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Journal of Chromatography B, 688 (1997) 239–243

JOURNAL OF CHROMATOGRAPHY B

Use of thiopropyl Sepharose for the synthesis of an adsorbent for the affinity chromatography of glutathione S-transferase

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Received 6 February 1996; revised 29 May 1996; accepted 10 June 1996

Abstract

Thiopropyl Sepharose 6B in the 2-thiopyridyl-activated form was used for the reversible immobilisation of reduced glutathione (GSH). The resulting affinity matrix was successfully tested as a sorbent for the partial purification of glutathione S-transferase (GST) from pig kidney. The specific elution of the enzyme was performed with 10 m*M* GSH in Tris–HCl buffer (pH 7.8), non-specific elution with 20 m*M* dithiotreitol (DTT) in the same buffer.

Keywords: Glutathione S-transferase; Enzymes

1. Introduction

The glutathione S-transferases (GST; EC 2.5.1.18) are a group of multifunctional, primarily cytosolic enzymes, that catalyse the nucleophilic attack of the thiol group of glutathione (GSH) on electrophilic centres in a wide variety of endogenous and exogenous organic molecules [1–3]. The reaction most frequently results in the covalent linkage of GSH, a tripeptide with the amino acid sequence γ-Glu–Cys–Gly on the second substrate, yielding a GSH conjugate, which is generally less toxic than the parent compound. This process thus leads to the detoxification of these compounds since resulting adducts are normally metabolised and excreted. GSTs are ubiquitous enzymes, their activity has been detected in several species ranging from microorganisms through fungi, plants, animals to man [4].

The GSTs from a variety of sources have been purified by conventional chromatographic procedures [5,6] as well as affinity chromatography [7,8], that has also been used for the examination and the measurement of the GST isoenzyme profile in animal and human tissues [9–11]. Because the expression of GST in cancerous and in normal tissues varies widely in both level and type, these techniques are very important in several aspects of cancer therapy.

Affinity chromatography of GST has usually been carried out with glutathione or S-glutathione derivatives irreversibly immobilised on agarose, cellulose, methacrylate or hydroxymethacrylate supports. In this paper we describe the preparation of an adsorbent for affinity chromatography of GST using the 2-thiopyridyl-activated thiopropyl Sepharose for reversible immobilisation of GSH through thiol–di-

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PII S0378-4347(96)00267-8

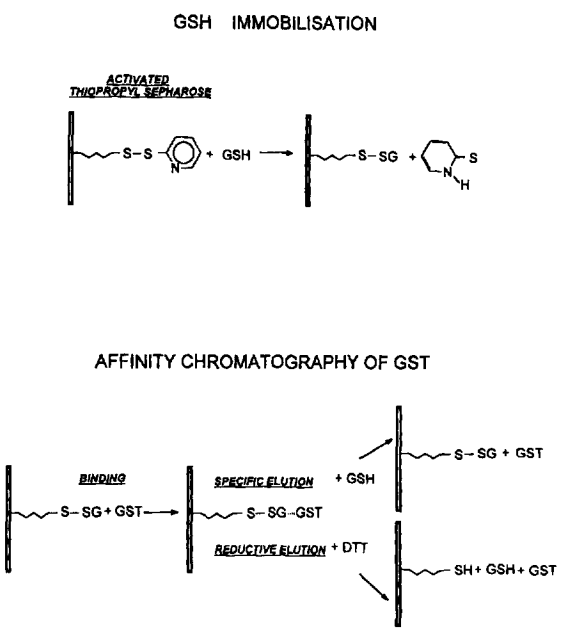

Fig. 1. Reaction scheme for purification of glutathione S-transferase (GST) on glutathione-thiopropyl Sepharose column.

sulphide exchange. The resulting affinity matrix was used for the partial purification of GST from pig kidney (Fig. 1).

2. Experimental

2.1. Materials

Pig kidneys were obtained from the slaughter house and were stored at −60°C. GSH, DTT, 2,2′-dipyridyldisulphide, 1-chloro-2,4-dinitrobenzene, Coomassie Brilliant Blue G-250 and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA) and thiopropyl Sepharose 6B from Pharmacia (Uppsala, Sweden). The materials used for electrophoretic separations were mostly from Serva (Heidelberg, Germany). All chemicals were of analytical purity.

2.2. Methods

2.2.1. GSH Immobilisation

Activated thiopropyl Sepharose column (5 cm×0.5 cm I.D.) was equilibrated with 50 m*M* Tris–HCl buffer (pH 7.8) or 50 m*M* acetate buffer (pH 4.5), containing 1 m*M* EDTA. GSH (20 m*M*) was dissolved in 10 ml of the equilibration buffer and applied onto the thiopropyl Sepharose column at a flow-rate of 0.1 ml/min. The degree of substitution was measured by monitoring the absorbance of the released 2-thiopyridone using a molar absorption coefficient [12] of $8.08\times10^{3}\ M^{-1}\ cm^{-1}$. The unbound and non-covalently bound GSH was eluted with an equilibrating buffer containing 0.2 *M* NaCl. The presence of GSH in the eluate was checked by thiol titration with 2,2′-dipyridyldisulphide [13].

2.2.2. Partial purification of pig kidney GST

Pig kidney (5 g) was partially thawed and homogenised with 10 ml of 50 m*M* Tris–HCl buffer (pH 7.8) containing 1 m*M* EDTA. The homogenate was centrifuged for 30 min at 10 000 *g* and the supernatant was filtered through a 0.22-μm membrane filter to remove floating lipids. The supernatant was then applied to the thiopropyl Sepharose column (5 cm×0.5 cm I.D.) containing immobilised GSH equilibrated with the same buffer (flow-rate 0.1 ml/min). The unbound proteins were washed out with equilibrating buffer containing 0.2 *M* NaCl and the GST was eluted with the same buffer containing 10 m*M* GSH or 20 m*M* DTT.

The chromatographic purification was performed with the Pharmacia low-pressure chromatography system consisting of a P1 peristaltic pump, a UV 1 monitor with an HR-25 cell, a FRAC 100 fraction collector and an REC-481 recorder. The samples were directly loaded on the columns with the aid of the P1 pump. All chromatographic steps were carried out in a cold room at 4–6°C. GST activity of the eluted fractions was measured simultaneously. The active fractions were pooled and stored at −60°C.

2.2.3. Enzyme analysis

The enzymatic activity of GST was assayed spectrophotometrically by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with GSH at 340 nm [7]. The reaction mixture consisted of 1 m*M* 1-chloro-2,4-dinitrobenzene and 5 m*M* GSH in 0.1 *M* phosphate buffer (pH 6.5). The absorbance of the reaction was read for 5 min. Controls were run without enzyme and the background absorbance was subtracted from the experimental data. One unit of

GST activity (IU) is defined as the amount of enzyme catalysing the formation of 1 μmol S-2,4-dinitrophenylglutathione per min at 30°C.

The protein concentration was determined by the method of Bradford [14] using bovine serum albumin as a protein standard. The spectrophotometric measurements were done with a UV 3000 Shimadzu spectrophotometer (Japan).

SDS–PAGE was performed as described previously in a Mini Protean II electrophoresis cell (Bio Rad, Richmond, CA, USA) using an optimised gel system [15] a gradient of acrylamide (T=8–21%) and N,N′-methylenebisacrylamide (C=0.5–3%). The samples for electrophoresis were diluted with sample buffer and heated for 5 min at 100°C. The protein bands were visualized by silver staining [16]. The electropherograms were analysed in a GS-670 Imaging Densitometer (Bio Rad).

2.2.4. Reactivation of thiol gel

The gel was first incubated for 45 min with a 5 m*M* solution of DTT in 0.1 *M* phosphate buffer (pH 7.5) to reduce all aliphatic disulphides which were formed in the elution. Washing with buffer removed excess DTT. The gel was then incubated for 45 min in a solution of 20 m*M* of 2,2′-dipyridyldisulphide in 0.1 *M* phosphate buffer (pH 8.0) containing 30% ethanol. Excess reagent was finally removed by extensive washing with the same buffer containing ethanol. The reactivated gel was stored at 6°C in a 0.1 *M* acetate buffer (pH 4.5) containing 1 m*M* EDTA [17].

3. Results and discussion

The binding of molecules containing free thiol groups to the thiopropyl Sepharose matrix via formation of disulphide linkage is pH dependent. At lower pH values (4–5) a high degree of coupling is achieved, but a longer reaction time is required. A faster coupling rate is found at neutral pH, but this pH yields a lower degree of coupling. The pH variation of the coupling degree is caused by the increased stability and decreased reactivity of reduced thiol groups at lower pH. In order to obtain maximum immobilisation of GSH onto the column matrix, buffers of different pH were tested for coupling; 50 m*M* acetate buffer pH 4.5 or 50 m*M* Tris–HCl buffer pH 7.8. The quantitative displacement of the 2-thiopyridyl moiety by GSH is important for suppression of non-specific binding of free thiol-containing proteins to the affinity matrix through thiol–disulphide exchange. In both buffers almost the same degree of substitution was achieved (between 15–18 μmol GSH per ml of gel). This level of substitution is in agreement with the data given for the activated thiopropyl Sepharose by the manufacturer [18].

The resulting affinity matrix was used for the partial purification of GST from pig kidney (Fig. 2). An almost homogeneous preparation of GST was obtained using the specific elution with free ligand-

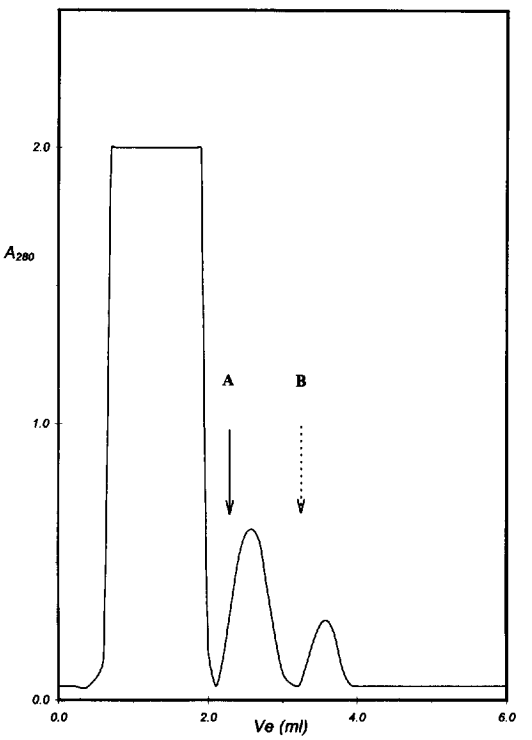

Fig. 2. Partial purification of the crude preparation of pig kidney GST on glutathione-thiopropyl Sepharose column. Approximately 50 mg of protein were applied on the column equilibrated with 50 m*M* Tris–HCl pH 7.8 containing 1 m*M* EDTA. After washing the column with equilibrating buffer, the same buffer containing 0.2 *M* NaCl (A) and 0.2 *M* NaCl with 10 m*M* GSH or 20 m*M* DTT (B) were applied at the positions marked with arrows (A) and (B). The flow-rate was 0.1 ml/min. V_E=elution volume; solid line: absorbance at 280 nm.

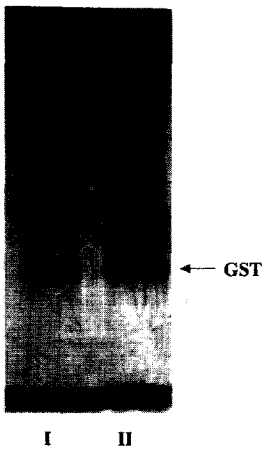

Fig. 3. SDS–PAGE electropherogram of GST elution with 10 m*M* of GSH (lane I) or by 20 m*M* of DTT (lane II) in 50 m*M* Tris–HCl pH 7.8 containing 0.2 *M* NaCl and 1 m*M* EDTA. Corresponding densitograms of lane I and II are shown below in (a) and (b), respectively.

GSH (Fig. 3a,b). The increase in specific activity was almost 150-fold, but the yield was only 50%. Improvement of the recovery was achieved, when the specific elution was replaced by elution under "reductive" conditions using DTT. This so-called "reductive" elution yielded approximately 100% recovery of activity, but the purification of GST was slightly poorer (120-fold) in that the resulting enzyme preparation contained a small amount of contaminant proteins (Fig. 3).

Binding capacity of the affinity matrix was determined through elution chromatography using a crude preparation of pig kidney GST. The glutathione affinity matrix prepared as described has a capacity of about 0.4 mg GST per ml of gel (using DTT for the reductive elution). It means that less than 0.1% of the immobilised GSH is accessible for the enzyme. The very small binding accessibility is probably due to steric exclusion. The binding capacity for GST is comparable with results achieved by using columns with covalently immobilised GSH on epoxy-activated matrices [8].

The extent of non-specific interaction between GST and thiopropyl Sepharose was determined using a matrix without immobilised GSH; both thiopropyl Sepharose in the 2-thiopyridyl activated form and in the thiol form were tested. The thiol form of thiopropyl Sepharose did not bind GST. The thiopropyl activated form did bind the enzyme, but through formation of mixed disulphide bond. Purification of GST was only 5-fold in contrast with that achieved with a glutathione-immobilised affinity

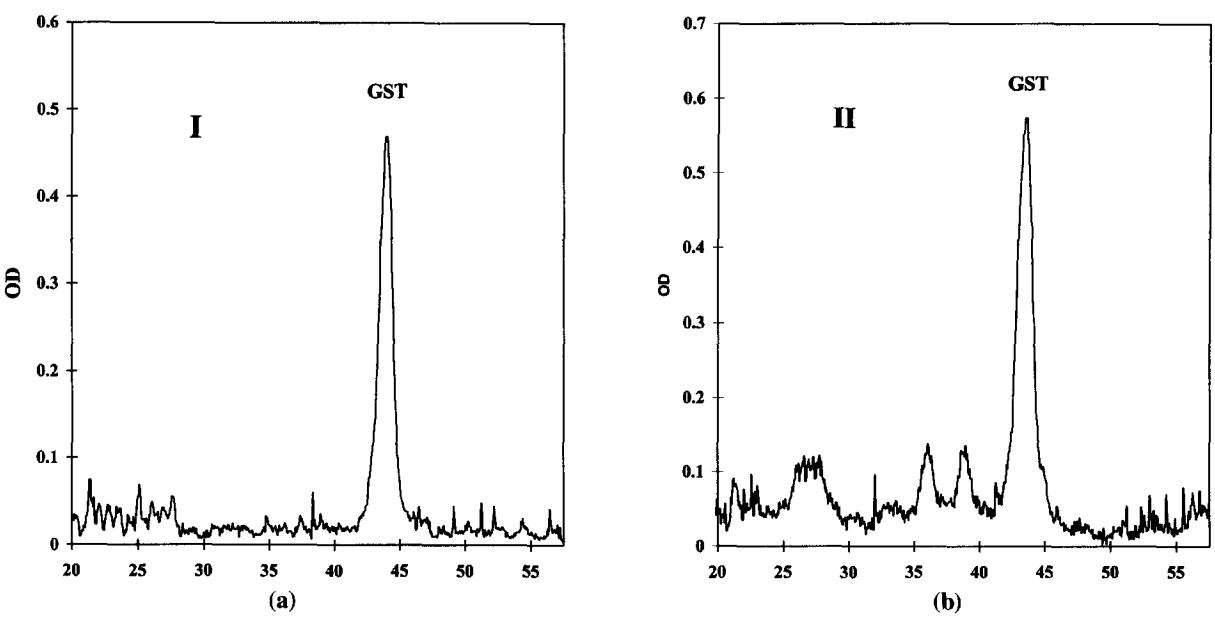

Fig. 3. (*continued*)

column. Reductive elution with DTT was used in both cases. These results also confirm that binding of GST on the glutathione affinity matrix is predominantly specific.

Since the GSH immobilisation is reversed by reductive elution with DTT, the affinity matrix has to be reactivated with 2,2′-dipyridyldisulphide followed by immobilisation of GSH before repeated use for GST purification. We have used the same matrix three times. The degree of GSH immobilisation, the binding capacity, the purification and the recovery of GST activity were in all cases completely reproducible.

The possibility of using this affinity matrix for purification of other enzymes exhibiting affinity interactions towards the glutathione structure was also tested, but unfortunately glyoxylase I and glutathione reductase did not bind to the affinity column.

4. Conclusion

Application of the activated thiopropyl Sepharose for the preparation of an affinity sorbent for GST has several advantages over the common alternative procedures so far published [7,8]. The preparation of the sorbent is simple and rapid, the sorbent is ready for use in 30 min in contrast to a time of up to several days required for GSH immobilisation on epoxy-activated matrices. The enzyme is eluted under mild non-denaturing conditions in a relatively small volume, retaining its activity. This system also provides an efficient tool for the screening of suitable glutathione derivatives as "ligands" for certain GST isoenzymes [19]. However, it may be emphasised that the absorption of the crude enzyme preparation must be performed in the absence of free low-molecular-mass thiols.

The GSH immobilisation as briefly described in this paper thus represents a fairly simple procedure for the rapid preparation of a stable matrix for the GST purification and offers a new and flexible tool for studies of this important enzyme. Since the GSH immobilisation during the GST elution is reversed, the matrix can be regenerated and thus re-used to immobilise different thiol-containing ligands as reactive centers for affinity chromatography or as a sorbent for covalent chromatography.

Acknowledgments

This work was supported by grant No. 302/96/1393 from the Grant Agency of the Czech Republic. We also thank the Benzina a.s. for partial support. The authors wish to thank Dr. M. Varad'a for densitometric measurements.

References

[1] W.B. Jacoby and W.H. Habig, in W.B. Jacoby (Editor), Enzymatic Basis of Detoxication, Vol. 3, Academic Press, New York, 1980, p. 63.
[2] B. Coles and B. Ketterer, CRC Crit. Rev. Biochem., 25 (1990) 47.
[3] C.B. Pickett and A.Y.H. Lu, Annu. Rev. Biochem., 58, (1989) 743.
[4] R.C. Fahey and A.R. Sunquist, Adv. Enzymol. Relat. Areas Mol. Biol., 64 (1990) 21.
[5] W.H. Habig, M.J. Pabst and W.B. Jacoby, J. Biol. Chem., 249 (1974) 7130.
[6] W.H. Habig and W.B. Jacoby, in W.B. Jacoby (Editor), Methods in Enzymology, Vol. 77, Academic Press, New York, 1981, p. 218.
[7] P.C. Simons and D.L. Vander Jagt, Anal. Biochem., 82 (1977) 334.
[8] P.C. Simons and D.L. Vander Jagt, in W.B. Jacoby (Editor), Methods in Enzymology, Vol. 77, Academic Press, New York, 1981, p. 235.
[9] J.B. Wheatley, M.K. Kelley, J.A. Montali, C.O.A. Berry and D.E. Smith Jr., J. Chromatogr. A, 663 (1994) 53.
[10] J.B. Wheatley, J.A. Montali and D.E. Smith Jr., J. Chromatogr. A, 676 (1994) 65.
[11] J.B. Wheatley, B. Hughes, K. Bauer and D.E. Smith Jr., J. Chromatogr. A, 676 (1994) 81.
[12] T. Stuchbury, M. Shipton, R. Norris, J.P.G. Malthouse, K. Brocklehurst, J.A.L. Herbert and H. Suschitzky, Biochem. J., 151 (1975) 417.
[13] K. Brocklehurst, J. Carlsson, M. Kiersten and E. Crook, Biochem. J., 133 (1973) 573.
[14] M.M. Bradford, Anal. Biochem., 72 (1976) 248.
[15] I. Matysková, J. Kovář and P. Racek, Biochim. Biophys. Acta, 839 (1985) 300.
[16] C. Damerval, Electrophoresis, 8 (1987) 158.
[17] L. Rydén and J. Carlsson, in J. Carlsson and L. Rydén (Editors), Protein Purification, p. 253.
[18] Pharmacia Fine Chemicals, Data Sheet, Media for Covalent Chromatography, Uppsala, 1984.
[19] V.M. Castro, M.K. Kelley, A. Engqvist-Goldstein and L.M. Kauvar, Biochem. J., 292 (1993) 371.