Phytochemistry, Vol. 25, No. 10, pp. 2261–2265, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

PROPERTIES OF GLUTATHIONE REDUCTASE FROM CHLOROPLASTS AND ROOTS OF PEA

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(*Revised received* 3 *February* 1986)

Key Word Index—*Pisum sativum*; Leguminosae; pea; enzymology; glutathione reductase; chloroplasts; roots.

Abstract—In *Pisum sativum*, about 90% of the glutathione in leaf and root is in the reduced form. GSSG reductase has been purified from chloroplasts and roots. The two enzymes had similar M_rs, but differed in pH optimum and sensitivity to inhibition by zinc, copper and ferrous ions. In comparison with the oxidized form, the reduced form of the enzymes had a much greater temperature instability, and was inhibited by *N*-ethyl maleimide.

INTRODUCTION

Glutathione has a widespread occurence in plants, and in other living organisms, and several roles for the compound have been suggested (for review see ref. [1]). Functions such as maintenance of the reduced state of proteins and other cell components, promotion of protein synthesis, and detoxification of H_2O_2 in chloroplasts all require a high proportion of the reduced form [1]. This is maintained by the enzyme glutathione reductase (EC 1.6.4.2) which converts the oxidized dimer (GSSG) to the reduced monomer (GSH). GSSG reductase from seedlings and leaves has been studied for a number of plant species [2–5]. In the leaf, a high proportion of the enzyme has been localized in the chloroplast [6, 7], and this is consistent with the presence of high levels of GSH in the organelle [7, 8], and its postulated role in detoxification of H_2O_2 [7, 9]. Some differences in properties of different preparations of leaf and chloroplast enzyme have been noted [10].

There is no detailed information about glutathione metabolism or GSSG reductase in the plant root. Therefore, we have investigated the glutathione levels and GSSG reductase of pea roots, as this may indicate the importance of GSH and glutathione function in the tissue. We have also purified the chloroplast enzyme, for comparison both with the root enzyme and with the preparations described by other workers.

RESULTS AND DISCUSSION

Distribution of glutathione and GSSG reductase in peas

Table 1 shows the content of reduced and oxidized glutathione in various tissues of 3-week-old pea plants. Levels of total glutathione in mature leaves (about 0.4 mM) were within the range of previously reported values [1]. Higher concentrations were found in growing leaves and apex, and some of this may be derived by transport from mature leaves [11]. About 90% of the total glutathione in the leaf is in the reduced form (94.5% on a molar basis). This is similar to the proportion of GSH reported for spinach chloroplasts [4]. GSSG reductase was also found in highest amounts in the young leaf tissues. This activity, accompanied by high levels of glutathione and a high GSH/GSSG ratio are consistent with the view that GSH has an important function in maintaining intensive protein synthesis [12].

On a fresh weight basis, lower levels of glutathione were present in the roots, but when expressed on a protein basis the values were as high as in the growing leaves. About 82% of the total was present as GSH, and an active GSSG reductase was present with an activity similar to that in leaves.

Enzyme purification

Purification of pea chloroplast and root GSSG reductases (Tables 2 and 3) achieved final specific activities of 2000 and 1780 nkat per mg protein respectively, with about 18% final recovery. Gel electrophoresis of the chloroplast enzyme showed one major and one minor band of protein: only the major band had GSSG reductase activity. The root enzyme gave one major band and two minor bands of protein, and again activity was associated only with the major band. The specific activity of both enzymes was close to that of spinach leaf [5] and rice embryo [13] GSSG reductase, about half that of the yeast [14] or rat liver [15] reductase, and five times higher than that reported previously for pea chloroplast reductase [10].

Molecular weight

Sephadex G-200 filtration indicated a similar M_r of around 135000 for both root and chloroplast GSSG reductase. A dimer structure and M_r ranging from 100000 to 145000 has been reported for the enzyme from yeast [14], rice embryo [13] and spinach leaf [4, 5]. In contrast, pea chloroplast enzyme was reported to be a tetramer of $\alpha_2\beta_2$ form with an M_r of 156000 [10].

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Table 1. Activity of glutathione reductase and glutathione levels in tissue of 3-week old pea plants

Tissue	GSSG-reductase			Glutathione content			Molar ratio [GSH]/[GSSG]
	(nkat /g fr. wt)	(nkat /mg protein)	Total (μg/g fr. wt)	Total (μg/mg prot)	GSH (μmol/g fr. wt)	GSSG (μmol/g fr. wt)	
Growing apex	57.5 ± 1.19	2.45 ± 0.08	508 ± 10.3	21.6 ± 1.07	1.48 ± 0.03	0.085 ± 0.002	17.4
Leaf, half-expanded	38.5 ± 0.82	1.78 ± 0.06	353 ± 9.1	16.4 ± 0.39	1.02 ± 0.02	0.064 ± 0.002	15.9
Leaf, mature	12.8 ± 0.82	0.92 ± 0.03	124 ± 5.5	8.8 ± 0.34	0.35 ± 0.015	0.025 ± 0.002	14.0
Root	4.7 ± 0.14	0.97 ± 0.04	96 ± 3.6	19.9 ± 0.95	0.25 ± 0.01	0.028 ± 0.001	9.2

Mean values for three experiments, ± standard error of mean.

pH effects

The pH optimum for root GSSG reductase was 7.7, with 50% activity at pH 6.1 and 9.1. The chloroplast enzyme had a broader optimum at pH 8.2, with 50% activity at 6.9, and 83% activity at pH 9.2 (measured in phosphate buffer). The relatively high optimum for chloroplast GSSG reductase suggests an adaptation to the pH of the stromal environment, and is similar to that of some other leaf or chloroplast preparations [4–6], but differs from that of another pea chloroplast study [10]. The pH optimum for the root enzyme is near to the values reported for animals [15] and yeast [16].

Kinetic properties

Data for root and chloroplast preparations are compared in Table 4. Both showed high affinity for GSSG and NADPH; the affinity of root enzyme for GSSG was about 3-fold higher than that of chloroplasts. In general the values were similar to those reported for most plant preparations compared in ref. [10], except for the spinach leaf enzyme which had an affinity for GSSG which was about 5–10-fold lower [4, 5].

The ratio of maximum enzyme velocity (estimated at saturating substrate concentration, 2 mM $NADP^+$, 30 mM GSH) for the forward–reverse directions was about 7:1 for both enzymes. When lower substrate concentrations were used, a ratio of 75:1 was obtained for spinach leaf enzyme [4]. This ratio, combined with the low affinities for GSH and $NADP^+$, confirms that the forward reaction will be greatly favoured under physiological conditions, and that the role of the enzyme is to maintain the glutathione of the cell in the reduced state.

Enzyme specificity

Both enzymes were capable of utilizing NADH as a substrate. However, in comparison with the activity obtained with NADPH, the activity with 0.1 mM NADH for root enzyme was under optimal conditions about 5%, but the chloroplast enzyme showed barely detectable (1.1%) activity with NADH. Maximum NADH-dependent activity for both enzymes was at pH 7.2–7.4. The specificity of the chloroplast GSSG reductase for NADPH is similar to that obtained for enzyme from pea and spinach leaf [4, 6], while the root enzyme specificity is nearer to that of animal preparations [15]. Neither enzyme showed activity when GSSG was replaced with L-cystine, cystamine or lipoic acid, as found by other workers [4, 5, 15].

Effect of inhibitors

The effects of several potential inhibitors, sulphydryl reagents and heavy metals, on the two pea enzymes was compared. Table 5 shows that both enzymes were strongly inhibited by *N*-ethyl maleimide, when pretreated for 3 min in the presence of NADPH. However, inhibition was not observed with preincubation in the absence of NADPH, even at 0.5 mM NEM. These results are in agreement with previous reports [2, 16] and indicate that a sulphydryl group is generated on the enzyme when it reacts with NADPH. Both pea enzymes had similar sensitivity to thiol reagents, but showed differences in response to heavy metals. Zinc (1.5 μM) gave only 8%

Table 2. Purification of glutathione reductase from pea chloroplasts

Step	Vol. (ml)	Total protein (mg)	Total activity (nkat)	Sp. act. (nkat/mg)	Purification (fold)	Recovery (%)
1. Chloroplast extract	1000	2200	4199	1.9	1	100
2. Ammonium sulphate	50	312	4016	12.8	7	95
3. Heat treatment	45	122	4099	33.6	18	97
4. Acetone	25	44	3460	78.6	41	82
5. First Sephadex G-200	20	11	2116	192.4	101	50
6. First DEAE Sephadex	15	1.4	1350	964.3	505	32
7. Second DEAE Sephadex	10	0.7	1050	1500	785	25
8. Second Sephadex G-200	15	0.4	800	2000	1047	19

Table 3. Purification of glutathione reductase from pea roots

Step	Vol. (ml)	Total protein (mg)	Total activity (nkat)	Sp. act. (nkat/mg)	Purification (fold)	Recovery (%)
1. Crude extract	2000	4700	3000	0.73	1	100
2. Ammonium sulphate	50	570	2700	4.7	6	90
3. Heat treatment	45	189	2616	13.8	19	87
4. Acetone	25	64	1716	26.8	36	57
5. First Sephadex G-200	20	16	1350	84	115	45
6. First DEAE-Sephadex	15	2.2	983	447	612	33
7. Second DEAE-Sephadex	10	1.0	717	717	982	24
8. Second Sephadex G-200	15	0.3	533	1778	2435	18

Table 4. Apparent K_m values for purified pea chloroplast and root glutathione reductase

Substrate	K_m (M) Chloroplast enzyme	K_m (M) Root enzyme
GSSG	2.8×10^{-5}	1.0×10^{-5}
NADPH	2.7×10^{-6}	2.3×10^{-6}
GSH	7.7×10^{-3}	8.2×10^{-3}
$NADP^+$	2.2×10^{-4}	2.1×10^{-4}

Table 5. Inhibition of glutathione reductase from pea chloroplasts and roots

Inhibitor	Concentration (μM)	Inhibition (% loss of activity) Chloroplast	Root
$ZnCl_2$	1.5	8	85
$CuSO_4$	10	15	75
$FeCl_2$	10	12	45
p-Hydroxymercuribenzoate	0.05	78	74
N-Ethyl maleimide	100	86	90

Enzymes were dialysed for 12 hr to remove EDTA, then incubated in Tris–HCl buffer, pH 8.0 for 3 min with 0.1 mM NADPH and the inhibitor at 20°. The reaction was then started by addition of GSSG.

inhibition of chloroplast GSSG reductase, whereas there was a loss of 85% of the activity of the root enzyme, and there were also differences with copper and iron. The differences between the two enzymes could result from metal ion effects at sites other than at the reactive sulphydryl groups thought to be involved in enzyme activity [2, 16]. However, the oxidized form of the enzyme was quite insensitive to metal ion inhibition, so the differences between chloroplast and root enzymes could reflect differences in the environment of the reactive sulphydryl groups. Magnesium, manganese or calcium (0.2 mM) had no inhibitory effect on either enzyme.

Both enzymes showed some end product inhibition with GSH, giving at 5 mM an inhibition of 25% (chloroplast) or 35% (root). The concentration of GSH in the chloroplast has been estimated to be up to about 4 mM [7, 8], although our observations show about half this concentration in pea chloroplasts [Bielawski and Joy, in preparation]. This suggests that end product inhibition by GSH is unlikely to play any major regulatory role *in vivo*.

Enzyme stability

Thermal stability of root and chloroplast GSSG reductase was strongly dependent on the state of enzyme

reduction, and also on pH. Figure 1A shows the changes in activity produced by incubation of the reduced and oxidized form of the enzyme for 30 min at different temperatures. The reduced form was highly unstable, even at 30°, while the oxidized form remained stable at 60–65°. In each treatment, the root enzyme was more stable than the chloroplast enzyme. The time course for inactivation at 70° of the enzyme alone (without GSSG or NADPH), or the oxidized form at two pH values is shown in Fig. 1B. Even in the absence of NADPH, the enzyme was less stable without GSSG, suggesting that the purified enzyme is at least partially in the reduced state. At pH 6, the enzymes showed a somewhat more rapid initial inactivation, and again the root enzyme was more stable. The reduced enzyme was also more unstable at pH 6 (Fig. 1C), with almost complete loss of activity in only 5 min at 40°. Inactivation by NADPH and stabilization by GSSG have also been reported for mouse liver [17] and yeast [18] enzyme, and this has been attributed to erroneous disulphide bridging [18]. In both reports, inactivation caused by NADPH could be reversed by GSSG, but in the present study reactivation was not observed with either pea enzyme. The observation that spinach leaf enzyme activity was decreased by addition of NADPH prior to assay [4] appears to be due to similar inactivation of the reduced form of the enzyme.

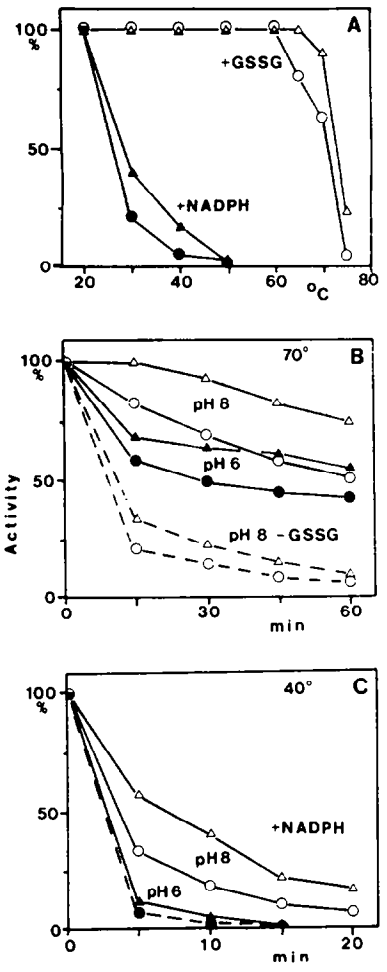

Fig. 1. Heat inactivation of glutathione reductase purified from pea chloroplasts (circles) and roots (triangles). All incubations were in 0.1 M phosphate buffer at the pH stated, and included 1 mM EDTA. (A) Enzymes held at the indicated temperature for 30 min, in presence of 2 mM GSSG (open symbols) or 0.1 mM NADPH (closed symbols); (B) Enzymes held at 70° for the indicated times in the presence of 2 mM GSSG at pH 8 (solid line, open symbols), pH 6 (closed symbols), or in absence of GSSG (dashed line); (C) Enzymes held at 40° for the indicated times in the presence of 0.1 mM NADPH, at pH 8 (open symbols) or pH 6 (closed symbols). 100% values were set as the activity of a sample removed at zero time, immediately after the addition of the enzyme to the appropriate incubation mixture, at the start of the temperature treatment. All activities were assayed at 20° immediately after treatment.

Conclusions

In general, the properties of the highly purified GSSG reductase from chloroplasts, described here, are similar to those of the enzymes from leaf or chloroplasts (including pea [6]) as discussed above. It is not clear why the properties of a pea chloroplast enzyme in a second report [10] were somewhat different in a number of respects, including pH optimum, effective inhibitory concentrations of *N*-ethyl maleimide and M_r. It is possible that exposure to different conditions during extensive purification may cause some alteration in enzymic properties. The considerable differences (in stability and sensitivity to inhibitors) between oxidized and reduced enzyme forms indicate that the redox state of the protein, and even order of addition of substrates [4], must be taken into consideration in any investigation of GSSG-reductase. The greater stability of the oxidized enzyme suggest that addition of GSSG during purification should be investigated as an aid to improving recovery. In a preliminary trial with root enzyme, recovery of activity in the heat treatment step was improved by up to 15% by inclusion of 2 mM GSSG during heating.

Pea roots contain substantial amounts of glutathione and an active GSSG reductase. This enzyme is not identical to the chloroplast enzyme, differing in pH optimum, sensitivity to heavy metal inhibition, relative activity with NADH and thermostability. Some of the glutathione in the root may be present as a transport or storage form of sulphur, as roots may lack the capacity to reduce sufficient sulphate for their own requirements and import glutathione from leaves [11]. However, the highly reduced state and activity of GSSG reductase suggest that in the root, GSH may also have a role in processes such as protein synthesis and maintenance of the reduced status of proteins and other components.

EXPERIMENTAL

Chemicals. Biochemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Plant material. Pisum sativum L. (cv. Little Marvel) seeds were germinated in vermiculite and grown hydroponically under conditions described previously [19]. Plants were used when *ca* 3 weeks old.

Enzyme purification. Throughout, buffers contained 1 mM EDTA, and all operations were carried out at 0–4°. Crude root extract: 1 kg of pea roots was washed in distilled water and ground in a Waring blender in 1 l. 0.1 M Pi buffer, pH 7.5. The

homogenate was pressed through four layers of cheesecloth and centrifuged at 27000 *g* for 20 min. Chloroplast crude extract: batches of mature leaves (2 kg total) were cut into chloroplast extraction medium (ratio 1:4, fr. wt:vol) containing 330 mM Sorbitol and 50 mM Tricine–KOH buffer, pH 7.8. Small batches were homogenized for 5 sec in a Waring Blender. The homogenate was filtered through two layers of cheesecloth and centrifuged at 250 *g* for 1 min. The resulting supernatant was then centrifuged at 2500 *g* for 1 min and the resulting chloroplast pellet was washed twice with extraction medium, spun down as before and then burst by resuspension in 0.05 M Pi buffer, pH 7.5 (vol. equivalent to 0.5 × fr. wt). After 5 min the suspension was centrifuged at 27000 *g* for 20 min and the supernatant obtained was used for further enzyme purification. The plant extracts were fractionated by addition of solid $(NH_4)_2SO_4$, and protein precipitating between 45% and 60% satn (278–390 mg/ml) was collected by centrifugation at 27000 *g* for 20 min, then dissolved in 50 ml 0.1 M phosphate buffer pH 8.0. This soln was heated in a water bath at 60° for 10 min then cooled to 4° and centrifuged as above. The supernatants from the heat treatment step was cooled to −4° (ice–NaCl), and cold Me_2CO (−21°) was added slowly while stirring until the concn of Me_2CO was 50%. The Me_2CO ppt was collected and resuspended in 25 ml Tris–HCl buffer, 50 mM, pH 8.0. The suspension was placed on a Sephadex G-200 column (2.5 × 100 cm) and eluted at a rate of 10 ml/hr with the same buffer. Active fractions from Sephadex G-200 were pooled and applied to a DEAE-Sephadex column (1.5 × 30 cm) equilibrated with 25 mM Tris–HCl buffer pH 8.0. Elution was effected by a linear NaCl gradient, 0–0.4 M, in the equilibration buffer Enzyme fractions (eluting at about 0.2 M NaCl) were collected and dialysed overnight against equilibration buffer. The preparation was rechromatographed by the same procedure and the most active fractions were pooled then protein was precipitated by addition of $(NH_4)_2SO_4$ (519 mg/ml). The ppt was dialysed in 50 mM Tris–HCl buffer, pH 8.0. This enzyme preparation was applied to a column (2.5 × 100 cm) of Sephadex G-200, equilibrated and eluted with 50 mM Tris–HCl buffer pH 8.0. Fractions containing the highest enzyme activity were combined and concd using $(NH_4)_2SO_4$ as described above. The final preparation was stable for at least 3 weeks at 0–2°.

Glutathione reductase assay. Enzyme activity was measured at 20° by decrease in absorbance at 340 nm due to NADPH oxidation. Reaction mixtures contained, in a vol. of 1 ml, the following final concns: GSSG (1 mM), NADPH (0.1 mM), EDTA (0.1 mM) and Tris–HCl buffer pH 8.0 (0.1 M). For crude extracts the reaction was started by GSSG addition but by NADPH addition for highly purified preparations.

Assay of glutathione. Glutathione was extracted and determined according to method of Tietze [20] as modified by Smith *et al.* [21]. Acid extraction prevented enzymic modification of glutathione during extraction. No precautions were taken to prevent autoxidation of GSH, which would increase the proportion of GSSG, but less than 2% of the GSH appears to be oxidized in this procedure [21]. The standard incubation mixture contained: 0.7 ml 0.1 M Pi buffer, pH 7.5 (containing 1 mM EDTA), 50 μl 20 mM 5,5'-dithiobis-(2-nitrobenzoic acid) dissolved in EtOH, 0.1 ml 2 mM NADPH, 0.1 ml standard (20–200 ng GSH or GSSG) or plant extract. The components were equilibrated in a cuvette for 3 min and reaction started by the addition of 50 μl (1 unit) of yeast glutathione reductase. The increase in absorbance at 412 nm was measured. After assay, each sample was standardized by addition of a known amount of GSH or GSSG followed by reassay.

Electrophoresis. Disc electrophoresis was performed in glass tubes (9 × 0.6 cm) essentially according to methods of Ornstein [22] and Davies [23]. All solns used in electrophoresis contained 1% (v/v) 2-mercaptoethanol to prevent formation of broad protein bands. Protein (10–50 μg) was applied to the gel and electrophoresis carried out for 2 hr, at 3 mA per tube. Protein was detected using Coomassie brilliant blue. Gels were stained for glutathione reductase activity by procedure of Kaplan [24].

Other methods. The protein content of the various enzyme preparations was determined by the method of Bradford [25].

M_r of the purified pea GSSG-reductase was estimated by Sephadex G-200 filtration based on the procedure of Andrews [26]. A Sephadex G-200 column (100 × 2.5 cm) was equilibrated with 50 mm Tris–HCl buffer, pH 8.0, containing 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA. Prior calibration of the column was with the following protein standards: β-amylase (200000), alcohol dehydrogenase (150000), albumin (66000), carbonic anhydrase (29000) and cytochrome *c* (12400).

Acknowledgement—The work was supported by a grant to KWJ from NSERC Canada.

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