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Purification and Characterization of Glutaredoxin (Thioltransferase) from Rice (*Oryza sativa* L.)[1]

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We purified and characterized glutaredoxin (thioltransferase), which catalyzes thiol/disulfide exchange reaction, for the first time in plants. The purification procedure employed an immunoabsorbent, antiglutaredoxin-Sepharose. Glutaredoxin was purified about 2,200-fold from rice bran and it appeared to be homogeneous on SDS-PAGE. MALDI-TOF mass spectrometry revealed that the protein has a molecular mass of 11,097.9 Da. Rice glutaredoxin consists of 105 amino acid residues, containing the tetrapeptide -Cys-Phe-Pro (Tyr)-Cys-, which constitutes the active site of *Escherichia coli* and mammalian glutaredoxins. Inactivation assay also indicated that cysteine residues are responsible for enzyme activity. Kinetic analyses revealed that the enzyme did not exhibit normal Michaelis-Menten kinetics. The enzyme has an optimum pH of about 8.7 with 2-hydroxyethyl disulfide as a substrate. In addition, rice glutaredoxin has dehydroascorbate reductase activity, like mammalian glutaredoxin.

Key words: dehydroascorbate reductase activity, glutaredoxin (thioltransferase), rice bran, thiol-disulfide interchange, thioredoxin.

Cellular disulfides, including protein disulfide and low-molecular-weight disulfide, can be reduced by a group of enzymes. Glutaredoxin, also known as thioltransferase (*1*), catalyzes the reduction of disulfide bonds of various protein and non-protein substrates in the presence of glutathione (*2-5*), and has been implicated in a large variety of biochemical reactions, such as the maintenance of a normal cellular thiol/disulfide ratio, the redox regulation of some enzyme activities, and the regeneration of oxidatively damaged proteins (*6-8*).

Two hydrogen donor systems are known for the NADPH-dependent reduction of ribonucleotides (*9*). One is the thioredoxin system composed of NADPH, thioredoxin, and thioredoxin reductase (*9*). The other is the glutaredoxin system composed of NADPH, glutaredoxin, glutathione, and glutathione reductase (*10*). Thioredoxin coupled to NADPH and thioredoxin reductase can reduce disulfides in protein substrates (*11*), whereas glutaredoxin coupled to NADPH and glutathione reductase uses the monothiol GSH to reduce disulfides (*12*).

Glutaredoxin and thioredoxin comprise a superfamily of ubiquitous heat-stable and small acidic proteins, containing an active site with the sequence -Cys-X-X-Cys- (*13*). Plant thioredoxin is involved in two enzyme systems (*14-16*). The NADP/thioredoxin (h type) system, which is analogous to bacterial and animal thioredoxins, is widely distributed in plant tissues (*17, 18*) and is located in the mitochondria, ER, and cytosol (*19*).

Glutaredoxin was originally discovered in a viable mutant of *Escherichia coli* (*10*) and it has been isolated and characterized from *E. coli* (*20, 21*), yeast (*22*), calf thymus (*23, 24*), bovine liver (*25*), rabbit bone marrow (*26*), pig liver (*27*), and rat liver (*3, 28*), as well as human erythrocytes and placenta (*8, 29*). The enzymatic properties, characteristics, and primary structures of bacterial and mammalian glutaredoxins have been studied extensively (*1*). However, plant glutaredoxin-thioltransferase has not been described. Here we describe the purification of glutaredoxin from rice bran to homogeneity, and the results of an examination of its primary structure and features.

EXPERIMENTAL PROCEDURES

Materials—Rice (*Oryza sativa* L. cv. Nipponbare) was obtained from an agricultural field in Shiga prefecture. Streptomycin sulfate and glutathione reductase [EC 1.6.4.2] were obtained from Sigma. Dehydro-L-ascorbic

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; EDTA, ethylenediaminetetraacetic acid; HEDS, 2-hydroxyethyl disulfide; IAA, iodoacetamide; DHA, dehydroascorbic acid; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; MALDI-TOF, matrix-assisted laser desorption ionization time of flight.

acid dimer was obtained from Fluka. GSH, lysylendopeptidase [EC 3.4.21.50] and *Staphylococcus aureus* V8 protease [EC 3.4.21.19] were obtained from Wako. NADPH, HEDS, IAA, and a Cosmosil 5C8-300 column were from Nacalai Tesque. CNBr-activated Sepharose 4B and a Hiload 26/10 Q Sepharose column were from Pharmacia Biotechnology. Bakerbond WP PEI and Butyl Scout columns were from J.T. Baker. A Superspher RP-18 column was from Merck. All other chemicals and reagents were of the highest purity available.

Preparation of Glutaredoxin from Rice Bran—All operations were performed at 4°C unless otherwise stated. Protein was determined according to the methods of Bradford (*30*) and Spector (*31*).

Step 1: One kilogram of rice bran was homogenized in a total volume of 4 liters of 10 mM $CaCl_2$ using a Polytron PT3000 (Kinematica AG) and adjusted to pH 6.0 with 1 M NaOH. After centrifugation for 30 min at 8,000 × *g*, the precipitate was discarded and the supernatant fraction (Fraction I) was saved.

Step 2: To stabilize glutaredoxin, fresh streptomycin sulfate (20% in water) was dropped into Fraction I using a pipette to give a final concentration of 0.8%. After an additional 30 min of stirring, the precipitate was removed by centrifugation. The supernatant fraction (Fraction II) was saved and adjusted to pH 7.5 with 1 M NaOH.

Step 3: Powdered solid ammonium sulfate was added with stirring to Fraction II to make 40% saturation. The mixture was stirred for 3 h and the precipitate was removed by centrifugation. The supernatant was further brought to 90% saturation by adding solid ammonium sulfate. The precipitate obtained after centrifugation was dissolved in about 200 ml of 50 mM Tris-HCl, pH 7.5, and dialyzed extensively against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 4°C for 2 days. Insoluble material was removed by centrifugation (Fraction III).

Step 4: Fraction III was adjusted to pH 9.0 with 1 M Tris base. A portion (about 30 ml) was loaded on a column of Hiload 26/10 Q Sepharose (FPLC), equilibrated with 10 mM Tris-HCl, pH 9.0 (solvent A), then eluted with a multiple linear gradient of 0 to 1 M NaCl in 10 mM Tris-HCl, pH 9.0 (solvent B). The elution protocol was as follows: 0-100 min, 100% solvent A, 0% solvent B; 100-300 min, 0-30% solvent B; 300-360 min, 30-50% solvent B; 360-390 min, 50-100% solvent B. The elution profile from 100-300 min, which afforded fractions 50-110, is shown in Fig. 1.

Purification of Glutaredoxin Using an Immunoabsorbent Column (Step 5)—Eight-week-old female BALB/c mice were immunized with 20 μg of recombinant rice glutaredoxin (*32*) in complete Freund's adjuvant (Sigma). Four days after a booster immunization, the mice were killed and the spleens were removed under aseptic conditions. Splenic lymphocytes were fused with P3×63AG8.653 mouse myeloma cells following a standard 50% polyethylene glycol fusion protocol. Hybridomas were selected in HAT medium containing 10% FBS. Hybridomas from wells containing supernatant with specific antibody were examined by limiting dilution. Ascitic fluid containing a monoclonal antibody was obtained from pristane primed mice that were injected intraperitoneally with the hybridoma cell line. The immunoglobulin fraction was purified by protein A Sepharose chromatography (Pharmacia).

The monoclonal antibody (3 ml, 1.28 mg/ml) was coupled with CNBr-activated Sepharose 4B (1 g) using the coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl), blocked with 50 mM Tris-HCl, pH 8.0, containing 1 M glycine and equilibrated with the RX buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 10 mM HEPES, pH 7.3) in a 15 ml column. The sample, 12 ml of fractions 86-90 eluted from a Hiload 26/10 Q Sepharose column, was fractionated with the immunoabsorbent column. Bound glutaredoxin was washed with the RX buffer, followed by 0.1% Triton X-100, then eluted with 0.1 M glycine-HCl, pH 2.5, containing 0.1% Triton X-100. The detergent was removed using a Bakerbond WP PEI Scout column.

Assay of Enzymic Activities—The assay for glutaredoxin is based on its GSH-disulfide transhydrogenase (thioltransferase) activity as described by Holmgren (*20*). Coupled with glutathione reductase, the rate of the glutaredoxin reaction was determined from the decrease in absorbance at 340 nm resulting from the oxidation of NADPH. The reaction proceeded at 25°C in 1.0 ml of a mixture containing 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA, 0.4 mM NADPH, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 100 μg/ml of bovine serum albumin, and 0.7 mM HDES in the absence or presence of rice glutaredoxin (~20 μl). One unit of glutaredoxin activity is defined as the amount required to oxidize 1 μmol of NADPH per min at 25°C.

Dehydroascorbate reductase activity was measured by means of the spectrophotometric assay described by Stahl *et al.* (*33*), which is based on the change in absorbance at 265.5 nm as dehydroascorbic acid is reduced to ascorbic acid. The reaction mixture consisted of 137 mM sodium phosphate, pH 6.8, 1 mM EDTA, 2 mM GSH, 1 mM dehydro-L-ascorbic acid, and rice glutaredoxin (~20 μl) in a total volume of 1.0 ml. The reaction was initiated by adding DHA and it was linear for up to 2 min at 30°C.

SDS-Polyacrylamide Gel Electrophoresis—Sample preparation and SDS-PAGE were conducted according to Laemmli (*34*), using a 3.5% stacking gel and a 14% separating gel at a constant current of 10 and 16 mA, respectively, in an AE 6540 electrophoresis apparatus (ATTO, Tokyo). The gel was stained with CBB staining solution.

Western Blotting—Western blotting proceeded according to Towbin *et al.* (*35*). The samples were resolved by 14% SDS-PAGE, then electroblotted at 100 mA for 40 min onto PVDF membrane (Millipore). The primary antibody was diluted 1,000-fold to form the enzyme-antibody immune complexes. Protein bands were visualized using alkaline phosphatase-labeled goat antimouse IgG.

Mass Spectrometry—MALDI-TOF mass spectrometry was performed using a Kompact MALDI IV mass spectrometer (Kratos, UK). The sample holder is a stainless steel slide (8.5 by 1.0 cm) on which 20 sample sites (1 × 1 × 0.5 mm each) are placed at regular intervals.

Structural Analysis—About 200 μg of purified rice glutaredoxin was reduced and alkylated using 1 M dithiothreitol and 2.5 μl of 4-vinylpyridine in the presence of 8 M urea, 1 M Tris-HCl, pH 8.5, and 4 mM EDTA for 2 h at 37°C. The alkylated glutaredoxin was purified on a Butyl Scout column and digested with lysylendopeptidase or *S. aureus* V8 protease. The digest was fractionated on a Butyl Scout or Cosmosil 5C8-300 column. Chromatography was performed with a linear gradient of acetonitrile from 10 to

45% in the presence of 0.1% trifluoroacetic acid.

The amino acid sequence was determined by automated Edman degradation using a PPSQ-10 (Shimadzu) or 476A sequencer (Applied Biosystems). To analyze the amino acid composition, aliquots containing peptides were hydrolyzed with HCl vapor at 150°C for 1 h and derivatized with phenyl isothiocyanate by the method of Bidlingmeyer *et al.* (*36*). Phenylthiocarbamyl amino acids were resolved on a Superspher RP-18 column.

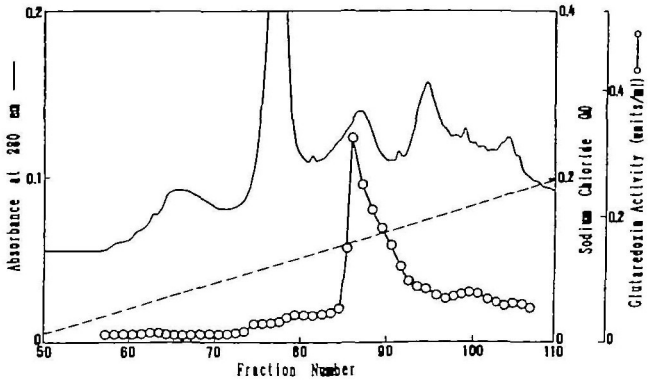

Fig. 1. **Hiload 26/10 Q Sepharose chromatography of rice glutaredoxin.** The elution profile of the protein extract (step 3) is presented with a linear gradient of NaCl from 0 to 0.2 M (- - -) using a Hiload 26/10 Q Sepharose column. The flow rate was 5 ml/min and fractions of 10 ml were collected. Protein absorbance at 280 nm —. Glutaredoxin activity (GSH-disulfide transhydrogenase activity) ○. For the elution protocol and assay of glutaredoxin activity see "EXPERIMENTAL PROCEDURES."

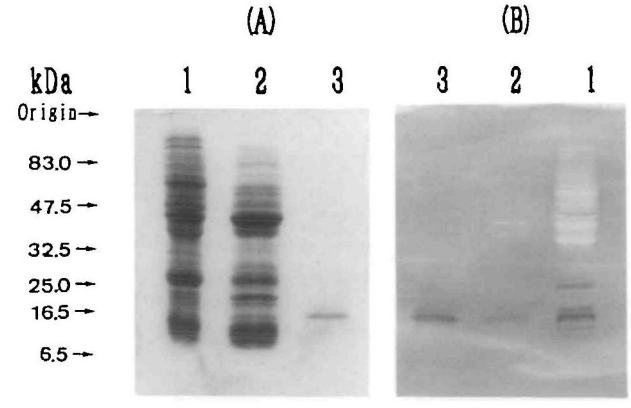

Fig. 2. **SDS-PAGE and Western blotting of glutaredoxin preparations from rice bran.** (A) SDS-PAGE of progressively purified rice glutaredoxin. The samples were concentrated after each step of purification and resolved by SDS-PAGE as described in "EXPERIMENTAL PROCEDURES." Lane 1, 15 μg of protein extract (step 3); lane 2, 10 μg of crude glutaredoxin (step 4); lane 3, 0.5 μg of purified glutaredoxin (step 5). (B) Western blotting of progressively purified rice glutaredoxin. Lane 1, 15 μg of protein extract (step 3); lane 2, 10 μg of crude glutaredoxin (step 4); lane 3, 0.5 μg of purified glutaredoxin (step 5). The other weak bands may be due to oligomers of rice glutaredoxin.

RESULTS

Purification of Glutaredoxin—Table I summarizes the results of purification of glutaredoxin from rice bran. The critical steps in the procedure were 4 and 5. As shown in Fig. 1, GSH-disulfide transhydrogenase activity was enriched in fractions 86-90 and the maximum activity was 0.32 unit/ml in fraction 87. The crude glutaredoxin was concentrated by a Hiload 26/10 Q Sepharose chromatography. After step 5, the enzyme had been purified more than 2,100-fold in an overall yield of 22.3% (Table I). The fraction obtained by immunoabsorbent chromatography migrated as a single band on SDS-PAGE (Fig. 2A, lane 3). MALDI-TOF mass spectrometry revealed that the protein has a molecular mass of 11,097.9 Da (Fig. 3).

Glutaredoxin Activity—Glutaredoxin has inherent GSH-

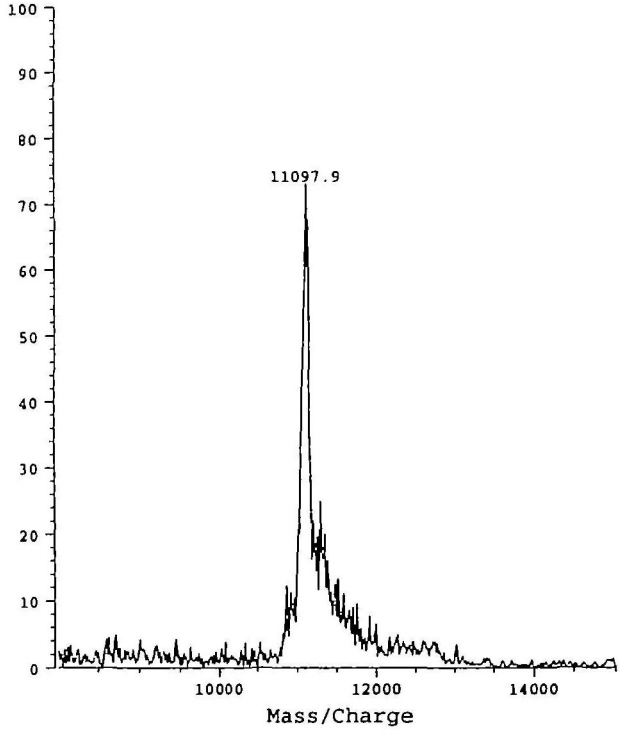

Fig. 3. **MALDI-TOF mass spectrometry of rice glutaredoxin.** About 10 pmol of rice glutaredoxin was examined as described under "EXPERIMENTAL PROCEDURES."

TABLE I. **Purification of glutaredoxin from 1 kg of rice bran.**

Purification step	Protein (mg)	Total activity (units*)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Crude extract	3,550	350	0.098	100	
2. Streptomycin sulfate	3,090	342	0.11	97.7	1.1
3. Ammonium sulfate (40-90%)	487	120	0.25	34.3	2.6
4. Hiload 26/10 Q Sepharose	48.3	88.1	1.82	25.2	18.6
5. Antiglutaredoxin-Sepharose 4B	0.37	78.2	211.4	22.3	2,157

* One unit corresponds to the amount of activity required to oxidize 1 μmol of NADPH per min at 25°C.

disulfide transhydrogenase activity (also named thioltransferase activity) (*20*), upon which we based its isolation and purification from rice bran. In step 1, the crude extract contained only 0.098 unit/mg of a specific activity. After step 4, the activity was enriched and the crude glutaredoxin had an activity of 1.82 units/mg. The final product had a specific activity of about 211.4 units/mg protein (Table I).

Primary Structure of Rice Glutaredoxin—A cDNA clone encoding rice glutaredoxin has been isolated and the amino acid sequence deduced (*32*). Here we investigated the primary structure of the mature protein. The protein was cleaved by lysylendopeptidase or *S. aureus* V8 protease and the cleavage products were separated by Butyl Scout or Cosmosil 5C8-300 chromatography (Fig. 4). The derived peptides (K1-K6 for lysylendopeptidase and V1-V6 for *S. aureus* V8 protease) were analyzed for amino acid composition and sequence. Although the N-terminus was blocked, the amino acid composition analysis of the N-terminal peptide (K1) indicated that it consisted of two alanine residues, one leucine and one lysine. Thus, the N-terminal sequence is Ala[1]-Leu[2]-Ala[3]-Lys[4]-, compared with the cDNA-derived sequence of -Ala[2]-Leu[3]-Ala[4]-Lys[5]-. The amino acid composition analysis of the peptide (K6) revealed that it did not contain lysine, and the sequencing showed that the C-terminus is -Ala[105].

Figure 5 displays the primary structure of mature rice glutaredoxin. Rice glutaredoxin is composed of 105 amino acid residues, corresponding to a molecular weight of 11,027.4. The tetrapeptide -Cys-Pro-Phe (Tyr)-Cys- of an active site identified in *E. coli* and mammalian glutaredoxins (*37-39*) was located at positions 22 to 25 in the polypeptide chain. The alignment of the protein sequence with those of *E. coli*, yeast and mammalian glutaredoxins showed only about 25, 37, and 39% homology, respectively.

The amino acid sequence deduced from the cDNA is composed of 112 amino acid residues starting at methionine and stopping at alanine (*32*). In contrast, in the mature protein the N-terminus begins with alanine located at position 2 and the C-terminus ends with alanine located at position 106 in the cDNA-derived sequence. Compared with the deduced amino acid sequence, the only difference was that the glycine residue located at position 34 is replaced by glutamic acid residue in the mature protein. We consider that this difference is due to either the presence of an isoform in rice glutaredoxin or misincorpo-

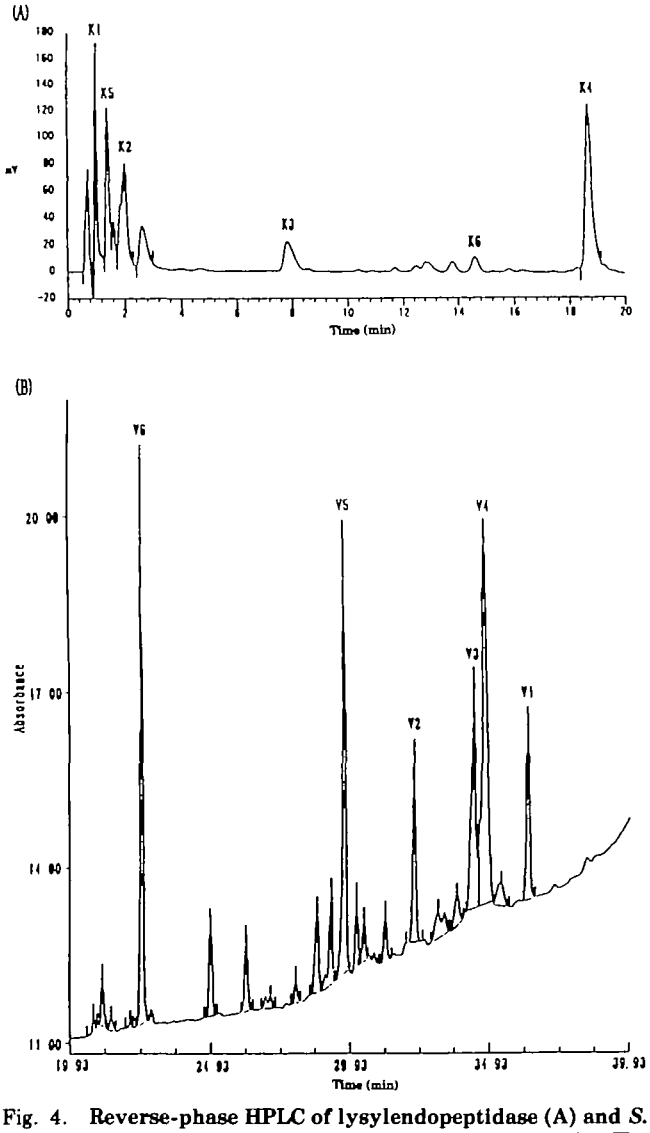

Fig. 4. **Reverse-phase HPLC of lysylendopeptidase (A) and *S. aureus* V8 protease (B) peptides from rice glutaredoxin.** The peptides digested with lysylendopeptidase were fractionated on a Butyl Scout column (peaks K). The peptides digested with *S. aureus* V8 protease were fractionated on a Cosmosil 5C8-300 column (peaks V).

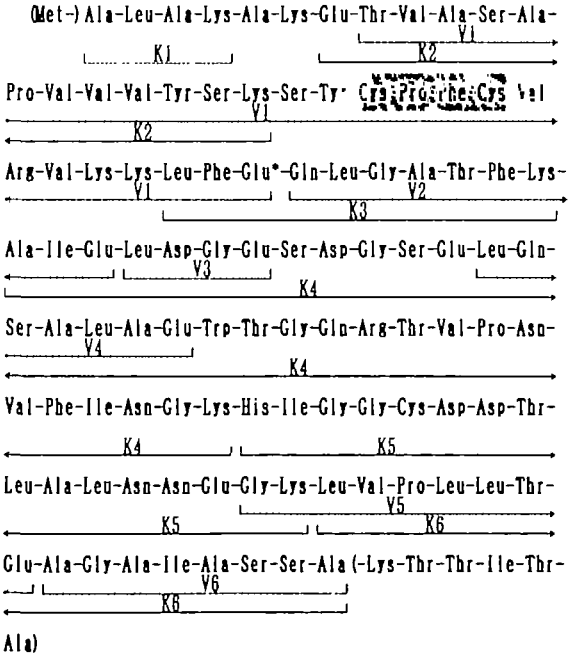

Fig. 5. **The primary structure of rice glutaredoxin.** The peptides isolated from digests with lysylendopeptidase (K) or *S. aureus* V8 protease (V) are shown (— and ·····) and numbered consecutively from the N-terminus. The sequence of the N-terminal peptide was determined from the composition of K1 peptide (·····), compared with the cDNA-derived sequence. The sequence -Ala[5]-Lys[6]- was also determined from the cDNA. All others were determined by direct amino acid sequencing of the peptides. The sequences in brackets were deduced from the cDNA. The glutamic acid (asterisk) is replaced by glycine in the cDNA-derived sequence. The putative tetrapeptide active site is shaded.

ration errors during construction of the cDNA library by the PCR technique (Taq DNA polymerase) (*32*).

Neutralization of the Activity by Antibody—The glutaredoxin activity in the protein extract was neutralized by adding antibody, as was that of crude and homogeneous rice glutaredoxins. About 85% of the activity of 0.2 μg of the purified glutaredoxin, 70% of the activity of 50 μg of the crude glutaredoxin, and 60% of the activity of 0.2 mg of the protein extract were neutralized by 15 μg/ml of the antibody (Fig. 6). Western blot assay using the antibody revealed the presence of a single immunoreactive polypeptide (Fig. 2B), which corresponded to the glutaredoxin band (11-12 kDa) resolved by SDS-PAGE (Fig. 2A, lane 3).

Kinetic Properties—We examined the kinetics of dependence on various concentrations of 2-hydroxyethyl disulfide under standard conditions. Figure 7 shows a plot of the glutaredoxin activity as a function of 2-hydroxyethyl disulfide concentration. The kinetic profile revealed that the enzyme did not display normal Michaelis-Menten kinetics. At higher concentrations of 2-hydroxyethyl disulfide, the enzyme activity was inhibited.

pH-Activity Profile—The activity of rice glutaredoxin as a function of pH was determined using the standard assay mixture. Figure 8 shows that the optimal pH of rice glutaredoxin is about 8.7. Most known glutaredoxins have basic pH optima and the optimal pH values of rat, bovine and pig thioltransferases are about pH 8.5, 8.5, and 9.0, respectively (*25, 27, 28*).

Inactivation of Glutaredoxin Activity—Inactivation of the glutaredoxin activity was tested by measuring the activity after incubating the enzyme with inhibitors. Iodoacetamide at 30 and 100 μM decreased the enzyme activity by about 70 and 95%, respectively (Table II). This finding indicates that cysteine residues are essential for the activity of rice glutaredoxin. Ca^{2+}, Mg^{2+}, and Mn^{2+} (1.0 mM) were not inhibitory, but 1.0 mM Cu^{2+} was effective. Rice glutaredoxin was very sensitive to H_2O_2, which oxidizes the reduced sulfhydryl groups.

Rice Glutaredoxin Has Dehydroascorbate Reductase Activity—Mammalian glutaredoxin has significant dehydroascorbate reductase activity, as reported by Wells *et al.* (*40*). We found that the specific dehydroascorbate reductase activity of purified rice glutaredoxin was about 91.7

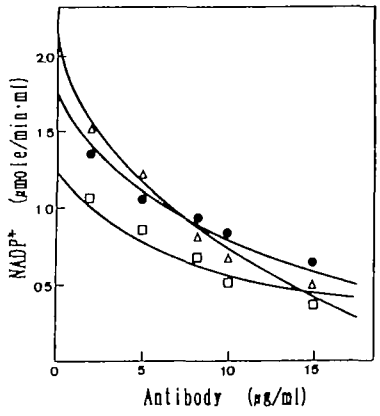

Fig. 6. **Neutralization of the glutaredoxin activity by antibody.** Progressively purified rice glutaredoxin samples were mixed with increasing amounts of the antibody in the presence of RX buffer. After a 5 min incubation at 37°C, reaction mixtures were diluted and the glutaredoxin activity was measured as described in "EXPERIMENTAL PROCEDURES." Open triangles, 0.2 μg of purified glutaredoxin (step 5); closed circles, 50 μg of crude glutaredoxin (step 4); open squares, 0.2 mg of protein extract (step 3).

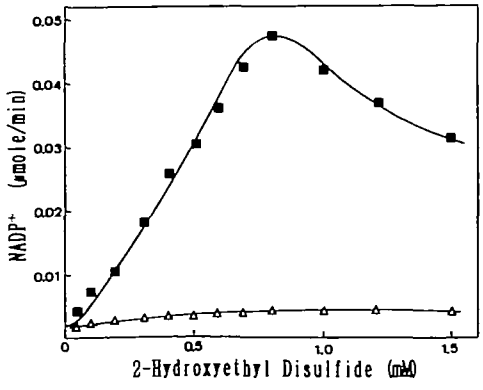

Fig. 7. **Kinetics of rice glutaredoxin activity on HEDS.** Glutaredoxin activity was assayed with various concentrations (■) in the range of 0-1.5 mM HEDS. Each assay contained 0.1 μg of purified glutaredoxin. For the assay of glutaredoxin activity see "EXPERIMENTAL PROCEDURES." △, velocity of spontaneous reduction.

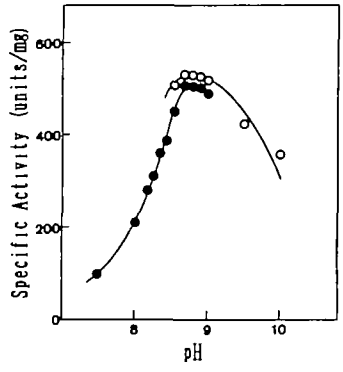

Fig. 8. **Dependence of the glutaredoxin-catalyzed reaction on pH.** Glutaredoxin activity was assayed in 0.1 M Tris-HCl buffer (●), pH 7.5 to 9.0 and in 0.1 M glycine-NaOH buffer (○), pH 8.6 to 10.0. Each assay mixture contained 0.13 μg of purified glutaredoxin. Details of the glutaredoxin assay are described in "EXPERIMENTAL PROCEDURES."

TABLE II. **Effect of inhibitors on the activity of rice glutaredoxin.**

Inhibitor	Concentration (mM)	Relative activity (%)
None	–	100
IAA	0.03	33
	0.1	5
H_2O_2	0.01	27
	0.1	3
$CaCl_2$	1.0	96
$MgCl_2$	1.0	92
$CuCl_2$	1.0	31
$ZnCl_2$	1.0	78
$MnCl_2$	1.0	90

Purified glutaredoxin (1.2 μg) was incubated with inhibitors at the indicated concentrations in a total volume of 100 μl for 10 min at 25°C, then the enzyme activity was assayed under standard conditions.

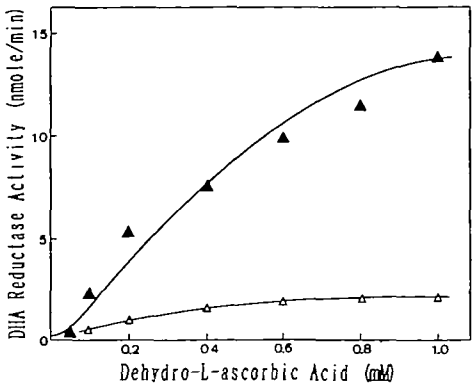

Fig. 9. **Kinetics of rice glutaredoxin activity on dehydro-L-ascorbic acid.** The standard assay of glutaredoxin activity included ~1.0 mM dehydro-L-ascorbic acid (▲). Each assay contained 0.6 μg of purified glutaredoxin. For the standard assay of activity see "EXPERIMENTAL PROCEDURES." △, velocity of spontaneous reduction.

units/mg protein. Figure 9 shows the kinetic behavior of the activity at various concentrations of dehydro-L-ascorbic acid.

DISCUSSION

Morell *et al.* have identified and partially purified glutaredoxin from spinach leaves (*41*). However in this study, we purified and characterized glutaredoxin from plant tissues for the first time. There is a glutaredoxin system not only in bacteria and animals, but also in plants, indicating that the system is ubiquitous.

An immunoabsorbent method using antiglutaredoxin-Sepharose proved to be efficient for the isolation and purification of glutaredoxin from rice bran. No thioredoxin bound to the antiglutaredoxin column, demonstrating that there the antiglutaredoxin antibody, did not cross-react with thioredoxin. The isolated protein was homogeneous on SDS-PAGE (Fig. 2A, lane 3) and MALDI-TOF mass spectrometry showed a molecular mass of 11,097.9 Da (Fig. 3). *E. coli* glutaredoxin has a molecular weight of 9,674 for the reduced form, making it one of the smallest known glutaredoxins (*21*). Mammalian glutaredoxin behaves as a slightly basic molecule with a molecular mass of 11-12 kDa (*1*).

Rice glutaredoxin consists of 105 amino acid residues, corresponding to a molecular weight of 11,027.4. We consider that the difference between this value and that of MALDI-TOF mass spectrometry was caused by modifications of the N-terminus and of a side chain. The N-terminus of rice glutaredoxin was found to be blocked. The N-terminus of all mammalian glutaredoxins is blocked by acetylated alanine (*26, 42-44*), and thus it seems reasonable that the N-terminus of rice glutaredoxin is also acetylated post-translationally. Another possibility might be that one of the glutamic acid (Glu) residues in the mature protein is converted to a γ-carboxyglutamic acid residue (*45*). If this were the case, the molecular mass of rice glutaredoxin would be 11,113.4 Da, corresponding to the value estimated by MALDI-TOF mass spectrometry within ±0.1%.

Kinetic analyses revealed that the enzyme did not display normal Michaelis-Menten kinetics (Figs. 7 and 9) and was inhibited at higher concentrations of substrate (Fig. 7). The kinetics of rat and pig thioltransferases are similar (*27, 28*). Gan and Wells reported that the Hill coefficient for rat thioltransferase was 2.5, consistent with substantial homotropic allostery (*27*). Rat and bovine thioltransferase are even inhibited at high concentrations of glutathione (*3, 25*). These results indicate that glutaredoxin-thioltransferase is an unusual enzyme. It seems probable that the regulation of allosteric activation and substrate inhibition of glutaredoxin are very important for controlling the ratio of cellular thiol and disulfide.

Wells *et al.* discovered that mammalian glutaredoxin possesses significant dehydroascorbate reductase activity (*40*). We found that rice glutaredoxin also has dehydroascorbate reductase activity, and its kinetics are shown in Fig. 9. The major ascorbate regeneration cycle of the cytoplasm is probably due to glutaredoxin coupled with the regeneration of GSH by NADPH catalyzed by GSSG reductase (*1*). Dehydroascorbate reductase has been identified in pig (*46, 47*), and isolated from pea and spinach (*48-50*). It would be of interest to compare the distributions of dehydroascorbate reductase and glutaredoxin in various cell systems.

Glutaredoxin is involved in glutathione-dependent deiodination of thyroxine to triiodothyronine (*51*), regulation of the activities of pyruvate kinase, papain, phosphofructokinase, and ornithine decarboxylase (*52-55*), and restoration of oxidatively damaged membrane proteins of human erythrocytes (*56*). *E. coli* and calf thymus glutaredoxins seem to play an important role in DNA synthesis (*23, 57*).

The reactions in which plant glutaredoxin is involved and the role it plays in plant cells should be investigated. The regulation of glutaredoxin expression relative to that of thioredoxin will be of particular interest.

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