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GÖKTUĞ ULUSU

MUSTAFA ERAT

MEHMET ÇİFTÇİ

HALİS ŞAKİROĞLU

EBUBEKİR BAKAN

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Research Article

Purification and Characterization of Glutathione Reductase from Sheep Liver

Göktuğ ULUSU
Department of Chemistry, Arts and Science Faculty, Atatürk University, Erzurum - TURKEY
Mustafa ERAT, Mehmet ÇİFTÇİ*
Biotechnology Application and Research Center, Atatürk University, Erzurum - TURKEY
*E-mail: ciftcim@atauni.edu.tr
Halis ŞAKİROĞLU
Department of Chemistry, Arts and Science Faculty, Atatürk University, Erzurum - TURKEY
Ebubekir BAKAN
Department of Biochemistry, Faculty of Medicine, Atatürk University, Erzurum - TURKEY

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Abstract: Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) was purified from sheep liver. The purification included 4 steps: preparation of homogenate, ammonium sulfate fractionation, 2', 5'-ADP Sepharose-4B affinity chromatography, and gel filtration chromatography. GR was obtained with a yield of 14.1% having a specific activity of 47.27 EU/mg proteins. Optimal pH, stable pH, and optimal temperature were 8.0, 5.5, and 60 °C, respectively. K_M and V_{max} values for NADPH and GSSG substrates were 0.0258 and 0.0239 mM, and 0.266 and 0.255 EU/ml, respectively. The overall purification was about 1477-fold for liver GR. The enzyme activity was measured spectrophotometrically at 340 nm. In addition, K_i values of 4.367 and 0.4055 mM were determined by means of Lineweaver-Burk graphs for $NADP^+$ and GSH, respectively.

Key Words: Glutathione reductase, purification, characterization, sheep, liver

Koyun Karaciğerinden Glutatyon Redüktaz Enziminin Saflaştırılması ve Karakterizasyonu

Özet: Glutatyon redüktaz (Glutatyon: $NADP^+$ oksidoredüktaz, EC 1.8.1.7; GR) enzimi koyun karaciğerinden saflaştırıldı. Saflaştırma işlemi, homojenatın hazırlanması, amonyum sülfat çöktürmesi, 2', 5'-ADP Sepharose-4B affinite kromatografisi ve jel filtrasyon kromatografisi şeklinde dört basamakta yapıldı. 47,27 EÜ/mg protein spesifik aktivitesine sahip olan GR enzimi % 14,1 verimle elde edildi. Enzim için optimum pH, stabil pH, optimum sıcaklık, sırasıyla 8,0, 5,5, 60 °C olarak bulundu. Ayrıca NADPH ve GSSG substratları için K_M ve V_{max} değerleri sırasıyla 0,0258, 0,0239 mM ve 0,266, 0,255 EÜ/ml olarak belirlendi. Tüm saflaştırma basamakları sonucu enzim 1.477 kat saflaştırıldı ve enzim aktivitesi spektrofotometrik olarak 340 nm'de ölçüldü. Buna ilaveten $NADP^+$ ve GSH için Lineweaver-Burk grafikleri yardımıyla K_i sabitleri sırasıyla 4,367 ve 0,4055 mM olarak bulundu.

Anahtar Sözcükler: Glutatyon redüktaz, saflaştırma, karakterizasyon, koyun, karaciğer

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 reduced form (GSH) in the presence of NADPH. In order to maintain a high ratio of [GSH]/[GSSG], the enzyme has a crucial role (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, in formation of the deoxyribonucleotid precursors of deoxyribonucleic acid (DNA), in maintaining the sulfhydryl groups of intracellular proteins and in protection of the cells against free radicals and reactive oxygen species such as H_2O_2, $O_2^{\cdot}$ and $^{\cdot}OH$ (2).

GR has been purified from many different sources such as rat liver (3,4), calf liver (5), gerbil liver (6),

human erythrocytes (7,8), bovine erythrocytes (9), porcine erythrocytes (10), sheep brain (11) and sheep liver (12), and its some characteristic properties have been determined.

Affinity chromatography, ion-exchange chromatography, hydrophobic and reversed phase chromatography and size-exclusive chromatography techniques have been used in order to purify the enzyme (13). Affinity chromatography on a 2', 5'-ADP Sepharose-4B column has been used for its efficient purification (14,15).

The aim of this study was to purify glutathione reductase (GR) from sheep liver and to investigate some characteristics of the enzyme.

Materials and Methods

Materials

Sephadex G-200, NADPH, GSSG, and protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. 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

A fresh sheep's liver was taken from Erzurum Slaughterhouse under cold conditions. After the liver was obtained it was washed in isotonic saline containing 1 mM EDTA and stored at –20 °C before use. Fifty grams of liver was first cut into small pieces and powdered. Then the powder was homogenized with 50 ml of 50 mM potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 13,000 rpm for 20 min and the precipitate was removed (6).

Ammonium Sulfate Fractionation and Dialysis

The homogenate was subjected to precipitation with ammonium sulfate (between 0% and 90%). Ammonium sulfate was slowly added to the homogenate by constantly stirring for complete dissolution. This mixture was centrifuged at 5,000 g for 15 min and the precipitate was dissolved in a minimum volume of 50 mM potassium phosphate buffer. The enzyme activity was determined in both the supernatant and precipitate for each respective precipitation. The enzyme was observed to precipitate at the 0-70% precipitation step. The resultant solution was clear and contained partially purified enzyme. It was then dialyzed at 4 °C in 10 mM Tris-HCl/1 mM EDTA (pH 7.5), for 2 h with 2 changes of buffer.

2', 5'-ADP Sepharose-4B Affinity Chromatography

Two grams of dried 2', 5'-ADP Sepharose-4B was used for a column (1 x 10 cm) of 10 ml bed volume. The gel was washed with 300 ml of distilled water to remove foreign bodies and air, suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0), and packed in the column. After precipitation of the gel, the column was equilibrated with 50 mM K-phosphate buffer including 1 mM EDTA, pH 6.0, by means of a peristaltic pump. The flow rates for washing and equilibration were adjusted to 20 ml/h. 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-acetate+0.1 M K-phosphate, pH 7.85. Washing was continued with 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 0.5 mM GSH and 0 to 1 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 (6,10,12).

Sephadex G-200 Gel Filtration Chromatography

Dried Sephadex G-200 (2 g) was used for a 165 ml-column (2 x 50 cm) volume. 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. Flow rate was adjusted to 15 ml/h by means of a peristaltic pump. Then the column was equilibrated with 50 mM Tris-HCl+50 mM KCl buffer, pH 7.0, until the final absorbance difference became 0 at 280 nm. The dialyzed sample was mixed with glycerol at a rate of 5%. The final sample was loaded onto the column and elutions were collected in 2 ml amounts. In each fraction, enzyme activity was determined at 340 nm. Active fractions were collected and stored at –20 °C for testing the enzyme purity by electrophoresis (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 50 mM Tris-HCl buffer pH 8.0, including 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. One enzyme unit is defined as the oxidation of 1 µmol NADPH per min under the assay condition.

Protein Determination

Quantitative protein determination was measured spectrophotometrically at 595 nm with bovine serum albumin being used as a standard (18).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine the enzyme purity, SDS-PAGE was carried out according to Laemmli's method (19). The acrylamyde concentrations of the stacking and the separating gels containing 0.1% SDS were 4% and 8%, respectively.

Stable pH Determination

For stable pH determination, equal volumes of the buffers (K-phosphate at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, and Tris-HCl at pH 7.5, 8.0, 8.5 and 9.0) and purified enzyme were mixed and kept in a refrigerator (+4 °C). The enzyme activity was assayed every 48 h.

Optimum pH Determination

In order to determine the optimum pH, K-phosphate and Tris-HCl buffers were used at pH 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 from 0 to 80 °C. The desired temperature was provided 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 Tris-HCl buffer, pH 8.0, in the range from 10 to 1000 mM.

Kinetic Studies

K_M and V_{max} values for NADPH and GSSG substrates were calculated from Lineweaver-Burk curves (20). Activities were assayed at 5 different NADPH concentrations with a fixed concentration of GSSG, and the same experiments were also performed at 4 different GSSG concentrations with a fixed NADPH concentration. The effects of $NADP^+$ and GSH on enzyme activity, which are products of the enzyme catalyzing reaction, were investigated. For this purpose, 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 in high NADPH (0.0150, 0.0312, 0.0625, 0.1250, 0.2500 and 0.5000 mM) and GSSG (0.0150, 0.0312, 0.0625, 0.1250, 0.2500 and 0.5000 mM) concentrations to determine the effect of substrates on enzyme activity. All kinetic studies were performed at 25 °C in 50 mM Tris-HCl buffer, pH 8.0.

Results

The Table shows purification characterized by a specific activity of 47.27 EU/mg protein, a yield of 14.1% and a purification coefficient of 1477. The elution profile of GR activity from 2',5'-ADP Sepharose-4B affinity and DEAE-Sephadex A50 ion exchange chromatography is shown in Figures 1a and 1b. At the end of the last step, the highly pure enzyme obtained exhibited a single band on SDS-PAGE (Figure 2). The optimum ionic strength of the enzyme was estimated to be 50 mM Tris-HCl buffer (Figure 3a). The enzyme was seen to show the highest activity at 60 °C (Figure 3b) after being tried between 0 and 80 °C. The optimal pH of G6PD was determined to be 8.0 using 50 mM Tris-HCl (Figures 4a and 4b). The stable pH of the enzyme was 5.5 in phosphate buffer (Figures 5a and 5b).

The Lineweaver-Burk graphs shown in Figures 6a and 6b were constructed for NADPH and GSSG. A K_M of 0.0258 mM and a V_{max} of 0.266 EU/ml were obtained for NADPH, and 0.0239 mM and 0.255 EU/ml for GSSG. K_i

Table. Purification scheme of GR from sheep liver.

Purification steps	Total volume	Activity (U/ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Homogenate	48	0.614	19.45	933.6	29.47	0.032	100	1.00
Ammonium sulfate precipitation (0-70%)	20	0.681	1.63	32.60	13.62	0.418	46.2	13
2',5'-ADP Sepharose-4B affinity chromatography	14	0.78	0.028	0.392	10.92	27.86	37.1	870.6
Sephadex G-200 gel filtration chromatography	16	0.26	0.0055	0.088	4.16	47.27	14.1	1477

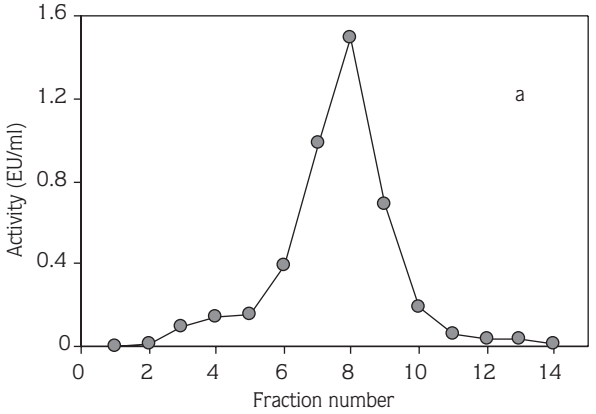

Figure 1a. Fractions obtained from 2',5'-ADP Sepharose-4B affinity column.

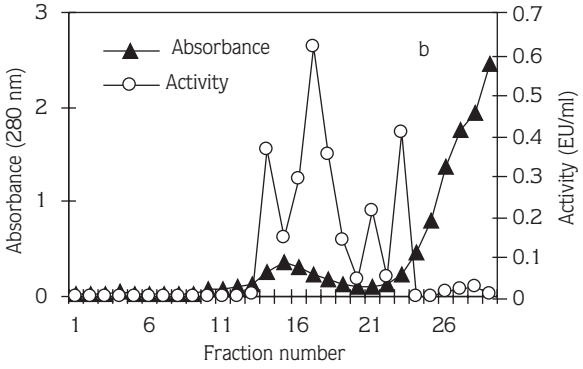

Figure 1b. Fractions obtained from Sephadex G-200 gel filtration column.

values were determined by means of Lineweaver-Burk graphs (4.367 and 0.4055 mM for $NADP^+$ and GSH, respectively) (Figures 7a and 7b).

Discussion

The undesirable biologic effects of oxidative agents, such as free radical and reactive oxygen species (ROS), are eliminated by enzymatic and nonenzymatic antioxidant defense systems. Enzymatic defense is provided by many enzyme systems such as glutathione reductase, glutathione peroxidase, glutathione S-transferase (GST), superoxide dismutase, catalase, aldoketoreductase 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 (21).

Glutathione reductase 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 (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). GSH is a product of the reaction catalyzed by glutathione reductase, and the GSH-related enzyme system is one of the most important protective systems in cells. Reduced glutathione is used in the detoxification of xenobiotics,

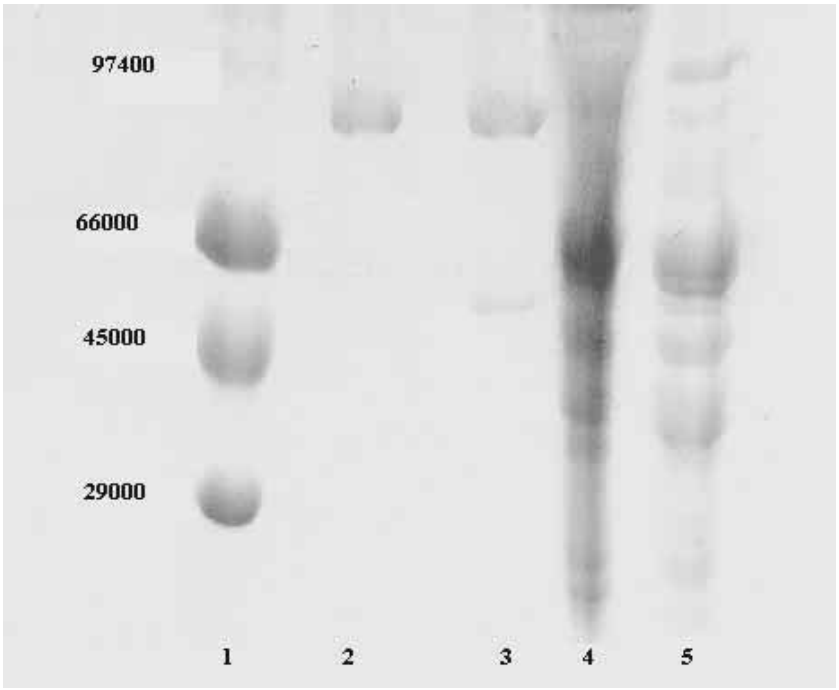

Figure 2. SDS-polyacrylamide gel electrophoresis of sheep liver GR. Lane 1: Standard proteins (rabbit phosphorylase b: 97,400 Da, bovine serum albumin: 66,000 Da, chicken ovalbumin: 45,000 Da, and bovine carbonic anhydrase: 29,000 Da); Lane 2: gel filtration chromatography sample; Lane 3: Affinity chromatography sample; Lane 4: ammonium sulfate precipitation; Lane 5: homogenate.

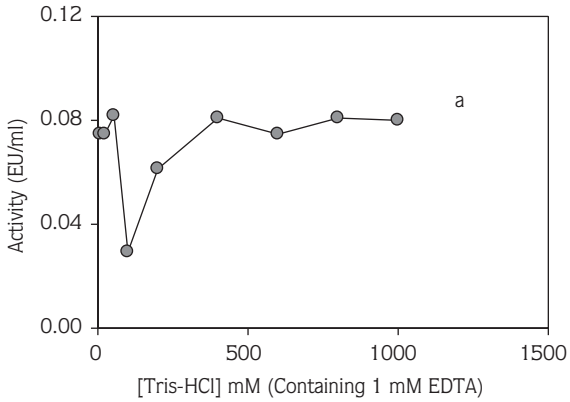

Figure 3a. Activity%-ionic strength graph of sheep liver GR enzyme.

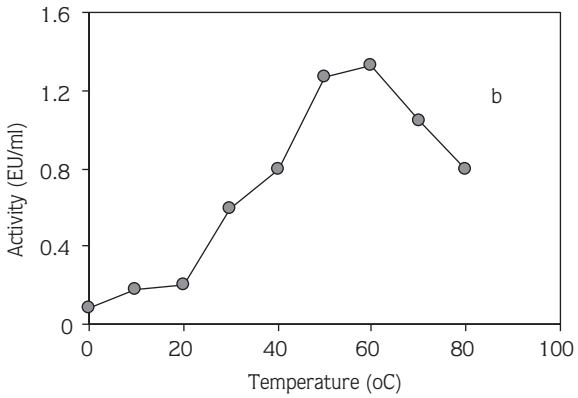

Figure 3b. Activity%-temperature graph of sheep liver GR enzyme.

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 its high nucleofilic potency. These reactions are catalyzed by glutathione transferases (GSTs) (22). These conjugates can be further metabolized into the corresponding cysteine conjugates. Cysteine conjugates can also be N-acetylated to yield mercapturic acids (23). In the other reaction of glutathione, disulfides present in

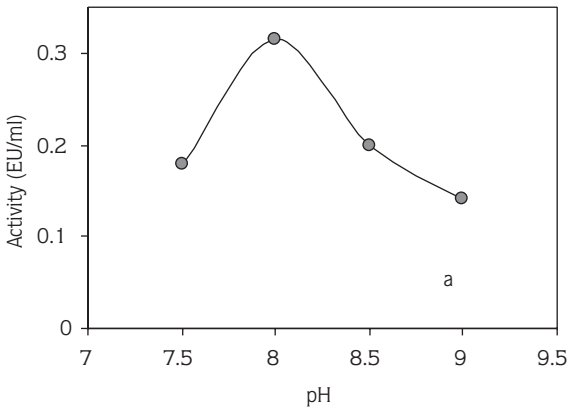

Figure 4a. Activity%-pH graph of sheep liver GR enzyme in Tris-HCl buffer.

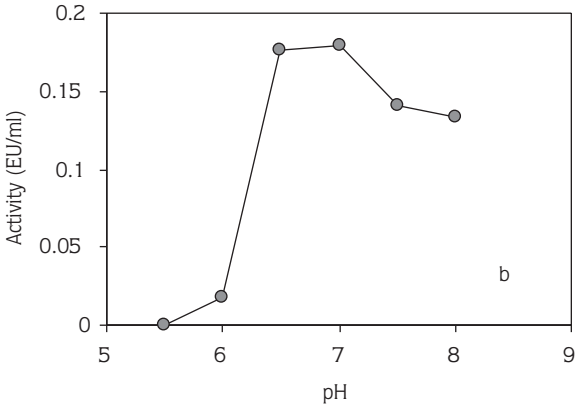

Figure 4b. Activity%-pH graph of sheep liver GR enzyme in K-phosphate buffer.

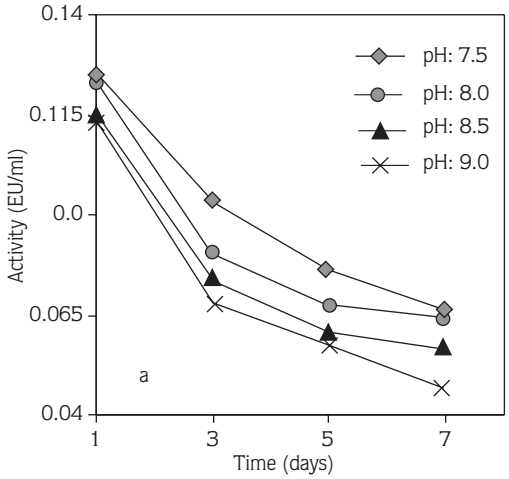

Figure 5a. Stable pH graph of sheep liver GR in Tris-HCl buffer.

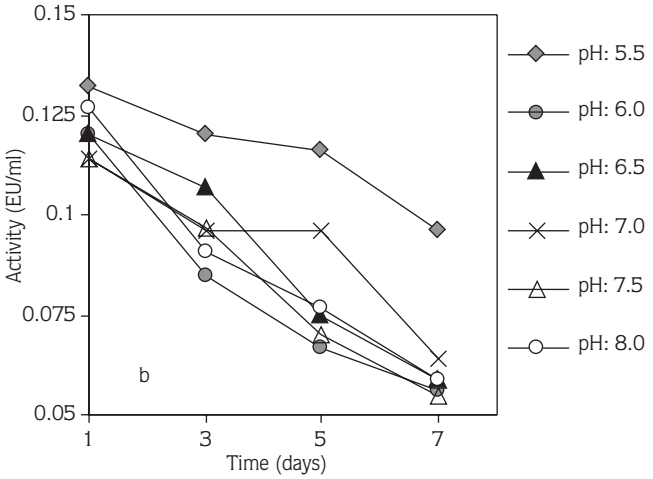

Figure 5b. Stable pH graph of sheep liver GR in K-phosphate buffer.

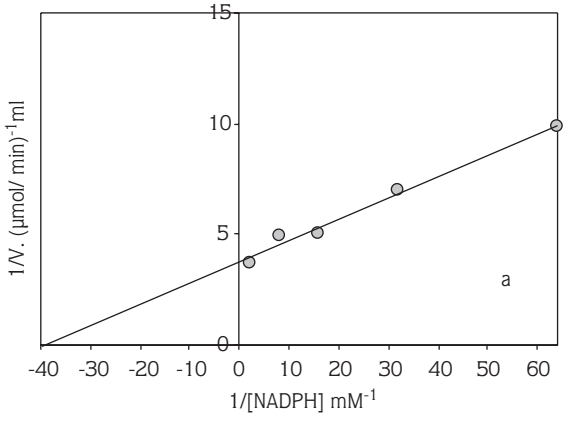

Figure 6a. Lineweaver-Burk graph in 5 different NADPH concentrations with 1 mM fixed GSSG.

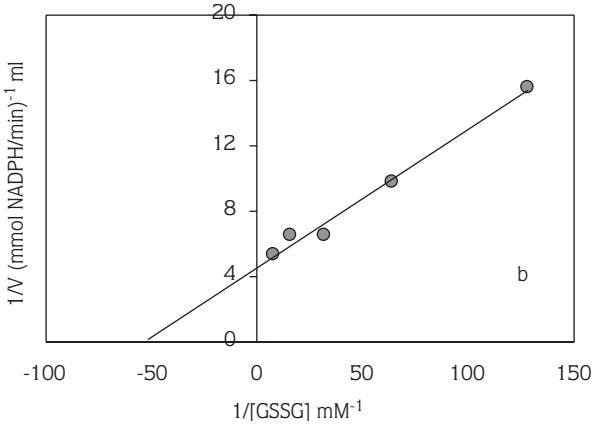

Figure 6b. Lineweaver-Burk graph in 5 different GSSG concentrations with 0.1 mM fixed NADPH.

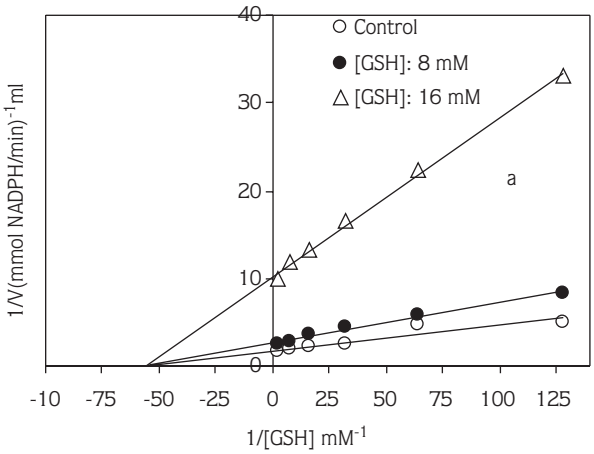

Figure 7a. Lineweaver-Burk graph in 6 different NADPH concentrations and in 2 defined different $NADP^+$ concentrations for determining K_i.

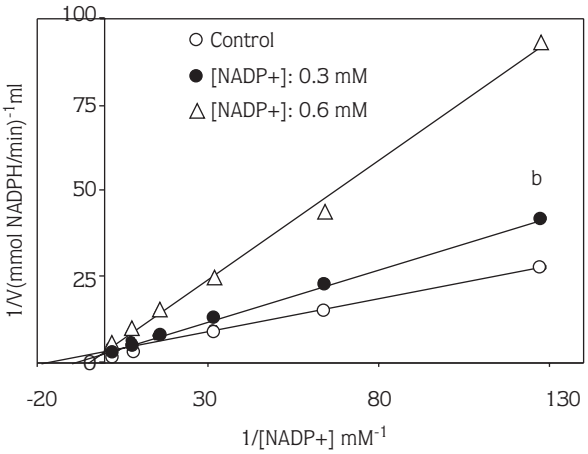

Figure 7b. Lineweaver-Burk graph in 6 different GSSG concentrations and in 2 defined different GSH concentrations for determining K_i.

the proteins are also converted into thiols by transhydrogenases. In these reactions, GSH is converted to GSSG, and high GSSG concentrations can also cause inhibition of some enzymes including protein synthesis (24).

The GR enzyme sample from the affinity column exhibited 2 bands on SDS-PAGE because pyridine nucleotides are able to bind not only glutathione reductase but also other several proteins (6). For that reason in this study, after 2', 5'-ADP Sepharose-4B affinity chromatography, Sephadex G-200 gel filtration chromatography was performed. Then in each fraction of gel filtration chromatography, absorbance and activity were measured at 280 and 340 nm, respectively. After that, active fractions were collected and stored at –20 °C for testing the enzyme purity by electrophoresis.

Investigators have used 3 or more steps for GR enzyme purification. These steps take a long time and thus result in a decline in enzyme activity, or low yield, during the procedure. We used only 2 chromatographic techniques: 2', 5'-ADP Sepharose-4B affinity chromatography by modification of washing solutions and Sephadex G-200 gel filtration chromatography. 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 period (only one full day).

To determine enzyme purity, samples from homogenate, ammonium sulfate, affinity and gel filtration were applied to SDS-PAGE. As seen in Figure 2, a standard sample was run in the first column, a gel filtration sample in the second column, an affinity sample in the third column, an ammonium sulfate sample in fourth column, and a homogenate sample in the fifth column. When the third column is compared with the fourth and fifth columns, it is found that the impurities are eliminated to a great extent. As seen in the second column, the enzyme is completely purified with gel filtration chromatography. Finally, the highly pure enzyme obtained exhibited a single band on SDS-PAGE (Figure 2, second column).

The stable pH profile of the enzyme was determined at 10 different pHs using 50 mM Tris-HCl and 50 mM K-phosphate buffer. The enzyme showed a maximum activity of 73.28% at the end of 7 days in 50 mM K-phosphate buffer (pH 5.5). Consequently, stable pH was regarded as 5.5 in 50 mM phosphate (Figure 5b).

The optimum pH activity was found to be 7.0 in 50 mM Tris-HCl buffer. Because enzyme activity in 50 mM Tris-HCl buffer (pH 8.0) is higher than in 50 mM phosphate buffer (pH 7.0), optimum pH was regarded as 8.0 (50 mM Tris-HCl) (Figures 4a and 4b). The result is similar to the activities of GRs obtained from porcine erythrocytes (10) and calf liver (5).

Enzyme activities were measured between 0 and 80 °C in order to search how temperature affects enzyme. As shown in Figure 3b, enzyme activity increases after 20 °C, and then it shows maximum activity at 60 °C. However, activity decreases sharply between 60 and 80 °C. Therefore, the optimum enzyme temperature could be regarded as 60 °C.

Enzyme activities were measured between 10 and 1000 mM Tris-HCl (containing 1 mM EDTA) in order to determine how ionic strength affects enzyme. As shown in Figure 3a, maximum enzyme activity was obtained in 50 mM Tris-HCl. Therefore, the optimum ionic strength of enzyme could be regarded as 50 mM Tris-HCl.

Furthermore, the affinity of enzyme with its own substrates was investigated at optimum pH and 25 °C by the help of Lineweaver-Burk plots (Figures 6a and 6b). K_M values of 0.0239 and 0.0258 mM were obtained for GSSG and NADPH, and 0.255 and 0.266 U/ml V_{max} values. According to these values, the K_M value for GSSG is smaller than the K_M value for NADPH. Thus, the affinity of GSSG to enzyme is greater than that of NADPH. Similar values were reported for GR from other sources (4,8,9,25).

As shown in Figures 7a and 7b both GSH and $NADP^+$, the products of the reaction catalyzed by GR, inhibits the enzyme noncompetitively and competitively, respectively. The K_i value for GSH is smaller than the K_i value for $NADP^+$, suggesting that GSH has more affinity to enzyme than does $NADP^+$. These results are different from those in previous reports (9).

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