Front. Biol. China 2007, 2(4): 391–396
DOI 10.1007/s11515-007-0059-6

RESEARCH ARTICLE

Cloning, expression and characterization of L-cysteine desulfhydrase gene from *Pseudomonas* sp. TS1138

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Abstract L-cysteine desulfhydrase (CD) plays an important role in L-cysteine decomposition. To identify the CD gene in *Pseudomonas* sp. TS1138 and investigate its effect on the L-cysteine biosynthetic pathway, the CD gene was cloned from *Pseudomonas* sp. TS1138 by polymerase chain reaction (PCR) method. The nucleotide sequence of CD gene was determined to be 1,215 bp, and its homology with other sequences encoding CD was analyzed. Then the CD gene was subcloned into pET-21a(+) vector and expressed in *Escherichia coli* (*E. coli*) by isopropyl-*β*-D-thiogalactopyranoside (IPTG) inducement. The recombinant CD was purified by Ni-NTA His-Bind resin, and its activity was identified by the CD activity staining. The enzymatic properties of the recombinant CD were characterized and its critical role involved in the L-cysteine biosynthetic pathway was also discussed.

Keywords L-cysteine desulfhydrase, L-cysteine, *Pseudomonas* sp., cloning, characterization

1 Introduction

L-cysteine was an important S-containing amino acid, which has significant applications in the pharmaceutical, food and cosmetic industries (Awano et al., 2005). Traditionally, industrial production of L-cysteine mainly depends on acid or alkali hydrolysis of hair. However, this technology creates many environmental issues such as high-energy cost, horrible odor and intractable waste products (Ryu et al., 1997). Since 1977, a microbial method for L-cysteine production, which uses a chemically synthesized substrate, DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC), has been developed with enzymes extracted from certain strains of bacteria, especially the genus *Pseudomonas* (Sano et al., 1977). It is a more economical, efficient and environmentally friendly way for L-cysteine production.

Previously, we investigated the process of the conversion of DL-ATC to L-cysteine using a new strain of *Pseudomonas* sp. TS1138, which was isolated from industrial wastewater (Liu et al., 2003). In previous study, we found that L-cysteine desulfhydrase (CD) played an important role in L-cysteine decomposition, resulting in the production of pyruvate, ammonia and hydrogen sulfide. Although this type of enzymatic activity has also been demonstrated in other bacteria, such as *Escherichia coli* (Awano et al., 2003; Awano et al., 2005), *Azotobacter vinelandii* (Zheng et al., 1993), *Fusobacterium nucleatum* (Fukamachi et al., 2002), *Treponema denticola* (Kurzban et al., 1999) and *Corynebacterium glutamicum* (Wada et al., 2002), few studies about the CD involved in the L-cysteine biosynthetic pathway in *Pseudomonad* have been reported.

In this study, we reported the identification and characterization of CD in *Pseudomonas* sp. TS1138 and investigated its important effect on the bioconvertion of DL-ATC to L-cysteine, which lays an experimental foundation for elucidating the metabolic pathway of L-cysteine in *Pseudomonas* sp. TS1138 and may benefit the construction of an engineering strain with high L-cysteine producing ability in future.

2 Materials and methods

2.1 Materials

Pseudomonas sp. TS1138 was isolated from industrial wastewater and cultured in ATC medium (Liu et al., 2003). *E. coli* DH5α and BL21 (DE3) were purchased from Invitrogen Biotechnology Co. Ltd. (Shanghai, China) and cultured in Luria Broth (LB) medium. The pBluescript SK (Stratagene, USA) vector, pET-21a (+) vector (Novagen, Germany) and

Translated from *Microbiology*, 2006, 33(4): 21–26 [译自: 微生物学通报]

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Ni-NTA His-Bind resin (Novagen, Germany) were purchased from Hope Biotech Co. Ltd (Tianjin, China). The enzymes and kits used for DNA manipulations were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). All other reagents used in this study were of analytical grade.

2.2 Cloning of the CD gene

Standard DNA manipulation procedures, such as extraction of genomic DNA from *Pseudomonas* sp. TS1138, restriction enzyme digestion, DNA ligation and transformation of *E. coli* cells, were performed with the method of Sambrook et al. (2001). Based on the CD gene sequence of *Pseudomonas putida* (*P. putida*) KT2440 (Nelson et al., 2002), primers were designed as follows: forward primer, 5′-CCG**GGATCC**AT-GAAGTTGCCGATCTACCTTG-3′; reverse primer, 5′-CCC**AAGCTT**TTAGTGGGCGGCCCACTC-3′), bearing *Bam*HI and *Hin*d III restriction sites (underlined in bold) respectively. The genomic DNA of *Pseudomonas* sp. TS1138 was used as a template to amplify the CD gene. Then, the about 1.2 kb fragment was ligated into the restriction sites of pBluescript SK vector for DNA sequencing.

2.3 Expression and purification of recombinant CD

The CD gene was subcloned into pET-21a(+) vector to generate pET-cd. *E. coli* BL21 (DE3) strain was transformed with the recombinant pET-cd and the positive clone was cultured at 37°C in 4 mL of fresh Luria-Bertani (LB) medium containing 100 µg/mL of ampicillin for 12−14 h. The freshly grown cultures were subcultured in larger volumes until an optical density (OD) value of 0.5 at 600 nm was obtained. The expression of recombinant protein was induced with 0.1 mmol/L IPTG at 20°C for 24 h. The cells were harvested by centrifugation at 4,000 g for 20 min at 4°C. The cell pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.4) and sonicated on ice. The lysate was centrifuged at 20,000 g for 20 min at 4°C to remove cellular debris. The recombinant protein was purified on the basis of its 6 × His-tag by affinity chromatography using a Ni-NTA His-Bind resin. Fractions collected from the Ni-NTA affinity column were subsequently dialyzed and stored at −20°C for future use.

2.4 Determination of CD activity

The CD activity was determined by quantifying the amount of pyruvate as described by Candurae (1959) with a slight modification. Briefly, 0.5 mL of crude enzyme extracts were incubated with 0.75 mL of 0.05% L-cysteine and 0.25 mL of 6% K_2HPO_4 at 37°C for 2 h. The reaction was terminated by the addition of 0.5 mL of 1% solution of 2, 4-dinitrophenylhydrazine in 2 M HCl at 37°C for 10 min. After further addition of 2 mL of 1.5 M NaOH for 20 min, the amount of pyruvate was determined by measuring the absorbance at 500 nM. One unit enzyme (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol pyruvate per min from L-cysteine under the above conditions.

2.5 CD activity staining

The CD activity was visualized by the staining procedure of Zdych et al. (1995). Samples (10–30 µg of protein in each lane) were separated by 8% native polyacrylamide gel electrophoresis (PAGE). The gel was stained by gently shaking in a CD activity staining solution (100 mM Tris-HCl, 10 mM EDTA buffer, pH 7.5, containing 50 mM L-cysteine, 20 mM pyridoxal phosphate and 1.6 mM $BiCl_3$) at room temperature for 2 h.

2.6 Enzymatic characterization of recombinant CD

The enzymatic properties of recombinant CD were determined by the methods described by Jin et al. (2004).

2.7 Quantification of L-cysteine

The amount of L-cysteine formed from DL-ATC was measured by Gaitonde's acid ninhydrin method (Gaitonde, 1967).

3 Results

3.1 Cloning and sequence analysis of CD gene

The polymerase chain reaction (PCR) amplification of the CD gene from *Pseudomonas* sp. TS1138 resulted in a fragment of the expected size of 1.2 kb (Fig. 1). The nucleotide sequence of the region essential for CD activity was determined by DNA sequencing, and a 1,215-bp open reading frame (ORF) was found. This ORF encodes a polypeptide composed of 404 amino acids. The nucleotide sequence of CD gene has been submitted to GenBank under an accession number of AY675347.

A homology search of nucleotide sequences was carried out using the BLAST program provided by the National Center for Biotechnology Information (NCBI). A high homology was achieved with the CD genes from *P. putida*, *P. syringae* and *P. aeruginosa* strains (88–99% sequence similarity); the CD genes from some Gram-negative bacteria (*Burkholderia pseudomallei*, *Azotobacter vinelandii*, *Erwinia carotovora* subsp., *E. coli* and *Vibrio vulnificuas*) showed a similarity of 73–89% respectively; and the CD genes from some Gram-positive bacteria (*Phtorhabdus lwninescens* subsp., *Bacillus cereus* and *Methanosarcina mazei*) and fungi (*Caenorhabditis elegans*, *Arabidopsis thaliana*, *Plasmodium yoelii yoelii*, *Dechloromonas aromatica* RCB and *Methanosarcina barkeri* str. fusaro) revealed a similarity of 19%–73% respectively. Figure 2 showed the CLUSTALW alignment of CD genes from different organisms.

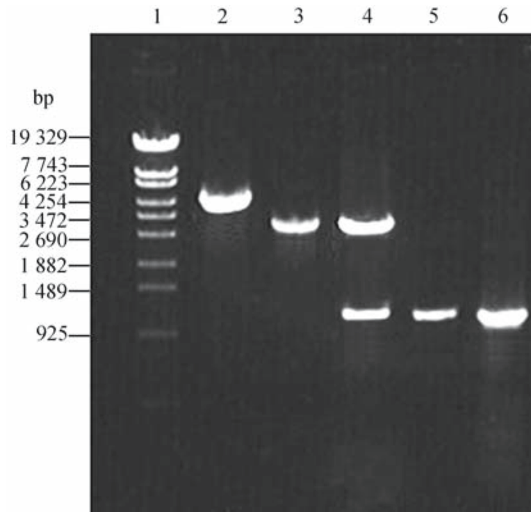

Fig. 1 Identification of the recombinant plasmid containing CD gene
Lane 1: DNA ladder; lane 2: recombinant plasmid digested by *Hin*dIII; lane 3: pBluescript SK plasmid digested by *Hin*dIII and *Bam*HI; lane 4: the recombinant plasmid digested by *Hin*dIII and *Bam*HI; lane 5: PCR product amplified from the recombinant plasmid; lane 6: PCR product amplified from the genomic DNA of *Pseudomonas* sp. TS1138.

The CD gene of *Pseudomonas* sp. TS1138 shared 98.3% homology with the sequence from *P. putida* KT2440. Besides, the 16SrRNA gene sequence of strain TS1138 (AY536741) was 99.0% identical with that of *P. putida* KT2440. These results suggest that strain *Pseudomonas* sp. TS1138 is perhaps a subspecies of *P. putida*.

3.2 Expression and purification of recombinant CD

The recombinant expression vector pET-cd was constructed and introduced into *E. coli* BL21 (DE3) for over-expression. Samples were analyzed by 12% SDS-PAGE and 8% native PAGE.

As shown in Fig. 3(a), lane 3, the recombinant CD was over-expressed in *E. coli* BL21 (DE3) and its activity was further confirmed by CD activity staining (Fig. 3 (b), lane 3).

The pET-21a(+) vector allows over-expression of heterologus proteins in *E. coli* and results in the addition of a short cleavable His-tag sequence at the C-terminus of the recombinant proteins. His-tags are unstructured and uncharged at physiological pH. They rarely alter or contribute to protein immunogenicity, and seldom interfere with protein structure,

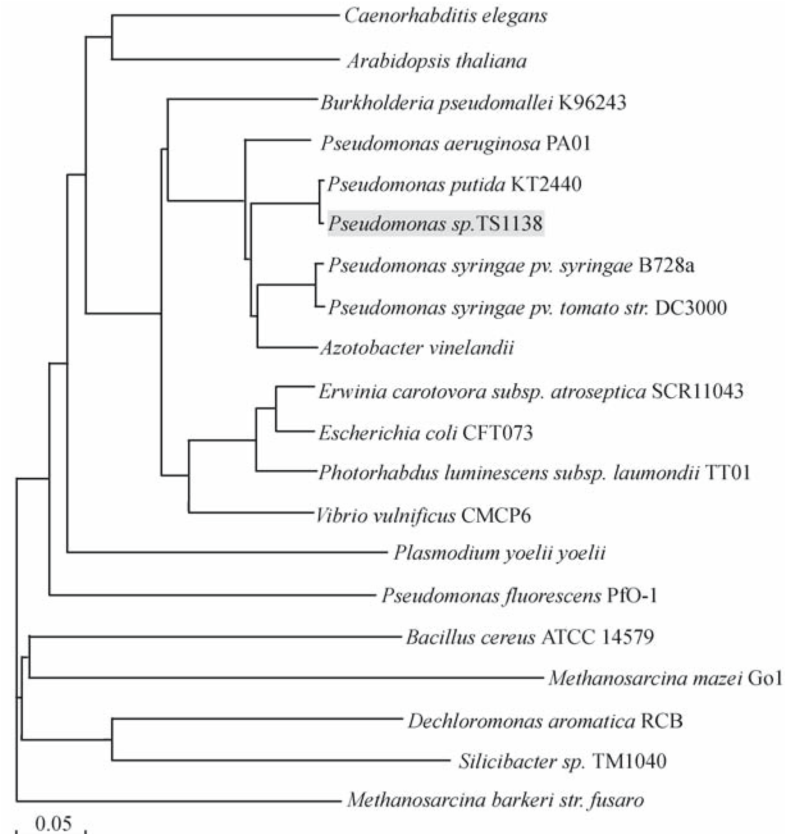

Fig. 2 Phylogenetic tree for CD genes from various organisms
The tree was constructed using CLUSTAL W program.

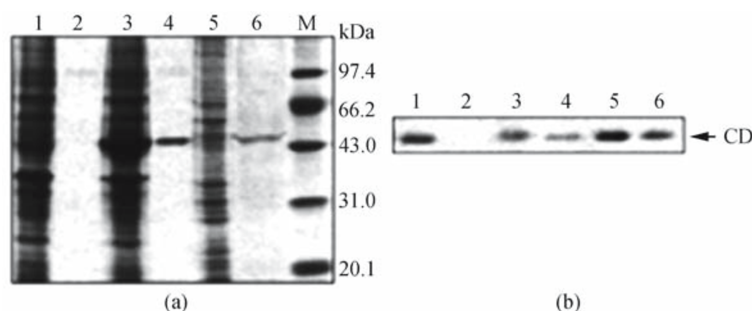

Fig. 3 SDS-PAGE (a) and CD activity staining (b) analysis
Lane 1: crude cell extracts of *E. coli* BL21(DE3) harboring pET-21a(+); lane 2: product purified from the crude cell extracts of *E. coli* BL21(DE3) harboring pET-21a(+) by Ni-NTA His-bind resin; lane 3: crude cell extracts of *E. coli* BL21(DE3) harboring pET-cd; lane 4: purified recombinant CD; lane 5: crude cell extracts of *Pseudomonas* sp. TS1138; lane 6: purified native CD; lane M: protein standards.

function or secretion. Due to the vector-encoded residues and the C-terminal His-tag, the apparent molecular weight of recombinant CD was about 53 kDa, a little larger than the expected value (48 kDa). However, it did not affect protein activity significantly.

The purification of recombinant CD was achieved in one step using Ni-NTA His-Bind resin (Fig. 3(a), lane 4). After native-PAGE and activity staining, CD activity was visualized in purified proteins (Fig. 3(b), lane 4). Although *E. coli* BL21(DE3) contains endogenetic CD (Fig. 3(a) and (b), lane 1), it did not interfere in the purification of recombinant CD, because there is no additional His-tag at the C-terminus of CD from *E. coli*.

3.3 Enzymatic characterization of recombinant CD

According to the methods previously described by Jin et al. (2004), the enzymatic activities of recombinant CD were characterized.

Incubation of recombinant CD with L-cysteine resulted in the formation of three end products: H_2S, NH_3 and pyruvate. D-cysteine, L-methionine, L-tyrosine, L-tryptophan, as well as DL-alanine and DL-serine, were not substrates for CD under the experimental conditions. Among all the SH-containing substrates, L-cysteine produced the largest amounts of NH_3, H_2S, and pyruvate, and was considered to be the primary substrate for CD. The K_m and V_{max} values were determined for CD activity with L-cysteine as a substrate, which were 2.63 mmol/L and 0.10 mmol/mL·min respectively.

The effects of pH and temperature on enzyme activity were tested (Fig. 4). For pH analysis, the purified recombinant CD was suspended in buffer at pH values from 3.0 to 11.0, and its strongest activity occurred at pH 7.5 (Fig. 4(a)). The pH tolerance test indicated that the CD activity was relatively stable between pH 6.0 to 9.0 after an incubation of 30 min (Fig. 4(a)). The enzyme was also sensitive to heat (Fig. 4(b)), with temperatures over 40°C significantly decreasing enzyme activity and 60°C for 30 min completely inactivating the enzyme. The optimum reaction temperature for CD was 35°C to 37°C.

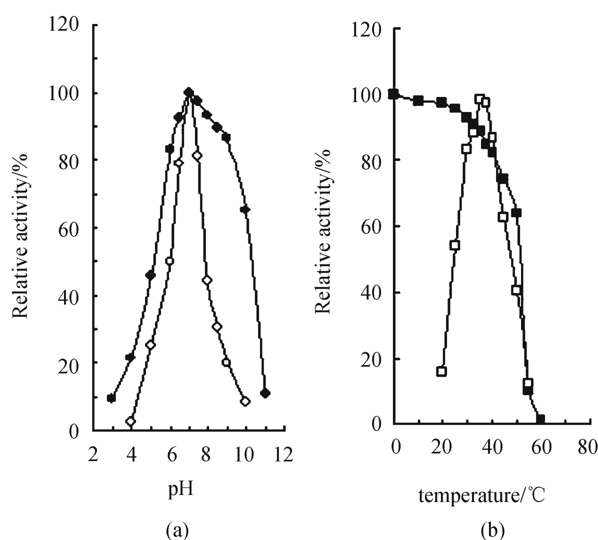

Fig. 4 Effects of pH (a) and temperature (b) on CD activity
Optimal reaction pH test (○); pH tolerance test (●); optimal reaction temperature test (□); temperature tolerance test (■).

The effects of selected metal ions and enzyme inhibitors on the activity of recombinant CD were also determined. Except for Zn^{2+} and Pb^{2+}, all the other metal ions (K^+, Li^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+} and Fe^{3+}) had no obvious effects on enzyme activity. The CD activity could be effectively inhibited by Zn^{2+} and activated by Pb^{2+}: lower than 40% and more than two folds respectively. Some enzyme inhibitors, such as phenylmethylsulphonyl fluoride (PMSF), hydroxylamine and ethylenediaminetetraacetic acid (EDTA), which were selected upon previously reported effects on proteases and other enzymes, were also investigated. As a result, 1 and 10 mmol/L of hydroxylamine both showed remarkably inhibitory effects on CD activity, which decreased to be 8.7% and 7.1% of the original activity, respectively.

3.4 Inhibitory effect of CD on the L-cysteine biosynthetic pathway

To find out whether CD was involved in L-cysteine decomposition in *Pseudomonas* sp. TS1138, the enzymatic conversion

experiment of DL-ATC to L-cysteine was carried out. The crude enzyme extracts of *Pseudomonas* sp. TS1138 were used as the enzyme source, in which 4 mL of crude enzyme extracts was incubated with 8 mL of substrate solution (0.75% DL-ATC, 11 mmol/L K_2HPO_4, 170 mmol/L NaCl, pH 7.5, containing 1 mmol/L of hydroxylamine and 5 mmol/L $ZnSO_4$ or not) at 37°C. At different time intervals, the concentrations of L-cysteine and pyruvate were measured.

As shown in Fig. 5, when hydroxylamine and $ZnSO_4$, which inhibit the CD activity, were added to the reaction mixture, the amount of L-cysteine increased significantly compared with those produced by the reaction without CD inhibitors at every time interval, and it had a maximal increase of 0.98 mmol/L of L-cysteine after 4 h reaction. On the other hand, the amount of pyruvate, which reflected the CD activity, reduced remarkably when the inhibitors were used.

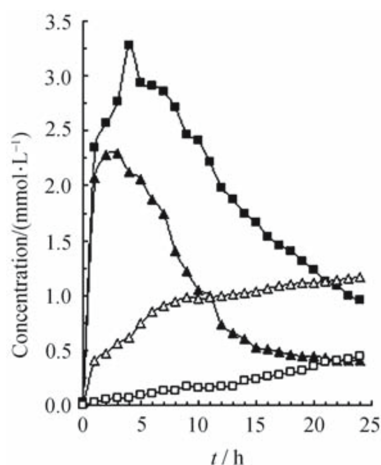

Fig. 5 Effect of CD on L-cysteine production
L-cysteine production at the presence (■) or without (▲) CD inhibitors; Pyruvate production at the presence (□) or without (□) CD inhibitors.

The results confirmed that CD played an important role in L-cysteine decomposition in *Pseudomonas* sp. TS1138.

4 Discussion

The bioconversion of DL-ATC is an effective method for the production of pharmaceutical-grade L-cysteine. It has been reported that the bacteria convert DL-ATC to L-cysteine through two pathways, the N-carbamyl-L-cysteine (L-NCC) pathway (Tamura et al., 1998) and the S-carbamyl-L-cysteine (L-SCC) pathway (Ryu and Shin, 1991). Several genes involved in the L-NCC pathway have been cloned from *Pseudomonas* sp. Strain ON-4a (Ohmachi et al., 2002) and strain BS (Shiba et al., 2002). However, the CD gene involved in this process, which is responsible for the decomposition of L-cysteine, is poorly understood and validated. In this study, we identified a CD gene from *Pseudomonas* sp. TS1138, and proved that the CD gene was involved in the conversion of DL-ATC to L-cysteine.

The 16SrRNA gene sequence of *Pseudomonas* sp. TS1138 is 99.0% identical with that of *P. putida* KT2440. Based on this, primers were designed according to the CD gene sequence of *P. putida* KT2440, and the CD gene of *Pseudomonas* sp. TS1138 was cloned successfully.

The CD gene was over-expressed in *E. coli* BL21 (DE3). In order to increase the solubility of the recombinant protein, cells were incubated at 20°C so as to lower down the rate of cell growth at lower temperature and simultaneously the incubation time was increased to 24 h to increase the cell mass. Under this condition, rate of protein synthesis is decreased and thus sufficient time for proper protein folding is provided, which increase the possibility for the protein to be present in the native form. Although *E. coli* was found to have CD activity and several genes have been identified previously (Awano et al., 2003; Awano et al., 2005), the recombinant CD could be purified by its 6 × His-tag which could remove the interference of CD from the host strain easily.

We investigated the enzymatic properties of the recombinant CD and confirmed that CD had an inhibitory effect on the L-cysteine production in *Pseudomonas* sp. TS1138. Zn^{2+} and hydroxylamine were found to inhibit CD activity significantly, and the presence of CD inhibitors could lead to a higher yield of L-cysteine. However, due to the remaining CD activity, the L-cysteine productivity would inevitably decrease. In addition, hydroxylamine also strongly inhibits the activities of L-cysteine-forming enzymes (Sano and Mitsugi, 1978), so a highest L-cysteine yield could not be achieved.

To further optimize L-cysteine production, CD should be eliminated from the conversion process. Pae et al. (1992) reported a CD gene mutant strain by ultraviolet mutagenesis, and its L-cysteine productivity was increased from 67% to 95%. It also has been demonstrated that disruption of CD genes was significantly effective at achieving L-cysteine overproduction in *E. coli* ((Awano et al., 2003; Awano et al., 2005), so we are presently continuing this research in order to construct a CD-gene-disruption strain to increase the L-cysteine production.

Acknowledgements This work was supported by Grant no. 30470053 from the National Natural Science Foundation of China and Grant no. 05YFJZJC00900 from the Natural Science Foundation of Tianjin, China.

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