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Araştırma Makalesi / Research Article

Cloning, Overexpression and Characterization of the FeSI Protein from *Azotobacter vinelandii* CA6

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Iron-sulfur protein; Nitrogen fixation; Nitrogenase; *Azotobacter vinelandii* CA6

Abstract

The [2Fe-2S] protein from *Azotobacter vinelandii* CA6, also known as Shethna protein I or FeSI protein, was cloned and overexpressed in *E. coli* and purified. SDS-PAGE analysis showed a band at ~11 kDa, the monomeric size of the protein, at each stage of the purification. Gel filtration profile of FeSI indicates it forms a dimer in its native state. The UV-visible spectrum showed absorbances at signature wavelengths, 344, 418 and 464 nm, due to the iron-sulfur cluster. The sequence of *A. vinelandii* CA6 FeSI protein are similar to the sequences of [2Fe-2S] ferredoxins from nitrogen-fixing *Clostridium pasteurianum* and *Aquifex aeolicus*, which is not a nitrogen fixer, including conserved cysteine residues. These suggest that FeSI may or may not be involved in nitrogen fixation as there is no evidence although the FeSI gene is present in the major *nif* gene cluster in *Azotobacter vinelandii* CA6. This study will be beneficial for understanding the function of FeSI in nitrogen fixation and the relations with other [2Fe-2S] proteins.

Azotobacter vinelandii CA6 FeSI Proteininin Klonlanması, İfadesi ve Karakterizasyonu

Anahtar kelimeler
Demir-kükürt proteini; Azot fiksasyonu; Nitrojenaz; *Azotobacter vinelandii* CA6

Öz

Bu çalışmada, *Azotobacter vinelandii* CA6'dan Shethna protein I veya FeSI proteini olarak da bilinen [2Fe-2S] proteini izole edilmiş, *E. coli*'de aşırı ifade edilmiş ve saflaştırılmıştır. SDS-PAGE analizi, saflaştırmanın her aşamasında proteinin monomer boyutu olan ~11 kDa'da bir bant göstermiştir. FeSI jel filtrasyon profili, doğal halde dimer olduğunu işaret etmektedir. UV-görünür spektrumunda, demir-kükürt kümesine özgü 344, 418 ve 464 nm dalga boylarında absorbanslar ölçülmüştür. *A. vinelandii* CA6 FeSI proteininin dizisi, korunmuş sistein rezidüleri dahil olmak üzere, nitrojen sabitleyici *Clostridium pasteurianum* ve bir nitrojen sabitleyici olmayan *Aquifex aeolicus*'tan elde edilen [2Fe-2S] ferredoksinlerin dizilerine benzemektedir. Bu bulgular, FeSI geninin *Azotobacter vinelandii* CA6'daki majör *nif* gen kümesinde bulunmasına rağmen hiçbir kanıt olmadığı için FeSI'in nitrojen fiksasyonuna dahil olabileceğini veya olmayabileceğini düşündürmektedir. Bu çalışma, FeSI'in nitrojen fiksasyonundaki işlevini ve diğer [2Fe-2S] proteinleri ile olan ilişkilerini anlamak için faydalı olacaktır.

1. Introduction

Azotobacter vinelandii CA6 has been studied for years as a model organism for nitrogen fixation (Shethna *et al.* 1968, Dean *et al.* 1993, Peters *et al.* 1995). Shethna *et al.* particularly isolated two iron-sulfur cluster containing proteins, called FeSI and FeSII, also known as Shethna proteins I and II, from *A. vinelandii* CA6, among the other iron-sulfur proteins (Shethna *et al.* 1968, Shethna *et al.* 1964). FeSII is determined to be protecting nitrogenase against inactivation by oxygen, by forming a complex with nitrogenase which does not possess a catalytic activity, but resilient to oxygen (Moshiri *et al.* 1994, Robson 1979, Schlesier *et al.* 2016). This is called the conformational protection whereas *A. vinelandii* CA6 has also a respiratory protection which occurs with a high respiratory rate to remove oxygen (Rapson *et al.* 2020, Zehr and Capone 2021). Besides FeSII, there is no study indicating the

relation of *A. vinelandii* CA6 FeSI with nitrogenase, despite its homology to the *Clostridium pasteurianum* [2Fe-2S] ferredoxin, which is known to be an interaction partner of the nitrogenase MoFe protein (Chatelet and Meyer 1999). Moreover, *Aquifex aeolicus* [2Fe-2S] ferredoxin is another homologue protein of *A. vinelandii* CA6 FeSI (Chatelet *et al.* 1999). All these three proteins are homodimers, have similar sequences and reveal a conservation of the four cysteine residues of the [2Fe-2S] cluster, which has also been confirmed by UV-visible and EPR spectroscopy and X-ray crystallography (Chatelet *et al.* 1999, Chatelet and Meyer 1999, Kabasakal *et al.* 2021, Shethna *et al.* 1964, Yeh *et al.* 2000). FeSI along with other homologue ferredoxins belong to the thioredoxin-like ferredoxin family. Thioredoxins can reduce/oxidize disulphide bonds with no iron-sulfur clusters (Saarinen *et al.* 1995). The sequences corresponding to the thioredoxin-like ferredoxin domains of multimeric hydrogenases (Appel and Schulz 1996, De Luca *et al.* 1998) and NADH-ubiquinone oxidoreductase of respiratory chains (Yano *et al.* 1994) are similar to the sequences of these iron-sulfur proteins. Among these three iron-sulfur cluster proteins, *C. pasteurianum* [2Fe-2S] protein has an additional cysteine residue apart from the four conserved cysteines. However, the importance of this cysteine residue is unknown, as it does not form a ligand with iron (Chatelet and Meyer 1999).

The nitrogenase structural genes, including MoFe, dinitrogen reducing domain of nitrogenase and most of the nitrogenase assembly components, are found in the primary *nif* cluster of genes. FeSI is found in the *nif* gene cluster, following the nitrogenase assembly factors *nifENX* (Setubal *et al.* 2009). The location of FeSI gene also supports the idea of the FeSI involvement in nitrogen fixation.

Here in this report, the recombinant production of *A. vinelandii* CA6 FeSI in *E. coli* and its purification, biochemical and spectroscopic characterization, also comparison with other [2Fe-2S] ferredoxins from *C. pasteurianum* and *A. aeolicus* are given.

2. Material and Methods

2. 1 Cloning, Expression and Purification

Cloning of the *fesI* gene into a pRSET-A vector (Thermofisher) containing a 6xHis tag was performed by Gibson assembly method (Gibson 2011). First, the gene was isolated by PCR amplification from the *Azotobacter vinelandii* CA6 (NCBI:txid1283331) genomic DNA, purchased from DSMZ German Collection of Microorganisms and Cell Cultures, GmbH. Primers were designed for the vector and the *fesI* gene to be assembled with complementary ends. They are given in Table 1. A 50 µl PCR medium contains 10 µl 5X Q5 reaction buffer, 1 µl 10 mM dNTP mix, 2.5 µl of 10 µM primers, 10 µl 5X Q5 High QC enhancer (New England Biolabs), 0.5 µl Q5 Polymerase (New England Biolabs), 0.5 µl (average) of DNA template and 24.5 µl water. The following PCR conditions were used: Initial denaturation, 98 °C for 30 seconds, thermocycling (35 cycles), denaturation, 98 °C for 10 seconds, annealing, 72 °C for 30 seconds, extension, 72 °C for 2 minutes and final extension, 72 °C for 2 minutes.

Table 1. Primers designed for Gibson Assembly cloning.

Vector	**Forward**	5’-GGATCCACGCGGAACCAGACC-3’
	Reverse	5’-GCCCGAAAGGAAGCTGAGTTGGCT-3’
Genomic DNA	**Forward**	5’-GGTCTGGTTCCGCGTGGATCCATGGCC AAACCCGAGTTCCATATC-3’
	Reverse	5’AGCCAACTCAGCTTCCTTTCGGGCCTACC AGATCTCGGCAGGGGT-3’

The PCR products were run on agarose gel and the bands corresponding to the vector and *fesI* gene were cut. DNA samples were extracted using the protocol given by PEQLAB Gel Extraction Kit. 5 µl of solution containing 50-100 ng vector and 2-3 fold excess insert (*fesI* gene) was added into 15 µl Gibson reaction mixture (New England Biolabs). It was

incubated at 50 °C for one hour and 5 µl of the reaction product was transformed into NEB-10-beta cells. The purified plasmid sizes were checked by agarose gel analysis and confirmed by restriction enzyme digest at 37 °C using BamHI-HF enzyme and the plasmids with right sizes were sent to sequencing (Eurofins Sequencing).

The pRSET-A plasmid containing *fesI* gene was transformed into *E. coli* KRX (Promega) cells. Cells inoculated with a 10 ml starter culture were cultured in 1 L Terrific Broth at 37 °C until they reach an OD between 0.6-0.8. The induction was initiated with 0.1% (w/v) rhamnose at 18 °C. After 18 h induction, cells were centrifuged at 4,000 g for 20 min to remove supernatant and resuspended in 50 ml of 50 mM Tris-HCl, pH 7.9, 150 mM NaCl. Cell wall disruption was performed by sonication for 5 min with 1 sec pulses (Sonics VCX 130 Vibra-Cell Processor). Cell debris was removed by centrifugation at 20,000 g for 30 min and the supernatant was filtered through a 0.22 µm filter. Affinity purification of the FeSI protein was performed by loading the filtered cell lysate onto a 5 ml nickel-NTA column and eluting with 500 mM imidazole in the resuspension buffer. For the His-tag was removal from the N-terminal end of the protein, the eluted protein was incubated with 50 U of thrombin at 4 °C overnight. The cleaved and concentrated protein sample was filtered in a microfuge column (ProteusTM protein clarification mini spin-columns, Generon). Filtered protein (volume <1 ml) was loaded to an HPLC (High Performance Liquid Chromatography) system (Jasco HPLC systems, USA) with 1 ml injection loop onto a gel filtration column (Superdex200 HiLoad 16/600, GE Lifesciences, UK) pre-equilibrated with 50 mM Tris-HCl pH 7.9, 150 mM NaCl and a flow rate of 1 ml/min. The eluted samples were monitored at 280 nm and collected in 2 ml fractions. Finally, the fractions corresponding to the FeSI peak were pooled and concentrated to ~15 mg/ml with a 15 ml centrifugal filter concentrator (Millipore Amicon MWCO (molecular weight cut-off) 10 kDa) and kept in the -80 °C freezer for further experiments. Purification was monitored by analysing with SDS-PAGE.

2.2 Spectroscopic Measurements

UV-visible spectra of the purified FeSI were recorded on a UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan) for both oxidised and reduced proteins of 0.3 mg/ml. 1 mM sodium dithionite ($Na_2S_2O_4$) (Sigma) was used for reducing 0.3 mg/ml purified FeSI.

2.3 Protein Sequence Alignments

Protein sequences of [2Fe-2S] ferredoxins *from A. vinelandii* CA6*, C. pasteurianum* and *A. aeolicus* were accessed by their UniProtKB IDs, P82802, P07324 and O66511, respectively. The sequence alignment of three proteins were performed using online Clustal Omega tool of EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute).

3. Results and Discussion

3.1 Cloning and Purification of A. vinelandii CA6 *FeSI*

The FeSI encoding gene (~325 kb) was cloned into pRSET-A vector using a restriction enzyme-free method, namely Gibson assembly, from the *Azotobacter vinelandii* CA6 genomic DNA and first transformed into NEB-10-beta (New England Biolabs) cells. Plasmid size was checked by agarose gel analysis after digestion with the BamHI-HF restriction enzyme. Once the size of the plasmid was confirmed, it was sent to sequencing. The correct plasmid was then transformed into KRX cells and the protein was overexpressed.

According to the primary sequence of FeSI (UniProtKB - P82802) and previous studies, the monomeric size of the protein is 11.395 kDa (Chatelet and Meyer 1999). The elution volume of a single peak on the gel filtration column denotes that the native molecular weight of the protein is ~ 23 kDa (Figure 1) (The calibration curve for the gel filtration column is given in the Supplementary Material), so it points out the FeSI forms a dimer like [2Fe-2S] ferredoxins from *C. pasteurianum* and *A. aeolicus* (Meyer *et al.* 1994, Yeh *et al.* 2000). Also, a band corresponding to ~ 11.5 kDa was observed by SDS-PAGE (Figure 1). Along with the red colour

recognized by eye, the presence and purity of the protein was confirmed by absorbances at 344 nm, 418 nm and 464 nm in the spectrum of FeSI, caused by the Fe-S chromophore. The pure FeSI protein has A344/A280, A418/A280 and A464/A280 ratios of 0.65, 0.49 and 0.38, respectively.

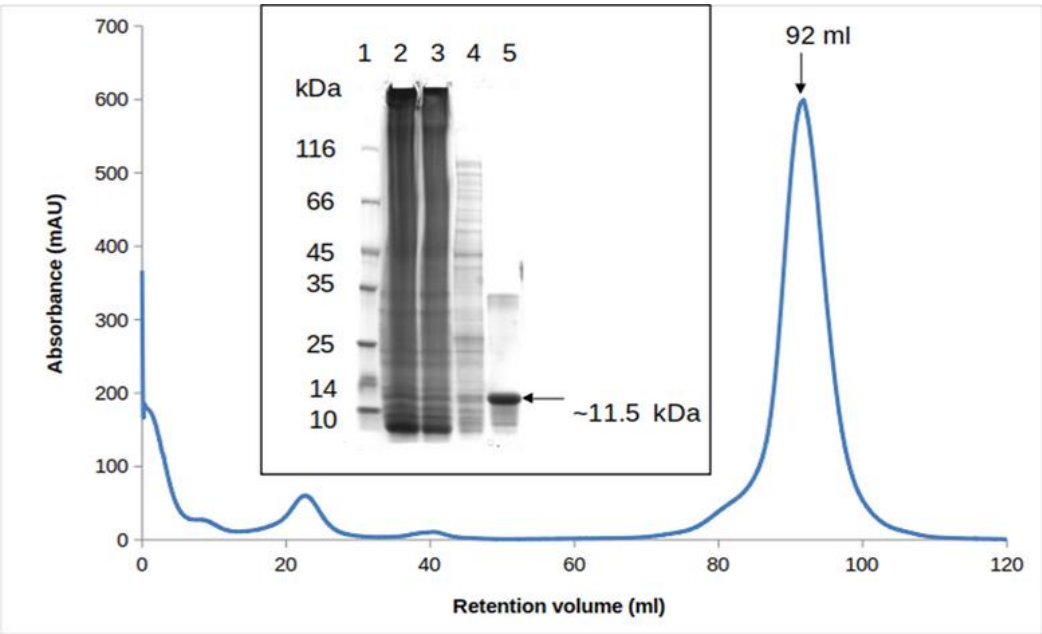

Figure 1. Gel filtration profile of the purified FeSI, measured at 280 nm. The elution volume of the peak indicates a dimer. Inset: SDS-PAGE gel of the purification stages, 1: Protein marker, 2: Cell lysate, 3: Flowthrough of the nickel affinity column, 4: Wash with 30 mM imidazole, 5: Elution with 500 mM imidazole.

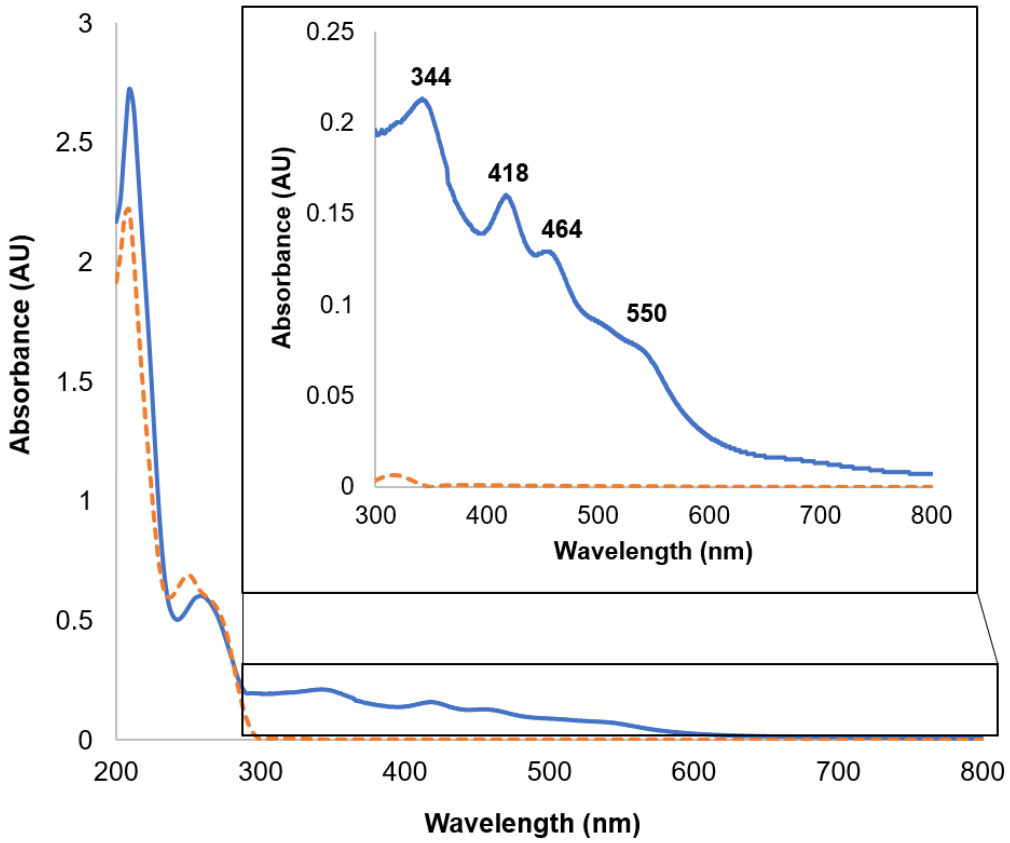

Figure 2. UV-visible absorption spectra of oxidized (solid line) and reduced (dashed line) FeSI proteins. Inset: Close view of the 300-800 nm region where the FeSI has signature peaks at 344, 418, 464 and 550 nm wavelengths.

3.2 Spectroscopic Measurements

A. vinelandii CA6 FeSI has signature peaks in the UV-visible absorption spectrum, compared to its reduced form (Figure 2). It shows absorbances at characteristic wavelengths, 344, 418, 464 and 550 nm due to the [2Fe-2S] clusters, which are also consistent with the natively purified FeSI (Shethna *et al.* 1968). Moreover, the UV-visible spectrum of *A. vinelandii* CA6 FeSI is very similar to spectra of [2Fe-2S] proteins from the nitrogen-fixing bacterium *C. pasteurianum* and non-nitrogen-fixing bacterium *A. aeolicus* (Meyer *et al.* 1994, Yeh *et al.* 2000).

3.3 Comparison with other [2Fe-2S] Proteins

Shethna protein I may have evolutionary relations with both [2Fe-2S] ferredoxins from *C. pasteurianum* and *A. aeolicus*. These three proteins have highly similar sequences, with conserved four cysteine residues and iron-sulfur clusters (Fig. 3). *A. vinelandii* CA6 FeSI has a sequence similarity with the [2Fe-2S] ferredoxins from *C. pasteurianum* and *A. aeolicus* of 35 and 44%, respectively. *C. pasteurianum* ferredoxin has an additional cysteine (Cys14), however, it was shown that it does not coordinate with the iron-sulfur cluster (Meyer *et al.* 1994). These similarities may explain both why *A. vinelandii* CA6 FeSI may not be involved in nitrogen fixation as *A. aeolicus* is not a nitrogen-fixing bacterium (Chatelet *et al.* 1999) and, on the contrary, *A. vinelandii* CA6 FeSI may be involved in nitrogen fixation due to the fact that [2Fe-2S] ferredoxin from *C. pasteurianum* interacts with nitrogenase specifically. The latter idea is supported with the presence of the FeSI-encoding gene within the *nif* gene cluster (Setubal *et al.* 2009). Three negatively charged residues (glutamates) play the role in the interaction between *C. pasteurianum* [2Fe-2S] protein and MoFe protein (Glu31, Glu34 and Glu38) and the reason for the non-interacting of *A. vinelandii* CA6 FeSI with nitrogenase may be the lack of these residues which are proposed to be essential for it (Golinelli *et al.* 1997). FeSI has Gly31, Asn34 and Gln38 at those positions. Apart from the sequential comparison and biochemical tests, there is no information about the structural similarity of these two proteins.

Figure 3. The sequence alignment of the *A. vinelandii* CA6 FeSI, [2Fe-2S] ferredoxins from *C. pasteurianum* and *A. aeolicus*. Stars and dots denote identities and similarities, respectively. Conserved cysteines (C11, C24, C56 and C60) are highlighted with boxes.

4. Conclusion

FeSI and FeSII proteins from *A. vinelandii* CA6 were discovered in the early years during the investigation of nitrogen fixation. FeSII has been shown to protect nitrogenase against oxygen damage. FeSI is different from FeSII by its cysteine ligand positions and according to sequential similarities, it seems more related to [2Fe-2S] ferredoxins from either *C. pasteurianum* or *A. aeolicus*. It may play a role in nitrogen fixation due to its genomic location and transcript data, as well as similarity with the *C. pasteurianum* [2Fe-2S] protein whereas it lacks acidic residues proposed to be interacting with nitrogenase. Moreover, it has a

high similarity with the [2Fe-2S] protein from *A. aeolicus*, which is not a nitrogen fixer. Taken together, the real function of *A. vinelandii* CA6 FeSI is still unknown and further research is believed to be useful for elucidating its function and possible related functions of [2Fe-2S] proteins.

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