwise stated, all reagents were of analytical grade and were purchased from Beijing Chemical Co (PRC.). Phenyl-Sepharose FF, DEAE-Sepharose FF, and Sephacryl S-200 were purchased from GE Healthcare (USA).

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Microorganism. *Microbacterium hydrocarbonoxydans* strain L29-9 was cultivated as previously described (Li *et al.,* 2006).

Enzyme purification. The cells were harvested by centrifugation, and re-suspended in 30 mM sodium dihydrogen phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT, then the cells were disrupted by sonication for 8 min at 400 W. Cell debris was removed from the resultant lysate by centrifugation at 15 000 *x g*, 4 °C for 20 min.

Active enzyme was precipitated with ammonium sulphate at 65% of saturated concentration. All further purification steps were carried out at 4 °C on a GE AKTA FPLC system (USA). The eluted proteins were monitored at 280 nm.

The ammonium sulphate-precipitated protein was dissolved in buffer A (30 mM Tris, 5 mM EDTA, 0.1 M ammonium sulphate, pH 8.0) and applied to a Phenyl-sepharose FF column which previously equilibrated with buffer A. Proteins were eluted with a linear ammonium sulphate gradient (0.1–0 M) with ten column volumes. The active fractions were pooled and applied to a DEAE–sepharose FF column equilibrated with buffer B (30 mM Tris, 5 mM EDTA, pH 8.0). Proteins were eluted with a linear NaCl gradient (0-2 M) with ten column volumes. Fractions with enzyme activity were pooled and freeze dried.

The freeze-dried fractions were dialyzed against buffer B (30 mM Tris, 5 mM EDTA, pH 8.0) and concentrated by freeze drying, and then applied to a Sephacryl S200 column equilibrated with buffer B. The active fractions were pooled and stored at -20 °C.

SDS-PAGE. SDS-PAGE was performed according to Laemmli *et al.* (1970).

Determination of the N-terminal amino acid sequence. The purified (–) gamma-lactamase on SDS-PAGE gel was transferred to a PVDF membrane (Bio-Rad). After blotting, the PVDF membrane was stained and the (–) gamma-lactamase band was cut out. N-terminal Edman sequencing was performed on an Applied Biosystems Procise 419 sequencer (USA).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The (–) gamma-lactamase protein spots were excised, and in-gel protein digestion were performed as described previously (Shevchenko *et al.,* 1996). Dried tryptic peptide mixtures were dissolved in 3 µl of saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and were spotted on a MALDI plate. MS and MS^2 spectra were acquired with a MALDI-TOF-TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer, USA) in the positive reflectron mode with a 200 Hz Nd-YAG 355 laser. Conversion of time-of-flight (TOF) to mass (Da) for the monoisotopic ions, $[M + H]^+$ was based on calibration of the instrument with a peptide standard kit (Applied Biosystems) containing des-Arg^1-bradykinin (m/z 904), angiotensin I (m/z 1,296), Glu^1-fibrinopeptide B (m/z 1,570), ACTH (1–17, m/z 2,093), ACTH (18–39, m/z 2,465), and ACTH (7–38, m/z 3,657). The resulted MS (PMF) and MS^2 spectra were searched using MASCOT software incorporated in the Applied Biosystems GPS v3.5 Explorer against the protein sequence database.

(–) Gamma-lactamase activity assays. Racemic gamma-lactam (0.5 mg) was added into 100 µl enzyme solution and transformed at 30 °C, 200 rpm for 30 min. Reaction mixture was extracted with 1 ml ethyl acetate and analyzed by chiral-HPLC. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 µmol of substrate per min.

Calculation of enantioselectivity. The enantioselectivity for the transform was expressed by enantiomeric excess (*e.e.S*) and enantiomeric ratio (*E*-value) (Chen *et al.,* 1982).

$$e.eS = \frac{S_+ - S_-}{S_+ + S_-} \times 100\%$$

$$E = \frac{ln[(1-c)(1-eeS)]}{ln[(1-c)(1+eeS)]}$$

where *c* stands for conversion, S_+ and S_- stand for (+) gamma-lactam and (–) gamma-lactam respectively.

Determination of protein concentration. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Analytical chiral-HPLC. The reaction mixture was extracted with 1 ml ethyl acetate. Ethyl acetate extract (10 µl) was applied to a DAICEL CHIRALPAK AS-H column and was eluted with a mobile phase (80% acetonitrile, 20% isopropanol; v/v). The UV absorption of gamma-lactam was monitored at 230 nm. Benzamide (internal standard), (+) and (–) gamma-lactam have retention times of 8.7, 11.8 and 13.9 minutes respectively at a flow rate of 0.6 ml min^{-1}.

RESULTS AND DISCUSSION

Purification and N-terminal amino acid sequence

The gamma-lactamase from *M. hydrocarbonoxydans* L29-9 was purified to electrophoretic homogeneity by chromatographic methods described above. SDS-PAGE showed the apparent molecular weight of the purified lactamase was about 31 kDa (Fig. 1, lane 5). The (–) gamma-lactamase was 330-fold purified with a recovery of 5.5% and the purified (–) gamma-lactamase had a specific activity of 61.3 ± 2.5 U mg^{-1} (Table 1). As shown in Fig. 2, a 93% enantiomeric excess (*e.e.*) value for unreacted (+) gamma-lactam was determined at 65% conversion. The enantioselectivity factor (E) of the purified enzyme was determined as 9.5 ± 0.8 for unreacted (+) gamma-lactam. The apparent K_m was 2.3 ± 0.2 mM and the V_{max} was 80.0 ± 15.4 U mg^{-1} respectively.

MALDI-TOF-MS analysis results showed (–) gamma-lactamase had no sequence identity to known proteins in the Swiss-Prot database. The N-terminal sequence (10 aa) of the (–) gamma-lactamase was GCITVGNENS, which demonstrated 90% identity to a gamma-lactamases from *Aureobacterium* sp. (Line *et al.,* 2004), and 90% identity to a co-factor free haloperoxidase from uncultured *Microbacterium* sp. (AY155546.1).

TABLE 1 - Purification of (–) gamma-lactamase from *M. hydrocarbonoxydans*

Step	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	5430	0.186	1001.0	100	1
Phenyl-Sepharose FF	62	1.1	68.2	6.8	5.9
DEAE–Sepharose FF	15	4.35	65.3	6.5	23.3
Sephacryl S-200	0.9	61.3	55.2	5.5	329.6

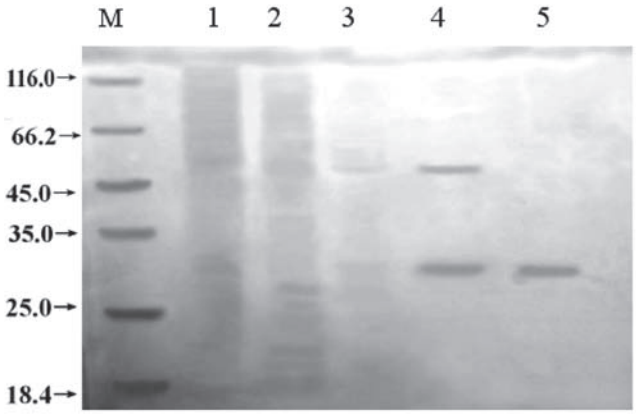

FIG. 1 - SDS-PAGE electrophoresis of (–) gamma-lactamase. M: protein marker, lane 1: cell-free extract, lane 2: ammonium sulphate fractionation, lane 3: phenyl–sepharose hydrophobic interaction chromatography, lane 4: DEAE-sepharose ion-exchange chromatography, lane 5: sephacryl S-200.

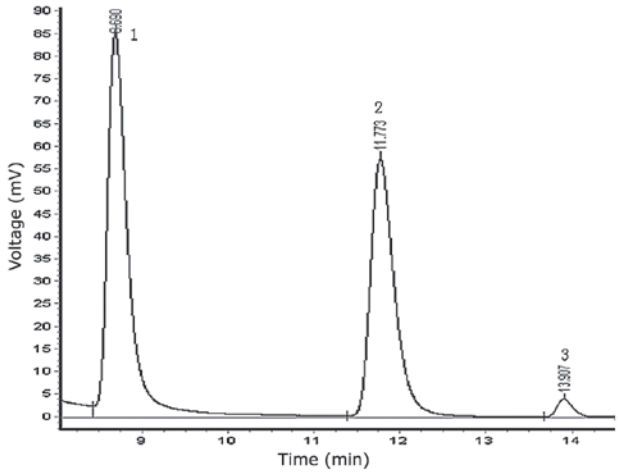

FIG. 2 - Chiral-HPLC analysis of resolution of racemic gamma-lactam by purified (–) gamma-lactamase. Peak 1: benzamide, peak 2: (+) gamma-lactam, peak 3: (–) gamma-lactam.

General properties of the purified gamma-lactamase

Maximum activity was found at pH 8.0 in 0.1 M TE buffer for gamma-lactam. The activity was measured at various temperatures, and the initial velocity of the hydrolysis reaching a maximum at 30 °C. The thermostability of the enzyme was relatively weak, and it remained 70% active after incubation for 30 min at 50 °C. The enzyme was completely inhibited by 5 mM phenylmethylsulfonyl fluoride. This indicates the involvement of a serine in the enzymic activity. DTT (1 mM) and 10 mM EDTA had no inhibitory effect. Metal ions (1 mM) including Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+} showed no effect on enzyme activity either.

DISCUSSION

During the purification of (–) gamma-lactamase, many protein peaks that had (+) gamma-lactam activities were also obtained. It seemed both (–) and (+) lactamase exist widely in cells of *M. hydrocarbonoxydans*. (+) Gamma-lactamase has relatively high activity but rapidly loses its activity during purification or storage. (-) Gamma-lactamase is more stable during the purification process. ESI-MS mass spectrometry and N-terminal sequence results indicated that (–) gamma-lactamase might be a new lactamase. Gene clone and expression of the (–) gamma-lactamase from *M. hydrocarbonoxydans* are in progress in our laboratory.

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