Journal of Chromatography, 194 (1980) 95–99
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,717

Note

Affinity chromatography of rat liver lactate dehydrogenase on the Remazol derivative of bead cellulose

DANICA MISLOVIČOVÁ, PETER GEMEINER*, ĽUDOVÍT KUNIAK and JIŘÍ ZEMEK
Institute of Chemistry, Slovak Academy of Sciences, 809 33 Bratislava (Czechoslovakia)
(Received January 2nd, 1980)

Much attention has been paid to anthraquinone–triazine derivatives, which have been used as general affinants in the affinity chromatography of enzymes[1,2] and other proteins[3]. The most frequently used carriers of these affinants are polysaccharides such as agarose and dextran. However, some of their hydrodynamic properties and the economy of their use are not always advantageous.

Cibacron Blue F3G-A has been used most frequently as an anthraquinone–triazine affinant. So far, experiments using powdered cellulose as a carrier of Cibacron Blue for the affinity chromatography of enzymes and serum proteins have not offered encouraging results[2–4]. The properties of macroporous bead cellulose, however, differ from those of cellulose. Its superior hydrodynamic properties, regular geometric shape and high porosity[5] on the one hand and its low price on the other have made it attractive for chromatographic procedures.

Previous experiments with Cibacron Blue have shown that the functional part of this large molecule, resembling the structure of nucleotides, has an anthraquinone arrangement[6]. The affinity of some enzymes, mainly NAD(P)-dependent dehydrogenases of animal origin, *e.g.*, lactate, malate and glyceraldehyde-3-phosphate dehydrogenases, towards this arrangement of the molecule is related to the presence of a "dinucleotide fold" super-secondary structure in these enzymes[1,2,6]. Our results have also indicated that some simpler anthraquinone arrangements, even without the triazine part, in the form of water-insoluble derivatives show higher affinities towards the given enzymes than the Cibacron Blue derivatives of these polysaccharides[7]. This paper deals with the application of one of these compounds, Remazol Brillant Blue R, as a derivative of bead cellulose for the affinity chromatography of lactate dehydrogenase from rat liver extracts.

EXPERIMENTAL

Preparation of the Remazol Brillant Blue derivative of bead cellulose

The Remazol Brillant Blue (RBB) derivative of bead cellulose (20–320 μm) was prepared by suspending 5 g of bead cellulose (0.61 g of dry cellulose) in 10 ml of 0.25 *M* sodium hydroxide solution followed by reaction, under continuous stirring, with 0.1 g of the dye EE AB 505 Remazol Brillant Blue R, C.I. Reactive Blue 19 (Farbwerke Hoechst, Frankfurt/Main, G.F.R.) at 25 °C for 1 h. The product was

0021-9673/80/0000–0000/$02.25 © 1980 Elsevier Scientific Publishing Company

washed thoroughly with distilled water until the washings were colourless, then distilled water containing 0.02% of sodium azide was added and the slurry was stored at 0–4 °C. The amount of dye bound to the dry cellulose was determined to be 65 μmole/g from the visible absorption of the solvent phase after the coupling reaction and using a molar absorptivity of 5930 l/mole·cm for RBB in water at 590 nm.

Affinity chromatography of lactate dehydrogenase from rat liver homogenate

In the preparation of 20% ethanol–10 m*M* Tris buffer extract from rat livers[8], a 20 m*M* Tris–hydrochloric acid buffer of pH 7 containing 2 m*M* of EDTA was used instead of 0.5 *M* sodium chloride solution. Livers were obtained from male white rats (Wistar). Two procedures were applied in the purification of crude lactate dehydrogenase (LDH) by affinity chromatography on the RBB derivative of bead cellulose.

Procedure 1. A 10-ml volume of the RBB derivative of bead cellulose was washed with 25 ml of a 0.1% solution of bovine serum albumin, 100 ml of 10 m*M* Tris–hydrochloric acid buffer (pH 7.5) containing 1 m*M* of EDTA and 2 m*M* of 2-mercaptoethanol (solution A), 100 ml of 1 *M* sodium chloride solution and finally with 200 ml of solution A. To a column thus prepared (10 ml; 20 × 0.9 cm), a portion of rat liver extract (25 ml) containing 2-mercaptoethanol at a final concentration of 2 m*M* and with the pH adjusted to 7.5 was applied. The column was then washed with 230 ml of solution A. The bound LDH was eluted with 120 ml of solution A containing 1 m*M* of reduced nicotinamide adenine dinucleotide (NADH) (solution C).

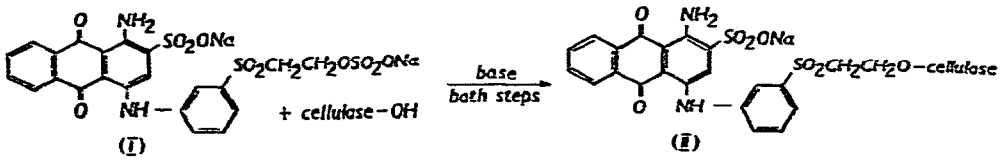
Procedure 2. This was the same as procedure 1 except that after washing with solution A elution was carried out with 100 ml of solution A containing 1 m*M* of nicotinamide adenine dinucleotide (NAD) (solution B) and finally with 160 ml of solution C.

For repeated applications, the column was finally washed, in both instances, with 60 ml of 1 *M* sodium chloride solution.

Fractions of 10 ml were collected at a flow-rate of 12 ml/h and suitable aliquots from each fraction were assayed for LDH activity and protein concentration. The catalytic activity of LDH was determined spectrophotometrically[9] and the protein concentration with bovine serum albumin as the standard[10]. Aliquots from the effluent with the highest specific activity were examined by electrophoresis on polyacrylamide gel. Slab gel electrophoresis was performed on SDS polyacrylamide gel (10%, w/w)[11] (80 V, 30 mA) for 3.5 h at 25 °C, without addition of thiol. A 10–20-μg amount of sample was applied. Standard proteins, bovine pancreas ribonuclease and chymotrypsin, hen ovalbumin and bovine serum albumin were supplied by Calbiochem (San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

Etherification of the cellulose hydroxyl groups with Remazol Brillant Blue R (I) proceeds in two steps[12] (summarized in Scheme 1) and, in contrast with Cibacron Blue F3G-A[1–4], under mild reaction conditions. Alkali metal hydroxides promote the reaction. The vinylsulphonyl group formed in the first step etherifies the polysaccharide in the second step and the product is formed with a high degree of conversion. The RBB derivative of the bead cellulose (II) had a high stability.



(I) (II)

Scheme 1.

Of the several anthraquinone–triazine derivatives of bead cellulose, including Cibacron Blue F3G-A, the RBB derivative has been shown to be the best for chromatographic purposes [7].The RBB derivative of the bead cellulose used was found to have a binding capacity of 1.5 mg/ml of rabbit muscle LDH (Biochemica Boehringer, Mannheim, G.F.R.) or 0.6 mg/ml of bovine serum albumin (Sevac, Prague, Czechoslovakia).

In previous experiments it was shown[1,2,6,7] that NADH was the best eluent of LDH from the antraquinone–triazine derivatives of polysaccharides. The immediate elution of LDH from the RBB derivative of bead cellulose was also achieved when a 1 m*M* solution of NADH in equilibrium buffer (solution C) was used. The purification factor of LDH from the rat liver extracts may be increased by prior elution with 1 m*M* NAD (solution B), but part of the LDH is also released. Successive elution with 1 m*M* NADH, on other hand, resulted in an almost 25-fold enrichment of LDH (Table I).

The differences between elution in two steps (procedure 2) and a single elution with 1 m*M* NADH (procedure 1) are also apparent from Fig. 1. Procedure 2 gives a purer fraction of LDH. The LDH monomer, having a molecular weight of 35,000, is within the range of simple polypeptides of chymotrypsin (molecular weight 22,600) and ovalbumin (molecular weight 45,000).

The purification factor of LDH achieved by affinity chromatography of rat liver extract by the procedure 2 is higher than that achieved in four steps of the traditional seven-step procedure[8]. In addition to the practical advantage of time saving,

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURES

Step No.	*Step*	*Volume (ml)*	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
1	Procedure 1: ethanol–Tris buffer extract	25	310	473	1.5	1.0	100
2	RBB–bead cellulose, affinity elution, NADH eluate	120	18	431	24.0	15.7	91
1	Procedure 2: ethanol–Tris buffer extract	25	319	452	1.4	1.0	100
2	RBB–bead cellulose, affinity elution, NADH eluate after NAD elution	160	8	266	33.3	23.4	59

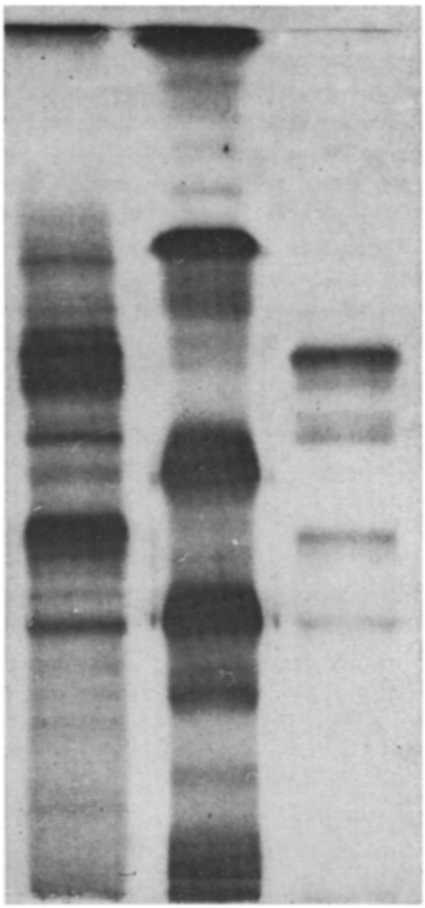


Fig. 1. Slab polyacrylamide gel electrophoresis of samples obtained from the affinity chromatography of extract of rat liver. Left, aliquot from fraction 24 (procedure 1); centre, mixture of standard proteins in the direction of electrophoresis (chymotrypsin, ovalbumin and bovine serum albumin); right, aliquot from fraction 34 (procedure 2).

the higher yields achieved are of importance. Thus, with procedure 1 a 91% yield of LDH was obtained, compared with 58% with the four-step procedure[5]. It is also of importance that the RBB derivative of bead cellulose can be used several times after previous elution with 1 *M* sodium chloride solution.

A similarly successful isolation of LDH from rabbit muscle was achieved[7]. Finally, it may be suggested that the proper selection of anthraquinone–triazine affinants linked to bead cellulose could enable us to use these products for the affinity chromatography of some other enzymes.

ACKNOWLEDGEMENT

The authors express their gratitude to Dr. J. Štamberg for supplying the samples of bead cellulose.

REFERENCES

1 E. Stellwagen, *Acc. Chem. Res.*, 10 (1977) 92.
2 R. S. Beissner and F. B. Rudolph, *J. Chromatogr.*, 161 (1978) 127.
3 S. Angal and P. D. G. Dean, *Biochem. J.*, 167 (1977) 301.
4 H.-J. Böhme, G. Kopperschläger, J. Schulz and E. Hoffmann, *J. Chromatogr.*, 69 (1972) 209.
5 J. Peška, J. Štamberg, J. Hradil and M. Ilavský, *J. Chromatogr.*, 125 (1976) 455.
6 R. S. Beissner and F. B. Rudolph, *Arch. Biochem. Biophys.*, 189 (1978) 76.
7 P. Gemeiner, D. Mislovičová, J. Zemek and Ľ. Kuniak, *Collect. Czech. Chem. Commun.*, submitted for publication.
8 W. T. Hsieh and C. S. Vestling, *Biochem. Prep.*, 11 (1966) 69.
9 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Vol. 1, Verlag Chemie, Weinheim, 2nd ed., 1970, p. 441.
10 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
11 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
12 J. F. Kennedy, *Advan. Carbohydr. Chem. Biochem.*, 29 (1974) 350.