Journal of Chromatography, 150 (1978) 279–283

CHROM. 10,446

Note

Interaction of glycosidases with insolubilized ovalbumin

R. T. DEAN*

Department of Experimental Pathology, University College Hospital Medical School, University Street, London WC1E 6JJ (Great Britain)

(Received July 25th, 1977)

Several adsorbents for affinity chromatography of N-acetylhexosaminidases[1–4] and other mammalian glycosidases[5–7] have been described recently. The preparations of most chemically stable adsorbents require quite tedious chemical syntheses to obtain modified substrates or inhibitors that can be coupled to solid phase supports, such as Sepharose 4B[8]; while some are classical immuno-adsorbents consisting of insolubilized antibodies specific for the enzyme[9–11] or multiple form[12] required. Few exploitations of natural substrates for affinity chromatography of glycosidases have been reported, but a glycopeptide from cartilage proteoglycan has been insolubilized, and shown to bind hexosaminidase A but not B (ref. 13). The purifications achieved from crude extracts by means of the listed procedures range from 3–1000 fold, but none of the products of one-step affinity chromatography seems to be pure protein. Indeed, several of the adsorbents have been operated under conditions favouring non-specific interactions, such as ion exchange (operation at low ionic strength[5,6]; see also discussion in ref. 14) and hydrophobic interaction (use of hydrophobic spacer arms[15]). Therefore recycling on the same[16], or more probably, different adsorbents will be required for complete purification of the enzymes concerned.

This paper describes an addition to the armoury of adsorbents for glycosidases, which may be of use in such purifications, in combination with other adsorbents. Unlike most of the adsorbents mentioned above, all the materials for preparation of this ovalbumin–Sepharose 4B conjugate, are readily available, without complex syntheses or purifications. Ovalbumin is a glycoprotein with free N-acetylglucosamine termini, and thus potentially a substrate for N-acetylhexosaminidase.

EXPERIMENTAL

Ovalbumin (Sigma) was covalently linked to Sepharose 4B using cyanogen bromide[8]. Sepharose was activated with 50 mg of cyanogen bromide per gram Sepharose 4B at pH 11, and coupling was performed (4°, 16 h) after washing the Sepharose with 100 mM sodium hydrogen carbonate. After reaction, the gel was washed with 0.5 M sodium chloride until the E_{280} reached zero, and stirred with

* Present address: Division of Cell Pathology, Clinical Research Centre, Watford Road, Harrow, Middlesex HA3 8AE, Great Britain.

0.2 *M* glycine (4°, 16 h) to block any remaining activated sites. The amount of ovalbumin bound was determined by difference; the preparation used contained approximately 10 mg of ovalbumin per ml packed Sepharose.

To obtain a soluble extract containing hexosaminidase and other glycosidases, a homogenate of rat liver (five passes of a tight Dounce) in 100 m*M* sodium acetate buffer, pH 4.5, containing 100 m*M* sodium chloride (1 g liver per 5 ml homogenate) was sonicated (4 × 15 sec, with cooling; MSE 150 W disintegrator) and sedimented (100,000 *g*, 60 min) at 4°. The supernatant was used for affinity chromatography.

Ovalbumin–Sepharose (10 ml) was packed in a 1.5 × 1.0 cm column, and equilibrated with 100 m*M* sodium acetate buffer (pH 4.5) and 100 m*M* NaCl (to suppress ionic interactions). The enzyme sample was run on (flow-rate 20 ml/h) and the column washed with four bed volumes of the equilibrating buffer (the pH of which is near the pH optimum for hexosaminidase activity). Elution was then usually continued with a mixture of 100 m*M* Tris–HCl (pH 7.8) and 100 m*M* sodium chloride, though other procedures are described in the results section.

N-Acetylhexosaminidase and β-galactosidase were assayed against their fluorigenic umbelliferone substrates, used at 0.2 m*M* in each case. Both assays were performed at pH 5.0 (100 m*M* sodium acetate buffer containing 0.1% Triton X-100) and 37°, in a reaction volume of 0.2 ml (ref. 17). The unit of enzyme activity is that amount which releases 1 nmole product per hour under the defined conditions.

RESULTS AND DISCUSSION

Initially, out of 6500 units of hexosaminidase, applied to the column, 1430 units remained bound after the wash, which brought the E_{280} of the eluate to that of the equilibrating buffer mixture. This activity could be largely eluted by the pH 7.8 buffer (at which pH hexosominidase A and B are inactive), with a total recovery of the enzyme activity of 95%.

The profile of a representative subsequent run is shown in Fig. 1. Table I indicates that a seven-fold purification was achieved in this run. The specific activity of the peak tube was higher by approximately five times, as the protein and enzyme curves are not parallel, indicating the presence of proteins other than hexosaminidase. Hexosaminidase was not adsorbed by a control column prepared from activated Sepharose as described above, but omitting the ovalbumin during the coupling step.

Even when small amounts of hexosaminidase were applied, 10% or more of the enzyme emerged in the washing eluate, but the "capacity" of the column was taken as the amount of enzyme bound from 6500 units of enzyme. During repeated use of the column, the capacity fell rapidly: by the fifth run it was only 10% of that initially. This may have been due to either glycosidase or protease activity during the running of the column.

Hexosaminidase was also adsorbed when the column was run with 0.5% Triton X-100 in all buffers, and eluted by the pH 7.8 buffer containing Triton. In contrast, the detergent has been used to elute some glycosidases from other adsorbents[13].

Alternative eluents were assessed for their ability to remove bound enzyme. The enzyme was not eluted by 10 m*M* N-acetylglucosamine in the pH 4.5 buffer, but was eluted by the substrate *p*-nitrophenyl-β-D-N-acetylglucosaminide (2.5 m*M*) at that pH. The enzyme so eluted was dialysed at pH 4.5 to remove the substrate

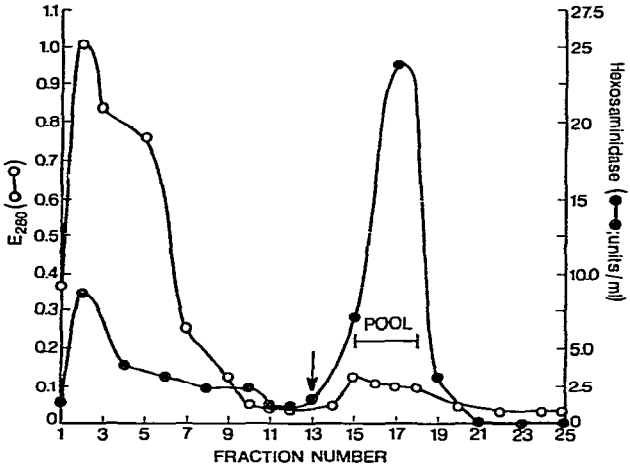

Fig. 1. Affinity chromatography of hexosaminidase on ovalbumin–Sepharose. A 0.7-ml portion of a soluble extract of rat liver (see Experimental) was applied to the column which was then washed and eluted as described in Experimental. Fractions of 3.8 ml were collected. The arrow indicates the application of a mixture of 100 m*M* Tris–HCl (pH 7.8) and 100 m*M* sodium chloride, and the horizontal bar the fractions pooled as the purified hexosaminidase sample.

before assay with the umbelliferone, and was found to have a higher specific activity than that eluted by the pH shift (purification of 15–20 fold in three runs, over the enzyme extract). An intermediate degree of purification was obtained by elution with a pH gradient in acetate from pH 4.5 upwards (hexosaminidase was eluted between pH 5.5 and 6.0).

Recycling of the purified enzyme from one column run, gave a further increase in specific activity, particularly when different elution methods were used in each run (pH, substrate), so that the maximum increase in specific activity achieved by a pair of runs was thirty-fold.

Thermal inactivation of the purified enzyme (pH 4.5, 50°, 30 min; 30–40% inactivation with three preparations) indicated the presence of heat-labile components[18]. Hexosaminidase C has not been noted in rat-liver tissue yet[19,20], but the multiple form composition of this tissue needs further study. However, the almost quantitative binding of small amounts of hexosaminidase suggests that the column binds both forms A and B.

Thus a specific interaction between hexosaminidases and ovalbumin–Sepharose occurs; whether or not this is productive enzyme–substrate binding is not clear. Bovine hexosaminidase does not seem to hydrolyse the ovalbumin carbohydrate moiety[21] although slow hydrolysis of this substrate by purified hen oviduct hexosaminidase has been demonstrated[22].

The column also absorbed β-galactosidase effectively: in one run (Table I) about 60% of the enzyme applied was bound and eluted at pH 7.8, with a purification of about six-fold. This enzyme was not eluted by the hexosaminidase substrate. The nature of this interaction is unclear since galactose is not a major component of the ovalbumin glycopeptide.

Other acid glycosidases for which the heterogeneous carbohydrate side-chains

TABLE I

CHROMATOGRAPHY OF GLYCOSIDASES ON OVALBUMIN-SEPHAROSE

Protein was determined by the method of Lowry *et al.*[27]. The results for the chromatogram are illustrated in Fig. 1. The relative specific activity (RSA) is the ratio of the specific activity of an enzyme in a column pool to its specific activity (SA) in the starting material.

Substance	*Protein*		*Hexosaminidase*			*β-Galactosidase*		
	Total amount (mgs)	*Concentration (mg/ml)*	*Total activity (units)*	*SA units/mg protein)*	*RSA*	*Total activity (units)*	*S.A. (units/mg protein)*	*RSA*
Soluble enzyme extract applied to column	19.6	28.0	260	13.3		25.2	1.29	
Material washed through column by the equilibrating buffer (100 m*M* sodium acetate, pH 4.5, 100 m*M* NaCl) (fractions 1–12)	—*	—	70	—	8.1	—		
Purified enzyme pool (fractions 15–18)	1.91	0.124	180	94.2	7.1	15.1	7.91	6.1
Total recoveries of enzymes (percentage recoveries in brackets)			250 (96%)			23.2 (92%)		

* — = Not determined.

are substrates (such as mannosidases) may be adsorbed, together with some proteinases and glycosyltransferases. Indeed, ovalbumin–Sepharose has been efficiently used as a substrate for galactosyltransferase[9]. The pH of adsorbtion and elution will allow some control of specificity: thus the recently discovered neutral endoglycosidase[23] will probably be adsorbed only at pH's near neutrality. The breadth of the specificity of the present adsorbent may therefore be comparable to, but probably less extensive than, that of concanavalin A–Sepharose, which binds many glycoproteins, and has given good purifications of mixtures of glycosidases[24–26].

Ovalbumin–Sepharose may be useful in conjunction with other adsorbents, in the purification of single glycosidases. In addition, it may be applicable to the study of the enzyme–substrate interaction of glycosidases and glycoproteins.

REFERENCES

1 B. Geiger and R. Arnon, *FEBS Lett.*, 45 (1974) 276.
2 E. E. Grebner and I. Parikh, *Biochim. Biophys. Acta*, 350 (1974) 437.
3 E. Bamberg, F. Dorner and W. Stoehl. *Experientia*, 31 (1975) 516.
4 M. Pokorny and C. P. J. Gleudemans, *FEBS Lett.*, 50 (1975) 66.
5 R. G. Harris, J. J. M. Rowe, P. S. Stewart and D. C. Williams, *FEBS Lett.*, 29 (1973) 189.
6 J. N. Kanfer, G. Petrovich and R. A. Mumford, *Anal. Biochem.*, 55 (1973) 301.
7 C. A. Mapes and C. C. Sweeley, *J. Biol. Chem.*, 248 (1973) 2461.
8 P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.*, 22 (1971) 345.
9 R. T. Dean, *Ph.D. Thesis*, University of Cambridge, 1973.
10 R. T. Dean, *Biochem. J.*, 138 (1974) 407.
11 F. E. Brot, J. H. Glaser. K. J. Roozen and W. S. Sly, *Biochem. Biophys. Res. Commun.*, 57 (1974) 57.
12 G. D. Vladutiu, P. J. Carmody and M. C. Rattazzi, *Prep. Biochem.*, 5 (1975) 147.
13 G. Dawson, R. L. Popper and A. Dorfman, *Biochem. Biophys. Res. Commun.*, 54 (1973) 1102.
14 R. T. Dean, *Biochem. J.*, 138 (1974) 395.
15 P. O'Carra, S. Barry and E. Corearay, *FEBS Lett.*, 43 (1974) 169.
16 I. P. Trayer and R. L. Hill, *J. Biol. Chem.*, 246 (1971) 6666.
17 T. J. Peters, M. Muller and C. de Duve, *J. Exp. Med.*, 136 (1972) 1117.
18 D. Robinson and J. L. Stirling, *Biochem. J.*, 107 (1968) 321.
19 D. Robinson and J. L. Stirling, *Biochem. J.*, 115 (1969) 39P.
20 R. T. Dean, in J. T. Dingle and R. T. Dean (Editors), *Lysosomes in Biology and Pathology*, Vol. 4, North Holland, Amsterdam, (1975) pp. 349–382.
21 E. Werries and L. Fressman, *Hoppe-Seyler's Z. Phys. Chem.*, 355 (1974) 1479.
22 A. L. Tarentino and F. Maley, *Arch. Biochem. Biophys.*, 147 (1971) 446.
23 M. Nishigaki, T. Muramatsu and A. Kobata, *Biochem. Biophys. Res. Commun.*, 59 (1974) 638.
24 S. Bishayee and B. H. Bachawat, *Biochim. Biophys. Acta*, 334 (1974) 378.
25 D. K. Banerjee and D. Basu, *Biochem. J.*, 145 (1975) 113.
26 E. Beutler, E. Giunto and W. Kuhl, *J. Lab. Clin. Med.*, 85 (1975) 672.
27 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.