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Affinity purification of *Schistosoma japonicum* glutathione-S-transferase and its site-directed mutants with glutathione affinity chromatography and immobilized metal affinity chromatography

Hsiu-Mei Chen[a,*], San-Lin Luo[a], Kai-Ti Chen[a], Chong-Kuei Lii[b]

[a]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 106, Taiwan*

[b]*Department of Nutrition, Chung Shan Medical College, Taichung 402, Taiwan*

Abstract

A C-terminally polyhistidine-tagged protein of *Schistosoma japonicum* glutathione-S-transferase, named as SjGST/His, and its Cys85→Ser, Cys138→Ser, and Cys178→Ser site-directed mutants were prepared and highly expressed in *Escherichia coli*. Both immobilized metal affinity chromatography (IMAC) and glutathione (GSH) affinity chromatography were used to purify these four enzymes. All of them were purified with equal efficiency by Ni^{2+}-chelated nitrilotriacetic acid agarose gel, but not by GSH Sepharose 4B gel. The protein amounts of wild-type and Cys85→Ser enzymes purified by the latter gel were three to seven-fold greater than those of the other two enzymes purified by the same gel, while their specific activities were two-fold lower, presumably because of the occurrence of noncovalent aggregation. Both purification methods yielded highly pure enzymes, while there were minor amounts of inter- and intra-disulfide forms in the IMAC purified enzymes except for the Cys85→Ser mutant. Addition of dithiothreitol to GSH-affinity purified enzymes shifted all of their mass spectra of matrix-assisted laser desorption/ionization-time of flight mass spectrometry toward low molecular-mass regions, while addition of GSH to IMAC purified enzymes shifted the spectra toward high molecular-mass regions. The shift values of wild-type enzyme were larger than those of the three mutants, indicating that the Cys85, Cys138, and Cys178 residues were S-thiolated by GSH during the GSH-affinity purification. This result was confirmed by isoelectric focusing. These findings suggest that IMAC is more efficient than the conventional GSH-affinity system for the purification of SjGST/His enzyme, especially for its mutants and fusion proteins. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Affinity chromatography; *Schistosoma japonicum*; Immobilized metal affinity chromatography; Gluatathione-S-transferase; Enzymes

1. Introduction

Glutathione-S-transferases (GSTs) are a family of multifunctional proteins that can catalyze the nucleophilic addition of the thiol group of glutathione (GSH) to a variety of electrophiles or bind a range of hydrophobic ligands [1]. They are important therapeutic targets in disease [2]. For example, *Schistosoma japonicum* GSTs, the major detoxification enzymes in *S. japonicum*, have promising vaccine potential against Schistosomiasis [3,4], and their inhibitors are novel antischistosomal drugs [5]. The gene of M_r 26 000 GST from *S. japonicum* (SjGST) has been cloned [3,6], highly expressed in various host cells, and successfully used as a gene fusion system for high expression and affinity purification

*Corresponding author.

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of recombinant proteins [7,8]. The protein contains four cycteine residues, with only the Cys169 residue buried inside the molecule and the other three, Cys85, Cys138, and Cys178, located on the surface of the protein. The structure of the recombinant protein produced from *Escherichia coli* indicates that neither intra- nor inter-disulfide linkage exists in the active dimeric enzyme [5].

GSTs are conventionally purified with GSH affinity chromatography [7] or other GSH-derivative affinity systems where reduced GSH is routinely used as an eluting agent. However, there are several drawbacks with such purification systems. First, most of the GST mutants, which are the subjects of a wide range of investigations involving the functional roles of the residues of GSTs, cannot be readily purified by this method because their GSH binding affinity may be affected by the mutations. Second, because recombinant SjGST loaded onto the GSH affinity gels tends to form soluble aggregates as a result of the oxidation of the free thiol groups [9], high concentrations of reduced GSH or other reducing thiols are usually required for efficient elution. Protein S-glutathiolation, the formation of mixed disulfides on protein thiol groups with GSH, has been reported for a large variety of proteins [10,11]. Although the effect of the linkage of GSH to SjGST is not clear, the phosphatase activity of carbonic anhydrase III is known to be increased by monoglutathiolation but decreased by diglutathiolation [12]. In addition, it has been reported that the activities of Class Pi GSTs are sensitive to S-thiolation [13–15]. Finally, if there is glutathione disulfide (GSSG) contamination in the GSH elution buffer, the possibility of protein S-gluathiolation increases [10].

Recently, immobilized metal affinity chromatography (IMAC), one of the most widely used purification systems for recombinant proteins, was used to purify different recombinant GSTs [16,17]. Metal binding sites, such as spatially adjacent superficial His residues [16] or a poly-His tag [17], were genetically engineered into human GST M1-1 and SjGST, respectively, to facilitate their purification with IMAC. Both studies showed that GSTs could be readily purified with this system as long as they contained accessible metal coordinating sites.

In this study, we investigated the performance of both the GSH affinity chromatography and the IMAC on the purification of SjGST and its site-directed mutants. A six-His tag was engineered to the C-terminal end of SjGST to produce the metal-binding fusion protein, SjGST/His [18]. The three superficial Cys residues of SjGST/His, Cys85, Cys138, and Cys178, were individually substituted with Ser. These mutants are particularly important in elucidating the functional roles of these Cys residues in enzyme activity and in probing the occurrence of oxidative aggregation and protein S-thiolation.

2. Experimental

2.1. Materials

E. coli BL21(DE3) was purchased from NovaGen (Madison, WI, USA). The plasmid pGSTH carrying the SjGST/His gene was constructed in this laboratory by cloning the SjGST gene fragment of pGEX-5X-2 (Pharmacia Biotech, Uppsala, Sweden) into the multiple cloning sites of pET-30b (Novagen) [18], which are located before the gene of the six-His tag. Enzymes for DNA manipulation were either from New England BioLabs (Beverly, MA, USA) or from AGS (Heidelberg, Germany). GSH Sepharose 4B gels and Ni^{2+}-chelated nitrilotriacetic acid (Ni^{2+}-NTA) agarose gels were from Pharmacia Biotech and QIAGEN (Santa Clarita, CA, USA), respectively. 1-Chloro-2,4-dinitrobenzene (CDNB) was from Wako (Osaka, Japan). GSH and imidazole were from Sigma (St. Louis, MO, USA). Ampholyte was from Pharmacia LKB (Piscataway, NJ, USA) and Gelbond was from Serva (Westbury, NY, USA). Sinapinic acid was from Hewlett-Packard (Böblinger, Germany).

2.2. Site-directed mutagenesis of SjGST/His

Site-directed mutagenesis of the SjGST/His gene on the pGSTH plasmid was performed following the method of Kunkel et al. [19]. The synthetic oligonucleotides used as primers to carry out the reactions are listed in Table 1. The primers were designed so that an extra restriction site (Table 1) was created in each mutation. The plasmids containing the mutated SjGST/His genes were screened by restriction diges-

Table 1
Synthetic oligonucleotides used as primers for site-directed mutagenesis

SiGST/His mutants	Oligonucleotide [a]	Created restriction site
Cys85→Ser	CACAACATGTTGGGTGG**A**T**CC**CCAAAAGAGCGTGCA	*Bam* HI
Cys138→Ser	AATGTTCGAAGATCG**C**TTAAGTCATAAAACATATTTAAATGGTGAT	*Afl* II
Cys178→Ser	CGTTCCCAAAATTAGT**A**AG**C**TTTAAAAAACGTATTGAAGCT	*Hind* III

[a] Bold letters: the substituted nucleotides; underlines: the mutant amino acid codons; upper lines: the created restriction site.

tions, sequenced, and transformed into *E. coli* BL21(DE3).

2.3. Enzyme Production

E. coli BL21(DE3) was the host strain used to produce wild-type (WT) and mutant SjGST/His enzymes. A single colony of this strain containing either WT or mutated pGSTH plasmids was used for inoculating Luria–Bertani (LB) broth containing 30 μg/ml kanamycin. After incubating overnight at 37°C, the culture was diluted 25-fold with the same medium in a shaking flask. At an $A_{600\ nm}$ of 0.7–0.8, the expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M*, and incubation was then continued for another 3 h. Cells were harvested by centrifugation followed by purification as described below.

2.4. GSH affinity purification of WT and mutant SjGST/His

Cell pellets harvested from 300 ml culture were suspended in 15 ml phosphate-buffered saline (PBS; 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4) and disrupted by sonication. The clear cell lysate was applied to a column packed with 200 μl GSH Sepharose 4B gels, followed by three washes with at least 10 bed volumes of PBS. Then, the protein was eluted with 400 μl GSH elution buffer (30 m*M* GSH, 50 m*M* Tris–HCl, pH 8). To remove GSH, the eluted protein was diluted four times with PBS and dialyzed against at least 2000 volumes of PBS with M_r cut off (MWCO) 3500 membranes.

2.5. IMAC purification of WT and mutant SjGST/His

Cell pellets harvested from 30 ml culture were suspended in 1.5 ml NTA loading buffer (0.1 *M* NaH_2PO_4, 0.1 *M* Tris–HCl, pH 8) and sonicated. Then, the clear cell lysate was mixed with 100 μl of 50% Ni^{2+}-NTA gel slurry, shaken gently at room temperature for 15 min, and centrifuged. The resulting pellet was washed three times with 10 bed volumes of NTA loading buffer containing 20 m*M* imidazole. To elute the protein, 100 μl NTA elution buffer (250 m*M* imidazole, 0.1 *M* NaH_2PO_4, 0.1 *M* Tris–HCl, pH 8) was used.

2.6. Assays

SjGST/His activities were determined with 1 m*M* GSH and 1 m*M* CDNB as substrates at pH 6.5 and 25°C [1]. One unit (U) of WT or mutant SjGST/His was defined as the amount of enzyme required to produce 1 μmol of product per minute. Protein concentration was determined by the method of Bradford [20] with bovine serum albumin used as a standard.

2.7. Molecular mass measurements

The molecular masses of proteins were measured by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS), HP G2025A, which incorporated a 337 nm nitrogen laser with a desorption/ionization pulse width duration of 3 ns and an electron multiplier detector. The instrument was operated in positive ion reflection mode with an acceleration potential of +28 kV. The mass scale was calibrated using peptide standard with a matrix of sinapinic acid. Sinapinic acid was also

used as the matrix for each sample, and an average of 155 laser shots were taken.

2.8. Isoelectric focusing (IEF)

For IEF, an equal amount of protein from each sample was applied onto a 0.8 mm thin gel containing 4% acrylamide and 2% LKB preblended pH 3.5–10 or pH 5–8 ampholyte. The gel was prefocused for 10 min at 4°C before sample loading and samples were focused for 60 min at the same temperature with a voltage limited to 1500 V and a current and power limited to 2.75 mA/cm and 1.125 W/cm gel width, respectively. After IEF, the gel was stained with Commassie blue.

3. Results and discussion

3.1. Affinity purification of WT and mutant SjGST/His enzymes

The same purification conditions were used for all enzymes in both the GSH and the Ni^{2+}-NTA affinity purification methods. The gel amounts used in both methods were much less than those required for the optimal binding, and significant amounts of enzymes were therefore still observed in the flow-through and the wash fractions (results not shown). 30 m*M* GSH and 250 m*M* imidazole were used to elute proteins from the GSH and the Ni^{2+}-NTA gels, respectively, because they yielded the best elution efficiency. Table 2 shows the values of total protein and specific activities of WT and mutant SjGST/His enzymes purified from the GSH and the Ni^{2+}-NTA affinity gels. The protein amounts and specific activities recovered from the Ni^{2+}-NTA gels were similar for the four enzymes, indicating that the production, activity, and metal binding affinity of SjGST/His were not affected by the mutations. The presence of high imidazole concentration (250 m*M*) or the addition of 20 m*M* β-mercaptoethanol in the NTA elution buffer had no effect on enzyme activity. On the other hand, the values in the GSH affinity purification were dramatically different among the four different enzymes. The protein amounts of Cys138→Ser and Cys178→Ser mutants recovered from the GSH gels were much less than those of the other two enzymes, implying that the ability of SjGST/His to bind with GSH affinity gels could be affected by these mutations. However, the specific activities of these enzymes were higher than those of the other two and close to the results in Ni^{2+}-NTA. The four-fold dilution and subsequent dialysis of WT and Cys85→Ser SjGST/His enzymes increased their specific activities to 11.1 and 11.6 U/mg, respectively, suggesting that a portion of the eluted enzymes were in inactive aggregated forms. The aggregation might have resulted from a high density of adsorbed proteins on the GSH affinity gels, which were subsequently eluted in high concentrations (67–82 mg/ml).

Table 2
Total protein and specific activity of WT and mutant SjGST/His purified by GSH affinity chromatography and IMAC[a]

SjGST/His form	Crude extracts: Specific activity (U/mg)	GSH gels[b]		Ni^{2+}-NTA gels[c]	
		Total protein (mg)	Specific activity (U/mg)	Total protein (mg)	Specific activity (U/mg)
WT	3.2	26.8	6.3	3.6	14.0
Cys85→Ser	2.1	32.9	6.9	4.6	12.5
Cys138→Ser	5.3	4.4	16.1	3.7	12.1
Cys178→Ser	4.7	8.3	12.1	4.2	12.6

[a] Data are reported as an average of three runs.
[b] The enzyme was purified from 300 ml cell cultures with 200 μl GSH Sepharose 4B gels.
[c] The enzyme was purified from 30 ml cultures with 50 μl Ni^{2+}-NTA gels.

3.2. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of WT and mutant SjGST/His enzymes

Fig. 1 shows the SDS–PAGE electrophoretic patterns of WT and mutant SjGST/His enzymes purified by using the two different affinity purification methods. All the enzymes purified from the GSH adsorbents showed only a single band (G1) under both the reducing and the nonreducing conditions (Fig. 1a), suggesting that the formation of inactive oxidative aggregates between the SjGST/His monomers was eliminated at 30 m*M* GSH elution. This implies that noncovalent aggregation was responsible for the decrease in the activities of eluted WT and Cys138→Ser SjGST/His enzymes from the GSH affinity adsorbents. For the IMAC purified enzymes (Fig. 1b), the patterns under reducing conditions were the same as those in Fig. 1a. However, several minor forms of oxidative aggregates at higher molecular masses (G3) were observed under nonreducing conditions, suggesting that intermolecular disulfide linkages formed between SjGST/His monomers when the enzymes were highly expressed in *E. coli*. The extra minor bands (G2) located below the major bands in WT, Cys138→Ser, and Cys178→Ser SjGST/His enzymes represented intramolecularly oxidized monomers which had higher mobility in SDS-PAGE than the reduced forms [21–23]. Therefore, the disappearance of this band in the Cys85→Ser mutant suggests that the intramolecular disulfide linkage may occur between Cys85 and other Cys residues.

3.3. MALDI-TOF-MS measurements of WT and mutant SjGST/His enzymes

Fig. 2 shows the MALDI-TOF-MS spectra of WT SjGST/His enzyme from different preparations. Only the spectra of the monomer forms are presented here because the signal intensities of dimeric forms were very low. The spectrum of the protein monomer eluted from the GSH affinity adsorbents showed a peak value around 29 001 m/z. The addition of 30 m*M* dithiothreitol (DTT) to the protein sample shifted the spectrum to the left, reducing the molecular mass of the peak value to 28 487. In contrast, for the WT enzyme purified from the Ni^{2+}-NTA gels, the spectrum showed a peak value of around 28 425 and the addition of 30 m*M* GSH shifted the spectrum to the right with a peak value at 29 031. We routinely observed similar spectral shifts for WT enzyme. In addition, we examined the molecular masses of WT enzyme eluted with only 10 m*M* GSH from GSH-affinity gels and the dialyzed WT enzyme and obtained spectra similar to Fig. 2a. These results

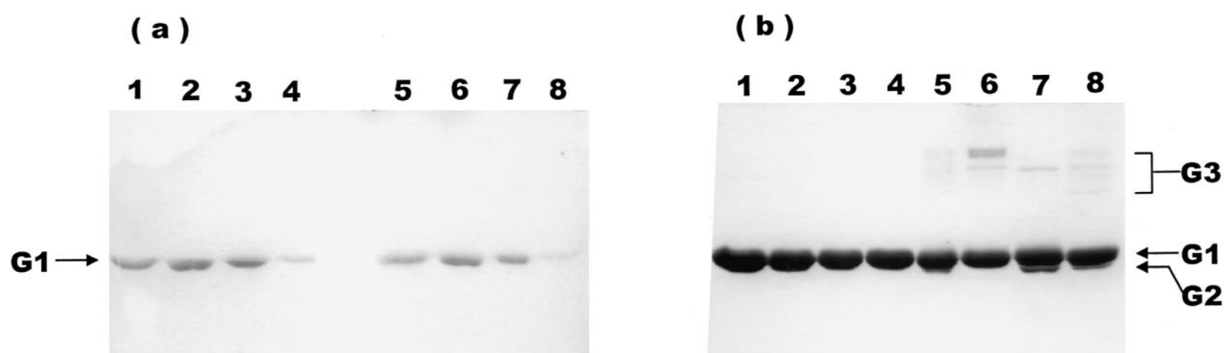

Fig. 1. SDS–PAGE of WT and mutant SjGST/His purified by (a) GSH Sepharose-4B gels and (b) Ni^{2+}-NTA gels under reducing (lane 1–4) and nonreducing (lane 5–8) conditions. (a) 2 μg and (b) 15 μg of protein was applied to each lane on 0.75 mm thick 12.5% polyacrylamide gels. For reducing electrophoresis, the protein was analyzed in the presence of 1% β-mercaptoethanol. Lanes 1, 5=WT, 2, 6=Cys85→Ser, 3, 7=Cys138→Ser, 4, 8=Cys178→Ser. The reduced, intra-disulfide linked, and inter-disulfide linked forms of SjGST/His are designated as G1, G2, and G3, respectively.

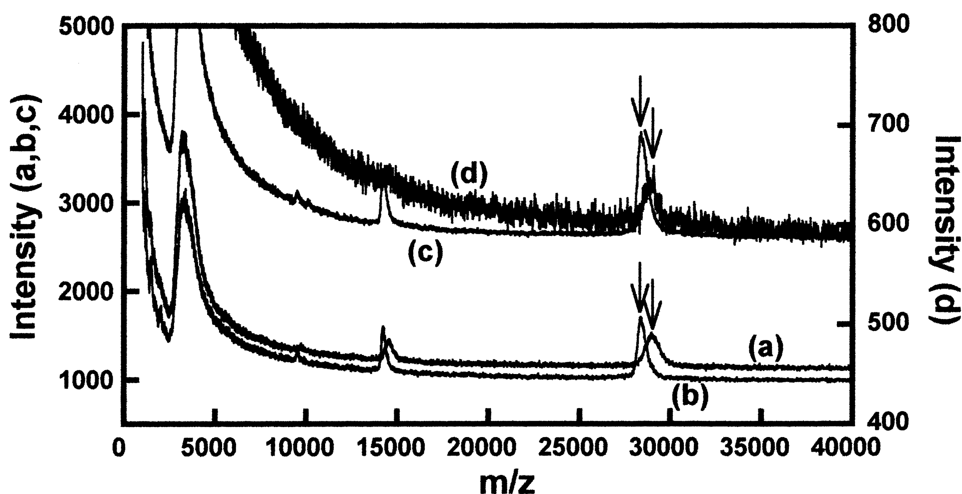

Fig. 2. MALDI-TOF mass spectra of WT SjGST/His. (a) Enzyme eluted from GSH gels. (b) 30 m*M* DTT + enzyme eluted from GSH gels. (c) Enzyme eluted from Ni^{2+}-NTA gels. (d) 30 m*M* GSH + enzyme eluted from Ni^{2+}-NTA gels. Arrows indicate the monomers of the enzyme at different states.

imply that the WT SjGST/His enzyme purified from GSH-affinity gels was S-thiolated with eluting GSH, while that from Ni^{2+}-NTA gels was not. The spectra obtained in this study were not narrow and sharp at all, presumably because of the existence of several different mixed-disulfide forms.

The shift-patterns of the MALDI-TOF-MS spectra of the three mutant SjGST/His enzymes were similar to those of the WT form. Table 3 summarizes the peak values of their monomer forms in each spectrum. As described above, the addition of DTT to all of the GSH-affinity purified enzymes decreased their measured molecular masses, while the addition of GSH to all the IMAC purified enzymes increased the values. The shift values of the WT enzyme were larger than those of the mutant enzymes. In addition, for all of the three mutant enzymes, the molecular masses of the DTT-treated GSH-affinity purified forms and IMAC purified forms were close to those of WT enzyme of the same preparations, while their oxidized forms were smaller. These results suggest that the Cys85, Cys138, and Cys178 residues were located on the enzyme surface and were susceptible to oxidative modification by GSH. The theoretical molecular masses of the reduced monomers of WT and any one of the mutant SjGST/His enzymes are 28 217 and 28 201, respectively, based on the amino acid sequences of the cloned protein monomers. That is, the measured molecular masses of the enzymes eluted from Ni^{2+}-NTA adsorbents were higher than the theoretic values. This suggests that it is possible that the crude enzymes produced by *E. coli*

Table 3
MALDI-TOF-MS measurements of WT and mutant SjGST/His purified with either GSH Sepharose 4B gels or Ni^{2+}-NTA gels

Purification gels	States	Monomer of SjGST/His form (M_r)			
		WT	Cys85→Ser	Cys138→Ser	Cys178→Ser
GSH	Purified form	29 001	28 841	28 848	28 835
	Purified form +30 m*M* DTT	28 487	28 416	28 474	28 522
Ni^{2+}-NTA	Purified form	28 425	28 503	28 451	28 472
	Purified form +30 m*M* GSH	29 031	28 730	28 658	28 690

BL21(DE3) were somewhat S-thiolated by molecules smaller than GSH. The same phenomena were also observed for in vivo S-thiolation by GSH for a variety of proteins produced by *E. coli* [24], *Saccharomyces cerevisiae* [25], and other cells [26,27], as well as the S-thiolation by other intracellular low-molecular-mass thiols [26,28].

We also analyzed the amino acid compositions of dialyzed WT SjGST and SjGST/His enzymes eluted from GSH-affinity adsorbents and obtained higher amounts of glutamic acid and glycine than the theoretical values predicted from the sequences of unmodified enzymes in both enzymes (data not shown). The amount of cysteine obtained was not higher than the theoretical value because it was unstable in direct acid hydrolysis. However, these results were sufficient to imply that the enzymes had been S-thiolated by GSH during the purification. For the IMAC-purified enzymes, the results of amino acid analysis were not much different from the values predicted from the unmodified enzyme sequences. Peptide digestions, more accurate molecular mass measurement, and amino acid analyses may be helpful in further identifying the small thiol molecules which are linked with crude SjGST/His enzymes in *E. coli* BL21(DE3) in vivo.

3.4. IEF of WT and mutant SjGST/His enzymes

Recently, IEF analysis has been applied to study the occurrence of thiol–disulfide exchanges between protein thiol and GSH of several proteins [27,29]. Based on the presence of one negative charge on GSH (an NH_3^+ and a COO^- group from the glutamate and a COO^- from the glycine residue of GSH), the isoelectric point (p*I*) of a protein can be changed once it reacts with GSH. Otherwise, the presence of acidity in the α-carboxyl group in the glutamate residue of GSH ($pK_a \approx 2.8$) may also manifest a change to GSH modified protein on IEF gels. Therefore, we used IEF to verify the S-glutathiolation of SjGST/His enzymes. DTT and GSSG were each used to react with the enzyme samples before loading on IEF gels to confirm the modification. Figs. 3 and 4 show the IEF results of WT and mutant SjGST/His enzymes purified from GSH and Ni^{2+}-NTA gels, respectively. All of the GSH-affinity purified enzymes displayed many isoforms over a

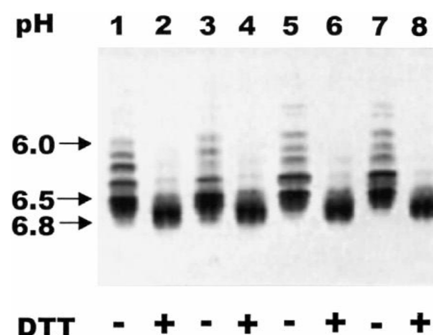

Fig. 3. IEF of WT (lanes 1, 2), Cys85→Ser (lanes 3, 4), Cys138→Ser (lanes 5, 6), and Cys178→Ser (lanes 7, 8) SjGST/His purified by GSH Sepharose-4B gels. 20 μg of protein was applied to each lane. Lanes 1, 3, 5, 7=purified native enzymes; 2, 4, 6, 8=purified enzymes treated with 30 m*M* DTT at 37°C for 30 min.

wide pH range (p*I*≈6.0–6.8) under nonreducing conditions on the IEF gels. However, all of them shifted to a narrower and more basic pH range (p*I*≈6.5–6.85) after the DTT treatment. This suggests that the SjGST/His enzymes were S-thiolated by GSH during the GSH-affinity purification. The IEF patterns of the mutant enzymes were not clearly different from that of the WT enzyme because of the extreme multiplicity of the mixed-disulfide forms. On the other hand, the IEF isoforms of SjGST/His enzymes purified from Ni^{2+}-NTA adsorbents (Fig.

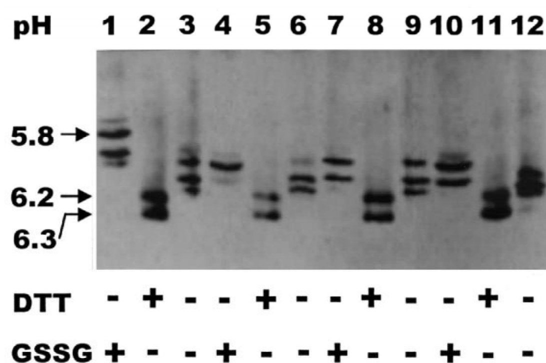

Fig. 4. IEF of WT (lanes 1–3), Cys85→Ser (lanes 4–6), Cys138→Ser (lanes 7–9), and Cys178→Ser (lanes 10–12) SjGST/His purified by Ni^{2+}-NTA gels. 10 μg of protein was applied to each lane. Lanes 1, 4, 7, 10=purified enzymes treated with 30 m*M* GSSG at 37°C for 30 min; 2, 5, 8, 11=purified enzymes treated with 30 m*M* DTT at 37°C for 30 min; 3, 6, 9, 12=purified native enzymes.

4) were less than those in Fig. 3. All of the untreated enzymes were unexpectedly focused at slightly more acidic regions than the enzymes treated with DTT, suggesting that the enzymes were S-thiolated in vivo by negatively charged small unknown molecules. All of the DTT-treated enzymes shifted to two distinct p*I* values (p*I*≈6.2 and 6.3), indicating that all the enzymes had two reduced isoforms. One of them was the native form, and the other might have resulted from the deamidation of Asn144 residue on the Asn144–Gly145 bond in the enzymes. Deamidation of an Asn residue that is followed by a Gly residue is a common non-catalytic reaction in aged or heat-treated proteins under neutral or alkaline conditions [30]. It results in the formation of either an Asp–Gly or isoAsp–Gly bond and consequently increases the acidity of the p*I* value of the enzymes [31]. Treatment with GSSG clearly shifted the p*I* value of the IMAC-purified WT enzyme by about 0.2 but did not shift the p*I* value of the mutant enzymes. However, the IEF patterns of the treated mutant enzymes were somewhat different from those of the untreated ones, suggesting the occurrence of disulfide exchanges.

4. Conclusion

In conclusion, we have used the WT and mutant SjGST/His enzymes to compare the GSH affinity and the IMAC purification systems. The IMAC system purified the mutant enzymes as efficiently as the WT enzyme, while the efficiency of the GSH-affinity system was greatly dependent on the enzyme structure. Mutant SjGST/His enzymes whose GSH binding sites have been affected by the mutations cannot be readily purified with the GSH-affinity gels. The noncovalent aggregation of the purified enzymes was not as prevalent in the IMAC system as in the GSH affinity system. In addition, use of GSH as an eluting agent in the GSH affinity purification system covalently modified the superficial Cys residues, Cys85, Cys138, and Cys178, of SjGST/His through S-thiolation or thiol–disulfide exchange. Such modifications may affect on the enzyme activity or stability because of the existences of a bulky charged GSH group. Therefore, the Ni^{2+}-NTA purification system is better for the purification of SjGST/His enzymes. The results of this study also suggest that for the GSH-affinity purification of GST fusion proteins, S-thiolation by GSH or oxidative aggregation should be cautiously examined, especially in cases where the target protein needs structurally correct disulfide linkages or unlinked reduced Cys residues for activity.

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