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Purification of Multiple Forms of Glutathione Reductase from Pea (*Pisum sativum* L.) Seedlings and Enzyme Levels in Ozone-Fumigated Pea Leaves[1]

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ABSTRACT

Glutathione reductase was purified from pea seedlings using a procedure that included 2′,5′-ADP Sepharose, fast protein liquid chromatography (FPLC)-anion exchange, and FPLC-hydrophobic interaction chromatography. The purified glutathione reductase was resolved into six isoforms by chromatofocusing. The isoform eluting with an isoelectric point of 4.9 accounted for 18% of the total activity. The five isoforms with isoelectric points between 4.1 and 4.8 accounted for 82% of the activity. Purified glutathione reductase from isolated, intact chloroplasts also resolved into six isoforms after chromatofocusing. The isoform eluting at pH 4.9 constituted a minor fraction of the total activity. By comparing the chromatofocusing profile of the seedling extract with that of the chloroplast extract, we inferred that the least acidic isoform was extraplastidic and that the five isoforms eluting from pH 4.1 to 4.8 were plastidic. Both the plastidic (five isoforms were pooled) and extraplastidic glutathione reductases had a native molecular mass of 114 kD. The plastidic glutathione reductase is a homodimer with a subunit molecular mass of 55 kD. Both glutathione reductases had optimum activity at pH 7.8. The K_m for the oxidized form of glutathione (GSSG) was 56.0 and 33.8 μm for plastidic and extraplastidic glutathione reductase, respectively, at 25°C. The K_m for NADPH was 4.8 and 4.0 μm for plastidic and extraplastidic isoforms, respectively. Antiserum raised against the plastidic glutathione reductase recognized a 55-kD polypeptide from purified antigen on western blots. In addition to the 55-kD polypeptide, another 36-kD polypeptide appeared on western blots of leaf crude extracts and the purified extraplastidic isoform. The lower molecular mass polypeptide might represent GSSG-independent enzyme activity observed on activity-staining gels of crude extracts or a protein that has an epitope similar to that in glutathione reductase. Fumigation with 75 nL L^{-1} ozone for 4 h on 2 consecutive days had no significant effect on glutathione reductase activity in peas (*Pisum sativum* L.). However, immunoblotting showed a greater level of glutathione reductase protein in extracts from ozone-fumigated plants compared with that in control plants at the time when the target concentration was first reached, approximately 40 min from the start of the fumigation, and 4 h on the first day of fumigation.

Reduced GSH is the major nonprotein sulfhydryl compound in all living organisms, including plants. GSH content and metabolism in plants are affected by developmental and environmental conditions (29). It has been implicated in numerous metabolic processes (27), of which its action as an antioxidant is particularly important (16). Several studies have documented the accumulation of GSH in plants exposed to oxidative stress conditions (7, 9, 22, 25, 31).

The enzyme GR[2] (NAD(P)H:oxidized-GSH oxidoreductase, EC 1.6.4.2) catalyzes the reduction of GSSG to GSH and maintains a high cellular GSH/GSSG ratio (17). Under normal conditions, GSH and GR are involved in the detoxification of H_2O_2 generated in the light by the Mehler reaction in chloroplasts. Cytoplasmic forms of GR have also been identified from mustard cotyledons and pea (*Pisum sativum* L.) leaves (10, 11). Increased GR levels have been correlated with tolerance to low temperature stress (9, 15), drought (14), air pollutants (22, 26, 30), and oxygen-enriched air (13). Recently, Kunert et al. (20) have reported that in *sodB* mutant *Escherichia coli* lacking iron superoxide dismutase activity, overproduction of GR provided partial protection against methylviologen. Neither GSH content nor the ratio of GSH/GSSG changed in *sodB* mutants, suggesting the possibility of GR reacting directly with toxic oxygen species.

GR has been purified from chloroplasts (4, 8, 19, 23, 28), leaf homogenates (17, 32), roots (4), and seeds (18, 24). Multiple forms of GR were found in several plant species. Bielawski and Joy (4) have reported differences in the isozymes present in roots and chloroplasts. Cold-hardened spinach had increased GR activity and additional isozymes with altered kinetic behavior when compared with nonhardened tissue (15). In situ photooxidation of plastids was used to distinguish plastidic and cytosolic isoforms of GR in mustard cotyledons (10). The cytosolic and plastidic isoforms were also differentially regulated by phytochrome. Purified GR from pea leaves was resolved into eight isoforms with pI values from 6.5 to 5.2 on two-dimensional gels (11). Cell fractionation and organelle purification methods revealed that five chloroplastic isoforms have pI values between 5.6

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[2] Abbreviation: GR, glutathione reductase; pI, isoelectric point; FPLC, fast protein liquid chromatography; CSTR, continuously stirred tank reactor; 0 (t), time when target concentration was first reached, approximately 40 min from the start of fumigation.

and 6.3, whereas the pI values of three mitochondrial forms are between 6.3 and 6.5.

We report here the purification and characterization of multiple forms of GR from pea seedlings using the technique of chromatofocusing. We are interested in understanding the role of GR in protection against air pollution stress and have examined the activity and protein levels of GR in pea seedlings after fumigation with ozone.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* L. cv Progress No. 9) plants were grown in pots of Pro-Mix BX (Primier Brands Inc., Stamford, CT) in a greenhouse at 25/20°C (day/night) under a 16-h photoperiod. The plants were watered daily and were fertilized once with Peter's Plant soluble food (20–20–20 N:P:K) when they were 10 d old. Two 3-week-old plants were used in all enzyme purification experiments.

Enzyme Purification

FPLC was conducted at room temperature and all other steps in purification were performed at 4°C.

Pea shoot and leaf tissue (1 kg) were ground to a fine powder in liquid N_2 using a prechilled mortar and pestle. The powder was homogenized in 2 L of 50 mM Pipes buffer (pH 6.8), 1 mM EDTA, 6 mM L-cysteine hydrochloride, 0.3% Triton-X 100, 1% PVP, mol wt 10,000, and 1% polyvinylpolypyrrolidone using a Polytron homogenizer (Brinkman Instruments Co., Upton, NY) at maximum speed for 3 min in 30-s intervals. The homogenate was filtered through eight layers of cheesecloth. The residue was reextracted with the homogenization buffer. The filtrates were pooled and centrifuged for 30 min at 22,000*g* in a fixed angle rotor (Beckman JA-14).

The supernatant was brought to 35% saturation with ammoniun sulfate and stirred for 30 min. After centrifugation at 22,000*g* for 30 min, the supernatant was brought to 80% saturation with ammonium sulfate and stirred for 1 h. The solution was centrifuged at 22,000*g* for 30 min and the precipitate dissolved in buffer A (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1 mM DTT). The solution was made 1 M with respect to ammonium sulfate and stirred for 15 min. The solution was centrifuged at 22,000*g* for 20 min and the residue was discarded. The supernatant was brought to 3 M saturation with ammonium sulfate. The precipitate was removed by centrifugation at 22,000*g* for 20 min and resuspended in buffer A. The solution was dialyzed against two changes of buffer A.

After dialysis, the sample was applied to a column of 2′,5′-ADP-Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Anderson et al. (2). Affinity-bound GR fractions were pooled and dialyzed against buffer B (20 mM Tris-HCl [pH 7.5], 1 mM EDTA). The sample was filtered through a 0.45-μm acrodisc (Gelman Sciences Inc., Ann Arbor, MI) and chromatographed on an anion-exchange column (Mono Q HR 5/5, Pharmacia) as described by Anderson et al. (2).

The fractions containing GR activity were pooled and made 1.5 M with respect to ammonium sulfate. The sample was applied to a hydrophobic interaction column (Phenyl-Superose HR 5/5, Pharmacia) equilibrated with 1.5 M ammonium sulfate in buffer A (buffer C). Fractions containing GR activity were pooled, dialyzed against buffer B, and applied to 2′,5′-ADP Sepharose 4B equilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted with a linear gradient of 1 to 10 mM $NADP^+$. The fractions containing GR activity were pooled and applied to a chromatofocusing column (Mono P HR 5/20, Pharmacia), which separates molecules on the basis of their pI. The column was injected with 1 mL of 5 M NaOH and equilibrated with start buffer, 0.025 M bis-Tris, pH 5.5. The sample was eluted in a pH gradient formed by eluting with Polybuffer 74 (Pharmacia), pH 4.0 (1:7, v/v). The flow rate was 1 mL/min and 1-mL fractions were collected. The factions were assayed for activity and pH.

Protein Determination

Protein was determined by the method of Bradford (6) using lyophilized BSA as a standard.

Preparation of Intact Chloroplasts

Protoplasts were isolated following the procedure of Alscher and Strick (1). Chloroplasts were released by passing the protoplast through a 30-μm nylon mesh attached to a 1-mL syringe. Chloroplasts were further purified by centrifugation through 40% Percoll. Intact chloroplasts were identified by measuring uncoupled ferricyanide-dependent oxygen evolution. Preparations used in the experiment were approximately 90% intact.

Purification of GR from Chloroplasts

Chloroplasts were extracted for GR purification as described for the leaf with a Polytron for 1 min. The purification steps included affinity chromatography using 2′,5′-ADP Sepharose, FPLC anion-exchange chromatography, and the purified enzyme was resolved into isoforms by FPLC chromatofocusing. The chromatofocusing profile of chloroplast GR was compared with the chromatofocusing profile of pea seedling extract.

Preparation of the Antiserum

After comparing the chromatofocusing profiles of pea seedling extracts with those of chloroplast extracts, fractions 52 through 70 in seedling extract were determined to be the plastidic form and fractions 47 through 51 were determined to be the extraplastidic form. Antibodies to purified plastidic GR (390 μg protein) were generated by Cocalico Biologicals (Reamstown, PA). The rabbit was given booster injections at 14 and 21 d and serum was collected at 6 and 10 weeks after the initial injection.

Gel Electrophoresis and Western Blotting

SDS-PAGE (10 and 12% gels) and native-PAGE (7.5% gels) were run using the discontinuous buffer system of Laemmli (21). Proteins were visualized on gels using silver

stain (Bio-Rad, Richmond, CA). Low-range (14.4–97.4 kD) molecular mass standards (Bio-Rad) were used as protein markers. Western blotting was performed as described by Anderson et al. (2). Immunodetection was carried out by probing the blots with antiserum of plastidic GR and using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad). Color development was performed using 100 mL of PBS containing 2.8 mM 4-chloro-1-naphthol (Bio-Rad) and 0.3% H_2O_2. Antiserum for plastidic GR was used at a dilution of 1:2000 (v/v).

GR activity was detected on native PAGE gels in 100 mL of Tris (pH 7.5) containing 10 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, 0.4 mM NADPH. Gels were developed for GR activity with and without GSSG.

Enzyme Assays

GR assays were carried out using a Beckman DU 65 spectrophotometer equipped with a Peltier temperature controller by monitoring GSSG-dependent NADPH oxidation at 340 nm. Assays were performed at 25°C in a 1-mL reaction mixture of 50 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 150 μM NADPH, and 500 μM GSSG.

The K_m values of chloroplastic and extraplastidic GR for GSSG and NADPH were each determined in the presence of a saturating concentration of the other substrate (150 μM NADPH and 500 μM GSSG). Limiting substrate concentrations varied between 2 and 100 μM for NADPH and 5 and 200 μM for GSSG. Each reaction was conducted using 127 ng of purified plastidic GR and 277 ng of extraplastidic GR. Duplicate assays were run for each concentration and data were analyzed by iterative fitting of the reaction rates to the rate equation using a computer program (PENNZYME, University of Pennsylvania).

pH Optima

The pH optimum was determined for both purified chloroplastic and extraplastidic GR at pH 5.5 to 6.5 using 50 mM Mes, pH 6.0 to 7.0 using 50 mM bis-Tris, pH 7.0 to 8.0 using Hepes, pH 7.5 to 9.3 using 50 mM Tris buffer. Each reaction was conducted using 127 ng of purified plastidic GR and 277 ng of extraplastidic GR.

Molecular Mass Determination

The mass of both native plastidic and extraplastidic enzymes was determined by gel filtration chromatography using a 1.5 × 30 cm Superose 6 FPLC column. The column was precalibrated with gel filtration molecular mass standards (Bio-Rad). The subunit molecular mass of plastidic GR was determined by SDS-PAGE.

Ozone Fumigation, Enzyme Extraction, and Western Blotting

For ozone fumigation studies, pea seeds were planted and grown in "cone-tainers" (Cone-Tainer Nursery, Canby, OR) containing Pro-Mix BX in a growth chamber at 25/20°C (day/night) under a 16-h photoperiod (approximately 300 μE m^{-2} s^{-1} PAR at plant level). The seedlings were watered and fertilized as described previously. Three-week-old plants were fumigated with ozone for 4 h on each of 2 consecutive days. Seedlings were acclimatized by placing in CSTR chambers 3 d before exposure to ozone. A 16-h photoperiod (approximately 400 μE m^{-2} s^{-1} PAR at plant level) was supplied with a mercury vapor lamp. Temperature in the chambers was 20°C at night and was between 24 and 27°C during the day. RH was maintained between 50 and 70%. Stomatal conductance was measured with LI-6200 portable photosynthesis system (Li-Cor, Lincoln, NE) to ensure that ozone entered into the leaves. Ozone was generated from oxygen by UV irradiation (Welsbach Laboratory Ozonator model T-408, Welsbach Ozone Systems Corporation, Philadelphia, PA) and monitored with a Teco UV Ozone Analyzer (model 49s, Thermo Electron Corporation, Hopkinton, MA) calibrated with a photocal 3000 Automated Ozone Calibrator (Columbia Scientific Industries, Austin, TX). Fumigation began 7 h after the onset of illumination and lasted 4 h after the target concentration (75 nL L^{-1}) was attained [0 (t) = time when target concentration was first reached, approximately 40 min from the start of the fumigation]. Ozone concentration within the CSTR chambers was controlled by a computerized system. Control pea seedlings were placed in CSTR chambers in similar conditions without ozone. The experiments were repeated twice and three plants were collected for each time period in each experiment.

Leaf tissue (0.2 g) was homogenized in 1.5 mL of 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 17,000g for 10 min and the supernatant was used for enzyme assays. GR assays and western blotting were carried out as described above.

RESULTS

Protein Purification

GR was purified both from pea seedling extract (Table I) and from isolated intact chloroplasts. Purified GR from pea seedling extract was resolved into six distinct isoforms by chromatofocusing (Fig. 1A). Purified GR from isolated intact chloroplasts revealed an isoform profile similar to that of the seedling extract (Fig. 1B) upon chromatofocusing. The least acidic isoform of chloroplast extract was a minor constituent

Table I. *Purification of GR from Pea Seedlings*

Purification Step	Specific Activity	Total Activity	Purification Factor	Recovery
	units/mg protein	*units*		%
3 M $(NH_4)_2SO_4$, dialyzed	0.600	1370	1	
2′,5′-ADP Sepharose	26	1300	43	95
Mono Q, pH 7.5	56	1280	93	93
Phenyl Superose	243	790	405	58
Affinity NADP-gradient Mono P	256	700	426	51
Plastidic GR (fractions 52–70)	400	200		15
Extraplastidic GR (fractions 47–51)	165	46		3

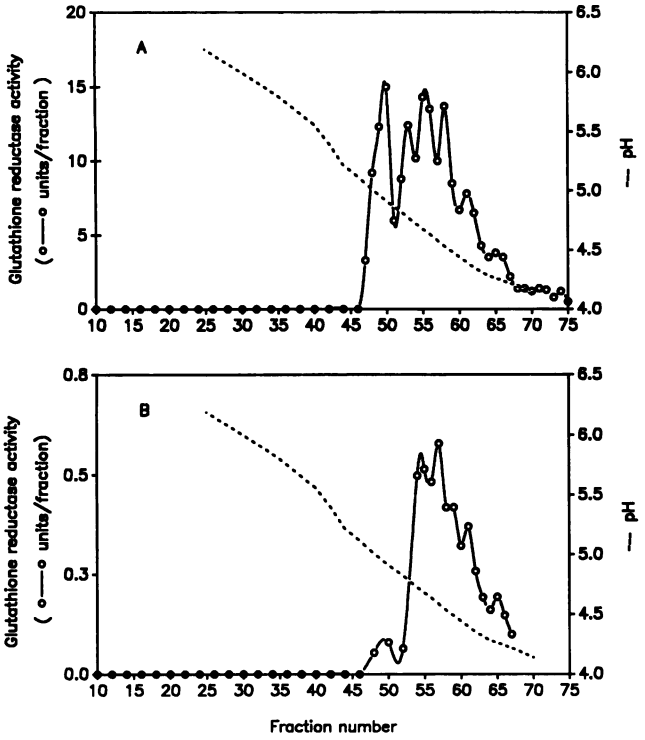

Figure 1. Elution profile of purified GR activity from pea seedling extract and from isolated chloroplast extract on Mono P HR 5/20 (Pharmacia). A, Elution profile of purified GR from pea seedling extract. The Mono P column was injected with 1 mL of 5 M NaOH and equilibrated with 25 mM bis-Tris, pH 5.5. A 4-mL sample of GR eluted from the 2',5'-ADP Sepharose 4 B column was applied to the column and eluted with Polybuffer 74, pH 4.0 (1:7 dilution, v/v). The flow rate was 1 mL/min and 1-mL fractions were collected and assayed for GR activity (O) and pH (–). B, Elution profile of GR from Percoll-purified pea chloroplasts. A 4-mL sample of GR eluted from Mono Q HR 5/5 column was applied to the Mono P column and eluted as described above.

(approximately 5%) of the total activity and the more acidic five isoforms were the most abundant ones. By comparing the isoform profiles of seedling extract with isolated chloroplast extract, we determined that the five more acidic isoforms were plastidic and the least acidic isoform was extraplastidic. The extraplastidic isoform in isolated, intact chloroplasts could be a contamination by the enzyme adhering to the chloroplast envelope. The putative plastidic isoforms (fractions 52–70) had pI values ranging from 4.1 to 4.8 (Fig. 1A) and the extraplastidic isoform (fractions 47–51) had a pI value of 4.9 (Fig. 1B). On native-PAGE gels stained for GR activity, the putative isoforms from the seedling extract (Fig. 2A) and the chloroplast extract (Fig. 2B) migrated as separate entities supporting their distinct identity. Pooled fractions of the putative plastidic forms and the extraplastidic form were used for further characterization.

Purified plastidic and extraplastidic GR had a specific activity of 400 and 165 units mg^{-1} protein, respectively (Table I). Affinity chromatography with 2',5'-ADP-Sepharose 4B resulted in 43-fold purification of GR and 95% of the protein containing GR activity was eluted with a 5-mL pulse of 10 mM $NADP^+$. Anion exchange with a Mono Q column yielded 93% of the recovery of the protein with GR activity and 93-fold purification. Further purification with Phenyl-Superose yielded 58% of GR protein and 405-fold purification. When fractions containing GR activity were pooled and reapplied to 2',5'-ADP-Sepharose and eluted with 1 to 10 mM $NADP^+$ gradient, the purification factor and percentage recovery were 426 and 51, respectively. Approximately 18% of the enzyme activity was recovered after chromatofocusing with a Mono P HR 5/20 column. The putative plastidic form accounted for approximately 82% and the extraplastidic form 18% of total activity recovered in this purification of GR from pea seedlings.

Molecular Mass Determination

The native molecular mass determined by Superose-6 chromatography was approximately 114 kD for both plastidic and extraplastidic forms. The subunit molecular mass as

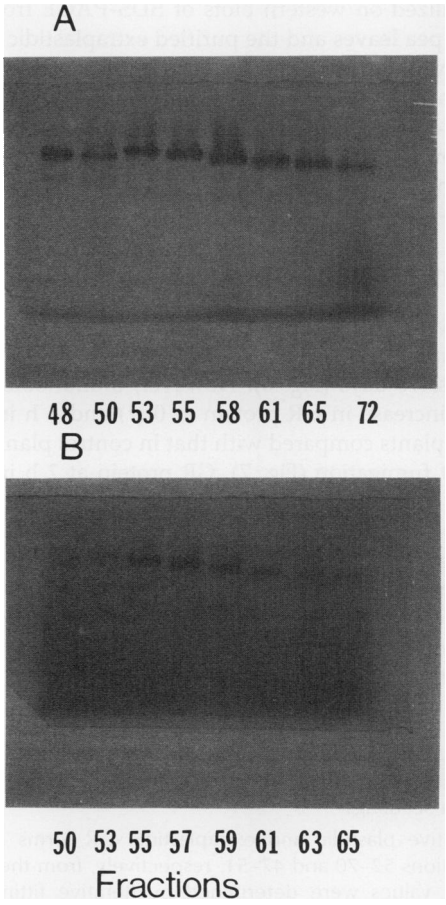

Figure 2. A, GR activity stain on 7.5% native-PAGE gels for fractions eluted from Mono P column. Fractions 47–51 represent the putative extraplastidic GR form and fractions 52–70 represent the putative plastidic GR form. Pea seedling extract; fractions 48, 50, 53, 55, 58, 61, 65, and 72 represent 125, 100, 125, 150, 250, 200, 150, and 75 ng protein, respectively. B, Percoll-purified chloroplast extract; fractions 50, 53, 55, 57, 59, 61, 63, and 65 represent 50, 50, 100, 125, 125, 100, 75, and 75 ng protein, respectively.

determined by SDS-PAGE was approximately 55 kD for the plastidic form, suggesting that the protein is a dimer composed of two identical subunits with several isoforms distinguishable by charge (Fig. 2).

Kinetic Parameters

The kinetic properties of the plastidic and extraplastidic forms appear to differ with respect to GSSG (Table II). The plastidic form had a K_m of 56 μM, whereas the extraplastidic form had a K_m of 33.8 μM for GSSG. For NADPH, the plastidic form had a K_m of 4.8 μM and the extraplastidic form had a K_m of 4.0 μM. Both plastidic and extraplastidic forms had a pH optimum of 7.6 with 50% of the activity ranging between 6.4 and 8.6.

Immunological Characterization

Antiserum made against plastidic GR recognized a single band with an apparent molecular mass of 55 kD from purified antigen on western blots of SDS-PAGE (Fig. 3). Two bands were visualized on western blots of SDS-PAGE from crude extracts of pea leaves and the purified extraplastidic GR (Fig. 4). Activity stains of native-PAGE gels using leaf crude extracts (data not shown) and chloroplast crude extracts (Fig. 5) showed one band of GSSG-dependent and a second band of GSSG-independent enzyme activity. It is possible that the second band that appears on the western blots is related to this GSSG-independent enzyme activity.

Effects of Ozone Fumigation

GR activity did not change significantly in 3-week-old plants after fumigation with 75 nL L^{-1} ozone for 4 h on each of 2 successive days (Fig. 6). However, immunoblotting revealed an increase in GR protein at 0 (t) and 4 h in ozone-fumigated plants compared with that in control plants on the first day of fumigation (Fig. 7). GR protein at 2 h in ozone-fumigated plants was similar to that in control plants. Interestingly, a similar pattern of change was observed in the lower molecular mass protein that cross-reacts with plastidic GR antiserum. Immunoblotting also revealed a cyclical change in GR protein in both control and ozone-fumigated plants. Ozone fumigation had no significant effect on stomatal conductance (data not shown).

Table II. *K_m Values of Purified Putative Plastidic and Extraplastidic GR from Pea Seedlings*

The putative plastidic and extraplastidic GR forms represent pooled fractions 52–70 and 47–51, respectively, from the Mono P column. K_m values were determined by iterative fitting of the reaction rates to the rate equation using Pennzyme (University of Pennsylvania).

Substrate	K_m	
	Plastidic GR	Extraplastidic GR
	μM	
GSSG	56.0	33.8
NADPH	4.8	4.0

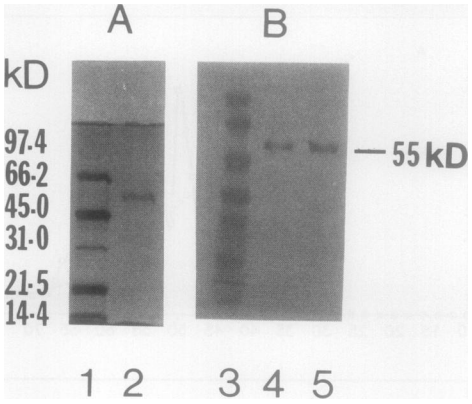

Figure 3. A, Electrophoretic separation of putative plastidic GR from pea seedling extract. Silver staining of 12% SDS-PAGE gel. Lane 1, SDS-PAGE low molecular mass standards; lane 2, 150 ng of purified putative plastidic GR. B, Western blot (10% SDS-PAGE gel) of putative plastidic GR from pea probed with antiserum raised against purified plastidic GR. Lane 1, Prestained SDS-PAGE low molecular mass standards; lanes 2 and 3, 170 and 340 ng of purified putative plastidic GR, respectively.

DISCUSSION

The protein purification procedure with chromatofocusing as the final step resolved six isoforms of GR from pea seedling extracts. Five plastidic isoforms have pI values between 4.1 and 4.8 and the extraplastidic isoform had a pI value of 4.9. Both the chromatofocusing profiles and migration pattern of five plastidic isoforms on activity-stained gels of purified GR from pea seedling extract and Percoll-purified chloroplasts were similar. Edwards et al. (11) also reported the presence of five chloroplastic GRs from isolated and Percoll-purified chloroplasts. However, their pI values are 1.5 pH higher than those reported in the present study. Chromatofocusing also enabled the resolution of an extraplastidic form with a pI higher than plastidic isoforms that may be the cytosolic form.

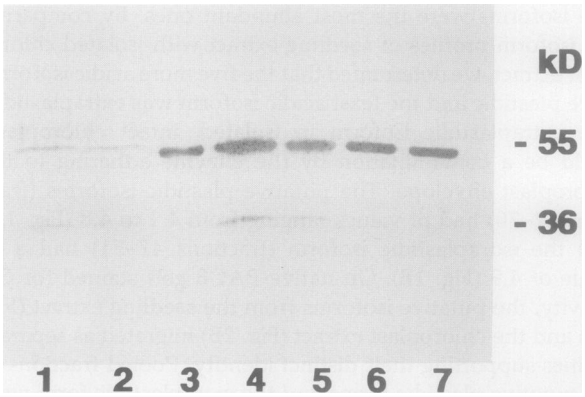

Figure 4. Western blot (10% SDS-PAGE gel) probed with antiserum raised against purified putative plastidic GR from pea. Lanes 1 and 2, Eighteen and 13.0 μg of protein of crude leaf extract, respectively; lanes 3, 6, and 7, 0.5, 1.5, and 1.0 μg of purified putative plastidic GR, respectively; lanes 4 and 5, 2.5 and 2.0 μg of purified putative extraplastidic GR, respectively.

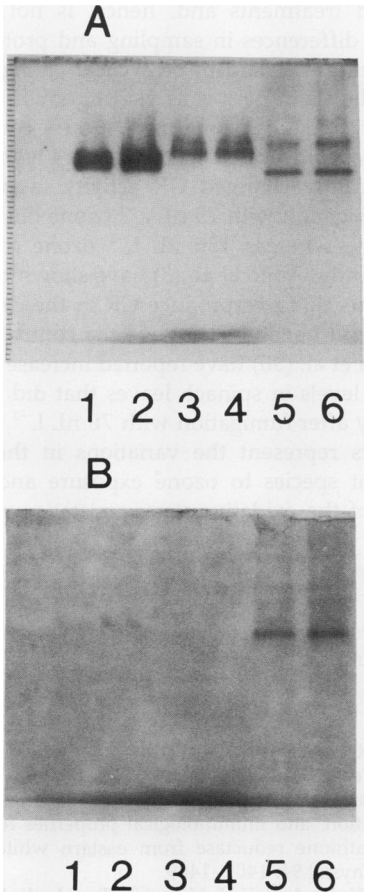

Figure 5. Activity staining of purified GR from pea seedling extracts and GR from crude extracts of isolated chloroplasts on 7.5% native-PAGE gels. A, Activity staining in the presence of GSSG. B, Activity staining in the absence of GSSG. Lanes 1 and 2, Ten and 20 ng of putative plastidic GR, respectively; lanes 3 and 4, 10 and 20 ng of putative extraplastidic GR, respectively; lanes 5 and 6, 35 and 70 ng of chloroplast extract, respectively.

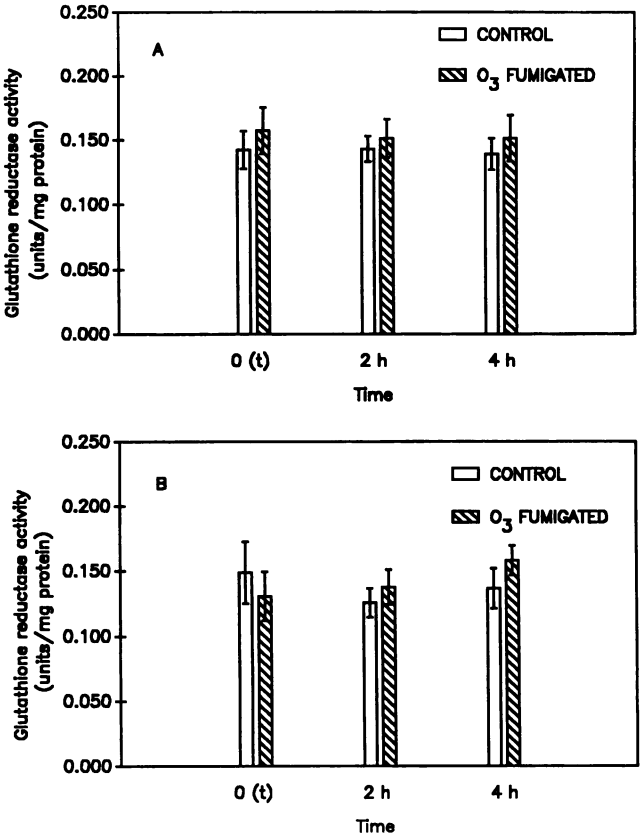

Figure 6. GR activity in peas fumigated with 75 nL L^{-1} of ozone. A, First day of fumigation; B, second day of fumigation. Values represent the mean ± SE of three replicates from each of two experiments.

In contrast with this result, Edwards et al. (11) reported that the cytoplasmic forms in crude extracts had lower pI values than chloroplastic forms. We did not detect GR activity eluting with a pI of greater than 6.0, which should specify the mitochondrial form (11). However, there may be multiple isoforms eluting with the extraplastidic GR in our study (Fig. 1A). Because the mitochondrial enzyme activity contributes only 2 to 3% of the total activity, our procedures would not have detected this activity.

The six isoforms observed by chromatofocusing are distinguishable on the basis of their pI values but not on the basis of molecular mass. This property is consistent with the report of multiple isoforms of GR observed on two-dimensional gels of pea leaf extract (11). Chromatofocusing yields sufficient protein to characterize the individual isoforms. The existence of multiple isoforms distinguishable by their pI values has been reported for other plant enzymes (5, 33). The differences in the charge of isoforms could be due to posttranslational processing of a single gene product (12). Edwards et al. (11) have ruled out glycosylation and phosphorylation, the common types of posttranslational modifications, as the factors contributing to the presence of multiple isoforms of GR in peas. They also concluded that neither oxidation reduction nor carboxymethylation are involved in the occurrence of multiple GR isoforms. The multiple isoforms may represent a small gene family encoding the protein.

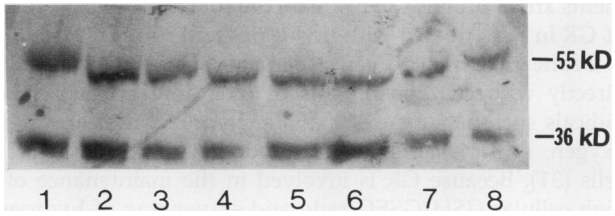

Figure 7. Western blot (10.0% SDS-PAGE gel) of leaf GR in peas exposed to 75 nL L^{-1} of ozone. Crude extracts were probed with antiserum raised against the putative plastidic GR. Fifteen micrograms of protein were loaded in each lane. Lanes 1, 3, and 5 represent control plants at 0 (*t*), 2 h, and 4 h of first day of fumigation, respectively; lanes 2, 4, and 6 represent ozone-fumigated plants at 0 (*t*), 2 h, and 4 h of fumigation, respectively. Lanes 7 and 8 represent control and ozone-fumigated plants, respectively, at 0 (*t*) of second day of fumigation.

The native enzyme of plastidic and extraplastidic forms had a molecular mass of 114 kD as determined by gel filtration chromatography (Superose 6, FPLC). Purified plastidic GR had a subunit molecular mass of 55 kD as deduced from the SDS-PAGE gels. Antiserum raised against pea plastidic GR in the present study recognized one band with a molecular mass of 55 kD, as did antiserum raised against purified leaf GR (11). These results indicate that pea GR is a homodimer as suggested by Halliwell and Foyer (17). Gels stained for GR activity (Fig. 5) show that the purified GR isoforms after chromatofocusing migrate faster than the GR from the crude extract of isolated chloroplasts. The Polybuffer in the purified isoforms might influence mobility by conferring conformational changes in the protein. The migration of purified GR after Mono Q and gel filtration chromatography was similar to that of GR from crude extracts of isolated chloroplasts (data not shown). The cross-reactivity of the antiserum with a lower molecular mass protein on western blots of crude leaf extracts and the purified extraplastidic GR but not the plastidic GR suggests that this lower molecular mass protein is of cytosolic origin. It is not established that this protein is related to the GSSG-independent activity observed on activity stain gels (Fig. 5).

Plastidic and extraplastidic GR had similar pH optima and K_m values for NADPH. The extraplastidic form had higher apparent affinity (lower K_m) for GSSG than did the plastidic form, a difference consistent with the probable distribution of GSH in the cell. Edwards et al. (11) have also reported that the mitochondrial form had higher affinities for both the substrates than did the chloroplast enzyme. The K_m value of the plastidic form for GSSG is similar to that reported for pea chloroplast enzyme by Connel and Mullet (8).

The multiple forms of GR pose the issue of whether there is differential regulation of these isoforms in response to specific environmental cues. There are examples where different isoforms of GR appear to respond differentially to environmental signals. Cold-hardened spinach leaves had higher GR activity and additional forms of GR compared with those present in the nonhardened leaves (15). The activity of plastidic GR from mustard cotyledons was sensitive to photooxidative treatment, whereas the activity of the cytoplasmic form was not affected (10). These two isoforms were also differentially regulated by phytochrome. Experiments are in progress to evaluate the role of various isoforms of GR in peas treated with herbicides and sulfur dioxide.

Ozone is known to cause damage to plants by reacting directly with cell constituents or by forming reactive free radicals such as superoxide anion, hydroxyl radical, singlet oxygen, and oxidants such as hydrogen peroxide in plant cells (31). Because GR is involved in the maintenance of a high cellular GSH/GSSG ratio and scavenging of hydrogen peroxide through the ascorbate-GSH cycle (17), this enzyme could play a critical role in protection against oxidative stress such as that caused by ozone. In the present investigation, fumigation of peas with 75 nL L^{-1} ozone for 4 h on 2 consecutive days did not cause any significant change in GR activity. An increase in GR protein was observed in fumigated plants compared with that in control plants at 0 (t) and 4 h but not at 2 h on the first day of fumigation. This representative pattern of change in GR protein over time was observed in replicated treatments and, hence, is not due to plant variation or differences in sampling and protein extraction. The underlying mechanism of cyclical change observed in GR protein levels in peas is as yet unknown. Neither fumigation with 75 nL L^{-1} ozone for 4 h on 4 consecutive days nor fumigation with 150 nL L^{-1} ozone for 4 h on 2 consecutive days significantly changed GR activity in peas (data not shown). Fumigation with 75 nL L^{-1} ozone did not cause any visible injury, whereas 150 nL L^{-1} ozone caused necrotic lesions. Recently, Aono et al. (3) have shown that transgenic tobacco plants that overproduce GR in the cytosol were no more resistant to ozone than were the control plants. However, Tanaka et al. (30) have reported increase in GR activity and protein levels in spinach leaves that did not show any visible injury after fumigation with 70 nL L^{-1} ozone for 2 d. These results represent the variations in the response of various plant species to ozone exposure and indicate the complexity of the oxidative stress resistance mechanisms in plants.

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