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A Second Thioltransferase of *Schizosaccharomyces pombe* Contains Glutathione S-transferase Activity

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Two types of the thioltransferase (also called glutaredoxin) have been previously detected in the cytosolic extract of *Schizosaccharomyces pombe*, a fission yeast. Previously, the one with a smaller molecular mass (14 kDa) was purified and characterized. In the present study, the second thioltransferase was purified. The purification procedure included ammonium sulfate fractionation (40–80%), Sephadex G-200 gel filtration, DEAE-cellulose ion-exchange chromatography, Sephadex G-50 gel filtration, and glutathione-agarose affinity chromatography. The purified enzyme showed a single band on SDS-PAGE, and its molecular mass was determined to be 23 kDa. It utilizes various compounds as substrates, including 2-hydroxyethyl disulfide. Interestingly, we found that the purified thioltransferase also contains significant glutathione S-transferase activity.

Keywords: Glutaredoxin, Glutathione S-transferase, *Schizosaccharomyces pombe*, Thioltransferase.

Introduction

Thioltransferase (TTase), also known as glutaredoxin (Grx), is a monomeric enzyme that catalyzes the reduction of low molecular weight disulfides and some protein disulfides in the presence of reduced glutathione and glutathione reductase. It has been shown to couple the oxidation of NADPH to the reduction of ribonucleotide (Holmgren, 1979), sulfate (Tsang, 1981), and methionine sulfoxide (Fuchs, 1977). Mammalian TTase was identified as a dehydroascorbate reductase (Wells *et al.*, 1990). Recently, TTase was shown to catalyze the reduction of alloxan to dialuric acid by glutathione (Washburn and Wells, 1997).

TTase is a small protein containing the active site -Cys-Pro-Tyr-(Phe)-Cys- which is conserved from *E. coli* to mammals (Wells *et al.*, 1993). TTases have been isolated and characterized from a number of prokaryotic and eukaryotic species, including *Escherichia coli* (Höög *et al.*, 1983), *Saccharomyces cerevisiae* (Gan *et al.*, 1990), bovine (Hatekeyama *et al.*, 1984), pig (Gan and Wells, 1987), rat (Axelsson *et al.*, 1978), human (Padilla *et al.*, 1995), kale (Sa *et al.*, 1998a), and Chinese cabbage (Cho *et al.*, 1998; 1999). TTase cDNAs were isolated and their nucleotide sequences were determined from pig (Yang *et al.*, 1989), human (Padilla *et al.*, 1995), and rice (Sha *et al.*, 1997). The yeast *Saccharomyces cerevisiae* has been reported to contain two TTase genes, which are required for protection against reactive oxygen species (Luikenhuis *et al.*, 1998). In v-jun-transformed chicken embryo fibroblasts, the expression of TTase mRNA could be induced, indicating that TTase is a direct target of v-Jun (Goller *et al.*, 1998). Recently, OxyR transcription factor, which is activated through the formation of a disulfide bond, has been found to be deactivated by TTase (Zheng *et al.*, 1998). TTase was detected within HIV-1, and was shown to implicate in the regulation and/or maintenance of protease activity in HIV-1 infected cells (Davis *et al.*, 1997).

Previously, we purified and characterized a 14 kDa TTase from *Schizosaccharomyces pombe* (Kim *et al.*, 1998). This TTase could catalyze the reduction of various disulfide compounds such as *S*-sulfocysteine, L-cysteine, and insulin. It was also found to contain reducing activity for non-disulfide substrates such as dehydroascorbic acid and alloxan. In this article, we report the purification of a second, larger TTase from *Schizosaccharomyces pombe*. Interestingly, this second enzyme was found to contain glutathione S-transferase activity.

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Materials and Methods

Chemicals Bovine serum albumin (BSA), insulin (bovine pancreas), *S*-sulfocysteine, L-cysteine, glutathione (reduced), glutathione reductase (yeast), *S*-hexylglutathione, NADPH, dithiothreitol, Tris, adenine, leucine, uracil, alloxan, oxytocin, 1-chloro-2,4-dinitrobenzene (CDNB), Sephadex G-200, Sephadex G-50, and glutathione-agarose were purchased from Sigma Chemical Co. (St. Louis, USA). Dehydroascorbic acid and 2-hydroethyl disulfide (HED) were from Aldrich Chemical Co. (Milwaukee, USA). DEAE-cellulose was a product of Whatman International Ltd. (Maidstone, England). Molecular weight standards for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad Laboratories (Richmond, USA). All other chemicals and reagents were of the highest grade commercially available.

Cell growth, harvest, and disruption Wild-type *Schizosaccharomyces pombe* was grown for three days at 30°C in YEALU medium using a shaking incubator. YEALU medium was composed of 30 g glucose, 5 g yeast extract, 75 mg adenine, 75 mg leucine, 75 mg uracil, per liter. The yeast cells were harvested by centrifuging the culture, and the frozen packed cells were these resuspended in 20 mM Tris buffer (pH 8.7), and 2 mM EDTA [buffer A] and disrupted by a glass bead beater and sonicator. The supernatant (Fraction 1) was obtained after centrifugation.

Enzyme assay Since thioltransferase contains transhydrogenase activity, its activity was measured spectrophotometrically at 340 nm using glutathione reductase (GR) as a coupling enzyme (Holmgren, 1979; Sa *et al.*, 1998b; Park *et al.*, 1999). In a total volume of 400 μl, two cuvettes each contained 1 mg/ml of BSA, 10 mM GSH, 60 μg/μl yeast glutathione reductase, 4 mM NADPH, 1 M Tris·HCl, and 20 mM EDTA, pH 8.0. To each cuvette was added 40 μl of 15 mM 2-hydroxyethyl disulfide (HED) dissolved in 5 mM Tris·HCl, pH 8.0. The absorbance at 340 nm was monitored for several minutes to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between GSH and HED. Enzyme was added to the sample cuvette and water to the control cuvette. The change in the absorbance at 340 nm was recorded with time and the activity was expressed as nmoles/min.

Glutathione S-transferase (GST) activity was measured by spectrophotometric assay according to the method of Habig *et al.* (1974). The reaction mixture (1.0 ml) contained 100 mM potassium phosphate buffer (pH 6.5), 2.0 mM CDNB, 5.0 mM glutathione (GSH), and enzyme source. The change in the absorbance was recorded with time and GST activity was expressed as pmoles/min.

Purification All purification procedures were carried out at 4°C. Dilute protein solutions were concentrated by centrifugation with Amicon Centriprep. Dialysis was done with Spectrum membrane tubing (MW_{CO}: 6000–8000). Solid ammonium sulfate was added to the crude extract (Fraction 1) to achieve a concentration of 40% saturation, and the mixture was stirred on ice for 1 h. The supernatant was recovered after centrifugation for 30 min at 12,000 rpm in a Sorvall GSA rotor. Solid ammonium sulfate was added to the supernatant to a final concentration of 80% saturation. After 1 h incubation on ice, the resulting precipitate was collected by centrifugation as described, and resuspended in buffer A (Fraction 2). The suspension was dialyzed overnight against three changes of 3 L of the same buffer. Fraction 2 was then loaded onto a Sephadex G-200 column (2.5 × 67 cm) equilibrated with butter A, and active fractions were collected (Fraction 3). Fraction 3 was loaded onto a DEAE-cellulose column (2.5 × 8 cm) equilibrated with buffer A. The column was washed with buffer A until the protein content of the effluent returned to baseline levels. Elution was carried out with a linear gradient of 0 to 0.5 M NaCl in buffer A and then active fractions were pooled (Fraction 4). Fraction 4 was loaded onto a Sephadex G-50 column (1.25 × 50 cm) equilibrated with buffer A and active fractions were collected (Fraction 5). Fraction 5 was loaded onto a glutathione-agarose affinity column (1 × 4 cm) equilibrated with buffer A. Elution was performed with step gradients of 5, 10, and 20 mM *S*-hexylglutathione in buffer A. Fractions eluted with 5 mM *S*-hexylglutathione containing thioltransferase activity were pooled (Fraction 6).

Determination of protein concentration Protein contents in fractions 1–6 were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. During purification, absorbance at 280 nm was measured to determine protein contents in chromatographic fractions.

SDS-polyacrylamide gel electrophoresis Electrophoresis on vertical 13% polyacrylamide slab gels (8 × 10 cm) or pre-cast gels was performed in the presence of SDS by the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 or silver nitrate.

Results and Discussion

Purification of a second TTase from *S. pombe* Previously, a TTase from *S. pombe* was purified to electrophoretic homogeneity by the combination of ion-exchange chromatography and gel filtration (Kim *et al.*, 1998). Since two peaks possessing TTase activity appeared in the elution profile of DEAE-cellulose ion-exchange chromatography, the original TTase was purified from the pool of the major peak. However, it was proved that there were at least two kinds of TTase in *S. pombe*. In this study, the second TTase was purified. In the first chromatographic step, the fraction obtained from ammonium sulfate fractionation was subjected to a Sephadex G-200 gel filtration column, generating two peaks of TTase activity (Fig. 1). The minor peak was collected and purified by DEAE-cellulose ion-exchange chromatography (Fig. 2). This fraction was loaded onto a Sephadex G-50 gel filtration column whose elution profile showed that its activity peak appeared to coincide almost with the protein peak (Fig. 3). However, these fractions were further subjected to affinity chromatography on glutathione-agarose. Transhydrogenase activity was eluted with buffer A containing 5 mM *S*-hexylglutathione. Its purity was examined on SDS-polyacrylamide gel electrophoresis (Fig. 4). The final step with the affinity

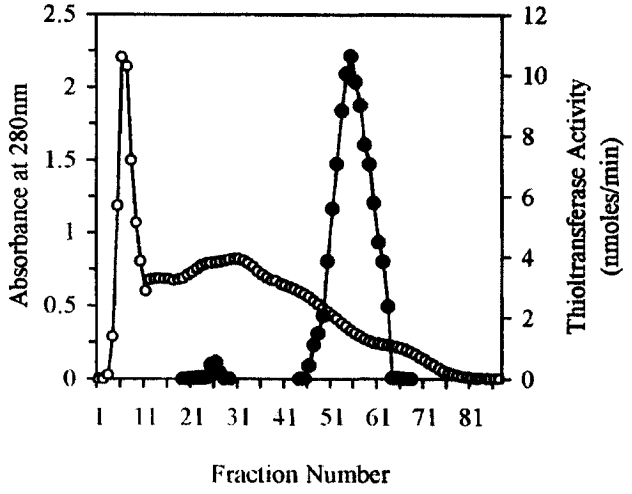

Fig. 1. Chromatographic profile of Fraction 2 on a Sephadex G-200 column (2.5 × 67 cm). Fractions were collected and absorbance at 280 nm (–○–) and thioltransferase activity were determined. Thioltransferase activity was represented as nmoles/min (–●–).

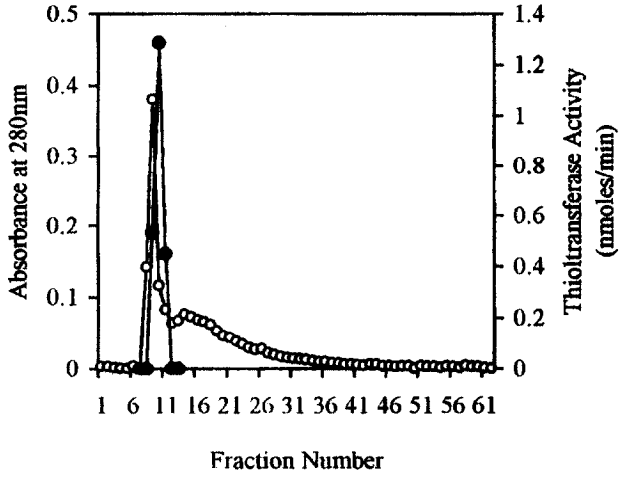

Fig. 2. Chromatographic profile of Fraction 3 on a DEAE-cellulose chromatography column (2.5 × 8 cm). Fractions were collected and absorbance at 280 nm (–○–) and thioltransferase activity were determined. Thioltransferase activity was represented as nmoles/min (–●–).

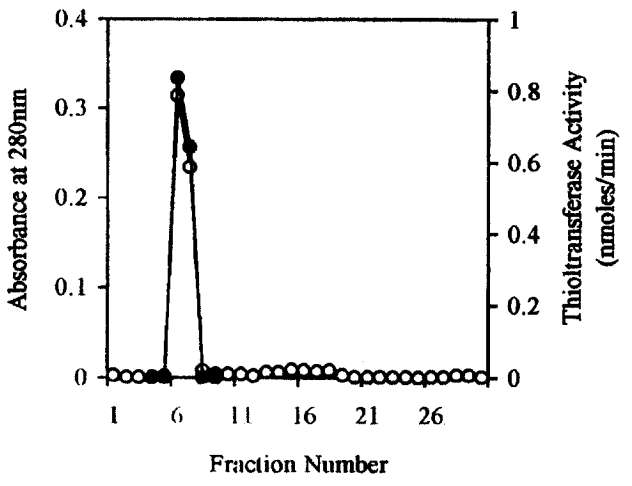

Fig. 3. Chromatographic profile of Fraction 4 on a Sephadex G-50 column (1.25 × 50 cm). Fractions were collected and absorbance at 280 nm (–○–) and thioltransferase activity were determined. Thioltransferase activity was represented as nmoles/min (–●–).

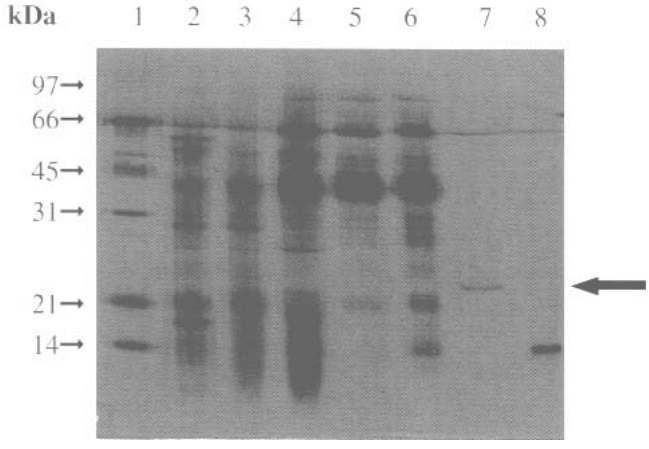

Fig. 4. Electrophoretic patterns of the second thioltransferase purified from *Schizosaccharomyces pombe*. Each fraction obtained during the purification procedure was analyzed on 13% SDS-PAGE and stained with silver nitrate. Lane 1, protein makers; Lane 2, Fraction 1 (crude extract); Lane 3, Fraction 2 (ammonium sulfate fractionation); Lane 4, Fraction 3 (Sephadex G-200 gel filtration); Lane 5, Fraction 4 (DEAE-cellulose ion-exchange chromatography); Lane 6, Fraction 5 (Sephadex G-50 gel filtration); Lane 7, Fraction 6 (glutathione-agarose affinity chromatography); Lane 8, the first thioltransferase purified from *Schizosaccharomyces pombe* (Kim *et al.*, 1998).

column showed a single protein band on SDS-PAGE, indicating that this last fraction was in a homogeneous state, confirming that a second TTase was successfully purified from *S. pombe*, the molecular weight of which was clearly different from the first TTase.

To verify the identity of the second TTase, individual components were deleted in a complete assay (data not shown). The results indicated that the full transhydrogenase activity of the second TTase absolutely required HED, GSH, and glutathione reductase. The molecular weight of the second TTase from *S. pombe* appeared to be about 23,000 Da on SDS-PAGE (Fig. 4). Its molecular size is apparently much larger than that of the first TTase (14,000 Da), and is comparable to that of Grx 2 from *E. coli* (Áslund *et al.*, 1994) and TTase CC-2 from Chinese cabbage (Cho *et al.*, 1999).

Characteristics of the TTase activity Substrate specificity of the second TTase was examined under saturating concentrations of substrates (Table 1). The enzyme could utilize HED, *S*-sulfocysteine, and insulin as substrates, with *S*-sulfocysteine appearing to be a superior substrate. However, it could not utilize cyclic peptide oxytocin as a substrate. Also, it did not show reducing activity for non-disulfide compounds such as dehydroascorbic acid and alloxan, indicating that the second TTase does not contain dehydroascorbate reductase activity, unlike the first. Transhydrogenase activity of the second TTase was examined under various concentrations of HED. Kinetic parameters were obtained by Lineweaver-Burk plot. As shown in Table 2, its K_m and V_{max} values were 1.28 mM and 0.47 nmoles/min, respectively. The K_m value of the first TTase was 0.03 mM for HED (Kim *et al.*, 1998), indicating that the first TTase has a much higher affinity for HED than the second TTase. The pH optimum of a second TTase from *S. pombe* was measured over pH 5–10.5 using three different buffer systems (Fig. 5) and was identified as pH 8.5. The first TTase has a pH optimum of 9 (Kim *et al.*, 1998). Similarly, most known TTases have a basic pH optimum and the optimal pH values of pig, bovine, and rat TTases are 8.5, 8.5, and 9.0, respectively (Hatekeyama *et al.*, 1984; Gan and Wells, 1986; 1987).

GST activity It was found that the second TTase of *S. pombe* contained significant GST activity, when CDNB was used as a substrate (Fig. 6). GST activity of the second TTase was enhanced with increased concentrations of reduced glutathione (Fig. 7). The optimum pH of glutathione S-transferase activity of the second TTase was measured over pH 5–10.5 using three different buffer systems (Fig. 8). Its optimum pH was 8.5, unlike the first

Table 1. Disulfide reducing activity of the purified thioltransferase against various disulfide compounds.

Substrate	Thioltransferase activity[a] (nmoles/min)	Relative activity (%)
Disulfide compounds		
HED	0.129	100
S-Sulfocysteine	0.855	665
Insulin	0.154	120
Oxytocin	–[b]	0
Non-disulfide compounds		
Dehydroascorbic acid	–[b]	0
Alloxan	–[b]	0

[a] Thioltransferase activity was measured by the standard assay method with various disulfides and non-disulfide compounds.
[b] Not utilized as substrates for the second thioltransferase of *S. pombe*.

Table 2. Kinetic parameters of the second thioltransferase from *S. pombe*.

Substrate	K_m (mM)	V_{max}	Catalytic efficiency (V_{max}/K_m)
TTase activity		(nmoles/min)	
HED	1.28	0.47	0.37
GST activity		(pmoles/min)	
CDNB	1.62	4.06×10^{-4}	2.51×10^{-4}
GSH	2.20	27.7×10^{-4}	12.59×10^{-4}

TTase and GST activities were assayed as described in Materials and Methods. The K_m and V_{max} values were obtained from Lineweaver-Burk plots.

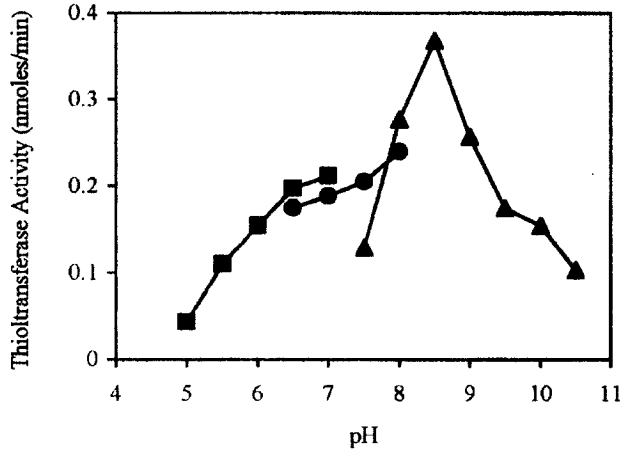

Fig. 5. Effect of pH on the activity of *S. pombe* thioltransferase. The initial velocities of thioltransferase reaction were monitored in a standard assay system with a buffer system of 0.1 M sodium acetate (pH 5.0–7.0, –■–), 0.1 M sodium phosphate (pH 6.5–8.0, –●–), or 0.1 M Tris-Cl (pH 7.5–10.5, –▲–).

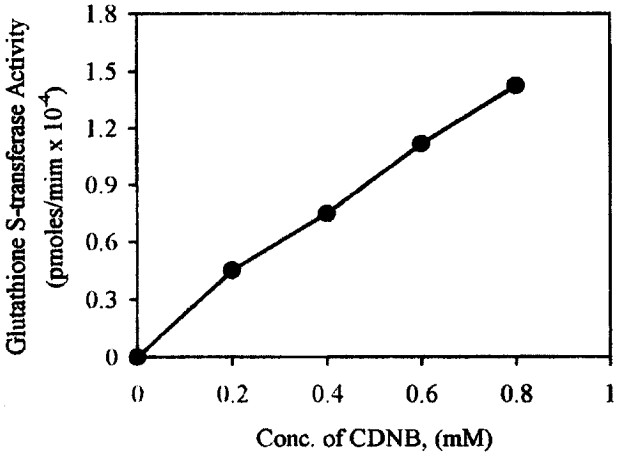

Fig. 6. The glutathione S-transferase activities of the second thioltransferase purified from *Schizosaccharomyces pombe*. Glutathione S-transferase activity was assayed by the standard method described in Materials and Methods, using various concentrations of substrate (CDNB).

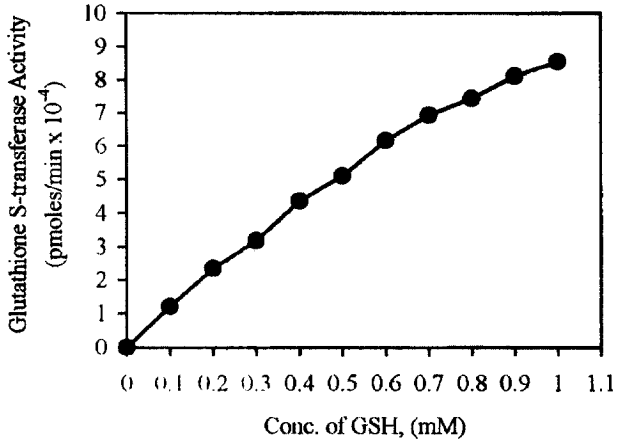

Fig. 7. Effects of GSH concentrations on the glutathione. S-transferase activity of the second thioltransferase from *Schizosaccharomyces pombe*. Glutathione S-transferase activity was assayed by the standard assay method described in Materials and Methods, using various GSH concentrations.

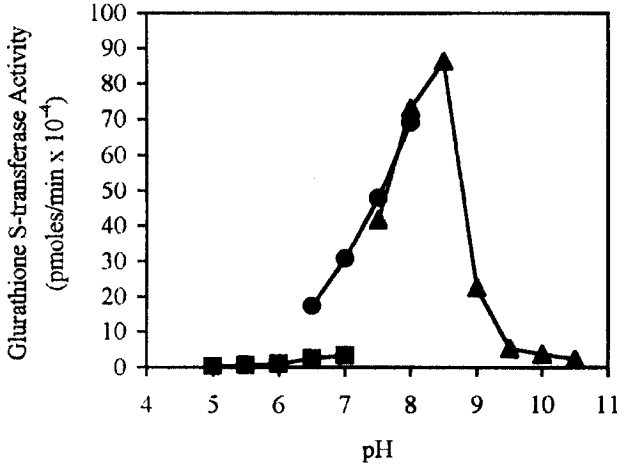

Fig. 8. Effect of pH on the glutathione S-transferase activity of *S. pombe* thioltransferase. Glutathione S-transferase activity was assayed by the standard method describe in Materials and Methods, with a buffer system of 0.1 M sodium acetate (pH 5.0–7.0, -■-), 0.1 M sodium phosphate (pH 6.5–8.0, -●-), or 0.1 M Tris-Cl (pH 7.5–10.5, -▲-).

TTase from *S. pombe* which did not contain GST activity (data not shown). The second TTase had K_m values of 1.62 mM and 2.20 mM for CDNB and GSH, respectively (Table 2). Since various thiol compounds were found to activate several TTases, the effects of a few thiol compounds were tested on the GST activity of the second TTase (Table 3). An 1.4-fold enzymatic activation was observed in the presence of 0.5 mM L-cysteine. However, L-cysteine had no effect on the transhydrogenase activity of the first TTase from *S. pombe* (Kim *et al.*, 1998). It was also shown that various metal ions slightly inhibited the

Table 3. Effects of thiol compounds on the glutathione S-transferase activity of *S. pombe* thioltransferase.

Thiol compound	Concentration (mM)	Total activity (pmoles/min)	Relative activity (%)
None	–	3.20×10^{-5}	100.0
Dithiothreitol	0.5	3.70×10^{-5}	115.6
L-Cysteine	0.5	4.52×10^{-5}	141.0
Mercaptoethanol	0.5	3.03×10^{-5}	94.6

Table 4. Effects of metal ions on the GST activity of the second *S. pombe* thioltransferase.

Metal ion	Concentration (mM)	GST activity (pmoles/min)	Relative activity (%)
None	–	1.63×10^{-4}	100.0
$AlCl_3$	1	0.49×10^{-4}	29.8
$CaCl_2$	1	0.45×10^{-4}	27.7
$CdCl_2$	1	0.45×10^{-4}	29.8
$HgCl_2$	1	0.94×10^{-4}	57.4
$MnCl_2$	1	0.42×10^{-4}	25.5
$ZnCl_2$	1	0.49×10^{-4}	29.8

Purified thioltransferase was incubated with metal ions at the indicated concentrations for 20 min at 30°C, and then the enzyme activity was assayed using the procedure described in Materials and Methods.

GST activity of the second TTase from *Schizosaccharomyces pombe* (Table 4).

One interesting aspect of the second TTase was its significant GST activity, indicating that one protein may contain both TTase and GST activities. However, the physiological reasons remain to be elucidated. The budding yeast *Saccharomyces cerevisiae* is also known to contain two kinds of TTases (Luikenhuis *et al.*, 1998), and in *E. coli* there are three kinds of glutaredoxins (thioltransferases, Áslund *et al.*, 1994). Although the function of multiple TTases in the cells remains unknown, it is presumed that each has different substrate specificities. Further investigation will be performed to explain the meaning of GST activity contained in the second TTase of *S. pombe*.

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