Journal of Chromatography, 130 (1977) 446–450

CHROM. 9503

Note

Enzyme purification by affinity chromatography using a non-covalently bound adsorbent

ANDRAS G. LACKO
Texas College of Osteopathic Medicine, North Texas State University, Denton, Texas 76203 (U.S.A.)
and
TIMOTHY F. CHEN
Department of Chemistry, North Texas State University, Denton, Texas 76203 (U.S.A.)
(Received June 29th, 1976)

Affinity chromatography is perhaps the most widely used modern technique for the isolation of biologically active materials. The pioneering work of Porath *et al.*[1,2] followed by further development of the method by Cuatrecasas[3] provided a technique of wide-ranging importance. The most commonly used procedure is to couple the affinity adsorbent to an agarose backbone[3] using the cyanogen bromide activation procedure[1]. An "extending arm" could be used in addition to increase the distance between the backbone and the group providing the specific binding capacity. An excellent example of such an approach is the use of serum albumin as an intermediary binder between estradiol and agarose for the isolation of estradiol binding protein from uterine tissue[4].

The present report describes an approach where a macromolecular complex is non-covalently bound to a lipophilic backbone providing an efficient surface for the enzyme substrate interaction to take place during affinity chromatography of lecithin:cholesterol acyltransferase (LCAT).

MATERIALS AND METHODS

Materials

Human plasma was obtained from local blood banks, from outdated lots of blood. Dodecylamine was the product of Aldrich (Milwaukee, Wisc., U.S.A.). Sepharose 4B was purchased from Pharmacia (Piscataway, N.J., U.S.A.). Cyanogen bromide was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). Polyethylene glycol (M.W. approx. 6,000) was obtained from Matheson, Coleman & Bell (East Rutherford, N.J., U.S.A.).

Methods

Dodecylamine agarose was prepared according to Deutch *et al.*[5] using the coupling procedure of March *et al.*[6]. Cholesterol determinations were carried out according to Parekh and Young[7]. Disc gel electrophoresis was performed according to Weber *et al.*[8]. Assays for LCAT were performed as follows. Human whole plasma

is heated at 60° for 30 min. Any precipitate which may form during this process is removed by centrifugation*. Human serum albumin (5% in 0.01 *M* Tris·HCl, 0.005 *M* EDTA, 0.15 *M* NaCl, pH 7.4) is also heated for 30 min at 60°[9]. A portion of this heated serum albumin solution is labeled according to the technique of Stokke and Norum[10] to contain about 4 μCi of [^{3}H]-cholesterol per ml in a stable emulsion. One part of heated plasma is mixed with one part of labeled and two parts of unlabeled serum albumin solution and once again heated for 30 min at 60° to inactivate the residual LCAT activity in the preparation. The above mixture is finally combined with four parts of a buffer solution (0.01 *M* Tris·HCl, 0.005 *M* EDTA, 0.15 *M* NaCl, pH 7.4) to form the LCAT substrate.

Substrate (200 μl) is then incubated with 5–20 μl of enzyme at 37°. The time of incubation is selected to give less than 2% conversion of free to esterified cholesterol.

The reaction is stopped by 4 ml of chloroform–methanol (2:1) and filtered on a sintered glass funnel (coarse). A 2-ml wash with chloroform–methanol is combined with the filtrate. The lipid extract is then reduced to dryness under a stream of air and dissolved in a minimum amount of heptane. The heptane solution is then spotted on silica gel thin-layer plate with plastic backing (Bakerflex-1B2) and the chromatogram is developed in a system of light petroleum (b.p. 30–60°)–diethyl ether–acetic acid (90:10:1). The spots corresponding to cholesterol and cholesterol ester are cut out and placed in scintillation vials for counting as described previously[11].

The percent cholesterol esterified is then computed and the rate of esterification is calculated by multiplying the percent esters generated by the initial cholesterol concentration. One unit of enzyme esterified 1 nmole of cholesterol per h as suggested by Albers *et al.*[12].

RESULTS AND DISCUSSION

A dodecylamine–agarose (DDA-agarose) column was prepared using 100 ml of Sepharose 4B[5]. When 50 ml of human plasma is applied to such a column, no LCAT activity is seen in the effluent. Upon washing with 0.01 *M* Tris, 0.005 *M* EDTA, buffer pH 7.4 or the same buffer containing 3 *M* NaBr or 5 m*M* sodium taurocholate, only traces of the enzyme activity may be recovered from the column. When, however, a DDA-agarose column was apparently saturated with plasma lipoproteins, it became a very efficient column resin for LCAT. Fig. 1 shows the processing of 100 ml of human plasma on such an affinity column. Nearly all the plasma proteins are eluted with a buffer containing 0.01 *M* Tris, 0.005 *M* EDTA, and 0.15 *M* NaCl while the LCAT activity is retained on the column. Upon changing the buffer to lower ionic strength (0.01 *M* Tris, 0.005 *M* EDTA, no NaCl), the enzyme activity is quantitatively recovered. The enzyme peak yields a preparation which is approx. 300-fold purified.

The lipoprotein saturated DDA-agarose is prepared the following way. Human plasma is treated with a 50% solution of polyethylene glycol (in water) to achieve a 4% concentration in the plasma. The precipitate thus generated is removed by low-speed centrifugation** and the supernatant is applied to the DDA-agarose column. When the cholesterol concentration in the effluent is the same as that of the material applied there, the column is considered saturated and it is washed

* 2000 *g* for 10 min.

** 8000 *g* for 30 min.

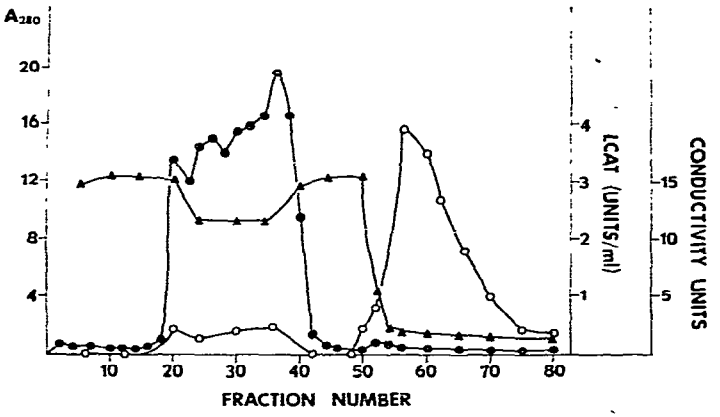

Fig. 1. Affinity chromatography of human plasma LCAT on a DDA-agarose column. ●, A_{280}; ○, LCAT activity; ▲, conductivity.

with 0.01 *M* Tris·HCl, 0.005 *M* EDTA, 0.15 *M* NaCl, pH 7.4) and it is ready for affinity chromatography.

Fig. 2 shows that most of the contaminants except high-density lipoprotein (HDL) and serum albumin are removed from the plasma as the result of DDA-agarose affinity chromatography.

LCAT has been one of the more difficult enzymes to purify in a stable and homogeneous form. Early attempts began in about 1963[13] and even the two latest reports[11,14] fall short of providing an efficient method for LCAT purification. Observations in our laboratory[15] indicate that the stability of the enzyme may depend on its concentration in solution. The early reports on the instability of LCAT[16,17] may have thus been due to the small amount of enzyme and to the dilute solutions of it that were obtained upon purification.

Preliminary studies indicate that the procedure shown in Fig. 1 could be scaled up substantially so that larger amounts of homogeneous enzyme preparations could eventually be obtained. Because of the larger amounts isolated, the enzyme concentration will be presumably high enough to ensure the stability of LCAT in solution.

Akanuma and Glomset[18] were the first to show that HDL covalently bound to Sepharose by the method of Porath *et al.*[1] could be used for the affinity chromatography of LCAT. This procedure has been utilized by other investigators[11,19] in attempting to purify LDAT. One of the serious problems with this method is the deterioration of the adsorber upon repeated use whether taurocholate[11] or increase in pH[19] is used to elute the enzyme. A significant advantage of the DDA-agarose column (saturated with plasma lipoproteins) is that it may be fully regenerated by simply washing with 0.01 *M* Tris, 0.005 *M* EDTA, 0.15 *M* NaCl and thus used in a cyclic operation indefinitely.

The potential significance of affinity chromatography using non-covalently bound adsorbents as described above could go well beyond the purification of LCAT. Presumably almost any mixture of lipids or lipoproteins (or individual molecular species) may be attached to such a lipophilic backbone and used to purify a whole host of enzymes. In addition, the studying of enzyme–substrate and (or) lipid–protein interactions by such affinity columns could have wide-spread use in biochemistry.

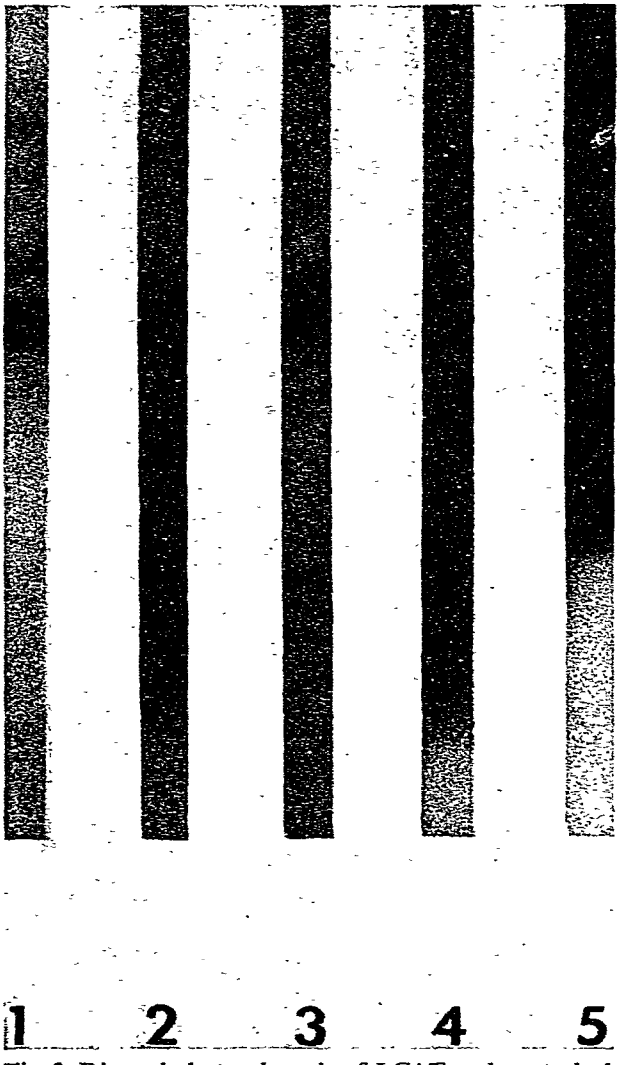

Fig. 2. Disc gel electrophoresis of LCAT and control plasma fractions in the presence of sodium lauroyl sulfate according to Weber *et al.*[8]. 1 = Human serum albumin (HSA); 2 and 4 = high-density lipoprotein and HSA; 3 = LCAT recovered after purification on DDA-Agarose column (40 μl); 5 = crude plasma fraction applied to DDA-Agarose column (5 μl).

ACKNOWLEDGEMENTS

This work was supported by grant No. HL-19142 from the National Heart and Lung Institute (U.S. Public Health Service) and from Faculty Research funds provided by the Texas College of Osteopathic Medicine.

The technical assistance of Gary L. Polk and Ilse Nelson and the secretarial assistance of Katherine Dorr are gratefully acknowledged.

REFERENCES

1 J. Porath, R. Axen and S. Ernbäck, *Nature (London)*, 215 (1967) 1491.
2 J. Porath, *Nature (London)*, 218 (1968) 834.
3 P. Cuatrecasas, *Methods Enzymol.*, 36 (1972) 29.
4 V. Sica, E. Nola, G. A. Puca, I. Parikh and P. Cuatrecasas, *J. Biol. Chem.*, 248 (1973) 6543.
5 D. G. Deutch, D. J. Fogelman and K. N. von Koulla, *Biochem. Biophys. Res. Comm.*, 50 (1973) 758.
6 S. C. March, I. Parikh and P. Cuatrecasas, *Anal. Biochem.*, 60 (1974) 149.
7 A. C. Parekh and D. M. Young, *Anal. Chem.*, 42 (1970) 1423.
8 K. Weber, J. R. Pringle and M. Osborn, *Methods Enzymol.*, 26 (1972) 3.
9 L. Wallentin and O. Vikrot, *Scand. J. Clin. Lab. Invest.*, 35 (1975) 661.
10 K. T. Stokke and K. R. Norum, *Scand. J. Clin. Lab. Invest.*, 27 (1971) 21.
11 A. G. Lacko, H. L. Rutenberg and L. A. Soloff, *Clin. Chim. Acta*, 39 (1972) 506.
12 J. J. Albers, V. G. Cabana and Y. D. B. Stahl, *Biochemistry*, 15 (1976) 1084.
13 J. A. Glomset and J. L. Wright, *Biochim. Biophys. Acta*, 89 (1964) 266.
14 K. G. Varma, A. G. Lacko, J. S. K. David and L. A. Soloff, *Circulation* 51, Suppl. 2 (1975) 278.
15 A. G. Lacko, unpublished observations.
16 K. K. W. Ho and A. V. Nichols, *Biochim. Biophys. Acta*, 231 (1971) 185.
17 C. J. Fielding and P. E. Fielding, *FEBS Lett.*, 15 (1971) 355.
18 Y. Akanuma and J. A. Glomset, *Biochem. Biophys. Res. Comm.*, 32 (1968) 639.
19 A. G. Lacko, K. G. Varma, H. L. Rutenberg and L. A. Soloff, *Scand. J. Clin. Lab. Invest.* 33, Suppl. 137 (9174) 29.