Expression, Purification, and Characterization of a [Fe₂S₂] Cluster Containing Ferredoxin from *Acidithiobacillus ferrooxidans*

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Abstract The [2Fe-2S] cluster containing ferredoxin has attracted much attention in recent years. Genetic analyses show that it has an essential role in the maturation of various iron–sulfur (Fe-S) proteins and functions as a component of the complex machinery responsible for the biogenesis of Fe-S clusters. The gene of ferredoxin from *A. ferrooxidans* ATCC 23270 was cloned, successfully expressed in *Escherichia coli*, and purified by one-step affinity chromatography to homogeneity. The MALDI-TOF MS and spectra results of the recombinant protein confirmed that the iron–sulfur cluster was correctly inserted into the active site of the protein. Site-directed mutagenesis results revealed that Cys42, Cys48, Cys51, and Cys87 were ligating with the [Fe₂S₂] cluster of the protein.

Introduction

Ferredoxins are small, acidic, electron transfer proteins that are ubiquitous in biological redox systems. They have either a [4Fe-4S], [3Fe-4S], or [2Fe-2S] cluster, whose reduction potential is highly negative (–300 mV or less) [1, 2]. Among them, ferredoxins with one [2Fe-2S] cluster per molecule are present in plants, animals, and bacteria, and they form a distinct 2Fe-2S ferredoxin family [3, 4].

The [2Fe-2S] cluster containing ferredoxin from *Escherichia coli* has attracted much attention in recent years. Genetic studies have shown that they have a crucial role in the biogenesis of Fe-S clusters. The gene encoding bacterial ferredoxin is located in the *isc* (iron–sulfur cluster) operon, in which eight genes are clustered [5, 6]. The *fdx* gene, together with *iscS*, *iscU*, *iscA*, *hscB*, and *hscA* in the operon, is crucial for the efficient biosynthesis of Fe-S clusters in *E. coli*. Overexpression of the entire operon improves the cellular ability for Fe-S cluster biosynthesis, resulting in a significant increase in the yield of recombinant Fe-S proteins [7, 8]. The biosynthesis of the Fe-S cluster therefore is a complex process that involves numerous components, including the ferredoxin. Similar machinery has been identified in yeast mitochondria [9].

Acidithiobacillus ferrooxidans is one of the most studied bacteria that thrives in acidic mine drainage, which obtains energy through the oxidation of the ferrous ion to the ferric ion, with molecular oxygen as the terminal electron acceptor [10]. There are some iron-sulfur proteins such as HiPIP or Iro protein which may be involved in the electron transfer, but the mechanisms for the iron-sulfur cluster assembly of these proteins are not clear so far.

In this study, the gene of ferredoxin from *A. ferrooxidans* ATCC 23270 was cloned, successfully expressed in *E. coli*, and purified by one-step affinity chromatography to homogeneity. As far as we know, this is the first report of the expression in *E. coli* of ferredoxin from *A. ferrooxidans*.

Materials and Methods

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. HB101 competent cells and *E. coli* strain

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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. *Taq* DNA polymerase, T4 DNA ligase, and restriction enzymes came from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources.

Cloning of the Ferredoxin Gene from *A. ferrooxidans* ATCC 23270

Genomic DNA from *A. ferrooxidans* ATCC 23270 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 polymerase chain reaction (PCR) 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′-CGCGCGAATTCAGGAGGAATTTAAAAT GAGAGGATCGCATCACCATCACCATCACACAAAA ATACGCGTCCTGCCGAACCCGGAAG-3′, containing a *Eco*RI site (GAATTC), a ribosome binding site (AGGA GGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2–11 of mature ferredoxin. The sequence of the reverse primer was 5′-CTGCAGGTCGACTTAG TGCCGCTCCTTTTCCAGATTGATGGTGTAT-3′, containing a *Sal*I site (GTCGAC), a stop anticodon (TTA), and anticodons for the last 10 amino acids of mature ferredoxin. PCR amplification was performed using *Taq* DNA polymerase, and samples were subjected to 25 cycles of 45 s of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of elongation at 72°C in a Mastercycler Personal of Eppendorf Model made in Germany. 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 [11], resulting in the pLM1::FDX plasmid. The isolated pLM1::FDX plasmid was then transformed into *E. coli* strain BL21(DE3) competent cells for expression purposes.

Construction of *A. ferrooxidans* Ferredoxin Mutant Plasmids

A QuikChange mutagenesis kit (Stratagene) was applied for constructing the pFDX (C42A), pFDX (C48A) pFDX (C51A), and pFDX (C87A) mutant expression plasmids. The plasmid pLM1::FDX was used as a template for constructing mutant expression plasmids through PCR. The following primer and their antisense primer were synthesized to introduce the mutated sequence:

C42A: 5′ ACATCGAGCATGCCGCCGAGATGTCCT GCG 3′, codon TGC for cysteine (C) was changed to codon GCC for alanine (A)

C48A: 5′ AGATGTCCTGCGCCGCCACTACCTGTCA TG 3′, codon TGC for cysteine (C) was changed to codon GCC for alanine (A)

C51A: 5′ GCGCCTGCACTACCGCTCATGTGATTCT GC 3′, codon TGT for cysteine (C) was changed to codon GCT for alanine (A)

C87A: 5′ CCTCCCGCCTCAGTGCCCAGGCGAAAGT GA 3′, codon TGC for cysteine (C) was changed to codon GCC for alanine (A)

Polymerase chain reaction amplification was performed using *Pfu* DNA polymerase and samples were subjected to 13 cycles of 0.5 min of denaturation at 95°C, 1 min of annealing at 61°C, and 12 mins of elongation at 72°C in a Mastercycler Personal of Eppendorf Model made in Germany. *Dpn*I restriction enzyme was used to digest the parental supercoiled double-stranded DNA. The constructed mutant plasmids were transformed into HB101 competent cells for screening purposes. The positive colonies with the mutant plasmids were identified by sequence analysis. The isolated mutant plasmids were transformed into *E. coli* strain BL21(DE3) competent cells for expression.

Expression of Recombinant Ferredoxin Wild Type and Its Mutant Proteins in *E. coli*

The *E. coli* strain BL21(DE3) cells with pLM1::FDX plasmid or mutant plasmids were grown at 37°C in 500 mL of terrific broth (TB) 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 mM isopropyl-D-thiogalactopyranoside (IPTG) overnight with shaking at 180 rpm. 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 mM potassium phosphate, pH 7.4, 0.5M NaCl), incubated with 5 mg lysozyme at room temperature for half an hour, and then stored at –80°C for purification.

Purification of Ferredoxin Wild Type and Its Mutant Proteins

The cells were lysed by sonication four times for 30 s each time using a 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. The Hi-Trap column was first equilibrated with 0.1M 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 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 by 5 column volumes of wash buffer (20 mM potassium phosphate, pH 7.4, 0.5M NaCl, 50 mM imidazole), and, subsequently, the protein was eluted with an elution buffer (20 mM potassium phosphate, pH 7.4, 0.5M NaCl, 500 mM imidazole). The Bradford's method was used to determine the protein content, with bovine serum albumin as the standard [12]. The eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 18% of acrylamide according to Laemmli [13]. The gels were stained with Coomassie brilliant blue R-250. The purified proteins were then dialyzed against a 20 mM potassium phosphate buffer, pH 7.4, 5% glycerol and then stored in a –80°C freezer.

MALDI-TOF MS of the Ferredoxin

The molecular mass of the purified ferredoxin was determined using an Ultraflex^{TM} TOF/TOF spectrometer (Bruker Daltonics) equipped with nitrogen laser (337 nm) and operated in reflector/delay extraction mode. An accelerating voltage of 25 kV was used for this study. The matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) result was obtained in the linear positive mode using α-cyano-4-hydroxy-cinnamic acid (saturated solution in 50% acetonitrile with 0.1% trifluoroacetic acid) as the ultraviolet (UV)-absorbing matrix. Prior to MALDI-MS analysis, the protein samples were diluted to a concentration of ∼1 μM in 20 mM potassium phosphate buffer containing 0.5M NaCl, pH 7.4. Theoretical molecular mass was calculated by the Compute pI/Mw tool from Expasy Proteomics Server of the Swiss Institute of Bioinformatics (SIB).

UV-Vis Scanning and EPR Spectra

Ultraviolet–visible spectra scanning was carried out at 25°C on a Techcomp UV-2300 spectrophotometer. The samples of wild-type ferredoxin or the mutant proteins (10 μM) were prepared in 20 mM phosphate buffer containing 0.5M NaCl, pH 7.4. X-band electronic paramagnetic resonance (EPR) spectra were recorded at 100 K on a JEOL

JES-FE1XG spectrometer. Parameters for recording the EPR spectra were typically 15–30 mT/min sweep rate, 0.63 mT modulation amplitude, 9.153 GHz frequency, and 4 mW incident microwave power; the sweep time was 2 min. The samples were diluted to 5 μM in 20 mM phosphate buffer containing 0.5M NaCl, pH 7.4.

Results and Discussion

Cloning of the Ferredoxin Gene and Construction of the Mutant Plasmids

The PCR technique was used to successfully add six continuous histidine residues to the N-terminal of the ferredoxin from *A. ferrooxidans* ATCC 23270, which greatly accelerated the protein purification process. Mutant expression plasmids of pFDX (C42A), pFDX (C48A), pFDX (C51A), and pFDX (C87A) were constructed, and its sequences were verified for the presence of the directed mutation and the absence of PCR-generated random mutations by DNA sequencing. The plasmids were then transformed into *E. coli* BL21(DE3) for expression.

Expression and Purification of Ferredoxin Wild Type and It Mutant Proteins

A nickel metal-affinity resin column was used for single-step purification of His-tagged ferredoxin wild type and its mutant proteins. The wild-type ferredoxin was observed to be a red-brown protein, indicating that the [Fe$_2$S$_2$] cluster is still bound to the protein after purification. The ferredoxin was observed in reduced state after purification according to UV-Vis scanning and EPR spectra. The final protein yield after affinity chromatography is 7.3%, the result suggests that the T7 polymerase promoter/BL21(DE3) expression system is an ideal system for expressing the ferredoxin in high yield; the leader peptide and hexahistag in the N-terminal might be responsible for the efficient expression of the ferredoxin. The ferredoxin mutant proteins were also purified following the same method as for the wild-type protein and were obtained as soluble proteins. The purified proteins were dialyzed against a 20 mM potassium phosphate buffer, pH 7.4, 5% glycerol as soon as possible after the purification.

The purities of the purified ferredoxin and its mutant proteins were further examined by SDS-PAGE and single bands corresponding to the 13- kDa protein were observed with >95% purity (Fig. 1). The stability of the wild-type ferredoxin was tested on the basis of its [Fe$_2$S$_2$] cluster stabilization and the His-tagged protein proved to be highly stable. The wild-type protein could be stored at 4°C for 1
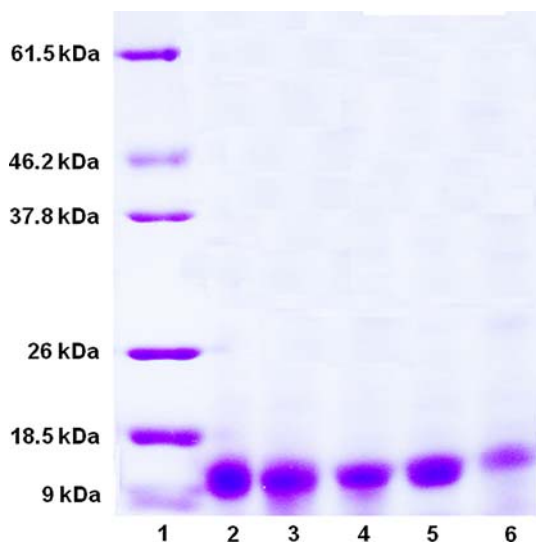
Fig. 1 Coomassie blue-stained SDS-PAGE of the purified ferredoxin from *A. ferrooxidans* ATCC 23270 and its mutant proteins. Lane 1, molecular mass standards; lane 2, purified ferredoxin wild type; lane 3, purified ferredoxin C42A mutant; lane 4, purified ferredoxin C48A mutant; lane 5, purified ferredoxin C51A mutant; lane 6, purified ferredoxin C87A mutant

month without significant change of activity. The recombinant ferredoxins from *E. coli* and humans have previously been produced in *E. coli*, all of the recombinant proteins were purified to holoproteins containing the $[Fe_2S_2]$ cluster [6, 14, 15].

MALDI-TOF MS of the Ferredoxin

The MALDI-TOF MS result of the recombinant ferredoxin is shown in Fig. 2. The molecular mass obtained by MALDI-TOF MS for the ferredoxin was 13,623.97 Da.
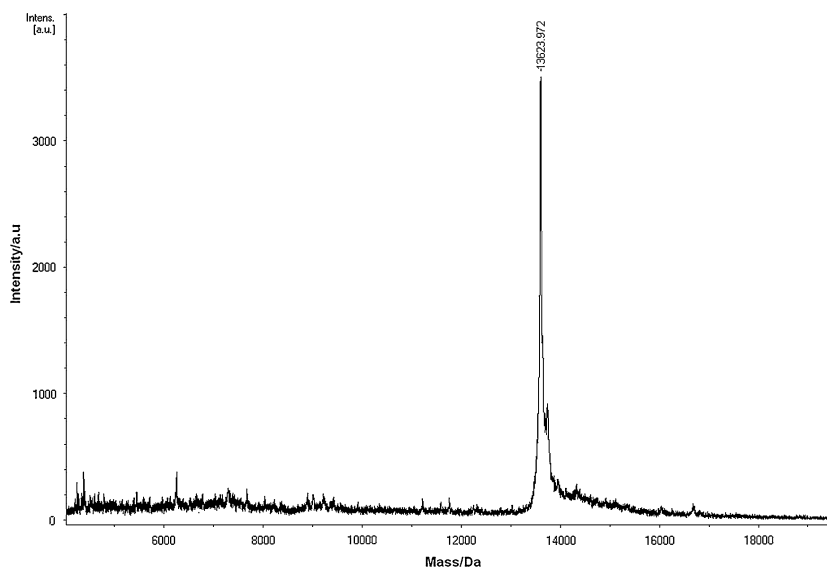
The protein sequence of the ferredoxin with the His-tags in the N-terminal plus the $[Fe_2S_2]$ cluster has a theoretical average molecular mass of 13,625.86 Da, which was in agreement with the experimental result. The results further confirmed that the recombinant ferredoxin was correctly folded and the $[Fe_2S_2]$ cluster was also correctly inserted into the active site of the protein when expressed in *E. coli*.
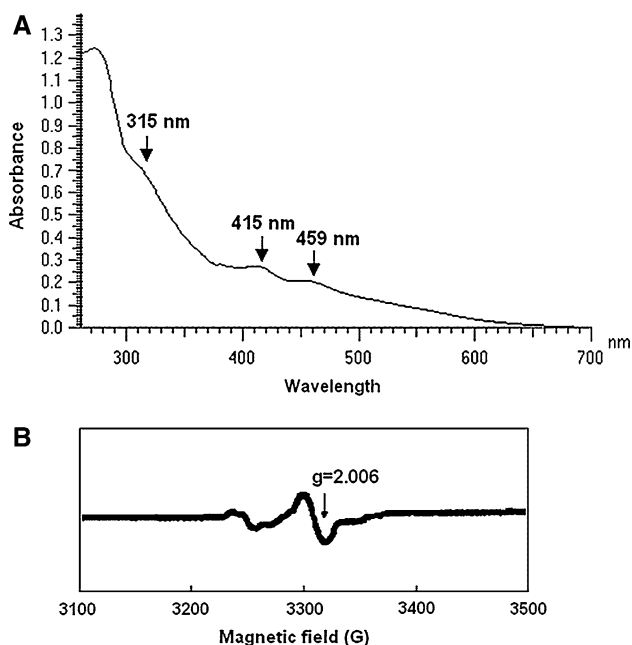
UV Scanning and EPR Spectra of the Ferredoxin

The UV-visible spectra of the recombinant ferredoxin is shown in Fig. 3A. The spectra were similar to those previously reported for the ferredoxins from various sources [6, 15]. There are three major absorption peaks, corresponding to 315 nm, 415 nm, and 459 nm, respectively, which is typical for proteins containing the $[Fe_2S_2]$ cluster.

The EPR spectra of the recombinant ferredoxin are shown in Fig. 3B. The recombinant ferredoxin exhibited a typical $S = 1/2$ EPR signal, indicating the presence of the $[Fe_2S_2]^{1+}$ cluster. The ferredoxin from various sources also showed similar EPR signals [6, 14, 15].

Role of Conserved Cysteines of Ferredoxin in Iron–Sulfur Cluster Binding

Sequence alignment for ferredoxins from various sources showed that the four cysteines Cys42, Cys48, Cys51, and Cys87 were highly conserved residues (Fig. 4); it was reported that the conserved cysteines of the ferredoxin family might be ligating with the $[Fe_2S_2]$ cluster [16]. To confirm this, mutations for these four cysteines of ferredoxin from *A. ferrooxidans* were created, and C42A, C48A, C51A, and C87A mutant plasmids were expressed

Fig. 2 MALDI-TOF MS of the ferredoxin from *A. ferrooxidans* ATCC 23270

absence of the $[Fe_2S_2]$ cluster in the mutant proteins. EPR results also confirmed that the $[Fe_2S_2]$ clusters were not incorporated in the mutant proteins, as shown in Fig. 5B. The results strongly suggested that the four conserved cyceines Cys42, Cys48, Cys51, and Cys87 were crucial residues for iron–sulfur cluster binding, and removal of the sulfhydryl group of the cysteines resulted in $[Fe_2S_2]$ cluster loss.

In ferredoxins, the iron–sulfur clusters are bound to the protein by covalent bonds between iron atoms and the sulfur atoms of the thiolate side chains of the four cysteine residues. Noncysteinyl ligands have been reported in other classes of iron–sulfur proteins. The $[Fe_2S_2]$ cluster in Rieske-type proteins is coordinated by the sulfur atoms from each of two cysteines and by one of the imidazolering nitrogen atoms from each of two histidines [17, 18], whereas for aconitase the $[Fe_4S_4]$ cluster is coordinated by one carboxyl oxygen, one hydroxyl oxygen from the substrate, and one oxygen from a water molecule [19, 20].

In summary, we report here the first expression in *E. coli* of His-tagged ferredoxin from *A. ferrooxidans* ATCC 23270. All of the probed properties of the recombinant protein support that the $[Fe_2S_2]$ cluster was correctly incorporated in the active site of the protein. Site-directed mutagenesis results revealed that Cys42, Cys48, Cys51, and Cys87 were ligating with the iron–sulfur cluster.

Fig. 3 (**A**) UV-Vis scanning of the recombinant ferredoxin from *A. ferrooxidans* ATCC 23270. (**B**) EPR spectra of the recombinant ferredoxin from *A. ferrooxidans* ATCC 23270

in *E. coli* and then purified by affinity chromatography to homogeneity (Fig. 1). To our surprise, all four mutant proteins had no color after purification, and UV-Vis scanning of the mutant proteins showed that there were no absorptions between 300 and 500nm compared to that of wild-type ferredoxin (shown in Fig. 5A), indicating the


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fdx_A.ferreoxidans    MTKIRVLPNPEVCPEGAEFDAEPGETIIAAALRNDIHIEHACEMSCACTTCHVILRAGFD 60
fdx_E.coli            MPKIVILPHQELCPDGAVLEANSGETILDAALRNGIEIEHACEKSCACTTCHCIVREGFD 60
fdx_A.hydrophila      MPKLIILPHPELCPDGAALEGATGETILDIALRNGIEIEHACEKSCACTTCHCVVREGFD 60
fdx_S.baltica         MPQLVFLPHAELCPDGAVLEANVGETILDVALRNGINIEHACEKSCACTTCHCIVREGFN 60
fdx_P.aeruginosa      MPQIVILPHADHCPEGAVFEAKPGETILDAALRNGIEIEHACEKSCACTTCHVIVREGLD 60
fdx_T.denitrificans   MPQLIVLPHVELCPEGIVIEAKSGETICDTLLANGVEIEHACEKSCACTTCHVVVREGFN 60
fdx_M.bacterium       MPKIIILPHHDLCPEGDIIEAKKGTSVCDALLSNNIHIEHACDQVCACTTCHVIIREGLQ 60
fdx_P.cryohalolentis  MPKITVLPHHEICPKGIEVELAAGENLCKALLEKGIKIEHACEMSKACTTCHVVVRKGFN 60

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fdx_A.ferreoxidans    SLVPAEEKEEDLLDKAWGLEPTSRLSCQAKVTNTQLVIEIPKYTINLEKERH 112
fdx_E.coli            SLPESSEQEDDMLDKAWGLEPESRLSCQARVTDEDLVVEIPRYTINHAREH- 111
fdx_A.hydrophila      SLEPSDELEDDMLDKAWGLEPESRLSCQARLADEDLVVELPKYTLNLASERH 112
fdx_S.baltica         DLEPSDELEDDMLDKAWGLEPESRLSCQAKVVDTDMVIEIPKYTVNMVSEGN 112
fdx_P.aeruginosa      SMEPSDELEDDMLDKAWGLEPDSRLSCQAVVADEDLVVEIPKYTINQVSEGH 112
fdx_T.denitrificans   SLAPSSEDEDDLLDKAWGLEPQSRLSCQARIANEDLVIEIPKYTINMVKEGH 112
fdx_M.bacterium       SLNPSDENEDDMLDKAWGLSPQSRLSCQAIVSDHDLTVEIPKYTINLAKEG- 111
fdx_P.cryohalolentis  SLDEMDDIEADLLDRAWGLEPDSRLSCQVMLEDDDLTIEIPKYTLNHAKENH 112

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Fig. 4 The sequence alignment of ferredoxin from *A. ferrooxidans* and other sources. A. ferreoxidans: ferredoxin from *A. ferrooxidans* ATCC 23270; E. coli: ferredoxin from *Escherichia coli*; A. hydrophila: ferredoxin from *Aeromonas hydrophila* ATCC 7966; S. baltica: ferredoxin from *Shewanella baltica* OS155; P. aeruginosa: ferredoxin from *Pseudomonas aeruginosa* PAO1; T. denitrificans: ferredoxin from *Thiobacillus denitrificans* ATCC 25259; M. bacterium: ferredoxin from *Methylophilales bacterium* HTCC2181; P. cryohalolentis: ferredoxin from *Psychrobacter cryohalolentis* K5. Residues conserved in all sequences are marked with an asterisk. Residues not conserved in all sequences but conserved in some sequences are marked with colon or period based on the degree of conservation. The highly conserved cysteine residues are marked in red
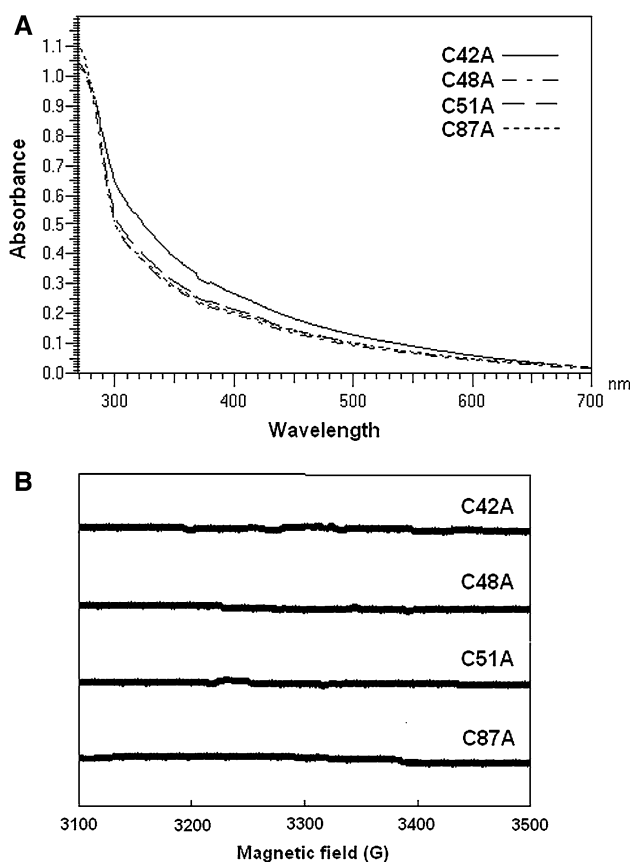
Fig. 5 **(A)** UV-Vis scanning of ferredoxin C42A, C48A, C51A, and C87A mutant proteins. **(B)** EPR spectra of ferredoxin C42A, C48A, C51A, and C87A mutant proteins

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