

## Purification and Identification of Glutathione S-transferase in Rice Root under Cadmium Stress

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**Abstract:** Cadmium (Cd) contamination in paddy soils poses a serious threat to the production and quality of rice. Among various biochemical processes related to Cd detoxification in rice, glutathione S-transferase (GST) plays an important role, catalyzing Cd complexation with glutathione (GSH) and scavenging reactive oxygen species (ROS) in cells. In this study, a hydroponic experiment was conducted to investigate the response of GST isozymes in rice roots upon Cd exposure. Results showed that the GST activity in rice roots was clearly enhanced by 50  $\mu\text{mol/L}$  Cd treatment for 7 d. The GST isozymes were purified by ammonium sulphate precipitation, gel filtration chromatography and affinity chromatography. After being separated by SDS-PAGE and visualized by silver staining, GSTU6 was identified by in-gel digestion, MALDI-TOF-MS analysis and peptide mass fingerprint. The results confirm the vital function of tau class rice GST in Cd detoxification.

**Key words:** rice; glutathione S-transferase; cadmium stress; purification; identification; isozyme

Glutathione S-transferases (GSTs, EC 2.5.1.18), encoded by a large family of genes, play a central role in the crucial processes of detoxification in plant cells (Edwards et al, 2000). Most GSTs described to date are dimers composed of 22–30 kDa subunits, and each subunit has a glutathione (GSH) binding site (G-site) and an adjacent electrophilic substrate binding site (H-site) (Dixon et al, 2002). These enzymes are mainly found in the cytosol of organisms and may be divided into several classes such as phi, tau, theta, zeta, lambda and dehydroascorbate reductase, among which phi and tau classes are specific in plants (Banerjee and Goswami, 2010). Based on protein sequence alignments, 40 GST genes are assigned to the phylogenetic class tau, 16 to phi, 3 to zeta, and 2 to theta in rice (Soranzo et al, 2004). In addition, these GSTs distribute in different organs and tissues of rice, and the expression of specific GSTs vary differently during plant development (Soranzo et al, 2004).

Rice is an important crop sustaining human society, providing food for almost half of the global population. However, contamination of paddy fields by heavy

metals such as cadmium (Cd) due to industrial and agricultural activities has become a global environmental problem, posing risk to human health if the level of this toxic metal in rice grains exceeds the maximum limits (e.g., 0.4 mg/kg for polished rice proposed by the Codex Alimentarius Commission of the Food and Agriculture Organization). To safeguard the rice grain quality, a thorough understanding of Cd movement inside the rice tissues is required.

Recently, it has been pointed out that Cd accumulation in grains may be controlled by the root-to-shoot translocation via the xylem (Uraguchi et al, 2009) and by the capacity to retain Cd in roots (Nocito et al, 2011). Cadmium sequestration in plant roots could be enhanced when Cd was bound to thiol compounds such as phytochelatin (Wong and Cobbett, 2009). Additionally, our previous research confirmed that Cd toxicity and translocation was reduced when the thiol content and GST activity in roots increased (Hu et al, 2009; Bai et al, 2011). GST may catalyze Cd complexation with GSH (Adamis et al, 2004), thereby alleviating Cd toxic effects and promoting Cd retention in plant roots (Dixit et al, 2011). However, except for a few reports on rice GST activity (Zhang and Ge, 2008; Zhao et al, 2009; Dubey et al, 2010) and gene

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expression (Dubey et al, 2010; Moons, 2003) under metal stress, the number and identity of GST isozymes that participate in Cd detoxification and sequestration still remain unknown.

Therefore, in this study, we aimed to purify and identify GST isozymes in rice seedlings upon Cd exposure using chromatography, electrophoresis and mass spectrometry methods.

## MATERIALS AND METHODS

### Chemicals

Glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), trypsin, phenylmethyl sulfonylfluoride (PMSF) and tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. The protein marker for SDS-PAGE was from Fermentas. All other reagents were of analytical grade.

### Rice culture

Rice seeds of N07-63 were obtained from Jiaying Academy of Agricultural Sciences, Zhejiang Province, China. The seeds were sterilized with 15% hydrogen peroxide for 10 min, rinsed, and germinated in the dark. Rice seedlings were hydroponically cultured to the three-leaf stage, and then transferred to plastic containers with 7 L nutrient solution. The composition of the culture solution was (g/L):  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  67.44,  $(\text{NH}_4)_2\text{SO}_4$  37.73,  $\text{KH}_2\text{PO}_4$  8.79,  $\text{K}_2\text{SO}_4$  12.20,  $\text{CaCl}_2$  22.15,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  81.13,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  1.4409,  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  0.0736,  $\text{H}_3\text{BO}_3$  0.9152,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.0352,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.0314,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  4.98,  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$  7.38 and  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  14.21. Rice seedlings were divided into two groups: control (CK) and 50  $\mu\text{mol/L}$  Cd treatment (supplied with  $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ ) for 7 d. The culture solution was renewed every 3 d and its pH was adjusted to 5.5–6.0 using 0.1 mol/L HCl or NaOH. After harvest, shoots and roots were separated and frozen in liquid nitrogen before GST activity analysis and GST isozyme purification. Since previous studies showed that the GST activity was much higher in rice roots than in shoots under Cd stress (Zhang and Ge, 2008; Zhao et al, 2009), we chose roots for GST purification in this study.

### Crude extract preparation

Root samples were homogenized with a ratio of 5:1 in 0.2 mol/L prechilled Tris-HCl buffer (pH 7.8, containing 5 mmol/L DTT, 1 mmol/L EDTA and 1 mmol/L PMSF). The crude extract was filtered through four

layers of cheese cloth and then centrifuged for 30 min (4 °C, 20 000  $\times g$ ) by Beckman Coulter Avanti J-25 (the United States) to obtain crude enzyme extract. One part of the extract was determined for the GST activity and the protein content. The rest of supernatant was processed for further purification.

### Purification of rice GST

Proteins in the rice samples were salted out in two steps. Firstly,  $(\text{NH}_4)_2\text{SO}_4$  was added to the concentration of 40%. After incubation on ice for 1 h, the pellet was collected by a 30-min centrifugation (4 °C, 20 000  $\times g$ ). Secondly, more proteins were precipitated by adding  $(\text{NH}_4)_2\text{SO}_4$  to 85% in the supernatant from the first step and centrifuging (4 °C, 20 000  $\times g$ ) again. Pellets from the two steps were combined and suspended in 10 mmol/L phosphate buffer saline (PBS) buffer (pH 7.4, containing 5 mmol/L DTT, 1 mmol/L EDTA and 150 mmol/L NaCl).

### Salt removal

The samples were desalted using a HiTrap column, with an equilibrium buffer of 10 mmol/L PBS (pH 7.4, containing 5 mmol/L DTT, 1 mmol/L EDTA and 150 mmol/L NaCl). Proteins were eluted from the HiTrap column by a 35-mL gradient from 150 to 0 mmol/L NaCl in 10 mmol/L PBS (pH 7.4, containing 5 mmol/L DTT and 1 mmol/L EDTA). Elution profiles were determined by monitoring the absorbance at 280 nm and enzyme activity using CDNB as a substrate.

### Gel filtration chromatography

The proteins were separated based on the molecular weight (MW) by loading the samples to a gel filtration column (Sephacrose G-75). The column was equilibrated with 10 mmol/L PBS (pH 7.4). Elution profiles were determined by monitoring the absorbance at 280 nm and the enzyme activity was determined using CDNB as a substrate.

### Affinity chromatography

The protein fractions with high GST activity were pooled together and further purified by a GSH affinity matrix using a wash-batch method. Approximately 200  $\mu\text{L}$  wet volume of GSH-agarose gel (Sigma) was mixed with the sample (9 mL) and incubated for 30 min at 4 °C. After centrifugation, the gel was washed with 12 mL of 10 mmol/L PBS (pH 7.4). The bound proteins were eluted from the gel by washing with 16 mL of 50 mmol/L Tris-HCl buffer (pH 8.0, containing

30 mmol/L GSH, 5 mmol/L DTT and 1 mmol/L EDTA).

### Enrichment of GST enzyme

The eluent from the affinity chromatography was concentrated using a freeze-drying equipment (Heto PowerDry LL3000, China). The GST activity was also determined using CDNB as a substrate.

### SDS-PAGE, tryptic digestion and mass spectrometry

The crude protein extract, fractions precipitated by  $(\text{NH}_4)_2\text{SO}_4$ , and fractions washed off and eluted from the GSH gel matrix were analyzed for protein molecular masses by denaturing SDS-PAGE, using the method of Laemmli (1970) in 13% gels. Protein bands were visualized by silver staining and in-gel tryptic digestion was performed on the proteins purified by the affinity chromatography.

Protein bands were excised, dehydrated in acetonitrile, and then reduced and alkylated. Gel pieces were thoroughly washed, dried in a Speedvac (Eppendorf Concentrator 5301, Canada), reswollen with 10  $\mu\text{g}/\text{mL}$  trypsin solution (in 25 mmol/L ammonium bicarbonate) at 4 °C for 30 min and then incubated at 37 °C for 12 h. Trifluoroacetic acid (TFA) was added to a final concentration of 0.1% to stop the reaction.

Digests were immediately spotted onto 600  $\mu\text{m}$  anchorchips (Bruker Daltonics). The Bruker peptide calibration mixture was spotted for external calibration of spectrum to a mass tolerance within 0.1 Da. All samples were allowed to air-dry at room temperature, and 0.1% TFA was used for on-target washing. Mass analysis was carried out on a time-of-flight Ultraflex II mass spectrometer (Bruker Daltonics). Peptide mass maps were acquired in a positive reflection mode, averaging 800 laser shots per MALDI-TOF spectrum.

The protein identity was confirmed by peptide mass

fingerprint using the UniProtKB/Swiss-Prot database (version 72). The following search parameter criteria were used: significant protein MOWSE score at  $P < 0.05$ , minimum mass accuracy 100 mg/kg, trypsin as enzyme, one missed cleavage site allowed, cysteine carbamidomethylation, acrylamide modified cysteine, methionine oxidation and similarity of pI and relative molecular mass specified, and minimum sequence coverage of 15%.

### Enzyme assay and protein determination

Protein content and GST activity toward CDNB were determined in each step of GST purification. Protein content was determined by Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard. GST activity was determined according to Habig et al (1974). A total of 0.5 g of fresh sample and 2 mL extractant (phosphate buffer solution 0.1 mol/L, pH 6.5 and 1 mmol/L EDTA) were homogenized. The suspension was centrifuged at  $12\,000 \times g$  for 15 min at 4 °C. The reaction mixture (3 mL) included 100 mmol/L potassium phosphate buffer (pH 6.5), 0.1 mmol/L CDNB, 0.1 mmol/L GSH and 0.1 mL of enzyme extract. The increase in absorbance at 340 nm was measured over a time period of 5 min (25 °C,  $E = 9.6 \text{ mmol}/(\text{L} \cdot \text{cm})$ ). One unit (U) of GST was defined as the conjugation of 1.0  $\mu\text{mol}$  of CDNB with reduced GSH per minute at pH 6.5 at 25 °C.

## RESULTS

### GST activities during purification

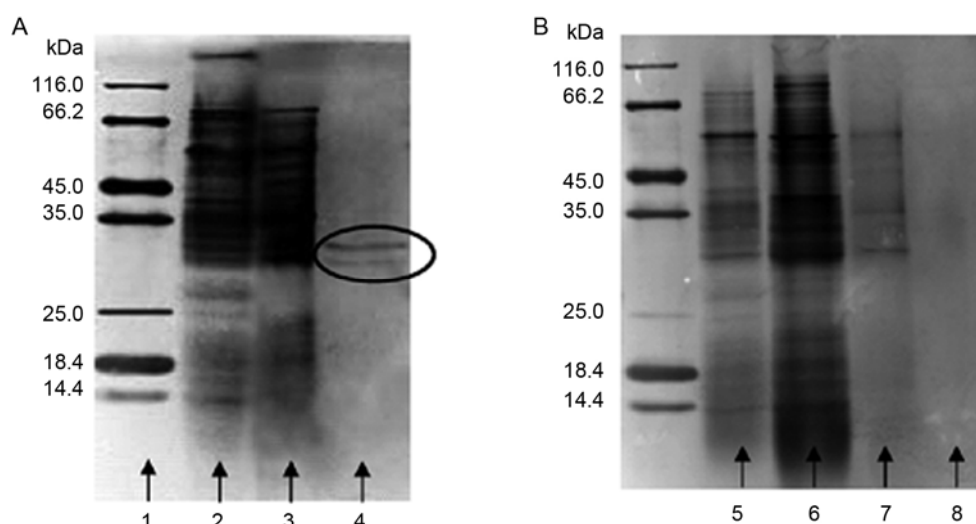
For all samples in the purification steps, the GST activities in the Cd-treated samples were consistently higher than those of the control (Tables 1 and 2). As

**Table 1. Purification of GST from rice roots without Cd treatment.**

Step	Total protein (mg)	Total unit (nmol/min)	Specific activity [nmol/(min·mg)]	Recovery rate (%)	Purification fold
Crude extract	49.07	4 575	93.2	100	1.00
Precipitation	38.81	4 023	103.7	88	1.11
Gel filtration chromatography	7.58	2 457	324.1	54	3.48
Affinity chromatography	0.11	170	1 625.0	4	17.43

**Table 2. Purification of GST from rice roots after Cd treatment.**

Step	Total protein (mg)	Total unit (nmol/min)	Specific activity [nmol/(min·mg)]	Recovery rate (%)	Purification fold
Crude extract	61.95	16 266	262.6	100	1.00
Precipitation	40.76	11 533	282.9	71	1.08
Gel filtration chromatography	7.53	4 112	564.6	25	2.15
Affinity chromatography	0.18	564	3 068.0	4	11.68



**Fig. 1.** SDS-PAGE analysis of glutathione S-transferase (GST) purification from Cd stressed (A) and untreated (B) rice roots.

Lane 1, Protein molecular weight markers; Lanes 2 and 5, Crude extracts for the Cd treated and control samples, respectively; Lanes 3 and 7, Fractions passed through the affinity column for the Cd treated and control samples, respectively; Lane 6, Fractions precipitated by  $(\text{NH}_4)_2\text{SO}_4$  for the control sample; Lanes 4 and 8, Eluents from the glutathione matrix for the Cd treated and control samples, respectively.

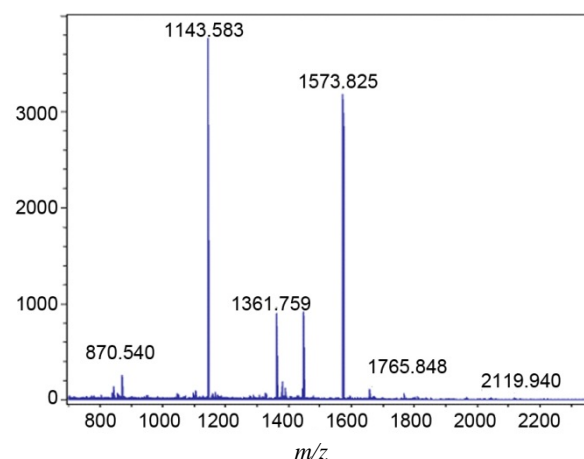
the purification continued, the GST activity declined but its specific activity increased due to the decrease in the amount of protein (Tables 1 and 2). At the end of affinity chromatography, the GST was purified 17.43-fold for the control and 11.68-fold for the Cd-treated sample.

### GST separation by SDS-PAGE

Proteins in the various steps in the GST purification were separated using SDS-PAGE (Fig. 1). Although there were bands within the 25–35 kDa range in crude extracts for both the control and Cd-treated samples, only for the latter could observe two bands with MW about 30–32 kDa in the eluent from the affinity chromatography. It indicates that, under the normal growth conditions, GST in the rice roots is not enough to be purified by the affinity chromatography.

### Mass spectrometry analysis and protein identification

The upper band of the Lane 4 (Fig. 1-A) was cut for in-gel digestion, after which the peptides were analyzed by the MALDI-TOF-MS (Fig. 2). With the peptide mass fingerprint through the UniProtKB/Swiss-Prot database, we identified the protein in the above mentioned band as rice GSTU6 (*Oryza sativa* subsp. *japonica*). Three major peaks corresponded to the following peptides: RTPALAAWEERF ( $m/z$  1 143.583), KGVVPDDADKLLLEFRQ ( $m/z$  1 573.825) and KLLGVWSSPYAIRV ( $m/z$  1 361.759) (Fig. 2). Interestingly, the theoretical MW of GSTU6 is 25 697



**Fig. 2.** MALDI-TOF mass spectrum of peptides after in-gel digestion of the upper band in Lane 4 of Fig. 1-A.

Da, but the MW shown in the SDS-PAGE migrated to about 32 kDa. Therefore, posttranslational modifications (PTM) in the amino acid sequence may have occurred for the rice GSTU6, but the nature of this PTM is yet to be determined. Moreover, after extensive searching in the protein database, no protein could be assigned to the band with lower MW in the Lane 4 of Fig. 1-A. Thus the nature of this protein was not studied further.

## DISCUSSION

Glutathione S-transferase is a well-known enzyme in the detoxification of xenobiotic substances (Wagner et al, 2002). It is normally induced by various abiotic stress,

such as herbicides, heat, ozone, metals and plant hormones (Marrs, 1996). According to Soranzo et al (2004), tau class GSTs are the most abundant in rice and their genes are up to 66% of all *osgst* genes. Their expression is often upregulated under different stress conditions such as salinity, pathogen infection and herbicide toxicity, suggesting that this class of GSTs may have a protective role in alleviating cellular damages caused by the above biotic and abiotic stress.

In this study, results of the enzyme activities (Tables 1 and 2) and expression (Fig. 1) showed that GST is clearly induced upon Cd exposure. These variations may be attributed to the change of GSH, which is required for the catalytic functions of many GSTs. Firstly, upon Cd exposure, GSH is consumed in the synthesis of phytochelatin, which forms complexes with Cd and subsequently sequesters into the vacuole (Nocito et al, 2011). Secondly, GSTs with peroxidase activity can use GSH as reductant for scavenging the reactive oxygen species formed upon Cd stress (Edwards et al, 2000; Dixon et al, 2002). Thus, the total and specific activities of GST were higher in the Cd treated sample than those in the control (Tables 1 and 2). Similar to other studies (Hong et al, 1999; Deng and Hatzios, 2002), the total activity of GST declined during the purification process (Tables 1 and 2). However, as the decrease of total proteins was more pronounced, the specific activity of GST in rice roots increased in the samples with and without the Cd treatments (Tables 1 and 2). The purification fold is the ratio of the specific activity of each purification step over that of the crude extract. The variations of the specific activities, therefore, may explain the difference of GST purification folds in Tables 1 and 2.

Most of the previous studies either focused on the dose-response relationship between stress and GST activity (Dixit et al, 2001; Iannelli et al, 2002; Zhang and Ge, 2008; Dixit et al, 2011), or followed the expression of individual GSTs at the mRNA level (Marrs and Walbot, 1997; Wagner et al, 2002; Moons, 2003). A few studies, however, attempted to purify and quantify GST isoforms at the protein level using proteomic technologies. For example, by using signature peptide and differential isotopic labeling techniques, eight GSTs were identified in *Arabidopsis* seedlings and the variations of their expression were monitored in response to copper and benoxacor treatments (Smith et al, 2004). In addition, Sappl et al (2004) identified 20 GSTs in *Arabidopsis* cell culture responding to salicylic acid treatment with a combination

of GST antibody detection, LC-MS/MS analysis of 23–30 kDa proteins and glutathione-affinity chromatography. In this study, a series of methods including gel filtration, affinity chromatography, SDS-PAGE, in-gel digestion and peptide mass fingerprint were applied to purify and identify rice GSTs. A tau class GST isozyme, GSTU6, was confirmed in the Cd-treated sample. Although the identification was based on the peptide mass fingerprint, the BLAST analysis of the three major peptide sequences ( $m/z$  1 143.583, 1 361.759 and 1 573.825; Fig. 2) confirmed that they were all from OsGSTU6 (data not shown). In plants, tau GST isozymes may protect cells from metal toxicity by catalyzing the conjugation of GSH with metals for subsequent vacuolar sequestration (Dixon et al, 2002; Moons, 2003; Ezaki et al, 2004). As mentioned before, 40 of 61 GST isozymes are from the tau class (Soranzo et al, 2004). Therefore, it is likely that they are more responsive upon metal exposure than other GST subgroups. However, it should also be aware that more proteomic investigation is needed to confirm the identity of all possible GST isozymes involved in the Cd detoxification in rice.

In summary, the chromatography, electrophoresis and mass spectrometry techniques were successfully applied for the purification and identification of GST isozymes in rice roots under the Cd stress. The confirmation of GSTU6 provides a preliminary insight into the Cd detoxification in rice. Further research is ongoing to elucidate more details of the expression and the subcellular location of GST isozyme(s) involved in the Cd sequestration in rice roots. The results may be useful for developing strategies to enhance Cd tolerance and to limit Cd mobility in rice.

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