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Expression of soluble native protein in *Escherichia coli* using a cold-shock SUMO tag-fused expression vector

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ABSTRACT

At present, approximately 30% of eukaryotic proteins can be expressed in a soluble form in *Escherichia coli*. In this study, a pCold-SUMOa plasmid was constructed in order to express heterologous proteins fused with SUMO by a cold-shock expression vector. The human cysteine desulfurase NFS1 and a chimeric cysteine desulfurase namely, EH-IscS were successfully expressed in *E. coli*. The proteins were particularly difficult to be produced functionally, due to their readily sequestered nature. The recombinant cysteine desulfurases that were generated by pCold-SUMOa exhibited higher activity, solubility and stability compared with the well-known plasmid pCold I. In contrast to the pCold TF plasmid, the SUMO tag conferred no biological activity with regard to the conformation of the cysteine desulfurases. Furthermore, the SUMO protease 1 can efficiently recognize the tertiary structure of SUMO and cleave it. The data indicate that the pCold-SUMOa vector is a promising tool for native eukaryotic protein production.

1. Introduction

Multiple studies have shown that iron-sulfur clusters are integral parts of diverse physiological processes including energy conversion, nitrogen fixation, intracellular iron homeostasis, RNA modification and protein translation, DNA damage repair and replication, and gene transcription [1–3]. Cysteine desulfurase was initially identified as a catalyst of the desulfurization of L-cysteine and as the main protein responsible for the transfer of sulfide to the iron-sulfur cluster assembly. The genes encoding the cysteine desulfurases are highly conserved from the prokaryotic to eukaryotic organisms. Human NFS1 contains two distinct NFS1 isoforms, which are produced *via* alternative utilization of the in-frame initiation codon [4]. Previous studies have investigated the crucial roles of NFS1 in the maintenance of cellular Fe-S homeostasis. Notably, the mutation (p.Arg72Gln) in NFS1 was associated with the loss of Fe-S clusters enzyme activities and the development of mitochondrial disease namely, autosomal recessive infantile mitochondrial complex II/III deficiency (IMC23D) [5]. In contrast to *Escherichia coli* (*E. coli*) cysteine desulfurase IscS, NFS1 requires the adaptor protein ISD11 in order to maintain its activity. The NFS1-ISD11 complex is essential for nuclear and mitochondrial iron-sulfur protein biosynthesis, moreover, the homozygous R68L ISD11 mutation causes the mitochondrial genetic disorder COXPD19 [6,7]. However, the homology of the eukaryotic ISD11 protein has not been identified in the prokaryote to date.

Our previous studies have demonstrated that ablation of iron chaperone proteins IscA/SufA and/or depletion of intracellular iron can disrupt the assembly of iron sulfur clusters, thus impeding sulfur delivery of IscS, and result in accumulation of red IscS intermediate in *E. coli* cells [8]. Whether the deficiency of accessible iron results in accumulation of the red NFS1-ISD11 complex in human or *E. coli* cells remains unknown. Moreover, the molecular mechanism and physiological significance of the accumulation of red-colored cysteine desulfurase in *E. coli* cells remain to be understood. Unfortunately, it is difficult to obtain functional proteins of NFS1 and ISD11 in *E. coli* cells, since the majority of these proteins are readily sequestered [9]. In addition, in order to further validate the binding site of red intermediate in IscS and the reason why NFS1 alone was not active. We have constructed two types of chimeric cysteine desulfurase derived from the IscS and NFS1.

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As we all know, *E. coli* is the most commonly used recombinant protein expression system, and is widely acknowledged in the fields of genetics, genome sequencing and physiology. According to statistical analysis, more than 60% of recombinant proteins and nearly 30% of recently approved recombinant therapeutic proteins are produced in *E. coli* expression systems [10]. This is attributed mainly to the fast growth of the bacteria that results in high yield of the target proteins. In addition, *E.coli* has been extensively used for a vast number of years and the recombinant technology is well known, whereas the applications of these bacteria are also based on their cost-effective nature. However, this expression system has certain disadvantages in the production of active eukaryotic proteins. Approximately 30% of the cloned genes can be expressed in soluble forms, most of which are degraded by endogenous proteases and/or form inclusion bodies. In some cases, the genes cloned cannot be expressed [10,11]. *In vitro* renaturation methods have been applied to convert inclusion bodies into a soluble form. However, the procedures are complicated and time-consuming and require laborious methodologies and additional costs for protein purification [11].

Previous study showed that the solubilizing tags (glutathione *S*-transferase (GST), small ubiquitin-like modifier protein (SUMO), and maltose-binding protein (MBP), *etc.*) could efficiently decrease the formation of inclusion bodies within the soluble proteins production [12], in the majority of the cases, although the tags require immediate removal for further applications. Notably, in genetically engineered pharmaceutical proteins, the final forms of the purified proteins must exert a native protein function [10]. This can be achieved by the use of a specific protease that can recognize the specific amino acid sequence between the tag and the protein-of-interest (POI) and digest it. The enzymes enterokinase, thrombin, factor Xa, tobacco etch virus (TEV) protease and Ulp1 are commonly applied to remove the fusion tags [13]. Among these proteases, Ulp1 protease that recognizes only SUMO tertiary structure and leaves the POI with its native N-terminal [12]. However, although a variety of techniques can be used to produce recombinant proteins, a universal method for the preparation of soluble, homogeneous and native N-terminus target proteins is not available to date. Therefore, the production of a prokaryotic expression plasmid that enhances the solubility of recombinant

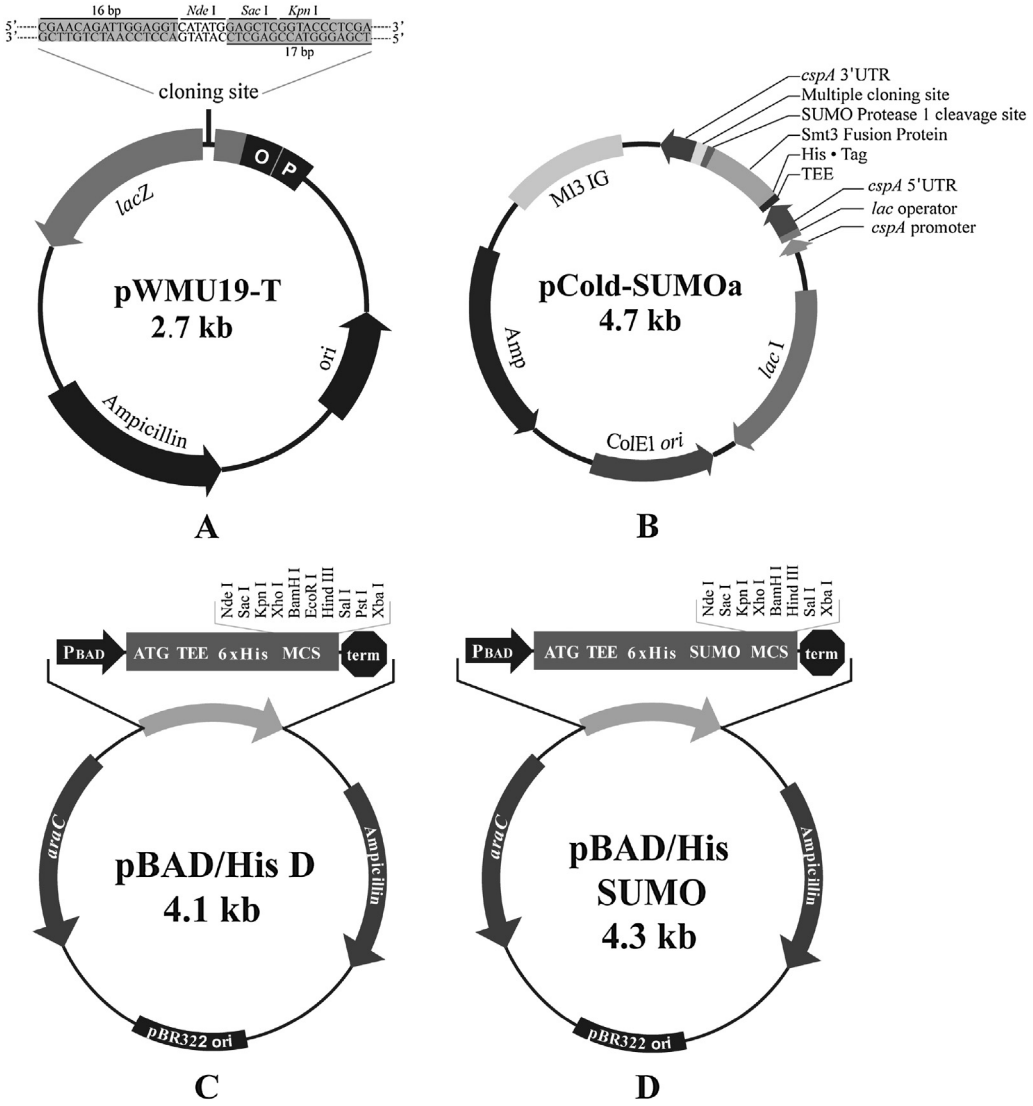

Fig. 1. Schematic illustration of the *E. coli* cloning and expression vector. (A) Schematic representation of the pWMU19-Tp sequencing vector; (B) Schematic representation of the pCold-SUMOa expression vector; (C) Schematic representation of the pBAD/His D expression vector; (D) Schematic representation of the pBAD/His SUMO expression vector.

exogenous proteins is considered a major research focus and possesses broad application prospects.

Recent studies have shown that a new bicistronic expression vector $pSUMO_{ulp1}$ that allows co-expression of any POI fused to both the SUMO and Ulp1 protease. In this issue, Ulp1 is able to cleave SUMO inside the bacteria and leave the POI free which is easy to be purified. But the use of $pSUMO_{ulp1}$ vector may still exhibit some deficiencies. Firstly, the C-terminus tagged six histidine of the target protein was evaluated as a non-natural protein; the second is the optimal growth or induction temperature of *E. coli* cells are inconsistent with the optimal temperature for Ulp1 protease activity, and if the digestion is not sufficient, the SUMO fusion protein and the POI will be purified simultaneously; the third is removing the SUMO tag will destabilize POI under certain conditions, so it must be cleaved in a specific reaction system that is conducive to enhancing the stability of the native POI *in vitro*.

Here, we have successfully constructed a novel cold-shock expression vector pCold-SUMOa which could overcome the aforementioned disadvantages. In this pCold-SUMO expression system, the T7 promoter expression system was replaced by the pCold cold-shock expression system which was clarified to increase the expression level and solubility of POI. Spontaneously, based on the seamless cloning technology, a pWMU-19T cloning vector suitable for pCold-SUMO system was constructed. In addition, the optimal reaction conditions for the Ulp1 protease *in vitro*, the purification scheme for native POI and reagent formulation to preserve Ulp1 protease and POI were also developed. Indeed, this system was further validated through the successful production of soluble recombinant NFS1 and EH-IscS proteins, while these two soluble recombined proteins could be used for further functional research. In conclusion, we suggest that the use of the pCold-SUMOa vector is a novel strategy for the generation of native eukaryotic proteins.

2. Materials and methods

2.1. Chemicals

The plasmids of pCold I, pCold TF and pCold-GST, restriction endonucleases and TALON Metal Affinity Resin were purchased from Takara corporation (Dalian, P. R. China). DNA polymerase and T4 DNA ligase were obtained from Vazyme biotechnology corporation (Nanjing, P. R. China). ClonFast kit was purchased from Obio technology corporation (Shanghai, P. R. China). All primers were synthesized by BGI corporation (Shenzhen, P. R. China). The remaining chemicals that were used were of analytical grade.

2.2. Strains and vectors

Strains and plasmids used in this study are described in Supplementary Table 1. The primers are listed in Supplementary Tables 2 and 3.

2.3. Construction of pWMU-19T and pCold-SUMOa vector

The *iscS* was amplified from the genome of *E. coli* MC4100. Each terminal of the amplified gene contains an *Nde* I enzyme site, which has the same homologous arms with the pCold-SUMOa plasmid digested by the *Nde* I restriction enzyme. The PCR product was cloned into the pUM19-T vector, which contains the mutated *Nde* I restriction enzyme site. Consequently, the IscS-pUM19-T plasmid was digested with *Nde* I. The linearized vector was self-looped with T4 DNA ligase. As a result, the cloning vector, pWMU19T was constructed (Fig. 1A).

The Smt3 fusion protein (SUMO) gene fragment was amplified from the pE-SUMOpro Kan vector. The plasmid pCold TF was digested with *Sal* I and was used as a template for the amplification backbone of the pCold-SUMOa. The pCold-SUMOa plasmid was finally constructed by the (Seamless Cloning Technology) SCT (Fig. 1B). pCold-SUMOa contains the same multi cloning sites with pCold TF with the exception of the *EcoR* I and *Pst* I, while the SUMO tag contains the latter two restriction sites.

The pBAD-His A plasmid was digested by *Nde* I and was used as a template to amplify the plasmid backbone. The pBAD plasmid was constructed by SCT according to the following characteristics: 1) The *Nde* I enzyme site (CATATG) of pBAD-His A plasmid was mutated into the sequence of CAGCTG; 2) The 319–470 bp sequence of pBAD-His A plasmid was replaced by the *Xba* I restriction endonuclease site and a stop codon. Therefore, the *Xba* I restriction site of the engineered pBAD His A plasmid could be used to construct a novel expression plasmid according to the experimental requirements using SCT (Fig. 1C and D).

2.4. Construction of recombinant plasmids

The plasmids NFS1 (55–457, in the absence of the transit peptide)-pET28b and EH-IscS-pET28b were obtained from Prof. HG. Ding of the Louisiana State University. EH-IscS includes the N-terminal domain amino acids 1–263 of *E. coli* IscS and the C-terminal domain amino acids 316–457 of human NFS1. The DNA fragment encoding *E. coli* IscS was amplified by the *E. coli* MC4100 chromosome DNA. The plasmids pCold I, pCold TFc and pBAD-HisD were digested with *Kpn* I, whereas the plasmids pCold-SUMOa, pBAD-SUMO and pET-SUMO were digested with *Nde* I. The plasmid pET28a-SUMO was digested with *BamH* I. All the plasmids were constructed with seamless cloning methods. The sequences of the plasmid constructs were confirmed by DNA sequencing.

2.5. Protein expression

All the protein expression experiments, unless stated, were conducted according to the following protocols: 1) Transformation of the expression plasmids into *E. coli* host strain BL21(DE3), and selection of the transformed vectors on Amp^+/LB plate; 2) Dilution of the cultured cells in 1:50 into fresh LB(Luria-Bertani) medium supplemented with ampicillin of 100 μg/ml overnight, and subsequent culture at 37 °C, with rotation at 250 rpm/min; 3) Leaving the culture solution at 15 °C for 30 min when its OD_{600} reaches to 0.4–0.5; 4) Addition of IPTG at the final concentration of 0.2 mM and subsequent culture with shaking at 15 °C for 24 h.

2.6. Purification of recombinant cysteine desulfurase by Ni^+ column

Purification of the recombinant cysteine desulfurases was conducted as previously described [14]. The purity of all the proteins was greater than 90% (> 90%) as demonstrated by the SDS-PAGE gel that was stained using Coomassie blue. The protein concentrations of IscS, EH-IscS, SUMO-EH-IscS, TF-EH-IscS, SUMO-NFS1 (55–457) and TF-NFS1(55–457) were measured from the absorption peak at 280 nm, and calculated with an extinction coefficient of 39.8 $mM^{-1}cm^{-1}$, 34.3 $mM^{-1}cm^{-1}$, 35.6 $mM^{-1}cm^{-1}$, 50.2 $mM^{-1}cm^{-1}$, 38.4 $mM^{-1}cm^{-1}$ and 53 $mM^{-1}cm^{-1}$, respectively. The UV–vis absorption spectra were measured in a Hitachi U3900 UV–vis spectrometer equipped with a temperature controller.

2.7. Enzyme activity measurement

The cysteine desulfurase activity assay was conducted as described in a previous study [8].

2.8. Aggregation analysis of recombinant cysteine desulfurase

The stability analysis of recombinant cysteine desulfurase was conducted as described in a previous study [6], although the buffer F composition was adjusted to 500 mM NaCl and 20 mM Tris-HCl (pH 8.0).

2.9. Substrate-binding assay

The substrate-binding assay was conducted as described in previous studies [4,15].

2.10. Cleavage of SUMO-EH-IscS fusion protein and purification of EH-IscS

IPTG (isopropyl β-*d*-thiogalactoside; 0.2 mM) was added to induce protein expression at 37 °C until the growth of *E. coli* cells reached an absorbance value of 1.0 (OD_{600}). Recombinant Ulp1 was purified as described previously [1]. Ulp1 protease was incubated with 100 μg control protein in the protease cleavage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) at 25 °C for 1 h, resulting in cleavage of approximately 95% and/or higher of the control proteins. The standard use of Minimum Ulp1 protease is defined as 1 unit of enzyme activity. The optimal reaction conditions for the Ulp1 protease included incubation of the His-tagged SUMO-EH-IscS and His-tagged Ulp1 protease at a ratio of 1 unit per 100–1000 μg of fusion proteins in the protease cleavage buffer at 25 °C for 1–2 h and/or at 4 °C overnight. Following TALON Metal Affinity Resin (Takara Bio Company) purification and subsequent removal of His-tagged proteins in the protease cleavage buffer by Co^{2+} magnetic beads, the mixture was analyzed by a 12% SDS-PAGE gel. The protein concentrations were determined by the BCA assay kit (Beyotime).

3. Results

3.1. Construction of a simple and efficient cloning vector based on SCT

Based on the TA connection and the blue-white spot screening method [16], traditional cloning vector construction exhibits certain defects namely, complicated operations and a high rate of false positive results. However, with the use of the latest SCT, the pMD19-T vector results in the transformation to the pWMU19-T vector. The pWMU19-T vector has the advantages of being a circular plasmid that can replicate in *E. coli*. Following digestion with a single restriction endonuclease, the linearized pWMU19-T vector was generated into a fragment with two short homology terminals (approximately 15–20 bp in size). The terminals were the same as the sequences that were produced in any expression vector digested by the same restriction enzyme at the multiple cloning site (Fig. 1A and B).

3.2. Construction of a new cold-shock expression vector based on SCT

To overcome the shortage of conventional techniques and achieve heterologous protein expression with high solubility and native N-terminal, we analyzed the existed commercial expression vector from *E. coli*. The promoter derived from the cold-shock gene *csp*A was fused with the solubility enhancing SUMO tag to construct the pCold-SUMOa plasmid (Fig. 1B). Following enzyme

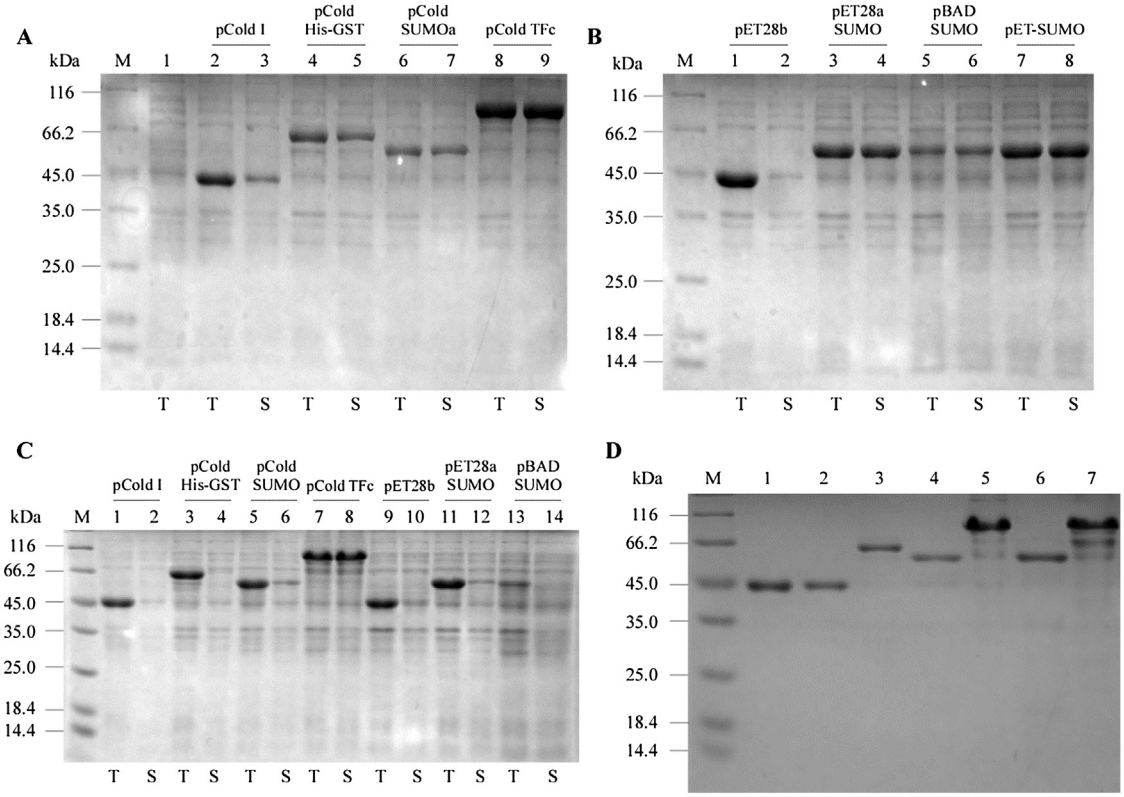

Fig. 2. Expression and purification of recombinant cysteine desulfurase in *E.coli* cells. The plasmids pET28b, pCold I, pCold-His-GST, pCold-SUMOa, pCold TFc, pET28a-SUMO, pBAD-SUMO and pET-SUMO were used to express EH-IscS (A–B) and NFS1 (C) proteins, respectively. Total cell protein was analyzed by 12% of SDS-PAGE gel (T) and the soluble (S) fractions were subjected to centrifugation. (A) Lane-1: un-induced control; (D) SDS-PAGE (12%) gel analysis of purified recombinant cysteine desulfurase. Lane-1: IscS, Lane-2: EH-IscS, Lane-3: GST-EH-IscS, Lane-4: SUMO-EH-IscS, Lane-5: TF-EH-IscS, Lane-6: human SUMO-NFS1(55–457), Lane-7: TF-NFS1(55–457). M: Protein molecular weight marker. The data are representative of three independent preparations.

digestion with *Nde* I, the same homology arms of the pCold-SUMOa and pWMU19-T plasmids were generated. The cloning and expression vectors can be constructed simultaneously provided that the 5′-end of DNA primers for the target gene amplification have the same homology sequences as the linearization vector. Thus, this method is considered a simpler, faster and highly cost-effective approach for vector construction compared to the traditional methods.

The expression of recombinant cysteine desulfurases fused with SUMO tag by a *ara*BAD promoter (P_{BAD}) was controlled in order to demonstrate the high performance of the constructed pCold-SUMOa plasmid (Fig. 1D). In addition, several well-documented plasmids were utilized, such as commercially available SUMO fusions and the Champion™ pET SUMO Protein Expression System (Invitrogen). The SUMOpro Protein and Peptide Expression Kit (Lifesensors) were further applied. And a highly active and specific SUMO protease 1 was used.

3.3. ***Enhanced solubility of recombinant cysteine desulfurase*** via ***fusion tags application***

During the NFS1(55–457) and EH-IscS expression in the pET28b vector, the proteins formed inclusion bodies (Fig. 2B and C). However, pCold (Takara) is a widely known vector that can increase the solubility of target proteins. A series of cold-shock expression vectors were used in order to obtain a soluble protein. The evaluation of the solubility of the tagged EH-IscS by SDS-PAGE indicated an improvement in the following order: TF > SUMO > GST > His (Fig. 2A). The expression of recombinant NFS1 (55–457) was transformed to inclusion bodies in pCold I, pCold-GST, and pET28b. It is important to note that the NFS1(55–457)-pCold-SUMOa and NFS1(55–457)-pET28a-SUMO indicated limited improvement with regard to the solubility compared with the NFS1(55–457)-pCold I, whereas the purity of SUMO-NFS1(55–457) was higher than 90% (>90%) as demonstrated by SDS-PAGE

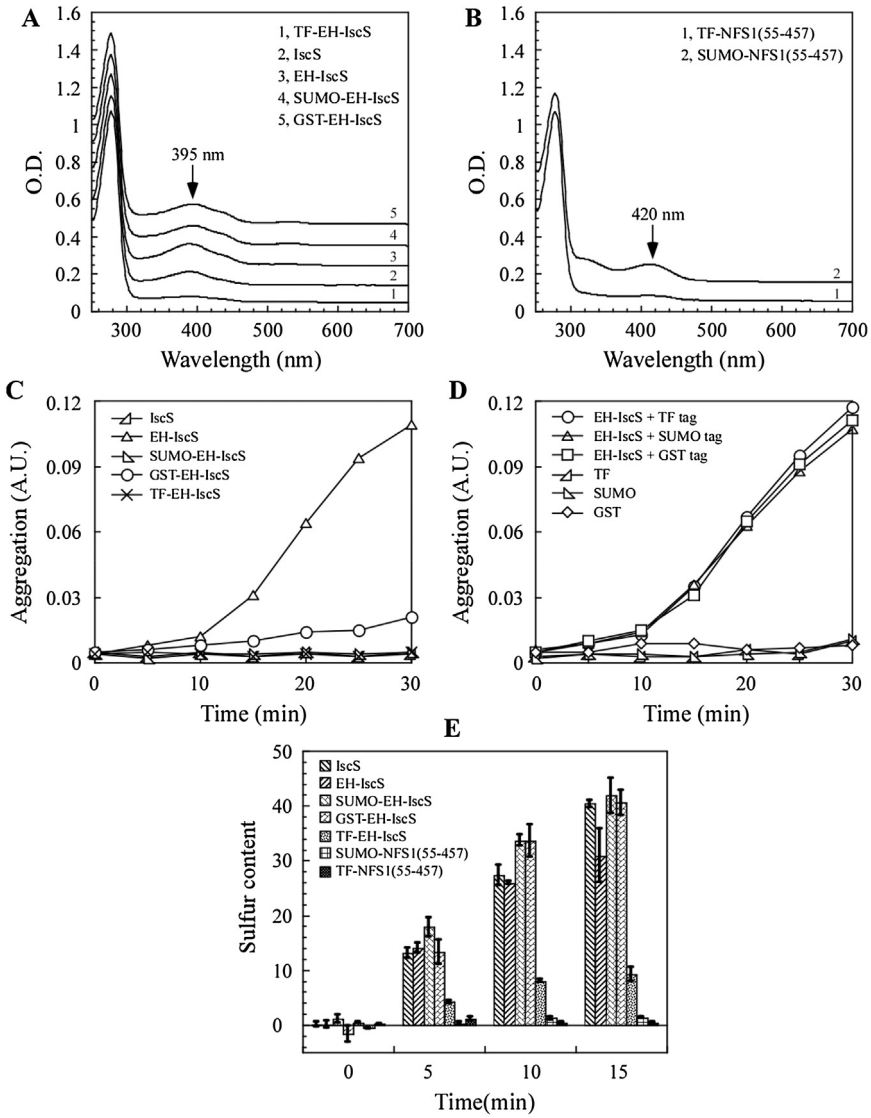

Fig. 3. Characterization of the recombinant cysteine desulfurases. (A) Purified TF-EH-IscS (20 μM), IscS (25 μM), EH-IscS (29 μM), SUMO-EH-IscS (28 μM) and GST-EH-IscS (13 μM) were subjected to UV–vis absorption measurements; (B) UV–vis absorption spectrum of purified human NFS1(spectrum 1, 2). The concentrations of TF-NFS1 and SUMO-NFS1 were 19 and 26 μM, respectively, as shown in (B); Fig. 3C-D. The stability of the purified recombinant cysteine desulfurase. (C) Effect of chaperone proteins co-expressed with EH-IscS; (D) Effect of the incubation of chaperone proteins with EH-IscS. The data are representative of three independent experiments; (E) The activity of purified recombinant cysteine desulfurases. Cysteine desulfurase activity of IscS, NFS1 and EH-IscS were measured using the sulfide detection method by Siegel [13]. At least three independent experiments were carried out for the cysteine desulfurase activity assay.

(Fig. 2D). Several studies that were conducted in order to express other inclusion bodies indicated a similar performance of SUMO [17,18]. Therefore, the data indicate that the pCold-SUMOa plasmid exhibits a promising application potential for the improvement of the solubility of the heterologous peptides. The *csp*A promoter can induce a high level of target protein expression compared with the commonly used *T7* and *ara*BAD promoter systems, while the yield of expression was estimated to 60% for the total cell protein content (Fig. 2C) [17]. Consequently, the pCold-SUMOa expression system is a more suitable expression system compared with the pET-SUMO and pBAD-SUMO plasmids as demonstrated by cell NMR (nuclear magnetic resonance) spectroscopy.

As shown in Fig. 2D, besides TF-NFS1(55-457), all the other purified recombinant cysteine desulfurase proteins were single band displayed on the SDS-PAGE gel. (Fig. 2).

3.4. Properties of the recombinant cysteine desulfurase

The UV–vis absorption indicated that the purified EH-IscS, GST-EH-IscS, SUMO-EH-IscS and IscS proteins exhibited the same pyridoxal 5′-phosphate (PLP) absorption peak at 395 nm (Fig. 3A). The UV–vis absorption spectra of the purified SUMO-NFS1 (55–457) exhibited the maximal absorption at 420 nm that resulted from the prosthetic group PLP. The proteins with this type of spectrum were yellow in color. In contrast to SUMO-NFS1, the absorption peaks of TF-EH-IscS and TF-NFS1(55–457) proteins at 395/420 nm were considerably weak (Fig. 3A and B). The effect of the TF tag (52 kDa) was considerably great with regard to the impairment of the conformation of the target proteins. The relative content of PLP in the recombinant cysteine desulfurases was calculated *via* the extinction coefficient and compared with that of IscS purified from *E. coli*. A limited number of studies were conducted to investigate whether the recombinant cysteine desulfurases fused with SUMO or GST tag would affect the combination with PLP, actually, when they were fused with TF tag caused a significant reduction of the PLP content, but not fused with SUMO or GST tag (Data were not shown).

During the process of protein purification and enzyme activity assay, floccose sediment was readily formed in the EH-IscS protein. The enzymatic reaction rate was notably reduced in the presence of the EH-IscS precipitate compared with the IscS. The effects of the chaperone proteins in the prevention of the aggregation of the recombinant cysteine desulfurases were investigated using by a light scattering method [6]. SUMO-EH-IscS, and/or TF-EH-IscS fusion proteins did not form aggregates during single incubation at 37 °C, *in vitro*. Concomitantly, the negative control EH-IscS incubation that contained the GST, SUMO and/or the TF tags did not prevent the EH-IscS from self-aggregation under the same conditions. Notably, EH-IscS fusion with the GST tag indicated partial aggregation suppression (Fig. 3C and D).

Cysteine desulfurase activity of EH-IscS and IscS was similar at the same molar concentrations (Fig. 3E). Interestingly, the activities of SUMO-EH-IscS and GST-EH-IscS fusion proteins were higher than those of the His-tagged control. Despite this activity increase, the TF-EH-IscS fusion protein indicated significantly lower activity. The data indicated that combination of NFS1 with its auxiliary protein namely, ISD11, is necessary for the retention of the activity, as demonstrated by separated SUMO-NFS1(55–457) and TF-NFS1(55–457) enzyme activity assays [9]. In conclusion, the aforementioned results further confirmed that the use of the SUMO tag as a fusion cofactor improved the solubility, enzyme activity and stability of the recombinant target proteins, and was superior to the GST and TF tags.

Furthermore, the formation of the active center of the recombinant cysteine desulphurases was investigated. The addition of 10 mM excess of free L-cysteine resulted in a decrease of the absorbance of PLP and an increase of the absorbance peak that corresponded to the formation of the L-cysteine-PLP ketimine adduct. The latter was formed in the purified IscS and EH-IscS forms (Fig.4A and B) as well as in the NFS1 (data not shown), indicating that both proteins were active towards L-cysteine. Furthermore, a new absorbance peak of the unstable reaction intermediates was observed that was higher for the SUMO-EH-IscS compared with that of the EH-IscS (Fig.4B and C). The formation of

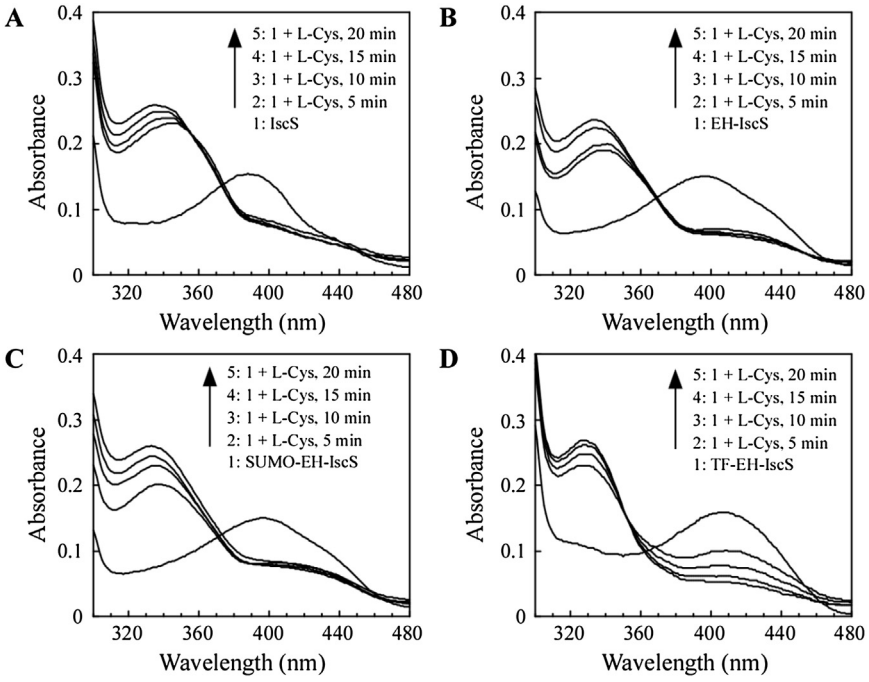

Fig. 4. Time-dependent spectral analysis of L-cysteine binding to recombinant cysteine desulfurase. (A–D) IscS, and EH-IscS were incubated with 10 mM L-cysteine at 4 °C for 20 min, resulting in the disappearance of the absorption peak at 395 nm, while a new absorption peak that corresponded to an unstable reaction intermediate appeared approximately at 330–340 nm. The data are representative of three independent experiments.

the intermediate absorption peak that was observed for the SUMO-NFS1 (55–457) protein at 330 nm was consistent with previous reports [4,15]. Therefore, the data suggested that the SUMO tag could promote appropriate folding of the peptides in order to produce soluble proteins. However, the absorption peaks of the TF-EH-IscS and TF-NFS1(55–457) were considerably weak. Moreover, the fusion of the TF tag in EH-IscS reduced the speed of the displacement reaction between L-cysteine and PLP, resulting in absorbance shifts with regard to TF-EH-IscS compared with those noted in EH-IscS (Fig.4D). In conclusion, the results suggested that the molecular weight of the TF tag (52 kDa) might interfere with the activity of the target proteins by sterically hindering the access of the substrate to the catalytic site, irrespective of the solubility enhancement conferred by the TF tag.

3.5. Digestion of SUMO fusion tag and purified target protein

Treatment of 1 mg SUMO-EH-IscS fusion protein with 1 U Ulp1 protease resulted in a complete and specific proteolysis (Fig. 5). The homogenous EH-IscS protein was obtained by TALON Metal Affinity Resin purification with no apparent protein precipitation. A purity of greater than 95% was achieved in a preparation of 5 to 6 mg of EH-IscS purified from 1 L LB medium.

A reagent formulation to preserve Ulp1 protease was further derived from these experiments and the exact conditions were as follows: 1.75 mg/ml Ulp1 protease in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20% glycerin, 1 mM benzamidine, 0.2 mM PMSF, 0.1 mM EGTA, 0.1% 2-mercaptoethanol. The formulation could be stored at −20 °C for 1 year.

4. Discussion

Previous study showed that all cysteine desulfurases contain conservative lysine and cysteine residues, which are indispensable for their catalytic activities [19]. Lysine residues can bind with cofactors, such as PLP in order to form a Schiff base. The cysteine residue is involved in the formation of a protein-bound cysteine persulfide intermediate, which serves as a sulfur donor and is subsequently incorporated to the biosynthesis of sulfur-containing biomolecules namely, biotin, thiamine, molybdopterin, and Fe-S clusters in proteins [20,21]. In addition, cysteine desulfurases act in the maintance of the balance of iron *in vivo*, and participate in the modification of tRNA and the phosphorothioation of DNA [22,23]. However, the specific catalytic mechanism of NFS1 was poorly understood, due to its insoluble status using the present production methods [4]. In a previous study, Marelja et al. co-expressed NFS1 (55–457) *via* a plasmid containing the *E. coli* chaperone GroEL in *E. coli* BL21(DE3) star cells, and a cold induction temperature was applied to increase the content of the soluble proteins. Saha et al. co-expressed NFS1 (55–457) with a pGro7 plasmid containing the groES-groEL chaperone, and further achieved the enhancement of the solubility of NFS1 (55–457) [6]. However, the aforementioned method required the use of a special chaperone plasmid and host strains that are time consuming and cost ineffective [4].

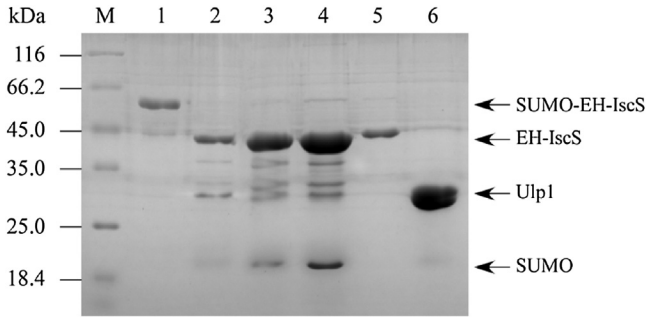

Fig. 5. SDS-PAGE gel (15%) analysis of SUMO-EH-IscS digested by Ulp1 protease and purified target proteins. M: Protein molecular weight marker; Lane1: purified SUMO-EH-IscS; Lanes 2-4: 100 μg, 500 μg and 1000 μg of SUMO-EH-IscS protein digested by Ulp1 (3.5 μg) protease, respectively; Lane-5: purified EH-IscS; Lane-6: Ulp1 protease. The data are representative of three independent experiments.

Until now, multiple methods were developed to increase the solubility of recombinant proteins in *E.* coli cells. The methodological strategies mainly include the following: (1) The addition of tags at the terminus of POI in order to enhance the solubility. This strategy has been used for the enzymes glutathione S-transferase (GST), SUMO, MBP, N-utilization substance A (NusA) and disulfide isomerase C (DsbC) that are well known for their solubility-enhancing fusion properties [10,11,13]; (2) The alteration of the expression vectors and conditions namely, the use of cold shock expression vectors from Takara [24], the addition of appropriate solvents (betaine, sorbitol) to the medium in order to promote the heterologous protein folding and/or control the rate of medium refilling required to reduce the rate of protein synthesis [25]; (3) The increase of the secretory form of the soluble protein *via* the addition of a signal peptide at the N-terminus of the target proteins, such as Sec translocase that is a translocon specifically involved in protein translocation across the cytoplasmic membrane by the Sec-system in *E. coli* [26,27]; (4) The directed evolution that results in soluble protein expression [28]. Furthermore, the development of a simple and efficient seamless DNA cloning method [29,30] can result in the fusion of two or more DNA fragments at the targeted junction sites in the absence of restriction enzymes. This method is appropriate for the simultaneous insertion of genes of interest into different vectors and it offers the selection of the most suitable expression vector from various types of constructs.

In the present study, a novel cold-shock expression vector pCold-SUMOa was constructed in order to obtain NFSI for further functional analysis. The production of the SUMO-NFS1(55–457) was greatly increased in *E. coli* (Fig. 3B) and the stability was significantly enhanced. Although the DNA sequence analysis revealed that the constructed recombinant plasmid contained the sequence of the *ISD11* gene, the recombinant ISD11(31–276) protein was undetectable in the cell extracts. According to the rare codons analysis, several rare codons were pinpointed in the ISD11 genetic sequence in *E. coli* that explains the lack of detection of ISD11(31–276) in the cell extracts.

A total of two types of chimeric cysteine desulfurases were constructed in order to address the aforementioned discrepancy and to analyze the function of the NFS1. One of the strategies was to fuse the N-terminal domain of human NFS1 (amino acids 55–315) with the C-terminal domain of *E. coli* IscS (amino acids 264–404) in order to form the chimeric cysteine desulfurase HE- IscS. The purity of the chimeric protein was further examined by SDS-PAGE and a number of protein bands were observed with the exception of the objective band with purity of lower than 40% (<40%). In addition, this protein exhibited no cysteine desulfurase activity (data not shown); An additional strategy was to fuse the N-terminal domain of *E. coli* IscS (amino acids 1–263) with the C-terminal domain of human NFS1 (amino acids 316–457) in order to construct the chimeric cysteine desulfurase EH-IscS. The aforementioned studies suggest that the EH-IscS that was fused with different tags for the control of the *csp*A promoter could generate soluble proteins, while the SUMO tag was the most effective expression tool for the production of the EH-IscS. This was due to the ability of the SUMO tag to significantly enhance the solubility of the EH-IscS protein and to further improve the stability and activity of the target proteins. The maximum yield of the isolated SUMO-EH-IscS was 12.1 mg/L in shake flask culture, and the final SUMO-NFS1 concentration produced by BL21(DE3) was 2.8 mg/L. A unique advantage of the SUMO tag is that it can be specifically cleaved by Ulp1 protease by the recognition of its

tertiary structure. Due to the high cleavage efficiency, the molecular mass proportion of Ulp1 and fused SUMO-protein was in the range of 1: 500–1000. Ulp1 could retain its activity at a maximum concentration of 2 M of urea [13].

Overall, the present study suggests that the pCold-SUMOa plasmid combined with seamless cloning technology may be efficient and useful for large-scale purification and tag removal in order to prepare native heterologous protein with a desired N-terminus.

5. Conclusions

Here we constructed a novel cloning vector namely, pWMU-19T based on SCT. Compared with TA cloning vector, pWMU-19T exhibits the characteristics of PCR products without adding base A reaction, the vector construction can be completed in 30 minutes, no positive clones need to bescreened by blue and white spots, as well as the positive clone rate is more than 90%. Moreover, the preparation method of the pWMU-19T is simple. Therefore, it is suitable for rapid cloningof genes and high-throughput preparation of gene libraries. Furthermore, we have successfully constructed a novel cold-shock expression vector pCold-SUMOa which is successfully to express human cysteine desulfurase NFS1 and a chimeric cysteine desulfurase EH-IscS in *E. coli*, which were difficult to be produced functionally becausemost of the protein was sequestered in inclusion bodies. Compared with the well-known pCold I, pCold-GST and pET28b plasmids, the newly recombinant cysteine desulfurase which generated in pCold-SUMOa has higher activity, solubility and stability. Meanwhile, unlike pCold TF plasmid, SUMO tag did not affect the protein conformation of the recombinant cysteine desulfurase. Furthermore, SUMO protease 1(Ulp1) was efficient to recognize the tertiary structure of SUMO, and the cleaving efficiency of SUMO-fusion is up to 95%.These data indicate that the pCold-SUMOa vector is applicable for the preparation of proteins with natural N-terminus in the field of bioengineering pharmaceutical and structural biology.

Conflict of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2018.e00261.

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