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Authors

Cáceres, Juan Carlos
Bailey, Clara A
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Selenocysteine substitutions in thiyl radical enzymes

Juan Carlos Cáceres[a], **Clara A. Bailey**[a], **Kenichi Yokoyama**[b], **Brandon L. Greene**[a,*]

[a]Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA, United States

[b]Department of Biochemistry, Duke University, Durham, NC, United States

Abstract

Cysteine thiyl radicals are implicated as cofactors in a variety of enzymatic transformations, as well as transient byproducts of oxidative stress, yet their reactivity has undermined their detailed study. Selenocysteine exhibits a lower corresponding selenyl radical reduction potential, thus taming this radical reactivity without significant steric perturbation, potentially affording a glimpse into otherwise fleeting events in thiyl radical catalysis. In this chapter, we describe a suite of fusion protein constructs for general and efficient production of site-specifically incorporated selenoproteins by a recently developed nonsense suppression technology. As a proof of concept, we produced *NikJ*, a member of the radical S-adenosyl methionine enzyme family involved in the biosynthesis of peptidyl nucleoside antibiotics. We place emphasis throughout the plasmid assembly, protein expression, and selenium quantitation on accommodating the structural and functional diversity of thiyl radical enzymes. The protocol produces *NikJ* with near quantitative selenocysteine insertion, 50% nonsense read-through, and facile protein purification.

1. Introduction

Amino acid radicals are essential cofactors in a number of enzymes and have been the subject of extensive study (Frey, 2001; Stubbe & van der Donk, 1998). Most elusive among the amino acid radicals are cysteine thiyl radicals, catalyzing remarkably diverse chemical transformations acting as H-atom acceptors, the product of H-atom donation, or as radical nucleophiles. When generated outside of their respective active site, free cysteine thiyl radicals rapidly and nonspecifically activate C—H bonds in peptides (Nauser, Casi, Koppenol, & Schöneich, 2008; Schöneich, 2011), reactivity that could potentially degrade the enzyme scaffold unless properly controlled. Therefore, enzymatic thiyl radical generation and catalytic outcomes must occur with high fidelity to maintain function over multiple turnovers. Targeting enzyme thiyl radicals continues to be an attractive therapeutic strategy (Greene et al., 2020), but rational design of mechanism-based inhibitors requires knowledge of the radical generation and active site chemistry, the details of which remain poorly understood for most enzymes. Thus far, the class II ribonucleotide reductase (RNR) from *Lactobacillus leichmannii* is the only example of an experimentally observed kinetically and chemically competent thiyl radical (Licht, Gerfen, & Stubbe, 1996).

*Corresponding author: greene@chem.ucsb.edu.

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Selenocysteine, a naturally occurring variant of cysteine where the sulfur atom is replaced with selenium, is a minimally invasive analog with opportunistic properties for experimental investigation of thiyl radical enzymes. The reduction potential of the selenocysteinyl radical is estimated to be 500 mV lower than that of the cysteinyl radical, corresponding to a 55 kJ/mol lower bond dissociation energy (Nauser, Dockheer, Kissner, & Koppenol, 2006), suggesting that selenocysteine may arrest radical chemistry long enough for direct experimental observation, analogous to other amino acid surrogates acting as radical traps (Minnihan, Seyedsayamdost, Uhlin, & Stubbe, 2011). The native translational machinery for site-selective selenocysteine incorporation into translating proteins (selenoproteins) is complex and difficult to repurpose for recombinant protein expression on the scale necessary for mechanistic studies (Serrão et al., 2018). Several approaches have been developed for recombinant selenoprotein expression that bypass the native machinery and are reviewed elsewhere (Metanis & Hilvert, 2014; Wang, Liu, Chang, Xu, & Wang, 2021). We have previously applied the method of Söll (Mukai, Sevostyanova, Suzuki, Fu, & Söll, 2018) for the recombinant expression of the *Escherichia coli* (*E. coli*) class Ia RNR α_2 subunit (Greene, Stubbe, & Nocera, 2019). This method employs an engineered allo-tRNA^{sec} that efficiently interacts with both *E. coli* serine aminoacyl tRNA synthetase and the elongation factor Tu and is cognate for the amber stop codon. Co-expression of *SelA* and *SelD* catalyzes the conversion of serine acylated allo-tRNA^{sec} to selenocysteine for amber codon suppression, and the tRNA, *SelA* and *SelD* are all encoded in the pSecUAG-Evol2 plasmid. RNR cloned into pET-14b and co-expressed with pSecUAG-Evol2 in ME6 cells generated selenocysteine substituted *E. coli* RNR at the thiyl radical cysteine site (C_{439}) with a yield of >2 mg protein per gram cell paste and 80–100% selenocysteine loading, but the expression and purification strategy was not designed for general application to diverse thiyl radical enzymes.

In this chapter, we describe the development of several “plug-and-play” fusion protein plasmids for the recombinant expression of site-specifically labeled selenoproteins. The constructs developed harbor C-terminal poly-histidine tags for immobilized metal ion affinity chromatography (IMAC), as well as removable fusion proteins, namely, the small ubiquitin-like modifier protein (SUMO, *Saccharomyces cerevisiae* SMT3) or maltose binding protein (MBP). All constructs can generate near native protein following purification and protease digestion with proteases that can also be recombinantly expressed targeting SUMO (Ulp1) (Guerrero, Ciragan, & Iwaï, 2015) or tobacco etch virus (TEV) protease recognition sites (van den Berg, Löfdahl, Härd, & Berglund, 2006). Lastly, the antibiotic selection markers have been altered for improved plasmid retention during scale-up, a persistent problem with prior constructions (Greene et al., 2019). Detailed instructions for Gibson assembly of arbitrary proteins of interest into the developed fusion protein expression vectors are detailed for ease of implementation. The radical S-adenosyl methionine (SAM) enzyme *NikJ* (Lilla & Yokoyama, 2016) serves as a test case, exemplifying several common obstacles to recombinant protein expression and non-sense suppression, including low overall expression and solubility, post-translational modifications (a 4Fe-4S cluster), oxygen sensitivity, and similar properties of the full-length and truncated protein products. We hope that these vector constructs and the methods detailed herein will expand the potential of selenocysteine incorporation in thiyl radical enzymes as a

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mechanistic probe, and contribute more broadly to nonsense suppression-based methods for unnatural amino acid incorporation in recombinantly produced proteins.

2. Recombinant expression of selenoproteins by non-sense suppression

2.1 Notes on choosing the right vector

Thiyl radical enzymes vary widely in size, tertiary and quaternary structure, solubility, post-translational modifications, cofactor composition, and in the role of dynamics in catalysis. This diversity of structure and function necessitates a nuanced vector design for expression and isolation of selenocysteine substituted proteins for mechanistic study. The ideal expression vector imbues the protein of interest (POI) with features that assist in soluble expression and purification, while not interfering with downstream enzymatic function, either by ease of removal or innocuity. To increase the solubility of recombinantly expressed proteins, tight control of expression or fusion to a soluble carrier protein, or both, have been shown to be effective (Guerrero et al., 2015; Raran-Kurussi, Keefe, & Waugh, 2015). Vector designs that improve purification efficiency generally rely on fusion of the POI to an affinity tag domain, either a polyhistidine tag (His-tag) for IMAC or fusion to a small molecule binding protein whose ligand can be functionalized onto a stationary phase. These protein or peptide fusion constructs are appended to the N- or C-terminus of the POI, which can interfere with function or protein–protein interactions. Additionally, nonsense suppression methods such as those described herein, generate both full-length and truncated protein products, thus N-terminal affinity tags may not be effective in discriminating between truncated and full-length protein.

No one expression vector solution is viable for all POI, and thus consideration of the functional properties of the POI is essential. The *E. coli* class Ia RNR α_2 subunit, for example, has been studied by selenocysteine substitution at the thiyl radical forming C_{439} by the method of Söll (Greene et al., 2019; Mukai et al., 2018). Using a C-terminal affinity tag may be useful in the purification of the full-length selenocysteine incorporated enzyme from the truncated impurity, but the C-terminal tail of the RNR α subunit is dynamic and contains two cysteines essential for the re-reduction of active site during turnover (Åberg et al., 1989; Booker, Licht, Broderick, & Stubbe, 1994; Greene et al., 2020). Fortunately, the central location of the target cysteine C_{439} in the overall protein sequence (761 residues total) destabilized the prematurely truncated 438-residue protein, and this impurity precipitates with the cell debris upon lysis, making an N-terminal tag more appropriate (Greene et al., 2019). Nevertheless, it has been shown that an N-terminal His-tag on RNR α_2 decreases its activity for unknown reasons (Minnihan et al., 2011). Thus, there is a need for modular strategies for N- and C-terminal fusion proteins that can be readily cleaved to produce the native or near-native POI.

The expression vector of choice must also be maintained and replicated by the host in concert with the pSecUAG-EVOL2 plasmid containing the selenocysteine biosynthetic machinery and UAG anti-codon allotRNA^{UTu} for successful expression of the selenocysteine loaded POI, and the two plasmids should be under orthogonal induction control as the selenocysteine-charged allo-tRNA^{UTu} must be present prior to POI mRNA translation. The pSecUAG-EVOL2 plasmid is induced by L-arabinose (*araBAD* promotor)

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and harbors a kanamycin (Kan) resistance cassette *KanR*. Previously pET and pUC vectors have been chosen for their relatively high expression levels, orthogonal origin of replication, and ampicillin selection marker (Evans et al., 2021; Greene et al., 2019; Mukai et al., 2018). We have previously described plasmid instability in scale-up expression due to seed culture β-lactamase activity and culture acidification during growth, resulting in loss of the pET vector (Greene et al., 2019). This can be partially ameliorated by washing the saturated inoculum cultures to remove excess β-lactamase, introducing buffer into the culture medium, and replacement of ampicillin by carbenicillin, but we have found more stable plasmid continuity with vectors that confer chloramphenicol resistance.

In light of the multitude of challenges and considerations necessary to produce recombinant selenoproteins by non-sense suppression methods at the scale necessary for *in vitro* study, we have designed a suite of vectors for efficient selenoprotein expression and purification designated pCm 1–6, shown in Fig. 1A. A user guide for selecting an appropriate vector for a given application is also provided in Fig. 1B. The library contains six plasmids with C-terminal 8× His-tags that are removable by proteases for applications where metal affinity tags may interfere with function or reconstitution. Plasmids pCm2, pCm3 and pCm5 contain the SMT3 SUMO fusion fragment, which acts as a low molecular weight soluble fusion protein and as a high-fidelity protease recognition site for Ubl-specific protease 1 (Ulp1), cleaving SUMO at its C-terminal GG*S motif. Plasmids pCm4–6 contain a maltose binding protein (MBP) domain, which may be more effective in maintaining POI solubility for troublesome proteins, and serves as an orthogonal affinity tag for purification on amylose resin (Duong-Ly & Gabelli, 2015; Raran-Kurussi et al., 2015). For all plasmids the entire C-terminal fused domain can be removed by TEV protease treatment. In total, these six plasmids should provide broad-spectrum support for selenoprotein expression and purification as demonstrated below. The pCm plasmids also show improved continuity in scale-up due to the introduced chloramphenicol resistance (*CmR*). A guide for Gibson assembly of these plasmids with arbitrary POI genes is also provided in Fig. 2. The vectors have been deposited in the Addgene database for accessibility, and both Ulp1- and TEV protease-expressing plasmids and protocols are also available.

3. Assembling pCm-based vectors and optimizing selenoprotein expression

The following protocol describes how to clone cDNA of a POI into the pCm plasmid family and test the expression and solubility of the resulting selenoprotein. We use as an example *Streptomyces tendae NikJ*, which catalyzes the SAM-dependent formation of octosyl acid 5′-phosphate (OAP) from 3′-enolpyruvyl uridine 5′-phosphate by a radical mechanism involving enantioselective substrate radical quenching by an H-atom transfer from C_{199}. The *NikJ* gene had previously been mutated by site-directed mutagenesis to convert the former cysteine coding TCG codon to the amber TAG codon for directed selenocysteine insertion ($C_{199}U$). Despite the low expression levels of $C_{199}U$ *NikJ*, its oxygen intolerance in both apo and holo form, and poor solubility during purification, the protein was expressed and recovered at a scale commiserate with detailed *in vitro* study. For a general thiyl radical enzyme or other POI the TGT/TGC→TAG mutation in the desired

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cysteine-to-selenocysteine mutation can be generated by site-directed mutagenesis before or after Gibson assembly. A detailed protocol for this site-directed mutagenesis is provided in the section “Mutate codon in desired position to TAG” of Chung et al. (Chung, Miller, Söll, & Krahn, 2021).

3.1 Materials

- Chloramphenicol (Cm)
- Kanamycin (Kan)
- Agar
- Agarose
- L-Arabinose (ara)
- Sodium selenite (Na_2SeO_3)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)
- 2× Laemmli Buffer
- S.O.C. medium
- Luria-Bertani (LB) medium
- Terrific broth (TB) medium
- *Dpn*I restriction enzyme (New England Biolabs)
- Phusion® High-Fidelity DNA Polymerase (New England Biolabs, or another high-fidelity polymerase)
- NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, or another Gibson assembly kit)
- InVision™ His-Tag In-Gel Stain (Thermo Scientific)
- Imperial™ Protein Stain (Thermo Scientific, or another protein stain)
- BugBuster® Protein Extraction Reagent (EMD Millipore)
- QIAprep® Spin Miniprep Kit (Qiagen, or another plasmid DNA isolation kit)
- 0.1 cm MicroPulser Electroporation Cuvettes
- Electrocompetent *E. coli* DH5α cells (Thermo Scientific)
- Electrocompetent *E. coli* BL21-AI™ cells (Thermo Scientific)
- pSecUAG-Evol2 (Addgene plasmid # 163148)
- pCm plasmid of choice (See Fig. 2, Addgene plasmids #174210, #174361, #174362, #174363, #174364, #174365)
- DNA of the protein of interest (POI) with an introduced TAG mutation for cysteine (TGT/TGC)
- Custom oligonucleotide primers (see below)

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- Thermocycler
- Temperature controlled incubator shaker capable of lower than 30°C temperatures
- MicroPulser Electroporator (Biorad)
- UV–Vis Spectrophotometer
- (Optional) SnapGene pro 5.3

3.2 Gibson assembly (2–3 days)

3.2.1 Design oligonucleotides to PCR amplify the POI gene (insert primers, Fig. 2) and the appropriately selected pCm plasmid (vector primers, Fig. 2) for Gibson assembly into the pCm vector of interest (Fig. 1). The primers listed in Fig. 2 can serve as a template, where the bold upper-case bases indicate the bases of the gene to be cloned, in this example *NikJ*. The number of overlapping base pairs necessary for proper annealing will depend on the GC content of the gene, but ~10 bp with 50% GC content has proven successful in our preparations. One primer pair should be obtained for the vector and another for the insert for each pCm-POI assembly

Note: The tool NEBuilder® HiFi DNA Assembly from the software SnapGene Pro can be used to design appropriate primers to clone the protein of interest in the open reading frame of the different pCm plasmids.

3.2.2 Amplify the pCm vector of choice and the gene of interest by PCR using the designed primers from step 3.2.1 and the Phusion® High-Fidelity DNA Polymerase, or other high-fidelity polymerase, according to the manufacturer's instructions. Resolve 3 μL of the PCR product by gel electrophoresis in an agarose gel to confirm the amplification of a single DNA fragment of the appropriate base pair composition

3.2.3 To avoid the transformation with template DNA, gel purify the pCm PCR amplicon or add 1 μL of *Dpn*I to digest any residual template plasmid in the samples according to the manufacturer's instructions

3.2.4 Assemble the vector and inserts using the NEBuilder® HiFi DNA Assembly Master Mix following the manufacturer's instructions. Transform 4 μL of the assembled product in 50 μL of electrocompetent *E. coli* DH5α cells by electroporation and outgrow cells in 1 mL of preheated S.O.C. media at 37°C for 1 h. Plate the competent cells in LB-agar plates supplemented with 30 μg/mL Cm and grow at 37°C until colonies appear

Note: Chemically competent cells can also be used with lower transformation efficiency.

3.2.5 Purify the assembled plasmid by Qiagen Spin Miniprep Kit according to the manufacturer's instructions from liquid culture growth of transformed colonies and confirm the correct gene insertion by sequencing the purified plasmid

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3.3 Expression and amber codon suppression optimization (6 days)

3.3.1 Once the cDNA has been correctly cloned and its sequence verified, co-transform >200 ng of the pCm plasmid and >200 ng of pSecUAG-Evol2 plasmid in electrocompetent *E. coli* BL21-AI™ cells by electroporation. Outgrow cells in preheated S.O.C. media at 37°C for 2 h and plate the transformed cells on LB-agar plates supplemented with 30 μg/mL Cm and 50 μg/mL Kan. Incubate the plates at 37°C overnight or until colonies appear

Note: The selection of a suitable *E. coli* strain for the expression of Sec containing enzymes prove to be a challenge as pSecUAG-Evol2 requires ara for induction and most commercial expression strains can metabolize ara, compromising its function in pSecUAG-EVOL2 induction. We have expressed *NikJ* in BL21, C41, C43 and BL21-AI *E. coli* cell lines, and only the BL21-AI strain, an *araB* knockout mutant unable to consume ara, produced the full-length protein at detectable levels. The T7 RNA polymerase of the BL21-AI strain is under the control of the *araBAD* promoter, providing more control over the expression of the POI in pCm vectors. Alternative strains of *E. coli* have also been used successfully (Evans et al., 2021; Mukai et al., 2018).

Note: We have observed low co-transformation efficiency for these plasmids; to improve the efficiency use high DNA concentration and recuperate the cells at 37°C with prewarmed S.O.C. medium for ≥2 h. Total DNA for transformation should not exceed 10% of total cell volume.

3.3.2 Test POI expression and amber codon suppression by inoculating four 5 mL cultures of TB medium, supplemented with 30 μg/mL Cm and 50 μg/mL Kan with a single colony each from step 3.3.1 and incubate overnight at 37°C and 200 rpm

Note: For *NikJ* the cultures were also supplemented with 1 mg/L $FeCl_3$ and 24.28 mg/L of L-cysteine, which did not affect expression or amber codon suppression.

3.3.3 Inoculate a 50 mL culture of TB medium supplemented with 30 μM of $NaSeO_3$, 30 μg/mL Cm and 50 μg/mL Kan with the overnight pre-culture from step 3.3.2, using enough cells to reach an OD_{600} of 0.01, and continue incubating the cultures at 37°C at 200 rpm

3.3.4 To test the expression of both the pSecUAG-EVOL2 and pCm plasmids induce the four cultures with the following ara and IPTG concentrations at OD_{600} = 0.5 and 1.0, respectively: no ara no IPTG (−ara, −IPTG), 1% *w*/*v* ara no IPTG (+ara, −IPTG), no ara 1.0 mM IPTG (−ara, +IPTG), and 1% w/v ara 1 mM IPTG (+ara, +IPTG). Collect 1 mL samples from each culture at OD_{600} = 1.0 prior to IPTG addition, spin down the cells by centrifugation, and flash freeze for storage at −80°C. Repeat the sample collection procedure ≥24 h after IPTG induction

3.3.5 Cell pellets collected before and ≥24 h after IPTG induction should be analyzed by SDS-PAGE to confirm expression. Resuspend the pre- and postinduction cell

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pellets to equivalent cell density; we routinely dilute the pellets from 1 mL samples in 100 μL of 1× Laemmli buffer per 1 unit of OD_{600}, and load 10 μL in a 15 μL well for 1 mm SDS-PAGE. Resolve the gel by electrophoresis, stain with InVision™ His-Tag In-Gel Stain according to the manufacturer's instructions, and image by UV transillumination. Next, stain the gel with Imperial™ Protein Stain or equivalent protein stain and image again. Compare the ratio of expression of full-length/truncated protein in the gel. Neither full-length or truncated POI should be expressed in the −ara, −IPTG or +ara, −IPTG cultures, only truncated protein should be observed in the −ara, +IPTG cultures, and an ideal ratio of ~1:1 full-length to truncated POI should be observed in the +ara, +IPTG. Fig. 3A shows a representative His-Tag Stain of *NikJ* expression levels in each of the 6 pCm vectors

Note: Specific His-tag detection is necessary as expression of the full-length protein might be low and difficult to observe in a Coomassie stained gel, as is the case for *NikJ*. We use InVision™ His-Tag In-Gel Stain for convenience; however, Western blotting can provide higher sensitivity for low expression proteins.

3.3.6 If full-length protein expression levels are low, repeat steps 3.3.1–3.3.5 varying the temperature, shaking speed, ara, and IPTG levels from 4 to 48 h after induction. Repeat these steps until an optimized full-length expression yield is realized or try cloning the POI in another plasmid

3.4 Cell lysis and solubility analysis (6 days)

3.4.1 Once the appropriate induction conditions have been determined, the relative solubility of the expressed fusion protein should be evaluated to improve the subsequent protein purification yield and long-term stability. Repeat protocol 3.3, inducing both pSecUAG-Evol2 and pCm-POI using the optimized conditions developed therein, and collect all of the final culture volume by centrifugation in a pre-weighed collection centrifuge tube. Measure the wet cell weight

3.4.2 To analyze the fusion protein solubility, resuspend 200 mg of the wet cell pellet from step 3.4.1 in 1 mL of BugBuster® Protein Extraction Reagent and follow the manufacturer's instructions to obtain soluble and insoluble protein preparations. Quantify the protein concentration in both soluble and insoluble fractions and dilute as necessary to achieve 2 mg/mL total protein concentration. Load equal amounts of samples from the soluble and insoluble proteins in an SDS-PAGE, resolve by electrophoresis, and stain the gel with InVision™ His-Tag In-Gel Stain. Image the His-tag stained gel for full-length protein distribution between soluble and insoluble fractions. Next, stain the total proteins with Imperial™ Protein Stain and after destaining, image total protein distribution. Compare the solubility of the different fusion proteins and choose the one with the highest full-length expression and solubility for scaling up the expression and to purify the full-length protein. Fig. 3B shows a representative solubility analysis for *NikJ* expressed from pCm1–6 vectors

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4. Milligram scale selenoprotein preparation

Most fusion proteins expressed in pCm plasmids are designed to be purified in 1–2 chromatographic steps. The first chromatographic step involves IMAC to separate the POI from other cellular proteins, yielding the full-length fusion protein free of truncated impurities. In a second step, the POI can be separated from the His-tag or additional fusion proteins by protease digestion, followed by a second chromatographic step that depends on the desired product. A His-tagged TEV protease will remove the C-terminal His-tag in all pCm constructs, and the SMT3 or MBP domain for pCm2 and pCm4/6, respectively. The C-terminal His-tag on a pCm2-expressed POI can also be removed by Ulp1 while retaining the solubilizing fusion protein SMT3. The His-tag-less POI can be isolated in a second IMAC step, this time as the flow through, removing the remaining tagged impurity and Ulp1 or TEV protease. Alternatively, solubilizing fusion proteins SMT3 and MBP-SMT3 can be removed by Ulp1 in pCm3 and pCm5, respectively, and the POI can be isolated by IMAC with a retained His-tag. Here, the Ulp1 is also retained on the IMAC column, and would require a subsequent purification step for removal (e.g., TEV protease followed by a third IMAC step). Additional purification steps may be necessary based on the properties of the protein, and the desired purity level. For plasmids pCm4, pCm5 and pCm6 amylose resin can be used to further purify the fusion protein.

Selenocysteine is prone to oxidation, forming diselenides, mixed selenosulfides, or selenic acids. To maintain the selenocysteine in the selenol form, it is best to remove oxygen altogether by performing purification under an inert atmosphere such as in a glove box. If a glove box is not available, excess reductant should be included in all steps along the purification process and in storage. Furthermore, both Ulp1 and TEV protease are cysteine proteases and require supplementing the protease buffer with reductant. Appropriate reductants include tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT) or β-mercaptoethanol (βME), the concentrations of which will depend on the purification step (see below).

Due to the uniqueness of the selenium atom in selenocysteine relative to the remaining 20 canonical amino acids, selenium quantitation by elemental analysis is a powerful technique for the analytical determination of selenocysteine content in the POI. Selenium quantitation is important as the biosynthesis of selenocysteine occurs on the serine acylated Ser-allo-tRNA^{Sec}, being catalyzed by *SelA* producing the Selenocysteine acylated Sec-allo-tRNA^{Sec} using as substrate selenophosphate produced by *SelD*. If the activity of SelA and SelD cannot keep up with the POI expression, amber codon suppression can occur with insertion of serine rather than selenocysteine. For some thiyl radical enzymes, this serine substituted protein may not be of significant concern. In the *E. coli* class Ia RNR the thiyl radical that initiates nucleotide reduction is generated by radical transfer from a stable diiron-tyrosyl radical cofactor in a second β_2 subunit. Neither radical transfer nor substrate reduction occur in the $C_{439}S$ α_2 mutant, and thus this impurity is experimentally “silent” both in activity assays and in radical trapping experiments (Greene et al., 2019). Conversely, in *PolH*, a functional homolog of *NikJ*, the $C_{209}A$ mutant retains ~40% of wild type activity, and both $C_{209}A$ and $C_{209}S$ mutants lose enantioselectivity as a result of the loss of the H-atom donor

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C_{209} (Lilla & Yokoyama, 2016). Thus, determining the effect of the *NikJ* $C_{199}U$ mutation on enzyme activity requires precise quantification of the contaminating $C_{199}S$ content.

Mass spectrometry is also a powerful tool for analyzing selenoproteins and determining semi-quantitatively the misloaded serine contamination. Intact or fragmented mass spectrometry and liquid chromatography-tandem mass spectrometry (LC MS/MS) can be used to detect the selenocysteine loaded protein by total mass or selenocysteine peptide detection. Importantly, it is not necessarily valid to assume that digested selenocysteine and serine peptide fragments will ionize similarly, thus the ratio of these peaks may not reflect the actual U/S ratio. This can be accounted for by authentic synthesis of the respective peptides and constructing a calibration curve (Lee et al., 2018).

Below, we describe a protocol for selenoprotein purification under an inert atmosphere and selenium quantitation by inductively coupled plasma optical emission spectroscopy (ICP-OES), again using $C_{199}U$ *NikJ* as an example. The purification and fusion tag removal can be accomplished in 1 day, minimizing the opportunities for selenocysteine oxidation or protein degradation. The purification yielded 0.4 mg/g cell paste. Following purification and ICP-OES selenium quantitation, the 4Fe-4S cluster of *NikJ* was reconstituted and the enzyme was determined to be active and enantioselective for the H-atom donation to the OAP radical intermediate, implying the $C_{199}U$ mutant is chemically competent and useful for subsequent investigations into the fate of thiyl (selenyl) radicals in this enzyme.

4.1 Materials

- Chloramphenicol (Cm)
- Kanamycin (Kan)
- Agar
- L-arabinose (ara)
- Sodium selenite (Na_2SeO_3)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)
- DL-Dithiothreitol (DTT)
- Streptomycin sulfate
- Lysozyme (chicken egg white, Sigma-Aldrich)
- Ribonuclease A (bovine pancreas, Sigma-Aldrich)
- 2× Laemmli Sample Buffer
- S.O.C. medium
- LB medium
- TB medium
- Buffer X (50 mM Tris pH 7.6, 300 mM sodium chloride, 5% *w*/*v* Glycerol, 1 mM DTT)

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- Lysis buffer (Buffer X, 1 mM PMSF)
- Buffer A (Equilibration/Wash buffer: Buffer X, 20 mM Imidazole)
- Buffer B (Elution buffer: Buffer X, 400 mM Imidazole)
- Imidazole
- InVision™ His-Tag In-Gel Stain (Thermo Scientific)
- Imperial™ Protein Stain (Thermo Scientific, or other Coomassie stain)
- Guanidinium chloride
- Trypsin (bovine pancreas, Sigma-Aldrich)
- α-Chymotrypsin (bovine pancreas, Sigma-Aldrich)
- Calcium chloride ($CaCl_2$)
- Trace-metal grade nitric acid
- Selenium Standard (TraceCERT, Sigma-Aldrich)
- Electrocompetent *E. coli* BL21-AI™ cells (Thermo Scientific)
- pSecUAG-Evol2 (Addgene plasmid # 163148)
- pCm plasmid from Gibson assembly
- Amicon® Ultra-15 Centrifugal Filter Units (EMD Millipore)
- Cytiva® HiTrap Desalting 5 mL columns with Sephadex G-25 resin
- HisPur™ Cobalt Resin column
- 0.1 cm MicroPulser Electroporation Cuvettes
- Temperature controlled incubator shaker capable of lower than 30°C temperatures
- Biorad® MicroPulser Electroporator
- UV–Vis Spectrophotometer
- Emulsiflex C3 French press or other cell lysis tool
- iCAP 6300 ICP-OES (Thermo Fisher Scientific) Optional
- Argon or Nitrogen gas
- Schlenk line
- Coy Lab’s Vinyl Anaerobic Chamber (<20 ppm oxygen)
- Polyhistidine-tagged Ulp1
- Polyhistidine-tagged TEV protease

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4.2 1.5 L protein expression (2–3 days)

4.2.1 Co-transform electrocompetent *E. coli* BL21-AI cells with >200 ng of the optimal Gibson assembled pCm plasmid and >200 ng of pSecUAG-Evol2 by electroporation. Outgrow cells in preheated S.O.C. media at 37°C for 2 h and plate the transformed cells in LB-Agar supplemented with 30 μg/mL Cm and 50 μg/mL Kan. Incubate the plates at 37°C until colonies appear.

4.2.2 Inoculate a 50 mL preculture of TB medium, supplemented with 30 μg/mL Cm and 50 μg/mL Kan with a single co-transformed colony and incubate overnight at 37°C

4.2.3 Inoculate a pre-warmed 1.5 L culture of TB medium in a 6 L flask, supplemented with 30 μM of $NaSeO_3$, 30 μg/mL Cm and 50 μg/mL Kan, with the overnight pre-culture from 4.2.2, using enough cells to reach an OD_{600} of 0.01

Note: $FeCl_3$ and L-cysteine are included for *in vivo* assembly of the *NikJ* 4Fe-4S cluster. These may be omitted for non-iron-sulfur proteins, but other supplements may be necessary for different selenoproteins of interest (*e.g.* metals, cofactors, or chelators).

4.2.4 Express the protein using the optimized conditions developed in step 3.3 on the 1.5 L scale. For *NikJ*, the expression was carried out at 37°C and 200 rpm shaking speed until the culture reached an OD_{600} of 0.5 (2–3 h after inoculation), then add 1% *w*/*v* of ara to induce the expression of the selenocysteine incorporation machinery by pSecUAG-Evol2 and the T7 RNA polymerase of the BL21-AI cells. Once an OD_{600} of 1.0 is reached (4–5 h after inoculation) induce with 1 mM IPTG, and lower the temperature to 30°C for 24 h with continued shaking, reaching a final OD_{600} of 8–10

4.2.5 Collect the cells by centrifugation and purify the protein immediately or flash freeze the pellet in liquid nitrogen and store at −80°C. Typical cell yields vary between 10 and 20 g wet cell weight per liter of TB medium

4.3 Protein purification (1 day)

4.3.1 Homogeneously resuspend the cell pellet in Lysis Buffer at 2.5–5 mL/g cell paste, then lyse the cells using an Emulsiflex C3 French Press under Argon as described by Schleicher et al. (Schleicher, Fritz, Seifert, & Steuber, 2020) by three passes at 14,000 psi. Centrifuge the lysate and centrifuge at 30,000×*g* for 30 min. Collect the supernatant (38–150 mL).

Note: Initial protein purifications can be carried out in Buffer X, but optimal buffer conditions should be determined once pure protein is obtained. For *NikJ*, all buffers were supplemented to a final concentration of 500 mM NaCl and 10% glycerol.

Note: All steps were carried out anaerobically, but the protocol described herein is more general. A reducing agent in the buffer is critical to keep the selenocysteine and native cysteines reduced in either case. An effective reductant

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system may include 0.5–2 mM TCEP, 1–10 mM DTT, or 5–20 mM βME. Reductants can poison metal affinity chromatography resins. Consult the resin manual to determine the appropriate reductant concentration.

Note: Alternative cell lysis protocols may be more appropriate for individual POIs and laboratory capabilities. We have observed similar results with BugBuster® Protein Extraction Reagent and ultrasonification, both of which can be adapted inside of a glove box to avoid oxygen exposure.

4.3.2 While stirring the supernatant from step 4.3.1, add a 1.5% *w*/*v* of streptomycin sulfate dropwise from a 6% w/v stock solution (0.2 volume equivalents), and incubate for ≥10 min. Centrifuge the solution at 30,000 × *g* for 30 min to remove DNA

4.3.3 Apply the clarified supernatant from step 4.3.2 to a Buffer A pre-equilibrated HisPur™ cobalt resin column at a flow rate of 1 mL/min to maximize resin adsorption of the His-tagged protein. Wash the column with >10 column volumes of Buffer A and then elute the protein with 3 column volumes of Buffer B. Pool fractions that contain protein as determined by the Bradford assay or absorbance at 280 nm and supplement with DTT to a final concentration of 10 mM

4.3.4 Concentrate the protein-containing solution in an Amicon® Ultra-15 centrifugal filter of appropriate molecular weight cutoff to ≤1.5 mL and buffer exchange the protein in a Buffer X pre-equilibrated HiTrap desalting 5 mL column. Quantify the protein concentration and use immediately, remove affinity tags and fusion proteins, or flash freeze in liquid nitrogen and store at −80°C. Fig. 4 shows a representative anaerobic purification of pCm2 expressed $C_{199}U$ *NikJ*

Note: For many proteins, purification to this point may be sufficient.

4.4 (Optional) fusion protein or His-tag removal (1–3 h)

Note: We produce and purified TEV protease by recombinant expression using the plasmid Addgene #125194 (van den Berg et al., 2006) and Ulp1 protease using the plasmid pFGET19_Ulp1 (Addgene Plasmid #64697) (Guerrero et al., 2015) according to the authors instructions. Both are commercially available in His-tagged form. Protease reaction times from other sources might differ from those reported herein.

4.4.1 For TEV protease digestion, add 1 mg of TEV protease per 10 mg of protein and incubate at room temperature for 30 min in Buffer X

Note: The POI sample for digestion must be previously desalted in Buffer X and free of imidazole.

4.4.2 Load the digested POI sample onto a HisPur™ Cobalt Resin column pre-equilibrated with Buffer A, wash with 2 CV of Buffer A, and collect all POI containing flow through. Follow the protocol in 4.3.4 to concentrate and buffer exchange the protein for storage

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4.4.3 For Ulp1 digestion add 1 mg of Ulp1 per 100 mg of protein and incubate at room temperature for 30 min in Buffer X

4.4.4 If digesting pCm2 load the digested POI sample onto a HisPur™ Cobalt Resin column pre-equilibrated with Buffer A, wash with 2 CV of Buffer A, and collect all POI containing flow through. Follow the protocol in 4.3.4 to concentrate and buffer exchange the protein for storage

Note: If digesting pCm3 or pCm5 the POI retains the C-terminal His-tag, and thus must be eluted from the IMAC column at high imidazole concentrations. The POI will likely co-elute with the His-tagged Ulp1, potentially requiring subsequent purification steps.

4.4.5 Analyze samples before and after digestion and the IMAC column flow through and high imidazole elution by SDS-PAGE to assess the purity of the produced protein. Fig. 5 shows a representative protease digestion for pCm2 and pCm5 by Ulp1 or TEV protease

4.5 Selenium quantification by ICP-OES (2 days)

Selenium quantitation for thiyl radical protein preparations by the aforementioned methods have typically yielded the $C_{199}U$ mutant *NikJ* with 0.4 mg/g of cell paste and 73% selenium incorporation. The protein was purified from cells expressing the pCm2 vector and digested with TEV protease to yield a >90% pure near-native protein as determined by SDS-PAGE (Fig. 4). Note: The limit of detection for the quantification of selenium using our ICP-OES method is 0.03 ppm, which corresponds to 2.7 nmol Se. In the following protocol, we supply enough protein to yield a theoretical concentration of 0.1 ppm Se, and our calibration curve spans 0–0.5 ppm. We require 7 mL of final solution to obtain method triplicates. Samples and standards should be prepared by weight. We routinely achieve >0.7 selenium atoms per protein monomer (>70%). The remaining <30% is assumed to be $C_{199}S$.

4.5.1 Dilute 8.9 nmol of purified protein in 132 μL of concentrated trace-metal grade nitric acid (to yield 2% nitric acid upon dilution) and allow to react at room temperature overnight. Heat samples to 90°C for 2 h and allow the tubes to cool to room temperature. Add 70 μL of trace-metal grade hydrogen peroxide and heat the sample at 90°C for an hour, until the solution is clear and there is no further bubbling.

4.5.2 Dilute the samples to 7 g final solution weight with water

4.5.3 Prepare method blank samples, one without protein and one with a wild-type protein, using the above digestion procedure

4.5.4 Prepare selenium standards (TraceCERT, Sigma-Aldrich) by diluting to a relevant range for the calibration curve in 2% nitric acid

4.5.5 Analyze prepared samples by ICP-OES according to the instrument standard operating procedure

4.5.6 Calculate the Selenium per protein monomer (Se/POI) by using the equation shown below

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$$\frac{Se}{POI} = \frac{(ppm\,Se) \times (g\,total\,solution)}{\left(10^6\right) \times \left(78.96 \frac{g}{\mathrm{mol}\,Se}\right) \times (mol\,protein\,in\,sample)}.$$

5. Evaluating the effect of cysteine-to-selenocysteine substitution

After successful expression and purification of a selenocysteine-substituted thiyl radical enzyme, the selenocysteine effect can be assessed in several ways including activity assays and spectroscopic analysis. In the case of the example *NikJ*, selenocysteine was introduced in the position of the C_{199} which is proposed to rapidly quench the substrate OAP radical enantioselectively (Lilla & Yokoyama, 2016). The pure C_{199}U *NikJ* was isolated with 0.8 Fe/monomer and reconstituted to 2.3 Fe/monomer, slightly below the wild type value of 3.0. The 4Fe-4S cluster reconstituted *NikJ* was determined to be catalytically active in the presence of SAM (0.5 mM), EP-UMP (0.5 mM), sodium dithionite (2 mM) and DTT (2 mM), producing enantiopure U-OAP from EP-UMP. Fig. 6 shows U-OAP formation by *NikJ* and C_{199}U *NikJ*, the mutant exhibits no detectable formation of epi-OPA (<5% epi-OAP), suggesting the selenocysteine may donate an H-atom from the selenol (R-SeH) form as opposed to electron transfer from the selenolate (R-Se^-, pK_a in solution of 5.2). Further investigations will shed light on the stability of the putative selenyl radical and the role of H-atom or electron transfer in C_{199}U *NikJ*.

Acknowledgments

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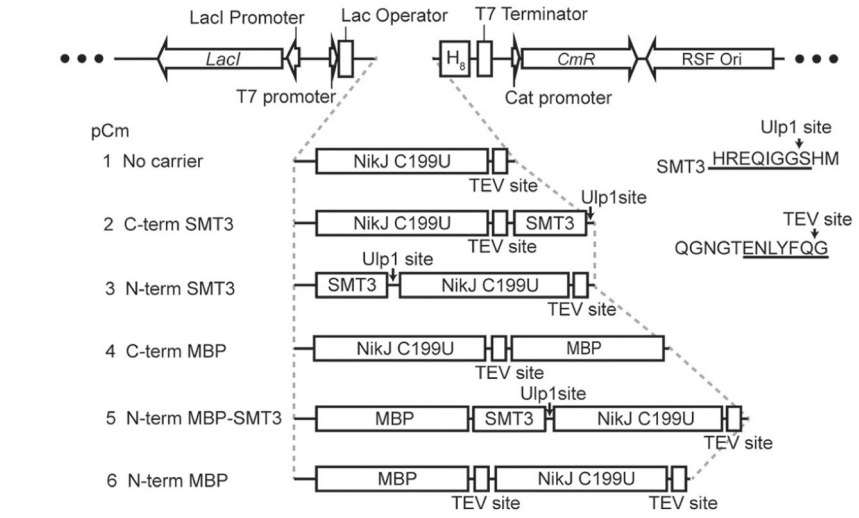
A



B

Criteria	Comments	Recommended plasmids	
		N-term His-Tag	C-term His-Tag
Will C-term modification affect function?	Yes: Avoid C-term tags or use a C-term cleavable tag. No: Use C-terminal tag to separate full-length and truncated.	pET 14-16, pET19, pET32	pET-20-22, pET32, pCm1-6
Will a His-tag interfere with function?	Yes: Ensure protease cleavable or avoid all together. No: Use any C-term His-tag from row 1.	pET 14-16, pET19, pET32	pCm1-6
Is expression low or inconsistent?	Yes: Use a stronger selection marker. No: Use any plasmid from row 2.		pCm1-6
Is solubility an issue?	Yes: Use MBP or SMT3 fusion proteins. No: Use any plasmid from row 3.		pCm2-6
Is IMAC insufficient?	Yes: Use MBP protein fusion and amylose affinity chromatography. No: Use any plasmid from row 4.		pCm4-6

Fig. 1.

Selenoprotein expression and purification strategy. (A) Schematic representation of pCm plasmids for selenoproteins expression and purification. Top: the common scaffold for all pCm plasmids with open variable region. Arrowed blocks represent coding sequences for: *LacI*, lactose operon repressor; H_8, octahistidine tag; *CmR*, chloramphenicol resistance gene. Bottom: the variable region of the pCm plasmids for fusion protein expression. SMT3, *Saccharomyces cerevisiae* SUMO homolog SMT3; MBP, maltose binding protein; TEV, tobacco etch Virus protease site; Ulp1, Ubl-specific protease 1; POI, protein of interest. A detailed view of the Ulp1 and TEV protease recognition sites is also provided. (B) User guide for selecting an appropriate plasmid for the expression and purification of a given selenocysteine mutant POI.

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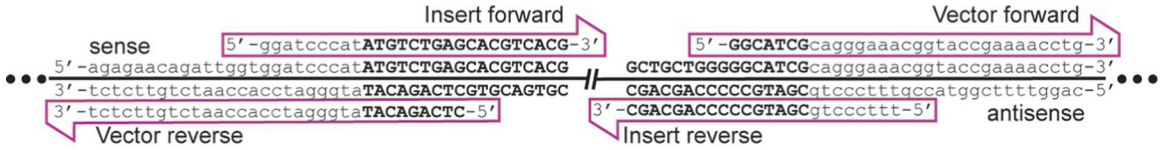

Plasmid	Primer Sense	Vector	Insert
pCm1	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-gagatatacc**ATGTCTGAGCACGTCACGC**-3′
	reverse	5′-**GCTCAGACAT**ggtatatctccttattaaagt taaacaaaattatttctacaggg-3′	5′-tttccctg**CGATGCCCCCAGCAGC**-3′
pCm2	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-gagatatacc**ATGTCTGAGCACGTCACGC**-3′
	reverse	5′-**GCTCAGACAT**ggtatatctccttattaaagt taaacaaaattatttctacaggg-3′	5′-gtttccctg**CGATGCCCCCAGCAGC**-3′
pCm3	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-ggatcccat**ATGTCTGAGCACGTCACG**-3′
	reverse	5′-**CTCAGACAT**atgggatccaccaatctgttctct-3′	5′-tttccctg**CGATGCCCCCAGCAGC**-3′
pCm4	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-gagatatacc**ATGTCTGAGCACGTCACGC**-3′
	reverse	5′-**GCTCAGACAT**ggtatatctccttattaaagt taaacaaaattatttctacaggg-3′	5′-tttccctg**CGATGCCCCCAGCAGC**-3′
pCm5	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-ggatcccat**ATGTCTGAGCACGTCACG**-3′
	reverse	5′-**CTCAGACAT**atgggatccaccaatctgttctct-3′	5′-tttccctg**CGATGCCCCCAGCAGC**-3′
pCm6	forward	5′-**GGCATCG**cagggaaacggtaccgaaaacctg-3′	5′-ttcagggg**ATGTCTGAGCACGTCACG**-3′
	reverse	5′-**CTCAGACAT**cccctgaaaatacaggttttcgaa-3′	5′-tttccctg**CGATGCCCCCAGCAGC**-3′

Fig. 2.

Gibson assembly design for the cloning of a *NikJ* homolog into pCm family of plasmids. Top: schematic of the designed primers annealed with the Gibson assembly product DNA. Bold uppercase bases correspond to NikJ-coding DNA and the lowercase bases to the vector DNA, primers are shown as arrows. Bottom: list of primers designed for cloning a *NikJ* homolog into pCm1–6. To design custom primers for the cloning of a POI cDNA replace the bold bases for the corresponding cDNA of the POI gene.

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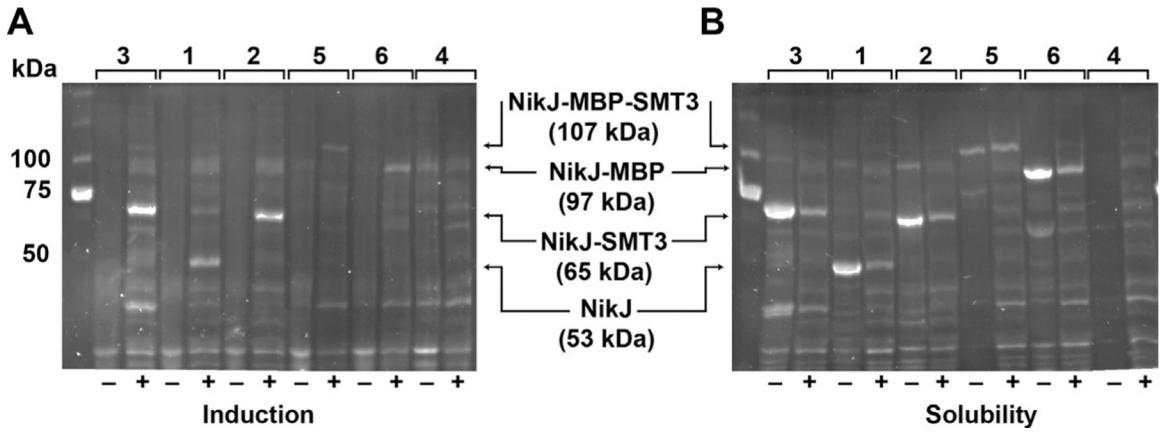

Fig. 3.

Expression and solubility of $C_{199}U$ *NikJ* from pCm1–6 vectors. (A) His-tag stained SDS-PAGE gel of co-transformed BL21-AI *E. coli* cells with pSecUAG-Evol2 and pCm plasmids 1–6. Lanes were loaded with culture samples pre- and 24 h post-IPTG induction. Numbers above lanes corresponds to pCm vectors according to Fig. 1A. (B) His-tag stained SDS-PAGE of protein extraction from samples from induced cultures in A, lane numbering corresponds to plasmids numbers on A and "−" sign indicates insoluble cell debris fraction and "+" sign soluble protein fraction.

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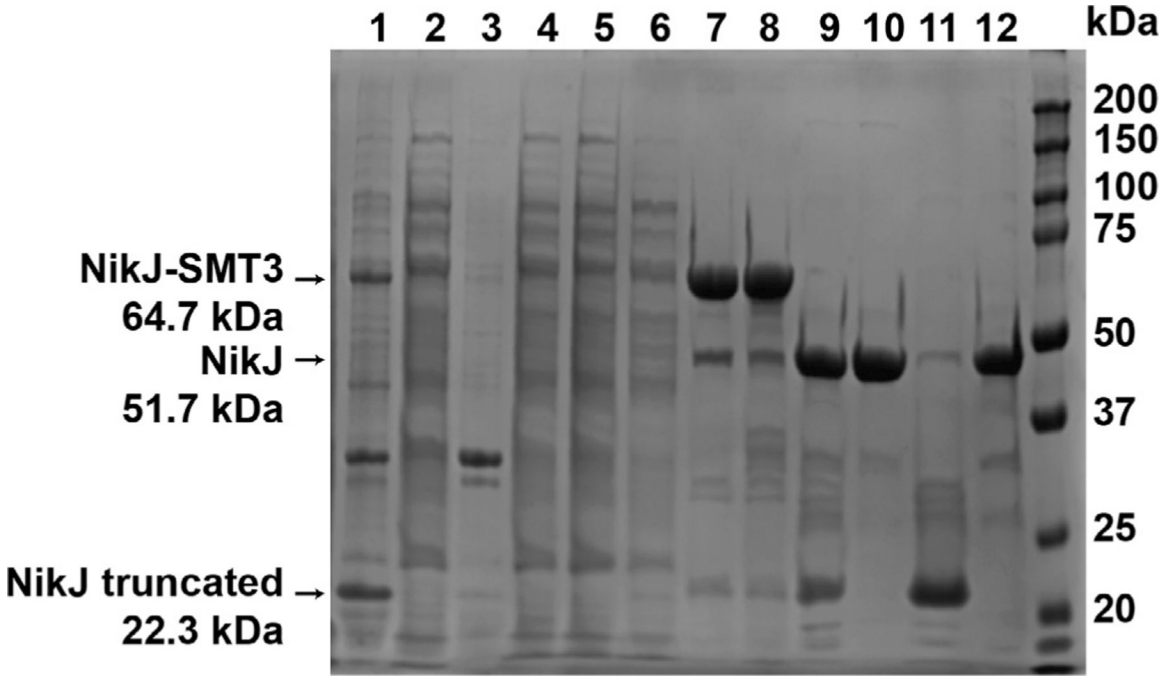

Fig. 4.

Selenoprotein purification and protease cleavage. A SDS-PAGE analysis of *NikJ*-SMT3 $C_{199}U$ purification. Lanes: 1, cell lysate debris; 2, cell lysate supernatant; 3, DNA precipitate; 4, DNA precipitation supernatant; 5, HisPur™ Cobalt Resin column flow through at 20 mM imidazole; 6, wash with 20 mM imidazole, 7, eluted *NikJ*-SMT3 $C_{199}U$ (400 mM imidazole); 8, 1:10 TEV digestion at time 0; 9, 1:10 TEV digestion at time 30 min; 10, TEV digested protein in the flow through of a HisPur™ Cobalt Resin column; 11, eluted TEV and SMT3 (400 mM imidazole), 12, concentrated and desalted purified *NikJ*.

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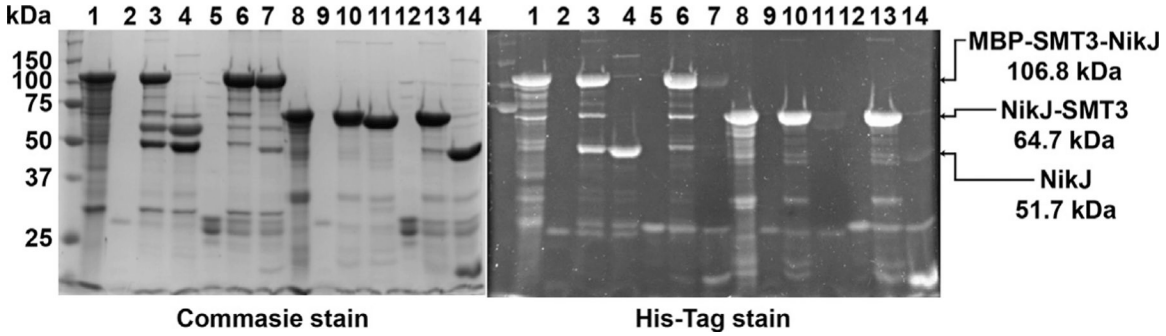

Fig. 5.

SDS-PAGE analysis of protease cleavage of partially purified MBP-SMT3-*NikJ*C_{199}U and *NikJ*C_{199}U-SMT3 stained with Coomassie stain and also His-Tag stain. Lanes: 1, MBP-SMT3-*NikJ*C_{199}U; 2, Ulp1 protease; 3, MBP-SMT3-*NikJ*C_{199}U + ULP1 protease 0 min incubation; 4, MBP-SMT3-*NikJ*C_{199}U + Ulp1 protease 30 min incubation; 5, TEV protease; 6, MBP-SMT3-*NikJ*C_{199}U + TEV protease 0 min incubation; 7, MBP-SMT3-*NikJ*C_{199}U + TEV protease 30 min incubation, 8, *NikJ*C_{199}U-SMT3; 9, Ulp1 protease; 10, *NikJ*C_{199}U-SMT3 + Ulp1 protease 0 min incubation; 11, *NikJ*C_{199}U-SMT3 + Ulp1 protease 30 min incubation; 12, TEV protease; 13, *NikJ*C_{199}U-SMT3 + TEV protease 0 min incubation; 14, *NikJ*C_{199}U-SMT3 + TEV protease 30 min incubation.

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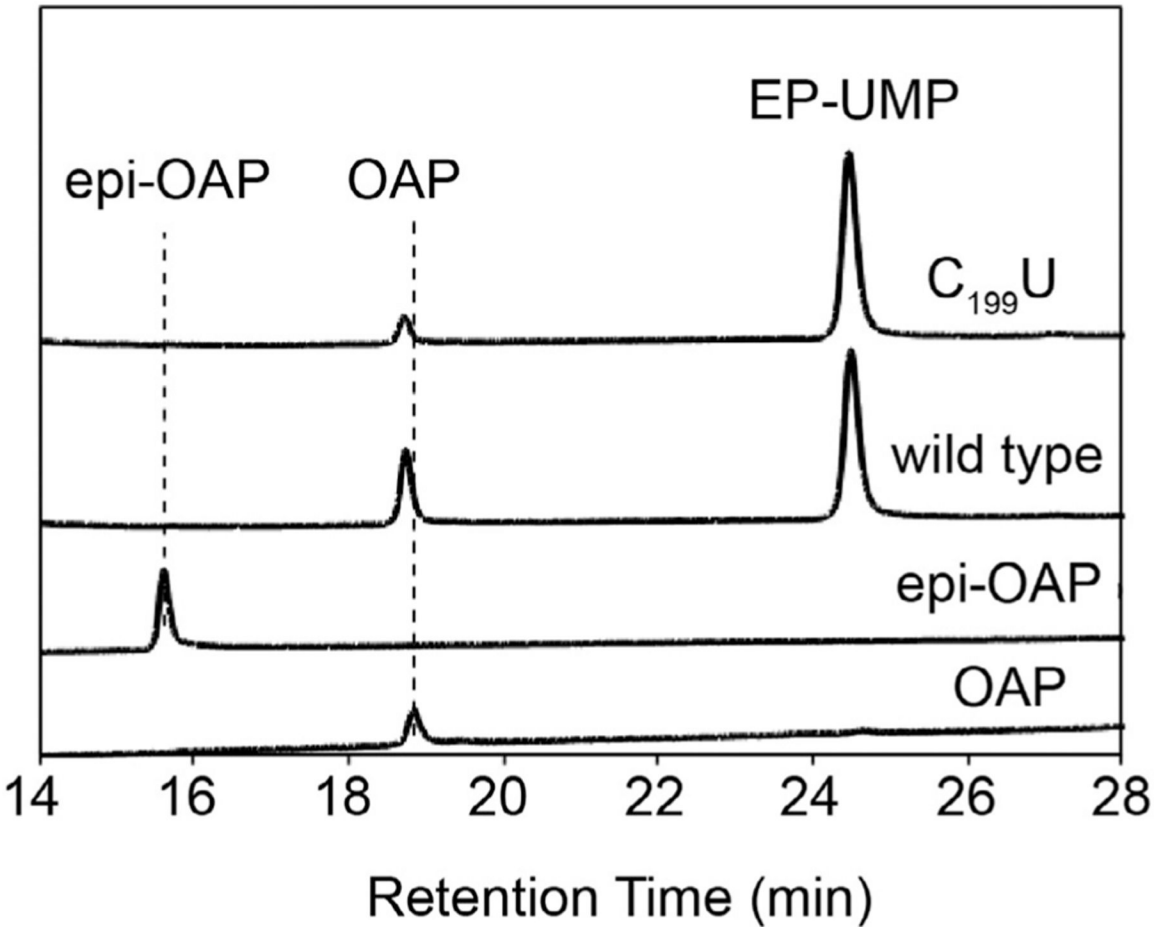

Fig. 6.

HPLC analysis of wild type and $C_{199}U$ *NikJ* activity. *NikJ* assays were performed with 10 μM of *NikJ* in the presence of SAM (0.5 mM), EP-UMP (0.5 mM), sodium dithionite (2 mM), DTT (2 mM), and 40 mM NaCl in 50 mM HEPES pH 7.0 or 50 mM MES pH 6.0. The reactions were initiated by the addition of the enzyme and incubated at 28°C for 1320 min. At each time point, 15 μL of the assay mix was mixed with equal volume of ethanol and stored at −20°C. After removal of the protein precipitation by centrifugation, the supernatant was diluted fourfolds in 10 mM ammonium acetate pH 6.0, and analyzed by HPLC (Dionex IC5000+, Thermo) equipped with a DNAPac PA-100 column (Thermo). The HPLC analysis was performed with a linear gradient of 10–300 mM ammonium acetate pH 6.0 over 40 min.