PURIFICATION OF GLUTATHIONE REDUCTASE FROM ERYTHROCYTES BY THE USE OF AFFINITY CHROMATOGRAPHY ON 2',5'-ADP-SEPHAROSE 4-B

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1. Introduction

Glutathione reductase (EC 1.6.4.2) has been purified from a variety of sources. However, the mammalian enzyme cannot easily be obtained in large quantities owing to the relatively low concentration of the enzyme in mammalian cells. The most successful purification procedure, which has been published so far, appears to be the preparation of glutathione reductase from human erythrocytes [1]. None the less, 4 weeks time and 10 purification steps are required to produce 40 mg of pure enzyme. This work has made possible the initiation of X-ray crystallographic investigations of glutathione reductase [2], and it is consequently important to investigate the chemical and physical properties of the enzyme for future correlation with the structure unravelled by X-ray diffraction methods. For such studies better purification methods are desirable.

The present paper shows that affinity chromatography on 2',5'-ADP-Sepharose 4-B, which has been designed for purification of $NADP^+$-linked enzymes [3], is a very efficient step in the purification of glutathione reductase from human or porcine erythrocytes. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), which has previously been purified simultaneously with glutathione reductase [1], can also be purified by the same technique.

2. Materials and methods

$NADP^+$ and NADPH were obtained from Sigma: GSSG from Boehringer. All other chemicals were standard commercial products of highest purity. The affinity matrix, 2',5'-ADP-Sepharose 4-B (lot no. 1858) was purchased from Pharmacia Fine Chemicals. The ligand was linked to agarose via the N^6 amino group in the adenine ring as described by Brodelius et al. [3]. Fresh pig blood was obtained from a local slaughterhouse. Human blood was outdated material from a blood bank. Erythrocytes were collected by centrifugation and washed 3 times with 0.9% NaCl containing 1 mM EDTA. Glutathione reductase activity was determined as previously described [4]. Glucose 6-phosphate dehydrogenase was assayed according to [5].

3. Results

A hemolysate was prepared by addition to 1 vol of washed erythrocytes: 3 vol of deionized water, 0.006 vol of toluene, and EDTA to a final concentration of 5 mM. After centrifugation, the stroma-free hemolysate could be chromatographed directly on a column of 2',5'-ADP-Sepharose 4-B. Fig.1 shows the results of such an experiment in which 50 ml of hemolysate prepared from human erythrocytes was applied to a 2.8 ml bed of the adsorbent equilibrated with 50 mM sodium phosphate (pH 7.6) containing 1 mM EDTA. Virtually all hemoglobin passed unretarded through the column, whereas glutathione reductase and glucose 6-phosphate dehydrogenase were adsorbed. The bed was washed with about 200 ml of the original buffer supported with 25 mM NaCl. Two consecutive pulses of 1 ml of 10 mM $NADP^+$ in the start buffer eluted 84% of the applied glutathione reductase activity in a large and a small peak (fig.1). The concentration of $NADP^+$ in the

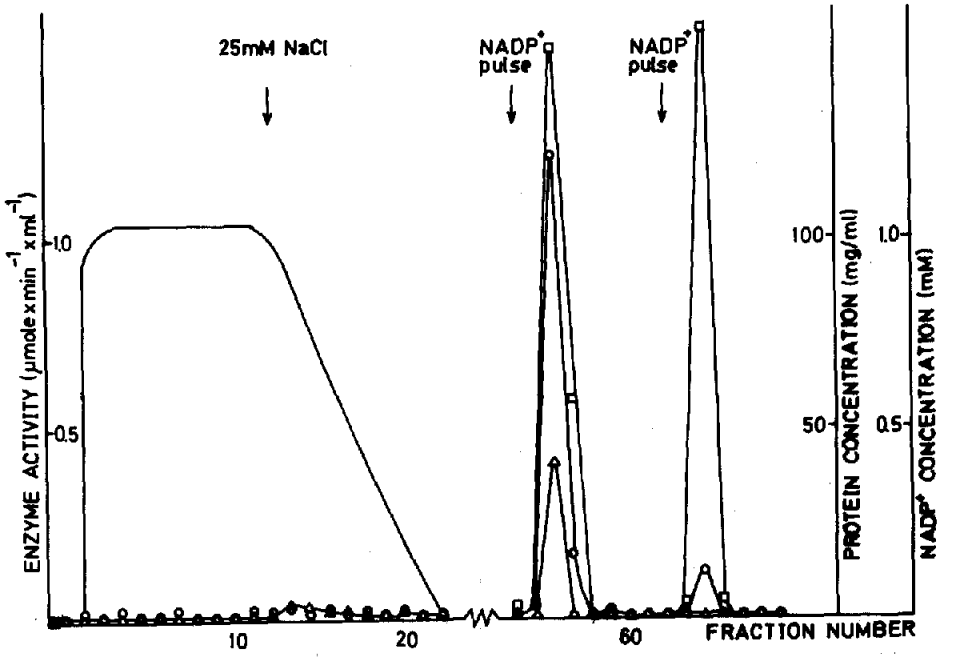

Fig.1. Affinity chromatography of human hemolysate on 2′,5′-ADP-Sepharose 4-B. A 50 ml sample was applied at 4°C to a column (2.8 ml) equilibrated with 50 mM sodium phosphate (pH 7.6) containing 1 mM EDTA. The flow rate was 3.8 ml × cm^{-2} × h^{-1} and the fraction volume was 4.6 ml. Washing was effected by 185 ml of start buffer containing 25 mM NaCl. Elution was carried out with two 1-ml pulses of 10 mM $NADP^+$ dissolved in the start buffer. Symbols: glutathione reductase activity (–○–); glucose 6-phosphate dehydrogenase activity (–△–); $NADP^+$ (–□–); protein (——).

effluent containing the two NADP(H)-dependent enzymes investigated was about 1.5 mM. After correction for the inhibitory effect of $NADP^+$ on glutathione reductase the specific activity was determined to be 21.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, which corresponds to a purification factor of 9760-fold in this single step. Glucose 6-phosphate dehydrogenase was purified 4860 fold. The smaller purification factor of this enzyme was due to a correspondingly lower yield, as compared to glutathione reductase. Pure human erythrocyte glutathione reductase has a specific activity of 240 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ [1] under assay conditions, which give somewhat higher values than our system. After correction for this difference it appears that the enzyme obtained after affinity chromatography is about 14% pure.

The purification of human glutathione reductase was not continued further, because our main interest has for a long time been focussed on the enzyme from porcine erythrocytes [6]. It appeared that the latter enzyme was equally amenable to purification by affinity chromatography on 2′,5′-ADP-Sepharose 4-B. $NADP^+$, which was used to elute the enzyme, was easily removed by adsorbing the enzyme on a hydroxyapatite column equilibrated with 10 mM sodium phosphate (pH 6.7) containing 1 mM EDTA. $NADP^+$ was washed out of the column with the buffer used for equilibration and the enzyme could then be eluted by increasing the phosphate concentration. This procedure also adds to the purification of glutathione reductase. Further purification steps involving chromatography on DEAE-cellulose and Sephadex G-150 gave a preparation of glutathione reductase having a specific activity of 125 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (table 1). Assuming that pure porcine enzyme has the same specific activity as the human enzyme, and considering that our assay gives 0.64 times less activity than the one used in [1], the purified enzyme is about 80% pure. This figure is consistent with the results of polyacrylamide gel electrophoresis, which showed one major band and some minor ones after staining for protein. The major

Table 1
Purification of glutathione reductase from porcine erythrocytes

Fraction	Volume (ml)	Total activity (μmoles/min)	Specific activity (μmoles × min^{-1} × mg^{-1})	Purification factor (-fold)
Hemolysate	1040[a]	202[a]	0.0016	1
2′,5′-ADP-Sepharose 4-B	75[a]	46.5[a]	(approx. 9)[b]	(approx. 6000)[b]
Hydroxyapatite	125	29.3	21.8	13 600
DEAE-cellulose	146	10.7	73.3	45 800
Sephadex G-150	43	7.2	125[c]	78 000

[a]Fractions from 7 experiments were combined. The yield after affinity chromatography is lower than a typical value owing to overloading of the column in some experiments.

[b]Estimated from a single experiment.

[c]Corresponding to 195 μmol × min^{-1} × mg^{-1} in the assay system of [1].

band also stained for glutathione reductase activity.

This purification scheme is presently being adapted for a large-scale procedure. A full description of the purification and characterization of the enzyme will be presented when this work has been completed.

4. Discussion

Purification of glutathione reductase by affinity chromatography has been attempted previously. In our laboratory a number of glutathione analogues, including GSSG and S-substituted GSH derivatives, have been tried as ligands (I. Carlberg and B. Mannervik, unpublished results, [4,7]) but none of the matrices were sufficiently efficient to solve the difficult purification problems encountered. Similar results were obtained by Harding using GSSG bound to Sepharose [8]. Somewhat better results were obtained in our laboratory [7] with $NADP^+$ coupled to succinylaminoethyl-Sepharose 4-B by the carbodiimide method (cf. [9]). However, the more well-defined matrix obtained by coupling N^6-(aminohexyl)-adenosine 2′,5′-bisphosphate to Sepharose [3] (marketed under the name of 2′,5′-ADP-Sepharose 4-B) seemed to be the most promising adsorbent, especially since glutathione reductase from a yeast extract had been purified 56-fold on this material [3]. The present investigation confirms this conclusion.

The findings that an ionic strength of up to 0.1 M did not elute glutathione reductase and that $NADP^+$ did release the adsorbed enzyme indicate strongly that the use of 2′,5′-ADP-Sepharose 4-B should be regarded as true affinity chromatography. The inherent ion-exchange properties of the ligand cannot contribute significantly to the experimental results obtained.

The several-thousand fold purification of glutathione reductase from erythrocytes in high yield by use of affinity chromatography may be regarded as a break-through in the preparation of the enzyme. It should also be noted that the above purification procedure involving chromatography on 2′,5′-ADP-Sepharose 4-B will also allow concurrent preparation of glucose 6-phosphate dehydrogenase (cf. [1]) and probably other $NADP^+$-linked enzymes (cf. [3]).

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References

[1] Worthington, D. J. and Rosemeyer, M. A. (1974) Eur. J. Biochem. 48, 167–177.

[2] Schulz, G. E., Zappe, H., Worthington, D. J. and Rosemeyer, M. A. (1975) FEBS Lett. 54, 86–88.
[3] Brodelius, P., Larsson, P.-O. and Mosbach, K. (1974) Eur. J. Biochem. 47, 81–89.
[4] Carlberg, I. and Mannervik, B. (1975) J. Biol. Chem. 250, 5475–5480.
[5] Cohen, P. and Rosemeyer, M. A. (1969) Eur. J. Biochem. 8, 1–7.
[6] Mannervik, B. (1969) Acta Chem. Scand. 23, 2912–2914.
[7] Carlberg, I. (1972) Thesis in candidate of philosophy examination, University of Stockholm.
[8] Harding, J. J. (1973) J. Chromatog. 77, 191–199.
[9] Larsson, P.-O. and Mosbach, K. (1971) Biotechnol. Bioeng. 13, 393–398.