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# One-step elimination of L-cysteine desulfhydrase from crude enzyme extracts of *Pseudomonas* sp. TS1138 using an immunomagnetic affinity matrix improves the enzymatic production of L-cysteine

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**Abstract**

In this study, a high efficiency immunomagnetic affinity matrix was developed to eliminate L-cysteine desulfhydrase (CD), which decomposes L-cysteine, in crude enzyme extracts from *Pseudomonas* sp. TS1138. After cloning and expression in *Escherichia coli*, recombinant CD was purified to raise polyclonal antibodies from mice. The anti-CD antibody was cross-linked to staphylococcal protein A-magnetic cellulose microspheres (MCMS) with dimethyl pimelimidate (DMP). The natural CD was eliminated from the crude enzyme extracts by treatment with the cross-linked antibody-protein A-MCMS, resulting in a high level of L-cysteine production. The conversion rate of DL-2-amino-$\Delta^2$-thiazoline-4-carboxylic acid (DL-ATC) to L-cysteine increased significantly from 61.9 to 96.2%. The cross-linked antibody-protein A-MCMS showed its durability after repetitive use, maintaining a constant binding capacity for CD during five cycles. This study may lead to a convenient and cost-efficient method to produce L-cysteine by enzymatic conversions.


*Keywords:* *Pseudomonas* sp. TS1138; L-Cysteine; L-Cysteine desulfhydrase; Immunomagnetic separation; Magnetic cellulose microspheres

## 1. Introduction

L-Cysteine, an important amino acid, has significant applications in the pharmaceutical, food and cosmetic industries [1]. 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 [2]. In addition, L-cysteine extracted from hair does not qualify for medical use because of sanitary problems. A microbial method for L-cysteine production, which uses a chemically synthesized substrate, DL-2-amino-$\Delta^2$-thiazoline-4-carboxylic acid (DL-ATC), has been developed with enzymes extracted from certain strains of bacteria, especially the genus *Pseudomonas* [3]. It is a more economical, efficient, and environmentally friendly way developed specifically for the production of pharmaceutical-grade L-cysteine.

Recently, 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 by our group [4]. In that same study, we found that L-cysteine desulfhydrase (CD) plays an important role in L-cysteine decomposition, resulting in the production of pyruvate, ammonia and hydrogen sulfide. This type of enzymatic activity has also been demonstrated in several mammalian tissues [5] and other bacteria, such as *Salmonella typhimurium* [6–8] and *Escherichia coli* (*E. coli*) [7]. To further optimize L-cysteine production, CD should be eliminated from the conversion process.

*Pseudomonas* sp. TS1138 is able to grow in minimal conditions, utilizing DL-ATC as its only source for carbon and nitrogen. Under this condition, L-cysteine is synthesized from DL-ATC and decomposed by CD to pyruvate, which is then transformed into acetyl-CoA and shuttled into the tricarboxylic acid cycle (TCA cycle). Based on this, the CD gene may be essential for *Pseudomonas* sp. TS1138. To date, construction of a CD gene-disrupted mutant strain has not been successfully achieved. Hence, it is necessary to develop an alternative method to improve L-cysteine production.

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Immunomagnetic separation (IMS), which utilizes magnetic microspheres (MMS) as the basis for separation, takes advantage of the high specificity of affinity chromatography and the high efficiency of magnetic response [9,10]. MMS can easily be collected when a magnetic field is applied. When coupled with appropriate ligands such as antigens or antibodies, they provide an effective tool for achieving rapid, simple and specific target protein separation even in large-scale preparation [11,12]. Conventional methods of immobilizing antibodies on MMS usually directly bind antibodies to MMS by chemical coupling. However, it often generates an affinity matrix with low affinity because of the multi-site attachment and orientation of the immunoglobulin molecule, which reduces the efficiency of the antibody–antigen interaction. Therefore, the MMS matrix with immobilized staphyloccocal protein A, which binds to the Fc fragment of the immunoglobulin, offers optimal stereo orientation of antibodies and exhibits great potential for further antigen capture [13].

In this study, we developed a high efficiency immunomagnetic affinity matrix for one-step elimination of CD from the crude enzyme extracts of *Pseudomonas* sp. TS1138, which subsequently resulted in a high level of L-cysteine production. Under optimized conditions, L-cysteine production was improved remarkably after the complete elimination of CD from crude enzyme extracts with cross-linked antibody-protein A-magnetic cellulose microspheres (MCMS). The method presented in this study can be considered as an efficient and convenient alternative way to improve L-cysteine production. Moreover, this method may also have potential applications in one-step protein purification after appropriate optimization.

## 2. Experimental

### 2.1. Materials

*Pseudomonas* sp. TS1138 was isolated from industrial wastewater and cultured in ATC medium [4]. *E. coli*, DH5α and BL21 (DE3), were purchased from Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The pET-21a (+) vector (Novagen, Germany), Ni-NTA His-Bind resin (Novagen, Germany) and pGEM-T easy vector (Promega, USA) 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). Dimethyl pimelimidate (DMP) (Pierce, USA), *O*-phenylenediamine (OPD, BR1965), diaminobenzidine (DAB) (Sigma, USA), Goat anti-mouse IgG conjugated with Horseradish Peroxidase (HRP) and Staphylococcal Protein A were purchased from TBD Bio. Co. Ltd. (Tianjin, China). Goat anti-mouse IgG and mouse IgG for standards were purchased from Sino-American Biotechnology Co. Ltd. (Luoyang, China). All other reagents were of analytical grade.

### 2.2. Cloning of the CD gene

Extraction of genomic DNA from *Pseudomonas* sp. TS1138, restriction enzyme digestion, DNA ligation and transformation of *E. coli* cells were performed by the method of Sambrook [14]. According to the CD gene sequence of *Pseudomonas putida* (*P. putida*) KT2440 [15], primers were designed as follows: forward primer, 5′-CCG***GAATTC***ATGAAGTTGCCGATCT-ACCTTG-3′; reverse primer, 5′-CCC***AAGCTT***TTAGTG GGCGGCCCACTC-3′, bearing *Eco*R I and *Hin*d III restriction sites (underlined in bold italic), respectively. The genomic DNA of *Pseudomonas* sp. TS1138 was used as a template to amplify CD gene. PCR was conducted using a thermal profile starting with 5 min denaturation at 95 °C, followed by 30 cycles of 94 °C (1 min), 56 °C (1 min), 72 °C (1 min) plus an additional extension time at 72 °C (10 min) in a T-gradient PCR thermocycler (Biometra, Germany). The amplification product was analyzed by 1% agarose gel electrophoresis. The specific 1.2 kb PCR product was purified using PCR gel purification kit. The purified DNA was ligated into the pGEM-T easy vector for DNA sequencing and was digested with *Eco*R I/*Hin*d III and ligated into the multiple cloning sites of the pET-21a (+) vector to create pET-cd. DNA sequencing was performed using an automatic DNA sequencer (ABI prism, USA).

### 2.3. Expression and purification of recombinant CD

*E. coli* BL21 (DE3) strain was transformed with the recombinant pET-cd plasmid. The transformed competent cells were 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 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) at 20 °C for 24 h. The cells were harvested by centrifugation at $4000 \times g$ 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 $20{,}000 \times g$ for 20 min at 4 °C to remove cellular debris. The recombinant protein was purified based on 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 overnight and stored at −20 °C for future use.

### 2.4. Production of polyclonal antibody against CD

Eight BALB/c mice (8-week-old females) were injected with the purified recombinant CD (20 μg/mouse) mixed with complete Freund's adjuvant at a 1:1 ratio. The mice were injected subsequently three more times with the protein (10 μg/mouse) mixed with incomplete Freund's adjuvant at 14-day intervals. One week after the fourth injection, mouse antisera were collected and stored at −20 °C.

### 2.5. Preparation of the cross-linked antibody-protein A-MCMS

MCMS were prepared by a suspension embedding procedure [16]. Briefly, purified cotton was reacted with NaOH and carbon

disulfide to prepare a viscose solution. 0.5 g of potassium oleate was dissolved in 180 mL of chlorobenzene and 40 mL of tetrachloromethane, and then 70 mL of viscose solution containing ferrofluids was added to the mixture. The mixture was stirred for 2 h at 90 °C. MCMS were harvested on a sieve, and then were washed with ethanol and finally with water. Further, MCMS were mixed with epichlorohydrin, ammonia hydroxide and glutaraldehyde in turn, to prepared GA-activated MCMS [17]. Such microspheres were supplied with sufficient free aldehyde groups and had a mean diameter of 150 μm.

For covalent binding of protein A, 1 mL of GA-activated MCMS was incubated with two volumes of 0.01 M sodium bicarbonate (pH 9.6) containing 10 mg of protein A overnight at 4 °C. The beads were washed and the reactive, uncoated surface was then blocked by the addition of 5 mL of 1 M glycine. The amount of protein A bound to the MCMS was calculated by comparing the content of protein A, which was determined by Lowry's method [18] before and after coupling.

One milliliter of mouse antisera containing 10.04 mg of anti-CD IgG was added to a test tube containing 1 mL of protein A-MCMS and incubated on a shaker at room temperature for 1 h. After magnetic separation, microspheres were washed six times with PBS. According to the method described by Schneider et al. [13], the anti-CD antibody-protein A-MCMS complex was washed with 20 volumes of 0.2 M triethanolamine (pH 8.2) and resuspended in the same buffer containing 20 mM DMP. The mixture was agitated gently at room temperature for 45 min and the reaction was stopped by resuspending in 20 volumes of 0.2 M triethanolamine (pH 8.2) for 10 min. After pre-elution with 0.1 M Glycine–HCl buffer (pH 2.5), the cross-linked antibody-protein A-MCMS was prepared and stored at 4 °C for future use.

### 2.6. Sandwich ELISA for quantification of anti-CD IgG

The quantification of anti-CD IgG in antisera was carried out by sandwich ELISA [19]. ELISA plates (Costar, USA) were coated with an optimal concentration of 10 μg/mL of goat anti-mouse IgG. The standard dilution mouse IgG (0.01–10 μg/mL) and serially diluted test samples were prepared, as determined in prior standardization experiments. Goat anti-mouse IgG conjugated with HRP was used as a tracer at 1:2000 dilution in PBS with 0.1% Tween 20 (PBST) and final developed with OPD substrate.

### 2.7. Preparation of crude enzyme extracts of Pseudomonas sp. TS1138

*Pseudomonas* sp. TS1138 was cultured in ATC medium at 28 °C for 16 h. The cells were harvested by centrifugation at 4000 × *g* for 10 min at 4 °C. The cell pellet was washed twice with glycine buffer (50 mM, pH 8.0) and resuspended in the same buffer. After sonication in ice, the resulting cell lysate was centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatants obtained were used as crude enzyme extracts.

### 2.8. One-step elimination of CD

The crude enzyme extracts of *Pseudomonas* sp. TS1138 were incubated with cross-linked antibody-protein A-MCMS by agitating gently at 4 °C for 3 h. After magnetic separation, the treated crude enzyme extracts were obtained and used for the future enzymatic assay immediately. The cross-linked antibody-protein A-MCMS binding with CD was washed three times with PBS buffer. Elution of the bound CD was conducted with two volumes of 0.1 M Glycine–HCl buffer (pH 2.5) for 10 min and repeated once. The eluted material was immediately brought to near neutrality (pH 7.4) by the addition of 200 μL of 1 M phosphate buffer. The eluates were pooled, dialyzed, freeze-dried and stored at −20 °C for future use.

### 2.9. Enzymatic conversion of DL-ATC to L-cysteine

The crude enzyme extracts of *Pseudomonas* sp. TS1138, in the presence or absence of CD, were used as the enzyme source. The conversion ability from DL-ATC to L-cysteine was assayed as follows: 20 mL of crude enzyme extracts was added into 40 mL of substrate solution (0.2% DL-ATC, 1.5% $K_2HPO_4$, pH 8.0) at 37 °C. At different time intervals, the concentrations of L-cysteine and pyruvate were measured. The reaction could be stopped by the addition of trichloroacetic acid to a final concentration of 2.5%. As a comparison, the conversion ability of the crude enzyme extracts with 0.5 mM hydroxylamine was used as a control.

### 2.10. Quantification of L-cysteine and determination of CD activity

The amount of L-cysteine formed from DL-ATC was measured by Gaitonde's acid ninhydrin method [20]. The CD activity was assayed by quantifying the amount of pyruvate as described by Candura [21] 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.

### 2.11. SDS–PAGE and western blot

Twelve percent polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed by the Laemmli method [22] and the gel was stained using 0.1% Coomassie Brilliant Blue R-250.

After separation by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane following the procedure developed by Towbin et al. [23]. The membrane was blocked with 5% dried skim milk in PBS overnight at 4 °C and incubated with antiserum to recombinant CD at a dilution of 1:10,000 for

2 h at room temperature. The membrane was washed six times with PBST and incubated with HRP-labeled goat anti-mouse IgG (1:2000) for 1.5 h at room temperature. Finally, the membrane was washed again with PBST and developed with DAB as the substrate.

### 2.12. CD activity staining

The CD activity was visualized by the staining procedure of Zdych et al. [24].

Samples (10–30 μg of protein in each lane) were separated by 9% native 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.

## 3. Results and discussion

### 3.1. Cloning, expression and purification of CD

The molecular mechanism of the conversion of DL-ATC to L-cysteine and the role of CD involved in this step were elucidated in Fig. 1. Previously, we identified and characterized the genes encoding ATC hydrolase and S-carbamyl-L-cysteine amidohydrolase, which shared extensively high homologies with related genes from *P. putida* KT2440 [4]. On the basis of the CD gene sequence of *P. putida* KT2440, primers were designed to clone the CD gene in *Pseudomonas* sp. TS1138.

The CD gene amplified from *Pseudomonas* sp. TS1138 was ligated into the pGEM-T easy vector for sequencing. The nucleotide sequence of the cloned CD gene was 1215 bp, which shared 98.3% homology with the sequence from *P. putida* KT2440 and has been submitted to GenBank under an accession number of AY675347. The recombinant expression vector pET-cd was constructed and introduced into *E. coli* BL21 (DE3) for overexpression.

As shown in Fig. 2A, lane 1, the recombinant CD was overexpressed in *E. coli* BL21 (DE3) and its activity was further confirmed by CD activity staining (Fig. 2C, lane 1). Due to some 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 alter protein activity significantly.

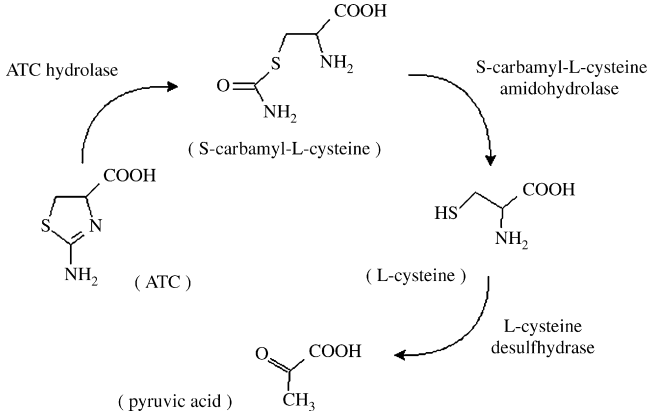


Fig. 1. The molecular mechanism of the conversion of ATC to L-cysteine in *Pseudomonas* sp. TS1138.

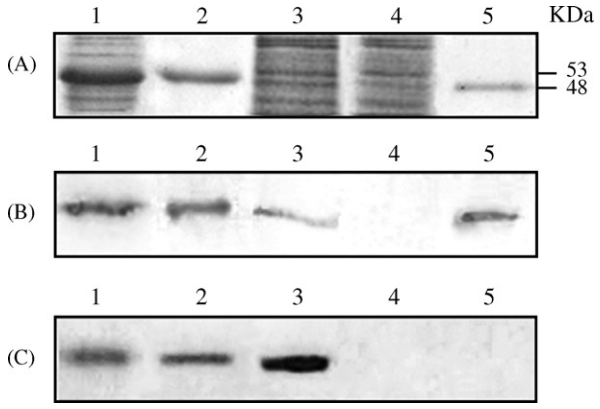


Fig. 2. SDS-PAGE, western blot and CD activity staining analysis. SDS-PAGE (A), western blot (B) and CD activity staining (C). Lane 1, crude cell extracts of *E. coli* BL21 (DE3) transformed with pET-cd; lane 2, purified recombinant CD; lane 3, crude enzyme extracts of *Pseudomonas* sp. TS1138; lane 4, crude enzyme extracts of *Pseudomonas* sp. TS1138 treated with cross-linked antibody-protein A-MCMS; lane 5, isolated CD by the cross-linked antibody-protein A-MCMS.

The purification of recombinant CD was achieved in one step using Ni-NTA His-Bind resin (Fig. 2A, lane 2,). After native-PAGE and activity staining, CD activity was visualized in purified proteins (Fig. 2C, lane 2).

### 3.2. Production of polyclonal antibody against CD

The purified recombinant CD was used to immunize eight mice to produce polyclonal antibody. As demonstrated in Fig. 2B, antisera obtained from these mice displayed very strong immunoreactivity and specificity to the lysate of *E. coli* containing pET-cd vector and the purified recombinant CD. Immunoreactivity against natural CD was also observed in the crude enzyme extracts of *Pseudomonas* sp. TS1138. Therefore, this result confirmed that the recombinant CD obtained from transformed *E. coli* was antigenically similar to the natural CD in *Pseudomonas* sp. TS1138. The polyclonal antibody against CD could be used for isolation of natural CD from the crude enzyme extracts of *Pseudomonas* sp. TS1138.

### 3.3. Preparation of cross-linked antibody-protein A-MCMS

The MCMS used in this study, with a mean diameter of 150 μm, are well shaped spheres with a porous surface and good intensity (Fig. 3). The magnetic content of the MCMS is 3.80%, and the homogenous distribution of $Fe_3O_4$ particles endows the MCMS a good performance in the magnetic response [17].

To develop an immunomagnetic separation matrix for one-step elimination of CD, protein A and anti-CD antibody were sequentially bound to GA activated MCMS. Protein A immobilized on MCMS owns high specificity of binding to the Fc fragment of immunoglobulin molecules from many mammals without interrupting its antigen-binding ability

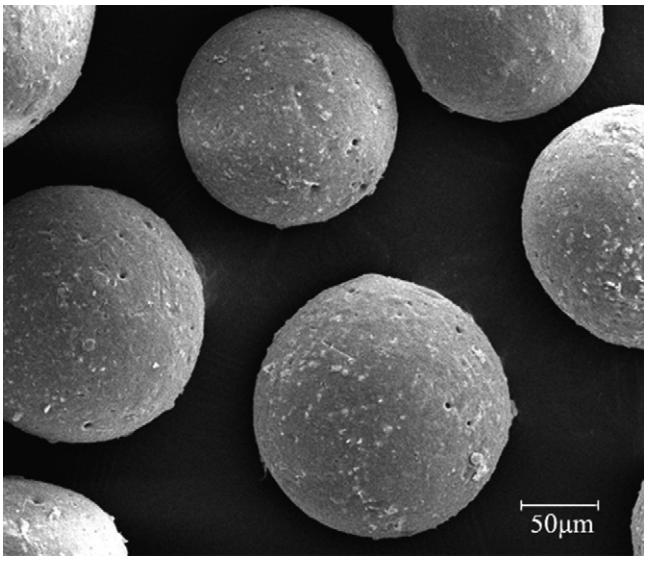


Fig. 3. Scanning electron microscopy (SEM) micrograph of MCMS.

making it possible to construct a suitable immunomagnetic affinity matrix for one-step purification.

As a result, protein A-MCMS was prepared by immobilizing 7.82 mg of protein A to 1 mL of MCMS, and then 9.26 mg of anti-CD antibody was bound to 1 mL of protein A-MCMS. Finally, a covalent cross-link, which stabilized the complex and overcame antibody leakage, was made between the antibody and protein A by treatment with DMP. This method allowed optimal stereo orientation of antibodies, which results in a maximum antigen binding efficiency.

### 3.4. Binding capacity of the cross-linked antibody-protein A-MCMS

The binding capacity of the prepared matrix was identified as the maximum protein amount of crude enzyme extracts in which CD activity was eliminated by the prepared magnetic matrix. Different protein amounts of crude enzyme extracts of *Pseudomonas* sp. TS1138 were incubated with 100 μL of cross-linked antibody-protein A-MCMS. To ensure complete antibody–antigen interaction and minimum loss of enzyme activity, the mixture was agitated gently at 4 °C for 3 h after optimization. The relative CD activity remaining in the treated crude enzyme extracts was determined, which further reflected the binding capacity of the matrix. As shown in Fig. 4, when the protein amount of crude enzyme extracts was less than 4 mg, no CD activity was detected. However, when an increasing protein amount of crude enzyme extracts (>4 mg) was applied, the relative CD activity rapidly increased, indicating that 100 μL of the matrix was able to completely eliminate CD from a maximum amount of 4 mg of crude enzyme extracts of *Pseudomonas* sp. TS1138.

### 3.5. One-step elimination of CD

To completely remove CD from the crude *Pseudomonas* sp. TS1138 enzyme extracts, 80 mg of protein in crude enzyme extracts were incubated with 2 mL of cross-linked antibody-protein A-MCMS. The mixture was agitated gently at 4 °C for 3 h. After magnetic separation, the treated crude enzyme extracts were used for the enzymatic assay. After extensive washing, the bound CD was eluted with 0.1 M Gly–HCl buffer (pH 2.5). As shown in Fig. 2A, only a single band (lane 5) from the concentrated eluates was visualized, which corresponded to the natural CD.

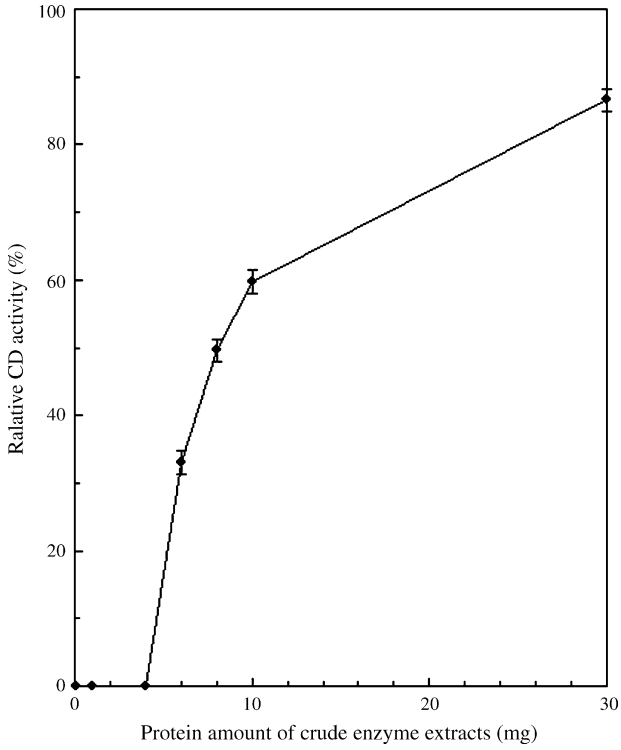


Fig. 4. Binding capacity of the cross-linked antibody-protein A-MCMS. The relative CD activity was calculated by the ratio of CD activity remaining in the treated enzyme extracts to that of the original enzyme extracts.

Western blot analysis (Fig. 2B) indicated that, compared to the original crude enzyme extracts, CD was completely removed after treatment with the cross-linked antibody-protein A-MCMS and the concentrated eluates contained highly purified CD.

Fig. 2C showed that no CD activity of crude enzyme extracts was detected after the elimination. The eluted CD also lost its activity during the elution and further concentration.

The results confirmed that in optimized conditions, CD could be completely eliminated from the crude enzyme extracts of *Pseudomonas* sp. TS1138 by one-step affinity purification with cross-linked antibody-protein A-MCMS.

### 3.6. Enzymatic conversion of DL-ATC to L-cysteine

To find out whether the CD-free crude enzyme extracts of *Pseudomonas* sp. TS1138 could result in a higher L-cysteine yield, the amount of L-cysteine converted by the crude enzyme extracts was measured. As shown in Fig. 5A, the amount of L-cysteine produced by the original crude enzyme extracts increased during the first 4 h and gradually decreased due to the decomposition of L-cysteine by CD. In

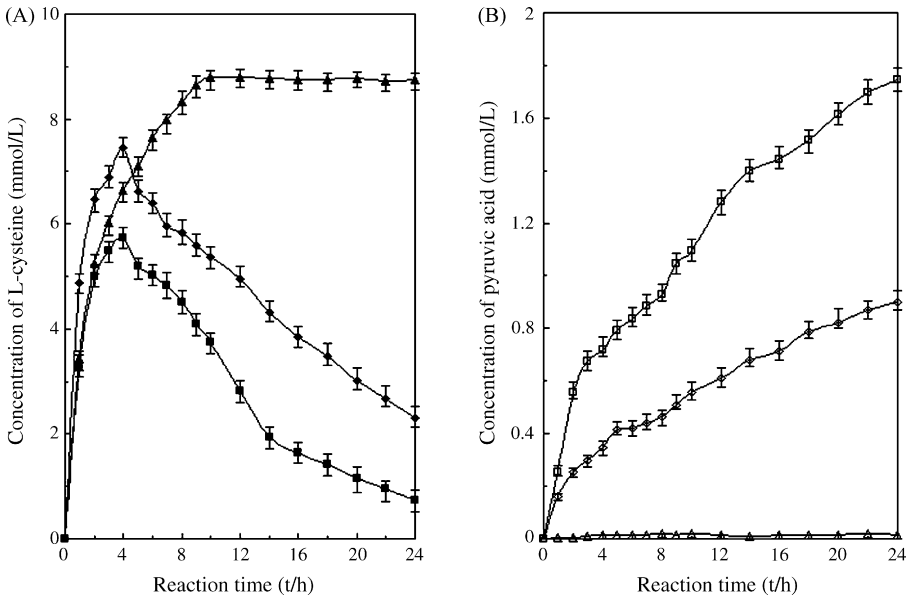


Fig. 5. Production of L-cysteine and pyruvate by different enzyme sources. Production of L-cysteine (A) and pyruvate (B). (■) and (□) indicate original crude enzyme extracts; (♦) and (◊) indicate crude enzyme extracts supplemented with hydroxylamine; (▲)and (△) indicate crude enzyme extracts treated with the cross-linked antibody-protein A-MCMS.

the fourth hour, it reached its highest L-cysteine yield of 62.7% (Table 1).

Hydroxylamine is an efficient inhibitor of CD [25]. However it also strongly inhibits the activities of L-cysteine-forming enzymes. As shown in Fig. 5A, when an optimal concentration of hydroxylamine was added to the reaction mixture, the amount of L-cysteine was increased compared with those produced by the original crude enzyme extracts at every time interval. After 4 h reaction, the highest L-cysteine yield of 81.6% was achieved (Table 1). However, L-cysteine production has reduced since then, probably due to the remaining activity of CD.

When the crude enzyme extracts were purified with cross-linked antibody-protein A-MCMS, the amount of L-cysteine increased significantly (Fig. 5A) and L-cysteine accumulated to a peak yield of 96.4% after 10 h reaction (Table 1), and remained at this high level in the following 14 h. Since L-cysteine has a feedback inhibitory effect to L-ATC hydrolase [26], it is expected that L-cysteine produced by the crude enzyme extracts may not reach a yield of 100%, even after complete depletion of CD.

Consistent with L-cysteine productivity of different enzyme sources shown in Fig. 5A, the pyruvate concentration presented in Fig. 5B, which reflected the CD activity, further confirmed that CD had been completely removed from the enzyme source by a one-step elimination with the cross-linked antibody-protein A-MCMS and L-cysteine was no longer decomposed during the enzymatic conversion.

### 3.7. Reutilization of the cross-linked antibody-protein A-MCMS

The durability of cross-linked antibody-protein A-MCMS was demonstrated by the repeated use of the matrix. For this

Table 1
Conversion rate of ATC to L-cysteine produced by different enzyme sources

| Time (h) | Conversion rate of ATC to L-cysteine[a] | | |
|---|---|---|---|
| | CR1 (%) | CR2 (%) | CR3 (%) |
| 2 | 54.8 ± 2.0 | 57.4 ± 1.9 | 70.7 ± 2.2 |
| 4 | 62.7 ± 1.7 | 72.7 ± 2.1 | 81.6 ± 2.1 |
| 6 | 55.0 ± 1.6 | 83.8 ± 2.2 | 69.9 ± 2.3 |
| 8 | 49.5 ± 2.2 | 91.2 ± 2.6 | 63.8 ± 2.2 |
| 10 | 40.9 ± 1.3 | 96.4 ± 1.8 | 58.8 ± 1.9 |
| 12 | 30.8 ± 1.5 | 96.2 ± 2.5 | 54.3 ± 2.2 |
| 24 | 8.1 ± 1.6 | 95.8 ± 2.1 | 25.2 ± 1.6 |

CR1: conversion rate of the original crude enzyme extracts. CR2: conversion rate of the crude enzyme extracts treated with the cross-linked antibody-protein A-MCMS. CR3: conversion rate of the crude enzyme extracts supplemented with hydroxylamine.

[a] The value of all parameters were measured at least three times (mean value ± S.D.).

Table 2
Durability of the cross-linked antibody-protein A-MCMS

| Number of use | Conversion rate[a] | | CD activity[a] | |
|---|---|---|---|---|
| | CR1 (%) | CR2 (%) | CA1 (U) | CA2 (U) |
| 1 | 62.7 ± 1.7 | 96.4 ± 1.8 | 5.63 ± 0.20 | N.D.[b] |
| 2 | 61.5 ± 2.0 | 95.6 ± 2.0 | 5.85 ± 0.13 | N.D. |
| 3 | 63.0 ± 2.3 | 97.0 ± 1.4 | 5.44 ± 0.21 | N.D. |
| 4 | 60.8 ± 1.9 | 96.2 ± 2.1 | 6.15 ± 0.17 | N.D. |
| 5 | 61.8 ± 1.8 | 96.0 ± 1.7 | 5.76 ± 0.16 | N.D. |
| Mean | 61.9 ± 1.1 | 96.2 ± 0.8 | 5.77 ± 0.38 | N.D. |

CR1: conversion rate of the original crude enzyme extracts. CR2: conversion rate of the crude enzyme extracts treated with the cross-linked antibody-protein A-MCMS. CA1: CD activity of the original crude enzyme extracts. CA2: CD activity of the crude enzyme extracts treated with the cross-linked antibody-protein A-MCMS.

[a] The value of all parameters were measured at least three times (mean value ± S.D.).

[b] Not detectable.

purpose, the one-step elimination of CD from 80 mg of protein in crude enzyme extracts was repeated five times. CD activity and L-cysteine production from treated enzyme sources were detected in each cycle compared with the initial one.

Table 2 shows that the cross-linked antibody-protein A-MCMS maintained a constant binding capacity for CD after repetitive use, resulting in almost identical yields of L-cysteine during the five cycles. The average conversion rate of DL-ATC to L-cysteine improved from 61.9 to 96.2% when the CD activity was removed by the cross-linked antibody-protein A-MCMS.

## 4. Conclusions

In this study, we present an efficient, reliable method for high-level production of L-cysteine in the enzymatic conversion of DL-ATC to L-cysteine with enzyme extracts from *Pseudomonas* sp. TS1138. The CD, which was responsible for the decomposition of L-cysteine, was cloned and expressed in *E. coli*. Highly specific polyclonal antibodies were raised in mice against purified recombinant CD. The cross-linked antibody-protein A-MCMS was generated and used for one-step elimination of CD from crude enzyme extracts of *Pseudomonas* sp. TS1138. The constructed immunomagnetic affinity separation matrix displayed high specificity, efficiency and durability for complete elimination of CD. The method described here may lead to a new approach to produce large-scale L-cysteine using enzymatic conversions with enzymes purified from *Pseudomonas* sp. TS1138 or other strains of bacteria. In addition, it may also provide an efficient and convenient alternative method to obtain virtually purified proteins in one step.

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## References

[1] N. Awano, M. Wada, H. Mori, S. Nakamori, H. Takagi, Appl. Environ. Microb. 71 (2005) 4179.
[2] O.H. Ryu, J.Y. Ju, C.S. Shin, Process Biochem. 32 (1997) 201.
[3] K. Sano, Y. Yokozeki, F. Tamura, N. Yasuda, I. Noda, K. Mitsugi, Appl. Environ. Microb. 34 (1977) 806.
[4] Y.S. Yu, Z. Liu, C.Q. Liu, Y. Li, Y.J. Jin, W.B. Yang, G. Bai, Biosci. Biotechnol. Biochem. 70 (2006) 2262.
[5] A. Kato, M. Ogura, H. Kimura, T. Kawai, M. Suda, J. Biochem. 59 (1966) 34.
[6] J.M. Collins, K.J. Monty, J. Biol. Chem. 248 (1973) 5943.
[7] G. Guarneros, M.V. Ortega, Biochim. Biophys. Acta. 198 (1970) 132.
[8] N.M. Kredich, L.J. Foote, B.S. Kreenan, J. Biol. Chem. 248 (1973) 6187.
[9] M. Ogiue-Ikeda, Y. Sato, S. Ueno, IEEE. Trans. Nanobiosci. 2 (2003) 262.
[10] D. Hanau, D.A. Schmitt, M. Fabre, J.P. Cazenave, J. Invest. Dermatol. 91 (1988) 274.
[11] C. Clarke, J. Titley, S. Davies, M.J. O'Hare, Epithelial Cell. Biol. 3 (1994) 38.
[12] G. Prioult, C. Turcotte, L. Labarre, C. Lacroix, I. Fliss, Int. Dairy J. 10 (2000) 627.
[13] C. Schneider, R.A. Newman, D.R. Sutherland, U. Asser, M.F. Greaves, J. Biol. Chem. 257 (1982) 10766.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, Plainview, New York, 2001, p. 27.
[15] K.E. Nelson, C. Weinel, I.T. Paulsen, et al., Environ. Microbiol. 4 (2002) 799.
[16] H. Fang, J. Wei, Y.T. Yu, Biomaterials 25 (2004) 5433.
[17] Y. Cao, G. Bai, J.Q. Chen, W. Tian, S.Q. Wang, W.B. Yang, J. Chromatogr. B. 833 (2006) 236.
[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[19] G. Bai, K. Fujiwara, H. Tanimori, T. Kitagawa, Biol. Pharm. Bull. 20 (1997) 1224.
[20] M.K. Gaitonde, Biochem. J. 104 (1967) 627.
[21] F. Candura, Boll. Soc. Ital. Biol. Sper. 35 (1959) 697.
[22] U.K. Laemmli, Nature 227 (1970) 680.
[23] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. U S A 76 (1979) 4350.
[24] E. Zdych, R. Peist, J. Reidl, W. Boos, J. Bacteriol. 177 (1995) 5035.
[25] K. Sano, K. Misugi, Agric. Bio. Chem. 42 (1978) 2315.
[26] O.H. Ryu, C.S. Shin, J. Microbiol. Biotechnol. 1 (1991) 50.