TÜBİTAK

Research Article

Turk. J. Vet. Anim. Sci.
2012; 36(5): 546-553

doi:10.3906/vet-1103-5

Purification and characterization of glutathione reductase from turkey liver

Pınar TAŞER, Mehmet ÇİFTCİ*
Department of Chemistry, Faculty of Science, Atatürk University, Erzurum - TURKEY

Received: 04.03.2011 ● Accepted: 03.01.2012

Abstract: This study aimed to purify glutathione reductase (GR) from turkey liver and investigate some of its characteristic features. The purification procedure comprised 2 steps: homogenate preparation and 2',5'-ADP Sepharose 4B affinity gel chromatography. Thanks to the 2 consecutive procedures, the enzyme, having the specific activity of 606.67 EU mg protein^{-1}, was purified with a yield of 10.75% and 2476-fold, and, in order to control enzyme purity, SDS-PAGE was done. Optimal pH, stable pH, optimal temperature, optimal ionic strength, molecular weight, turnover number of enzyme, and k_{cat}/K_M and K_M and V_{max} values for NADPH and glutathione disulfide substrates were also determined for the enzyme. In addition, K_i constants and the type of inhibition were determined by means of Lineweaver-Burk graphs obtained for such inhibitors as $NADP^+$ and glutathione. In conclusion, glutathione reductase enzyme was isolated and characterized from turkey liver for the first time, and some of its kinetic properties were determined.

Key words: Turkey, purification, glutathione reductase, liver

Introduction

Glutathione reductase (glutathione:$NADP^+$ oxidoreductase, E.C.1.8.1.7; GR), a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes, catalyzes the reduction of glutathione disulfide (GSSG) to a reduced form (GSH) in the presence of NADPH (nicotinamide adenine dinucleotide phosphate). The enzyme has a crucial role in maintaining a high [GSH]-to-[GSSG] ratio (1). GSH is the major nonprotein sulfhydryl compound in all living organisms and has been shown to be involved in the regulation of protein synthesis and enzyme organization, and in the formation of the deoxyribonucleotide precursors of deoxyribonucleic acid (DNA) (2). Other important properties of glutathione include being a reaction partner for the detoxification of xenobiotics, being a cofactor in isomerization reactions, maintaining the thiol redox potential in cells, and keeping sulfhydryl groups of intracellular proteins in the reduced form (3).

In metabolism, due to electron transport system activity, drugs, radiation, and poisons, oxygen (O_2) turns into superoxide radicals ($O_2^{\cdot}$), and then the superoxide radicals turn into hydrogen peroxide. If hydrogen peroxide is not deactivated, it turns into hydroxyl radicals and these radicals damage the structure of lipids, proteins, and DNA. This is why detoxification of hydrogen peroxide is important. This reaction is catalyzed by glutathione peroxidases in the presence of GSH. While hydrogen peroxide is being reduced to H_2O, GSH is oxidized to GSSG.

* E-mail: ciftcim@atauni.edu.tr

To continue performing this reaction GSSG must be reduced to GSH. GR is necessary for this reduction reaction and also protects cells against oxidative stress as an antioxidant (4).

GR has been purified from many different sources such as rat liver (5), calf liver (6), gerbil liver (7), human erythrocytes (8), and sheep brain (9), and some of its characteristic properties have been determined. In researching the literature, it was found that xanthine dehydrogenase (10), fructose-1,6-bisphosphatase (11), and aspartate aminotransferase enzymes (12) have been purified from turkey liver. In this study, GR was purified from turkey liver and its characteristic features were determined and compared with those of other species.

Affinity chromatography, ion-exchange chromatography, hydrophobic and reversed phase chromatography, and size-exclusive chromatography techniques have been used in order to purify the enzyme (10,13). Affinity chromatography on a 2',5'-ADP Sepharose 4B column has been used for its efficient purification (14,15). For this reason, in this study, we used this technique in order to purify the enzyme within a short time period lasting 7 to 8 h. The kinetic behaviors of the enzyme were also investigated.

Material and methods

Materials

Sephadex G-200, NADPH, GSSG, and protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co., and 2',5'-ADP Sepharose 4B was obtained from Pharmacia. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of the homogenate

After livers were obtained from adult turkeys, they were washed in isotonic saline containing 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (1,4-dithiothreitol), and 1 mM PMSF (phenylmethylsulfonyl fluoride) and stored at −80 °C before use. Fifty grams of liver was first cut into small pieces and powdered. The powder was then homogenized with 50 mL of 50 mM potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 13,000 rpm for 60 min, and the precipitate was removed and the sample was dialyzed (7).

2',5'-ADP Sepharose 4B affinity chromatography

A column (1 × 10 cm) of 10 mL in bed volume was made using 2 g of dried 2',5'-ADP Sepharose 4B. The gel was washed with 300 mL of distilled water to remove foreign bodies and air, suspended in 50 mM K-phosphate buffer containing 1 mM EDTA and 1 mM DTT (pH 6.0), and packed in the column. After precipitation of the gel, the column was equilibrated with the same buffer by means of a peristaltic pump. The flow rates for washing and equilibration were adjusted to 20 mL h^{-1}. The dialyzed sample obtained previously was loaded onto the 2',5'-ADP Sepharose 4B affinity column and the column was washed with 25 mL of 0.1 M K-acetate + 0.1 M K-phosphate, pH 6, and 25 mL of 0.1 M K-phosphate + 0.1 M KCl, pH 7.85. Washing continued with 30 mL of 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.0, until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted with a gradient of 0 to 1 mM GSH and 0 to 0.5 mM NADPH in 50 mM K-phosphate, containing 1 mM EDTA (pH 7.0). Active fractions were collected and dialyzed with equilibration buffer. All of the procedures were performed at 4 °C (7,16).

Activity determination

Enzymatic activity was determined spectrophotometrically with a Shimadzu spectrophotometer (UV-1208) at 25 °C, according to the method described by Carlberg and Mannervik (17). The assay system contained 100 mM K-phosphate buffer, pH 7.5, including 1 mM EDTA, 2 mM GSSG, and 2 mM NADPH. The definition of 1 enzyme unit was taken as the oxidation of 1 μmol of NADPH per minute under the assay conditions.

Protein determination

Quantitative protein determination was measured spectrophotometrically at 595 nm according to the Bradford method, with bovine serum albumin being used as a standard (18).

SDS-PAGE

To control the enzyme purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

carried out according to Laemmli's method (19). The acrylamide concentrations of the stacking and the separating gels were 3% and 8%, respectively, and they contained 0.1% SDS.

Stable pH determination

For stable pH determination, equal volumes of the buffers (K-phosphate at pH 6.0, 6.5, 7.0, and 7.5; and Tris-HCl at pH 7.5, 8.0, 8.5, and 9.0) and purified enzyme were mixed and kept in a refrigerator at 4 °C. The enzyme activity was assayed once at 12 h.

Optimum pH determination

In order to determine the optimum pH, K-phosphate and Tris-HCl buffers were used within pH ranges of 5.5 to 8.0 and 7.5 to 9.0, respectively.

Optimum temperature determination

For the determination of the optimum temperature, enzyme activity was assayed at different temperatures in the range of 0 °C to 80 °C. The desired temperature was provided by using a PolyScience bath (model 9105).

Ionic strength determination

For the determination of the optimum ionic strength, enzyme activity was determined using different concentrations of K-phosphate buffer, pH 7.5, in the range of 10 mM to 1000 mM.

Molecular weight determination

Sephadex G-200 gel filtration

The molecular weight of the enzyme was determined on the basis of Andrew's method (20). The enzyme-containing tube was first determined. The void volume was observed with Blue Dextran 2000. Bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa) were used as standards (Sigma: MW-GF-200).

SDS-PAGE

The subunit determination was made by SDS-PAGE (19). Porcine myosin (200 kDa), rabbit muscle phosphorylase (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa) were used as standards (Sigma: MW-SDS-200).

Kinetic studies

K_M and V_{max} values for NADPH and GSSG substrates were calculated by Lineweaver-Burk curves (21). Activities were assayed at 5 different NADPH concentrations with a fixed concentration of GSSG, and the same experiments were also done at 5 different GSSG concentrations with a fixed NADPH concentration. The effects of $NADP^+$ and GSH, which are products of the enzyme catalyzing reaction, were investigated on enzyme activity. To do this, activities were determined and Lineweaver-Burk graphs were drawn for the determination of K_i and inhibition type for NADPH and GSSG. GSSG and NADPH were used as substrates when the inhibitory effects of GSH and $NADP^+$ were determined, respectively. In addition, activities were measured at high NADPH (0.01, 0.015, 0.02, 0.04, and 0.08 mM) and GSSG (0.01, 0.016, 0.02, 0.024, and 0.03 mM) concentrations to determine the effect of substrates on enzyme activity. All kinetic studies were performed at 25 °C in 100 mM K-phosphate buffer, pH 7.5.

Results

The Table shows the purification characterized by a specific activity of 606.67 EU mg $protein^{-1}$, a yield of 10.74%, and a purification coefficient of 2476. The elution profile of GR activity from 2',5'-ADP Sepharose 4B affinity is also shown in the Table. At the end of the last step, a highly pure enzyme was obtained, which exhibited a single band on SDS-PAGE (Figure 1). The optimum ionic strength of the

Table. Purification steps of glutathione reductase enzyme.

Samples	Total volume (mL)	Activity (EU mL^{-1})	Protein (mg mL^{-1})	Total protein (mg)	Total activity	Specific activity (EU mL^{-1})	% Yield	Purification fold
Homogenate	45	0.734	3	135	33.03	0.245	100	1
Affinity chromatography	19.5	0.182	0.0003	0.00585	3.549	606.67	10.74	2476

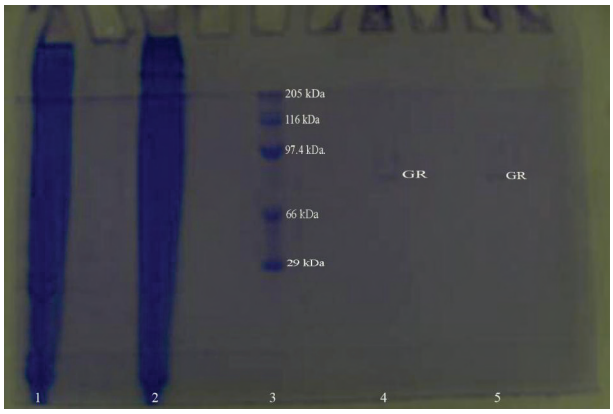

Figure 1. Purification control of homogenate and GR from affinity column by SDS-PAGE. Lines 1 and 2: homogenate, line 3: standard proteins (porcine myosin: 200 kDa, rabbit muscle phosphorylase: 97 kDa, BSA: 66 kDa, ovalbumin: 45 kDa, bovine erythrocytes CA: 29 kDa), lines 4 and 5: GR from affinity column.

enzyme was estimated to be 100 mM in K-phosphate buffer. The enzyme was seen to show the highest activity at 40 °C after being assayed between 0 °C and 80 °C (Figure 2). The optimal pH of GR was determined as 7.5 using 100 mM K-phosphate (Figure 3). The stable pH of the enzyme was 8.5 in Tris-HCl buffer.

The Lineweaver-Burk graphs are shown in Figures 4 and 5, which were constructed for NADPH and GSSG. A K_M of 0.17 mM and a V_{max} of 0.55 EU mL^{-1} were obtained for NADPH, and 0.03 mM and 0.228 EU mL^{-1} were obtained for GSSG. K_i values were also determined by means of Lineweaver-Burk graphs (0.37 ± 0.05 mM and 29.08 ± 14.9 mM for $NADP^+$ and GSH respectively) (Figures 6 and 7).

K_{cat}, or the turnover number of GR, was calculated as 8.290 s^{-1} by use of the V_{max} value for GSSG.

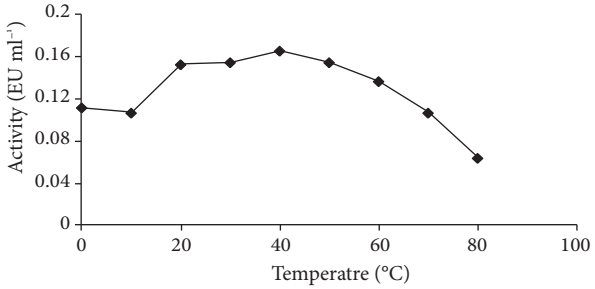

Figure 2. Temperature-activity graph for determining optimum temperature of turkey liver GR.

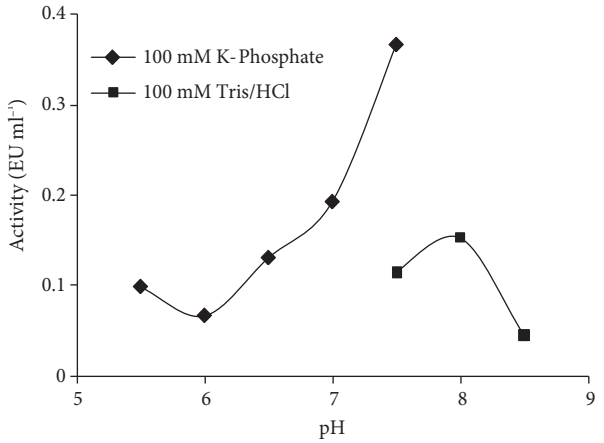

Figure 3. pH-activity graph as a result of optimum pH studies of turkey liver GR.

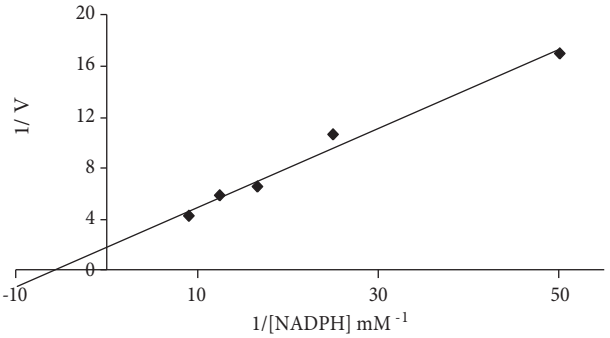

Figure 4. Graph for determining K_M and V_{max} values for NADPH.

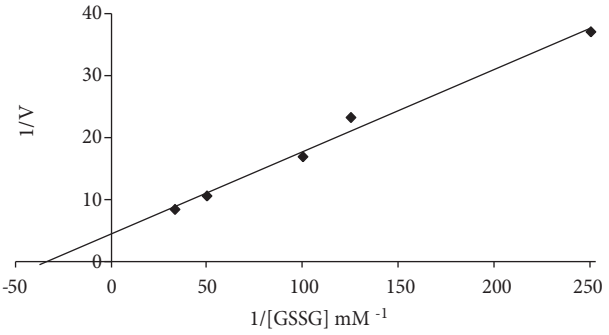

Figure 5. Graph for determining K_M and V_{max} values for GSSG.

Figure 1 exhibits the SDS-PAGE conducted for the purity and molecular weight of the enzyme. For the standard proteins and GR, R_f values were calculated, and an R_f-log M_W graph (Figure 8) was obtained according to Laemmli's procedure (19), showing a

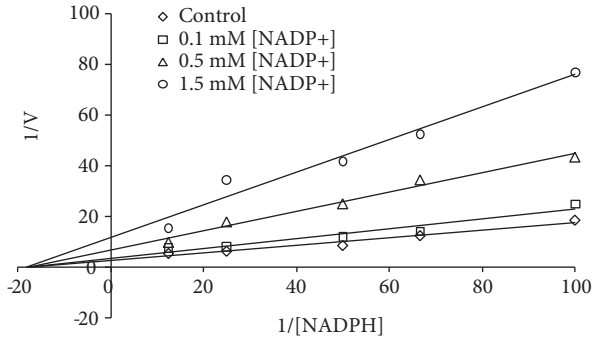

Figure 6. Graph for exhibiting inhibition type of $NADP^+$ on activity of turkey liver GR and determining K_i constant.

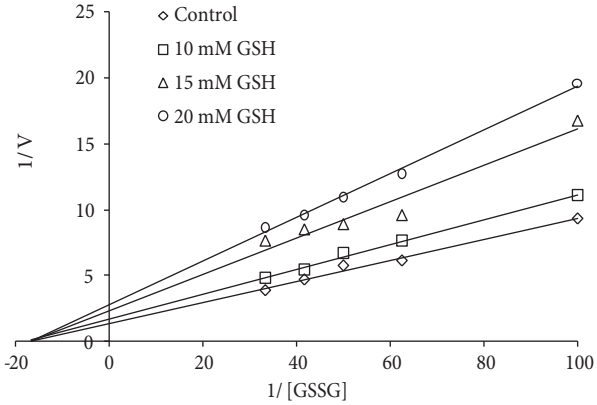

Figure 7. Graph for exhibiting inhibition type of GSH on activity of turkey liver GR and determining K_i constant.

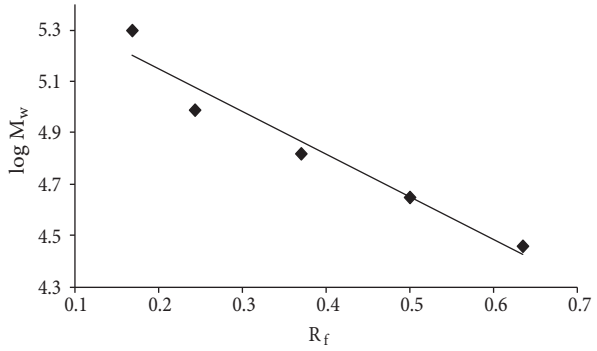

Figure 8. R_f-log M_W graph according to SDS-PAGE results (standard proteins: porcine myosin, 200 kDa; rabbit muscle phosphorylase, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; bovine erythrocytes CA, 29 kDa).

molecular weight of 65 kDa for GR. The molecular weight of the enzyme was also determined by gel filtration. A K_{av}-log M_W graph was obtained (Figure 9), which showed a molecular weight of 131 kDa for GR.

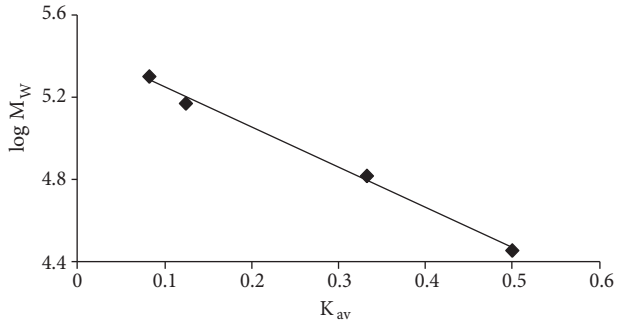

Figure 9. K_{av}-log M_W graph according to gel filtration chromatography results (standard proteins: bovine erythrocyte carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; yeast alcohol dehydrogenase, 150 kDa; sweet potato β-amylase, 200 kDa).

Discussion

The undesirable biological effects of oxidative agents such as free radicals and reactive oxygen species (ROS) are negated by enzymatic and nonenzymatic antioxidant defense systems. Enzymatic defense is provided by many enzyme systems such as GR, glutathione peroxidase, glutathione S-transferase (GST), superoxide dismutase, catalase, aldo-keto reductase, and DNA repair enzymes. Nonenzymatic antioxidant defense systems include many different agents like vitamins, transferrin, ceruloplasmin, lactoferrin, uric acid, taurine, GSH, cysteamine, cysteine, and thioredoxin (22).

GR catalyzes the reduction of GSSG in the presence of NADPH, with the latter being produced in the pentose phosphate metabolic pathway by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. GSH is a product of the reaction catalyzed by GR, and the GSH-related enzyme system is one of the most important protective systems in cells. Reduced glutathione is used in detoxification of xenobiotics, protection of the thiol groups of intracellular proteins, scavenging of H_2O_2 and other organic peroxides, and counteraction of oxidative events. The first step in the detoxification of xenobiotics consists of GSH conjugates owing to their high nucleophilic potency. These reactions are catalyzed by GSTs (23). These conjugates can be further metabolized into the corresponding cysteine conjugates. Cysteine conjugates can also be N-acetylated to yield mercapturic acids (24). In another reaction with glutathione, disulfides present

in the proteins are also converted into the thiols by trans-hydrogenases. In these reactions, GSH is converted to GSSG, and high GSSG concentrations can also cause inhibition of some enzymes including protein synthesis (25).

In this study, turkey liver GR enzyme was first isolated and characterized. A purification procedure was carried out by the preparation of the homogenate and affinity chromatography on 2',5'-ADP Sepharose 4B. Thanks to the 2 consecutive steps, the enzyme was purified by up to 2476-fold with a recovery ratio of 10.4% as compared with the homogenate. While washing was being done, the absorbances and activity of samples were measured spectrophotometrically at 280 and 340 nm, respectively. The enzyme was then eluted with a gradient of 0 to 1 mM GSH and 0 to 0.5 mM NADPH in 50 mM K-phosphate containing 1 mM EDTA (pH 7.0). In addition, during the purification process, GSH was added to the elution buffer for the protection of the enzyme activity. Finally, the obtained highly pure enzyme exhibited a single band on SDS-PAGE. Investigators have used 3 or more steps for GR enzyme purification. GR was purified from bovine liver in 2 chromatographic steps by 2',5' ADP-Sepharose 4B affinity and DEAE anion exchange chromatography with a 38.4% yield and by 5456-fold (26). Rainbow trout liver GR was purified with a yield of 41% and by 1654-fold by 2',5' ADP-Sepharose 4B affinity and Sephadex G-200 gel filtration chromatography (27). GR from mouse liver was purified with a yield of 66% and by 4700-fold by 8-amino-2'-phospho-adenosine diphosphoribose and N^6-adenosine 2',5'-bisphosphate-Sepharose (28). These steps take a long time and thus result in a decline in enzyme activity, or low yield, during the procedure. We used only a single chromatographic technique, 2',5'-ADP Sepharose 4B affinity chromatography, by modifying the washing solutions. These results mean that the procedure used in the purification is good enough to be used in other studies. This purification procedure also has the advantage of a short experimental time period (only 7 or 8 h).

Optimum temperature (Figure 2), optimum ionic strength, optimum pH (Figure 3), and stable pH were found as 40 °C (100 mM K-phosphate buffer), 7.5 (100 mM K-phosphate buffer), and 8.5 (100 mM Tris-HCl) for the enzyme, respectively. The stable pH profile of the enzyme was determined at 8 different pH levels using 100 mM Tris-HCl and 100 mM K-phosphate buffers. The enzyme was able to show a maximum activity of 62.69% at the end of 3.5 days in 100 mM Tris-HCl buffer (pH 8.5). The optimum pH activity was found to be 7.5 in 100 mM K-phosphate buffer. Because of the fact that enzyme activity in 100 mM K-phosphate buffer (pH 7.5) is higher than in 100 mM Tris-HCl buffer (pH 7.5), the optimum pH was accepted as 7.5 (100 mM K-phosphate) (Figure 3). This result is similar to those of GRs obtained from bovine liver (26), but is different from those obtained from rainbow trout liver (27) and calf liver (6).

The enzyme was seen to exhibit the highest activity at 40 °C in a study of temperatures between 0 °C and 80 °C. In previous studies, it was 50 °C for bovine liver (26) and 10 °C for rainbow trout liver GR (27). Our findings are different from these results. The optimum ionic strength of the enzyme was estimated to be 100 mM in K-phosphate buffer.

Figure 1 exhibits the SDS-PAGE done for the determination of the purity and molecular weight of the enzyme. For the standard proteins and GR, R_f values were calculated and an R_f-log M_W graph (Figure 8) was obtained according to Laemmli's procedure (19), showing a molecular weight of 65 kDa for GR. The molecular weight of the enzyme was also determined by gel filtration. A K_{av}-log M_W graph was obtained (Figure 9), which showed a molecular weight of 131 kDa for GR. When the results from gel filtration chromatography and SDS-PAGE were compared, it was seen that molecular weight by gel filtration was 2 times greater than by SDS-PAGE. We came to the conclusion that turkey liver GR is a homodimer. GRs of different origins have similar molecular weights as follows: 55 kDa (homodimer) and 53 kDa (homodimer) from mouse liver and rainbow trout liver by gel filtration and SDS-PAGE, respectively (27,28).

The k_{cat} and k_{cat}/K_M values of the enzyme were determined as well. K_{cat} is the number of substrate molecules that turn over to product per molecule enzyme per unit time. Thus, k_{cat} is sometimes called the turnover number, and it shows the efficiency of the enzyme. The k_{cat}/K_M value shows the specificity constant of the enzyme to its substrate. There are few

references in the literature to k_{cat} and k_{cat}/K_M values of GRs. When the k_{cat} value of GR was calculated and compared to the reference material, it was seen that the k_{cat} of turkey liver GR was greater than *Phaeodactylum tricornutum* GR (29) and *Taenia crassiceps* metacestode GR (30). K_{cat} is 8290 s^{-1} and k_{cat}/K_M is (2.76×10^8) s^{-1} M^{-1} for turkey liver, while these values are 190 s^{-1} and (3.17×10^6) s^{-1} M^{-1} for *Phaeodactylum tricornutum* GR and 5.1 s^{-1} and (3.40×10^5) s^{-1} M^{-1} for *Taenia crassiceps* metacestode GR.

K_M and V_{max} values were calculated for NADPH and GSSG by Lineweaver-Burk graphs (Figures 4 and 5). K_M constants were calculated as 0.03 mM and 0.17 mM and V_{max} values as 0.228 U mL^{-1} and 0.55 U mL^{-1} for GSSG and NADPH, respectively. These results show that the affinity of GSSG to the enzyme is higher than that of NADPH, which is in agreement with those reported for GR from some other sources (27,28).

The data given in Figures 6 and 7 show that both $NADP^+$ and GSH, the products of reactions catalyzed by GR, inhibit the enzyme noncompetitively. K_i values were estimated as 0.37 ± 0.05 mM for $NADP^+$ and 29.08 ± 14.9 mM for GSH, suggesting that $NADP^+$ has more affinity to the enzyme than GSH. These results are different from those in previous reports (26,27), because we studied a different source of GR. That is, the amino acid sequence, active site, and turnover number of enzymes can be different in various tissues. When other sources (GRs from bovine liver, rainbow trout liver, and mouse liver) are examined, it is seen that all K_i values differ from each other.

Acknowledgments

We would like to thank TÜBİTAK - BİDEB for supporting domestic graduate scholarship programs (Code: 2210).

References

1. Schirmer, R.H., Krauth-Siegel, R.L., Schulz, G.E.: In Dolphin, D., Poulson, R., Avramovic, O. Eds. Coenzymes and Cofactors, Vol. 3. John Wiley and Sons, New York. 1989; 553-559.
2. Gul, M., Kutay, F.Z., Temocin, S., Hanninen, O.: Cellular and clinical implications of glutathione. Ind. J. Exp. Biol., 2000; 38: 625-634.
3. Meister, A., Anderson, M.E.: Glutathione. Annu. Rev. Biochem., 1983: 52, 711-760.
4. Lehninger, A.L., Nelson, D.L., Cox, M.M.: Principles of Biochemistry. 2nd edn., Worth Publishers, New York. 2005.
5. Calberg, I., Altmejd, B., Mannervik, B.: Purification and immunological studies of glutathione reductase from rat liver. Evidence for an antigenic determinant at the nucleotide-binding domain of the enzyme. Biochim. Biophys. Acta, 1981; 677: 146-152.
6. Carlberg, I., Mannervik, B.: Purification and characterization glutathione reductase from calf liver. An improved procedure for affinity chromatography on 2',5'-ADP Sepharose 4B. Anal. Biochem., 1981; 116: 531-536.
7. Le Trang, N., Bhargava, K.K., Cerami, A.: Purification of glutathione reductase from gerbil liver in two steps. Anal. Biochem., 1983; 133: 94-99.
8. Krohne-Ehrich, G., Schirmer, R.H., Untucht-Grau, R.: Glutathione reductase from human erythrocytes. Isolation of the enzyme and sequence analysis of the redox-active peptide. Eur. J. Biochem., 1977; 80: 65-71.
9. Acan, N.L., Tezcan, E.F.: Sheep brain glutathione reductase: purification and general properties. FEBS Lett., 1989; 250: 72-74.
10. Cleere, W.F., Coughlan, M.P.: Avian xanthine dehydrogenases-I. Isolation and characterization of the turkey liver enzyme. Comp. Biochem. Physiol. B, 1975; 15: 311-322.
11. Han, P.F., Owen G.S., Johnson, J. Jr: Purification and properties of fructose 1,6-bisphosphatase from turkey liver. Arch. Biochem. Biophys., 1975; 1: 171-179.
12. Barra, D., Martini. F., Montarani. G., Doonan. S., Bossa. F.: Primary structure of mitochondrial aspartate aminotarnsferase from turkey liver. FEBS Lett., 1979; 108(1): 103-106.
13. Toribio, F., Martinez-Lara, E., Pascual, P., Lopez-Barea, J.J.: Methods for purification of glutathione peroxidase and related enzymes. J. Chromatogr. B Biomed. Appl., 1996; 684: 77-97.
14. Brodelius, P., Larsson, P.O., Mosbach, K.: The synthesis of three AMP-analogues: N6-(6-aminohexyl)-adenosine 5'-monophosphate, N6-(6-aminohexyl)-adenosine 2',5'-bisphosphate, and N6-(6-aminohexyl)-adenosine 3',5'-bisphosphate and their application as general ligands in biospecific affinity chromatography. Eur. J. Biochem., 1974; 47: 81-89.
15. Mannervik, B., Jacobsson, K., Boggaram, V.: Purification of glutathione reductase from erythrocytes by the use of affinity chromatography on 2', 5'-ADP-Sepharose 4-B. FEBS Lett., 1976; 66: 221-224.

16. Erat, M., Çiftçi, M.: In vitro effects of some antibiotics on glutathione reductase from sheep liver. J. Enzyme Inhib. Med. Chem., 2003; 18: 545-550.

17. Carlberg, I., Mannervik, B.: Glutathione reductase assay. Methods Enzymol., 1985; 113: 484-495.

18. 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: 248-251.

19. Laemmli, D.K.: Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature, 1970; 227: 680-683.

20. Andrews, P.: The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J., 1965; 96: 595-606.

21. Lineweaver, H., Burk, D.: The determination of enzyme dissociation constants. J. Am. Chem. Soc., 1934; 56: 658-666.

22. Meister, A.: Glutathione metabolism and its selective modification. J. Biol. Chem., 1988; 263: 17205-17208.

23. Beckett, G.J., Hayes, J.D.: Glutathione S-transferases: Biomedical applications. Adv. Clin. Chem., 1993; 30: 281-380.

24. Van Welie, R.T.H., Van Dijek, R.G.J.M., Vermeulen, N.P.E., Van Sittert, N.J.: Mercapturic acids, protein adducts, and DNA adducts as biomarkers of electrophilic chemicals. Crit. Rev. Toxicol., 1992; 22: 271-306.

25. Deneke, S.M., Fanburg, B.L.: Regulation of cellular glutathione. Am. J. Physiol., 1989; 257: L163-L173.

26. Ulusu, N.N., Tandoğan, B.: Purification and kinetic properties of glutathione reductase from bovine liver. Mol. Cell. Biochem., 2007; 303: 45-51.

27. Tekman, B., Ozdemir, H., Senturk, M., Ciftci, M.: Purification and characterization of glutathione reductase from rainbow trout (*Oncorhynchus mykiss*) liver and inhibition effects of metal ions on enzyme activity. Com. Biochem. Physiol. C, 2008; 148: 117-121.

28. López-Barea, J., Lee, C.: Mouse-liver glutathione reductase purification, kinetics, and regulation. Eur. J. Biochem., 1979; 98: 487-499.

29. Arias, D.G., Marquez, V.E., Beccaria, A.J., Guerrero, S.A., Iglesias, A.A.: Purification and characterization of a glutathione reductase from *Phaeodactylum tricornutum*. Protist, 2009; 161(1): 91-101.

30. Rendon, J.L., Arenal, I.P., Guevara-Flores, A., Uribe, A., Plancarte, A., Mendoza-Hernandez, G.: Purification, characterization and kinetic properties of the multifunctional thioredoxin-glutathione reductase from *Taenia crassiceps* metacestode (cysticerci). Mol. Biochem. Parasitol., 2004; 133: 61-69.