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Preparation of Selenocysteine-Containing Forms of Human SELENOK and SELENOS

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Abstract

Selenoprotein K (SELENOK) and Selenoprotein S (SELENOS) are the members of the endoplasmicreticulum-associated degradation (ERAD) complex, which is responsible for translocating misfolded proteins from the endoplasmic reticulum (ER) to the cytosol for degradation. Besides its involvement in the ERAD, SELENOK was shown to bind and stabilize the palmitoyl transferase DHHC6, and thus contributes to palmitoylation. SELENOK and SELENOS reside in the ER membrane by the way of a single transmembrane helix. Both contain an intrinsically disordered region with a selenocysteine (Sec) located one or two residues away from the C-terminus. Here, we describe the preparation of the Seccontaining forms of SELENOS and SELENOK. SELENOK, which contains no native cysteines, was prepared in an *E. coli* cysteine auxotroph strain by exploiting the codon and the insertion machinery of Cys for the incorporation of Sec. In contrast, the preparation of SELENOS, which contains functionally important cysteine residues, relied on *E. coli*'s native Sec incorporation mechanism.

Keywords

Selenoprotein; SELENOK; SELENOS; Membrane selenoproteins; Membrane enzymes; Selenocysteine; SelK; SelS

1. Introduction

Eight of the 25 human selenoproteins are integral membrane proteins [1]. Two of these, SELENOS (previously known as SelS [2]) and SELENOK (previously known as SelK [2]), participate in the degradation of misfolded proteins, a critical component of proteostasis [3]. The two proteins share a similar organization with a short luminal N-segment, a single pass transmembrane α -helix anchored to the ER, and a cytosolic intrinsically disordered region with the Sec one or two residues away from the C-terminus. Both the proteins bind to derlins, which are transmembrane proteins and essential constituents of the ERAD complex (Fig. 1) [4–6]. SELENOS recruits the valosin-containing p97, an ATPase that provides the energy to pull protein substrates out of the ERAD complex [7, 8]. It also interacts with additional p97 adaptors, substrate recruiters, and partners [9]. In contrast to SELENOS, SELENOK's specific role in the ERAD pathway is not yet known [1, 10]. In addition to its involvement in the ERAD, SELENOK, however, was shown to bind and stabilize the palmitoyl transferase DHHC6 [11]. SELENOK thus contributes to palmitoylation, i.e., the reversible attachment of fatty acids onto cysteine residues [12].

Since both SELENOS and SELENOK possess a transmembrane α -helix, their preparation requires their stabilization in a detergent environment that mimics the native cellular membrane [13]. Their expression is also nonstandard, as heterologous expression of membrane proteins is well known to be restricted by the host's limited membrane area, and by improper folding or lack of stability in the non-native membrane [14]. Furthermore, the two proteins are intrinsically disordered and as such they are prone to proteolytic cleavage in host organisms [15]. All these challenges were addressed by expressing both SELENOK and SELENOS in the robust host *E. coli* using a fused protein partner to increase solubility and prevent integration into *E. coli* membranes [10, 16–18]. For both the proteins the incorporation of Sec is essential to maintain their functional integrity [10, 11, 17, 18]. Because SELENOK contains no cysteine, but SELENOS has two cysteines, one of which is critical for its function, different strategies were employed to incorporate the single Sec in the respective protein. In cases of Cys-less proteins, such as SELENOK, there is no need to use the low efficiency, cellular Sec-insertion machinery to incorporate Sec. Instead, a higher yield can be obtained by exploiting the native Cys incorporation machinery for Sec insertion. To that end the Sec codon is mutated to Cys and a cysteine auxotroph *E. coli* strain is employed to prevent Cys biosynthesis [19]. The cysteine auxotroph is then grown in defined growth medium that is depleted of Cys and enriched in L-selenocystine [20]. Misloading the cysteinyl-tRNA with Sec instead of Cys leads then to successful Sec incorporated into the protein. To maximize the yield while minimizing the undesired incorporation of Cys the selenocystine is added after inducing expression. This also allows keeping Sec's concentration in the growth medium low in order to minimize the impact of selenocystine's cytotoxicity. However, this approach cannot be used for SELENOS because here the Sec is paired with a Cys and both must be incorporated in a site-specific manner for SELENOS to retain functionality. Therefore, a genetic incorporation method that harnesses *E. coli*'s native Sec incorporation machinery was used [16]. This requires that an RNA element, termed Sec insertion sequence (SECIS), is inserted 11 nucleotides after the UGA codon into the mRNA [21]. If this stem-loop structure occurs in the coding region of the mRNA, this element will dictate the amino acid sequence downstream from the Sec. However, in SELENOS the Sec is located at the C-terminus, and thus the SECIS element can be placed in the 3' untranslated region. In addition, co-expression with the *selA*, *selB*, and *selC* genes (encoding selenocysteine synthase, a Sec-specific elongation factor and tRNA^{Sec}, respectively) further increases the efficiency of Sec insertion [22]. It is also beneficial to induce protein expression at a late exponential phase, in which the level of release factor 2 is decreased [23]. The lower concentration of release factor 2 reduces the frequency of wrongful termination of protein translation at the UGA codon because of its misreading as a stop codon. This approach allowed the expression and purification of the cytosolic segment of SELENOS, which was abbreviated as cSELENOS [17, 18]. In contrast, the expression of full-length SELENOS at a late exponential phase led to a low yield. Strategies for the preparation of the full-length SELENOS involve native chemical ligation similar to the one described in the chapter about the preparation of SELENOM. In the case of cSELENOS, the genetic incorporation yielded a large fraction of truncated protein (over 95% of all proteins) in which the Sec was not integrated because protein translation was prematurely terminated. This truncated form, cSELENOS 188, differs from cSELENOS by only two amino acids. The truncated form contains a single cysteine and thus forms

intermolecular disulfide bonds between proteins. Fortunately, this leads to the aggregation of the truncated form, which greatly aids the purification of the homodimeric, Sec-containing cSELENOS via size exclusion chromatography. The expression and purification strategies for both cSELENOS and SELENOK are summarized in Fig. 2.

2 Materials

2.1 General Materials

1. LB Medium: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 2 g/L glucose.
2. Isopropyl- β -D-galactoside (IPTG).
3. Antibiotics: ampicillin, kanamycin, and chloramphenicol.
4. Protease inhibitors: phenylmethylsulfonyl fluoride (PMSF) and benzamidine.
5. Detergents: Triton X-100 and n-dodecyl- β -D-maltoside (DDM).
6. Ethylenediaminetetraacetic acid (EDTA).
7. Maltose.
8. D-desthiobiotin.
9. Chromatography media: Amylose resin (NEB), StrepTrap™ HP column, HiTrap™ SP HP column, HiPrep™ 16/60 Sephacryl S-100 HR size exclusion column, Superdex™ 200 10/300 GL size exclusion column, and HiTrap™ desalting column (all from GE Healthcare Life Sciences).

2.2 Stock Solution for the Defined Medium (Adapted from Studier's MDAG Medium [24])

1. 1 M MgSO₄: Dissolve 12 g MgSO₄ in 100 mL water and then autoclave.
2. 1000× trace metal: stock solutions of different trace metals should be made separately and mixed together before use. The specific components of the trace metal solutions are listed in Table 1. FeCl₃ is dissolved in 0.12 M HCl. All other metals are dissolved in water.
3. 40% Glucose Solution: dissolve 40 g glucose in 72 mL water and adjust the final volume to 100 mL, then perform filter sterilization using steriflip (with a 0.22 μ m pore size).
4. 25% Aspartate Solution: dissolve 25 g aspartic acid and 8 g NaOH in 84 mL water and adjust the final volume to 100 mL and the pH to 7.0, then autoclave.
5. 50× M (Salt Solution): dissolve 17.75 g Na₂HPO₄, 17 g KH₂PO₄, 13.4 g NH₄Cl, and 3.55 g Na₂SO₄ in 80 mL water and adjust the final volume to 100 mL and the pH to 7.0, then autoclave.
6. 17 Amino Acids Mixture (except cysteine, tyrosine, methionine): 10 mg of each amino acid/mL. To make 100 mL stock of the amino acid solution, dissolve 1 g of each amino acid (Na-Glu, Asp, Lys-HCl, Arg-HCl, His-HCl, Ala, Pro, Gly,

Thr, Ser, Gln, Asn, Val, Leu, Ile, Phe, Trp) in water. The amino acid solution is sterilized using steriflip (with a 0.22 μ m pore size).

7. 2.5% Methionine Solution: dissolve 2.5 g methionine in 100 mL water, then autoclave.
8. 1000 $\times$ Vitamin Stock: prepare the mixture from individual vitamin stock solutions and then sterilized it using steriflip (with 0.22 μ m pore size). The specific components of individual vitamin solutions are listed in Table 2.

2.3 Defined Growth Medium

Mix together different amount of stock solutions (as tabulated in Table 3) with autoclaved water to make defined growth medium immediately prior to use. See Table 3 for specific formulations.

2.4 Buffers for SELENOK Purification

1. Lysis buffer: 50 mM Na₃PO₄, 200 mM NaCl, 1 mM EDTA, 0.2% (v/v) Triton X-100, 1 mM PMSF, and 1 mM benzamidine, pH 7.5.
2. Amylose wash buffer: 50 mM Na₃PO₄, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 0.067% DDM.
3. Amylose elution Buffer: 50 mM Na₃PO₄, 200 mM NaCl, 20 mM maltose, 1 mM EDTA, and 0.067% DDM, pH 7.5.
4. StrepTrap wash buffer: 50 mM Na₃PO₄, 200 mM NaCl, and 0.067% DDM, pH 7.5.
5. StrepTrap elution buffer: 50 mM Na₃PO₄, 200 mM NaCl, 2.5 mM D-desthiobiotin, and 0.067% DDM, pH 7.5.
6. Trypsin digestion buffer: 50mMTris-HCl, 20mMNaCl, and 0.067% DDM, pH 8.0.

2.5 Buffers for cSELENOS Purification

1. Lysis buffer: 50 mM Na₃PO₄, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mM benzamidine, pH 7.5.
2. Amylose wash buffer and SP column buffer A: 50 mM Na₃PO₄, 200 mM NaCl, and 1 mM EDTA, pH 7.5.
3. Amylose elution buffer: 50 mM Na₃PO₄, 200 mM NaCl, 20 mM maltose, and 1 mM EDTA, pH 7.5.
4. SP column buffer B: 50 mM Na₃PO₄, 1 M NaCl, and 1 mM EDTA, pH 7.5.
5. Trypsin digestion buffer: 50mMTris-HCl, and 20mMNaCl, pH 8.0.

3 Methods

3.1 Expression and Purification of SELENOK

3.1.1 Clone SELENOK U92C into pMAL-c5X Expression Vector

1. The gene of *Homo sapiens* SELENOK (UniProtKB-Q9Y6D0) is codon optimized for expression in *E. coli*.
2. The expression vector pMAL-c5X is designed to produce fusion proteins with the cytoplasmic maltose binding protein (MBP) and was obtained from New England Biolabs (NEB).
3. In order to facilitate purification, a hexahistidine tag was introduced into MBP between I3 and E4 by standard site directed mutagenesis [16].
4. A linker sequence (NSSS) and a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) were inserted immediately after MBP using *Ava*I and *Bam*HI restriction sites. The TEV cleavage site was followed by a Met to introduce an *Nco*I restriction site (CCATGG).
5. SELENOK U92C was cloned into this modified pMAL-c5X vector after the TEV protease cleavage site using *Nco*I and *Bam*HI restriction sites.
6. To assist in purification, an eight amino acids StrepII tag (WSHPQFEK) was inserted between the TEV protease cleavage site and SELENOK U92C. This vector was named pMALMBP-SELENOK U92C.
7. The final pMAL-MBP-SELENOK U92C vector is used to transform the *E. coli* cysteine auxotroph strain BL21(DE3) with the genotype *selB::kan cys51E*, referred to as BL21 (DE3)cys (see **Note 1**) [19].

3.1.2 Expression of SELENOK

1. For optimal yield, freshly transform pMAL-MBP-SELENOK U92C expression vector into BL21(DE3)cys cells. Plate the transformed cells evenly onto LB agar plates supplemented with 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 50 µg/mL L-cysteine, and incubate overnight at 37 °C (see **Note 1**).
2. Pick up 2–3 colonies from the plate to grow overnight in 200 mL LB medium supplemented with 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 50 µg/mL L-cysteine at 37 °C.
3. The next morning, transfer 10 mL of overnight culture to 1 L fresh LB medium supplemented with 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 50 µg/mL L-cysteine.

The optical density (OD) at 600 nm wavelength of the growth medium after the addition of overnight culture should not exceed 0.1.
4. Grow cells at 37 °C while shaking at 250 rpm.
5. Harvest cells when the OD at 600 nm reaches 1.5 (about 6 h) by centrifugation at 4000 × *g* for 20 min at 4 °C.

6. After discarding the supernatant, resuspend the cell pellet in the centrifuge bottles with 100 mL of ice cold, autoclaved water per bottle. This step is necessary for changing the growth medium into a defined mixture and removing residual Cys in the medium. The resuspension process should be carried out on ice.
7. Centrifuge for 10 min at $4000 \times g$ at 4 °C.
8. Resuspend the cell pellet with 100 mL cold water on ice as detailed in **step 6** (see **Note 2**).
9. Centrifuge for 15 min at $4000 \times g$ at 4 °C and discard the supernatant.
10. Collect the cell pellet after centrifugation. Dissolve the pellet in 100 mL of defined medium on ice.
11. Transfer 100 mL of the resuspended cell pellet into 900 mL of defined medium, which has been supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin.
12. Grow cells at 37 °C for 30 min and then decrease the temperature to 18 °C. Allow the cells to recuperate at that temperature for an additional 30 to 60 min. The OD at 600 nm should be close to 1.5 at the end of the incubation period.
13. Add 0.5 mM IPTG and 200 µM L-selenocystine to the growth medium to induce expression (see Note 3).
14. Grow cells overnight at 18 °C while shaking at 250 rpm (between 20 and 24 h).
15. Harvest cells by the centrifugation at $4000 \times g$ for 20 min at 4 °C and decant the supernatant.
16. Immediately flash freeze the cells in liquid N₂, unless you intend to purify the protein straightaway.

3.1.3 Purification of SELENOK—SELENOK purification is carried out using two chromatographic steps (Fig. 2a). First, the fusion protein MBP-SELENOK is purified by amylose affinity chromatography. Then, the fusion partner MBP is cleaved off by TEV protease. Finally, MBP and TEV protease are removed from SELENOK by strep tag affinity purification.

1. Resuspend the cell pellet with 30–50 mL of lysis buffer (add PMSF just before you lyse the cells). Lyse the cells using a high pressure homogenizer (e.g., EmulsiFlex-C5, Avestin) operating continuously at 9000 psi performing at least 5 cycles. Keep the homogenizer on ice to minimize SELENOK denaturation (see **Notes 4** and **5**).
2. Immediately afterward centrifuge the cell lysate for 60 min at $12,000 \times g$ or higher. Collect the supernatant.
3. To prepare the amylose affinity column preequilibrate the column with 5 column volumes (CV) of amylose loading buffer. Subsequently, load the column with the

supernatant and collect the flow-through. The binding capacity for amylose resin is around 3 mg MBP-SELENOK fusion protein per milliliter of resin. For 1 L of the medium, 10 mL of amylose resin will suffice (*see Note 6*).

4. Wash the amylose affinity column with 10 CV of amylose washing buffer.
5. Elute MBP-SELENOK fusion protein with 3 CV amylose elution buffer.
6. Estimate the concentration of eluted fusion protein MBPSELENOK by measuring its absorbance at 280 nm and using an extinction coefficient of 88,810 $\text{M}^{-1} \text{cm}^{-1}$.
7. Now perform TEV protease cleavage of MBP-SELENOK. The molar ratio of TEV protease to MBP-SELENOK fusion protein should be around 1:20. Add the desired amount of TEV protease to the eluted protein (in elution buffer). Let the TEV cleavage reaction occur overnight (12–14 h) at 4 °C while gently stirring (*see Notes 7–9*).
8. Evaluate the purity and percentage of successful TEV cleavage by running a 16% Tris-Glycine gel. A typical result is shown in Fig. 3.
9. If the TEV cleavage is successful, proceed to run a 5 mL Strep-Trap™ HP column to remove MBP and TEV. The binding capacity of StrepTrap™ HP column is around 7 mg of protein per milliliter of resin.
10. Before loading the StrepTrap™ HP column with the TEV cleavage mixture, remove aggregates by centrifuging the mixture at $10,000 \times g$ for 10 min or filtering using a 0.22 μm syringe filter.
11. To prepare the StrepTrap™ HP column preequilibrate it with 5 CV of StrepTrap washing buffer.
12. Load the supernatant onto the StrepTrap™ HP column and collect the flow-through.
13. Wash the StrepTrap™ HP column with 10 CV of StrepTrap washing buffer.
14. Elute SELENOK using 3 CV StrepTrap elution buffer. The yield of SELENOK is around 2 mg proteins from 1 L defined growth medium.
15. Estimate the purity of the protein by SDS-PAGE. Typically, it is higher than 95% at this point (*see Note 10*). A typical result is shown in Fig. 4.
16. If necessary, perform size exclusion chromatography. To that end, concentrate SELENOK to 5 mg/mL and then filter the sample using a 0.22 μm syringe filter. Load the sample onto a HiPrep™ 16/60 Sephacryl S-100 HR size exclusion column or a Superdex™ 200 10/300 GL size exclusion column and elute with amylose wash buffer. The molecular weight of the SelK DDM complex is around 90 kDa. Collect the fractions containing SELENOK and check the purity by running a 16% Tris-Glycine SDS-PAGE.
17. Approximate the Sec incorporation ratio by intact protein mass spectrometry (*see Notes 11 and 12*). To this end, first reduce SELENOK (10 μM) by adding 5 mM

dithiothreitol (DTT) and incubate at room temperature for 20 min. Inject the protein sample into the mass spectrometer (e.g., a Xevo G2-S QTOF, Waters), which is coupled with a reverse phase HPLC column such as an ACQUITY UPLC Protein BEH C4 reverse-phase column (300 Å, 1.7 µm, 2.1 mm × 150 mm). Use an acetonitrile gradient from 5 to 95% with 0.1% formic acid, over a running time of 5 min at a constant flow rate of 0.5 mL/min. Acquire the data from *m/z* 350 to 2000, at a rate of 1 s/scan. Typically, about 70–80% of the protein contains Sec at position 92 while the remaining contains Cys. A typical, deconvoluted mass spectrum is shown in Fig. 5 (see **Note 13**).

18. Concentrate SELENOK (using for example an Amicon ultra centrifugal filter unit with a membrane molecular weight limit of a 100 kDa) to 5 mg/mL and then add 10% glycerol and flash freeze in liquid N₂. For long-term storage, keep the protein at 80 °C (see **Note 14**).

3.1.4 Tandem Mass Sequencing of SELENOK—To confirm the presence of Sec in SELENOK, it is digested by trypsin protease and subjected to peptide sequencing.

1. Exchange the buffer of the SELENOK solution into the trypsin digestion buffer using a 5 mL HiTrapTM desalting column.
2. Reduce SELENOK by adding 5 mM DTT and incubate at room temperature for 20 min.
3. Boil at 95 °C for 5 min. Cool down the sample to room temperature, add 20 mM iodoacetamide (IAM), and incubate for 20 min to alkylate the Sec.
4. Add 0.5 µg trypsin into 100 µL of alkylated SELNOK (1 mg/ mL) from **step 3** and digest at 37 °C for 16 h.
5. Sequence the Sec-containing peptide using, for example, a Q Exactive Orbitrap interfaced with Ultimate 3000 LC system. 5 µL of trypsin-digested protein from step 4 are loaded on an Ace ultracore super C18 reverse-phase column (75 mm × 2.1 mm) via an autosampler. The samples are analyzed using a 45 min gradient going from 5% B to 95% B at a flow rate of 0.2 mL/min. Solvent A is water containing 0.1% formic acid and solvent B is acetonitrile containing 0.1% formic acid. A typical result is shown in Fig. 6.

3.2 Expression and Purification cSELENOS

3.2.1 Design of Constructs for Expression

1. The gene of *Homo sapiens* SELENOS (UniProtKB-Q9BQE4) is codon optimized for expression in *E. coli*. The Sec codon must remain UGA.
2. The expression vector pMAL-c5X, designed to produce fusion proteins with the MBP, was obtained from NEB.
3. A hexahistidine tag was introduced into MBP between I3 and E4 to facilitate purification by standard site directed mutagenesis [16].

4. A linker sequence NSSS and TEV protease cleavage site (ENLYFQS) was inserted immediately after MBP using *Ava*I and *Bam*HI restriction sites. The TEV cleavage site was followed by a Met to introduce an *Nco*I restriction site (CCATGG).
5. The cytoplasmic portion of SELENOS (residues 52–189, abbreviated as cSELENOS) was cloned into this modified pMAL-c5X vector using *Nco*I and *Bam*HI sites. To allow Sec encoding, an *E. coli* formate dehydrogenase H SECIS element was also included immediately after the stop codon [25]. This vector was named pMAL-MBP-cSELENOS.
6. The plasmid of pMAL-MBP-cSELENOS is used for cotransformation of *E. coli* BL21(DE3), along with the pSUABC plasmid (see Note 15) expressing *E. coli* SelA, SelB, and SelC under the control of their endogenous promoters [22].

3.2.2 Expression of cSELENOS

1. Cotransform pMAL-MBP-cSELENOS and pSUABC plasmids into *E. coli* BL21(DE3) and plate the transformed cells evenly onto LB agar plates supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, and incubate overnight at 37 °C (see Note 16).
2. Pick up 2–3 colonies from the agar plate to grow overnight in the 200 mL LB medium supplemented with 100 µg/mL ampicillin with 34 µg/mL chloramphenicol at 37 °C.
3. The following morning, transfer 10 mL overnight culture to the 1 L defined medium supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The OD at 600 nm of the medium after the addition of overnight culture should not exceed 0.1.
4. Grow cells at 37 °C while shaking at 250 rpm.
5. Lower the growth temperature to 18 °C when the OD at 600 nm reaches 2.0. Then let the cells recover for 30–60 min at 18 °C.
6. To induce expression, add 0.5 mM IPTG, 10 µM Na₂SeO₃, and 100 µg/mL L-cysteine to the growth medium. A typical OD at the point of induction is 2.2–2.4.
7. Grow cells at 18 °C while shaking at 250 rpm for 20–24 h.
8. Harvest cells by a 15 min centrifugation at 5000 × *g* at a temperature of 4 °C and subsequently discard the supernatant.
9. Immediately freeze cells in liquid N₂, unless you intend to purify the protein straightaway.

3.2.3 Purification of cSELENOS—cSELENOS purification is carried out using three chromatographic steps (Fig. 2b). First, the fusion protein MBP-cSELENOS is isolated by amylose affinity chromatography. Next, the fusion partner MBP is cleaved off by TEV protease. MBP, TEV, and aggregated truncated SELENOS (cSELENOS 188) are then removed by cation exchange chromatography. Finally, the purity with respect to cSELENOS

is increased by separating it from the truncated form, cSELENOS 188, using size exclusion chromatography.

1. Resuspend the cell pellet with 30–50 mL of lysis buffer (add PMSF just before you lyse the cells). Lyse the cells using a high pressure homogenizer (EmulsiFlex-C5, Avestin) operating continuously at 9000 psi for at least 5 cycles. Keep the homogenizer on ice to minimize cSELENOS denaturation.
2. Immediately centrifuge the cell lysate for 60 min at $12000 \times g$ and collect the supernatant.
3. To prepare the amylose affinity column preequilibrate it with 5 CV of amylose loading buffer. Subsequently, load the supernatant onto the column and collect flow-through. The binding capacity for amylose resin is typically around 7 mg MBP-cSELENOS fusion protein per milliliter of resin. For 1 L of the medium, 30 mL of amylose resin should thus suffice.
4. Wash the amylose affinity column with 10 CV of amylose washing buffer.
5. Elute MBP-cSELENOS with 3 CV of amylose elution buffer.
6. Estimate the concentration of the eluted MBP-cSELENOS by measuring the UV absorbance at 280 nm using an extinction coefficient of $81,945 \text{ M}^{-1} \text{ cm}^{-1}$.
7. Cleave MBP-cSELENOS using TEV protease. The molar ratio of TEV protease to MBP-cSELENOS fusion protein should be around 1:20. Add the desired amount of TEV protease to the eluted protein (in elution buffer). Perform TEV cleavage reaction overnight (12–14 h) at 4°C while gently stirring.
8. Evaluate the purity and success of TEV cleavage by running a reducing 16% Tris-Glycine gel. A typical result is depicted in Fig. 7.
9. When the cleavage by the TEV protease is complete, proceed to remove MBP and TEV protease using a 5 mL HiTrapTM SP HP column. The binding capacity for HiTrapTM SP HP column is about 40 mg cSELENOS per milliliter of resin.
10. Centrifuge the TEV cleavage mixture at $10,000 \times g$ for 10 min or filter it using a $0.22 \mu\text{m}$ syringe filter prior to loading it onto the HiTrapTM SP HP column.
11. To prepare the HiTrapTM SP HP column preequilibrate it with 5 CV of SP column buffer A.
12. Load the supernatant onto the HiTrapTM SP HP column and collect the flow-through.
13. Wash the HiTrapTM SP HP column with 10 CV of SP column buffer A.
14. Elute cSELENOS with a gradient of SP column buffer B from 0 to 100% over 15 CV.
15. Evaluate the purity of different fractions by nonreducing 16% Tris-Glycine SDS-PAGE. A typical result is shown in Fig. 8. Note that cSELENOS elutes at a lower

salt concentration than the truncated form cSELENOS 188 (see **Notes 17** and **18**).

16. Approximate the ratio of cSELENOS to cSELENOS 188 by intact protein mass spectrometry. First, reduce cSELENOS (10 μ M) by adding 5 mM DTT and incubate at room temperature for 20 min. Inject the protein sample into the mass spectrometer (e.g., a Xevo G2-S QTOF, Waters), which is coupled with a reverse phase HPLC column such as an ACQUITY UPLC Protein BEH C4 reverse-phase column (300 Å, 1.7 μ m, 2.1 mm $\times$ 150 mm). Use an acetonitrile gradient from 5 to 95% with 0.1% formic acid, over a running time of 5 min at a constant flow rate of 0.5 mL/min. Acquire the data from m/z 350–2000, at a rate of 1 s/scan.
17. Combine fractions that contain cSELENOS, concentrate them to 5 mg/mL, and filter the sample with a 0.45 μ m syringe filter. Load the sample onto a HiprepTM 16/60 Sephacryl S-100 HR size exclusion column and elute with SP column buffer A. Collect fractions containing the cSELENOS and evaluate the purity by 16% Tris-Glycine SDS-PAGE. cSELENOS is usually eluted at a later time than the truncated form cSELENOS 188. A typical result is depicted in Fig. 9. About 0.6 mg of cSELENOS can be obtained from 1 L of growth medium (see Note 19).
18. Approximate the ratio of cSELENOS to cSELENOS 188 in different fractions by mass spectrometry as detailed in **step 16**. typical result is shown in Fig. 10 (see also **Notes 20** and **21**).
19. Collect all fractions containing cSELENOS and concentrate to 5 mg/mL and then add 10% glycerol and flash freeze with liquid N₂. For long-term storage, store the protein at –80 °C.

3.2.4 Tandem Mass Sequencing of cSELENOS—To confirm the presence of Sec in cSELENOS, it is digested by trypsin protease and subjected to peptide sequencing.

1. Exchange cSELENOS into the trypsin digestion buffer using a 5 mL HiTrapTM desalting column.
2. Reduce cSELENOS by adding 5 mM DTT and incubate the mixture at room temperature for 20 min.
3. Boil at 95 °C for 5 min. Cool down the sample to room temperature, add 20 mM IAM, and incubate for 20 min to alkylate the Sec and Cys.
4. Add 0.5 μ g trypsin into 100 μ L alkylated cSELENOS (1 mg/ mL) solution from **step 3** and digest at 37 °C for 9–10 h.
5. Sequence the Sec-containing peptide using a Q Exactive Orbi-trap interfaced with Ultimate 3000 LC system. Load 5 μ L of trypsin-digested protein on an Ace ultracore super C18 reverse-phase column (75 mm $\times$ 2.1 mm) via an autosampler. The samples are analyzed using a 45 min gradient going from 5% B to 95% B at a flow rate of 0.2 mL/min. Solvent A is water containing 0.1%

formic acid and solvent B is acetonitrile containing 0.1% formic acid. A typical result from this procedure is shown in Fig. 11.

4 Notes

Notes Regarding SELENOK Expression and Purification

1. BL21(DE3)cys cells require 25 µg/mL kanamycin and 50 µg/ mL L-cysteine for growth. The strain can be requested from Dr. Marie-Paule Strub at the NIH laboratory of molecular biophysics. Other cysteine auxotroph *E. coli* strains are available from the *E. coli* Genetic Stock Center. A regular BL21(DE3) *E. coli* strain can also be employed instead of BL21(DE3)cys, but the resulting sample contains equal amounts of cSELENOK and cSELENOK U92C.
2. Washing the cells with water when transferring from LB to defined growth medium is essential for removing residual Cys and reaching high Sec incorporation levels.
3. L-selenocystine is not soluble at neutral pH. It could be dissolved in 1 M HCl or 1 M NaOH to prepare a 100 mM stock. Alternatively, the powder can be directly added to the growth medium.
4. Add the protease inhibitor PMSF just before cell lysis. PMSF is easily degraded in aqueous solutions, so the stock solution is prepared in methanol or isopropanol. Beware of toxicity.
5. SELENOK has autoproteolytic activity and therefore all the purification steps should be carried out on ice or at 4 °C.
6. Save all fractions (flow-through, wash out, and elution) until you have verified by gel that the binding capacity of the column was not exceeded. If you find that there is fusion protein in fractions other than the elution, estimate the amount and rerun **steps 3–8** of Subheading 3.1.3.
7. TEV protease was prepared in house using a pMHTDelta238 plasmid following published procedures [26]. The plasmid was purchased from the Protein Structure Initiative: Biology Materials Repository.
8. If cloudiness is observed in the TEV cleavage mixture, remove aggregates by centrifuging the mixture at $10,000 \times g$ for 10 min or filtering using a 0.22 µm syringe filter.
9. Following cleavage of the fusion protein by TEV protease, SELENOK retains the sequence GWSHPQFEK in its N-terminus.
10. Following purification there may be a small amount of two truncated forms of SELENOK: one form exhibiting a truncation between H79 and L80 with a molecular weight of 10,128 Da and another form that terminates even earlier between P66 and P67 with a molecular weight of 8647 Da. Both truncated proteins appear to be caused in vivo by protease activity in *E. coli*.

11. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is the preferred method to quantitatively determine the concentration of selenium in the protein sample.
12. The ratio of SELENOK to SELENOK U92C is estimated from their relative intensities in the mass spectrum. For reliable estimation the ion counts must be kept well below the saturation level of the mass detector.
13. If Sec incorporation is low, the most likely reason is that residual Cys is not completely removed when the cells are transferred from the LB medium to defined medium or that the cells were not allowed sufficient time to consume the internal Cys pool. To improve the ratio of Sec over Cys incorporation the concentration of L-selenocystine in the defined medium can be increased up to 300 mM; however this will reduce yield.
14. When concentrating membrane proteins avoid sharp gradients in detergents and proteins. Frequently mix the concentrate in concentrator devices. Do not exceed a concentration of 8 mg/ mL.

Notes Regarding cSELENOS Expression and Purification

15. The pSUABC plasmid is available upon request from Prof. E. S. J. Arner at the Karolinska Institutet [22, 27].
16. The pMAL-MBP-cSELENOS and pSUABC plasmids can be co-transformed into electrocompetent BL21(DE3) cells following standard protocols for electro transformation. Alternatively, it can be done sequentially by transforming pSUABC plasmid into chemically competent BL21(DE3) cells first, making the cells containing pSUABC plasmid to be chemically competent and then transforming them with the pMALMBP-cSELENOS plasmid [27].
17. cSELENOS 188 tends to aggregate in the absence of reducing agents because of the formation of intermolecular disulfide bonds. It binds the HiTrapTM SP HP cation exchange column tighter and thus requires for elution at higher concentration of salt than cSELENOS.
18. Only 5% of the total expressed protein is the cSELENOS. After the purification by ion exchange chromatography, cSELENOS can be enriched up to 20% of the total protein.
19. We found that compared to the TB growth medium, the use of the LB medium results in a higher yield.
20. Since cSELENOS 188 aggregates it either elutes through the size exclusion column's void volume or runs ahead of the cSELENOS. Consequently, cSELENOS can be separated from the truncated form cSELENOS 188 based on size and can be enriched up to 70–90% after size exclusion chromatography.
21. Only cSELENOS, but not cSELENOS 188, is enzymatically active. Thus, it is possible to test its presence in different fractions by using an insulin reductase assay instead of employing mass spectrometry [17].

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Fig. 1. SELENOK and SELENOS interact with multiple proteins in the ERAD complex to coordinate degradation of misfolded proteins

Fig. 2. Schematics illustrating the strategies for expression and purification of SELENOK and SELENOS. **(a)** SELENOK is expressed by supplementing the growth medium with selenocystine and purified by amylose and StrepTrap™ HP affinity columns. **(b)** cSELENOS is expressed using Sec genetic incorporation machinery and purified by amylose, HiTrap™ SP cation exchange, and HiPrep™ 16/60 Sephacryl S-100 HR size exclusion columns

Fig. 3. Purification of MBP-SELENOK by amylose affinity chromatography and its subsequent cleavage by TEV protease as monitored by 16% reducing Tris-Glycine SDS-PAGE

Fig. 4. Purification of SELENOK by StrepTrap™ HP column monitored by 16% nonreducing Tris-Glycine SDS-PAGE

Fig. 5. Deconvoluted mass spectrum of reduced SELENOK. In addition to SELENOK (11,609 Da calculated weight), also a small amount of SELENOK U92C with a calculated molecular mass of 11,563 Da is present

Fig. 6.

Identification of Sec in SELENOK. (a) Mass spectrum of Sec-containing peptide from SELENOK with the sequence GPSPPPMAGGU(IAM)GR, where IAM stands for iodoacetamide and U for Sec. The pattern is indicative of selenium isotopic abundance. The theoretical M/Z of the triply charged peptide GPSPPPMAGGU(IAM)GR is shown on *right* and the experimental data on *left*. (b) MS/MS spectrum of the peptide from panel a confirms Sec is present in this peptide

Fig. 7. Purification of MBP-cSELENOS by amylose affinity chromatography and subsequent cleavage by TEV protease as monitored by 16% nonreducing TrisGlycine SDS-PAGE. *Lane 1:* Amylose column elution; *Lane 2:* TEV protease cleavage mixture

Fig. 8.

Purification of cSELENOS by cation exchange chromatography. (a) Elution profiles of cSELENOS from a HitrapTM SP HP ion exchange column under nonreducing conditions. The mixture of MBP-cSELENOS following cleavage by TEV protease was loaded to remove the TEV protease and MBP. Only cSELENOS and cSELENOS188 bind the column. Peak 1, which elutes with 300 mM NaCl, contains the majority of the Sec-containing form. Peak 2, which elutes with 600 mM NaCl, contains cSELENOS 188. cSELENOS 188 bind tighter because it forms oligomers by mixed disulfide bonds. (b) 16% nonreducing Tris-Glycine SDS PAGE analysis of cSELENOS/cSELENOS188 mixtures following separation by Hitrap SP FF column. The gel on the *left* shows different fractions under the first peak; the gel on the *right* shows different fractions under the second peak. Peak 1 contains less of the higher oligomeric forms

Fig. 9.

Separation of the cSELENOS from cSELENOS 188 by size exclusion chromatography. **(a)** Elution profiles of cSELENOS from a HiprepTM 16/60 Sephacryl S-100 HR size exclusion column under nonreducing conditions. Peak 2, which eluted last, contains cSELENOS. **(b)** SDS-PAGE analysis of cSELENOS/cSELENOS 188 mixtures following separation by HiprepTM 16/60 Sephacryl S-100 HR size exclusion chromatography. Lanes 1 and 2 were run under nonreducing conditions: lane 1, cSELENOS first peak; lane 2, cSELENEOS second peak; lane M, protein molecular mass standards (the molecular mass in kilodaltons is noted on the right). Lanes 3 and 4 were run under reducing conditions: lane 4, cSELENOS first peak; lane 5, cSELENOS second peak. The image was modified from ref. 17

Fig. 10. Deconvoluted mass spectrum of reduced cSELENOS after the purification by HiprepTM 16/60 Sephacryl S-100 HR size exclusion column. cSELENOS was enriched from cSELENOS 188 to over 50%. The calculated molecular mass of cSELENOS 188 is 15,093 Da and that of cSELENOS is 15,300 Da

Fig. 11.

Identification of Sec in SELENOS. (a) Mass spectrum of Sec-containing peptide from cSELENOS with the sequence RGPSSGGU(IAM)G, where IAM stands for iodoacetamide and U for Sec. The pattern is indicative of selenium isotopic abundance. The theoretical M/Z of the triply charged peptide RGPSSGGU(IAM)G is shown on *right* and the experimental data on *left*. (b) MS/MS spectrum of the peptide from panel a confirms Sec is present in this peptide

Table 1

Trace metal solutions necessary to prepare 100 mL of 1000× trace metal mixture [24]

Components	Stock concentration (M)	Volume to add (mL)
FeCl ₃	0.1	50
CaCl ₂	1	2
MnCl ₂	1	1
ZnSO ₄	1	1
CoCl ₂	0.2	1
CuCl ₂	0.1	2
NiCl ₂	0.2	1
Na ₂ MoO ₄	0.1	2
Na ₂ SeO ₃	0.1	2
H ₃ BO ₃	0.1	2
H ₂ O	–	36

Table 2

Vitamin solutions for preparing 100 mL of 1000× vitamin mixture [24]

Components	Stock concentration (mM)	Volume to add (mL)
Nicotinic acid	10	2
Pyridoxine	10	2
Thiamine	10	2
<i>p</i> -Aminobenzoic acid	10	2
Pantothenate	10	2
Folic acid	0.1	5
Riboflavin	0.1	5
Vitamin B12	5	4
H ₂ O	–	76

Table 3

Composition of 1 L of defined growth medium

Medium component	Volume to add (mL)
1 M MgSO ₄	2
1000× trace metal	0.2
40% Glucose solution	10
2.5% Methionine solution	8
25% Aspartate solution	12
17 amino acids	20
50× M	20
1000× vitamin	1
H ₂ O	926.8