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Sheep brain glutathione reductase: purification and general properties

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Sheep brain glutathione reductase was purified about 11000-fold with an overall yield of 40%. The method included ammonium sulphate fractionation, heat denaturation, 2′,5′-ADP Sepharose 4B and Sephadex G-200 chromatography steps. Specific activity at the final step was 193 IU/mg. The M_r of the enzyme was found to be 116000 by gel filtration chromatography. On SDS-PAGE, two identical subunits of M_r 64000 were obtained. From the spectral data, about 2 mol FAD per mol of enzyme were calculated.

Glutathione reductase; Enzyme characterization; (Sheep brain)

1. INTRODUCTION

GSSGR (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) has been purified from a number of different organisms. The enzyme isolated from other sources has been reported to have an M_r value ranging between 100000 and 125000, consisting of two identical subunits, each containing an FAD ring [1–3].

The enzyme is responsible for maintaining glutathione in the reduced state. By coupling with the glutathione peroxidase reaction, GSSGR prevents cellular damage by oxidative stress. Brain is the organ with the highest risk of oxidative stress [4]. The aim of this study is to purify and characterize GSSGR from sheep brain, in which GSSGR is implied to have important functions.

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Abbreviations: GSSGR, glutathione reductase; GSSG, oxidized glutathione; GSH, reduced glutathione

2. MATERIALS AND METHODS

2.1. *Materials*

Fresh sheep brains were obtained from an Ankara slaughterhouse. All the other reagents were analytical grade.

2.2. *Enzyme assay*

GSSGR assays were carried out spectrophotometrically, with a Beckman spectrophotometer model 25, at 37°C, modifying the method of Staal et al. [5]. The assay system contained 100 mM sodium phosphate buffer, pH 6.8, 5 mM EDTA, 1 mM GSSG and 0.12 mM NADPH. A unit of activity is defined as the oxidation of 1 μmol of NADPH per min under the assay conditions.

2.3. *Protein assay*

Protein concentrations were determined by the method of Bradford [6].

2.4. *Purification of GSSGR*

Sheep brains were homogenized with 3 vols 50 mM sodium phosphate, pH 7.5, containing 1 mM EDTA (buffer A) and centrifuged at 14000 × *g* for 30 min. Ammonium sulphate fraction (35–55%) of the supernatant was dissolved in and dialyzed against the same buffer. The dialysate was heated for 1 h at 65°C. Precipitate was removed by centrifugation and the supernatant was applied to a column of 2′,5′-ADP Sepharose 4B (1 × 5 cm), equilibrated with buffer A. Activity was eluted with 10 ml of buffer A containing 1 mM NADPH and 1 mM GSH. Active fractions were loaded onto a column of Sephadex G-200 (1.6 × 75 cm) equilibrated with buffer A. The enzyme eluted

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Table 1

Summary of the purification steps

Steps	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
14000 × *g* supernatant	500	4700	80.5	0.017	1	100
Ammonium sulphate fractionation (35–55%)	26	1872	58.5	0.031	1.82	73
Heat treatment (1 h at 65°C)	24.5	46.3	57.1	1.233	72.5	72
2′,5′-ADP Sepharose 4B	6.3	0.38	35	92.592	5450	44
Sephadex G-200	31.6	0.17	31.9	193.330	11355	40

was concentrated and stored at −30°C after addition of 1 mg albumin per ml.

2.5. *SDS-polyacrylamide gel electrophoresis*

The method was carried out on 7.2% acrylamide slabs using modified Laemmli buffer system [7]. Coomassie Brilliant Blue R250 was used for staining.

2.6. *Molecular mass determination*

Molecular mass of the enzyme was estimated by Sephadex G-200 gel filtration according to the method of Andrews [8].

3. RESULTS AND DISCUSSION

The results of the purification are summarized in table 1. In the 2′,5′-ADP Sepharose 4B step, NADPH was used for the elution of GSSGR. NADPH has been reported to be an inhibitor of the enzyme [2,9]. GSH was added to the buffer system to protect the enzyme. The aggregated forms of the erythrocyte enzyme have been reported especially upon exposure to NADPH, due to the oxidation of thiols at the active site [2]. For this reason, we thought that this effect was also overcome by the addition of GSH. Sephadex G-200 chromatography was used to eliminate NADPH completely (fig.1). The purified enzyme preserved as described retains its activity for about 2 months. The entire procedure of purification takes 4 working days.

The molecular mass of the enzyme was found to be 116 kDa by gel filtration chromatography (fig.2). On SDS-PAGE, a single band was obtained. From the calibration curve, the subunit molecular mass was estimated to be 64 kDa (fig.3). The ratio of A_{280} to A_{460} was found to be 4.92.

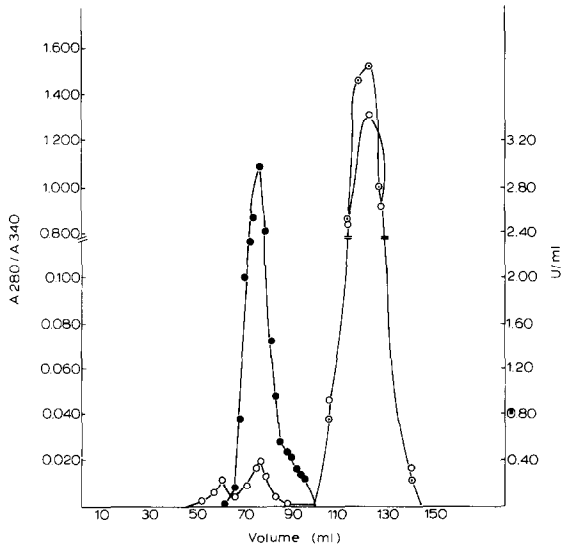

Fig.1. Elution profile of Sephadex G-200 chromatography: (●—●) enzyme activity; (○—○) A_{280} readings; (○—○), (⊙—⊙) A_{340} readings.

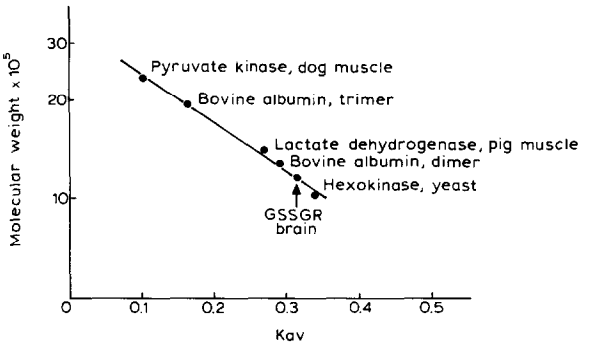

Fig.2. Calibration curve of M_r determination by gel filtration chromatography.

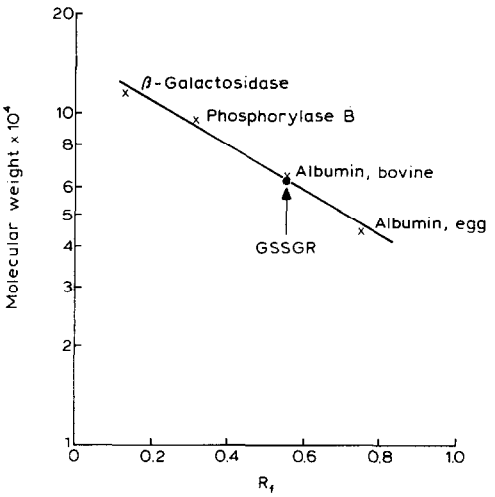

Fig.3. Calibration curve of subunit molecular mass determination by SDS-PAGE.

Employing the extinction coefficient of 11.3 $mM^{-1} \cdot cm^{-1}$ at 462 nm for enzyme-bound FAD [10], 2.36 mol of FAD per mol of enzyme was calculated. These results are in agreement with those reported for GSSGR from other sources [1–3,5].

Under the assay conditions, reaction is essentially in favour of reduction of GSSG. The reverse reaction was only 0.5% of that of the forward reaction. The enzyme also uses NADH with a specific activity of 8 IU/mg under the same conditions. Work on elucidating the kinetic properties and reaction mechanisms of the enzyme is now in progress.

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