Effect of Polyamines on Glutathione Reductase Activity in Spinach

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The effects of polyamines (putrescine, spermidine and spermine) on glutathione reductase (glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) activity of spinach leaves (*Spinacia oleracea* L. cv. Gladiator) were investigated under *in vivo* and *in vitro* conditions. Spinach was grown in sand culture under controlled conditions for 30 d. In *in vivo* assays 30-day-old plants were sprayed with polyamines once, and leaves were harvested 1, 5, 10 and 15 d after treatment. The three polyamines decreased the GR activity to different degrees, depending on time after application, type of compound and their concentration. In order to study whether or not polyamines can exert a direct effect on GR, the enzyme was partially purified from spinach leaves and incubated with polyamines in the reaction medium. Under these *in vitro* conditions, GR was inhibited by polyamines in a polyamine type- and concentration-dependent manner. Interestingly, spermine exerted the most intense inhibitory effect in both *in vivo* and *in vitro* experiments. It is proposed that the early decrease of glutathione reductase activity in leaves treated with polyamines can be due to a direct interaction of these compounds with the enzyme.

Key words: Glutathione Reductase, Polyamines, Spinach

Introduction

Glutathione reductase (glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) is a flavin-containing enzyme that catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH) according to

$$GSSG + NADPH + H^+ \rightleftharpoons NADP^+ + 2\ GSH.$$

This reaction involves two steps: first the enzyme is reduced by the electron transferred from NADPH to FAD and from there to the active disulfide, 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 Massey, 1989). GR is a member of an interesting group of flavoenzymes that have a redox cystine residue in their active sites.

Glutathione reductases have high specificity for their substrates, although some glutathione conjugates and mixed glutathione disulfides can also be reduced (Gaullier *et al*., 1994). Most GRs can catalyze 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 ($< 10\ \mu$M), there is considerable variation in their affinity for GSSG (from 10 to 7300 μM). GR isoforms have different substrate affinity and this has been proposed to regulate the GR activity in response to stress conditions (Edwards *et al*., 1994).

Glutathione reductase has a key role in the antioxidant 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 (Casano *et al*., 1999; Lascano *et al*., 1998).

Polyamines (PAs) are widely distributed in living cells and are implicated in the regulation of a wide range of processes such as growth, cell division, DNA replication and cell differentiation

Abbreviations: GR, glutathione reductase; PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine.

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(Galston and Kaur-Sawhney, 1995; Marton and Morris, 1987). The PAs spermidine (Spd) and spermine (Spm) and the diamine putrescine (Put) are basic, small molecules involved in plant growth and development (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1995; Marton and Morris, 1987; Tiburcio *et al*., 1997). It has been suggested that they perform many physiological effects by binding to negative charges of phospholipids and DNA, thereby modulating the function of nucleus and membranes (Kaur-Sawhney *et al*., 1978). Put, Spm, and Spd are found in a wide range of organisms from bacteria to plants and animals. They occur in both free as well as conjugated forms and titres, depending on external conditions such as light and temperature (Galston and Flores, 1991). Increased PA titres have been noticed during sprouting in potato tubers (Galston and Flores, 1991) and Jerusalem artichoke (Bagni and Pistocchi, 1991; Bagni *et al*., 1981), seed germination (Gallardo *et al*., 2003; Puga-Hermida *et al*., 2003), and root and shoot formation (Mengoli *et al*., 1992). The conjugated PAs are known to be associated with the physiology of flowering in higher plants (Botha and Whitehead, 1992; Slocum *et al*., 1984).

Polyamines are involved in plant responses to biotic and abiotic stresses. Conjugated PAs have been shown to accumulate in incompatible interactions between plants and a variety of pathogens, while changes in the diamine catabolic enzyme diamine oxidase suggest a role for this enzyme in the production of hydrogen peroxide during plant defense responses (Tiburcio *et al*., 1993). Recently, a role for Spm in the hypersensitive response of barley to powdery mildew and particularly of tobacco to TMV has been suggested (Cowley and Walters, 2002; Walters, 2003). Polyamine metabolism has long been known to be related to various kinds of abiotic stress including osmotic stress (Aziz *et al*., 1997), salt stress (Aziz *et al*., 1998), acid stress (Shen *et al*., 1994), heavy metals (Groppa *et al*., 2001, 2003) and UV radiation (Kramer *et al*., 1991). Importantly, both the response of plants to and the damages caused by biotic and abiotic stresses involve changes in the level of reactive oxygen species and oxidative degradation of some cellular components. A number of experiments have suggested polyamines as efficient antioxidants in many experimental systems, exerting this effect through the protection of cellular components such as cell membranes, nucleic acids and polyunsaturated fatty acids from oxidative damage (Kitada *et al*., 1979; Lovaas, 1991, 1996; Tadolini *et al*., 1984). The effective antioxidant performance evidenced by polyamines is in relation to the type of the radical produced (Lovaas, 1997), which largely depends on the stress factor or the severity of the stress.

The nature of the antioxidant action of PAs remains to be elucidated. It is possible that PAs function as metal chelators thus diminishing the metal-catalyzed production of reactive oxygen atoms (Lovaas, 1996). Alternatively, PAs could control the level of reactive oxygen atoms by modulating the activity of some antioxidant enzymes. The aim of the present work was to assess this second possible mechanism of PA action in the antioxidant protection of plants by studying the effects of PAs on the activity of GR, one of the most important antioxidant enzymes. GR activity was decreased in leaves sprayed with PAs, probably as a consequence of direct inhibition of the enzyme by PAs, as indicated by results from *in vitro* assays. The early decrease of GR activity induced by PAs suggests a possible role of these compounds in the transient increase of the reactive oxygen content which elicits the defensive response.

Materials and Methods

Growth conditions

Seeds of spinach (*Spinacia oleracea* L. cv. Gladiator) were grown in sand culture supplied with standard Hoagland's nutrient solution every 2 d. They were maintained in a growth chamber under controlled conditions (12 h light/22 °C and 12 h dark/18 °C) with a light intensity of 400 μmol m^{-2} s^{-1}.

In vivo experiments

After 30 d of growth, spinach leaves were slowly sprayed with 0.01 mM, 0.1 mM or 1 mM Put, Spd and Spm for 24 h. During this period each plant received 2 mL of the corresponding PA solution. After 1, 5, 10 and 15 d, leaves were harvested, frozen in liquid nitrogen and stored at −80 °C for enzyme activity determination in crude extracts.

In vitro experiments

The effect of PAs on partially purified GR was assayed by including different PAs in the reaction medium of enzyme activity (see below) at concentrations ranging from 0.01 to 1 mM.

Table I. Partial purification of spinach GR. The enzyme was purified from leaves of 30-d-old plants. For details see Materials and Methods.

Purification step	Total volume [mL]	Activity[a] [EU/mL]	Total activity[a] [EU]	Protein [mg/mL]	Total protein [mg]	Specific activity[a] [EU/mg]	Recovery (%)	Purification (fold)
Crude extract	50	0.120	6.00	1.81	90.50	0.066	100.0	1
Ammonium sulfate precipitation (30–70%)	8	0.480	3.84	2.42	19.36	0.198	64.0	3
Sephadex G 200 gel filtration chromatography	11	0.128	1.41	0.04	0.44	3.200	23.5	48.48

[a] EU, enzyme unit. One enzyme unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per min under the assay conditions.

Crude extracts

Frozen leaves were ground to powder in liquid nitrogen, and then resuspended in the extraction buffer [50 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride, Tris-HCl, pH 7.6, containing 1 mM EDTA], giving 1 g leaves/5 mL. The suspension was centrifuged at 4 °C for 30 min at 24,000 × *g* and the supernatant was used as a crude extract.

Sephadex G-200 gel filtration chromatography

5 g dried Sephadex G-200 were used for a 150 mL column. The gel was incubated in distilled water at 90 °C for 5 h. After removal of the air in the gel, it was loaded onto the column (1.5 × 75 cm). The flow rate was adjusted to 15 mL/h by means of a peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH 7.0, until the final absorbance difference became zero at 280 nm and the pH value became same with that of the equilibration buffer. The sample from precipitation was mixed with 50 mM glycerol and loaded onto the column. Elutions were collected as 2 mL fractions in Eppendorf tubes with equilibration buffer containing 50 mM glycerol. Activity values were determined in each fraction at 340 nm, and active fractions (11 mL) were put together. In each sample obtained from purification steps, activity and protein concentrations were determined and calculated values are showed in Table I.

Enzyme assay

The enzymatic activity was measured spectrophotometrically at 25 °C, according to the method of Lamotte *et al.* (2000). The reaction medium contained 0.75 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH in a total volume of 1 mL. The decrease in absorbance at 340 nm was followed with a Shimadzu spectrophotometer UV-(1208) after the addition of the enzyme. One enzyme unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per min under the assay conditions.

Protein determination

Protein concentrations were estimated from measurements of absorbance at 595 nm according to Bradford's method (1976), with bovine serum albumin as a standard.

Statistical analysis

Results are expressed as the mean ± SD, $n = 3$. Differences between means (test *vs.* control) were analyzed by Student's *t*-test.

Results and Discussion

In previous reports, PAs have been tested as antioxidants using *in vitro* assays, by exogenous addition to the incubation medium of leaf sections or by spraying the plants. It has been proposed that PAs can prevent the increase of active oxygen species and diminish oxidative damage by chelating divalent metal ions, which could reduce both the generation of reactive oxygen species and metal-catalyzed oxidations of cellular components (Groppa *et al.*, 2001, 2003; Ha *et al.*, 1998; Lovaas, 1996, 1997; Velikova *et al.*, 2000). However, a relatively low level of active oxygen atoms can also be maintained by increasing the activity of the anti-

Table II. Effects of PAs on spinach GR activities in *in vivo* experiments. Each value is the average ± SD of three independent repetitions.

Polyamine	Concentration	Time after treatment [d]							
		1		5		10		15	
		Glutathione reductase activity							
	[mM]	[EU/mg prot.]	(%)	[EU/mg prot.]	(%)	[EU/mg prot.]	(%)	[EU/mg prot.]	(%)
Putrescine	0 (control)	0.22 ± 0.03	100	0.20 ± 0.03	100	0.19 ± 0.01	100	0.18 ± 0.03	100
	0.01	0.19 ± 0.00	86.9	0.13 ± 0.01	64.9**	0.16 ± 0.01	84.2*	0.18 ± 0.01	99.5
	0.1	0.18 ± 0.01	81.6	0.15 ± 0.01	76.2	0.14 ± 0.01	73.7**	0.13 ± 0.01	72
	1	0.14 ± 0.01	64.6**	0.15 ± 0.01	75.7	0.19 ± 0.01	97.4	0.16 ± 0.01	85.2
Spermidine	0 (control)	0.22 ± 0.03	100	0.20 ± 0.003	100	0.19 ± 0.01	100	0.18 ± 0.03	100
	0.01	0.18 ± 0.01	78.5*	0.19 ± 0.01	94.5	0.14 ± 0.01	74.2**	0.16 ± 0.005	89.6
	0.1	0.16 ± 0.01	72.6*	0.17 ± 0.02	84.2	0.16 ± 0.01	85.3	0.15 ± 0.007	81.3
	1	0.14 ± 0.01	62.3**	0.13 ± 0.003	63.9*	0.19 ± 0.004	99.5	0.18 ± 0.013	98.9
Spermine	0 (control)	0.22 ± 0.03	100	0.20 ± 0.03	100	0.19 ± 0.02	100	0.18 ± 0.03	100
	0.01	0.15 ± 0.03	66.8*	0.19 ± 0.01	89.1	0.19 ± 0.02	97.9	0.16 ± 0.01	85.7
	0.1	0.15 ± 0.02	68.6*	0.19 ± 0.03	97.5	0.19 ± 0.02	99.5	0.12 ± 0.01	67*
	1	0.12 ± 0.02	55.6**	0.15 ± 0.02	72.8	0.18 ± 0.02	93.7	0.18 ± 0.02	99

* $P \leq 0.05$, ** $P \leq 0.01$, with respect to control.

oxidant defense system (Scandalios, 1993). In this paper we explored this second possibility by studying the effect of PAs on the activity of GR. This flavin enzyme is a key component of the antioxidant system, since the maintenance of high levels of reduced glutathione is a prerequisite for the efficient scavenging and degradation of reactive oxygen species and the normal functioning of the cell (Mullineaux and Creissen, 1997; Mullineaux *et al.*, 1996).

In a series of *in vivo* experiments we tested the effects of external applications of different concentrations of the PAs Spm and Spd and the diamine Put to leaves of spinach plants, grown under controlled conditions. Leaf samples were collected and GR activity was determined in crude soluble protein extracts at different time intervals (Table II). During this period, control plants showed a slow decrease of GR activity, probably due to the onset of leaf senescence, as indicated by previous reports (Casano *et al.*, 1999). In addition to age-dependent changes, all the PAs decreased GR activity to different degrees with respect to controls, depending on the PA type and concentration. Spm was by far the most effective, producing ca. 50% inhibition of the enzyme when applied at 1 mM concentration. Interestingly, the impact of the PA-induced decrease of GR activity seemed to be mainly restricted to the first 24 h after treatment. Thereafter, only marginal effects could be observed, suggesting that the GR activity slowly recovered from the initial inhibition, reaching levels similar to the controls at the end of the experimental period.

The dynamics of PA effects observed in *in vivo* experiments suggest that these compounds could directly interact with the GR protein, reducing its catalytic capacity. This hypothesis was tested through *in vitro* assays, in which partially purified GR was exposed to the action of PAs up to levels of 1 mM, during the activity assay. Considering that levels of total PAs in tobacco leaves range from 0.12 to 2.6 μmol per g of fresh weight (Paschalidis and Roubelakis-Angelakis, 2005), and that most of leaf PAs and GR accumulate in chloroplasts, it is plausible to estimate that the enzyme could be exposed *in vivo* to PAs levels as high as those used in our experiments. As shown in Fig. 1, GR was inhibited by PAs in a PA type- and concentration-dependent manner. The diamine Put had only a marginal effect, as reflected by a high value of the calculated I_{50} of ca. 35 mM, while Spd and especially Spm strongly reduced the GR activity. Pending on a more detailed study with GR protein purified to a higher extent, our results argue in favour of a direct inhibition of the enzyme, probably by binding of PAs to the GR moiety. PAs are basic molecules, which are positively charged at

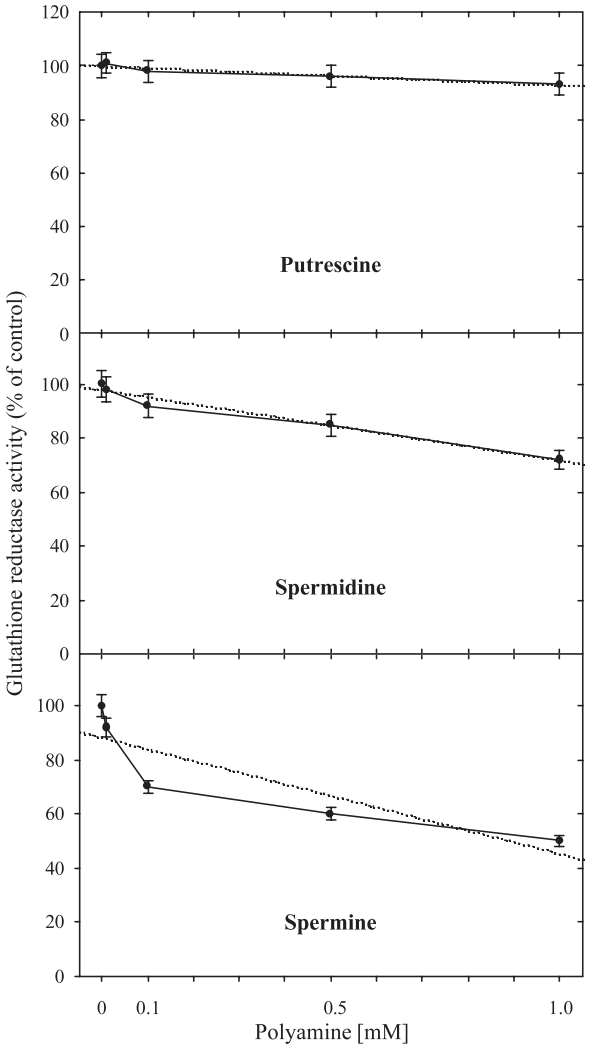

Fig. 1. *In vitro* effect of PAs on GR activity. The effect of PAs on partially purified GR was assayed by including different PAs in the reaction medium at the indicated concentrations. Dotted lines depict estimated linear regression curves. For details see Materials and Methods.

physiological pH. Therefore, they can bind to negatively charged amino acid residues of many proteins, including GR. Accordingly, PAs have been shown to bind to many types of proteins, including a number of enzymes whose activities are directly modulated by polyamine binding (Walters, 2003).

It is interesting to note that Spm was the most effective PA in both *in vivo* and *in vitro* experiments. Moreover, similar GR inhibition levels were measured with 0.1 and 1 mM Spm in both types of experiments, indicating that the rapid decrease of the GR activity observed in leaves treated with Spm could be attributed, at least in part, to a direct interaction between the PA and enzyme. As previously pointed out, PAs have been found to prevent oxidative damage of cells. How is this function compatible with the concomitant inhibition of one of the most important antioxidant enzymes? To answer this question it is important to note that even when an elevated level of reduced gluthatione is necessary to collaborate in the antioxidant defense and to maintain cell functions, a transient decrease in the gluthatione reduction state seems to be part of the redox-sensing system involved in the antioxidant response (Creissen *et al.*, 1999; Foyer and Noctor, 2005). Since the inhibitory effect of PAs on GR is mainly limited to the first hours after treatment, we speculate that the transient decrease of reduced glutathione could be involved in the perception-transduction chain of events that modulate the level of antioxidant defenses.

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