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Purification of cholesterol-esterifying enzymes from rat liver cytosol by high-performance liquid chromatography

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Abstract

Three enzymes esterifying cholesterol with long-chain fatty acids were purified approximately 31 000-fold to apparent homogeneity from the cytosol of normal rat liver. The enzymatic activity was tested by incubation of active fractions with tritiated cholesterol and separation of newly formed esters from non-reacted cholesterol by a passage through silica gel cartridges with subsequent assay for radioactivity by liquid scintillation. For the purification of enzymes, active proteins were precipitated by $(NH_4)_2SO_4$ to 35% saturation. The bulk of inactive proteins was removed by size-exclusion chromatography on TSK G3000 SW. The active fraction was subsequently separated on Separon HEMA BIO 1000 DEAE in gradients of 0–500 m*M* KCl into three enzymatic activities differing in their retention and these proteins were finally purified by affinity HPLC on columns of cholesterol immobilized on HEMA BIO 1000 E-H. Final purified enzymes showed the same single band in polyacrylamide gel electrophoresis corresponding to 16.5 kDa. Combination of individual enzymes did not increase the overall yield of cholesteryl esters but the reaction-rate was significantly accelerated. These proteins are apparently subunits of a larger complex (M_r 65 000) that can be demonstrated by electrophoresis in the absence of 2-mercaptoethanol. Results presented in this paper indicate that because of good and rapid separation of active proteins, HPLC may be a method of choice for enzyme purifications.

Keywords: Cholesterol; Steroids; Enzymes

1. Introduction

Cholesteryl 14-methylhexadecanoate (CMH), a lipid isolated and identified in our laboratory [1] plays an important role in protein synthesis and is required as a cofactor by several enzymes and protein factors involved in this process (see, for review Ref. [2]).

The level of this particular ester is significantly increased in the blood serum of cancer patients [3] and its synthesis (as well as that of some other cholesteryl esters) in the liver is enhanced in relation to the growth of experimental tumors in rats [4]. Of several cholesterol-esterifying activities present in the liver cell, only that in the cytosol is affected by the malignant growth [4].

This paper describes methods based predominantly on HPLC which were developed for the purification of this enzymatic system to apparent homogeneity. It provides evidence that preparative HPLC may have

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some advantages over the classical techniques for enzyme purifications.

2. Experimental

2.1. Biological materials

Female random-bred Wistar rats, 150–200 g, were fed a standard pelleted diet and given water ad libitum. Rats were killed by breaking their necks, livers were quickly excised and placed in 2 volumes of ice cold medium A (50 m*M* Tris–HCl buffer, pH 7.5, 5 m*M* $MgCl_2$, 25 m*M* KCl, 250 m*M* sucrose and 7 m*M* 2-mercaptoethanol). The tissue was finely minced with scissors and homogenized four times for 1 min with 1-min intervals at an intermediate speed in an Ultra-Turrax homogenizer.

2.2. Chemicals and radiochemicals

All chemicals were purchased from Sigma Aldrich (Prague, Czech Republic). Chemicals were of AR or ACS purity. [1α,2α(*n*)-^{3}H]Cholesterol (49 Ci/mmol) was a product of Amersham (Amersham, UK). It was dissolved in acetone and diluted with non-labeled cholesterol (Merck, Darmstadt, Germany) to a specific radioactivity of 10 Ci/mmol. The final adjustment was carried out by diluting one part of this solution with 9 parts of water and this final solution was kept at −20°C.

2.3. High-performance liquid chromatography

The system used consisted of Beckman Model 114M solvent modules equipped with a Model 210A sample injection valve and a Model 450 system organizer for gradient elution. The 2082.1 variable wavelength-detector equipped with the Apex integrating software were products of ECOM (Prague, Czech Republic). Stainless steel columns packed with TSK G3000SW (700×20 mm I.D., 20 μm), Ultraspherogel SEC 3000 (300×7 mm I.D., 5 μm) and Ultraffinity EP (50×4.6 mm I.D., 10 μm) were purchased from Beckman (San Ramon, CA, USA). Separon HEMA-BIO 1000 DEAE (250×8 mm I.D., 10 μm) and Separon HEMA-BIO 1000 E-H (an epoxy-activated copolymer of 2-hydroxymethyl methacrylate and ethylene dimethyl acrylate) (100× 10 mm I.D., 10 μm) columns were products of Tessek (Prague, Czech Republic). Silicagel cartridges (SILICA-cart 10×10 mm I.D., 60 μm) were from the same source. Immediately before use, the cartridges were prewashed with 5 ml of 15% (v/v) diethyl ether in *n*-heptane.

2.4. Immobilization of cholesterol

Affinity columns of any origin were washed with 100 ml of acetone at a flow-rate of 1.0 ml/min at ambient temperature. Thereafter, the flow-rate was decreased to 0.20 ml/min, the temperature increased to 45°C and 20 ml of 1–2.5 m*M* cholesterol in acetone was recycled through the column for 48 h. The column was then washed with 100 ml of acetone at a flow-rate of 1.0 ml/min at ambient temperature followed by 100 ml of 100 m*M* 2-mercaptoethanol to deactivate residual active groups. Finally, columns were equilibrated against 20 m*M* Tris–HCl buffer. As calculated from the difference of cholesterol contents in the starting solution and in that after recycling of the column, columns prepared in this way contained 18–45 μmol of cholesterol bound/column. Columns containing up to 80 μmol cholesterol/column could be prepared by increasing the sterol concentration in the recycling solution. However, these very high loads resulted in a significantly prolonged elution (and hence considerably increased volumes) of enzymes without a significantly increased capacity of the column. The binding capacity of both types of columns used was at least 2 mg of protein/column.

2.5. Incubations

Standard incubation mixtures were prepared in Eppendorf microtubes and contained in a final volume of 0.1 ml: 20 m*M* Tris–HCl buffer, pH 7.5, 15 m*M* $MgCl_2$, 7 m*M* 2-mercaptoethanol, quantities of enzyme protein as indicated for individual experiments, 0.5 μg of total phosphatidyl cholines in 5 μl of 10% ethanol, and 15 pmol of [^{3}H]cholesterol in 5 μl of 10% acetone (see above). Blank samples of the same composition but with no enzymes added were incubated in experimental series. All mixtures were

incubated in duplicates at 37°C for 180 min (unless indicated otherwise), then chilled in ice, 10 μl of Blue Dextran (10 mg/ml of water) were then added (to facilitate the latter visualisation of the lower layer) followed by 250 μl of diethyl ether–ethanol (2:1, v/v). Mixtures were then thoroughly vortex-mixed for 1 min and left to stand for 10–20 min at room temperature. Thereafter, 100 μl of the upper organic layer were aspirated, transferred to another tube and left to evaporate at room temperature overnight.

Separation of radioactive cholesteryl esters from free cholesterol was done as described earlier. This method gave satisfactory recoveries of cholesteryl esters and blank values (free non-adsorbed radioactive cholesterol) did not exceed 2–4% of the total input of radioactivity [5]. Briefly, the residue was dissolved in 100 μl of chloroform and 75 μl of this solution was added to 2 ml of 15% diethyl ether in *n*-heptane (v/v) at the top of prewashed silica gel cartridges. This solution was sucked through the minicolumns using a Dorcus vacuum manifold (ECOM) and the columns were washed with an additional 3 ml of the same solvent mixture. Both eluates were collected directly in 7 ml of Sigma fluor scintillation cocktail for non-aqueous samples and the radioactivity was assayed in a LKB scintillation counter with an efficiency of 41%. The radioactivity of blank incubates (representing 2–4% of the total input) was subtracted from that of experimental mixtures to obtain net activities. The specific activities of the individual enzymatic fractions was calculated using the saturation curves obtained using 5–10 increasing quantities of enzyme protein.

2.6. Chemical methods

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli [6] and slab gels were stained with silver [7]. Proteins were determined as described by Bradford [8]. For a rapid check for protein contents during enzyme fractionation the method of Warburg and Christian [9] was also used.

2.7. Purification of enzymes

All procedures were performed at 0–4°C, except for HPLC where the columns were eluted at ambient temperatures. However, the fractions were chilled to 0°C during their collection. If required, enzyme fractions (including purified enzymes) in the standard buffer were stored at −20°C. Repeated freezing and thawing (up to 4–5 times) did not significantly affect the enzymatic activity. The standard buffer used throughout the isolation was 20 m*M* Tris–HCl buffer, pH 7.5.

A scheme of the purification as well as its results are presented in Table 1.

2.7.1. Step I: Cytosol

The liver homogenate prepared as described in Experimental was centrifuged at 150 000 *g* for 60 min in a Beckman Type 50Ti rotor. The upper 2/3

Table 1
Purification of cholesterol-esterifying enzymes from 100 g of rat liver

No.	Step	Protein (mg)	Total activity (U)	Yield (%)	Specific ativity (pmol/mg)	Purification (-fold)
I	Cytosol	8140	5664	100	0.69	1
II	25%$(NH_4)_2SO_4$	1164	4434	78.3	3.81	5.5
III	Size-exclusion	17.2	4076	72.0	237	343.5
IV A	Ion-exchange	1.6	1764	31.1	1102	1597
IV B	Ion-exchange	0.5	724	12.8	1448	2098
IV C	Ion-exchange	1.08	1386	24.5	1283	1859
V A	Affinity	0.025	502	8.9	20 080	29 101
V B	Affinity	0.025	542	9.6	21 680	31 420
V C	Affinity	0.045	946	16.7	21 022	30 466

1 unit of enzyme activity = 1 pmol of cholesterol esterified in standard assay conditions; specific activity is pmol of cholesterol esterified by 1 mg of protein,

of the supernatant was aspirated (omitting the uppermost lipid layer) and pooled,

2.7.2. Step II: Precipitation with ammonium sulphate

Solid $(NH_4)_2SO_4$ was slowly added under constant stirring up to a 35% saturation. After the salt has been dissolved, the suspension was stirred for additional 60 min and then centrifuged at 12 000 *g* for 15 min in a Beckman Type 35 rotor. Supernatants were discarded, the pellets were suspended in the standard buffer and dialyzed against the same buffer overnight. The next day, the solution was clarified by centrifugation at 12 000 *g* for 5 min and the pellets were discarded.

2.7.3. Step III: Size-exclusion chromatograph

Step II proteins were applied in 2-ml portions (up to 110 mg of protein) onto the TSK G3000SW column equilibrated against the standard buffer and the column was eluted with the same buffer at a flow-rate of 2.0 ml/min. The absorption was continuously monitored at 280 nm and fractions 4 ml/2 min were collected. Portions (50 μl) of each second fraction were tested for activity in the standard assay. Activity was present in a small peak appearing after the bulk of protein has been eluted (Fig. 1). Active fractions were pooled and proteins precipitated with $(NH_4)_2SO_4$. The precipitates were centrifuged, pellets resuspended, dialyzed and the enzyme solutions were clarified as described for Step II.

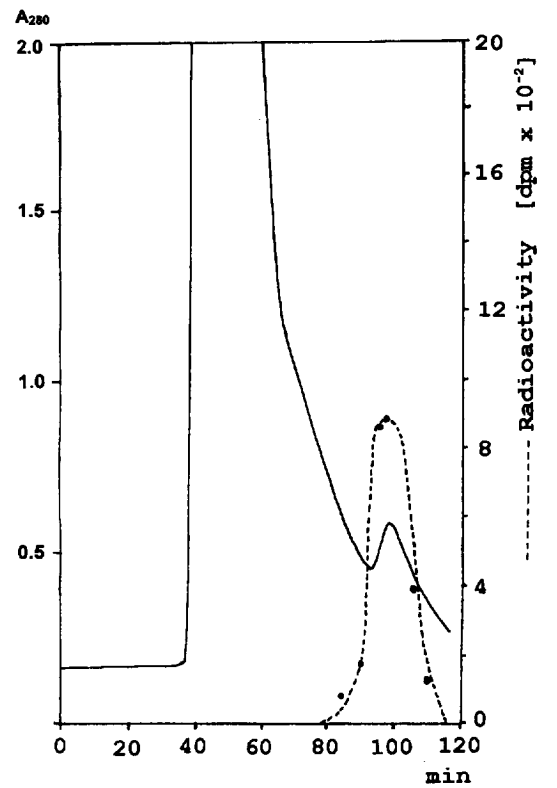

Fig. 1. Separation of step II enzymes on TSK G3000 SW; 105 mg of Step II protein were separated by size-exclusion as described in Section 2.

2.7.4. Step IV: Ion-exchange chromatography

Step III proteins (1.5 ml, approx. 9–12 mg of protein) were injected onto the HEMA-BIO DEAE column equilibrated against the standard buffer. The column was eluted with a discontinuous linear gradient 0–500 m*M* KCl of the following composition: 0 KCl (20 min), 0–100 m*M* KCl (5 min), 100 m*M* KCl (10 min), 100–500 m*M* KCl (20 min), 500 m*M* KCl (10 min). The eluate was continuously monitored at 215 nm, fractions of 4.0 ml/4 min were collected and 50 μl portions were tested in the standard assay. The activity was separated into three distinct fractions (Fig. 2), which were separately pooled and processed further by $(NH_4)_2SO_4$ precipitation, as described for step II. Because of low yields of all step IV enzymes, these proteins were usually pooled from 4–5 enzyme isolations for further purification.

2.7.5. Step V: Affinity chromatography

Each fraction of step IV enzymes was injected in 300–500 μl portions onto columns of immobilized cholesterol. The column was eluted at a flow-rate of 0.50 ml/min with the standard buffer at ambient temperature. The absorption was continuously recorded at 215 nm. After the non-adsorbed protein had been eluted, the column temperature was increased to 45°C (within 3 min). After approximately 2 min of elution with the standard buffer at this temperature, the active fraction started to elute (Fig. 3). Adsorbed fractions from several runs were pooled and processed further as described for step I.

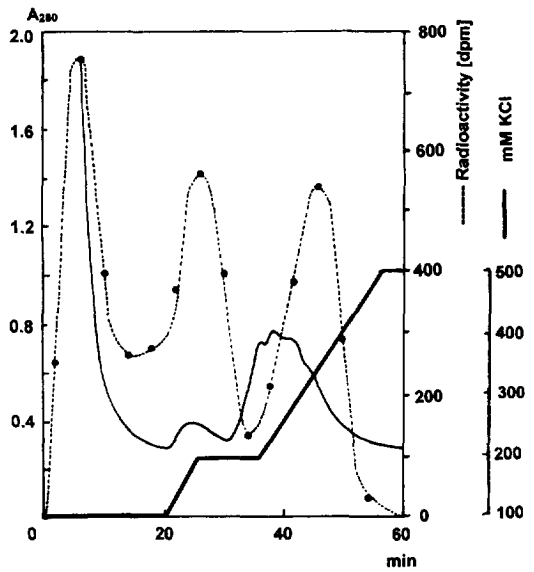

Fig. 2. Separation of step III enzymes on Separon HEMA BIO 1000 DEAE; 10 mg of step III proteins were separated by ion-exchange chromatography as described in Section 2.

3. Results

PAGE in denaturating conditions showed the presence of a single band. Its position was identical in all three step V enzymes and corresponded to an apparent molecular mass of 16.5 kDa (Fig. 4). If 2-mercaptoethanol was added to electrophoresis buffer, an additional faint band appeared of an apparent molecular mass of 65 kDa (results not shown). These results were consistent with those of size-exclusion HPLC on Ultraspherogel SEC 3000. All step V enzymes gave the same symmetrical peak corresponding to an apparent molecular mass of approx. 15–18 kDa (results not shown).

Combination of two step V enzymes, and even the addition of all three enzymes together, did not increase the yield of cholesteryl esters synthesized. In the experiments in which the fraction A enzyme was gradually added to reach saturation and this saturating quantity was supplemented by further

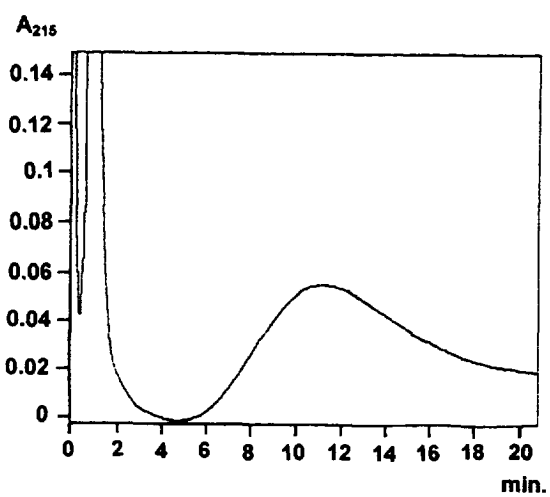

Fig. 3. Separation of step IV enzymes on cholesterol immobilized on HEMA-BIO 1000 E-H; 1.5 mg of step IV C proteins were eluted using the standard buffer at ambient temperature up to 4 min and the eluted protein was discarded. At 5 min the column temperature was increased to 45°C and the fraction eluted between 6–18 min was collected. Details are described in Section 2.

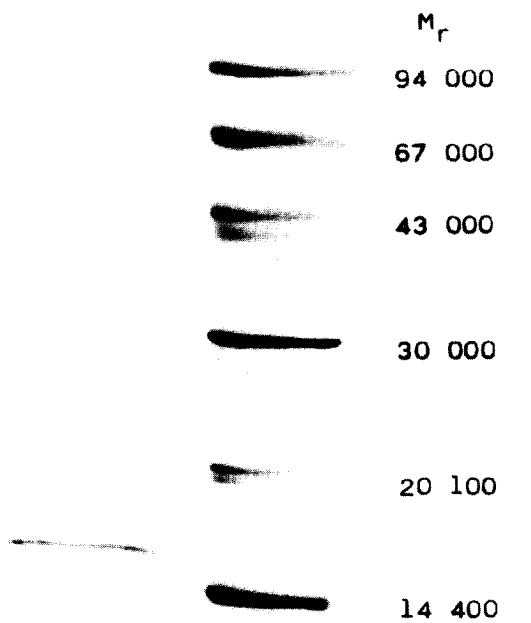

Fig. 4. PAGE of step V C enzyme (left). Standard proteins (right) were: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa. Electrophoresis was performed in the absence of 2-mercaptoethanol.

addition of B or C enzymes, no increase of activity was found in mixtures containing both enzymes. In the presence of an excess of the second enzyme, a drop of activity was even found, apparently due to hypersaturation (Fig. 5). The same results were obtained with any other enzyme combination. Conversely, gradual addition of a second enzyme to subsaturating amounts of a first one increased the activity. However, in no case could values higher than those obtained with saturating quantities of a single enzyme be demonstrated (data not shown).

However, combination of two or even three enzymes resulted in a significantly enhanced reaction rate. Whereas incubation of approx. 2 h was required for maximum yields with individual enzymes, the incubation time was shortened to approximately 60 min with enzymes added in combination (Fig. 6).

Purified enzymes required the optimum concentration of cholesterol in the range of 140–160 n*M* (Fig. 7). The enzymatic activity was significantly enhanced by both Mg^{2+} as well as by 2-mercaptoethanol (Fig. 8). Addition of KCl in concentrations of 0–150 m*M* was without any effect (results not shown).

More purified enzyme fractions (i.e., step IV and V enzymes) showed an absolute requirement for phosphatidylcholines. Both cholates and acyl-CoA were not effective as substrates (results not shown).

4. Discussion

Evidence is provided by the results presented in this paper that for the purification of at least some enzymes, HPLC may possess some advantages over conventional techniques of low-pressure chromatog-

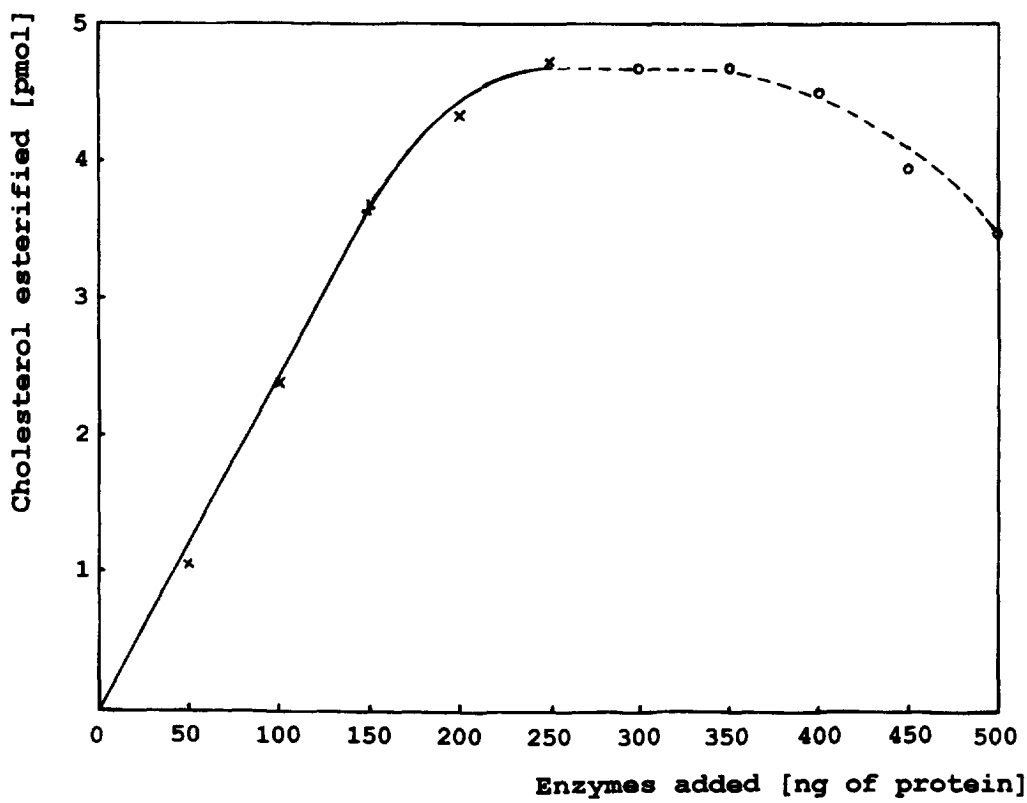

Fig. 5. Effect of additions of step V C enzyme to saturating quantities of step V A enzyme. Incubation mixtures composed as described in Section 2 contained increasing amounts of enzyme A (×). After the saturation level (250 ng) was reached, this quantity of A enzyme was maintained and incubates were, in addition, supplemented with enzyme C (○) (up to 250 ng). Mixtures were incubated at 37°C for 180 min and processed further as described in Section 2.

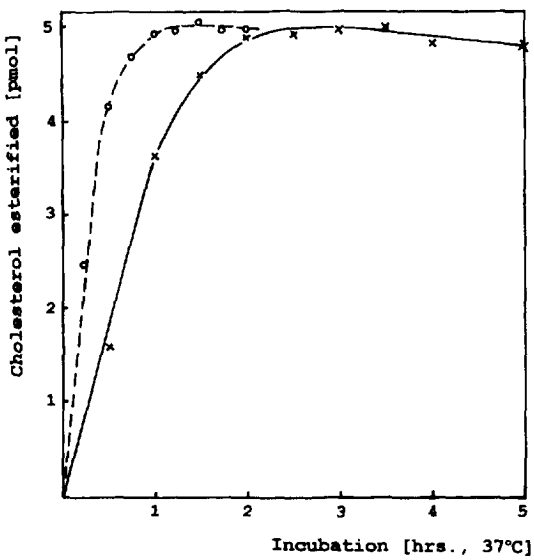

Fig. 6. Effect of the incubation time on the esterification of cholesterol. Mixtures composed as described in Section 2 contained 220 ng of step V C protein (×) or 140 ng of step V B together with 130 ng of step V C protein (○) and were incubated at 37°C for the time indicated.

raphy. Thus satisfactory separations are obtained very rapidly excluding the necessity of cooling the columns during the work. Retention volumes are highly reproducible and the time-consuming testing

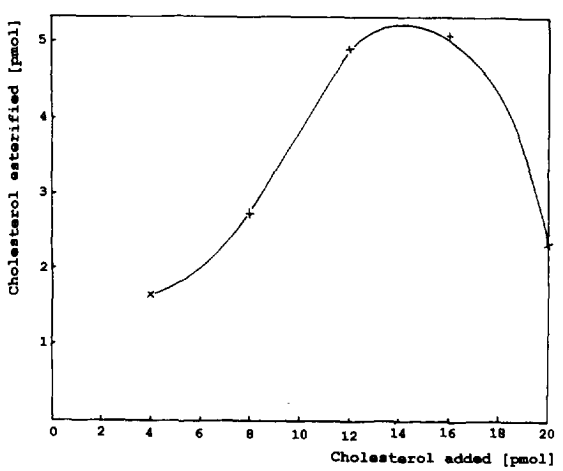

Fig. 7. Effect of the concentration of cholesterol on the activity of its esterification. Mixtures composed as described in Section 2 contained 220 ng of step V B enzyme protein and 0.5 μg of total phosphatidylcholines from egg-yolk and amounts of [^{3}H]cholesterol as indicated.

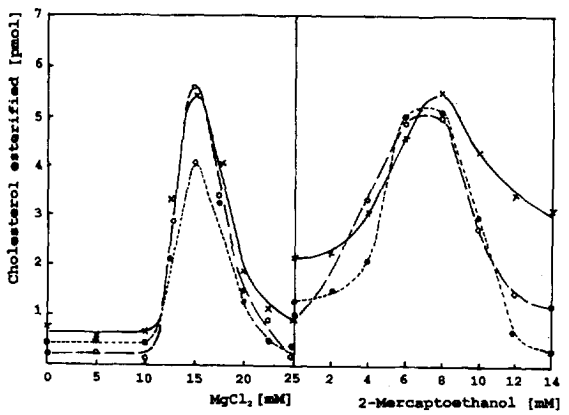

Fig. 8. Effect of Mg^{2+} (left) and 2-mercaptoethanol concentration (right) on the esterification of cholesterol. Mixtures composed as described in Section 2 contained (left) 7 m*M* 2-mercaptoethanol and concentrations of $MgCl_2$ as indicated, or (right) 15 m*M* $MgCl_2$ and 2-mercaptoethanol as indicated. The following quantities of step V proteins were added: A (●), 230 ng, B (×), 225 ng and C (○), 235 ng.

of activity in many fractions in isolations with subsequent batches of enzymes is therefore not necessary. Conversely, the limited capacity of even preparative columns does often require repeated chromatography of the same material. A disadvantage of affinity chromatography in our experiments was the larger volume in which the active fraction was eluted. The reason of this cannot be explained at the moment.

An almost absolute requirement of enzymes described in this paper for phosphatidylcholines indicates that they belong to the family of lecithin–cholesterol acyl transferases (LCAT) (EC 2.3.1.43) [10].

Enzymes of this group were found to occur in several tissues and, in particular, in body fluids (see, for review Ref. [10]). The presence of such enzymes in the liver has been reported by Russell et al. [11] and it has been suggested that they may be precursors of blood plasma LCAT [12]. Unfortunately, no attempts have been described so far on the purification and characterization of these liver enzymes so that a comparison with the preparations described here is scarcely possible.

Of all members of the LCAT family, the enzyme occurring in the blood plasma of humans and experimental animals has been investigated to a great

detail [10]. This enzyme was purified to a degree comparable with that of our enzymes (34 700-fold) [13]. It is a glycoprotein with an apparent molecular mass of 65–66 kDa [13,14] and in this respect significantly differs from the enzymes described in this paper. Blood plasma LCAT requires the addition of substrate in the form of lipoproteins [10] or lecithin–cholesterol vesicles with additional components [15]. On the contrary, a simple solution of phosphatidylcholines in organic solvents served as a satisfactory substrate for enzymes described here.

An addition of both Mg^{2+} and 2-mercaptoethanol simultaneously was almost absolutely required for enzymic activity. No explanation could be formed as yet for this particular combination, nevertheless, the latter compound apparently affects intramolecular-SH groups in individual enzymes since this effect was present if single enzymes were used.

Sufficient information is apparently not available at the moment as to why three cholesterol-esterifying enzymes are present in rat liver cytosol. All these enzymes have an identical molecular mass, substrate requirements and reaction products but differ in their charge, as revealed by their separation by ion-exchange chromatography. Although the activity of a single enzyme in subsaturating quantities may be increased by the addition of another enzyme, the overall yield of cholesteryl esters cannot be increased over that obtained in the presence of saturating amounts of a single enzyme alone. These facts suggest that these individual enzymes may in fact be subunits of a larger complex, which becomes dissociated during the purification procedure. It cannot be excluded that high pressures used in HPLC may have such a dissociating effect. In the presence of 2-mercaptoethanol, PAGE revealed the presence of an additional protein, the molecular mass of which may correspond to a tetramere of individual enzymes. If the yields of the individual enzymes at the end of the purification are considered, then the tetramere would be composed of one molecule each of A and B enzyme and two molecules of fraction C. We are, however, well aware that such an assumption based only on the yields would be very poor and rather speculative and that further experiments are necessary to settle this problem. However, our recent findings showing significantly different activities of individual enzymes when serum of cancer patients was used as a source of substrates (J. Hradec, data not shown) seem to support the subunit concept.

Acknowledgments

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