Glutathione Reductase in Wheat Grain. 1. Isolation and Characterization

Frédéric de Lamotte, Nicolas Vianey-Liaud, Marie-Pierre Duviau, and Károly Kobrehel*

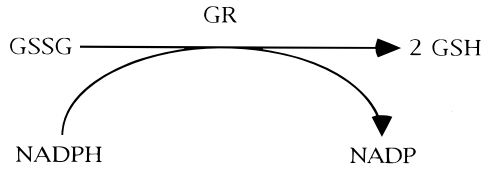
Unité de Biochimie et Biologie Moléculaire des Céréales, INRA, 2 place Viala, 34060 Montpellier Cedex 01, France

Durum wheat (*Triticum durum*, Desf.) endosperm of mature kernels contained a single form of glutathione reductase (GR); it appeared about the 18th day after anthesis while another isoform, present at the early stages of grain development, disappeared between the 20th and 30th days after flowering. The form that was present at grain maturity was isolated and characterized. It was composed of two monomers, each one having an apparent molecular mass of about 60 kDa. The K_m values for NADPH and for GSSG were 3.7 and 9.1 μM, respectively, and the V_m values for NADPH and for GSSG were 594 and 575 μkat·mg^{-1} protein, respectively. The pH_i of the enzyme was situated between pH 4.4 and 4.5. At a constant temperature of 25 °C, the optimum GR activity was found to be between pH 7.5 and 8.0. It was relatively resistant to high temperatures and was very resistant to very low temperatures.

Keywords: *Glutathione reductase; wheat; glutathione; enzyme; proteins*

INTRODUCTION

The biochemical function of glutathione reductase (GR)(EC 1.6.4.2) is to reduce the oxidized form of glutathione (GSSG). The reaction requires the presence of NADPH, as shown below:



In fact, GR reduces the disulfide bond that links together two molecules of reduced glutathione (GSH), which is a tripeptide (g-L-glutamylcysteinylglycine).

The physiological role of GR in the cell is not yet clearly established, but, obviously, it is related to the function of glutathione. Glutathione was shown to be involved in the regulation of protein synthesis (Alscher, 1989; Rennenberg, 1982) as well as in the defense mechanisms of plants against different stresses (Levitt, 1980; Smith et al., 1990; Alscher, 1989).

In wheat kernel, glutathione occurs in three different forms: oxidized, reduced or linked to proteins (GS-Pr) (Sarwin et al., 1992; Schofield and Chen, 1995), and it seems to have a considerable effect on the characteristics of dough during wheat processing (Coventry et al., 1972; Bloksma, 1974). Accordingly, GR should also be involved in all functions, both physiological and technological, where glutathione is involved. In this regard it seemed warrant to undertake a study of wheat GR. Here we present the results on GR isolation from durum wheat endosperm and on the characterization of the purified enzyme.

* To whom correspondence should be addressed. Tel: 33 4 99 61 23 88. Fax: 33 4 99 61 23 48. E-mail: kobrehel@ensam.inra.fr.

MATERIALS AND METHODS

Plant Materials. Glutathione reductase (GR) was isolated from a semolina of durum wheat (*Triticum durum*, Desf.), cultivar Primadur. Semolina was obtained in a pilot mill.

Reagents and Chemicals. All chemicals were obtained from commercial sources. Nicotinamide adenine dinucleotide phosphate (NADPH), alkaline phosphatase, nitro blue tetrazolium (NBT), bromochloroindolyl-phosphate-*p*-toluidine salt (BCIP), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), polyoxiethylene-sorbitan monolaurate (Tween 20), reduced glutathione (GSH), oxidized glutathione (GSSG), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) came from Sigma−Aldrich Co. (St. Louis, MO); sodium dodecyl sulfate (SDS), acrylamide and bisacrylamide from BDH (BDH ltd, Poole, England); ammonium persulfate, *N*,*N*-dimethylformamide (DMF) and 2-mercaptoethanol (2-ME) from Merck Chemical Co. (Darmstadt, Germany). All chemicals were of the highest quality available.

Protein Extraction and Determination. Wheat proteins were extracted from semolina with a 25 mM Tris-HCl buffer, pH 7.5, containing EDTA (1 mM) and 2-ME (10 mM). The semolina/buffer ratio was of 1:3 g/mL. The mixture was stirred for 2 h at 4 °C and then centrifuged at 4 °C for 15 min at 3800*g*.

The protein content of the extracts was determined with the Bradford micromethod (Bradford, 1976), using BSA as a standard.

GR Activity Measurement. GR activity was determined spectrophotometrically (Kontron Uvikon 940). The oxidation of NADPH was monitored at 340 nm at 25 °C. The method described by Conn and Vennesland (1951) was modified as follows. A 100 μL portion of protein extract, containing various but determined amount of protein, was added to 25 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 1 mM GSSG, and 100 μM NADPH (final concentrations). The final volume was 1 mL. Total activity was expressed in μkatals and specific activity in μkat·mg^{-1} protein.

Electrophoresis of Proteins. SDS-PAGE analyses were performed according to Laemmli (1970), the acrylamide concentration of the stacking and the separating gels were 4% and 17.5%, respectively. Non-denaturing (native) polyacrylamide gel electrophoresis (PAGE) analyses (without SDS) were performed by using separating gels of 7.5% and the migration

10.1021/jf0003808 CCC: $19.00
Published on Web 09/07/2000

buffer was completed with 10 mM 2-ME to avoid protein aggregation.

GR Activity Detection on PAGE. The revelation of GR activity, after electrophoretic separation of the proteins in non denaturing conditions on polyacrylamide gel, was performed by using two different methods as described by Kaplan (1968) and by Foyer et al. (1991), respectively.

Determination of pH_i. A chromatofocalization method was used on a Poly Buffer Exchange (PBE) column (Amersham Pharmacia-Biotech) under the conditions recommended by the supplier.

Western Blots. Polyclonal GR antibodies were raised in rabbits with durum wheat GR, which was isolated in this study. GR was detected on nitrocellulose membranes after Western blotting. All operations were carried out at room temperature unless otherwise stated. Following electrophoresis, transfer to nitrocellulose (Protran BA 79 0.1 μm, Schleicher et Schuell GmbH, Dassel, Germany) was carried out with a Bio-Rad system with 25 mM Tris, 140 mM glycine, supplemented with 20% methanol as transfer buffer. After transfer, nitrocellulose was incubated overnight at 4 °C in TBS-Tween (20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0,1% Tween 20) containing 1% BSA. The membrane was then incubated for 3 h with the rabbit antibody against wheat GR (1:5000 dilution) in a solution containing 1% BSA in TBS-Tween and then washed for 3 × 10 min in TBS-Tween containing 1% BSA. The membrane was then incubated again for 1 h with secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate from Sigma−Aldrich). After this step, the membrane was washed twice in TBS-Tween containing 1% BSA. The alkaline phosphatase reaction was developed in 10 mL of revealing buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl), 33 μL NBT (100 mg/mL in 70% DMF), and 33 μL BCIP (50 mg/mL in 70% DMF).

Chromatographic Separations of Proteins. After ammonium sulfate fractionation (Figure 1: 0−40%, 40−80% saturation in extraction buffer) and dialysis against buffer A (30 mM Tris-HCl, pH 7.5, containing 1 mM EDTA) several chromatographic techniques were used in order to achieve GR purification. For the first chromatographic separation a 2.5 cm × 25 cm Q-Hyper-D (Sepracor) column was used. The loaded column was washed with buffer A, then proteins were eluted in three steps (with 200 mL KCl 75 mM, with 500 mL KCl gradient from 75 to 200 mM and with 200 mL KCl 1 M, all these solutions in buffer A) at a 200 mL/h flow. Fractions containing GR were dialyzed against buffer A and loaded on a Affi-Gel, Blue (Bio-rad) column (2.5 cm × 25 cm). Proteins were eluted with a linear gradient of NaCl in buffer A (750 mL, from 0 to 1 M NaCl) at a 50 mL/h flow. GR containing fractions were dialyzed against buffer B (100 mM KH_2PO_4, pH 7.0, 10 mM 2-ME) and loaded on a Phenyl-Sepharose (Amersham Pharmacia Biotech) column (1.5 cm × 20 cm), previously equilibrated with buffer B containing 1.4 M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient (560 mL, from 1.4 to 0 M $(NH_4)_2SO_4$) at a 80 mL/h flow. The active fractions were dialyzed against buffer A. All these chromatographic separations were carried out at 4 °C. The last purification step was achieved at 25 °C with a Q8HR (Waters) column (0.5 × 5 cm). Proteins were eluted with a linear gradient of KCl in buffer A (120 mL, from 0 to 2 M KCl) at a 60 mL/h flow.

RESULTS

Isolation of GR. The scheme of successive steps of GR isolation from durum wheat semolina is presented in Figure 1. The amount of protein extracted was 6.2 mg·g^{-1} of semolina, and the total GR activity in the crude extract was 3.0 μkat·g^{-1} of semolina, which corresponded to a specific GR activity of 0.4 μkat·mg^{-1} of protein (Table 1). The specific activity of the supernatant, obtained after precipitation of the crude extract with 40% ammonium sulfate, increased to 0.5 μkat·mg^{-1} of protein. At that step about 37% of proteins of the crude extract were eliminated.

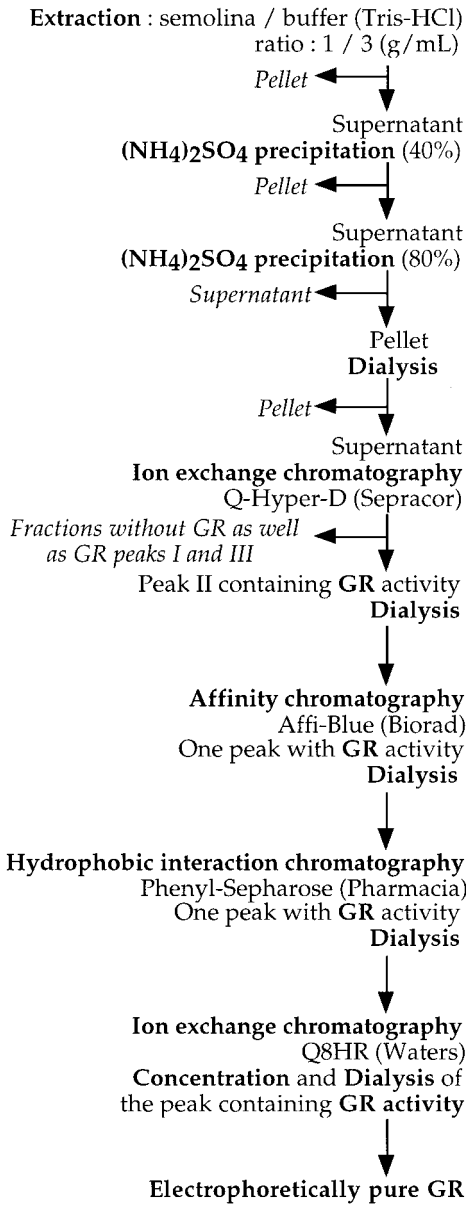


Figure 1. Scheme of GR isolation from durum wheat semolina.

Table 1. GR Isolation Steps from Durum Wheat Endosperm

purification steps	protein mg.g^{-1} semolina	activity total μkat.g^{-1} semolina	activity specific μkat·mg^{-1} protein	recovery (total activity, %)
crude extract	6.2	3.0	0.4	100
precipitation (40%)	3.9	2.0	0.5	66
precipitation (80%)	2.1	1.8	0.9	59
Q-Hyper-D	0.02	1.5	65.3	50
Affi-Gel	2.4×10^{-3}	0.7	305.5	23
phenyl-seph.	5.5×10^{-4}	0.3	592.8	10
Q8HR	2.0×10^{-4}	0.2	1040.0	7

After precipitation, when the ammonium sulfate concentration of the solution was increased up to 80%, no GR activity could be detected in the supernatant, and it was discarded. The pellet was dissolved and dialyzed, the specific GR activity of the solution was 0.9 μkat·mg^{-1} of protein, and it contained about 34% of the initially extracted proteins. This solution was used for the further isolation of GR by means of chromatographic techniques.

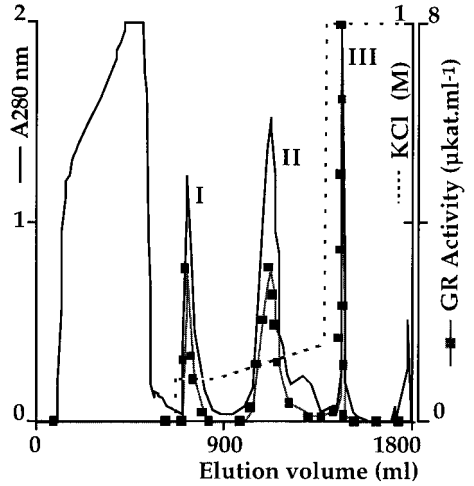


Figure 2. Q-Hyper-D (Sepracor) ion-exchange chromatograph. Ammonium sulfate precipitated proteins (see Figure 1) were eluted in three steps: with KCl 75 mM; with a KCl gradient from 75 to 200 mM and with KCl 1 M; all these solutions in 30 mM Tris-HCl buffer, containing 1 mM EDTA, pH 7.5, at a 200 mL/h flow.

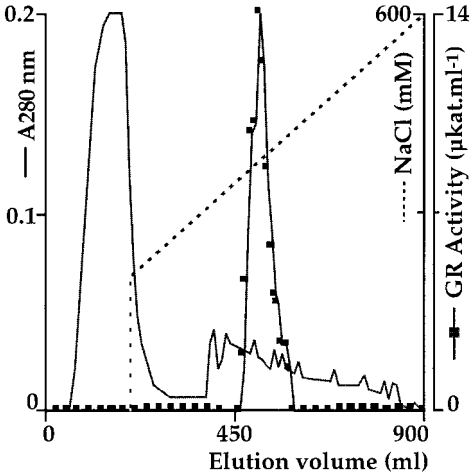


Figure 3. Affi-Blue (Bio-Rad) affinity chromatograph. Proteins were eluted with a linear gradient of NaCl (750 mL, from 0 to 1 M NaCl) in 30 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, at a 50 mL/h flow.

At the first chromatographic step, using a Q-Hyper-D ion-exchange column, three GR activity peaks were obtained (Figure 2). Peaks I, II, and III contained 24, 56, and 20%, respectively, of the total GR activity recovered at that stage. Peak II was saved for further purification while peaks I and III were discarded as well as the tail of the peak II, fractions containing very low GR activity. At each chromatographic step that followed—affinity (Figure 3), hydrophobic interactions (Figure 4), and anion-exchange chromatography (Figure 5)—one single GR activity peak was obtained.

From the beginning to the end of the purification the specific activity increased about 2600-fold, from 0.4 μkat·mg^{-1} in the crude extract to 1040 μkat·mg^{-1} for the purified enzyme. About 7% of the initial total activity of the crude extract was recovered. The yield in protein and in activity at the successive purification steps is summarized in Table 1.

After the last chromatographic step, the isolated GR was electrophoretically homogeneous, it showed a single protein band when submitted to SDS-PAGE analysis (Figure 6).

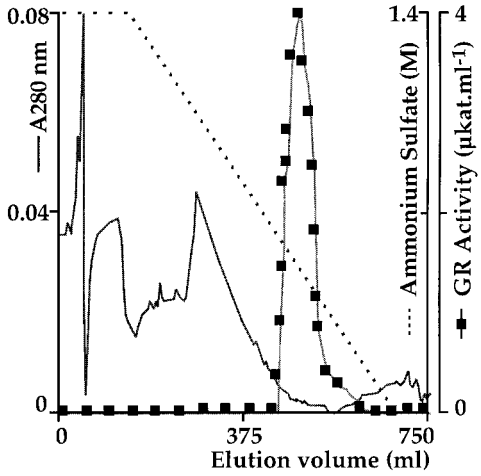


Figure 4. Phenyl-Sepharose (Amersham Pharmacia Biotech) hydrophobic interaction chromatograph. Column equilibrated with a 100 mM KH_2PO_4 buffer, 10 mM 2-ME, pH 7.0, containing 1.4 M $(NH_4)_2SO_4$. Proteins eluted with a linear gradient (560 mL, from 1.4 to 0 M $(NH_4)_2SO_4$) at a 80 mL/h flow.

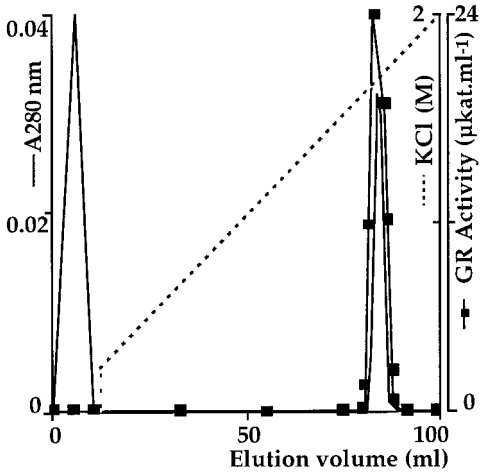


Figure 5. Q8HR (Waters) ion-exchange chromatograph. Proteins were eluted with a linear gradient of KCl in buffer A (120 mL, from 0 to 2 M KCl) at a 60 mL/h flow.

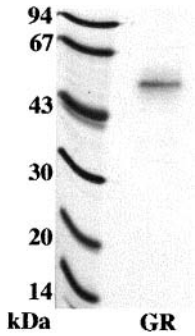


Figure 6. SDS-PAGE analysis of purified glutathione reductase.

Detection of GR with GR Antibodies. GR isolated from wheat endosperm was used to raise polyclonal antibodies in rabbits. The antibodies were purified against the isolated, pure wheat GR before being used for GR detection. The Western blot analysis showed that durum wheat endosperm contained a single GR form (result not shown).

Activity Detection of GR on PAGE. In agreement with the results of SDS-PAGE and Western blot analy-

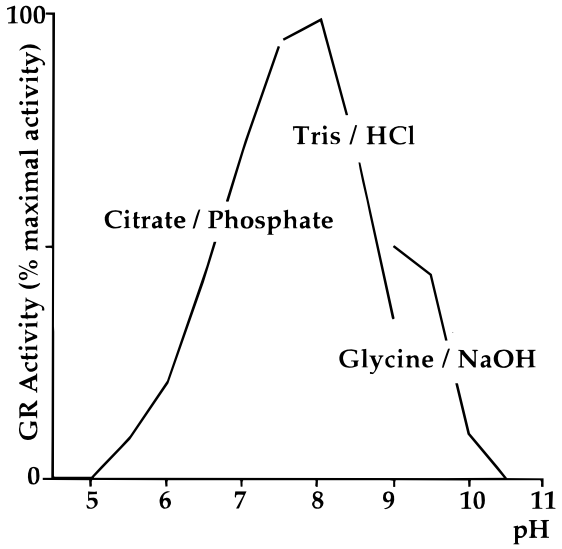


Figure 7. Effect of pH on wheat endosperm GR activity (pH 4.5−7.5: citrate−phosphate 100 mM; pH 7.5−9.0: Tris-HCl 100 mM; pH 9.0−10.5: glycine−NaOH 200 mM).

ses, in wheat endosperm a single GR form was found with both activity detection methods used, as described by Kaplan (1968) and by Foyer et al. (1991), respectively (results not shown). The purified GR yielded also a single GR isoform.

Characterization of the Isolated GR. *Apparent Molecular Mass (MM).* The apparent molecular mass of about 110 and 60 kDa was found by using gel filtration chromatography (Sephadex G-150 from Amersham Pharmacia Biotech) and SDS-PAGE, respectively. SDS-PAGE analysis of the isolated GR fraction yielded a single band (Figure 6), and a single activity peak was obtained at the last purification step of Q8HR ion exchange chromatography (Figure 5). Together, these results suggest that wheat endosperm GR is composed of two subunits, both having a molecular mass of about 60 kDa. Similar results were reported for GR in rice grain and in pea; the former was found to be a 2 × 52 kDa homodimer (Ida and Morita, 1971) and the latter a 2 × 55 kDa dimer (Madamanchi et al., 1992).

Isoelectric pH (pH_i). The chromatofocalization method used showed that the pH_i of isolated GR from durum wheat endosperm is between pH 4.4 and 4.5. This is close to the value reported for pea GR (Madamanchi et al., 1992).

Optimum pH of GR Activity. GR activity was measured between pH 5.0 and pH 10.5 at 25 °C (Figure 7). In agreement with earlier reported results for wheat germ GR (Conn and Vennesland, 1951), the optimum GR activity was found to be between pH 7.5 and 8.0. However, about 10% of the maximum activity was still present both at pH 5.5 and at pH 10.0. This broad range of activity could be of both physiological and technological importance as it ensures GR to remain active during and after various technological treatments.

Determination of K_m and V_m Values. The K_m values of wheat endosperm GR for NADPH and for GSSG were 3.7 and 9.1 μM, respectively. The calculated V_m values for NADPH and for GSSG were 594 and 575 μkat·mg^{-1} protein, respectively. The highest affinity of GR toward NADPH than toward GSSG seems to be a general feature in plants, in our knowledge no exception to this rule has been reported in the literature.

Table 2. pH Effect on Kinetic Constants

	pH 6.5	pH 7.5	pH 8.5
K_m NADPH[a]	8.8	3.9	4.7
V_m NADPH[b]	0.14	0.31	0.22
efficiency (Vm/Km)	0.0159	0.0795	0.0468
K_m GSSG[a]	26.8	10.5	21.5
V_m GSSG[b]	0.15	0.31	0.22
efficiency (V_m/K_m)	0.0056	0.0295	0.0102

[a] μM. [b] μkat·mg^{-1} prot.

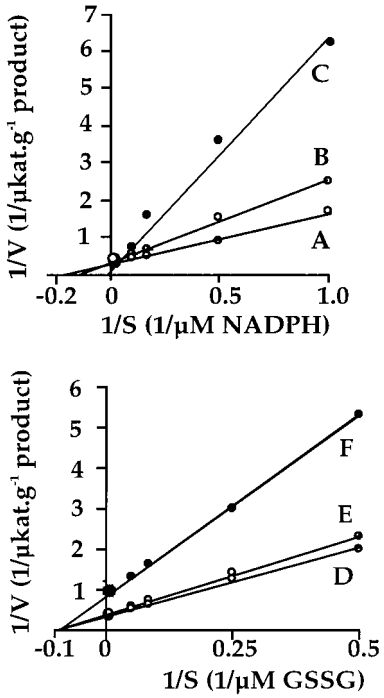


Figure 8. Effect of NADP and of GSH on the K_m and V_m of wheat GR. A, B, and C were obtained by using 0, 0.1, and 1 mM NADP, respectively, and D, E, and F by using 0, 1, and 10 mM GSH, respectively.

To study the effect of pH on K_m and V_m, the pH values of 6.5, 7.5, and 8.5 were chosen (Table 2). The kinetic of the enzyme was Michaelic. The highest affinity toward the substrates—thus the highest efficiency of the enzyme (V_m/K_m)—was found at pH 7.5. At higher and at lower pH than 7.5 the V_m decreased toward both GSSG and NADPH, while K_m increased in both cases. These results are in agreement with those found for corn GR (Turner and Pollock, 1993).

Effect of the Enzyme Reaction Products. GR activity was measured at two concentrations of $NADP^+$ with variable concentrations of NADPH and at two concentrations of GSH with variable concentrations of GSSG. The curves obtained showed that at variable NADPH concentrations $NADP^+$ was a competitive inhibitor, while GSH was a noncompetitive inhibitor toward GSSG (Figure 8). Similar inhibitory effect was already reported both for $NADP^+$ (Wingsle, 1989) and for GSH (Mahan and Burke, 1987; Wingsle, 1989; Sexton and Mutus, 1995).

Influence of Temperature upon GR Activity. Results showed a relatively high resistance of GR to high temperatures (Figure 9). Like GR from pea (Bielawski and Joy, 1986), the oxidized form appeared more heat resistant than the form reduced with 2-ME. In relation to thermoresistance, we found that the activity of wheat GR was not affected by exposure to very low temperatures. Freeze-drying or even repeated freezing and

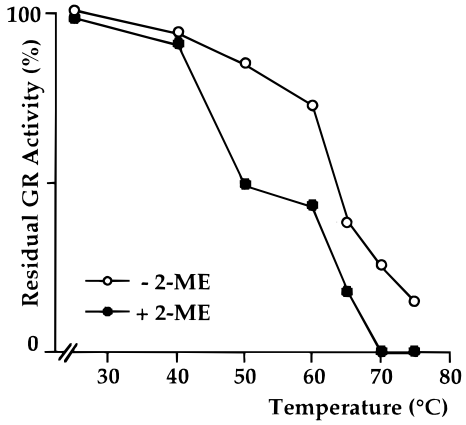


Figure 9. Effect of temperature on the isolated wheat GR activity in the presence and in the absence of 2-ME. GR samples were incubated for 10 min in buffer A with or without 10 mM 2-ME at 25, 40, 50, 65, 70, and 75 °C and then cooled to 4 °C. The GR activity was measured spectrophotometrically after centrifugation 15 min at 14000*g*.

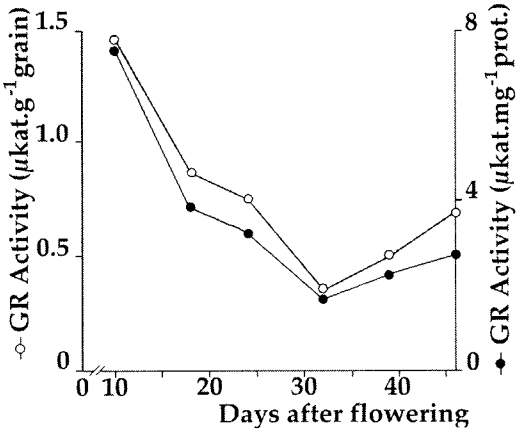


Figure 10. Evolution of total and specific GR activities during wheat grain development (measurements were performed three times).

subsequent thawing of the enzyme did not alter significantly its activity.

GR during Grain Development. The presence of a single GR form in mature wheat endosperm raised the question whether a single GR form exists throughout the wheat grain development. To address this question, samples were harvested at different stages of grain development. The evolution of GR activity was monitored spectrophotometrically, by Western blot analysis and by GR activity detection on native polyacrylamide gel, after electrophoretic separation of the proteins.

The highest GR activity was found at the earliest stage of grain development, when the first measurements, the 10th day after anthesis, were performed. This was followed by a progressive decrease of activity until about the 30th day after anthesis. Then, from that time on, until grain maturity, a slight increase of activity was observed again but without reaching the level of activity found sooner, after anthesis (Figure 10). The last period of time corresponded to the progressive desiccation of the kernels. All along grain development the evolution was similar for both total and specific activities.

The detection of GR activity, by using Western blot, showed the presence of two isoforms (Figure 11). One isoform with strong intensity was revealed at the early

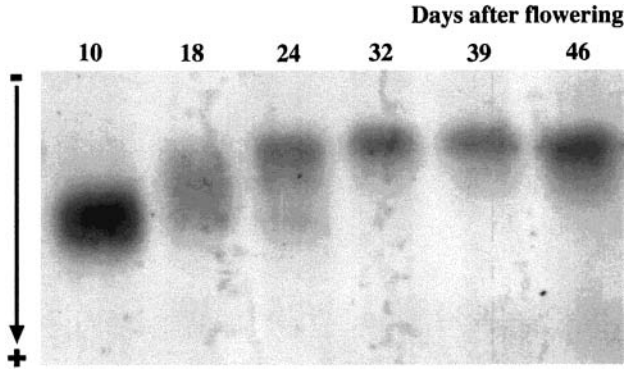


Figure 11. Western blot detection of GR composition during wheat grain development.

stage of grain development. It disappeared between the 20th and 30th days after flowering. The second isoform, which was present at grain maturity and of which isolation and characterization are presented in this work, appeared about the 18th day after anthesis and its intensity increased progressively to reach the maximum at grain maturity.

The activity detection of GR on native polyacrylamide gel, after electrophoretic separation of the proteins, confirmed the results obtained by Western blot analysis. One GR isoform was detected at the earlier period of grain development, and it disappeared progressively while another isoform appeared later which intensity increased until grain maturity. The two methods, described by Kaplan (1968) and by Foyer et al. (1991), respectively, gave similar results (results not presented).

GENERAL DISCUSSION AND CONCLUSIONS

The different approaches used to detect the presence of GR—Western blot analysis and two methods of activity detection on PAGE—concordantly and conclusively showed that, at grain maturity, wheat endosperm contained a single GR form. Results also suggested that the enzyme, like many other plant GRs, was a dimer of similar molecular masses. The detection of three peaks having GR activity, by using the ion-exchange chromatographic column of Q-Hyper-D, were probably due to minor modifications of the enzyme that occurred during the isolation. The fact that wheat endosperm contains a single GR form, implies that varietal differences regarding GR activity could be only quantitative. This could be of technological interest in connection with the role attributed to glutathione during wheat processing. However, varietal differences in this regard are yet to be confirmed.

Altogether, the characterization of the isolated wheat GR showed in many respects—such as apparent molecular mass, optimum pH of activity, pH_i, K_m, and V_m values, resistance to high temperatures—very similar properties to GRs known from different plant sources.

The total and specific GR activities changed in parallel during grain development (Figure 10), suggesting that the two isoforms present at the different stages of grain development were probably two different enzymes that could have different characteristics. Based upon the present results, however, no suggestion can be made regarding possible, different physiological functions for the two forms. From a technological point of view only the second form, which is present in the mature kernel, seems to be of interest. In this regard at least two different characteristics of the wheat endosperm GR are

to be emphasized. Results showed a relatively high resistance of GR to high temperatures and it was especially resistant to very low temperatures, including deep-freezing and thawing. Moreover, wheat endosperm GR remained active at very wide range of pH. All these characteristics can enhance the role played by GR during wheat processing, especially during dough formation. While, in this regard, the study of the role of glutathione has attracted quite a lot of attention, interestingly, the possible involvement of GR has not yet been addressed.

ABBREVIATIONS USED

GR, glutathione reductase (EC 1.6.4.2); GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; EDTA, ethylenediamine tetraacetic acid.

LITERATURE CITED

Alscher, R. G. Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Plant*. **1989**, *77*, 457−464.

Bielawski, W.; Joy, K. W. Properties of glutathione reductase from chloroplasts and roots of pea. *Phytochemistry* **1986**, *25*, 2261−2265.

Bloksma, A. H. Thiol and disulfide groups in dough rheology. *Cereal Chem*. **1974**, *52*, 170−183.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

Conn, E. E.; Vennesland, B. Enzymatic reduction of glutathione by triphosphopyridine nucleotide (coenzyme II). *Nature* **1951**, *167*, 976−977.

Coventry, D. R.; Carnegie, P. R.; Jones, I. K. The total glutathione content of flour and its relation to the rheological properties of dough. *J. Sci. Food Agric.* **1972**, *23*, 587−594.

Foyer, C. H.; Lelandais, M.; Galap, C.; Kunert, K. J. Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. *Plant. Physiol.* **1991**, *97*, 863−872.

Ida, S.; Morita, Y. Studies on respiratory enzymes in rice kernel. Part. VIII. Enzymatic properties and physical and chemical characterization of glutathione reductase from rice embryos. *Agric. Biol. Chem*. **1971**, *35*, 1550−1557.

Kaplan, J. C. Electrophoretic study of glutathione reductase in human erythrocytes and leucocytes. *Nature* **1968**, *217*, 256−258.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Levitt, J. In *Responses of plants to environmental stresses*; Levitt, J., Ed.; Academic Press: New York, 1980; Vol. I, pp 248−249.

Madamanchi, N. R.; Anderson, R. G.; Alscher, R. G.; Cramer, C. L.; Hess, J. L. Purification of multiple forms of glutathione reductase from pea (*Pisum sativum* L.) seedlings and enzyme levels in ozone-fumigated pea leaves. *Plant. Physiol*. **1992**, *100*, 138−145.

Mahan, J. R.; Burke, J. J. Purification and characterization of glutathione reductase from corn mesophyll chloroplasts. *Physiol. Plant*. **1987**, *71*, 352−358.

Rennenberg, H. Glutathione metabolism and possible biological roles in higher plants. *Phytochemistry* **1982**, *21*, 2771−2781.

Sarwin, R.; Walther, C.; Laskaxy, G.; Butz, B.; Grosch, W. Determination of free reduced and total glutathione in wheat flours by an isotope dilution assay. *Z. Lebensm. Unters. Forsch*. **1992**, *195*, 27−32.

Schofield, J. D.; Chen, X. Analysis of free reduced and free oxidised glutathione in wheat flour. *J. Cereal Sci*. **1995**, *21*, 127−136.

Sexton, D. J.; Mutus, B. Glutathione reductases from a variety of sources are inhibited by physiological levels of glutathione. *Comp. Biochem. Physiol*. **1995**, *103*, 197−201.

Smith, I. K.; Polle, A.; Rennenberg, H. Glutathione. In *Stress respons in plants: Adaptation and acclimation mechanisms*, *Plant Biology Series*; Alscher, R. G., Cummings, J. R., Eds.; Wiley-Liss: New York, 1990; Vol. 12, pp 201−237.

Turner, L. B.; Pollock, C. J. The effects of temperature and pH on the apparent Michaelis constant of glutathione reductase from maize (*Zea mays* L.). *Plant. Cell Environ*. **1993**, *16*, 289−295.

Wingsle, G. Purification and characterization of glutathione reductase from scots pine needles. *Physiol. Plant*. **1989**, *76*, 24−30.

Received for review March 23, 2000. Revised manuscript received July 11, 2000. Accepted July 11, 2000.

JF0003808