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Purification and enzymatic characteristics of cysteine desulfurase, IscS, in *Acidithiobacillus ferrooxidans* ATCC 23270

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Abstract: A cysteine desulfurase protein, IscS, was encoded by the operon iscSUA in *Acidithiobacillus ferrooxidans*. The gene of IscS from *Acidithiobacillus ferrooxidans* ATCC 23270 was cloned and expressed in *Escherichia coli*. The protein was purified by one-step affinity chromatography to homogeneity. The final protein yield after affinity chromatography was 12.9%. The enzyme was characterized for thermal stability, pH and kinetic parameters. The molecular mass of recombinant IscS was 46 ku by SDS-PAGE. The optimum pH was 8.0−8.5. The enzyme had a temperature optimum at 30 ℃ and was relatively stable at 40 ℃, with 67% loss of activity. 1,5-I-AEDANS significantly inhibited IscS activity. Kinetic parameters K_m and V_{max} were found to be 0.11 mmol/L and 2.57 μmol/(L·min).

Key words: *Acidithiobacillus ferrooxidans*; IscS; purification; optimum pH; optimum temperature; inhibition; kinetics

1 Introduction

Iron-sulfur proteins are widely distributed in almost all organisms and play essential roles in energy metabolism, DNA repair, transcriptional regulation, and biosynthesis of nucleotides and amino acids[1−2]. Three separate systems including ISC (iron-sulfur cluster) [3], NIF(nitrogen fixation)[4], and SUF(sulfur)[5] have been identified for bacterial Fe-S clusters biosynthesis. Of these systems, ISC appears to provide a fundamental Fe-S cluster biosynthesis pathway. The *isc* operon (*iscRSUA-hscBA-fdx*) encoded the components of the ISC machinery, which are conserved from bacteria to higher eukaryotes and, in *Escherichia coli*[6].

Three isc genes, iscS, iscU and iscA, have been the targets of recent investigations. IscU is characterized as an Fe-S cluster assembly scaffold protein[7−8]. The function of IscA remains controversial in the biogenesis of Fe-S proteins. It might function as an iron donor or an alternate scaffold for Fe-S clusters synthesis[9−10]. IscS is a cysteine desulfurase which is a pyridoxal 5′-phosphate(PLP)-dependent enzyme. IscS catalyzes the conversion of L-cysteine to L-alanine and sulfan sulfur via the formation of a protein-bound cysteine persulfide intermediate on a conserved cysteine residue, which is the major cellular catalyst for the mobilization and distribution of sulfur from cysteine for a number of different biosynthetic pathways[3, 11−12].

Acidithiobacillus ferrooxidans is a motile, Gram-negative, chemolithotrophic bacterium that utilizes Fe(Ⅱ), H_2S ,S^0, reduced inorganic sulfur compounds and molecular hydrogen as energy sources. The oxidation of sulfur produces sulfuric acid which is responsible for some of the interesting characteristics of *At. ferrooxidans*. The presence of sulfuric acid strongly acidifies the environment. Therefore, *At. ferrooxidans* is an acidophile by necessity, growing within a pH range of 1.3−4.5. It is generally found in acidic environments such as mining dumps and acid mine drainages. This organism plays a key role of microbial communities taking part in the bacterial-chemical processes of bioleaching under

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mesophilic conditions.

2 Experimental

2.1 Materials

Acidithiobacillus ferrooxidans ATCC 23270 was obtained from the American Type Culture Collection. A HiTrap chelating metal affinity column was purchased from GE healthcare LTD. Top10 competent cells, *E. coli* strain BL21(DE3) competent cells came from Invitrogen Life Technologies. The Plasmid Mini kit, a gel extraction kit and synthesized oligonucleotides were obtained from Sangon Company of Shanghai, China. L-cysteine, PLP, Taq DNA polymerase, T4 DNA ligase and restriction enzymes came from MBI Fermentas, Germany. 1,5-I-AEDANS, *N*-Ethylmaleimide *N*,*N*-dimethyl-*p*-phenylenediamie, and D-cysteine came from Sigma. All other reagents were of research grade or better and were obtained from commercial sources.

2.2 Cloning of ISCS gene from *A. ferrooxidans* ATCC 23270

Genomic DNA from *A. ferrooxidans* was prepared using the EZ-10 spin column genomic DNA isolation kit from Bio Basic Inc., according to the manufacturer's instructions for bacterial DNA extraction. This genomic DNA was used as a template for PCR reaction. The gene was amplified by PCR using primers that were designed to add six continuous histidine codons to the 5′ primer. The sequence of the forward primer was 5′-CGCGCGA-ATTCAGGAGGAATTTAAAATGAGAGGATCGCAT CACCATCACCATCACATCGAGGGAAGGTTGCGG ATCGATCCGGATCGTCCCATTTAT-3′, containing an *EcoR*I site (GAATTC), a ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2−11 of mature IscS. The sequence of the reverse primer was 5′-CTGCAGGGAT-CCTTAGTGCGCGGCCCACTGAATGCTGTGGATA-TCAAT-3′, containing a *BamH*I site (GGATCC), a stop anticodon(TTA), and anticodons for the last 11 amino acids of mature IscS protein. PCR amplification was performed using *Taq* DNA polymerase, and samples were subjected to 25 cycles of 45 s of denaturation at 94 ℃, 1 min of annealing at 65 ℃, and 2 min of elongation at 72 ℃. The amplification products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The resulting PCR product was gel purified, double digested and ligated into a pLM1 expression vector, resulting in the pLM1::IscS plasmid [13]. The identified positive colony was grown in LB medium containing ampicillin (50 mg/L), and the plasmid pLM1:IscS was isolated from harvested bacteria cells using a plasmid extraction kit. The isolated pLM1::IscS plasmid was then transformed into *E. coli* strain BL21 (DE3) competent cells for expression purposes.

2.3 Expression of soluble recombinant protein IscS in *E. coli*

We use the following procedure for expression of soluble IscS protein. The *E. coli* strain BL21(DE3) cells with pLM1::ISCS plasmid were grown at 37 ℃ in 500 mL of LB medium containing ampicillin (100 mg/L) to an optical density at 600 nm (OD_{600}) of 0.6. At this point, the cells were incubated at room temperature with the addition of 0.5 mmol/L isopropyl-D-thiogalactopyranoside (IPTG) overnight with shaking at 180 r/min. The cells were harvested by centrifugation and the cell pellet was washed with an equal volume of sterile water. The cells were again harvested by centrifugation, suspended in start buffer (20 mmol/L potassium phosphate, pH 7.4, 0.5 mol/L NaCl), incubated with 5 mg lysozyme at room temperature for 0.5 h, and then stored at −80 ℃ for purification.

2.4 Purification of *A. ferrooxidans* ATCC23270 IscS

The cells were lysed by sonication four times for 30 s each time (150 W Autotune Series High Intensity Ultrasonic sonicator equipped with a 8 mm diameter tip). The insoluble debris was removed by centrifugation and the clear supernatant was used for protein purification. A Hi-Trap column (6E Healthcare Ltd) was first equilibrated with 0.1 mol/L nickel sulfate to charge the column with nickel ions followed by 5 column volumes of MiliQ water to remove unbound nickel ions from the column, and then by 5 column volumes of start buffer (20 mmol/L potassium phosphate, pH 7.4, 0.5 mol/L NaCl) to equilibrate the column. The clarified sample was applied to the Hi-Trap column after filtering it through a 0.45 μm filter. The column was washed with 5 column volumes of start buffer followed with 5 column volumes of wash buffer (20 mmol/L potassium phosphate, pH 7.4, 0.5 mol/L NaCl, 50 mmol/L imidazole), and subsequently the protein was eluted with an elution buffer (20 mmol/L potassium phosphate, pH 7.4, 0.5 mol/L NaCl, 500 mmol/L imidazole). The method of Bradford was used to determine the protein content with BSA as standard. We used the SDS-PAGE with 15% (volume fraction) acrylamide to analyze the eluted fractions[14]. The gels were stained with Coomassie Brilliant Blue R−250. The purified enzyme fractions were combined and dialyzed against a 20 mmol/L potassium phosphate buffer, pH 7.4, 5% (volume fraction) glycerol, and then stored at −80 ℃.

2.5 UV-Vis scanning

Ultraviolet-visible spectra scanning was carried out

at 25 ℃ on a Techcomp UV−2300 spectrophotometer. The samples of IscS (10 μmol/L) were prepared in 20 mmol/L phosphate buffer containing 0.5 mol/L NaCl, pH 7.4. L-Cysteine (1.0 mmol/L) and D-Cysteine (1.0 mmol/L) were added in two samples of wild-type IscS separately and incubated for 30 min. Blank sample was also performed on other identical solutions lacking cysteine.

2.6 Assay of *A. ferrooxidans* IscS activity

The activity of *A.ferrooxidans* IscS was determined by measuring the rate of production of sulfide from L-Cysteine incubated under anaerobic conditions in l mL of 50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L $MgCl_2$, 1.0 mmol/L L-Cysteine, 5 mmol/L dithiothreitol (DTT), and the sample containing the enzymatic activity. The incubations were carried out in autosampler vials sealed at the start of the incubation with Teflon-lined caps. Control assays were also performed on other identical solutions lacking cysteine.

Then we used the following method to determine the sulfide concentration. First, 100 μL of 30 mmol/L $FeCl_3$ in 1.2 mol/L HCl were added to the vial to stop the reaction. Second, 100 μL of 20 mmol/L *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 mol/L HCl was added to generate the methylene blue. After mixing, the test tube was incuated for 30 min at room temperature in the dark. Samples were centrifuged to remove solid sulfur, and spectrophotometric measurements of the methylene blue formed were done at 670 nm. A calibration curve had been made previously using Na_2S [11]. As this method does not take account of the loss of sulfide through reactions with sulfur, the activities determined are underestimated.

2.7 Enzyme kinetics

2.7.1 Effect of pH and temperature

The IscS activity reaction was taken in different pH values from 5.5 to 10.2. Appropriate buffers (0.2 mol/L acetic acid, pH 5; 0.2 mol/L phosphate, pH 5−7 and 0.05 mol/L Tris-HCl, pH 7−10) were used to determine the optimum pH. The optimum pH values obtained from this assay were used in all the other experiments.

The effect of temperature on IscS activity was measured within 10−50 ℃. We used temperature controlled water bath to reach these temperatures.

2.7.2 Thermal inactivation

Heat treatments of IscS wild type were carried out at 35, 45, 55, 65 and 75 ℃ for varied periods of time in a temperature controlled water bath; 0.5 mL of enzyme solution was placed in a pre-warmed tube at the specified temperature and 0.01 mL liquor was withdrawn at various time intervals, cooled and assayed for residual activity. The stability of the enzyme was expressed as percentage of residual activity and was calculated by comparison with untreated enzyme.

2.7.3 Effect of inhibitors

N-ethylmaleimide, 1,5-I-AEDANS, allylglycine, SDS and urea were evaluated for their effectiveness as inhibitors of IscS activity, using the sulfide production assay. The results were reported as percentage of L-cysteine inhibition.

2.7.4 Determination of K_m and v_{max}

IscS activity was assayed in buffers at optimum pH value and ambient temperature. The K_m value and maximum velocity v_{max} were determined by the Lineweaver-Burk plot[15].

2.8 Molecular structure modeling of IscS from *A. ferrooxidans* ATCC23270

A preliminary model for the structure of IscS was constructed using the approach of comparative protein modeling, applied by the Modeller module of Insight II software (Accelrys software Inc.) running on Dell Precision 470 workstation with Redhat Linux system. The predicted protein structure was carried out on the basis of the sequences of the IscS from *A. ferrooxidans* and *E. coli*, because their sequence identity is relatively high (66.2%). The template structure of the IscS from *E. coli* (PDB entry code 1P3W) refined to 2.3 Å was used for modeling. We used the GenDoc Software for sequence alignment of template and target sequences.

3 Results and discussion

3.1 Cloning of *A. ferrooxidans* ATCC 23270 IscS gene

PCR technique was used to successfully add six continuous histidine residues to the N-terminal of the IscS from *A. ferrooxidans* ATCC 23270, which greatly accelerated the protein purification process. The transformant was grown in LB medium and the corresponding plasmid was isolated and re-transformed to *E. coli* BL21(DE3) for expression.

3.2 Expression and purification of IscS from *A. ferrooxidans* ATCC 23270

Optimum expression of IscS protein from *A. ferrooxidans* ATCC 23270 in *E. coli* BL21(DE3) was conducted with 0.5 mmol/L IPTG at room temperature (25 ℃) rather than at 37 ℃. Induced cells showed a characteristic intense yellow color due to the presence of the pyridoxal 5′-phosphate (PLP) cofactor in the overproduced cysteine desulfurase.

Nickel metal-affinity resin column was used for single-step purification of His-tagged IscS protein. The wild-type IscS protein also had an intense yellow color, due to the presence of the PLP cofactor in the purified

protein. The yellow color was also reported in other IscSs from *E. coli*, *B.pertussis*, and *Geobacillus stearothermophilus* V[3, 16−17].

The final protein yield after affinity chromatography was 12.9%, which suggests that the T7 polymerase promoter/BL21(DE3) expression system is an ideal system for expressing the IscS in high yield. The purified protein were dialyzed overnight against a potassium phosphate buffer, pH 7.4, 5% glycerol as soon as possible after the purification. The addition of 5% glycerol was essential for maintaining the long-term stability of the protein. The purities of the purified IscS was further examined by SDS-PAGE and single band corresponding to the 46 ku protein was observed with ＞ 95% purity (Fig.1). The wild-type protein could be stored at 4 ℃ for 2 months without significant change of activity.

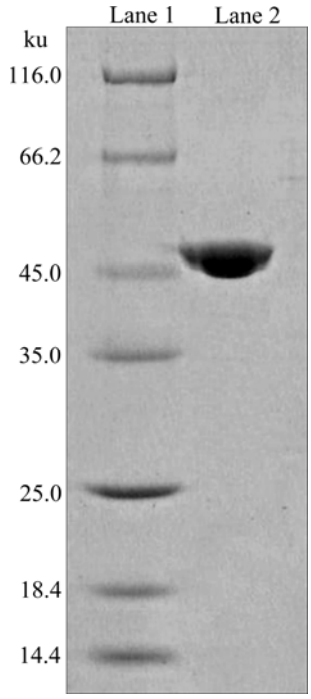

Fig.1 Coomassie blue-stained SDS-PAGE of purified IscS from *A. ferrooxidans* ATCC23270: Lane 1—Molecular mass standards; Lane 2—Purified IscS

The protein purification is shown in Table 1. The final yield from the purification, based on the desulfurization of L-cysteine to give hydrogen sulfide, was 39%, and the total activity of purified IscS was 1 475.4 nm of hydrogen sulfide produced per minute, and the specific activity was 148 nmol of hydrogen sulfide produced per minute per milligram of protein (Table 1).

3.3 UV-visible light scanning of IscS

The UV-visible spectrum of the recombinant IscS is shown in Fig.2. The wild-type IscS belonged to a family of PLP-dependent enzymes and exhibited the UV-visible spectrum characteristic of other cysteine desulfrases, with an absorbance maximum for PLP centered at (420±1) nm. The spectra were similar to those previously reported for the IscS and NifS from various sources.

Table 1 Purification summary of IscS from *A. ferrooxidans* ATCC 23270

Purification step	Total protein/ mg	Yield/ %	Total activity/ (nmol·min^{-1})	Specific activity/ (nmol·min^{-1}·mg^{-1})
Crude extract	154.1	100.0	4 317.2	22.4
Affinity chromatography	19.9	12.9	1 475.4	148.0

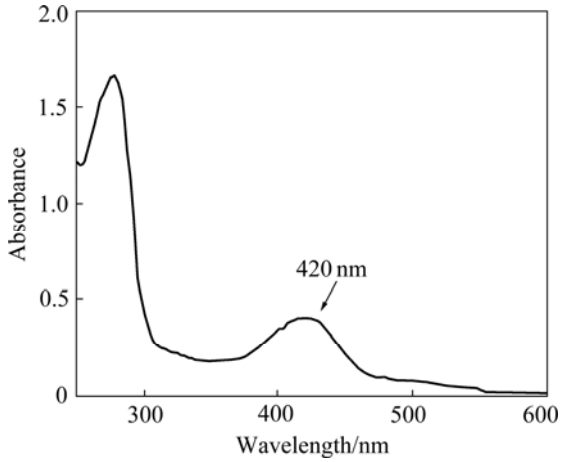

Fig.2 UV-visible absorbance spectrum of purified IscS from *A. ferrooxidans* ATCC23270 (Characteristic absorption peak of PLP is indicated by arrowhead)

The results of incubation of L-cysteine and D-cysteine with IscS separately for 10 min are very distinct. The optical spectrum of IscS exhibited a decrease in absorbance at (416±1) nm which was incubated in the presence of L-cysteine. However, there was no obvious change on the spectrum in the addition of D-cysteine. The results suggested that the L-cysteine was the only substrate for this enzyme and the PLP participated in the cysteine desulfuration reaction. Our IscS activity assay in which the substrate L-cystein was replaced by D-cysteine has the identic results (data not show). All the results were also in agreement with the previous reports[3, 17−18].

3.4 Enzyme kinetics

3.4.1 Effect of pH and temperature

The pH optimum for enzyme-catalyzed desulfuration of L-cysteine in various buffers was found to occur at pH 8.0−8.5. At pH above 10.0 or below 5.0, the activity decreased very rapidly. This value was close to the optimal pH of IscS from *Geobacillus*

stearothermophilus V[11]. The effect of pH on cysteine dusulfurase activity is shown in Fig.3. The rapid inactivation of the enzyme above pH 8.0 or below 5.0 may be due to conformational changes in the enzyme under the acidic or alkaline conditions.

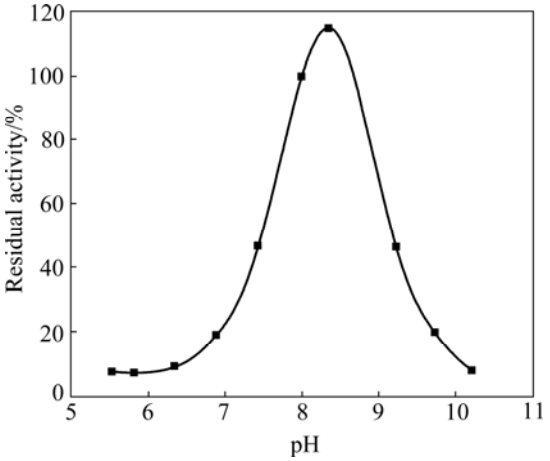

Fig.3 Effect of pH on IscS activity

The activity of IscS was measured at different temperatures at optimum pH for 2 h. The enzyme showed the highest activity at 30 ℃. The optimal temperature was different from the *Geobacillus stearothermophilus* V IscS[18]. Nevertheless, the optimum temperature of 30 ℃ was the same as the optimum growth temperature of *Acidithibacillus ferrooxidans*. The effect of temperature on cysteine dusulfurase activity is shown in Fig.4.

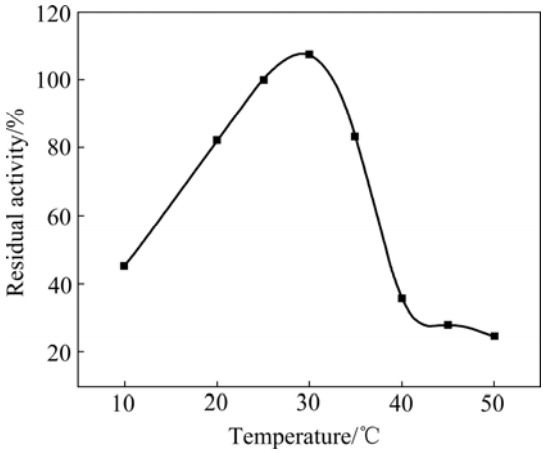

Fig.4 Effect of temperature on IscS activity

3.4.2 Thermal inactivation kinetics

The enzyme was incubated at different temperatures (35−65 ℃) for 2 h and, after cooling, the residual enzyme activity was measured (Fig.5). The residual activity of the enzyme exceeded 95% when it was incubated at 35 ℃ for 2 h. However, the residual activity of the enzyme was only 33% when it was incubated at 40 ℃ for 2 h. The deactivation was faster when incubated at 55 ℃; no activity occurred virtually after 30 min. Consequently, enzyme was stable at 35 ℃ but unstable at temperatures above 55 ℃. The decrease in percentage of residual activity at higher temperatures is due to the unfolding of the tertiary structure.

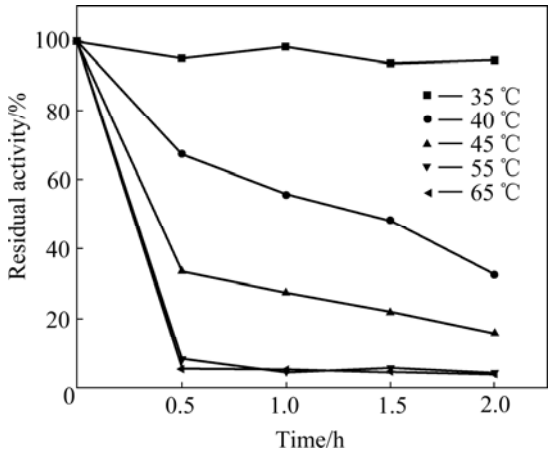

Fig.5 Thermal stability of IscS at varied temperatures (35−65 ℃) versus time

3.4.3 Effect of inhibitors

The effects of various inhibitors on the purified IscS are shown in Table 2. The results were reported as percentage of L-cysteine inhibition. It was markedly inhibited by 1,5-I-AEDANS. *N*-ethylmaleimide, allylglycine and urea showed minimum inhibition. *N*-ethylmaleimide was reported to be a strong inhibitor of NIFS[11]. It was also reported that L-lylglycine was a membrane permeable suicide inhibitor on IscS, which forms covalent adduct with the active site cysteine of IscS in *E. coli*, resulting in enzyme inactivation[12].

Table 2 Effect of inhibitors on IscS activity

Inhibitor	Percentage of inhibition/%	
	[Inhibitor]/[IscS]=1	[Inhibitor]/[IscS]=10
SDS	47.1	97.8
Urea	1.4	3.0
Allylglycine	4.2	2.9
N-ethylmaleimide	18.9	33.8
1,5-I-AEDANS	68.5	–

If the IscS was incubated under anaerobic conditions in the presence of 5 mmol/L dithiothreitol, and then the the reduced enzyme was incubated with equal amounts of 1,5-I-AEDANS, the inhibition of IscS was up to 90%. However, the inhibition of the other inhibitors had no obvious change after the same operation. The reciprocal of the amount of 1,5-I-AEDANS required to inactivate 1 mol IscS was measured. Results of typical experiment are presented in Fig.6. The result of a tryptic digest prepared from the

inactivated protein indicated that the cysteine (Cys 331) was modified by 1,5-I-AEDANS[3,19]. Clearly, the cysteine residue in IscS participates in the desulfurization reaction in the presence of L-cysteine.

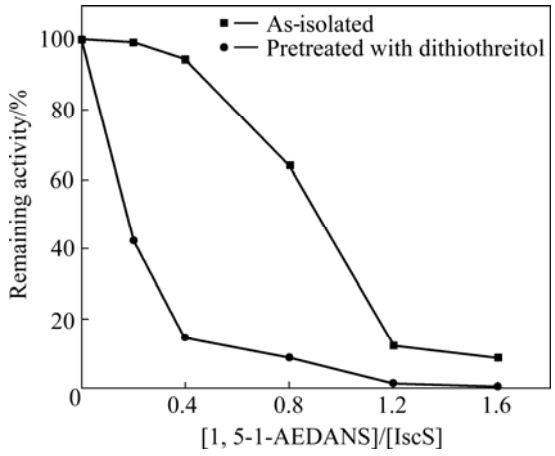

Fig.6 Effect of 1,5-I-AEDANS on IscS activity with and without prior incubation with dithiothreitol (Protein was assayed for activity after treatment with 1,5-I-AEDANS using sulfide production assay)

3.4.4 K_m and v_{max} of IscS from *A.ferrooxidans* ATCC 23270

K_m and v_{max} values of cysteine desulfurase IscS were calculated from the Lineweaver–Burk graphs and the results are shown in Table 3 and Fig.7. The K_m for IscS from *A. ferrooxidans* ATCC 23270 was 0.11 mmol/L and v_{max} was 2.57 μmol/(L·min). The result shows that the substrate-binding site of IscS has a high affinity for L-cysteine.

Table 3 Detection results for K_m and V_{max} of cysteine desulfurase IscS

Serial number	[L-cysteine]/ (mmol·L^{-1})	[L-cysteine]$^{-1}$/ (L·mmol^{-1})	v^{-1}/ (min·mmol^{-1}·L)
0	0	–	–
1	0.01	100	4 439.6
2	0.02	50	2 472.5
3	0.03	33.3	1 800.2
4	0.04	25	1 429.2
5	0.05	20	1 189.7

3.5 Molecular modeling of IscS from *A. ferrooxidans*

Among all the available molecular structures of IscS, the IscS from *E. coli* was identified as the best structural template for modeling the molecular structure of IscS from *A. ferrooxidans* ATCC 23270. This model was refined by MM optimization and MD simulation.

The modeled overall structure of IscS from *A. ferrooxidans* ATCC 23270 was shown to be a monomer

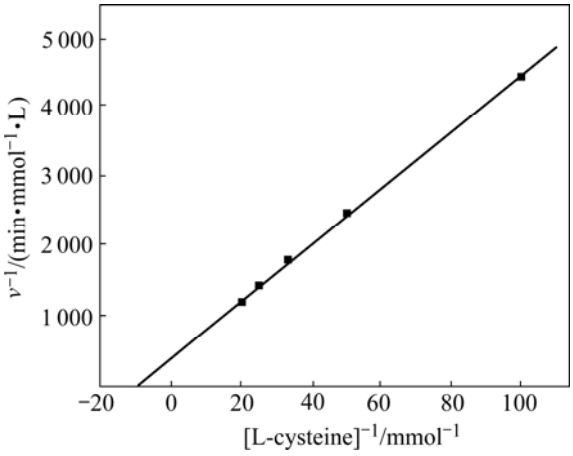

Fig.7 K_m and v_{max} of cysteine desulfurase IscS (Lineweaver–Burk)

(Fig.8). Sequence alignment for IscS from various sources showed that the five residues His106, Lys208 and Cys331 were highly conserved residues(Fig.9). It is reported that the lysinyl (Lys208 in *A. ferrooxidans* ATCC 23270) and cysteinyl (Cys331 in *A. ferrooxidans* ATCC 23270) residues are considered to be the probable PLP-binding and active-site cysteinyl residues [11,17−18]. Our molecular modeling of IscS from *A.ferrooxidans* also supports these points: the PLP group interacted with Lys208 by forming an internal aldimine Schiff base; the imidazole ring of His106 was positioned directly above the pyridine ring of PLP, which was likely to function as an acid-base catalyst in several protonation/ deprotonation steps during catalysis. So His106 was also significant for the catalytic reaction. Therefore, we will choose these residues for site-directed mutagenesis to study their functions for the catalytic reaction in the next investigation.

4 Summary

We report here the first overexpression of *E.coli* of His-tagged IscS protein from *A. ferrooxidans* ATCC23270. There are partial characteristics of IscS: the optimum reaction temperature of cysteine desulfurase was 30 ℃, the optimum reaction pH was 8.0−8.5. The enzyme was stable at 35 ℃ but unstable at temperatures above 55 ℃. The inhibiting effect of inhibitors was 1,5-I-AEDANS ＞ SDS ＞ *N*-ethylmaleimide ＞ allylglycine＞urea. The K_m for IscS from *A. ferrooxidans* ATCC 23270 was 0.11 mmol/L and v_{max} was 2.57 μmol/(L·min).

Abbreviations

Abbreviations used are as follows: *A. ferrooxidans*, *Acidithiobacillus ferrooxidans*; Amino acids; C, Cys, cysteine; H, His, histidine; K, Lys, lysine; PLP, pyridoxal

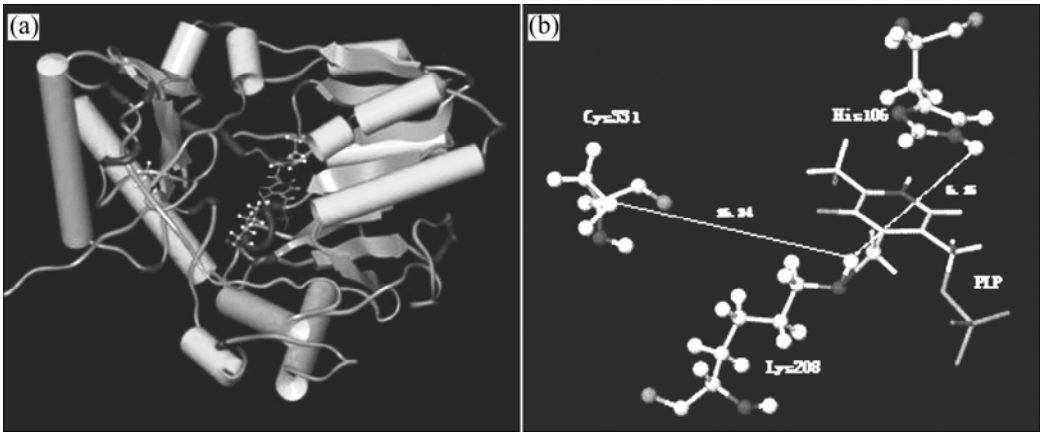

Fig.8 Overall structure of IscS from *A. ferrooxidans* ATCC 23270 (a) and modeled IscS structure (b) showing active site

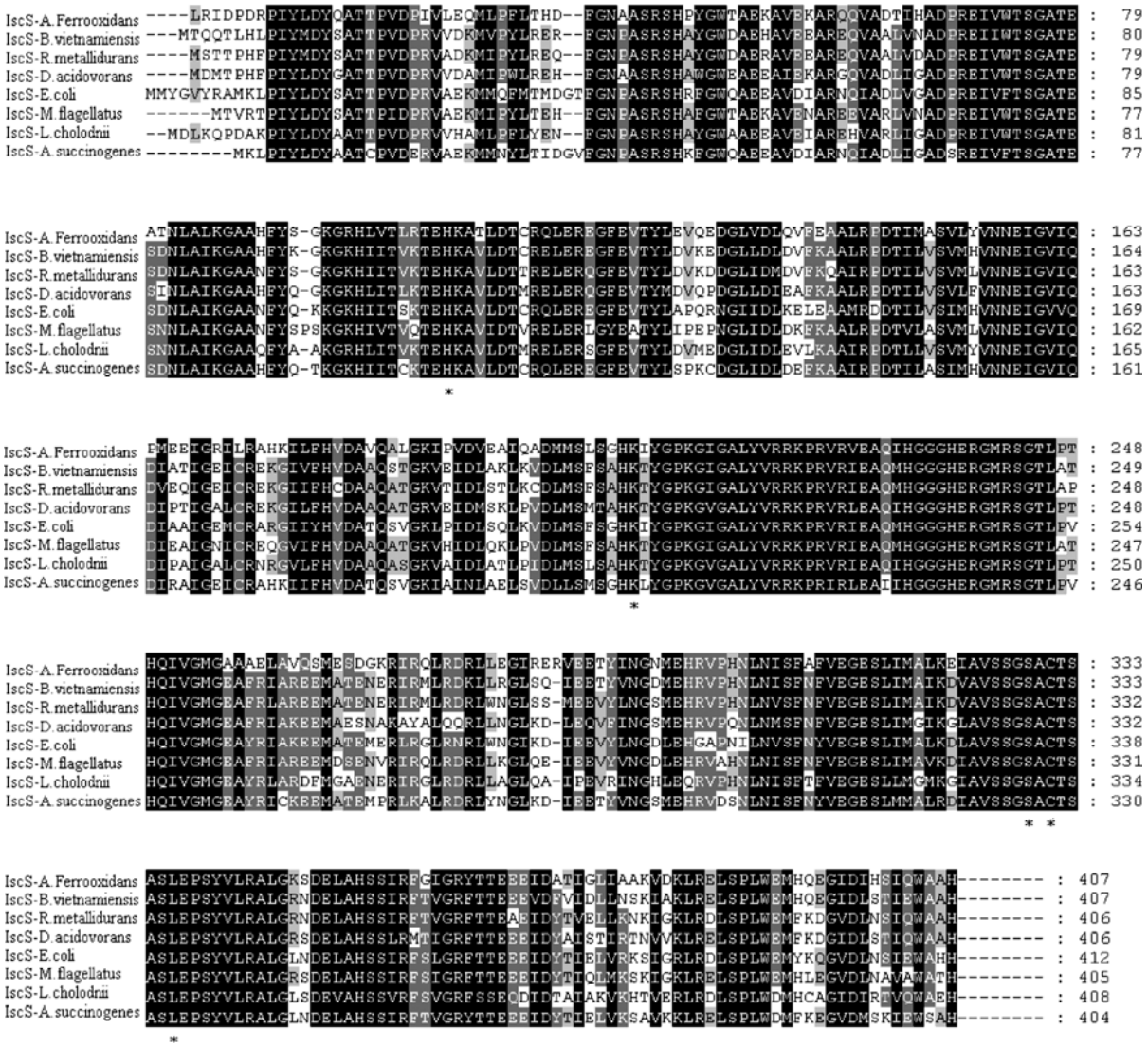

Fig.9 Sequences alignment of IscS from *A.ferrooxidans* and other sources (Sequence alignment was made by GenDoc software): *A.ferrooxidans*—IscS from *Acidithiobacillus ferrooxidans* ATCC 23270; *B.vietnamiensis*—IscS from *Burkholderia vietnamiensis* G4; *R.metallidurans*—IscS from *Ralstonia metallidurans* CH34; *D.acidovorans*—IscS from *Delftia acidovorans* SPH-1; *E.coli*—IscS from *Escherichia coli*; *M.flagellatus*—IscS from *Methylobacillus flagellatus* KT; *L.cholodnii*—IscS from *Leptothrix cholodnii* SP-6; *A.succinogenes*—IscS from *Actinobacillus succinogenes* 130 Z. Some important residues which are conserved are marked with *.

5′-phosphate-dependent; 1,5-I-AEDANS, *N*-iodoacety-*N*′-(5-sulfo-1-naphthay)ethylenedialmine; IPTG, isopropyl-D-thiogalactopyranoside; DTT, dithiothreitol; IscA, iron-sulfur cluster assembly protein A; IscS, iron-sulfur cluster assembly protein S; IscU, iron-sulfur cluster assembly protein U; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; UV/Vis, ultraviolet-visible spectroscopy.

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