Cysteine-free glutathione-S-tranferase as a tool for thiol-specific labeling of proteins

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Glutathione-S-transferase (GST) is a detoxification enzyme that interacts with glutathione in a highly specific manner, enabling *Schistosoma japonica* GST to be used as a tag for efficient affinity purification (1). Fusion of GST to target proteins can significantly increase their solubility (2), which is a property that has allowed the mechanism of amyloid formation by polyglutamine proteins to be studied in vitro (3).

Numerous applications require proteins to be chemically modified at specific amino acid residues. Cysteine residues are relatively rare, highly reactive, and can be modified with very high selectivity. Thus, thiol-specific reagents provide the best means to modify proteins selectively at defined sites to obtain detailed structural and functional information, as a recent study of the yeast PSI prion protein has demonstrated (4). The application of this technique to GST fusion proteins is limited by the presence of four cysteines within GST, two of which readily react with thiol-specific reagents. To overcome this limitation, we constructed a GST mutant in which all the cysteines are substituted with serines (GST C4S).

The introduction of point mutations in proteins can result in significant changes in their structural and/or functional properties. The crystal structure of *S. japonica* GST (5) shows that none of the four cysteine residues are directly involved in glutathione binding or at positions where their exchange with serines is expected to cause steric clashes. Therefore, we expected GST C4S to have very similar biochemical and biophysical properties compared with the wild-type protein but a significantly reduced ability to interact with thiol-specific reagents, making it useful for the purification and site-specific labeling of fusion proteins.

We used the plasmid pGEX-6P-1 (GenBank® accession no. U78872; Amersham Biosciences, Freiburg, Germany) as a template and sequentially introduced four single nucleotide substitutions by a modified PCR mutagenesis protocol (6). Briefly, the plasmid was amplified using *Pfu* polymerase (Stratagene, La Jolla, CA, USA) to generate the mutated full-length linear plasmid as the product. Table 1 shows the 5′ phosphorylated primers containing the desired mutations that we used. The template was then digested with *Dpn*I, and the product was circularized by ligation. The final plasmid pGEXC4S-6P-1 encodes GST with the cysteines substituted with serines, GST C4S. The presence of the mutations was verified by DNA sequencing.

GST and GST C4S expression was induced in *Escherichia coli* SURE® competent cells (Stratagene) at 28°C with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h in 2× TY media. The cells were lysed for 30 min at 4°C in Buffer A [50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol (DTT)] containing 0.5 mg/mL lysozyme, 0.1% Triton X®-100, and 0.1 U/µM Benzonase® (Merck, Darmstadt, Germany), followed by sonication and centrifugation at 100,000× *g*. Supernatants were loaded onto Glutathione-Sepharose™ 4B columns (Amersham Biosciences). The beads were sequentially washed with Buffer A/0.1% Triton X-100 and Buffer A/0.5 M NaCl. Proteins were eluted with 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, 10% glycerol, 0.1 mM DTT, and 20 mM reduced glutathione, dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 10% glycerol, 0.1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and stored at -80°C. The purity of GST and GST C4S was very high (Figure 1A). The yields of both proteins were tens of milligrams per liter of culture, showing that the introduction of serine residues does not interfere with the ability of the protein to bind glutathione. Results from our laboratory show that GST C4S is as efficient for the expression and purification of fusion proteins as GST (C. Langer and R. Boteva, unpublished data).

Both GST and GST C4S interact to a similar extent with commercially available antibodies (Figure 1B), demonstrating that there are no major changes in epitope exposure.

The tryptophan fluorescence spectra of GST and GST C4S in 20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$ at 30°C are identical (Figure 1C) and their far UV circular dichroism (CD) spectra at the same conditions are also very similar (data not shown). Upon gel filtration chromatography at pHs between 5 and 9, both proteins behave like dimers with apparent molecular masses of 54 kDa

Table 1. Mutagenesis Primers

Primer Name	Sequence
GSTC85SF	5′-GGTT**C**TCCAAAAGAGCGTGCAGAG-3′
GSTC85SR	5′-ACCCAACATGTTGTGCTTGTCAGC-3′
GSTC138SF	5′-CATAAAACATATTTAAATGGTG-3′
GSTC138SR2	5′-A**G**ATAAACGATCTTCGAACATTTTCAGC-3′
GSTC169,178SF	5′-GT**C**CCTGGATGCGTTCCCAAAATTAGTTT**C**TTTTA-AAAAACGTATTGAAGC-3′
GSTC169,178SR	5′-ATTGGGTCCATGTATAAAACAACATCAAGAGCG-3′

The mismatching bases have been bolded and underlined.

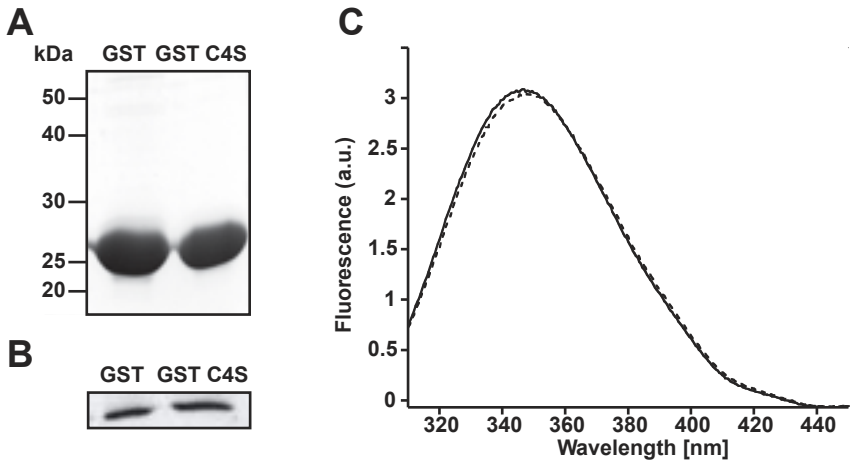

Figure 1. Characterization of GST and GST C4S. (A) Purified GST and GST C4S (20 µg each) separated by SDS-PAGE. (B) Purified GST and GST C4S (1 ng each) separated by SDS-PAGE, transferred to nitrocellulose membrane, and detected by immunoblotting using 1/2000 diluted goat anti-GST antibody (Amersham Biosciences). (C) Tryptophan fluorescence of GST (solid line) and GST C4S (dashed line) (2 µM each). Tryptophan residues were excited at 295 nm. GST, glutathione-S-transferase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; a.u., arbitrary unit.

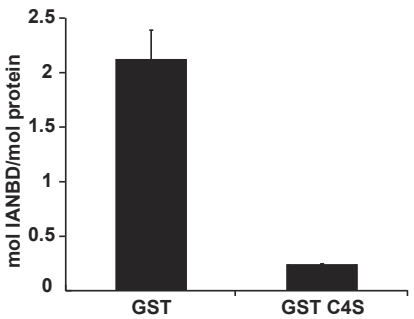

Figure 2. Fluorescent labeling of GST and GST C4S. Data show molecules of IANBD incorporated per molecule of protein. The average of three experiments is shown together with the standard deviation. GST, glutathione-S-transferase; IANBD, *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole.

at pH 7.4 (data not shown), which is in accordance with the previous studies of GST (7). These results demonstrate that the substitution of all four cysteine residues with serines does not result in significant changes in the secondary, tertiary, or quarternary structure of the protein.

The abilities of GST and GST C4S to interact with thiol-specific reagents were compared by incubating 20 µM of each protein with 400 µM *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester; Molecular Probes, Eugene, OR, USA) in 20 mM Tris-HCl, pH 7.5, 150 mM KCl at 4°C for 2 h, followed by gel filtration on a Bio-Spin™ 30 desalting column (Bio-Rad Laboratories, Munich, Germany) to remove the free dye. The protein-to-dye ratio was determined spectroscopically from protein and IANBD absorbance at 280 and 480 nm, respectively. GST bound approximately two molecules of IANBD, which suggests that two of the four cysteines are highly reactive. In contrast, only trace amounts of dye were found in GST C4S under the same conditions (Figure 2), most probably due to nonspecific, noncovalent binding.

The dramatically reduced affinity of GST C4S for thiol-specific reagents and almost identical biophysical and biochemical properties of GST and GST C4S suggest that the mutant protein can be used as a tag for affinity purifications, and all previously developed reagents and procedures can be applied without any significant changes. GST C4S fusions could be very useful in studies on structure, function, and interactions of proteins containing cysteine residues with low reactivity, especially when highly selective and specific thiol labeling is required, such as in fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy, fluorescence and anisotropy decay measurements, and cross-linking. It is therefore hoped that the novel GST C4S will increase the range of applications of GST in biochemistry and biophysics.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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