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Expression of selenocysteine-containing glutathione *S*-transferase in *Escherichia coli* ☆

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Abstract

Evolution of a probable 'glutathione-binding ancestor' resulting in a common thioredoxin-fold for glutathione *S*-transferases and glutathione peroxidases may possibly suggest that a glutathione *S*-transferase could be engineered into a selenium-containing glutathione *S*-transferase (seleno-GST), having glutathione peroxidase (GPX) activity. Here, we addressed this question by production of such protein. In order to obtain a recombinant seleno-GST produced in *Escherichia coli*, we introduced a variant bacterial-type selenocysteine insertion sequence (SECIS) element which afforded substitution with selenocysteine for the catalytic Tyr residue in the active site of GST from *Schistosoma japonica*. Utilizing coexpression with the bacterial *selA*, *selB*, and *selC* genes (encoding selenocysteine synthase, SelB, and $tRNA^{Sec}$, respectively) the yield of recombinant seleno-GST was about 2.9 mg/L bacterial culture, concomitant with formation of approximately 85% truncation product as a result of termination of translation at the selenocysteine-encoding UGA codon. The mutations inferred as a result of the introduction of a SECIS element did not affect the glutathione-binding capacity ($K_m = 53\,\mu M$ for glutathione as compared to 63 μM for the wild-type enzyme) nor the GST activity ($k_{cat} = 14.3\,s^{-1}$ vs. $16.6\,s^{-1}$), provided that the catalytic Tyr residue was intact. When this residue was changed to selenocysteine, however, the resulting seleno-GST lost the GST activity. It also failed to display any novel GPX activity towards three standard peroxide substrates (hydrogen peroxide, butyl hydroperoxide or cumene hydroperoxide). These results show that recombinant selenoproteins with internal selenocysteine residues may be heterologously produced in *E. coli* at sufficient amounts for purification. We also conclude that introduction of a selenocysteine residue into the catalytic site of a glutathione *S*-transferase is not sufficient to induce GPX activity in spite of a maintained glutathione-binding capacity.

Keywords: Glutathione *S*-transferase; Selenoprotein; Selenocysteine; Protein expression; Enzyme redesign

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) constitute a family of multifunctional proteins able to detoxify endogenous and xenobiotic electrophiles by conjugation with glutathione (GSH) [1,2]. Known GST structures consist of two domains, a conserved GSH-binding site at the N-terminus and a hypervariable hydrophobic xenobiotic substrate-binding site at the C-terminus [1–3]. According to sequence, immunological, kinetic, and tertiary/quaternary structural properties,

☆ *Abbreviations:* SECIS, selenocysteine insertion sequence; GST, glutathione *S*-transferase; sjGST, GST from *Schistosoma japonica*; GPX, glutathione peroxidase; Sec, selenocysteine; Seleno-GST, selenium-containing GST; FDH, formate dehydrogenase H.

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GSTs are classified into the α, μ, π, θ, κ, ζ, ω, σ, and β classes [3]. The GSH-binding domains typically belong to the thioredoxin-fold superfamily of protein domains [1,4], which among other enzymes also includes glutaredoxins, protein disulfide isomerases, and glutathione peroxidases. This possibly suggests the evolution of GSTs and GPxs from a common 'glutathione-binding protein' ancestor. In the active site of GSTs, the catalytic residue is typically either Tyr or Ser, presumed to act by inducing and stabilizing a reactive thiolate at the conjugating sulfur of the GSH substrate [1,3].

Glutathione peroxidase (GPX, EC1.11.1.9) is a well-known selenoprotein [5–7] which functions as an antioxidant to protect from oxidative damage by catalyzing the reduction of a variety of hydroperoxides, using glutathione as the reducing substrate and a reactive selenocysteine residue in the active site. Since the classical 'cytosolic GPX' was discovered in 1957 [8], four types of selenium-containing GPXs have been found in vertebrates [8–10]. Structural determinations, detailed kinetic studies, and modeling of enzyme–substrate complexes have suggested how the selenocysteine (Sec) residue plays its critical role in the catalytic cycle by utilizing the specific redox properties of selenium [11,12].

In all organisms known to have selenoproteins, Sec is encoded by an opal codon, UGA, which usually functions as a stop codon [6,13–15]. The alternative decoding of UGA as a codon for Sec incorporation is a complex co-translational process [14]. In *Escherichia coli*, this process involves a *cis*-acting element, the so-called SECIS (selenocysteine insertion sequence) element forming a structural feature of the mRNA following the UGA codon, and four *trans*-acting factors, SelA, SelB, SelC, and SelD [6,14–16]. The SelC factor is a selenocysteine-specific tRNA originally charged with a seryl moiety (seryl-tRNA^{Sec}), which is converted to selenocysteinyl-tRNA^{Sec} by the action of selenocysteine synthetase (SelA). The active selenide is donated by selenophosphate, synthesized from selenide and ATP by selenophosphate synthetase (SelD). SelB is the selenocysteine-specific elongation factor, which achieves specificity by interaction with both selenocysteinyl-tRNA^{Sec} and the SECIS element of the mRNA. Thereby the quaternary complex SelB·GTP·Sec-tRNA^{Sec}. SECIS is formed and Sec may then be inserted by SelB at the ribosome into the growing selenopolypeptide, at the site corresponding to the selenocysteine-encoding UGA codon. In prokaryotes, the SECIS element is located immediately downstream of the UGA within the open reading frame (ORF) and thereby the SECIS element must be unfolded upon Sec insertion in order to encode the amino acid residues following the Sec [17–19]. In archaea and eukaryotes, the SECIS elements must not necessarily unfold upon Sec insertion, as they are instead located in the 3′ untranslated region of the selenoprotein-coding mRNA [20,21]. The bacterial SECIS element is rather species-specific [22] while the SECIS elements in eubacteria and eukaryotes are somewhat less species-restricted [23]. The SECIS element of the fdhF mRNA, encoding the 80 kDa subunit of formate dehydrogenase H in *E. coli*, has been characterized in significant detail [17]. The minimal requirement for SECIS function is the upper stem–loop structure consisting of 17 nucleotides, which is the region that binds to and thereby recruits SelB, and this stem–loop structure must be located 11 nucleotides downstream of the UGA codon [18,19].

It is chemistry, not binding specificity, that is the dominant factor in evolution of new enzymatic activities [24]. As a consequence, enzymes with similar folds can catalyze very different chemical reactions as a result of different catalytic groups in their active sites. Both GSTs and GPXs have a GSH-binding site [1], but to a great extent due to the use of different catalytic residues, i.e., Tyr (or Ser) on one hand and Sec on the other, they have completely different catalytic mechanisms [4]. However, some overlap between their activities may exist in some aspects, beyond their shared GSH-dependency, since certain GSTs were reported to display GPX activity towards organic hydroperoxide [25]. We thereby wanted to ask whether it could be possible to convert a GST protein to an enzyme having GPX activity, by introducing a Sec residue into the active site. Separate strategies may be attempted to introduce a Sec residue into a protein. Two methods constitute either chemical modification [26,27] or the use of selenium substitution to a cysteine auxotroph strain [28,29], but neither of these methods can achieve truly site-directed substitution. We previously reported the conversion of GST to a selenium-containing GST (seleno-GST) with GPX activity using chemical modification [27]. However, the non-directed substitution hampered any further structure–function characterization of the seleno-GST thus produced. Use of peptide ligation mediated by Sec is also hampered due to the limitations for the size of selenopeptide prepared by chemical synthesis [30,31]. Another method is to achieve heterologous expression of a recombinant selenoprotein, using the selenoprotein synthesis machinery of *E. coli.* This has been rather successfully used for production of mammalian thioredoxin reductase, which has a selenocysteine residue close to the C-terminus, but the method is more difficult when the Sec residue is internally situated, since the essential SECIS element will impose mutations in the resulting protein [32,33]. We nonetheless used this strategy in the present study and report that a GST could be successfully engineered into a selenium-containing enzyme replacing its native catalytic residue Tyr7 with Sec. This seleno-GST is the first recombinant selenoprotein heterologously produced in *E. coli* with an internal Sec residue, that has been completely purified. We also show that the SECIS element did not affect the GSH-binding capacity and although the approach failed to engineer a novel GPX activity, this production may serve as a

promising starting point for further studies involving recombinant selenoproteins.

Materials and methods

Materials. The pGEX-2T plasmid, the goat anti-sjGST polyclonal antibody, and glutathione–Sepharose 4B were purchased from Pharmacia. The secondary rabbit anti-goat IgG was from Dingguo (Beijing, China). Restriction endonucleases, T4 DNA ligase, and Pyrobest DNA polymerase were from TaKaRa (Dalian, China). NADPH, 1-chloro-2,4-nitrobenzene (CDNB), cumene hydroperoxide, and yeast glutathione reductase were from Sigma. Butyl hydroperoxide was from Fluka. The [^{75}Se]selenite was obtained from the Missouri University Research Reactor (MURR), USA.

Growth media and bacterial strains. E. coli. strain JM109 was used for cloning and propagation of plasmids and expression studies were preformed in *E. coli* strain BL21 (DE3). Bacteria were grown in 2× YT medium with ampicillin (100 μg/ml) for cells with pGEX-2T or pPelB, and/or chloramphenicol (34 μg/ml) for cells with the pSUABC plasmid carrying the accessory *sel* genes [32].

Construction of plasmids. The plasmid pGEX-2T was a commercial vector from Pharmacia, which carries glutathione *S*-transferase gene from the helminth *Schistosoma japonica* (sjGST). Overlap PCR was used for mutagenesis of the Tyr7 codon UAU to a Sec codon (UGA) and for introducing the minimal SECIS element. For this, we used six primers, including two pairs of complementary oligonucleotides (see Table 1 for sequences of primers). Primers P1 and P2 were used to amplify the 1.9 kb DNA fragment from pGEX-2T carrying the GST open reading frame and introducing suitable restriction sites (nucleotides given in italics for P1 and P2 in Table 1 introduced *Eco*RV and *Eco*RI cleavage sites). The SECIS element was introduced into sjGST using primers S1 and S2 whereas mutagenesis of Tyr7 to Sec was performed with primers T1 and T2. The 1.9 kb PCR product was cut with *Eco*RV and *Eco*RI endonucleases and ligated back to the purified 3.0 kb fragment of pGEX-2T. In order to make the seleno-GST a fusion protein, we subsequently synthesized another pair of primers, P3 and P4, to amplify the seleno-GST gene and introducing *Bam*HI and *Eco*RI restriction sites. This PCR product was then cut with *Bam*HI and *Eco*RI and inserted into the pGEX-2T plasmid downstream of the wild-type sjGST gene. To provide additional nucleotides at the N-terminus of the seleno-GST for fusion with the signal peptide of pelB, primers P5 and P6 were designed to amplify the seleno-GST gene, which was then ligated into plasmid pPelB by digestion with *Eco*RI and *Sal*I. As a result, three separate mutants of sjGST could be expressed—namely GST-U (sole mutation of Tyr by Sec), GST-S (containing the minimal SECIS but with intact Tyr7), and seleno-GST (containing the Sec-for-Tyr mutation and the SECIS element), as well as a fusion of the seleno-GST construct at the C-terminal side of sjGST were engineered.

Expression and analysis of recombinant protein. Transformed BL21 (DE3) cells expressing sjGST or GST-S were grown in liquid 2× YT media containing ampicillin (100 μg/ml) at 37 °C to an A_{600} of 0.6–0.8 and induced with 1 mM IPTG for 4 h at 30 °C. Transformed BL21 (DE3) cells expressing seleno-GST or seleno-GST were grown at 37 °C to an A_{600} of 2.4 and induced with 1 mM IPTG for 18 h at 24 °C.

Protein concentration was determined using a Bio-Rad protein assay with BSA as standard, essentially following the method of Bradford [34]. Western blotting was performed on the nitrocellulose membranes according to the manufacturer's instructions (GST Handbook, Pharmacia) with small modifications: the goat anti-GST antibody was used with 1: 10,000 dilution and 1% Tween 20 was included in all solutions. The second antibody was a horse anti-goat IgG-horseradish peroxidase (HRP) conjugate. For ^{75}Se labeling, the procedure previously described [32] was utilized.

Purification of enzyme. The seleno-GST fusion product was purified from the soluble protein fraction of bacterial extracts using a glutathione–Sepharose 4B column and Waters HPLC system. All operations were carried out at 0–4 °C, unless otherwise noted. As a first step a batch purification of the seleno-GST fusion was made from the crude extracts of *E. coli* using GSH–Sepharose 4B, as follows: 1 L of bacterial culture was collected, resuspended in 30 ml buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 1.8 mM KH_2PO_4, pH 7.3), and then disrupted by sonication. The supernatant was collected by centrifugation and added to 10 ml of 50% slurry GSH–Sepharose 4B, equilibrated with buffer A. After incubation with gentle agitation at room temperature for 30 min, the GSH–Sepharose matrix was collected by centrifugation and washed four times with 50 ml buffer A. The bound fusion protein was then eluted from the GSH–Sepharose 4B with 10 ml buffer B (10 mM GSH, 50 mM Tris–HCl, pH 8.0) and collected as the supernatant after a centrifugation. After incubation with 8 M urea for 30 min at room temperature, the supernatant was fractionated using Waters HPLC system fitted with a TSK 2000SWXL gel column (7.8×300 mm, TOSOH, Japan). The column had been equilibrated with buffer C (20 mM sodium phosphate, 8 M urea) and elution was performed using 15 ml buffer. Fractions (0.7 ml) were collected manually and diluted 10-fold with buffer D (50 mM sodium phosphate, pH 6.5, 0.1 M NaCl, 2 mM EDTA, and 2 mM DTT) without urea to allow for renaturation. Finally, the fractions containing target protein, as detected at double-wavelength of 254 and 280 nm, were purified by HPLC with a TSK 2000SWXL column, equilibrated with buffer E (20 mM sodium phosphate, 2 mM EDTA, pH 7.0), in order to remove any trace DTT. The wild-type sjGST and its mutant GST-S and seleno-GST were also first partially purified using a GSH–Sepharose 4B batch approach, as in purification of the seleno-GST fusion. Then the GSH present in the eluted supernatant fraction was removed by HPLC with the TSK 2000SWXL column equilibrated and eluted with buffer E. The enzymes were concentrated by reverse dialysis against PEG20000. All of the purified protein fractions were

Table 1
Primers for PCR amplification

Primer	Sequence[a]	Orientation[b]
P1	5′-AT*GAATTC*CTGGGGATCCACG-3′	+
P2	5′-TGCG*GATATC*TCGGTAGTGGG-3′	−
S1	5′-TTAAGGTTGCAGGTCTGCACCCTCGACTTCTTTT-3′	+
S2	5′-GGGTGCAGACCTGCAACCTTAATTTTCCAATAACCTAGT-3′	−
T1	5′-CTATACTAGGTTGATGGAAAATTAAGG-3′	+
T2	5′-TTAATTTTCCATCAACCTAGTATAGGG-3′	−
P3	5′-ATTC*GGATCC*CCTATACTAGGTTG-3′	+
P4	5′-ACC*GAATTC*TTATTTGGAGGATGG-3′	−
P5	5′-CAGTATT*GAATTC*CCCTATACTAGG-3′	+
P6	5′-CAG*GTCGAC*TTTTGGAGGATGG-3′	−

[a] Nucleotides in italics correspond to the restriction sites. Underlined nucleotides correspond to the SECIS element and Sec codon UGA.
[b] +, primer directed towards the 3′ end of *sjGST*; −, primer oriented in the opposite direction.

frozen in batch immediately after purification and stored at −78 °C. Purity was >90% as judged by SDS–PAGE electrophoresis.

CD and fluorescence measurements. Circular dichroism spectra were measured at 25 °C using spectropolarimeter JASCO J-810 with a 0.5 mm path length. Each spectrum was the average of three scans using a band width of 0.5 nm and an integration time of 1 s with the concentration of protein being 0.52 mg/ml. Measurements of intrinsic protein fluorescence were performed at 25 °C using spectrofluorometer SHIMADZU RF-5301PC. The fluorescence was measured at 341 nm upon excitation of tryptophans at 295 nm. The concentration of protein was in this case 1 mg/ml and all fluorescence measurements were repeated at least three times. All protein samples for CD or fluorescence measurements were in 100 mM sodium phosphate buffer, pH 7.0.

Assay of enzymatic activity. The activity of enzymes was measured using an UV/visible spectrophotometer SHIMADZU UV-3100. The GST activity of sjGST and its mutant GST-S was measured as described by Habig and Jakoby [36]. The reaction was carried out at 30 °C in 1 ml solution containing 100 mM sodium phosphate buffer, pH 6.5, 1 mM GSH, and 0.5–5 μg enzyme. After preincubation for 3 min, 1 mM CDNB was added, and then the increase of absorbance resulting from dinitrophenyl conjugation with glutathione was recorded at 340 nm for 3 min. The appropriate controls for non-enzymatic reaction were performed and subtracted from the catalyzed reaction (typically <25% of the enzymatic reaction). Kinetic constants for GSH were determined by varying [GSH] from 0.04 to 1 mM and holding [CDNB] at 4 mM, using the enzyme amounts given in the figure legends.

The GPX activity was measured according to Wilson's method [35]. These reactions were carried out at 37 °C in 700 μl solution containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM GSH, 1 U GSH reductase, and 0.5–100 μg enzyme, following consumption of NADPH by decrease in absorbance at 340 nm.

Results

Design of sjGST

As starting protein for this work, we selected glutathione *S*-transferase from the helminth *S. japonica* (sjGST, Accession No. 1GTB) because its chemical properties and crystal structure have been investigated in detail [37–39] and it can be expressed in *E. coli* with a high yield [40]. Furthermore, sjGST can be purified rapidly and easily from cell lysates using a glutathione–Sepharose column. Because expression of a eukaryotic selenoprotein in *E. coli* requires the introduction of a bacterial-type SECIS element immediately adjacent to the UGA codon inside the ORF [18,22], this necessitates a change of amino acid sequence of some residues on the C-terminal side of the Sec residue. The minimal fdhF SECIS [18,19], consisting of 17 nucleotides, was thus introduced into the sjGST gene 11 nucleotides downstream of the UGA codon (Fig. 1A).

The analysis of potential secondary structures adopted by the mRNA of seleno-GST sequence and calculation of energies was carried out using the 'MFOLD' program [41], suggesting that the minimal fdhF-SECIS could be stably folded (data not shown). Compared with the wild-type sjGST, the introduction of the SECIS element resulted in six sequential point mutations, namely G12V, L13A, V14G, N15L, P16H, and T17P (Fig. 1). As suggested from the crystal structure of sjGST [38,39], the former three mutations should reside in a loop between the second structure β1 and α1, whereas the last three mutations should reside in helix of α1. Two conserved residues (Gly12 and Leu13) were mutated. In order to assess whether these mutations affected activity, a control mutant was made in which the SECIS element was introduced into the open reading frame, but the catalytic Tyr residue was kept intact. In order to produce the selenocystine-containing variant, the catalytic residue Tyr7 codon UAU was however mutated to the opal UGA codon, which should direct Sec substitution for Tyr7, when made in concert with the introduction of the here described SECIS element.

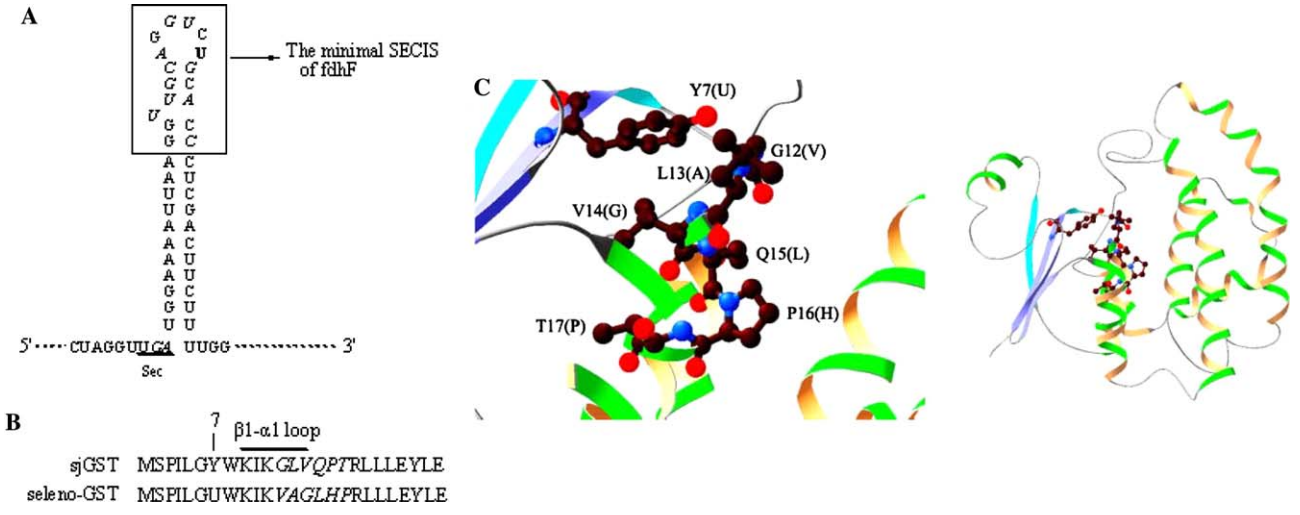

Fig. 1. (A) The minimal SECIS element (boxed) engineered into the sjGST ORF at a distance of 11 nucleotides from the codon UGA coding Sec by mutating the corresponding nucleotides. (B) Alignment of the partial amino acid sequences of sjGST and seleno-GST where substitutions are made as a result of the SECIS element. U represents Sec and nucleotides and amino acids mutated as a result of the introduction of the SECIS element are highlighted in italics. (C) Schematic representation of the crystal structure of sjGST [38] showing the proposed location of the mutated amino acid residues in the tertiary structure of the protein.

Expression of seleno-GST

The bacteria transformed for expression of seleno-GST were grown in 2× YT medium complemented with 5 μM sodium selenite. To examine production of seleno-GST, protein extracts were analyzed by SDS–PAGE, ^{75}Se-labeled autoradiogram as well as Western blotting. As shown in Fig. 2B, no protein band corresponding to seleno-GST (~27 kDa) could be detected on a Coomassie-stained gel and even with coexpression of genes selA, selB, and selC, there was no detectable band neither in the Coomassie-stained gel (Fig. 2A, lane 5) nor in the autoradiogram showing ^{75}Se-incorporation (Fig. 2B, lane 5). Since the UGA is positioned close to the N-terminus (Sec7) in seleno-GST, this makes it impossible to detect any truncation product in a conventional protein gel. We therefore inserted the seleno-GST gene into the pGEX-2T plasmid between *Bam*HI and *Eco*RI sites downstream of the wild-type sjGST gene to obtain a fusion of sjGST (with Tyr7) and seleno-GST (with Sec7). Thus, the presence of any truncation product could be detected both using Coomassie-staining and Western blot against sjGST, while synthesis of the full-length Sec-containing protein could be assessed using Coomassie-staining, Western blot as well as ^{75}Se-labeling. The molecular weight of the sjGST/seleno-GST fusion protein is 52 kDa and production of this protein was clearly revealed as a ^{75}Se-labeled product increased in intensity upon co-transformation with *selA*, *selB*, and *selC* (Fig. 2B, lanes 7 and 8). As evident from Fig. 3, both the UGA-truncation product produced from the fusion gene (i.e., wild-type sjGST, 28 kDa) and the Sec-containing full-length sjGST/seleno-GST fusion protein (52 kDa) could be detected at significant amounts on both the Coomassie-stained gel (Fig. 3A), as well as using Western blot against the sjGST epitope (Fig. 3B).

Expression of the sjGST/seleno-GST fusion helped to overcome the block in translation initiation and enabled clear detection of both truncation product and the full-length selenoprotein, but made purification of an isolated seleno-GST difficult. Therefore, we constructed another expression vector using the pPelB plasmid, which enables fusion with a signal peptide of 22 amino acids at the N-terminus [42]. The resulting vector was named pPelB-seleno-GST and was co-transformed into BL21 cell with pSUABC plasmid. This resulted in higher expression levels than direct expression of seleno-GST and the correct product could be positively identified by Western blotting using the whole cell lysate (Fig. 4).

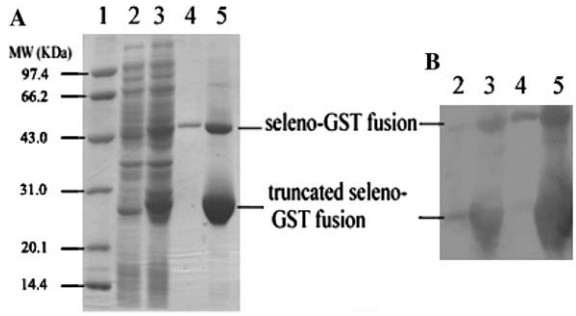

Fig. 3. Immunoblot analysis with sjGST antibody. (A) 12% SDS–PAGE gel stained with Coomassie blue. Lane 1, Protein marker; lane 2, lysates of BL21 cells with selA, selB, and selC genes without IPTG induction; lane 3, lysates of BL21 cells coexpressing seleno-GST fusion with selA, selB, and selC genes with IPTG induced; lane 4, fraction of seleno-GST fusion from HPLC; and lane 5, fractions from GSH–Sepharose 4B. (B) Western blot from the SDS–PAGE gel (A).

Purification and analysis of kinetic parameters

All of the enzymes (sjGST, GST-S, and seleno-GST) were subsequently purified to near homogeneity, including the seleno-GST which was expressed using the pPelB approach (Fig. 4). The yield of seleno-GST was about 2.9 mg/L of culture. As shown in Fig. 6, the truncated product resulting in wild-type sjGST produced by UGA truncation of the sjGST/seleno-GST fusion was

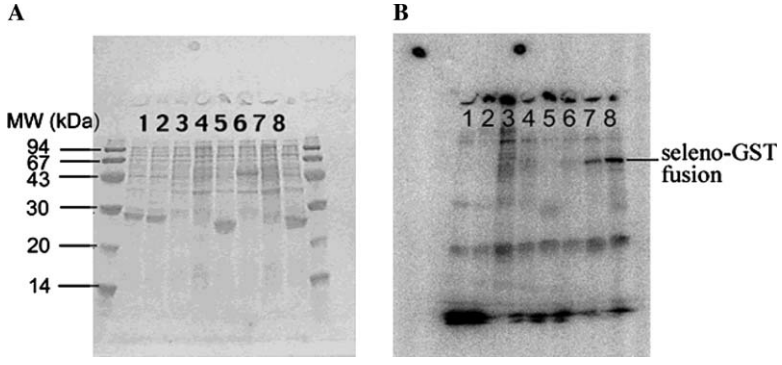

Fig. 2. Detection of the recombinant proteins produced in *E. coli*. (A) Coomassie-stained gel from an SDS–PAGE analysis of the lysates of *E. coli* carrying plasmid pGEX and/or pSUABC. Lane 1, sjGST; lane 2, GST-S; lane 3, GST-U; lane 4, seleno-GST; lane 5, coexpressing seleno-GST with selA, selB, and selC genes; lane 6, sjGST–sjGST fusion; lane 7, seleno-GST fusion; and lane 8, coexpressing seleno-GST fusion with selA, selB, and selC genes. In (B), the autoradiogram showing ^{75}Se-labeling is shown, using the same SDS–PAGE gel as shown in (A).

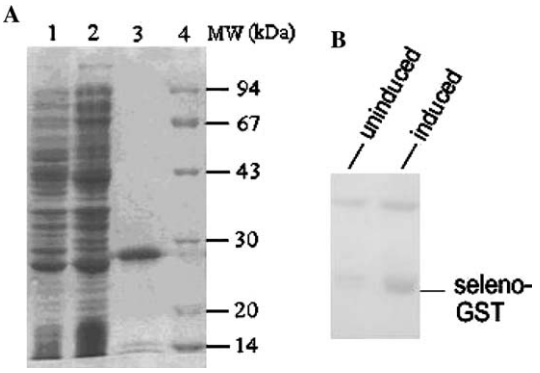

Fig. 4. SDS–PAGE Western-blot analysis of purified seleno-GST and lysates of *E. coli* cells transformed with pPelB-seleno-GST. (A) Lane 1, lysates of BL21 cells with selA, selB, and selC genes without IPTG induction; lane 2, lysates of BL21 cells coexpressing seleno-GST fusion with selA, selB, and selC genes with IPTG induced; lane 3, purified seleno-GST eluted from GSH–Sepharose 4B; and lane 4, Protein marker. (B) Western-blot analysis of lysates of *E. coli* cells.

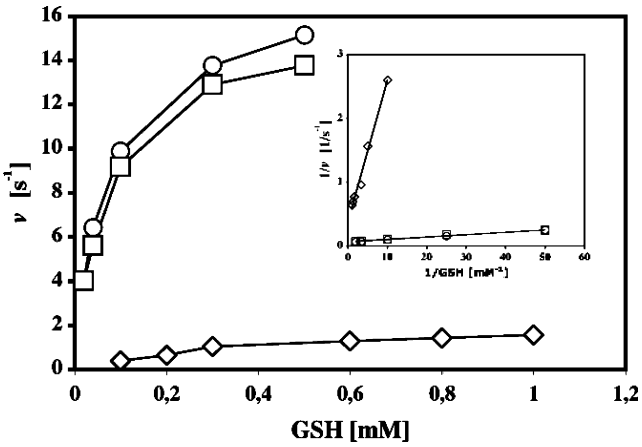

Fig. 6. Kinetics of the catalyzed conjugation reaction between CDNB and GSH for sjGST (○), GST-S (□), and seleno-GST (◇). The velocities (V) of the reaction are expressed as M product formed per M enzyme per second. The data are means for at least three separate measurements with standard errors less than 5%. The inset displays the Lineweaver–Burk plot used for determination of kinetic parameters given in Table 2.

also purified over the GSH–Sepharose. If the purification yield is considered to be the same for the full-length and the truncated product, this could be used to estimate the read-through efficiency of Sec. Hence, we performed a serial dilution of the proteins purified from the sjGST/seleno-GST fusion approach and analyzed this on SDS–PAGE. From the result shown in Fig. 5, the relative concentration of the full-length product over the truncated UGA-product suggested an efficiency of selenocysteine incorporation at about 13%. About 0.924 mg of full-length Sec-containing sjGST/seleno-GST fusion protein could be purified from 1 L of bacterial culture. The final yield of pure seleno-GST using the pPelB-approach was about 0.25 mg purified protein obtained from 1 L of bacterial culture.

To assess any signs of effects on the overall protein structure by introducing the mutations resulting from the need of a SECIS element, measurements of intrinsic fluorescence and circular dichroism were performed using the GST-S and sjGST proteins. Far-UV circular dichroism measurements gave highly similar spectra of GST-S compared to those of the wild-type sjGST, suggesting that the overall fold was maintained (data not shown). Also the fluorescence spectra showed no difference between these proteins using the maximum emission wavelength at 341 nm (not shown).

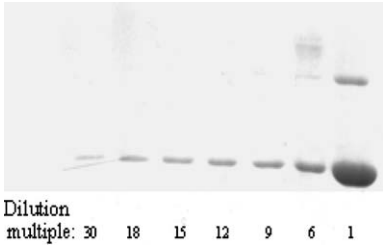

Fig. 5. Serial dilutions of fractions from GSH–Sepharose 4B. The upper band corresponds to the full-length sjGST/seleno-GST fusion protein, while the lower band represents the sjGST part of the fusion protein resulting from UGA termination.

We next initiated determination of kinetic parameters for the purified wild-type sjGST and the GST-S and seleno-GST mutants using the CDNB substrate in the typical GST assay. As illustrated in Fig. 6, the GST activity was *not* affected by the introduction of mutations as a result of the SECIS element, whereas substitution of Tyr7 for Sec clearly hampered the GST activity. The resulting kinetic parameters are given in Table 2. In comparison to sjGST, the mutant GST-S had essentially the same K_m for glutathione, k_{cat} and thereby similar catalytic efficiency (k_{cat}/K_m) as the wild-type enzyme (Table 2). In contrast, the k_{cat}/K_m of the seleno-GST was severely decreased (about 60-fold) as a result of both decreased k_{cat} and increased K_m. This result was not surprising, as the catalytic Tyr residue had been exchanged for a Sec, which can hardly function as a base stabilizing a reactive thiolate of the GSH substrate. However, the maintained activity of the GST-S showed that the SECIS element did not affect GSH binding and since Sec is commonly utilized in peroxidase reactions, we next wished to assess any GPx activity in the

Table 2
Kinetic parameters of the wt sjGST and mutant GST-S and seleno-GST

Enzyme	GSH		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
sjGST	0.063 ± 0.008	16.6 ± 1.1	263.2 ± 3.0
GST-S	0.053 ± 0.005	14.3 ± 1.2	270.3 ± 3.4
Seleno-GST	0.57 ± 0.025	2.6 ± 0.1	4.5 ± 0.22

purified proteins, based on the reasoning further outlined in the Introduction. GPX activity of all three enzymes was hence determined but no GPX activity could be detected for any of these proteins, using three standard peroxidase substrates (hydrogen peroxide, butyl hydroperoxide, and cumene hydroperoxide). Hence, a maintained GSH binding in a glutathione *S*-transferase with substitution of the catalytic Tyr residue for Sec is not sufficient for the introduction of a novel GPx activity.

Discussion

Although the mechanism of bacterial translation of selenoproteins has been well elucidated [6,13–16], expression of heterologous selenoproteins in *E. coli* has been hampered due to the low inherent efficiency of selenocysteine incorporation and the species-specific requirement of a bacterial SECIS element [22,32,33,43]. Analysis of the natural FDH-H SECIS element has revealed that the Sec incorporation is a process with very low efficiency (~2–5%) and even though coexpression with SelA, SelB, and SelC is utilized, the efficiency still remains rather low, although it was shown to be increased to about 7–10% [43]. That figure agrees well with the 13% efficiency that we report here, using the sjGST/seleno-GST fusion approach. In spite of this rather low efficiency of Sec incorporation at an internal position in a selenoprotein, we were here able to produce recombinant heterologous selenoproteins in *E. coli* at a yield sufficient for analysis of the purified enzymes. The yield per liter of bacterial culture for the sjGST/seleno-GST fusion protein and the seleno-GST produced using the pPelB signal peptide was 0.924 and 0.247 mg, respectively.

The introduction of a SECIS element had no major effect on the second structure and global conformation of sjGST, as suggested by the circular dichroism (CD) spectra and intrinsic fluorescence intensity data and the intact GST activity. This was a promising result, as it infers that introduction of a Sec residue by introduction of a SECIS element within an open reading frame may be a productive approach for the synthesis of synthetic selenoproteins with internal Sec residues. The second question we addressed in this study—whether a GST protein could be turned into a GPx by substitution with Sec—did not give an affirmative result. After substituting Sec for the catalytic residues Tyr7, the K_m for GSH increased more than 10-fold compared to the GST-S protein, which was a surprise. Possibly the difference of side chain structure between Tyr and Sec is sufficiently large to affect the local conformation of amino acid residues at the active site and decrease hydrogen bond interactions for binding of the GSH substrate. Alternatively, as the seleno-GST completely failed to display GPX activity, it may indicate that the Sec residue makes rather stable selenenylsulfide derivatives with GSH, which may possibly be attacked and reduced by a second molecule of GSH, but at a low rate, resulting in a distorted apparent "K_m" and a lack of activity. In fact, we find it difficult to explain the residual, albeit low, GST activity of the seleno-GST (Table 2). Most likely the Sec residue is involved in a number of different reactions, the nature of which must be more thoroughly studied in order to reveal their true biochemical nature. To summarize this study, however, we may conclude that although we could not introduce a novel GPx activity by Sec insertion into a GST protein, we have shown that Sec-containing proteins may indeed be expressed in *E. coli* at a yield sufficient to analyze their kinetic properties, and that a SECIS element may be introduced into an enzyme without necessarily severely affecting the kinetic properties of that enzyme. We thereby propose that the method utilized herein for production of recombinant selenoproteins carrying internal Sec residues may be attempted as a general strategy in future work focused on enzymes and other proteins carrying internal Sec residues.

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