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Biochemical and molecular characterisation of wheat chloroplastic glutathione reductase

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Abstract

Wheat leaves contain two charge/mass-separable isoforms of glutathione reductase (GR, EC 1.6.4.2), one chloroplastic and the other probably cytosolic. The chloroplastic GR was purified to homogeneity, and its biochemical and molecular characterisation showed features very similar to the other plant GRs. In its native conformation the enzyme is composed by two subunits of 56 kDa and an associated polypeptide of 32 kDa, with an overall molecular mass of approximately 150 kDa. Optimum activity was observed at pH 8.00 and with an ionic strength between 60 to 100 mM. GR activity is highly sensitive to temperature changes, exhibiting an exponential increase up to 45 °C. It showed a high affinity for oxidised glutathione and an intermediate affinity for NADPH at pH 8.0. Inhibition tests with thiol and histidine modifiers demonstrated that -SH groups and histidine residues are essential for the catalytic properties of the enzyme. To study the origin of GR isoforms, the number of GR gene copies and the number and size of GR transcripts were determined. Southern analyses showed that wheat GR isoforms are encoded by multiple gene copies. However, a single size transcript of approximately 1.4 kb was observed, suggesting that different GR isoforms could be generated by post-transcriptional and/or post-translational modifications.

Additional key words: antioxidant, chloroplast, oxidative stress, *Triticum aestivum*.

Introduction

Glutathione reductase (GR) is a flavin-containing enzyme that catalyses the NADPH-dependent reduction of glutathione disulphide (GSSG) to reduced glutathione (GSH) according to the reaction:

$$GSSG + NADPH + H^+ \longleftrightarrow NADP^+ + 2\ GSH$$

This reaction involves two steps: first the enzyme is reduced by the electron transferred from NADPH to FAD and from there to the active disulphide, generating a protonated cystine and a thiolate anion. The catalytic cycle is completed by the reaction of the reduced enzyme with GSSG, via a thiol-disulfide interchange (Ghisla and Masey 1989). GR is a member of an interesting group of flavoenzymes that have a redox cystine residue in their active sites.

GRs have high specificity for their substrates, although some glutathione conjugates and mixed glutathione disulphides can also be reduced (Gaullier *et al.* 1994). Most GRs can catalyse the reduction of GSSG using NADH, but the efficiency is quite low (Halliwell and Foyer 1978). While most GRs have a high affinity for NADPH (< 10 μM), there is considerable variation in their affinity for GSSG (from 10 to 7300 μM). GR isoforms with different substrate affinity, have been proposed as a mechanism for the regulation of GR activity in response to stress conditions (Edwards *et al.* 1994).

As recently reviewed by Moullineaux and Creissen (1997), the native molecular mass of GR ranges from 60 to 190 kDa and its quaternary structure vary among species. Most of the GRs studied are homodimers. However, in pea and maize GR exists as an heterodimer

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Abbreviations: DEPC - diethyl pyrocarbonate; DTT - dithiothreitol; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidised glutathione; NEM - N-ethyl maleimide; PAGE - polyacrylamide gel electrophoresis; PCMB - *p*-chloromercuribenzoate; PMSF - phenylmethylsulfonyl fluoride; TLCK - Nα-*p*-tosyl-L-lysine chloromethyl ketone.
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(Kalt-Torres *et al.* 1984, Mahan and Burke 1987) and in *Chlamydomonas reinhardtii* it is a monomer (Takeda *et al.* 1993). Studies in cyanobacteria (Rendon and Mendoza-Hernandez 1989) and pea GRs (Edwards *et al.* 1990) indicate that GR dimers may be further assembled into tetramers, or even higher aggregation states, and that an equilibrium can be established among various higher-order states. The equilibrium between the different forms appears to be pH- and temperature-dependent, and this could be a mechanism for the regulation of GR activity (Moullineaux and Creissen 1997).

GR has a key role in the antioxidative system of plants, since it is involved in the maintenance of the redox state and the total level of glutathione and ascorbate, in the degradation of reactive oxygen species, in the regeneration of electron acceptors and in repairing oxidative damage. Several studies have shown increases in the GR activity and changes in the GR isoform pattern in plants under different oxidative stresses (Lascano *et al.* 1998, Casano *et al.* 1999). GR isoforms have been reported in many plant species, *e.g.*, mustard (Drumm-Herrel *et al.* 1989), pea (Edwards *et al.* 1994), eastern white pinus (Anderson *et al.* 1990), red spruce (Hausladen and Alscher 1994), and *Ch. reinhardtii* (Serrano and Llobell 1993). It has been postulated that GRs in plants are encoded by a multigene family (Edwards *et al.* 1990, 1994, Madamanchi *et al.* 1992). In red spruce subjected to freezing temperatures, two GR isoforms with different amino-terminal amino acid sequences were found, providing evidence that more than one GR gene exists in this species (Hausladen and Alscher 1994, Anderson *et al.* 1990). However, not all isoforms are encoded by different genes. In pea there are only two genes encoding several isoforms, which are probably the products of post-translation process (Madamanchi *et al.* 1992). It has been suggested that post-translational mechanisms such as glycosylation, phosphorylation, and different oxidation states of the enzyme, could give rise to charge-separable isoforms (Edwards *et al.* 1990). On the other hand, the occurrence of a partly unspliced GR RNA indicates that a differential post-transcription process may be involved in the generation of GR isoforms (Creissen and Moullineaux 1995). In wheat leves, GR presents at least two isoforms, one chloroplastic and the other presumably located in the cytosol (Lascano *et al.* 1998). They are important components of the defence against oxidative stress, however the main structural and biochemical properties of wheat GRs are not yet determined.

The aims of this work were to perform the biochemical characterisation of plastid GR purified from wheat (*Triticum aestivum* L. cv. Oasis) leaves and to study the possible causal relationship among the number of GR gene copies, the number and size of GR transcripts, and the presence of different GR isoforms in wheat leaves.

Materials and methods

Plants: Wheat seeds (*Triticum aestivum* L. cv. Oasis) were kindly provided by INTA Marcos Juarez Experimental Station. Plants were grown in vermiculite under continuous light (irradiance of 40 W m^{-2}) and temperature of 24 ± 1 °C, for 8 d. Thereafter, 3-cm subapical segments were cut from the primary leaves, extensively washed and immediately used for GR purification.

Glutathione reductase purification: All the steps were carried out at 4 °C. Leaf segments (300 g) were homogenised in 50 mM Tris-HCl, pH 7.8, and 10 mg cm^{-3} PVPP (1 g of tissue per 4 cm^3 of medium). The homogenate was filtered through four layers of *Miracloth* and then centrifuged at 10 000 *g* for 10 min. The supernatant was recovered and treated with ammonium sulphate and the precipitate produced between 40 and 90 % of saturation was collected by 10 min centrifugation at 10 000 *g*. The precipitate was dissolved in 25 cm^3 of 50 mM Tris-HCl, pH 7.8, and then dialysed against 5 dm^3 of the same buffer during 12 h (with one replacement). For the subsequent steps, we designed two slightly different protocols, based on Halliwell and Foyer (1978). In the first the dialysate (25 cm^3) was loaded onto a *DEAE-Sephacell* column (50 × 1.3 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.8. The enzyme was eluted with a 400 cm^3 linear gradient of KCl (0 - 350 mM) at 180 cm^3 h^{-1}. Fractions of 10 cm^3 were collected. Peak fractions with GR activity (50 cm^3 total volume) were loaded onto a 2'-5'-ADP agarose column (8.8 × 1.2 cm), washed with 30 cm^3 of 50 mM Tris-HCl, pH 7.8, and the enzyme was eluted with 20 cm^3 of 10 mM $NADP^+$ in 50 mM Tris-HCl, pH 7.8. The other protocol was similar, except that the ion exchange chromatography was replaced by gel filtration. After ammonium sulphate precipitation and dialysis, 5 cm^3 of dialysate was loaded onto a *Sephadex G-150* column (100 × 2.5 cm) equilibrated with 50 mM Tris-HCl, pH 7.8, and 100 mM NaCl. The elution was carried out with the same buffer, at a 43 cm^3 h^{-1} flux. This step was repeated five times to process the total volume of dialysate (25 cm^3). Fractions with GR activity were poolled and subjected to affinity chromatography as described above. The polypeptide patterns obtained after each step of purification were analysed by denaturing (SDS) PAGE (12.5 % acrylamide) according to Laemmli (1970) and by non-denaturing (ND) PAGE (10 % acrylamide) according to Davies (1971).

GR activity: Enzyme activity was assayed at 25 °C according to Schaedle and Bassham (1977), following the decrease in absorbance A_{340} due to NADPH oxidation. The reaction mixture contained 50 mM Tris-HCl, pH 7.8, 0.15 mM NADPH, 0.5 mM GSSG, 3 mM $MgCl_2$, 1 mM EDTA and 0 - 0.25 cm^3 protein sample. The reaction was started by the addition of NADPH.

GR activity in native gels: After ND-PAGE, GR activity was developed by incubating gels with a reaction mixture containing 250 mM Tris-HCl, pH 7.8, 4 mM GSSG, 0.3 mM NADPH, 1 mM EDTA, 3 mM $MgCl_2$, 0.35 mM 2,6-dichlorophenolindophenol (DCPIP) and 0.25 mM 2,5-diphenyltetrazolium bromide thiazolyl blue (MTT) (Madamanchi *et al.* 1992). Duplicate gels were incubated without GSSG to discard unspecific diaphorase bands. Typically, activity bands appeared after 20 to 30 min incubation at room temperature.

Protein content: It was determined according to Bradford (1976) with bovine serum albumin as a standard.

Characterisation of GR: The native molecular mass of the enzyme was determined through gel filtration chromatography on a *Sephacryl S-300-HR* (*Sigma*, St. Louis, USA) column (90 × 2 cm), equilibrated with 50 mM Tris-HCl, pH 7.8 and 100 mM NaCl. The column was calibrated with the following molecular mass markers: apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa).

The influence of ionic strength on GR activity was estimated by adding 50 - 400 mM KCl to the reaction mixture. The effect of pH on GR activity was determined within the 5.5 - 10 pH range. We used 50 mM Tris-maleic buffers for the 5.5 - 7.5 range, 50 mM Tris-HCl buffers for the 7 - 9 range and 50 mM Tris-glycine buffers for the 9 - 10 range. The thermal dependence of GR activity was estimated by varying the temperature of the standard reaction mixture within the range of 2 to 45 °C.

The *K*m for each of the two substrates of the enzyme (GSSG and NADPH) was calculated by plotting enzymatic activities against concentrations of one of the substrates while keeping the other constant and saturating. The double-reciprocal graph allowed the mathematical calculation of *K*m.

In other experiments, purified GR was preincubated in 50 mM Tris-HCl, pH 7.8, with each of the following compounds: GSSG (0.5 mM), NADPH (0.1 mM), GSH (1 mM), NADP (1 mM), iodoacetamide (1 mM), NEM (N-ethyl maleimide) (1 mM), PMSF (phenylmethyl-sulfonyl fluoride) (1 mM), TLCK (N(-*p*-tosyl-L-lysine chloromethyl ketone) (1 mM), $ZnSO_4$ (0.5 mM), DTT (dithiothreitol) (1 mM), β-mercaptoethanol (5 mM), PCMB (*p*-chloromercuribenzoate) (1 mM), leupeptin (0.1 mM) and DEPC (diethyl pyrocarbonate) (1, 2, 4 mM). After 30 min at 30 °C, the remnant GR activity was assayed under standard conditions.

Southern and Northern blot analysis: Copy number of wheat GR gene was determined by Southern blot according to Sambrook *et al.* (1989). DNA isolated from wheat leaves (Sambrook *et al.* 1989) was digested with EcoRI, PstI, XbaI and HindIII enzymes according to instructions of supplier (Boehringer Mannheim, Mannheim, Germany). The digestion products (10 μg of DNA) were separated in a 0.8 % agarose gel and transferred to a nylon membrane by capillary. The membrane was hybridized with a double-strand GR probe labeled with digoxigenine. The washing conditions after the hybridization were markedly stringent (0.3 mM sodium citrate, 3 mM NaCl, 0.1 % SDS and 65 °C). The double strand probe of GR gene was obtained by PCR from wheat cDNA. The cDNA was produced with the Super Script RNase H Reverse Transcriptase (*Finnizymes OY*, Espoo, Finland). RNA was isolated from 50-100 mg wheat leaf tissue using *Quiagen* Plant RNA mini extraction kit (*Quiagen*, Hilden, Germany). The primers were designed considering consensus regions from other well-known GR gene sequences. The primer sequences were: Forward: 5' GGC TGT GGG TGA TGT AAC GAA C 3' and Reverse: 5' CGC ATT GTC ACA AAC TCT TCA GC 3'.

Number and size of GR transcripts were determined by Northern blot following Sambrook *et al.* (1989). RNA electrophoresis was carried out in an agarose gel (1 %) under denaturing conditions in the presence of formaldehyde, transferred by pressure or capillary to a nylon membrane and hybridized with a single-strand probe labelled with digoxigenine. The washing conditions after the hybridization were markedly stringent (0.3 mM sodium citrate, 3 mM NaCl, 0.1 % SDS and 65 °C). The single strand GR probe (enriched in the strand complementary to transcripts) was obtained by an asymmetric PCR reaction on wheat cDNA, in which the relation between forward and reverse primers was 1 to 0.02, respectively. The probes were then purified from 1 % agarose gel using the *Quiaex II* kit (*Quiagen*, Hilden, Germany).

The wheat cDNA fragment used as GR probe was sequenced by Bio Resource Center of Cornell University (New York, USA). Sequence analysis of this fragment indicated a 60 % homology with respect to other GR known sequences.

Chloroplast isolation: Intact chloroplasts were obtained from primary wheat leaves according to Kalt-Torres *et al.* (1984). Briefly, 15 g of leaves were blended with 150 cm^3 of 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 0.15 % bovine serum albumin in 50 mM Tris-

HCl, pH 7.5, and filtered through 8 layers of cheesecloth. Crude chloroplasts were pelleted by centrifugation at 1 000 *g* for 3 min and resuspended in grinding medium without EDTA or albumin. Intact chloroplasts were separated by centrifugation (10 000 *g*, 20 min) in a continuous Percoll gradient (40 - 80 %). Chloroplasts with a protein to chlorophyll ratio of 10 or more banded as a sharp layer near the bottom of the tube. This band was collected, rinsed with a large volume of resuspension medium and resuspended in a hypotonic medium (resuspension medium without sorbitol). All steps were carried out at 4 °C.

Results and discussion

Purification and molecular mass estimation of GR: GR purification from wheat leaf segments was carried out by two slightly different protocols. In both cases an affinity chromatography was applied as the main step, using a column of 2′-5′ADP-agarose (Table 1).

Table 1. Purification of chloroplastic GR from wheat leaf. GR purification was carried out by two protocols which differed in the intermediate chromatographic step. In both protocols a final affinity chromatography was applied as the main step.

Step		Total protein [mg]	Total activity [nmol(NADPH) s^{-1}]	Specific activity [nmol(NADPH) mg^{-1}(protein) s^{-1}]	Purification (fold)
	whole leaf extract	3090	6183.3	3.6	1
	$(NH_4)_2SO_4$ 40 - 90 % of saturation	276	3216.6	13.2	3.6
Protocol 1	*DEAE-Sephacell*	75.1	2100.0	28.1	7.6
	2′5′-ADP-agarose	1.0	366.6	360.8	98.4
Protocol 2	*Sephadex G-150*	33.6	2633.3	78.3	21.4
	2′5′-ADP-agarose	0.79	1266.6	1600.2	436

In the first purification protocol an ionic exchange chromatography before the affinity chromatography was used, and the final enzyme preparation had a specific activity of 360 nmol(NADPH) mg^{-1}(protein) s^{-1}. Similar specific activities have been obtained in GR purified from pea and maize chloroplasts (Kalt-Torres *et al.* 1984, Mahan and Burke 1987). After the last purification step, a single protein band was found in ND-PAGE (data not shown), however in SDS-PAGE, besides a major band of approximately 56 kDa, another band of approximately 32 kDa was observed (Fig. 1*A*, *line 5*). The molecular mass of the major polypeptide is similar to the monomer of other plant GRs (Moullineaux and Creissen 1997). Other authors (Connell and Mullet 1986, Madamanchi *et al.* 1992) has also reported an analogous 32 kDa polypeptide, however it is not clear whether this polypeptide is part of GR molecule or it is a contaminant. Connell and Mullet (1986), who purified GR from pea chloroplasts, demonstrated that this polypeptide does not have enzymatic activity and it is not recognized by GR antibodies. However, when purifying six GR isoforms from pea leaves, Madamanchi *et al.* (1992) demonstrated the presence of the 32 kDa polypeptide by Western-blot.

With the aim to separate the 32-kDa polypeptide, we assayed an alternative purification strategy in which the ion exchange step was substituted by a gel filtration before the affinity chromatography. Using this second protocol we obtained a highly purified enzyme with a specific activity almost 4.5 times higher than that obtained with the first protocol (Table 1). However, in addition to the 56 kDa polypeptide, the 32 kDa polypeptide was still present in the highly purified GR (Fig. 1*A*), suggesting that it makes a part of the wheat GR. Moreover, the native molecular mass of the purified enzyme, determined by gel filtration, was approximately 150 kDa (Fig. 1B). In sum, these results suggest that the molecule of chloroplastic (see below) wheat GR would be a dimer, formed by two identical subunits of 56 kDa plus a bound polypeptide of 32 kDa.

Subcellular location of the purified enzyme: The subcellular location of the purified GR was determined by activity gels comparing total extract, purified chloroplasts and purified enzyme. Two activity bands were observed in the total extract. In purified chloroplasts, a single band that coincides with the higher mobility band of the total extract and with the purified enzyme band was observed (Fig. 2). These results indicate that the purification protocol(s) yielded an enzyme preparation which corresponded to the chloroplastic charge/mass-separable isoform of wheat GR. Accordingly, Stevens *et al.* (2000) observed that 2'-5'-ADP agarose has a very low affinity for the cytoplasmic GR isoform, and concluded that a GR preparation from crude extract using this chromatographic step should be mostly chloroplastic.

Effect of ion strength, pH and temperature on GR activity: In human erythrocytes, Worthington and Resemeyer (1976) found a marked dependence of GR

activity on the ion strength of reaction mixture, obtaining the maximum activity with salt concentrations around 100 mM. The effect of ion strength on wheat GR was assayed at different concentrations of KCl in the reaction medium. The maximum activity of the purified enzyme was obtained within the range from 50 to 100 mM KCl (Fig. 3*A*). Higher salt concentration did not produce further increases in enzymatic activity. Consistent with these results, Kalt-Torres *et al.* (1984) observed a requirement of 60 to 100 mM chloride salts to obtain the highest activity of chloroplastic GR from pea.

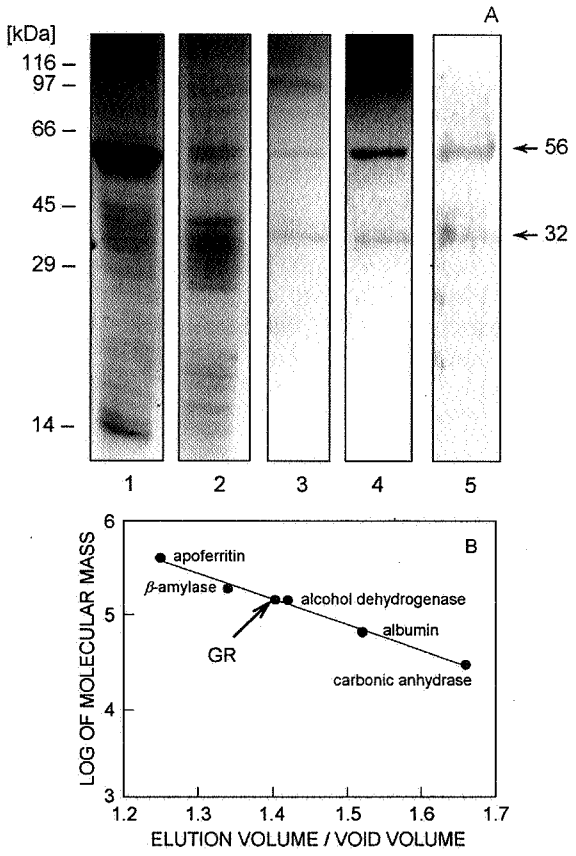

Fig 1. SDS-PAGE of the GR purification steps. After electrophoresis polypeptidic bands were stained with Coomassie Brilliant Blue R. Whole leaf extract (1); 40 - 90 % amonium sulfate precipitate (2); gel filtration on *Sephadex G-150* (3); affinity chromatography on 2′5′-ADP-agarose of protocol 2 (4); affinity chromatography on 2′5′-ADP-agarose of protocol 1 (5) (See Table 1 and Materials and methods). Molecular mass marker positions are indicated on the left. On the right side, arrows indicate GR polypeptide components (*A*). Native molecular mass estimation of GR by gel filtration using a column of *Sephacryl S-300* calibrated with the following molecular mass markers: apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). The *arrow* indicates the position corresponding to the peak fraction of GR, which was approximately coincident with that of alcohol dehydrogenase (*B*).

The effect of pH on purified GR activity was tested in the 5.5 - 10 range (Fig. 3*B*). Maximum GR activity was obtained between pH 7.5 and 8. Outside this pH range, a marked reduction in GR activity was found. This possible

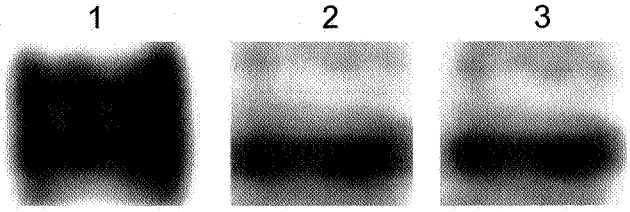

Fig. 2. Subcellular location of the purified GR. Whole leaf extract (20 μg protein) (1), intact purified chloroplasts (20 μg protein) (2), and purified enzyme (5 μg protein) (3) were run in ND-PAGE and GR isoforms were revealed by activity stain.

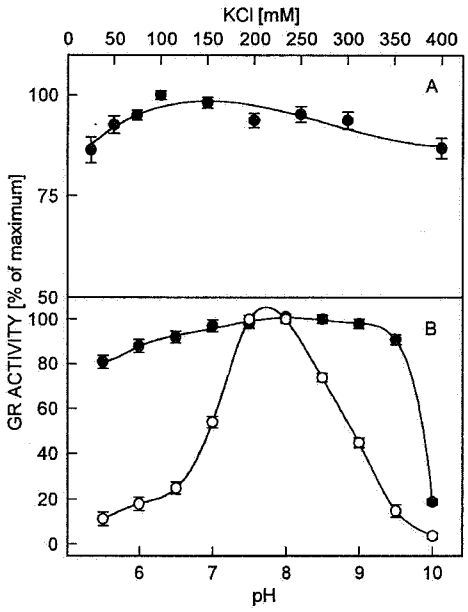

Fig. 3. Effect of ion strength (*A*) and pH (*B*) on wheat chloroplastic GR. Different ionic strenghts in the reaction mixture were created by KCl at the indicated concentrations. GR activity in reaction media with different pH (*open circles*) and GR activity assayed at optimum pH after a 30 min-preincubation at the indicated pH values and 30 °C (*closed circles*). Results represent the mean of three independent experiments with their correspondent standard deviation.

inactivating effect was tested by preincubating the enzyme in media of various pH values for 30 min, and then assaying remnant activity at optimum pH. By this procedure, irreversible inactivation was only observed at pH 10, which is denaturing condition for most proteins. Other pre-incubations outside the 7.5 - 8.0 range caused mostly reversible effect, and only a slight decrease in GR activity was found in acidic pH (Fig. 3*B*), indicating a high stability of GR protein within a wide pH range. The quite narrow range of optimal pH for GR activity fits well with the pH estimated for chloroplastic stroma under light

conditions (Heineke and Heldt 1988), and suggests that light could regulate chloroplastic GR activity via pH changes of the plastid matrix.

GR activity increased exponentially (r^2 = 0.99) as a function of the reaction temperature (Fig. 4) at least up to 45 °C and reached more than 400 % of the activity found at 25 °C. This great thermal plasticity of GR activity was also observed in other plants of temperate zones (Burke and Hatfield 1987, Mahan *et al.* 1990, Kidambi *et al.* 1990), and represents an evolutive advantage for plants adapted to climates with marked daily and seasonal variations of temperature. In addition, it is important to keep in mind that measurements of GR activity in the laboratory will be valid to make inferences about the "*in vivo*" GR activity of plants subjected to conditions which induce changes in leaf temperature, only if the reaction temperature is adjusted to the actual leaf temperature.

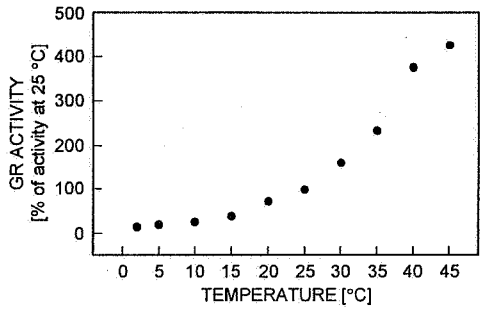

Fig. 4. Effect of temperature on wheat chloroplastic GR activity. The thermal dependence of GR activity was estimated by varying the temperature of the standard reaction mixture within the indicated range. Results represent the mean of four independent experiments with their correspondent standard deviation.

Kinetic constants: The *K*m for each substrate of the chloroplastic GR was estimated maintaining the other one in constant and saturating concentration. In the Fig. 5 it can be observed the representations of the double reciprocal plots. The mathematical calculation of the *K*m values for each substrate was carried out using the lineal regression equation of the double reciprocal plots. Estimated *K*m for GSSG and NADPH, were 40 and 15 µM, respectively (Fig. 5). When compared with other purified GRs (Moullineaux and Creissen 1997), it is evident that the affinity of the chloroplastic wheat GR is high for GSSG, and intermediate for NADPH.

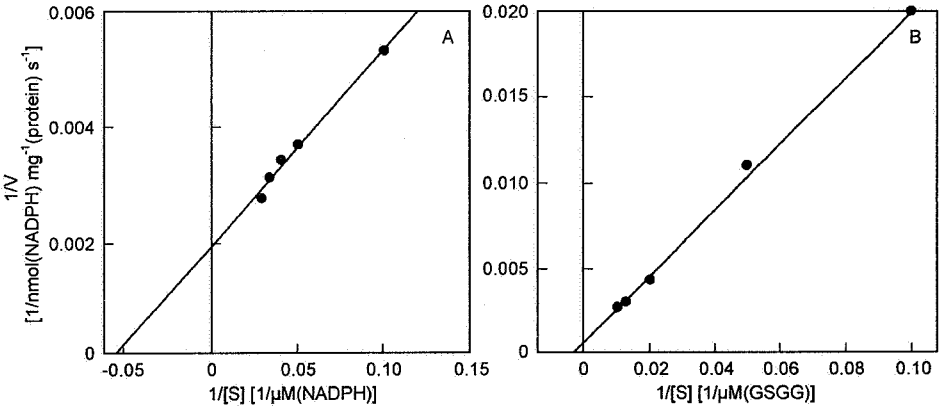

Fig. 5. Influence of the substrate concentration on purifed GR. The double-reciprocal plots for GSSG (A) and NADPH (B) were determined by assaying the enzymatic activity at different concentrations of one of the substrates while keeping the other constant and saturating.

Inhibition tests: When the enzyme was preincubated with its substrates it was observed that GSSG did not alter the GR activity, while NADPH caused an inhibition of approximately 50 % (Table 2). In a previous work we demonstrated that the inhibitory effect of preincubations with NADPH on purified wheat GR depend on the temperature, pH and the presence of GSSG in the preincubation medium (Lascano *et al.* 1999). Pre-incubations with the products of reaction, GSH and NADP, did not affect GR activity. Consistent with the result obtained with GSH, other thiol reducing agents, like DTT and β-mercaptoethanol, did not affect wheat GR activity. However, there are studies that show inhibitory effects of GSH and other disulfide bridge reducers on GR activity (Mahan and Burke 1987, Pinto *et al.* 1984).

Treatments with different inhibitors showed a marked effect of thiol-oxidants like NEM and PCMB, which cause about 90 % inhibition of GR activity. Likewise, leupeptin, a cystein-proteases inhibitor, caused an inhibition of 26 %. Another potent inhibitor of wheat GR is Zn^{2+} (94 % inhibition), which interacts with histidine and/or cysteine residues (Creighton 1993). Zn^{2+} inhibition was reversible and competitive with GSSG (data not shown). GSSG (0.5 mM) also suppressed the inhibitory effect of NEM. Similar results were obtained by other authors who reported the inhibitory effects of NEM and PCMB and the protective effects of GSSG (Kalt-Torres *et al.* 1984, Mahan and Burke 1987). DEPC, a potent histidine modifier, inhibited GR activity linearly in a

concentration-dependent way. The chemical modification induced by DEPC was reverted by incubations in presence of 100 mM histidine (data not shown). In sum, inhibitor assays indicate the involvement of essential thiol groups and histidine residues in the catalytic activity of chloroplastic wheat GR.

Table 2. Effect of different compounds on the GR activity. Purified chloroplastic GR (0.22 μg protein) was incubated at 30 °C in 50 mM Tris-HCl buffer, pH 7.8, with different chemicals cited below at the indicated concentrations. After 30 min remnant GR activity was measured by standard procedure. Initial values for controls were around 360 nmol(NADPH) mg^{-1}(protein) s^{-1}. Results represent the mean of four independent experiments, with standard deviation less than 10 %.

Compound	Concentration [mM]	Inhibition [%]
GSH	1	0
GSSG	0.5	0
NADPH	0.1	50
NADP	1	0
NEM	1	86
PCMB	1	96
$ZnSO_4$	0.5	94
Leupeptin	0.1	26
β-mercaptoethanol	5	0
DTT	5	0
TLCK	1	0
PMSF	1	12
DEPC	1	23
DEPC	2	60
DEPC	4	100

Number of GR gene copies and size of GR transcripts: The existence of chloroplastic and extra-chlorolastic isoforms of GR in wheat leaves (Fig. 2) and other species could be due to different GR genes. The number of GR gene copies in wheat was determined by Southern blot. These analyses showed that there are numerous copies of the GR gene(s) in wheat, as could be expected in a polyploid (hexapaloid) organism (Fig. 6*A*). However, when the number and size of GR transcripts of wheat leaves were determined by Northern blot analysis, using a highly specific single-strand probe, a single transcript band of approximately 1.4 kb was detected (Fig. 6*B*). These results agree with those of others authors, who found a unique transcript size in plants that contain more than one GR gene copy (Stevens *et al.* 1997). Although differences in nucleotide sequences among 1.4 kb GR transcripts cannot be ruled out, we speculate that a differential post-translational processing could be the most probable origin of the chloroplastic and extrachloroplastic isoforms of wheat GR.

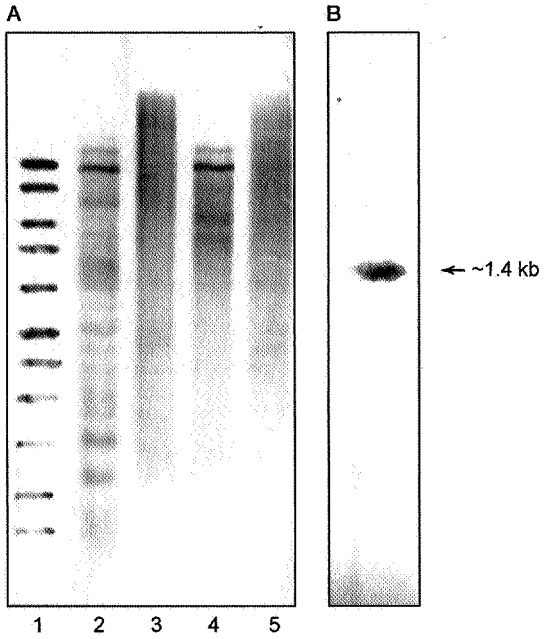

Fig. 6. Southern blot analysis of GR wheat genomic DNA. DNA samples digested with restriction endonucleases: EcoRI (2), PstI (3), XbaI (4) and HindIII (5), molecular mass markers (0.75 to 10 kbp) (1) (*A*). Northern blot analysis of GR. Position and size of GR transcript is indicated. Weak bands of higher molecular mass correspond to unspecific probe retention to some ribosomal RNAs, and were not observed in gels loaded with lower amounts of RNA (*B*).

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