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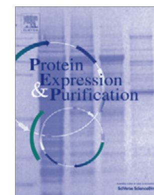

An efficient protocol for the production of tRNA-free recombinant Selenocysteine Synthase (SELA) from *Escherichia coli* and its biophysical characterization

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ABSTRACT

Selenocysteine Synthase (SELA, E.C. 2.9.1.1) from *Escherichia coli* is a homodecamer pyridoxal-5′-phosphate containing enzyme responsible for the conversion of seryl-tRNA^{sec} into selenocysteyl-tRNA^{sec} in the biosynthesis of the 21th amino acid, selenocysteine (Sec or U). This paper describes the cloning of the *E. coli selA* gene into a modified pET29a(+) vector and its expression in *E. coli* strain WL81460, a crucial modification allowing SELA expression without bound endogenous tRNA^{sec}. This expression strategy enabled the purification and additional biochemical and biophysical characterization of the SELA decamer. The homogeneous SELA protein was obtained using three chromatographic steps. Size Exclusion Chromatography and Native Gel Electrophoresis showed that SELA maintains a decameric state with molecular mass of approximately 500 kDa with an isoelectric point of 6,03. A predominance of α-helix structures was detected by circular dichroism with thermal stability up to 45 °C. The oligomeric assemblage of SELA was investigated by glutaraldehyde crosslinking experiments indicate that SELA homodecameric structure is the result of a stepwise addition of intermediate oligomeric states and not a direct monomer to homodecamer transition. Our results have contributed to the establishment of a robust expression model for the enzyme free of bound RNA and are of general interest to be taken into consideration in all cases of heterologous/homologous expressions of RNA-binding proteins avoiding the carryover of endogenous RNAs, which may interfere with further biochemical characterizations.

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Introduction

Selenocysteine (Sec) amino acid was discovered in the mid-1960 [1] as the 21th amino acid [2] present in the organisms of all three domains of life: bacteria, archaea, and eukarya. Being an additional amino acid residue to the canonical 20, it has a defined codon for incorporation. This codon is fixed in all selenocysteine-containing organisms as a UGA-Sec codon functionally differentiated from the normal UGA-Stop codon by the *cis*-signaling of the SECIS sequence (SElenoCysteine Incorporating Sequence) [3,4]. The initial steps of the selenocysteine synthesis pathway involve the charging of tRNA^{sec} (SELC) with serine by Seryl-tRNA Synthetase (SerRS), followed by the conversion of seryl-tRNA^{sec} into selenocysteyl-tRNA^{sec} by Selenocysteine Synthase (SELA, E.C. 2.9.1.1) [5]. SELA is a homodecamer of approximately 500 kDa exclusive of the bacteria domain, which converts seryl-tRNA^{sec} (Ser-tRNA^{sec}) into selenocysteyl-tRNA^{sec} (Sec-tRNA^{sec}) in a pyridoxal-5′-phosphate (PLP) dependent mechanism [5,6].

In eukarya and archaea the conversion from Ser-tRNA^{sec} to Sec-tRNA^{sec} occurs by a different pathway involving the sequential phosphorylation of serine and conversion to Sec-tRNA^{sec} by two enzymes, a phosphoseryl-tRNA^{sec} kinase (PSTK) and a Sep-tRNA:-Sec-tRNA synthase (SepSecS) [7].

Aiming to better understand the selenocysteine incorporation pathway, the *selA* gene was cloned into a modified expression vector and the protein was expressed in a specific *E. coli* strain (WL81460), whose tRNA^{sec} was deleted, allowing for the preparation of tRNA^{sec}-free SELA in large amounts. The native conformation and isoelectric point of purified protein were analyzed by biochemical methods and additional analyses were performed using fluorescence spectroscopy and circular dichroism, revealing that this expression method results in a protein consistent with the biologically active SELA for biochemical investigations.

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Materials and methods

Cloning, bacterial strains and growth conditions

The *selA* gene was amplified from *E. coli* K12 genomic DNA (DNAeasy Tissue Kit–Qiagen) by Polymerase Chain Reaction (PCR). Two oligodeoxynucleotide primers for PCR amplification, i.e. SELA-1 (5′-ACTGTATCATATGACAACCGAAACGCGTTTCCTCTATAG-3′) and SELA-2 (5′-TAGCTAAGCTTTCATTTCAACAACATCTCCAAAAAC CG-3′) with *Nde*I and *Hind*III restriction sites, respectively, were synthesized, based on the available sequence (Genebank accession number P0A821). PCR was carried out in a PTC-100 thermocycler (MJ Research Inc.) with 2.5 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer's conditions. The sample was subjected to 3 min denaturation at 96 °C followed by 30 cycles of denaturation at 96 °C for 0.5 min, annealing at 55 °C for 0.5 min and extension at 68 °C for 1.5 min.

The 1392pb product of *selA* gene was purified in a 1% agarose gel, digested with *Nde*I-*Hind*III restriction enzymes, cloned into *Nde*I-*Hind*III digested pET29a(+) vector and transformed into *E. coli* DH5α competent cells. *Escherichia coli* WL81460 strain, a derivate of FM433 strain carrying an in-frame deletion in the *selC* gene and kanamycin-resistant was a kind gift from Dr. August Böck [8]. This strain was modified for the expression of T7 RNA polymerase enzyme by a genome integration of the λDE3 prophage using Lambda DE3 Lysogenization kit (Stratagene) following the manufacturer's instructions. Plasmid pET29a(+) kanamycin resistance gene was substituted by ampicilin resistance with the pET-Duet-1 vector sequence using *Dra*III and *Sap*I restriction enzymes (Fermentas). The ampicillin-resistant pET29a(+) was transformed into *E. coli* WL81460(λDE3) competent cells and selected by 50 µg/mL ampicillin and 30 µg/mL kanamycin, in LB agar medium.

To overexpress the encoded SELA protein, 3 liters of *E. coli* WL81460(λDE3) cells were grown aerobically in LB selective medium to O.D.$_{600}$ of 1.0 at 37 °C. The culture was induced by the addition of 0.1 mM IPTG for 2 h at 37 °C and the cells were harvested by centrifugation at 4000*g* and stored frozen at −20 °C.

Purification and characterization of SELA protein

The frozen cells expressing the recombinant SELA were solubilized in buffer A (20 mM potassium phosphate pH 7.5, 10 µM PLP and 10 µg/mL lysozyme) and lised by ultrasound disruption (550 Sonic Dismembrator Fisher Scientific). The cell extract was clarified by centrifugation at 30,000*g* for 30 min and the supernatant was precipitated by the addition of ammonium sulfate to 25% saturation in ice and centrifuged at 20,000*g* for 20 min. The sediment was suspended in buffer A without lysozyme and desalted on a 5 mL Hi Trap Desalting column (GE) previously equilibrated with this buffer at a flow rate of 2.0 mL/min. The desalted fraction was applied to a 2.0 × 12.0 cm Hydroxyapatite ion exchange column (Bio-Rad) and SELA eluted as the flow through. The purified SELA was applied to a Hi Trap Q HP ion exchange column (GE) and eluted with 300 mM KCl in buffer A of a linear KCl gradient (0–1000 mM). The recombinant SELA protein was desalted again as described previously and the fractions collected were adjusted to 10% of glycerol and concentrated up to 8.0 mg/mL by ultrafiltration (10 kDa, Amicon).

Purified SELA was analyzed by size-exclusion chromatography in a Superdex 200 HL column (1.6 × 60 cm size) (GE) equilibrated with buffer A without PLP. The yellow color of the eluted SELA indicate that after purification the protein retains the bound PLP. Oligomerization states were verified by 4–15% native gradient gel electrophoresis in a *Phast System* (GE) using molecular weight standards containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (140 kDa). The concentration of pure SELA protein in the solution was determined spectrophotometrically at 280 nm by considering the predicted aromatic amino acids content and the predicted molar extinction coefficient (ε = 35410 M^{-1} cm^{-1}) obtained from *ProtParam* tools [9]. Isoelectric focusing was performed in the *Phast System* (GE) using precast *PhastGel IEF* gels (pI range 3–9) and pI markers according to the manufacturer's instructions.

Circular dichroism spectroscopy (CD) and Intrinsic fluorescence spectroscopy

Far UV CD spectra of recombinant SELA protein at 0.2 mg/mL in 20 mM potassium phosphate (pH 7.5) were recorded in a Jasco J-715 spectropolarimeter over a wavelength range of 190–250 nm by the signal averaging of 16 accumulations using a scanning speed of 100 nm/min, a spectral bandwidth of 1 nm, and a response of 0.5 s. The data analysis was performed using CDSSTR program and SP29 and SP37A were used as protein databases [10]. The thermal denaturation of SELA at 0.2 mg/mL concentration was performed in 20 mM potassium phosphate (pH 7.5) and 10 mM Dithiothreitol to promote denaturation by reduction of the three potential disulfides bounds. The thermal denaturation process was followed by measuring the decrease in the CD signal at 222 nm as a function of temperature in the range of 10 °C to 90 °C at 1° C min^{-1}. The measurements were recorded every 2 °C temperature increments after 90 s stabilization at each temperature.

Intrinsic fluorescence measurements were taken in a quartz 10 x 2 mm cuvette in an ISS-PC1 spectrofluorimeter at 25 °C using 0.1 mg/mL of SELA protein in 20 mM potassium phosphate, pH 7.5. The emission spectra were recorded in a 300–450 nm range using an excitation wavelength of 295 nm and a 305 nm filter cut-off in the emission. The results are an average of three independent experiments and each measurement was corrected for the buffer contribution.

Chemical crosslinking

Chemical crosslinkings were performed according to [11], with glutaraldehyde at 0.01, 0.02, 0.05, 0.07, 0.1, 0.2, 0.5, 1, 2, 3 and 5% and 30 µM of the recombinant SELA monomer during 3 h reactions at 25 °C. The reactions were analyzed by native and denaturing PAGE.

Results and discussion

Purification and oligomerization analysis of SELA protein

The initial experiments in our laboratory aiming to obtain the recombinant SELA from *E. coli* cells resulted in a preparation that contained a tightly bound nucleotide species, resistant to RNAse and DNAse treatment. Due to these results we have modified the expression of SELA to the *E. coli* WL81460 strain due to its deficiency in the production of SELA specific tRNA (seryl-tRNA^{sec}). For such purpose, we had to establish the λDE3 lysogen, generating the strain *E. coli* WL81460(λDE3) and change the antibiotic resistance of the pET29(+) construct from kanamycin to ampicillin. These characteristics allowed the production of a homogeneous sample of recombinant SELA, free of bound nucleotides for accurate biochemical and biophysical characterizations. We believe that this approach is relevant for the investigation of all tRNA-binding proteins for accurate biochemical characterizations.

The purified SELA protein from *E. coli* WL81460(λDE3) strains achieved high homogeneity after size exclusion chromatography using a Superdex 200 HL (1.6 x 60 cm) column (10,000 kDa to

600,000 kDa separation range). Recombinant SELA protein has a yellow color before and after size exclusion chromatography indicating the presence of a covalently bound PLP, as seen also by Forchhammer et al., 1991 [12]. The binding of seryl-tRNA^{sec} to the SELA PLP is essential to Sec synthesis.

The results showed a single peak that was eluted in the exclusion volume of approximately 48 mL, which corresponds to the decameric structure of SELA protein (Fig. 1A). SDS–PAGE analysis confirmed the purity of the sample and the approximate monomeric molecular mass of 50 kDa (Fig. 1A). The purified SELA protein oligomerization state was verified by native gradient gel electrophoresis (4% to 15%) (Fig. 1B). Both methods, i.e. Size Exclusion Chromatography and Native Gradient Gel Electrophoresis are consistent and show a calculated molecular mass of 600,000 kDa for the recombinant SELA protein, equivalent to a decamer. The experimental isoelectric point of 6.03 +/- 0.10 is consistent with the predicted value of 6.21. These results are in agreement with the data published by Forchhammer and co-workers [12] and Engelhart et al., [13], confirming that the recombinant SELA obtained by our procedure is equivalent to the native SELA.

The presence of endogenous RNA in the recombinant SELA at 2.0 mg/mL concentration was verified in the Qubit fluorometer (Invitrogen) using the Quant-iT RNA assay. A comparative analysis of recombinant SELA measured in the same protein concentration detected approximately 1 μg/mL RNA in SELA expressed in *E. coli* BL21(λDE3) while no signal was detected (below 20 ng/mL) in SELA expressed in *E. coli* WL81460(λDE3). This result is suggestive that the recombinant SELA binds tightly to endogenous SELC RNA.

Circular dichroism (CD) and thermal denaturation

To further characterize the recombinant SELA obtained, circular dichroism (CD) and Thermal Stability tests were performed. The far UV CD spectra at 25 °C displayed two negative bands at 222 and 208 nm and a positive band near 200 nm (Fig. 2A). The estimated α-helix and β-strands content ranged from 74% to 76%, and from 10% to 13%, respectively, the content of turns ranged from 2% to 4% and that of unrelated structures ranged from 9% to 12%. These results indicate that the purified *E. coli* SELA protein is formed mainly by α-helices with a significant β-strands core, consistent with the *Methanoccocus jannaschii* structure (GI: 78101334), in which the putative SELA of archaea is mostly formed by α-helices and has a β-strand core [12,14].

The temperature variation in the SELA secondary structure showed that the protein stability is maintained up to 45 °C. SELA has a thermal transition from 60 to 70 °C with a Tm of 66 °C and an evident plateau above 70 °C, in which all secondary structures are lost and the protein is completely denatured (Fig. 2B). These results indicate that the recombinant SELA, lacking the bound RNA, has a stable fold that withstands thermal degradation.

Intrinsic fluorescence spectroscopy

The tertiary structure of SELA protein from *E. coli* was also studied by tryptophan intrinsic fluorescence as an additional method to verify that the folding of the recombinant SELA is stable and consistent with the putative homologue from *M. jannaschii*. The SELA emission spectrum showed a maximum fluorescence wavelength at approximately 336 nm upon excitation at 295 nm (Fig. 2C). The high signal of fluorescence intensity obtained is an average of the contributions of the four tryptophan residues presented in each monomer of SELA protein and indicates that these residues are relatively buried in the structure of the protein.

Chemical crosslinking assays

Aiming at the characterization of the oligomeric assemblage of SELA, crosslinking experiments were conducted using the reagent glutaraldehyde in conjunction with denaturing and non-denaturing gel electrophoresis. The results (Fig. 3A and B) indicate that the crosslinking reaction occurred between SELA monomers. A strong band is visible with the expected molecular mass of 600 kDa (Fig. 3A) in the absence of glutaraldehyde. These results are consistent with our size exclusion chromatography data and those of Forchhammer, 1991 [12].

Native gel electrophoresis results (Fig. 3A) show that at glutaraldehyde concentrations above 1.0% three oligomeric states, smaller than the expected decamer (approximately 100, 200, and 300 kDa) are visible. The intensity of those oligomeric classes increases in function of the concentration of glutaraldehyde. These results are suggestive that SELA reaches the homodecameric structure by the stepwise addition of intermediate oligomeric

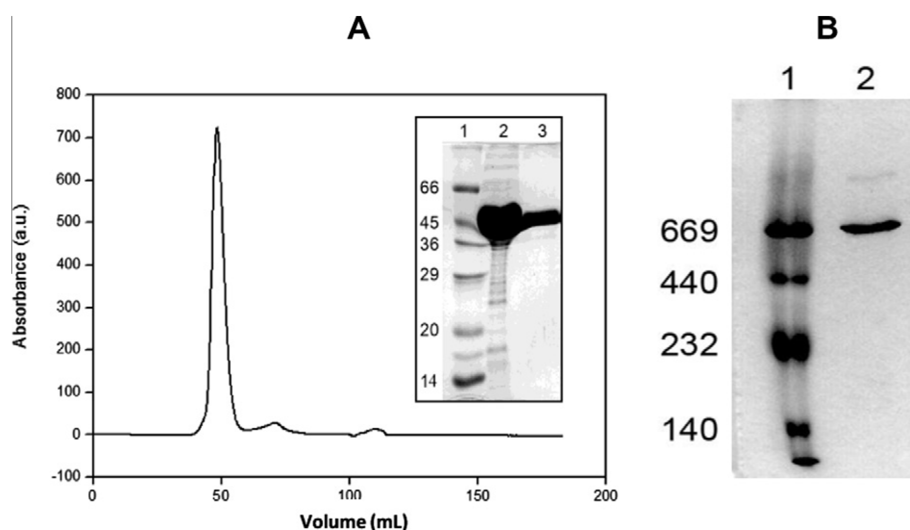

Fig. 1. Recombinant SELA purification protein using Superdex 200 HL (1.6 × 60 cm) column (GE). (A) Size Exclusion Chromatographic (SEC) profile of SELA protein monitored at 280 nm. Samples were analyzed by SDS–PAGE (15%). Lane 1, molecular-mass marker (kDa), Lane 2, Protein preparation applied to SEC, Lane 3, SELA protein eluted at approximately 50 mL. (B) Native gel electrophoresis of the purified recombinant SELA. Lane 1, molecular-mass marker (kDa), Lane 2, Recombinant SELA protein at 4 μM after Size Exclusion Chromatography.

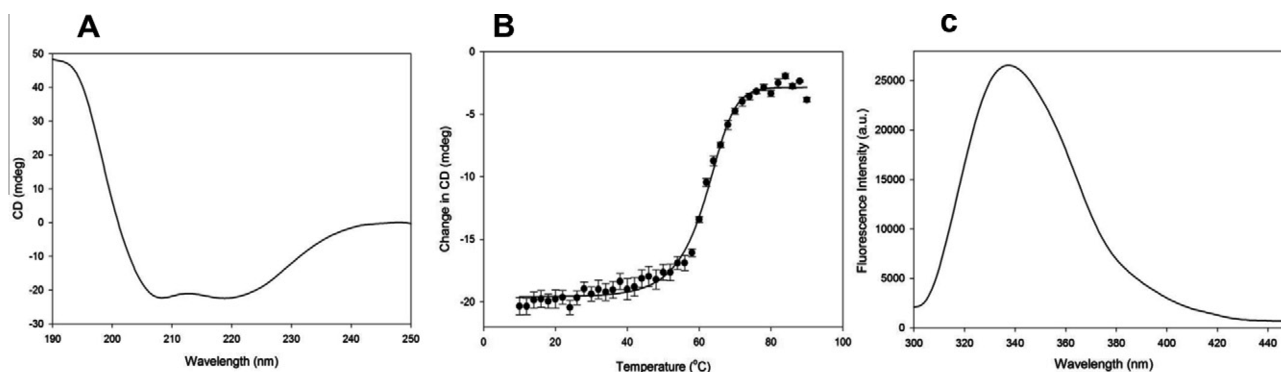

Fig. 2. Structural analysis of SELA protein. (A) CD spectra of SELA at 0.2 mg/mL in 20 mM potassium phosphate buffer (pH 7.5) at 25 °C. (B) Transition curve of the thermal denaturation of SELA protein monitoring the changes at 222 nm as a function of temperature (10–90 °C) with 0.2 mg/mL protein in buffer with 10 mM of DTT to avoid the interference of sulfide bonds during the denaturation process. The results are an average of three independent experiments. (C) Intrinsic fluorescence emission spectra of SELA protein at 25 °C using 0.1 mg/mL of SELA protein in 20 mM potassium phosphate, pH 7.5. The emission spectra in the 300–450 nm range were recorded using an excitation wavelength of 295 nm and a cutoff 305 nm filter in the emission.

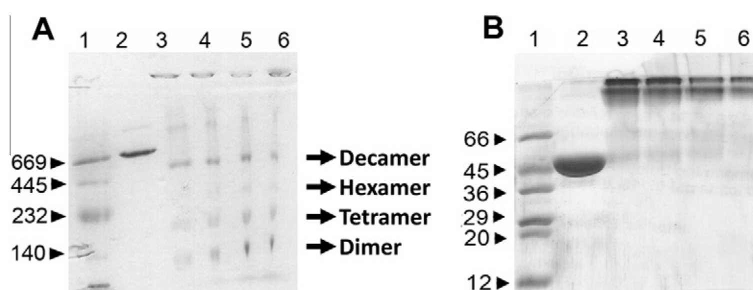

Fig. 3. Oligomeric assembly of SELA. (A) Native gel electrophoresis and B) SDS–PAGE, analysis of the recombinant SELA crosslinking with increasing concentrations of glutaraldehyde showing the sequential formation of oligomeric structures. Lanes 1, Molecular mass marker, Lanes 2, SELA without glutaraldehyde, Lanes 3 to 6, SELA with 1, 2, 3 and 5% glutaraldehyde show the formation of larger SELA complexes. The SDS–PAGE (B) confirms the absence of free SELA under the same crosslinking conditions under which SELA is present as a high molecular mass that does not enter the gel mech.

states, e.g., monomer–dimer–tetramer–hexamer–decamer, opposed to the transition from a monomer to a homodecamer. The SDS–PAGE analyses of the crosslinking reaction (Fig. 3B) are consistent with these results, revealing the absence of the free monomeric form.

Conclusions

Selenocysteine Synthase (SELA) from *E. coli* is a pyridoxal-5′-phosphate containing enzyme responsible for the formation of selenocysteyl-tRNA^{sec} from seryl-tRNA^{sec} and selenophosphate [15]. The expression of SELA in the WL81460(λDE3) strain of *E. coli* and the establishment of a purification protocol are of relevance since we have shown that the protein expressed in SELC containing *E. coli* cells binds and co-purifies endogenous RNA, probably tRNA^{sec}. Such a tRNA^{sec}-bound SELA protein may be heterogeneous in the number of tRNA molecules bound per decamer, compromising subsequent biochemical investigations. The analysis of the recombinant SELA, free of bound RNA is consistent with the results expected for the native protein by SDS–PAGE analysis, revealing a 50 kDa monomer. The native gradient gel electrophoresis demonstrated that a decamer is formed as a stable oligomeric state. The SELA biophysical analyses are consistent with the expected characteristics of *E. coli* SELA, regarding its isoelectric point and secondary structure elements content, as determined by circular dichroism studies. Two additional experiments, namely thermal denaturation assays and intrinsic fluorescence showed that the structure is compact and stably folded. Interestingly, the expression of the recombinant SELA in the SELC deficient *E. coli* strain WL81460(λDE3) allowed the observing the stepwise oligomeric assembly of the homodecamer. Our results of SELA characterization are consistent with the data previously published contributing to the establishment of a robust expression model for the enzyme free of bound endogenous tRNA^{sec}. The use of the previous expression methodology resulted in an RNA-bound recombinant SELA, which otherwise would interfere with the functional experimentation of the enzyme. These results are of general interest and should be taken into consideration in all cases of heterologous/homologous expressions of recombinant RNA-binding proteins to avoid the carryover of endogenous RNAs, which may interfere with further biochemical characterizations.

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