A Method for the Purification of Milligram Quantities of Stable Human Phosphatidylcholine–Cholesterol Acyltransferase

By KODUNGALLORE G. VARMA and LOUIS A. SOLOFF

Lipid Research Laboratory, Department of Medicine, Temple University Health Sciences Center, Philadelphia, PA 19140, U.S.A.

(Received 13 November 1975)

A method for processing 3 litres of human plasma for the purification of phosphatidylcholine–cholesterol acyltransferase is described. The method involves $(\text{NH}_4)_2\text{SO}_4$ fractionation, citric acid treatment, and DEAE-cellulose and hydroxyapatite chromatography. At this stage the enzyme preparation is purified approx. 8000-fold. This preparation appears to be free of lipoproteins as determined by immunoelectrophoresis against anti-human serum and is minimally contaminated with albumin (less than $30\text{ }\mu\text{g}/\text{mg}$ of enzyme protein) as determined by immunodiffusion. The activity of the enzyme was stable for 4 days, but most of its activity was lost after 20 days. On electrophoresis on 5% polyacrylamide gel, a fast-moving band with enzyme activity and a slow-moving band with no enzyme activity are observed. A faint band of albumin was also present. Extracts of enzymically active bands cut from ten gels and then pooled and extracted with $0.15\text{ M-NaCl}/4\text{ mm-sodium phosphate}$, pH 7.0, showed a single band on re-electrophoresis on 5% polyacrylamide gel.

Phosphatidylcholine–cholesterol acyltransferase (EC 2.3.1.43) is the enzyme responsible for the esterification of plasma cholesterol. In humans almost all plasma cholesterol ester is formed by the activity of this enzyme (Glomset, 1968). It has been suggested that the primary function of this enzyme is related to the maintenance of plasma lipoprotein structure during metabolism (Schumaker & Adams, 1970). A role for the enzyme in the transport of cholesterol from peripheral tissues to liver (Glomset, 1968) and in maintaining the integrity of plasma membrane has also been proposed (Glomset & Norum, 1973). Subjects who are congenitally devoid of this enzyme have abnormal plasma lipoproteins (Glomset *et al.*, 1970) and an unusual type of atherosclerosis, even when concentrations of blood cholesterol are far below normal values (Stokke *et al.*, 1974; Gjone, 1974). Patients with cholestasis also have abnormal plasma lipoproteins when the plasma has decreased cholesterol-esterifying activity *in vitro* (Simon & Kepkay, 1974). It appears therefore that phosphatidylcholine–cholesterol acyltransferase plays an important role in lipid metabolism.

At the present time the enzyme cannot be assayed. The measurement of cholesterol-esterifying activity of the plasma is used as an index of this enzyme's activity. Such measurements require heat-inactivated serum or temporary inhibition of enzyme present in plasma and the use of externally added radioactive cholesterol. The nature of the substrate under these conditions is poorly defined, and may vary from one plasma

sample to another, particularly for plasma from subjects with disorders of lipid metabolism (Norum, 1974). Further, radioisotopic methods depend on the assumption that complete equilibrium between radioactive cholesterol and plasma free cholesterol has occurred. G.l.c. methods cannot measure changes in free cholesterol during the first few minutes of incubation. The influence of cofactors and inhibitors of the enzyme in plasma are not taken into account when cholesterol-esterifying activity is used as a measure of the enzyme itself. Radioimmunoassay appears to be the method of choice to assay the enzyme in human plasma (Norum, 1974). Here we report a method suitable for processing large quantities of human plasma that yields milligram amounts of highly purified and stable enzyme suitable for the production of antibodies.

Material and Methods

Plasma

Human plasma (24 days old) in $0.02\text{ M-sodium citrate}/0.5\%$ dextrose was from Community Plasma, Philadelphia, PA, U.S.A. The cholesterol-esterifying activity of the plasma as described below was checked and compared with that of fresh human plasma. Only plasma showing greater than 75% of the activity of fresh human plasma was used for purification of the enzyme. Any sediment present in this plasma was removed by centrifugation at 2000 g at 4°C for 30 min.

[1,2-³H]Cholesterol was from New England Nuclear Corp., Boston, MA, U.S.A. It was more than 99% pure as checked by t.l.c. and the specific radioactivity was greater than 50 Ci/mmol.

Chemicals

Chemicals and reagents were from the following sources: enzyme-grade (NH₄)₂SO₄, J. T. Baker and Co., Phillipsburg, NJ, U.S.A.; human serum albumin, Cutter Laboratories, Berkeley, CA, U.S.A.; ovalbumin and lysozyme, Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose DE-52 (microcrystalline), Reeve Angel, Clifton, NJ, U.S.A.; hydroxyapatite-HT, Calbiochem, San Diego, CA, U.S.A.; reagents for polyacrylamide-gel electrophoresis, Eastman Kodak Co., Rochester, NY, U.S.A. All chemicals used were of analytical grade.

Enzyme assay

The rate of cholesterol esterification, as index of the enzyme activity, was assayed by a modification of the method of Glomset (1969). Fresh human serum was heated at 56–60°C for 30 min with mild shaking. An emulsion of [³H]cholesterol in 5% (w/v) albumin was prepared by the addition of [³H]cholesterol in acetone (Stokke & Norum, 1971). To 1 vol. of heated serum, 2 vol. of the above emulsion was added and the volume was made up to 8 vol. of fresh heated serum by the addition of 5% human serum albumin. The mixture was again heated at 56–60°C with mild shaking for 30 min. To this mixture was added an equal volume of 0.01 M-Tris/5 mM-EDTA/0.15 M-NaCl, pH 7.4. Any precipitate formed during the preparation of this mixture was removed by centrifugation at 4000g for 30 min, and the supernatant was stored at 0–4°C. It was used as a substrate for measuring the rate of cholesterol esterification. Substrate (200 µl, containing 0.08–0.1 µCi of [³H]-cholesterol) was incubated with 5–20 µl of the enzyme sample for 4 h at 37°C. The rate of cholesterol esterification of the original plasma was determined at the time when enzyme samples at different stages of purification were being assayed. Whenever it was found that the rate of esterification/ml of enzyme sample was more than that of the starting plasma, the sample was diluted with the substrate to avoid possible inactivation and to bring the rate of esterification below that of the plasma. The reaction was stopped by the addition of 4 ml of chloroform/methanol (2:1, v/v) and the lipids were extracted for 2 h at 25°C. The extract was filtered through a sintered-glass filter and the residue was washed with 2 × 2 ml of chloroform/methanol (2:1, v/v). The radioactivity in the free and esterified cholesterol was determined by liquid-scintillation counting (Lacko

et al., 1973). The rate of cholesterol esterification was calculated from the following formula

$$\frac{\mu\text{g of cholesterol esterified by 1 ml}}{\text{of enzyme preparation}} = \frac{A \times CE}{(CF + CE) \times v \times t}$$

were A = µg of cholesterol in the incubation mixture, CE = c.p.m. in the cholesterol ester spot of the chromatogram, CF = c.p.m. in free cholesterol spot of the chromatogram, *t* = time of incubation in h, *v* = volume of enzyme sample used in ml.

The cholesterol-esterifying activity of plasma is not constant from subject to subject. To facilitate comparisons of different preparations, cholesterol-esterifying activities of the preparation are expressed as plasma units (defined as µg of cholesterol esterified by 1 ml of plasma in 1 h). A blank containing an equal volume of 0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4, instead of the enzyme sample was included in all assays to estimate the cholesterol-esterifying activity of the substrate.

Free cholesterol was determined by the method of Parekh & Jung (1970); P₁ was assayed by the method of Bartlett (1959); protein was measured by the method of Lowry *et al.* (1951), with human serum albumin as the standard; protein concentration of column eluates was determined by measuring *E*₂₈₀ in a Gilford model 240 spectrophotometer.

Column chromatography

Precycling of DEAE-cellulose DE-52 with 0.5 M-NaOH and 0.5 M-HCl was performed according to the manufacturer's instructions. Hydroxyapatite was packed at 13.8 kPa (21 lb/in²) in a column (2.5 cm × 40 cm) to a height of 25 cm. With a hydrostatic head of 40 cm, the flow rate was 5–6 ml/h. The available column volume was 80 ml, as checked by running a solution of 0.1% Methyl Red through the column. The column was extensively washed (40 bed vol.) with 0.15 M-NaCl/4 mM-sodium phosphate buffer, pH 6.9, until the pH and the conductivity of the effluent was the same as those of the washing buffer. Details of the actual run are given in the legends of Figs. 2 and 3.

Polyacrylamide-gel electrophoresis

Disc electrophoresis of protein solutions was performed as described by Davis (1964) in a 5% gel. The proteins were fixed in 12.5% (w/v) trichloroacetic acid and then stained in 0.01% Coomassie Blue R-250 as described by Chrambach *et al.* (1967). Association between cholesterol-esterifying activity and protein band was ascertained by cutting the gel into 2 mm pieces. A portion of each 2 mm piece was assayed for esterifying activity and the remainder was stained with Coomassie Blue. In order to evaluate whether

one could obtain pure enzyme from the gels, ten gels (6mm×50mm) were run simultaneously. Approx. 100 µg of protein from hydroxyapatite fractions was put on each gel. After electrophoresis, 2mm bands were cut, and the bands showing enzyme activity were pooled, extracted with 0.15M-NaCl/4mM-sodium phosphate, pH 7.0, and re-run on 5% polyacrylamide gels to assess the purity of the preparation. Electrophoresis in the presence of sodium dodecyl sulphate was conducted by the method of Weber & Osborn (1969), except for the following details. A final concentration of 8M-urea in gels was obtained by adding urea to the polymerization mixture. The urea concentration of the denaturing buffer, 0.01M-sodium phosphate/1% sodium dodecyl sulphate/1% mercaptoethanol, was 6M. After denaturation of the protein samples overnight they were dialysed against a solution of 0.1M-sodium phosphate/0.1% sodium dodecyl sulphate/0.1% mercaptoethanol/8.0M-urea, pH 7.0. Human serum albumin, ovalbumin, lysozyme and apoprotein A-I [the major apoprotein of human high density lipoprotein, isolated by the method of Edelstein *et al.* (1972)] were used as standards of known molecular weight. Protein was stained by the method of Fairbanks *et al.* (1971). The R_F values of different proteins were determined by inserting a copper wire at the dye front at the end of electrophoresis run and determining the ratio of the distance travelled by the stained protein band to the distance travelled by the dye front (Hedrick & Smith, 1968).

Results

Stability of the enzyme at low pH

It has been claimed (Glomset, 1969) that phosphatidylcholine-cholesterol acyltransferase is rapidly inactivated below pH 6.0. We had the opportunity of checking the stability of this enzyme at low pH values while attempting to produce an immunoaffinity-chromatographic technique for purification of this enzyme. We found that the partially purified (150–300-fold) enzyme was stable down to a pH as low as 3.5 (0.1M-glycine/HCl). Further, we noted that more than 60% of the enzyme activity was precipitated when equal volumes of the enzyme in 0.01M-Tris/HCl/5mM-EDTA/0.15M-NaCl, pH 7.4, and 0.2M-glycine/HCl/2.0M-NaCl, pH 3.5, were mixed. Substituting 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ for 1M-NaCl likewise resulted in the precipitation of the enzyme when the pH was lowered to 4.0 (see Fig. 1).

Purification of the enzyme

All steps were carried out at 0–4°C or in an ice bath unless otherwise specified. The results are shown in Table 1.

Vol. 155

Ammonium sulphate fractionation

Satd. $(\text{NH}_4)_2\text{SO}_4$ solution (1.5 litres), pH adjusted to 7.0 with aq. 1M- NH_3 was added with stirring to 3.0 litres of plasma, and the mixture kept at 4°C overnight. The suspension was centrifuged at 5000g for 60min in a Sorvall RC-3 centrifuge. The supernatant, which contained all the esterifying activity (see Table 1), was subjected to acid treatment as described below.

Acid treatment

Citric acid solution (1M; about 200ml) was added in 10–20min from a burette to the above supernatant (approx. 4.5 litres) with vigorous stirring to bring its pH to 4.0. The solution was stirred for a further 90min and then centrifuged at 5000g for 60min. The residue was suspended in 800ml of 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ and the pH of the sample was raised to 7.4 by the addition of Na_2CO_3 . It was dialysed against several changes of 10vol. of 5mM-EDTA, pH 7.4, until the conductivity of the dialysis residue was less than 1.0mmho. The recovery of the enzyme at this stage was more than 50%.

DEAE-cellulose chromatography

The dialysis residue (1.5 litres) was applied to a DEAE-cellulose column equilibrated at pH 7.4 and

Fig. 1. Stability of the enzyme at different pH values

Citric acid solution (1.0M) was added to 30ml of supernatant fraction formed by treating plasma with 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ to adjust the pH to the values shown in the Figure. After leaving for 2h at 0–4°C, the preparation was centrifuged at 10000g for 15min. The residue, if any, was dissolved in 30ml of 0.01M-Tris/5mM-EDTA/0.10M NaCl, pH 7.4, and the pH of the sample was adjusted to pH 7.4 with satd. Na_2CO_3 . The volume of the sample was noted, and 10 µl was assayed for enzyme activity. Black bars, activity in residue; white bars, activity in supernatant.

Table 1. *Purification of phosphatidylcholine-cholesterol acyltransferase*

For details see the Results section.

Step	Total volume (ml)	Total plasma activity (units)	Total protein (g)	$10^3 \times$ sp. activity (units/g)	Yield (%)	Purification (fold)
Plasma	3000	3000	218.1	13.8	100	1
33%-Satd. $(\text{NH}_4)_2\text{SO}_4$ supernatant	4500	3643	162.9	22.3	121	1.62
Dialysed pH4 precipitate	1500	1667	34.7	48.1	56	3.5
DEAE-cellulose DE-52 pool	530	869	1.378	631	29	46
Hydroxyapatite	45	473	0.0041	115 000	15.8	8312

Fig. 2. *Purification of the enzyme by DEAE-cellulose chromatography*

The enzyme preparation (1660 plasma units) was put on a column (2.5 cm $\times$ 40 cm) of DEAE-cellulose DE-52. The column was eluted at pH 7.4 with a gradient of 700 ml of 1 mM-Tris/5 mM-EDTA/25 mM-NaCl plus 700 ml of 0.01 M-Tris/5 mM-EDTA/0.15 M-NaCl. The flow rate was 45 ml/h and 30 ml fractions were collected. ●, E_{280} ; ▲, cholesterol-esterifying activity; ■, NaCl concentration.

was eluted with a NaCl gradient (details are described in the legend of Fig. 2). More than 80% of the contaminating proteins emerged from the column before the enzyme started to leave the column. Fractions containing cholesterol-esterifying activity more than 34-fold purified were pooled, and concentrated by vacuum filtration to approx. one-twentieth of the original volume of the pooled fractions. The concentrated enzyme was dialysed against 3×50 vol. of 0.15 M-NaCl/4 mM-sodium phosphate, pH 6.9, over a period of 30 h before being applied to the hydroxyapatite column.

Hydroxyapatite chromatography

The dialysis residue was put on a hydroxyapatite column and was eluted successively with 1 column

vol. of increasing phosphate concentration at the same pH and NaCl concentration (see legend of Fig. 3 for details). The cholesterol-esterifying activity was eluted as a single peak at a phosphate concentration of 20–25 mM. The ratios of the enzyme activity to E_{280} for protein were constant on both sides of the peak.

Purity of the enzyme

Pooled hydroxyapatite fractions concentrated to 1.0 mg of protein/ml by vacuum filtration did not give any arc when tested against anti-human serum by immunoelectrophoresis (results not illustrated). Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis of these pooled fractions showed a major band of mol.wt. 88 000 and a faint band of

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pooled hydroxyapatite fractions of enzyme

Approx. 25 μ g of the enzyme was applied to the gel; (1) 88000 mol.wt. protein, (2) 70000 mol.wt. protein. Human serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 43000), apoprotein A-I (mol.wt. 28000) and lysozyme (mol.wt. 14400) were used as standards of known molecular weight.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of pooled hydroxyapatite fraction of enzyme

Polyacrylamide-gel electrophoresis was conducted in 5% gel. The sample was applied in 20% (w/v) sucrose over a stacking gel. (a) Enzyme (10 μ g); (b) enzyme (15 μ g) plus human serum albumin (10 μ g); (c) samples of enzymically active bands pooled and homogenized from ten gels.

Fig. 3. Purification of the enzyme by hydroxyapatite chromatography

The enzyme preparation (1000 plasma units) dialysed against 0.15M-NaCl/4mM-sodium phosphate buffer, pH6.9, was applied to a column (2.5cm×25cm). The enzyme was eluted with approx. 1 column vol. (80ml) of 4, 15, 30 and 60mM- P_i , pH6.9, containing 0.15M-NaCl. The P_i concentrations given are those of the eluates. Because of the dead space of the column, P_i concentrations were always less than those of the eluting buffer. The flow rate was approx. 5–6 ml/h, and 2.5 ml fractions were collected. ●, E_{280} ; ▲, cholesterol-esterifying activity; ■, P_i concentration.

mol.wt. 70000 (Plate 1). Polyacrylamide-gel electrophoresis on a 5% gel showed two major bands and a faint band with the same mobility as albumin (cf. Plate 2a and 2b). The cholesterol-esterifying activity was identified as the fast-moving band (R_F 0.67). Judging from the intensity of stain in the bands, the enzymically active band is probably more than 20000-fold purified over the starting plasma. Extracts of these bands from 10 gels when pooled and re-electrophoresed showed a single band with R_F 0.66 (Plate 2c).

Stability of the enzyme on storage

No serious loss of activity occurred during storage at 4°C for 100h. After 20 days 80% of the original activity was lost and 95% of the activity was lost after 30 days.

Discussion

Highly purified phosphatidylcholine-cholesterol acyltransferase has been obtained in several laboratories (Fielding & Fielding, 1971; Kostner, 1974; Lacko *et al.*, 1974). However, the methodology could not be adapted for large-scale purification of the enzyme, because all the investigators used a procedure involving ultracentrifugation at very high speeds for extended periods of time (100000g for at least 36h). Further, the preparation obtained by Fielding &

Fielding (1971), which was purified 2500-fold compared with fresh plasma, was stable for only 3h, and Kostner (1974) has as yet not published the details of his procedure or the stability of his enzyme preparation. The method described here provides a way of getting highly purified and stable enzyme in milligram amounts and involves only low-speed centrifugation, a procedure that makes it possible to process plasma in litre quantities.

It is difficult to compare the purity of the enzyme preparation obtained by our procedure with those obtained by Fielding & Fielding (1971) and by Kostner (1974), since none of their samples was tested for homogeneity. However, their preparations appear to be free of contaminating lipoproteins on the basis of the following evidence. Fielding & Fielding's (1971) preparation showed an absolute requirement for apoprotein A-I for the action of the enzyme phosphatidylcholine-cholesterol emulsion. Kostner (1974) observed a requirement for apoprotein AIII, one of the minor apoproteins of human high density lipoprotein, when phosphatidylcholine/cholesterol emulsions were used for the assay. Further, no apoproteins were observed in his preparation when it was subjected to polyacrylamide-gel electrophoresis. Although we have not tested our enzyme preparations from the hydroxyapatite column for the requirement for apolipoproteins as cofactors for action on phosphatidylcholine/cholesterol emulsions, our preparations appear to be free of lipoproteins as judged by

the following criteria. (1) Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis did not show bands corresponding to the molecular weight of apolipoproteins (7000–30000). (2) Immunoelectrophoresis of our enzyme concentrated to 1mg of protein/ml against anti-human serum did not give an arc, suggesting the absence of contaminating lipoproteins. The amount of albumin in our enzyme preparation was less than 3% (30 μ g/mg of enzyme protein) as determined by immunodiffusion. The slow-moving band is observed in 5% polyacrylamide gel (Plate 2) is as yet not identified. That it could be an inactive aggregate or a serum protein present in a minute amount is suggested by the non-reactivity of this protein towards anti-human serum when the enzyme was subjected to immunoelectrophoresis.

It is highly unlikely that the small amount of albumin present in our enzyme preparation would be sufficient to produce antibodies when injected into animals. Should this unlikely possibility occur or should the protein of the slow-moving band not be an aggregate of the enzyme and be immunogenic, it should be possible to adsorb out the anti-albumin and the antibody to the slow-moving band with pure albumin and with extracts of bands of the contaminating protein present in the polyacrylamide gel. Finally, the homogeneous gel bands already shown to have enzyme activity could be cut from several gels and then pooled and extracted with 0.015M-NaCl/4mM-sodium phosphate and used directly for chemical analysis and antibody production (Weintraub & Raymond, 1963).

We acknowledge the excellent technical assistance of Ms. Faye Baldwin and Ms. Margaret Nickel, and thank Ms. Elizabeth Wolf for typing the manuscript. This investigation was supported by the Council for Tobacco Research, U.S.A., and the U.S. Public Health Service grant no. HL 17431-01 from the National Institute of Health.

References

- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
 Chrambach, A., Reisfeld, R. A., Wycott, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154
 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
 Edelstein, C., Lim, C. T. & Scanu, A. M. (1972) *J. Biol. Chem.* **247**, 5842–5847
 Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
 Fielding, C. J. & Fielding, P. E. (1971) *FEBS Lett.* **15**, 355–358
 Gjone, E. (1974) *Scand. J. Clin. Lab. Invest. Suppl.* **137**, 155–167
 Glomset, J. A. (1968) *J. Lipid Res.* **2**, 1–65
 Glomset, J. A. (1969) *Methods Enzymol.* **5**, 543–548
 Glomset, J. A. & Norum, K. R. (1973) *Adv. Lipid Res.* **2**, 1–65
 Glomset, J. A., Norum, K. R. & King, W. (1970) *J. Clin. Invest.* **49**, 1827–1837
 Hedrick, J. L. & Smith, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155–164
 Kostner, G. (1974) *Scand. J. Clin. Lab. Invest. Suppl.* **137**, 19–21
 Lacko, A. G., Rutenberg, H. L. & Soloff, L. A. (1973) *Biochem. Med.* **7**, 178–183
 Lacko, A. G., Varma, K. G., Rutenberg, H. L. & Soloff, L. A. (1974) *Scand. J. Clin. Lab. Invest. Suppl.* **137**, 29–34
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Norum, K. R. (1974) *Scand. J. Clin. Lab. Invest. Suppl.* **137**, 7–13
 Parekh, A. C. & Jung, D. H. (1970) *Anal. Chem.* **42**, 1423–1427
 Schumaker, V. H. & Adams, G. H. (1970) *J. Theor. Biol.* **26**, 89–91
 Simon, J. R. & Kepkay, D. L. (1974) *Gastroenterology* **66**, 539–567
 Stokke, K. T. & Norum, K. R. (1971) *Scand. J. Clin. Lab. Invest.* **27**, 21–27
 Stokke, K. T., Bjehve, K. S., Blomhoff, J. P., Oystese, B., Flatmark, A., Norum, K. R. & Gjone, E. (1974) *Scand. J. Clin. Lab. Invest. Suppl.* **137**, 93–100
 Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
 Weintraub, M. & Raymond, S. (1963) *Science* **142**, 1677–1678