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Purification of Glutathione Reductase From Chicken Liver and Investigation of Kinetic Properties

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Abstract

Glutathione reductase was purified from chicken liver and some characteristics of the enzyme were investigated. The purification procedure was composed of four steps: preparation of homogenate, ammonium sulfate precipitation, 2',5'-ADP Sepharose 4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. Owing to the four consecutive procedures, the enzyme was purified 1714-fold, with a yield of 38%. Specific activity at the final step was 120 enzyme unit (EU)/mg of protein. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of the enzyme was found to be 100 kDa by Sephadex G-200 gel filtration chromatography, and the subunit molecular weight was found to be 43 kDa by SDS-PAGE. Optimum pH, stable pH, optimum ionic strength, and optimum temperature were 7.0, 7.4, 0.75 *M* Tris-HCl buffer including 1 m*M* EDTA, and 50°C, respectively. K_M and V_{max} values for NADPH and glutathione disulfide (GSSG) substrates were also determined for the enzyme.

Index Entries: Glutathione reductase; purification; chicken liver; enzyme; gel filtration chromatography; affinity chromatography.

Introduction

Glutathione reductase (glutathione:$NADP^+$ oxidoreductase; EC 1.8.1.7) is essential for the maintenance of cellular glutathione in its reduced form, which is necessary for the normal functioning of the cell. During the catalyzing reduction of glutathione disulfide (GSSG) to reduced glutathione

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(GSH), which is a tripeptide consisting of L-γ-glutamyl-L-cysteinylglycine, the enzyme uses NADPH or NADH as a reducing agent. GSH has an important role in the synthesis and degradation of proteins, regulation of enzymes, formation of the deoxyribonucleotide precursors of DNA, and protection of the cells against free radicals and reactive oxygen species *(1)*.

Glutathione reductase has been purified and characterized from numerous mammalian sources such as rat liver *(2,3)*, calf liver *(4)*, gerbil liver *(5)*, sheep brain *(6)*, human erythrocytes *(7,8)*, bovine erythrocytes *(9)*, and porcine erythrocytes *(10)*. It has also been isolated from nonmammalian and plant sources *(11–14)*. To purify the enzyme, affinity chromatography, ion-exchange chromatography, hydrophobic and reverse-phase chromatography, and size-exclusion chromatography techniques have been used *(15)*. Affinity chromatography on a 2',5'-ADP Sepharose 4B column has been used for its efficient purification *(16,17)*.

In this article, we report the purification of chicken liver glutathione reductase using a 2',5'-ADP Sepharose 4B affinity column and a Sephadex G-200 gel filtration column. In addition, we report the results of our investigation of some of the kinetic and characteristic properties of the purified enzyme.

Materials and Methods

Reagents and Chemicals

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

Preparation of Homogenate

Thirty-five grams of fresh chicken liver was washed in isotonic saline containing 1 m*M* EDTA; cut into small pieces; and homogenized in a Waring blender with 80 mL of 0.1 *M* Tris-HCl buffer (pH 7.4) containing 1 m*M* EDTA, 1 m*M* dithiothreitol, and 1 m*M* phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 26,000*g* for 60 min, and the precipitate was removed. This process was repeated two times and the supernatant was used as a crude extract. Ten milliliters of crude extract was separated, and ammonium sulfate precipitation (0–10, 10–20, 20–30, 30–40, 40–50, 50–60, and 60–70%) was performed on the sample with solid $(NH_4)_2SO_4$. For each respective precipitation, enzyme activity was determined both in the supernatant and in the precipitate. The enzyme was observed to precipitate at 30–70% saturation. Seventy milliliters of the remaining sample was brought to 30–70% ammonium sulfate saturation with solid $(NH_4)_2SO_4$. The precipitate was dissolved in a small amount of 0.1 *M* Tris-HCl buffer including 1 m*M* EDTA, pH 7.4, and then dialyzed against the same buffer *(5)*.

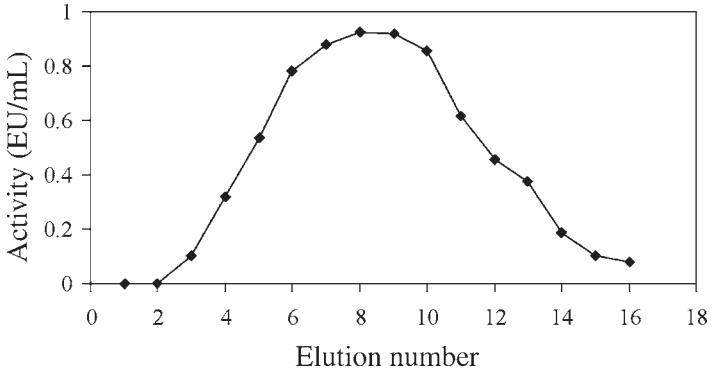

Fig. 1. Purification of glutathione reductase on 2',5'-ADP Sepharose 4B affinity column.

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

For a 10-mL bed volume, 2 g of dried 2',5'-ADP Sepharose 4B gel was used (Fig. 1). 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 a column. After precipitation of the gel, it was equilibrated with 50 m*M* K-phosphate buffer including 1 m*M* 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 washed successively with 25 mL of 0.1 *M* K-acetate + 0.1 *M* K-phosphate (pH 6.0) and 25 mL of 0.1 *M* K-acetate + 0.1 *M* K-phosphate (pH 7.85). The latter washing was continued with 50 m*M* K-phosphate buffer including 1 m*M* EDTA (pH 7.4) until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted successively with a gradient of 0–1 m*M* NADPH in a solution of 80 m*M* K-phosphate + 80 m*M* KCl + 10 m*M* EDTA (pH 7.85). Active fractions were collected and dialyzed with equilibration buffer. During all procedures, the temperature was kept at 4°C *(3,9,10)*.

Sephadex G-200 Gel Filtration Chromatography

Five grams of dried Sephadex G-200 was used for a 150-mL column size. The gel was incubated in distilled water at 90°C for 5 h. After removing the air in the gel, it was loaded onto the column (1.5 × 70 cm). The flow rate was adjusted to 15 mL/h by means of a peristaltic pump. The column was equilibrated with 50 m*M* Tris-HCl, 50 m*M* KCl buffer, pH 7.4 until the final absorbance difference became 0 at 280 nm and the pH value became the same as that of the equilibration buffer. The dialyzate from the affinity chromatography column was mixed with glycerol to form a mixture including 50 m*M* glycerol. The final sample was loaded onto the column and eluted with equilibration buffer including 50 m*M* glycerol. Two-milliliter elutions were collected in tubes. For each fraction, activity values were determined at 340 nm (Fig. 2). Active fractions were lyophilized and

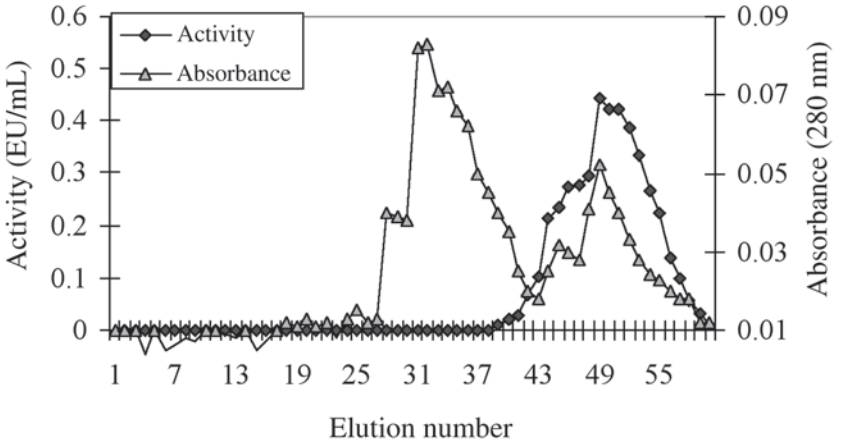

Fig. 2. Purification of glutathione reductase on Sephadex G-200 gel filtration column.

stored at –85°C in order to check the enzyme purity by electrophoresis and determine the kinetic properties of the enzyme.

To determine the native molecular weight of the enzyme, the same gel filtration column was used after washing and equilibrating. The native molecular weight of the enzyme was estimated on the same gel filtration column according to the method of Andrews *(18)*. At first, to establish the void volume, Blue Dextran (2000 kDa) was passed through the column; then, horse heart cytocrome-*c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa) were used as standard proteins (MW-GF-200; Sigma).

Determination of Activity

Enzymatic activity was measured spectrophotometrically at 25°C according to the method of Carlberg and Mannervik *(19)*. The assay system contained 0.75 m*M* Tris-HCl buffer, pH 7.0, including 1 m*M* EDTA; 1 m*M* GSSG; and 0.1 m*M* NADPH in a total volume of 1 mL. The decrease in absorbance at 340 nm was followed with a Shimadzu Spectrophotometer UV-(1208). The reaction was initiated by adding the enzyme solution. One enzyme unit is defined as the oxidation of 1 μmol of NADPH/min under the assay conditions.

Determination of Protein

Protein concentrations were estimated from measurements of absorbance at 595 nm according to Bradford's *(20)* method, with BSA as a standard. Qualitative protein determination was performed spectrophotometrically at 280 nm according to Segel's *(21)* method.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

To check the enzyme purity and determine the subunit molecular weight of the enzyme, sodium dodecyl sulfate polyacrylamide gel electro-

phoresis (SDS-PAGE) was performed using Laemmli's *(22)* method, with bovine carbonic anhydrase (29 kDa), chicken ovalbumin (45 kDa), and BSA (66 kDa) used as standard proteins (MW-SDS-200; Sigma). Acrylamide concentration of the stacking and separating gels was 4 and 10%, respectively, and 1% SDS was also added to the gel solution. The gel was stabilized in a solution containing 50% propanol + 10% trichloroacetic acid + 40% distilled water for 30 min. Staining was done for about 2 h in a solution of 0.1% Coomassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid + 39.9% distilled water. The gel was washed with several changes of the same solvent without dye until protein bands were cleared.

Determination of Optimum pH

To determine the optimum pH, enzyme activity was measured in 0.75 *M* Tris-HCl + 1 m*M* EDTA and 0.75 *M* potassium phosphate buffers over the pH ranges of 7.0–8.5 and 5.5–7.0, respectively.

Determination of Optimum Temperature

To determine the optimum temperature, enzyme activity was assayed in 0.75 *M* Tris-HCl buffer, pH 7.0, including 1 m*M* EDTA at different temperatures in the range from 5 to 70°C. The desired temperature was provided by using a Polyscience bath (model 9105).

Determination of Stable pH

To determine stable pH, equal volumes of the buffers (K-phosphate at pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; Tris-HCl at pH 7.5, 8.0, and 8.5) and purified enzyme were mixed and kept in a refrigerator (+4°C). The enzyme activity was assayed at the beginning and once every 2 d.

Kinetic Studies

To determine K_M and V_{max} values separately for NADPH and GSSG substrates, at optimum pH and 25°C the enzyme activity was measured at six different substrate cuvet concentrations for NADPH (0.006, 0.013, 0.025, 0.05, and 0.1 m*M*) with a constant GSSG (1 m*M*), and for GSSG (0.0625, 0.125, 0.25, 0.5, and 1 m*M*) with a fixed NADPH (0.1 m*M*) concentration. K_M and V_{max} values were calculated by means of Lineweaver-Burk graphs *(23)*.

Results

Chicken liver glutathione reductase was purified 1714-fold with a yield of 38%. The specific activity at the final step was 120 EU/mg of protein (Table 1). Figure 3 exhibits results of the SDS-PAGE performed to determine the purity and subunit molecular weight of the enzyme. For the chicken liver glutathione reductase and standard proteins, R_f values were estimated and an R_f–log MW graph was drawn according to the Laemmli

Table 1
Purification Scheme of Glutathione Reductase From Chicken Liver

Purification step	Total volume (mL)	Activity (EU/mL)	Total activity (EU)	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification (fold)
Homogenate	60	0.106	6.36	1.513	90.78	0.07	100	1
Ammonium sulfate precipitation (30–70%)	35	0.141	4.94	1.213	42.46	0.12	78	1.7
2',5'-ADP Sepharose 4B affinity chromatography	11	0.418	4.60	0.004	0.04	104	72	1485
Sephadex G-200 gel filtration chromatography	10	0.241	2.41	0.002	0.02	120	38	1714

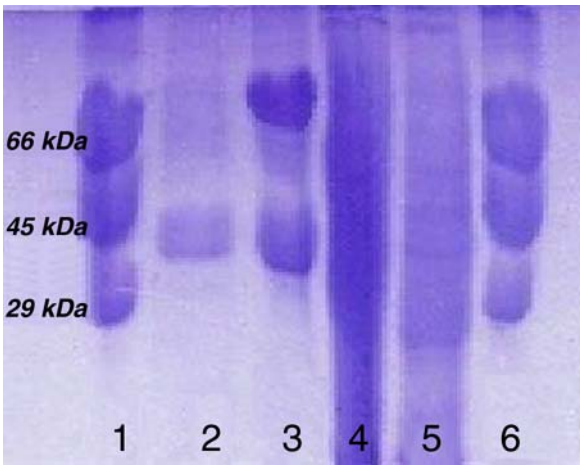

Fig. 3. SDS-PAGE: lanes 1 and 6, standard proteins; lane 2, purified glutathione reductase from Sephadex G-200 gel filtration column; lane 3, sample from 2',5'-ADP Sepharose 4B affinity column; lane 4, ammonium sulfate precipitate; lane 5, homogenate.

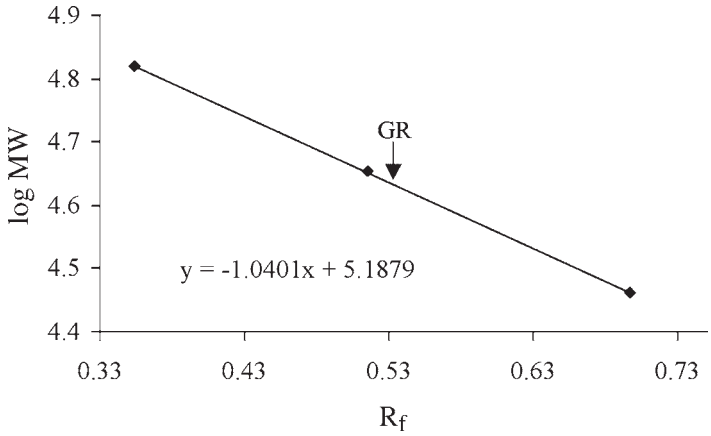

Fig. 4. Standard R_f–log MW graph of glutathione reductase (GR) using SDS-PAGE. Standards: BSA (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa).

procedure, showing a subunit mol wt of 43 kDa for the enzyme (Fig. 4). The native molecular weight of the enzyme was also determined by gel filtration chromatography. The K_{av}–log MW graph shows a mol wt of 100 kDa for the enzyme (Fig. 5). The optimum pH of the enzyme was determined to be 7.0 using 0.75 *M* Tris-HCl buffer including 1 m*M* EDTA (Fig. 6). The stable pH of glutathione reductase was 7.4 in Tris-HCl buffer (Fig. 7). The optimum ionic strength of the enzyme was 0.75 *M* Tris-HCl (Fig. 8). The enzyme showed the highest activity at 50°C (Fig. 9) in a study of temperature between 5 and 70°C.

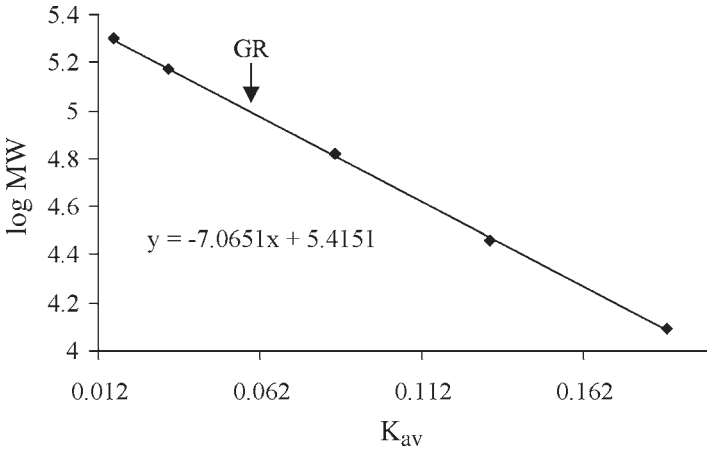

Fig. 5. Standard K_{av}–log MW graph of glutathione reductase (GR) using gel filtration. Standards: sweet potato β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), and horse heart cytocrome-*c* (12.4 kDa).

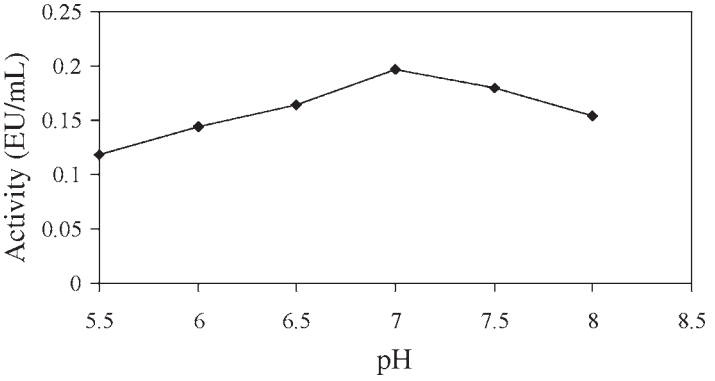

Fig. 6. Activity–pH graph of glutathione reductase.

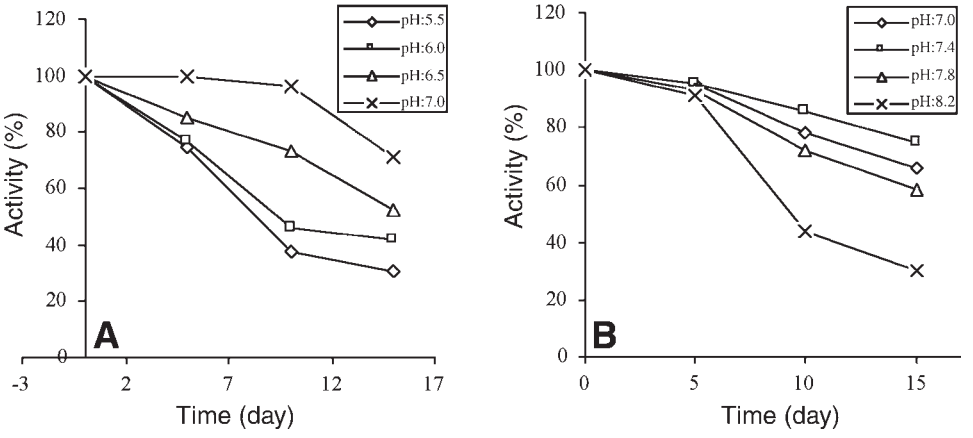

Fig. 7. Stable pH graph of glutathione reductase: **(A)** in Tris-HCl buffer; **(B)** in potassium phosphate buffer.

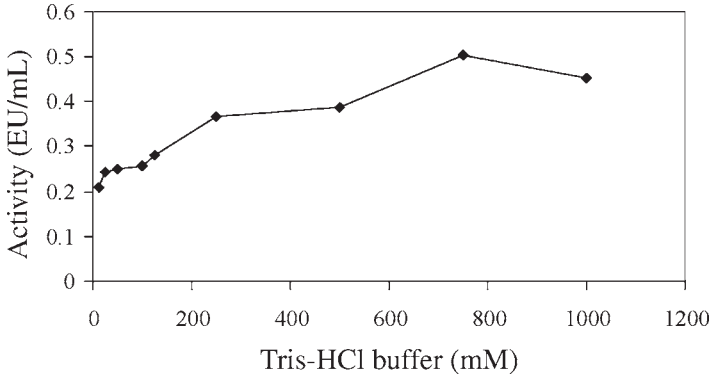

Fig. 8. Optimum ionic strength graph of glutathione reductase.

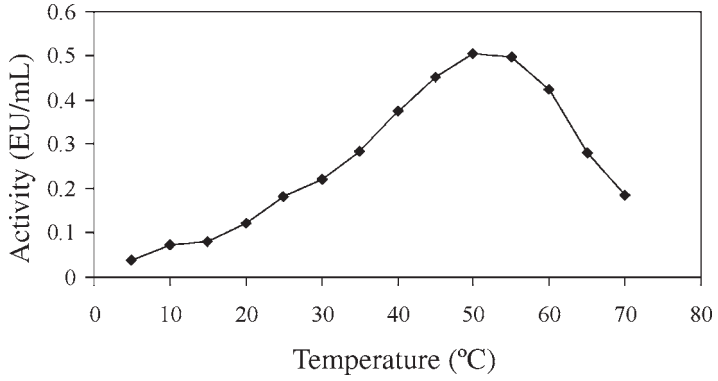

Fig. 9. Effect of temperature on glutathione reductase activity.

The Lineweaver-Burk graphs, which were constructed for NADPH and GSSG, are shown in Figs. 10 and 11. A K_M of 0.1252 m*M* and a V_{max} of 1.2656 EU/mL were obtained for NADPH and 0.1542 m*M* and 0.6677 EU/mL for GSSG.

Discussion

To our knowledge, chicken liver glutathione reductase was purified and characterized for the first time in the present study. The purification procedure consisted of preparation of homogenate, ammonium sulfate precipitation, 2',5'-ADP Sepharose 4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. The purification characteristics show that the procedure used is reliable enough to be used by other investigators. This purification also has the advantage of being relatively fast, taking only 1 d. The enzyme, having a specific activity of 120 EU/mg of protein, was purified at a 38% yield, 1714-fold. The specific activity and yield just mentioned for glutathione reductase is higher than that reported

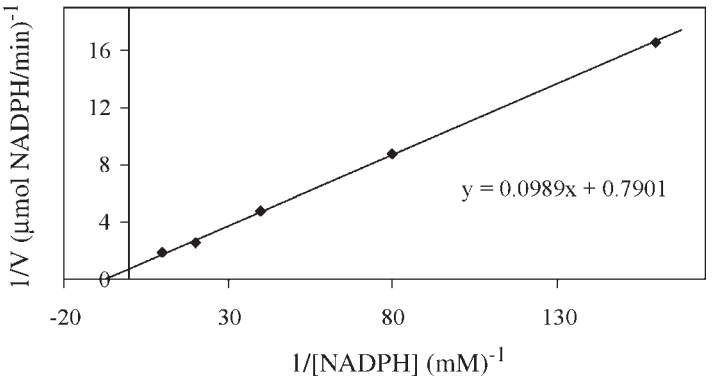

Fig. 10. Lineweaver-Burk graph of five different NADPH concentrations and at fixed GSSG concentration.

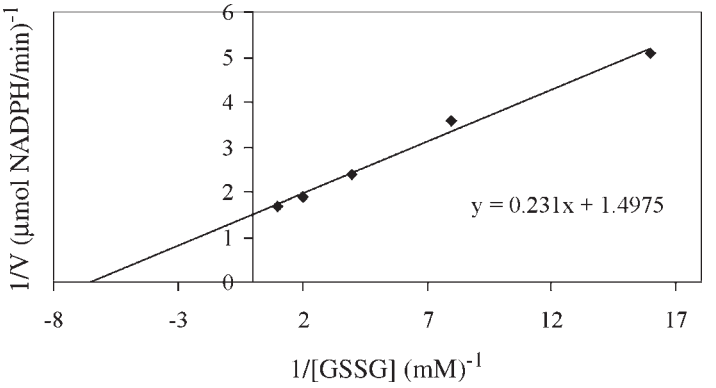

Fig. 11. Lineweaver-Burk graph of five different GSSG concentrations and at fixed NADPH concentration.

in some studies *(9)*. These results demonstrate that the purification method of glutathione reductase from chicken liver is convenient.

Figure 3 presents the results of the SDS-PAGE performed to determine the purity and molecular weight of the enzyme. A high purity of the enzyme was obtained. For glutathione reductase and standard proteins, the R_f values were calculated, and the R_f–log MW graph (Fig. 4) was obtained according to Laemmli's procedure, showing a mol wt of 43 kDa for chicken liver glutathione reductase. The native molecular weight of the enzyme was also determined by gel filtration chromatography. A K_{av}–log MW graph was obtained (Fig. 5), which showed a mol wt of 100 kDa for the enzyme. Because the molecular weight determined by gel filtration chromatography was approximately twice that by SDS-PAGE, native chicken liver glutathione reductase may be found as a dimer in an active state. Glutathione reductases of different origin have similar molecular weight, as follows: 100 kDa from human erythrocyte (dimer) *(24)*, 100 kDa from calf liver

(dimer) *(4)*, 103 kDa from porcin erythrocyte (dimer) *(10)*, 116 kDa from sheep brain (dimer) *(6)*, 125 kDa from gerbil liver (dimer) *(5)*, and 125 kDa from rat liver *(2)*.

The optimum pH of glutathione reductase was determined as 7.0 using 0.75 *M* Tris-HCl (Fig. 6), a result similar to that of previous studies *(4,5,9,25)*. The stable pH profile of the enzyme was estimated at seven different pH values using 50 m*M* Tris-HCl and 50 m*M* potassium phosphate buffer. The stable pH of the enzyme was determined to be 7.4 in Tris-HCl buffer. This pH was similar to that of bovine erythrocyte glutathione reductase *(9)*. The optimum ionic strength of the enzyme was determined to be 0.75 *M* potassium phosphate buffer. However, about 89% of the maximum activity was present in 1 *M* potassium phosphate buffer and 48% of the maximum activity was present in a 25 m*M* concentration. This concentration is higher than that of bovine erythrocyte glutathione reductase. The enzyme's highest activity point was at 50°C, after trials between 5 and 70°C. The optimum temperature determined as 50°C was similar to that of bovine erythrocyte glutathione reductase *(9)*.

The K_M for NADPH was lower than that for GSSG, suggesting a higher affinity of glutathione reductase to NADPH when compared with GSSG. The K_M values determined in our study were very similar to those obtained in rat liver *(2)*, calf liver *(4)*, and bovine erythrocytes *(9)*.

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