RESEARCH ARTICLE

Oxidation of the enzymes involved in nitrogen assimilation plays an important role in the cadmium-induced toxicity in soybean plants

K. B. Balestrasse · S. M. Gallego · M. L. Tomaro

Abstract Cadmium causes oxidative damage and hence affects nitrogen assimilation. In the present work we tested the relationship between the inactivation of the enzymes involved in nitrogen assimilation pathway (glutamine synthetase (GS)/glutamate synthase (GOGAT)) and the protein oxidation in nodules of soybean (*Glycine max* L.) plants under Cd^{2+} stress. Therefore, the effect of Cd^{2+} and reduced gluthatione (GSH) on GS and GOGAT activities, and protein abundance and oxidation were analyzed. Under the metal treatment, amino acids oxidative modification occurred, evidenced by the accumulation of carbonylated proteins, especially those of high molecular weight. When Cd^{2+} was present in the nutrient solution, although a decrease in GS and GOGAT activities was observed (17 and 52%, respectively, compared to controls), the protein abundance of both enzymes remained similar to control nodules. When GSH was added together with Cd^{2+} in the nutrient medium, it protected the nodule against Cd^{2+} induced oxidative damage, maintaining GS and GOGAT activities close to control values. These results allow us to conclude that the inactivation of the nitrogen assimilation pathway by Cd^{2+} in soybean nodules is due to an increment in GS and GOGAT oxidation that can be prevented by the soluble antioxidant GSH.

Keywords *Bradyrhizobium japonicum* · Cadmium · *Glycine max* L. · Nitrogen assimilation · Protein oxidation

Abbreviations

DAB-3,3′	diaminobenzidine
2,4-DNPH-2,4	dinitrophenylhydrazine
GOGAT	glutamate synthase (EC 1.4.1.14)
GS	glutamine synthetase (EC 6.3.1.2)
ROS	reactive oxygen species
TBS	Tris-buffered saline
TTBS	Tris-buffered saline plus Tween

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Introduction

The symbiotic association of leguminous plants with *(Brady)rhizobium* results in the formation of root nodules, which are able to reduce N_2 to NH_4^+ by the enzyme nitrogenase (EC 1.18.6.1). Ammonium is rapidly assimilated by glutamine synthetase (GS, EC 6.3.1.2), which catalyses the ATP-dependent amination of glutamate, producing glutamine (Lea and Miflin 2004). Glutamine is subsequently deaminated

by the enzyme glutamate synthase (GOGAT, EC 1.4.1.14) that catalyses the reductive transfer of the amido group of glutamine to the α-keto position of 2-oxoglutarate, yielding two molecules of glutamate (Lancien et al. 2000).

Cadmium is a potent poison for all living cells. Several studies suggest that oxidative stress is involved in Cd^{2+} toxicity, by either inducing oxygen free radical production, or by decreasing enzymatic and non-enzymatic antioxidants (Benavides et al. 2005). Oxidative stress is a condition referred to as an imbalance between oxidant generation and antioxidant systems. As a consequence of this phenomenon, an enhanced amount of cellular oxidation products is formed (compared to physiological levels). Reactive oxygen species (ROS) generated as by-products of cellular metabolism or from environmental sources cause modifications to the amino acids of proteins (Nyström 2005). Oxidatively modified proteins can undergo chemical fragmentation or form aggregates because of covalent cross-linking reactions and increase surface hydrophobicity, leading to a loss of function (Berlett and Stadtman 1997).

Reduced glutathione (GSH), a reductant molecule that protects thiol groups of enzymes and react with singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·), plays a pivotal role in protecting the plants from ROS injury (Gratão et al. 2005). Not only elevated levels of GSH are correlated with environmental stress tolerance, but also enhanced GSH synthesis seems to be an intrinsic response of plants to stress (May et al. 1998). Adaptation to grow in the presence of Cd^{2+} was linked to the capacity to maintain high intracellular GSH concentration (Gallego et al. 2005).

The effect of different Cd^{2+} concentrations on soybean nodule metabolism has been extensively studied in our laboratory (Balestrasse et al. 2001, 2003, 2004, 2005). Cadmium causes oxidative damage and hence affects nitrogen fixation and assimilation in roots and nodules of soybean plants. More detailed studies of Cd^{2+} effect on soybean nodules should be necessary in order to understand the mechanisms of metal toxicity. In the present work, we study the relationship between the GS/GOGAT pathway inactivation and protein oxidation in soybean nodules subjected to Cd^{2+} stress condition. The protective effect of the soluble antioxidant GSH was also analyzed.

Materials and methods

Plant material and growing conditions

Seeds of soybean (*Glycine max* L. cv. AG 445-RG, Nidera) were surface sterilized with 5% v/v sodium hypochlorite solution (25 g l^{-1} available chlorine) for 10 min and washed several times with distilled water. Seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and planted in vermiculite. After germination at 5 day, plants were removed from pots, roots were gently washed and transferred to separate containers for hydroponics. Plants were germinated and grown in a controlled climate room at 24 ±;2°C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 μmol m^{-2} s^{-1}. The hydroponic medium was nitrogen-free Hoagland nutrient solution containing 1.5 mM KH_2PO_4, 0.8 mM $MgSO_4·7H_2O$, 2.5 mM KCl, 0.8 mM $CaCl_2·2H_2O$, 5 mM Fe–EDTA, 1 μM $MnSO_4·H_2O$, 1 μM $ZnSO_4·7H_2O$, 12.5 μM H_3BO_3, 0.25 μM H_2MoO_4 and 0.25 μM $CuSO_4·2H_2O$ (pH 5.8) (Hoagland and Arnon 1957). Medium was continuously aerated, and replaced every 3 days. After 4 weeks, plants were treated with nutrient solution devoid of Cd^{2+} and GSH (control), or containing 200 μM $CdCl_2$, 200 μM $CdCl_2$ plus 300 μM GSH or 300 μM GSH. After 48 h of treatment nodules were isolated and used for determinations. Each experiment was repeated three times and consisted of five replicates (*n*=15).

Determination of Cd content

Nodule tissues without and with Cd^{2+} addition, and with Cd^{2+} plus GSH were thoroughly rinsed four times with distilled water containing 10 mM Na–EDTA to eliminate the metal that could be superficially adsorbed. Plant material was dried at 80°C during 48 h, weighed and ground to a fine powder. Cadmium determinations were made on HNO_3:$HClO_4$ (3:1 v/v) digests by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300).

Enzyme determinations

Plant material (0.3 g) for GS determination was extracted in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 2 mM EDTA,

and homogenized at 4°C. Following centrifugation (15,000 × g) for 30 min at 4°C, GS activity was measured in an assay buffer consisting of 50 mM Tris–HCl (pH 7.5), 4 mM ATP, 80 mM Na–glutamate, 30 mM MgSO$_4$, 10 mM NH$_2$OH and 30 mM cysteine (Kanamori and Matsumoto 1972). A standard curve was made using γ-glutamyl hydroxamate.

Extracts for determination of GOGAT activity were prepared from 0.3 g of nodules homogenized in 3 ml of extraction buffer containing 100 mM MES-NaOH buffer (pH 6.8), 100 mM sucrose, 2% (v/v) 2-mercaptoethanol, and 15% (v/v) ethyleneglycol at 4°C. Homogenates were centrifuged at 10,000×g for 20 min (4°C) and the supernatant fraction was used for the assays. GOGAT was determined in the homogenates by measuring the decrease in absorption at 340 nm due to NADH oxidation, in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.6), 0.1% (v/v) 2-mercaptoethanol, 100 μM NADH, 2.5 mM 2-oxoglutarate and 100 mM glutamine (Groat and Vance 1981).

Western-blot analysis for GS and GOGAT

Proteins from nodules were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN 3 System (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed in 7.5 and 12% gels (4% stacking gels), for GOGAT and GS respectively, and run according to Laemmli (1970). The separated polypeptides were transferred to a nitrocellulose membrane at 250 mA constant for 2 h in a Mini-Trans-Blot Electrophoretic System (Bio-Rad) according to the manufacturer's instructions. The membranes were washed in 25 mM Tris–HCl (pH 8.5), 192 mM glycine and 20% (v/v) methanol. The blots were blocked by incubation for 2 h in 2% (w/v) powdered non-fat dry milk dissolved in Tris-buffered saline (TTBS), (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20) before reaction with the appropriate antibody. The membrane was incubated with rabbit antibody against barley leaf NADH–GOGAT for GOGAT (dilution 1:1000), or rabbit antibody against GS purified from *P. vulgaris* root nodules (Cullimore and Miflin 1984) for GS (dilution 1:1000). Dilution of antibodies was in TTBS with 2% (w/v) powdered non-fat dry milk for NADH-GOGAT and GS. The blots were washed two times for 10 min

each with TTBS. Goat anti-rabbit horseradish peroxidase conjugate was used as secondary antibody. The blots were washed two times for 10 min before color development (ECL immunodetection system, ECL Western Blotting protocols, Dako).

Carbonyl groups determination

Protein oxidation was measured as total carbonyl groups content by reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) (Levine et al. 1990). Extracts (50 μg of protein) derivatized with 2,4 DNPH were separated by SDS-PAGE in 12% (w/v) running and 4% (w/v) stacking polyacrylamide gels (Laemmli 1970). Two gels were run simultaneously: one for protein staining with Coomassie Brilliant Blue R-250 and the other for immunodetection. Derivatized proteins were transferred onto nitrocellulose membranes and were detected with rabbit anti-DNP primary antibody from Sigma-Aldrich (St Luis, USA). Bands corresponding to oxidized proteins were visualized by secondary goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase and using 3,3'-diaminobenzidine (DAB) as a substrate.

Immunoprecipitation and immunochemical detection of GS and GOGAT carbonyl groups

Proteins (100 μg) derivatized with DNPH as mentioned above, were separated by affinity chromatography. Antibodies anti-DNP (50 μl) were linked to cyanogen bromide activated sepharose 4% agarose matrix (100 mg) from Sigma-Aldrich (St Luis, USA). Samples were incubated overnight at 4°C with excess of anti-DNP-agarose resin and then centrifuged for 5 min at 10,000 × g and at 4°C. Resin beads were washed 3 times with Tris-buffered saline (TBS), pellets were re-suspended in 50 μl of 100 mM glycine (pH 2.5). After centrifugation, pellets were discarded, the pH of the supernatants was adjusted to 6.8 and used for immunodetection of GS and GOGAT. DNPH derivatized proteins were separated by 12.5% (w/v) SDS-PAGE. After electrotransfer of the proteins to nitrocellulose membranes, GS and GOGAT were detected as described above. In all cases, membranes were photographed with a Fotodyn equipment, and the intensity of bands was analyzed

with GelPro software and expressed as arbitrary units based on absolute integrated optical density.

Protein determination

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Values indicate mean values ± s.e. Differences among treatments were analyzed by one-way ANOVA, taking $P<0.05$ as significant according to Tukey's multiple range test.

Results

Nodule cadmium concentrations

Since Cd^{2+} and GSH were added to the nutrient solution together, there could be a likelihood that GSH and Cd^{2+} interact outside the plant decreasing Cd^{2+} bioavailability for uptake. In order to verify this possibility, nodule Cd concentrations were determined. As shown in Table 1, the presence of GSH did not affect the cadmium uptake, and the levels of Cd^{2+} were similar to those found when the plants were treated only with 200 μM of the metal ion.

Effect of cadmium on the nitrogen assimilation pathway

The behavior of the enzymes involved in the nitrogen assimilation (GS and GOGAT) in soybean plant nodules was studied. In plants subjected to 200 μM

Table 1 Cadmium concentrations in soybean nodules

Treatments	Cd concentration μg/g DW
Control	6.52 ± 0.52
Cd^{2+}	80.31 ± 0.52*
Cd^{2+}+GSH	78.20 ± 0.52*
GSH	5.52 ± 0.43

Nodule tissues were used for the assays. Experiments were carried out as described in Materials and methods. Data are the means ± S.E.M. of three different experiments with five replicated measurements. *Significant differences $P < 0.05$ according to Tukey's multiple range test

Cd^{2+} during 48 h, GS and GOGAT activities significantly decreased (17 and 52%, respectively, compared to controls). When the nutrient solution was supplemented with 300 μM GSH (with and without Cd^{2+}), the enzyme activities remained similar to the controls (Table 2).

To test whether modification of the GS and GOGAT activities in nodules induced by Cd^{2+} treatment were due to protein abundance modification, SDS-PAGE and Western blots were performed. No significant differences were observed either in GS or in GOGAT protein content in nodules subjected to Cd^{2+} treatment (Figs. 1, 2). Similarly, no effect with GSH addition was observed.

Effect of cadmium on soluble proteins and carbonyl groups content

The effect of Cd^{2+} on soybean nodule proteins and their carbonyl groups content was evaluated. Soybean plants were treated with 200 μM Cd^{2+}, 200 μM Cd^{2+} plus 300 μM GSH or 300 μM GSH for 48 h. Although Cd^{2+} treatment altered total soluble protein content in the nodule (data not shown), the extracts had similar soluble protein profile in SDS-PAGE staining with Coomassie Blue (Fig. 3).

In nodules, Cd^{2+} treatment significantly increased carbonyl groups content (34% compared to control plants). This enhancement was totally prevented when GSH was included in the nutrient solution (Fig. 4a). When immunodetection of carbonyl

Table 2 Effect of cadmium and GSH treatments on ammonium assimilation enzyme activities

Treatment	GS (U/mg protein)[a]	GOGAT (U/mg protein)[b]
Control	0.757 ± 0.019	0.046 ± 0.002
Cd^{2+}	0.638 ± 0.027**	0.022 ± 0.002*
Cd^{2+}+GSH	0.776 ± 0.005	0.055 ± 0.007
GSH	0.736 ± 0.015	0.054 ± 0.006

Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control) or containing 200 μM Cl_2Cd, 300 μM GSH or 200 μM Cl_2Cd plus 300 μM GSH in hydroponic medium for 48 h. Enzyme activities were assayed as described in Materials and methods. [a]One unit of GS forms 1 μmol of γ-glutamyl hydroxamate per min under the assay conditions. [b]One unit of GOGAT oxidizes 1 μmol of NADH per min under the assay conditions. Data are the means ± S.E.M. of three different experiments with five replicated measurements. Each value represents $n=15$. *Significant differences $P<0.05$, **Significant differences $P<0.01$ according to Tukey's multiple range test
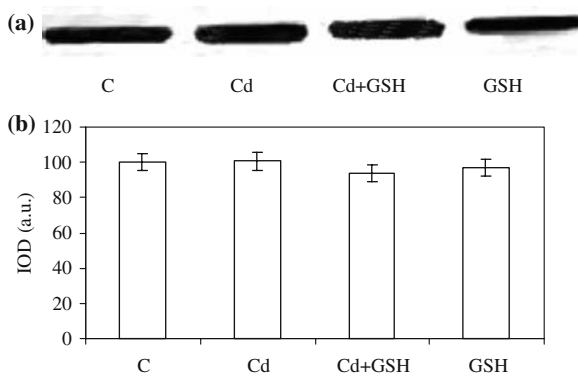
Fig. 1 Effect of cadmium and GSH treatments on GS protein abundance in soybean nodules. Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control), or containing 200 μM CdCl$_2$, 300 μM GSH or 200 μM CdCl$_2$ plus 300 μM GSH in hydroponic medium for 48 h. Proteins (40 μg per well) were separated by native-PAGE. (**a**) Western blot analysis of GS protein expression. (**b**) Densitometry was done to quantify GS protein expression, and expressed as arbitrary units (a.u.) based on absolute integrated optical density (IOD). The blot is representative of 3 blots with a total of 4–5 samples/group between the 3 blots
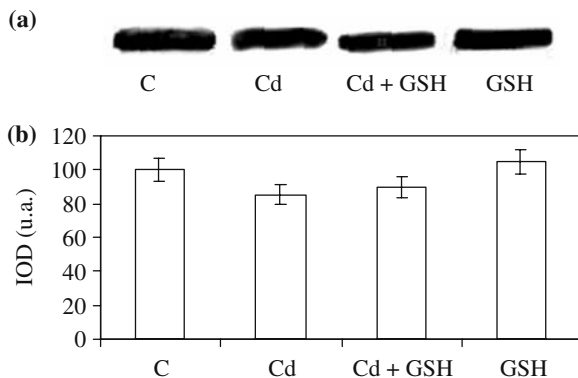

Fig. 2 Effect of cadmium and GSH treatments on GOGAT protein abundance in soybean nodules. Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control), or containing 200 μM CdCl$_2$, 300 μM GSH or 200 μM CdCl$_2$ plus 300 μM GSH in hydroponic medium for 48 h. Proteins (40 μg protein per well) were separated by native-PAGE. (**a**) Western blot analysis of GOGAT protein expression. (**b**) Densitometry was done to quantify GOGAT protein expression, and expressed as arbitrary units (a.u.) based on absolute integrated optical density (IOD). The blot is representative of 3 blots with a total of 4–5 samples/group between the 3 blots

residues was performed, the densitometric scanning was in agreement with the result obtained by spectrophotometric methods. Qualitative pattern of carbonylated proteins showed that Cd^{2+} ions increased

oxidation of proteins with molecular weight higher than 66 kDa (Fig. 4b).

GS and GOGAT protein oxidation

Taking into account the present results, the degree of GS and GOGAT oxidation was estimated. A significant oxidation of both proteins was observed (Fig. 5). This oxidation was completely prevented by GSH addition in the plant nutrient solution containing Cd^{2+}.

Discussion

Glutamine and glutamate synthetase are key enzymes in nitrogen metabolism of higher plants. GS and GOGAT form the GS/GOGAT cycle, considered the main pathway for the primary assimilation of ammonium (Lea and Miflin 2004; Suzuki and Knaff 2005). The present investigation reveals that during exposure to Cd^{2+}, GS and GOGAT activity levels were significantly diminished in soybean nodules. A decrease in these enzyme activities was observed in other species, such as maize (Boussama et al. 1999; Astolfi et al. 2004), pea (Chugh et al. 1992), bean (Gouia et al. 2000) and rice (Kumar and Dubey 1999; Chien et al. 2002), and reflects a general inhibition of primary nitrogen assimilation and even of the overall metabolic activity of the cell, because GS and GOGAT are also involved in recycling ammonium produced by photorespiration. But the mechanism of Cd^{2+} toxicity remained still unclear and no information about nodule response was available.

Cadmium is a powerful enzyme inhibitor (Das et al. 1997) and it has been demonstrated that it causes oxidative stress in plants (Benavides et al. 2005). Our previous results showed that Cd^{2+} produces oxidative stress and deleterious effects on the antioxidant defense system in soybean nodules (Balestrasse et al. 2001). In nodules exposed to Cd^{2+}, GS and GOGAT activities decreased, however neither decrease in the protein abundance nor fragmentation was observed. Although no loss of band staining in both enzymes in SDS-PAGE was found, GS and GOGAT protein oxidation were produced. Previous studies (Levine et al. 1981) suggested that
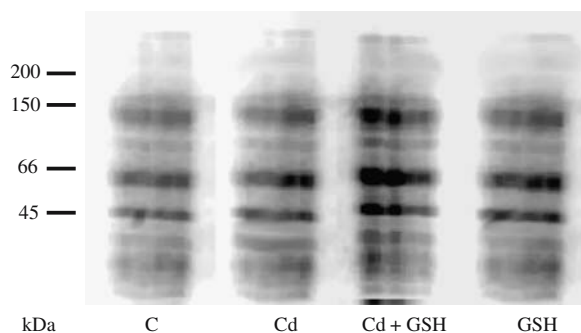
Fig. 3 Effect of cadmium and GSH treatments on total proteins in soybean nodules. Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control), or containing 200 μM CdCl$_2$, 300 μM GSH or 200 μM CdCl$_2$ plus 300 μM GSH in hydroponic medium for 48 h. Proteins (50 μg) were subjected to SDS-PAGE (12% w/v polyacrylamide) and stained with Coomassie Brilliant Blue R-250. The electrophoresis data shown are representative of two independent experiments with three replicates

the turnover of GS from *E. coli* occur in two steps. Oxidative modification of GS was implicated as the first step in the turnover of this enzyme in bacteria. Glutamine synthetase is oxidized, resulting in inactivation, loss of at least one histidine, and the generation of one or more carbonyl groups. In the second step, oxidized GS is degraded by specific proteases (Levine 1983; Rivett and Levine 1990). In the chloroplast, the stromal GS appears to be highly labile to ROS attack, because GS degradation in illuminated chloroplasts requires the function of the photosynthetic electron transport chain (Stieger and Feller 1997). Chloroplastic GS of wheat seedlings has been reported to be particularly prone to degradation under oxidative stress conditions (Palatnik et al. 1999). Moreover, by incubating of soybean root extracts enriched in GS, in a metal-catalyzed oxidation system to produce the hydroxyl radical, Ortega et al. (1999) have shown that GS was oxidized. Reactive oxygen species react with proteins and generate oxidation products such as carbonyl groups on the molecule (Chao et al. 1997). In barley leaves (*Hordeum vulgare* L.), GS decreased and GOGAT was absent under a high Cu concentration. The development of toxicity symptoms corresponded to an accumulation of Cu in the leaves and to a gradual increase in protein carbonylation (Demirevska-Kepova et al. 2004). Although Cd^{2+} is a redox inactive metal, it caused accumulation of oxidatively damaged proteins in
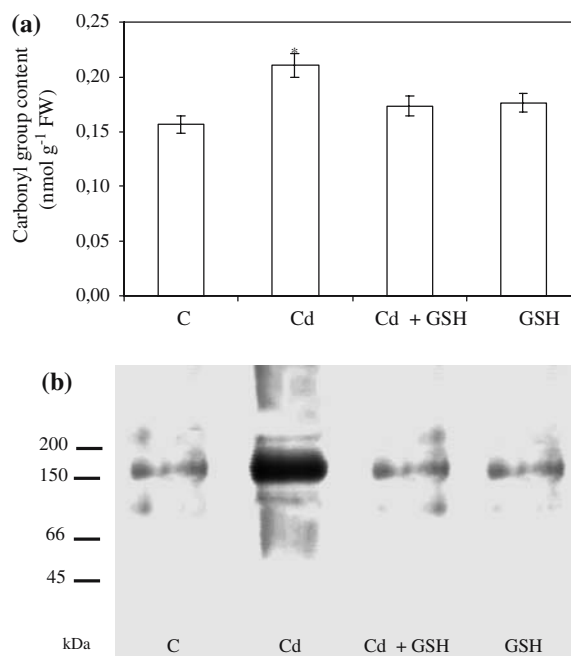

Fig. 4 Effect of cadmium and GSH treatments on proteins oxidation. Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control), or containing 200 μM CdCl$_2$, 300 μM GSH or 200 μM CdCl$_2$ plus 300 μM GSH in hydroponic medium for 48 h. (**a**) Total carbonyl groups content. Values are means ± s.e ($n=15$). * Significant differences $P<0.05$ according Tukeýs multiple range test. (**b**) The DNPH-derivatized proteins (50 μg of total protein) were subjected to a SDS-PAGE (12.5% w/v polyacrylamide). Western blotting was performed using anti-DNP antibody and bands were visualized by densitometry as described Material and methods. The positions of molecular mass markers (in kDa) are shown on the left. The blot is representative of 3 blots with a total of 4–5 samples/group between the 3 blots

soybean nodules, as it was observed in other plant species such as pea plants (Romero-Puertas et al. 2002). Cadmium exposure resulted in oxidation of high molecular weight nodule proteins, which included GS and GOGAT. GS of bacteroids from root nodules of *Glycine max* is a large protein of about Mr 760,000 consisting of 12 apparently identical Mr 63,000 subunits (Bhandari and Nicholas 1986). GOGAT of higher plants and alfalfa nodules is a monomer of about Mr 200,000 (Anderson et al. 1989). Besides, Ortega et al. (1999) have reported a dramatic increase in the level of the oxidized forms of GS subunits, suggesting that the oxidative modification may be an intermediate step in the turnover of GS in vivo. This fact, could be explaining, at least in
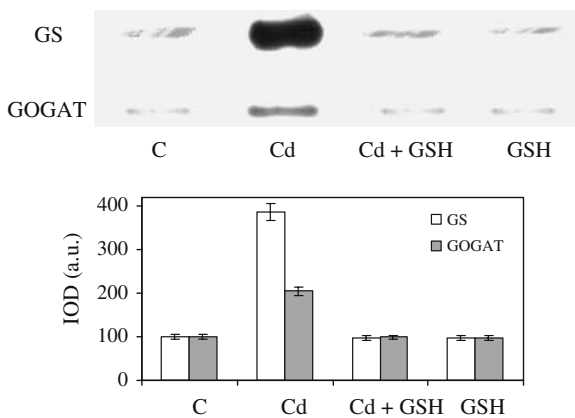
Fig. 5 Identification of GS and GOGAT oxidized proteins. Soybean (*Glycine max* L.) nodulated plants were treated with nutrient solution devoid of cadmium and glutathione (control), or containing 200 μM CdCl$_2$, 300 μM GSH or 200 μM CdCl$_2$ plus 300 μM GSH in hydroponic medium for 48 h. Nodule extracts reacted with 2,4 DNPH and derivatized proteins were immunoseparated with antibody anti-DNP, the DNPH-derivatized proteins (50 μg of total protein) were subjected to SDS-PAGE (12.5% w/v polyacrylamide). Western blotting was performed using anti-GS and anti-GOGAT antibodies. Bands were visualized by densitometry as described in Material and methods. The blot is representative of 3 blots with a total of 4–5 samples/group between the 3 blots

part, our results, which demonstrated the higher susceptibility to oxidation of GS as compared to GOGAT.

Protein carbonylation is an irreversible oxidative process and it would be expected to contribute to inhibition or the impairment of multiple enzymes, thus affecting cellular functions ranging from protein synthesis, energy production and cytoskeleton dynamics to signal transduction (Sohal 2002; Sohal et al. 2002). In the present study, the antioxidant glutathione protected the nodule against the deleterious effect of the metal. Reduced glutathione is the predominant non-protein thiol, redox-buffer, phytochelatin precursor and substrate for keeping the ascorbate in reduced form in the ascorbate–glutathione pathway (Gratão et al., 2005). Addition of GSH avoided accumulation of carbonylated proteins and hence prevented the oxidation of GS and GOGAT protein by Cd^{2+} treatment.

The protective effect of external GSH supply is apparently not due to interference with Cd uptake into the nodules. Therefore, it is likely that it is attributable to an elevated cellular GSH content in the nodules, due to uptake from the nutrient solu-

tion. Elevated cellular levels of GSH, which is the low-affinity substrate for phytochelatin synthase (Vatamaniuk et al. 2000), have been shown to enhance phytochelatin-based Cd sequestration and tolerance in Indian mustard and tobacco (Zhu et al. 1999; Pomponi et al. 2006). Alternatively, the enzyme protection might also directly result from the antioxidant activity of GSH as such, at least in part.

Although increments in protein carbonyl groups content and the inactivation of the nitrogen assimilation process in plants exposed to Cd^{2+} was previously described, there is no information about the possible mechanisms involved. The present study demonstrates that the decrease in activities of both enzymes of the nitrogen assimilation pathway, GS and GOGAT, was due to an oxidation of the proteins, more than a modification of the protein expression. In addition, these findings reinforce the notion that glutathione is a very important antioxidant in nodules, preventing the oxidation of proteins and protecting the nitrogen assimilation pathway.

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