Journal of Chromatography, 77 (1973) 191–199
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CHROM. 6444

AFFINITY CHROMATOGRAPHY IN THE PURIFICATION OF GLUTATHIONE REDUCTASE

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(Received November 1st, 1972)

SUMMARY

Three agarose-bound GSSG (oxidized glutathione) derivatives were prepared. Their ability to retain glutathione reductase from tissue extracts was compared. One derivative, GSSG–$NH(CH_2)_6NH$–agarose, was able to retain glutathione reductase from yeast, wheat germ, human lens, sheep retina and human red blood cell. The use of the derivative for the partial purification of glutathione reductase from human lens, sheep retina and human red blood cell is described.

INTRODUCTION

Affinity chromatography has been widely used in the purification of enzymes, binding-proteins, antigens and antibodies[1,2]. It appeared that glutathione reductase would lend itself to this technique; its substrate GSSG* has groups that can be readily attached to agarose, with or without spacer chains, by established procedures[3]. The preparation and properties of such agarose-bound GSSG derivatives are reported in this paper, together with the use of one derivative to achieve partial purification of glutathione reductase from several sources.

Glutathione reductase of lens and retina has not been purified previously, although some properties of the enzyme have been examined in extracts of bovine lens[4] and human cataractous lens[5]. Lens glutathione reductase is particularly interesting because of the decrease of reduced glutathione with age and with cataract formation in human lens[6]. This enzyme reaction is probably a major source of $NADP^+$ for the pentose phosphate pathway in the lens.

EXPERIMENTAL AND RESULTS

Commercial materials

Yeast glutathione reductase (E.C. 1.6.4.2) and NADPH were obtained from Boehringer Corp., London; Sepharose 4B and Sephadex G-25 from Pharmacia, London; GSSG, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and crude glutathione reductase from wheat germ from Sigma London Chemical Co., Kingston upon Thames; cyanogen bromide from Koch-Light Laboratories Ltd., Colnbrook. BDH Chemicals Ltd., Poole, supplied 1,6-diaminohexane, 6-amino-*n*-hexanoic acid, $NaBH_4$,

* GSSG = oxidized glutathione.

and most common chemicals. Aldrich Chemical Co. Inc., Milwaukee, Wisc., supplied 5,5'-dithiobis(2-nitrobenzoic acid).

Assay of glutathione reductase (E.C. 1.6.4.2)

The assay procedure of HORN[7] was used, except that 1 m*M* EDTA was also present in the assay mixture and serum was omitted.

In the present report a unit of glutathione reductase is the amount of enzyme which reduces 1 μmole of GSSG in 1 min at 25° under the assay conditions[8].

Protein estimations

Protein was estimated by the method of LOWRY *et al.*[9], as described by LAYNE[10].

Affinity column materials

GSSG–agarose was prepared by addition of GSSG (900 mg in 50 ml of 0.1 *M* sodium borate, pH 9.5) to Sepharose 4B (50 ml) that had been activated by cyanogen bromide (5 g), using the method of CUATRECASAS[3].

A second derivative of GSSG was prepared, using 1,6-diaminohexane as a 'spacer' between the agarose and the GSSG. Sepharose 4B (50 ml) was activated as before, 1,6-diaminohexane (11.6 g in 50 ml of 0.1 *M* sodium borate, pH 9.5) was added. GSSG was attached to the free amino groups of this derivative by addition of GSSG (0.5 g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (500 mg) to 100 ml of slurry at pH 4.7 (ref. 3). This derivative will be shown as GSSG–NH$(CH_2)_6$NH–agarose

A third derivative was prepared as above but using 6-amino-*n*-hexanoic acid as the spacer. This derivative will be shown as GSSG–CO$(CH_2)_6$NH–agarose.

The amount of bound GSSG in each of these derivatives was determined by measuring the glutathione, GSH, released by reduction. To 0.2 ml of settled bed of the derivative was added 2 ml of 1 *M* Tris, pH 9.0, containing 20 mg of $NaBH_4$. Reduction proceeded for 1 h at 20° before the pH was lowered to 4.0 to stop reaction. Glutathione was determined in the supernatant using the 5,5'-dithiobis(2-nitrobenzoic acid) reagent of ELLMAN[11], as described by HARDING[6]. This method does not measure GSSG that is attached to agarose by both 'ends' of the molecule, but such GSSG is unlikely to take part in enzyme binding.

The amount of GSSG bound to agarose in the three derivatives is shown in Table I. If each GSSG residue could bind one molecule of glutathione reductase, then even the GSSG–NH$(CH_2)_6$NH–agarose would bind up to 13.2 mg of glutathione reductase per ml of column bed; for the yeast enzyme this represents 1200 units/ml of column bed.

TABLE I

AMOUNT OF GSSG BOUND TO AGAROSE IN GSSG–AGAROSE DERIVATIVES

	Bound GSSG (μmole GSSG/ml settled bed)
GSSG–agarose	0.25
GSSG–NH$(CH_2)_6$NH–agarose	0.11
GSSG–CO$(CH_2)_6$NH–agarose	0.44

Lens extracts

Human lenses referred to as 'normal' were clear lenses obtained *post mortem*. For chromatography on the affinity columns, lenses (usually 10) were ground with five times their weight of water; insoluble protein was removed by centrifugation at 10,000 *g* for 20 min at 4°. In early experiments the supernatant was dialysed to remove low-molecular-weight compounds that might interfere with column binding (*e.g.* GSSG, NADPH); but this caused some loss of activity. In later experiments therefore low-molecular-weight compounds were removed from lens extracts on Sephadex G-25 (10.5 × 2 cm) by elution with 67 m*M* phosphate, pH 6.6, or 1 m*M* phosphate, pH 6.6.

Human haemolysate

Citrated whole blood less than one week old was obtained from the National Blood Transfusion Service, Churchill Hospital, Oxford. Haemolysates were prepared as described by STAAL *et al.*[8], and dialysed against twenty-five times the volume of water, two changes, for 20 h at 4°.

Sheep retina

The retinae of a sheep were removed and ground in an all-glass manual homogenizer with five times the weight of 67 m*M* phosphate, pH 6.6. Insoluble material was removed by centrifugation. The supernatant (2.8 ml) was dialysed, with stirring, against 2 l of water (three changes) for 2 h. Assays before and after dialysis showed that even this brief dialysis destroyed 40% of the glutathione reductase activity.

Elution of the affinity columns

GSSG–agarose. Yeast glutathione reductase (30 μl containing 2.7 units) was loaded onto a column of GSSG–agarose (12.6 × 1.7 cm) and eluted with 67 m*M*

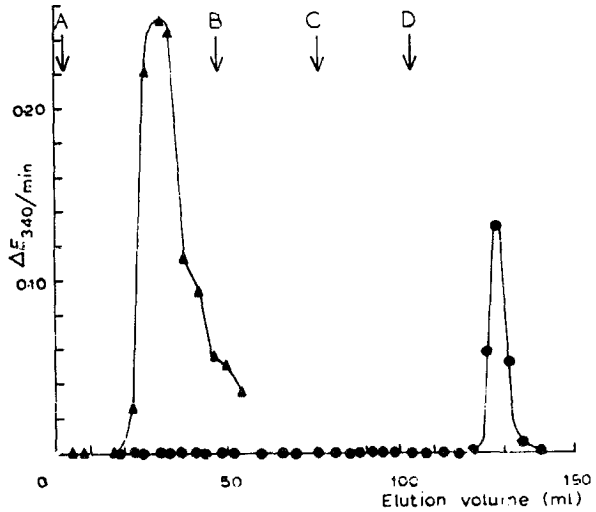

Fig. 1. Delay of yeast glutathione reductase on GSSG–NH$(CH_2)_6$NH–agarose compared with GSSG–agarose. ●—●, GSSG–NH$(CH_2)_6$NH–agarose; ▲--▲, GSSG–agarose. Eluant changes are indicated by arrows. A and C = 67 m*M* phosphate; B = 1 m*M* GSSG in 67 m*M* phosphate; D = 1 *M* NaCl in 67 m*M* phosphate. All pH 6.6.

phosphate, pH 6.6. The effluent fractions (about 3 ml) were assayed for glutathione reductase. All the activity was found after 30-ml elution (Fig. 1). Thus it appeared that glutathione reductase, at least that from yeast, was not bound to or delayed by GSSG–agarose.

GSSG–NH$(CH_2)_6$NH–agarose. Yeast glutathione reductase (30 μl containing 2.7 units) was diluted with 0.5 ml of 50 m*M* phosphate, pH 6.6, and loaded onto a column (12 × 1.7 cm) of GSSG-NH$(CH_2)_6$NH–agarose. Stepwise elution at pH 6.6 was as follows: (a) 50 ml of 67 m*M* phosphate; (b) 30 ml of 1 m*M* GSSG in 67 m*M* phosphate; (c) 30 ml of 67 m*M* phosphate; (d) 50 ml of 1 *M* NaCl in 67 m*M* phosphate; (e) 35 ml of 67 m*M* phosphate. Effluent fractions (about 3 ml) were assayed for glutathione reductase. No activity was released from the column by the first three eluants. All the activity was eluted as a single peak after 25 ml of 1 *M* NaCl in 67 m*M* phosphate, pH 6.6, had passed into the column (Fig. 1). Fig. 1 shows the elution of yeast glutathione reductase from GSSG–NH$(CH_2)_6$NH–agarose compared with its elution from a GSSG–agarose column of similar size. All the enzyme was retained by GSSG–NH$(CH_2)_6$NH–agarose until released by an increase in ionic strength of the eluting buffer.

The values of ΔE_{340}/min for the more active fractions (Fig. 1) were too great for accurate estimation and cannot be used to calculate recoveries. Recoveries quoted below are based on assays in pooled column fractions.

In three further runs with the yeast enzyme 98 to 100 % of the activity was held on the column and released only by an increase in ionic strength of the eluting buffer.

This column also retained 100 % of the glutathione reductase activity of an extract of normal human lens and of a crude wheat germ preparation.

After 4 to 6 runs during two months at room temperature, using yeast glutathione reductase, lens extracts and other tissue extracts, the columns could no longer bind all the enzyme applied. In a test run with the yeast enzyme only 40 % of the activity was retained. It seemed possible that binding ability was lost due to release of reduced glutathione, perhaps by protein thiol, so an attempt was made to regenerate the GSSG from. A solution (25 ml) of 5 m*M* GSSG in 50 m*M* phosphate made alkaline with 0.1 *M* NaOH to pH 9.5 was passed into each column. The flow was stopped and regeneration proceeded for 3 h at 20°, then the columns were washed through with 1 m*M* phosphate, pH 6.6.

The regenerated columns were tested by running each with yeast glutathione reductase. No activity was released during elution with 50 ml of 1 m*M* phosphate, pH 6.6; all the activity was eluted about 25 ml after starting elution with 1 *M* NaCl in 1 m*M* phosphate, pH 6.6. In later runs on the regenerated columns, 100 % of glutathione reductase was retained after applying either normal human lens extracts or sheep retina extracts.

Use of GSSG–NH$(CH_2)_6$NH–agarose in purification of glutathione reductase from normal human lens. Before choosing human lenses for this experiment the glutathione reductase content of lenses from several species was determined (Table II). Extracts of other lenses were prepared as described for human lens. Normal human lens had twenty times as much glutathione reductase as the most active of the other lenses (rat), so no attempt was made to isolate glutathione reductase from lenses other than human.

TABLE II

GLUTATHIONE REDUCTASE CONTENT OF ANIMAL LENSES

Result for human lens is expressed as mean ± S.D. for 20 determinations. Other results are single determinations.

Animal	*Age*	*Glutathione reductase (units/g wet wt.)*
Cow	adult	0.060
Rat	50 days	0.088
Sheep	adult	0.061
Pig	adult	0.036
Rabbit	2 months	0.038
Squid	—	not detectable
Haddock	—	0.055
Human (normal)	over 40 years	1.73 ± 0.25

Dialysed human lens extract (19 ml) containing about 5.3 units of glutathione reductase was applied to a column (12 × 1.7 cm) of GSSG–NH$(CH_2)_6$NH–agarose. The column was eluted in sequence with 180 ml of 67 m*M* phosphate, pH 6.6; 50 ml of 0.1 *M* NaCl in the phosphate buffer; 50 ml of 1 *M* NaCl in the phosphate buffer; and finally with 50 ml of the phosphate buffer itself. Flow-rate was about 20 ml/h; 3-ml fractions were collected. Elution of glutathione reductase was monitored by assay on 0.3-ml samples; protein was followed by absorbance at 280 nm of the column fractions. The elution profile is shown in Fig. 2. Although in other runs with lens extracts glutathione reductase was wholly retained by the column (for example, see below), this time a part of the activity emerged with little delay. The retained activity was eluted as two peaks, one 38 ml after starting elution with

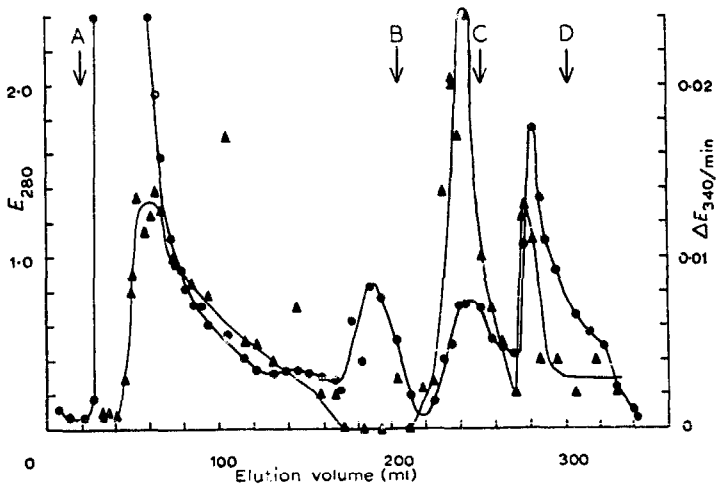

Fig. 2. Affinity chromatography of human lens extract on GSSG–NH$(CH_2)_6$NH–agarose. Column, 12 × 1.6 cm; flow-rate, 18 ml/h; ΔE_{340}/min is the decrease in extinction at 340 nm in the glutathione reductase assay brought about by a 0.3-ml sample of the effluent. ●, E_{280} for protein; ▲, ΔE_{340}/min for glutathione reductase. Eluant changes are indicated by arrows. A and D = 67 m*M* phosphate; B = 0.1 *M* NaCl in 67 m*M* phosphate; C = 1 *M* NaCl in 67 m*M* phosphate. All pH 6.6.

0.1 *M* NaCl in 67 m*M* phosphate, the other 25 ml after starting elution with 1 *M* NaCl in 67 m*M* phosphate. The effluent from 230 to 250 ml (Fig. 2) was pooled; it contained 17 % of the original glutathione reductase activity with a specific activity of 0.35 units/mg of protein compared with 0.004 units/mg of protein in the original extract. Thus, one pass through the column had improved the specific activity by a factor of 88, with 17 % recovery.

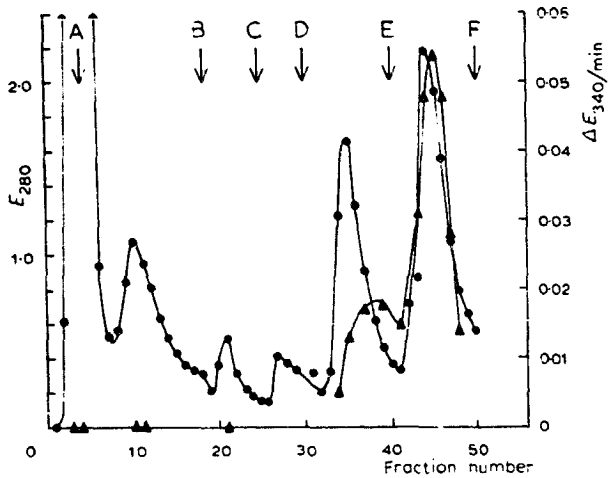

Fig. 3. Affinity chromatography of human lens extract on small column of GSSG–NH$(CH_2)_6$NH–agarose. Column, 2.7 × 1.7 cm; flow-rate, about 18 ml/h; fraction size, about 2.5 ml; ΔE_{340}/min as defined in Fig. 2. ●, E_{280} for protein; ▲, ΔE_{340}/min for glutathione reductase. Eluant changes are indicated by arrows. All are in 1 m*M* phosphate pH 6.6. In addition, B = 10 m*M* in NaCl; C = 30 m*M* in NaCl; D = 100 m*M* in NaCl; E = 1 *M* in NaCl.

In another experiment 15 ml of a human lens extract, freed of low-molecular-weight material on Sephadex G-25, was fractionated on a much smaller column (2.7 × 1.7 cm) of GSSG–NH$(CH_2)_6$NH–agarose. Column elution was at pH 6.6 as follows: 40 ml of 1 m*M* phosphate, 20 ml of 10 m*M* NaCl in 1 m*M* phosphate, 20 ml of 30 m*M* NaCl in 1 m*M* phosphate, 20 ml of 100 m*M* NaCl in 1 m*M* phosphate, 20 ml of 1 *M* NaCl in 1 m*M* phosphate, 20 ml of 1 m*M* phosphate. Effluent was monitored as above. The elution profile is shown in Fig. 3. All glutathione reductase activity was retained by the column and was only released when the salt concentration was raised to 100 m*M*. Most of the recovered activity was eluted by 1 *M* NaCl in 1 m*M* phosphate. On the other hand, most of the protein came through the column without delay and further amounts were released by each increase of ionic strength. Column fractions 43 to 47 (see Fig. 3) were pooled. They contained 35 % of the original glutathione reductase with a specific activity of 0.12 units/mg of protein, compared with 0.0035 units/mg of protein in the original extract. This represents a thirty-three-fold improvement of specific activity.

Use of GSSG–NH$(CH_2)_6$NH–agarose in purification of glutathione reductase from other tissues. Dialysed sheep retina extract (2.8 ml) was applied to the GSSG–NH$(CH_2)_6$NH–agarose column (12 × 1.7 cm) that had been regenerated by GSSG.

After the extract had soaked in, the column was eluted with 1 m*M* phosphate, pH 6.6, until E_{280} of the effluent was low (70 ml); further elution at pH 6.6 followed with 50 ml of 0.1 *M* NaCl in 1 m*M* phosphate, 50 ml of 1 *M* NaCl in 1 m*M* phosphate, and finally 50 ml of 1 m*M* phosphate. The effluent was passed through an ultraviolet absorptiometer (LKB Uvicord 4701 A) to monitor protein elution. Column fractions in the protein-containing regions were assayed for glutathione reductase. The undelayed protein and the protein eluted in 0.1 *M* NaCl in 1 m*M* phosphate had no glutathione reductase activity. The enzyme was eluted by 1 *M* NaCl in 1 m*M* phosphate. The active fractions were pooled and samples of the pool taken for protein and enzyme determinations. This pool had 0.11 units of glutathione reductase/mg of protein compared with 0.0019 units/mg of protein in the loaded extract and 0.0033 units/mg of protein in the undialysed extract. Thus a single run through GSSG–NH$(CH_2)_6$NH–agarose gave a fifty-six-fold improvement of specific activity; overall the improvement factor was 33. In this experiment no glutathione reductase was lost on the column.

Purification of glutathione reductase from a haemolysate was achieved with the use of a single increase in ionic strength.

The haemolysate (70 ml) was applied to the GSSG–NH$(CH_2)_6$NH–agarose column (12 × 1.7 cm) and allowed to run in. The column was eluted at pH 6.6 with 50 ml of 67 m*M* phosphate, followed by 50 ml of 1 *M* NaCl in 67 m*M* phosphate and then 50 ml of 67 m*M* phosphate.

Protein eluted as a peak, after 28 ml of 1 *M* NaCl in 67 m*M* phosphate had passed into the column, had glutathione reductase activity. The active fractions were pooled and examined for protein and enzyme activity. The pooled fractions contained 13 % of the original enzyme activity with specific activity improved by a factor of 60.

GSSG–CO$(CH_2)_6$NH–agarose. Yeast glutathione reductase (30 μl containing 2.7 units) was diluted with 0.5 ml of 1 m*M* phosphate, pH 6.6, and loaded on to a column (3 × 1.7 cm) of GSSG–CO$(CH_2)_3$NH–agarose. After the enzyme solution had soaked in, the column was eluted at pH 6.6 with 30 ml of 1 m*M* phosphate, then with 30 ml of 1 *M* NaCl in 1 m*M* phosphate. The effluent after elution with 1 *M* NaCl in 1 m*M* phosphate was pooled and assayed for glutathione reductase; enzyme activity was also assayed in some early fractions. Only 37 % of the original activity was delayed by the column.

The same column and elution procedure were used for application of a human lens extract. In this case all the glutathione reductase activity passed through the column undelayed.

A second run with human lens extract used a different portion of GSSG–CO$(CH_2)_6$NH–agarose. Again the glutathione reductase passed through undelayed.

DISCUSSION

The first derivative, GSSG–agarose, had GSSG attached directly to the agarose matrix, and did not bind glutathione reductase (yeast). It has been a common experience in affinity chromatography that a long chain between ligand and agarose matrix is necessary to allow binding[1–3, 12].

The third derivative, GSSG–CO$(CH_2)_6$NH–agarose, did not bind human lens

glutathione reductase and did not bind the yeast enzyme as well as GSSG–NH–$(CH_2)_6$NH–agarose. The GSSG–CO$(CH_2)_6$NH–agarose had four times as much attached GSSG as the GSSG–NH$(CH_2)_6$NH–agarose (Table I), so lack of binding is not due to lack of ligand. The length of the chain between the ligand and the agarose matrix is the same for both derivatives. It seems that the difference in enzyme binding must be related to the groups by which GSSG is attached to the 'spacer' chain. In GSSG–NH$(CH_2)_6$NH–agarose a carboxyl group of GSSG is involved in the attachment; in GSSG–CO$(CH_2)_6$NH–agarose an amino group is involved. Apparently the masking of one of the two amino groups of GSSG prevents binding of lens glutathione reductase. It is possible that both amino groups are involved in the binding of GSSG to lens glutathione reductase.

The second derivative, GSSG–NH$(CH_2)_6$NH–agarose, with a six-carbon chain between ligand and matrix was able to bind glutathione reductase from yeast, human lens, wheat germ, sheep retina and human red blood cells. Performance of GSSG–NH$(CH_2)_6$NH–agarose and GSSG–agarose is contrasted in Fig. 1. Surprisingly, yeast glutathione reductase was not released from the column by 1 m*M* GSSG. In all cases glutathione reductase could be released simply by an increase in ionic strength.

GSSG–NH$(CH_2)_6$NH–agarose was useful as a one-step partial purification procedure for glutathione reductase from a variety of tissues. Delay of inactive protein was presumably a reflection of the inevitable ion-exchange properties of these GSSG derivatives. The enzyme from human lens and retina has not been previously purified.

Glutathione reductase of red blood cell has been purified 47,000-fold to a final specific activity of 165 units mg with 5 % recovery using nine purification steps and five dialysis steps[8]. The liver enzyme has been purified 25,000-fold with 0.5 % recovery[13]. It is probable that affinity chromatography could beneficially replace one or more of the steps involved in these purifications. The present results for the sheep retina enzyme were particularly promising as the basis of a purification scheme.

Agarose-bound GSSG derivatives may be readily reduced to give agarose-bound reduced glutathione, which might be of use in the purification of glutathione peroxidase and glyoxalase.

ACKNOWLEDGEMENTS

I am grateful to Dr. A. PIRIE for helpful discussions and to Mr. K. C. RIXON for skilled technical assistance.

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