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PURIFICATION OF LIPOPROTEIN LIPASE FROM RAT POST-HEPARIN PLASMA

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SUMMARY

1. Lipoprotein lipase, which catalyses the hydrolysis of the triglyceride moiety of plasma low-density lipoproteins, has been highly purified from rat post-heparin plasma.

2. The product gave a single band after acrylamide gel electrophoresis, and specific activity was not further increased by acetone fractionation or by chromatography on Sephadex G-150.

3. The sedimentation coefficient was 4.85 S, and the apparent molecular weight was 72 600.

4. The temperature and pH optima and the stability of the purified enzyme have been determined. The stability of the plasma enzyme is the same as that previously reported for the enzyme in adipose tissue.

INTRODUCTION

Lipoprotein lipase is released into the bloodstream of laboratory rats and other mammals following the intravenous injection of heparin[1] and post-heparin plasma has been used as a convenient source of enzyme for the several partial purifications that have been reported[2–5]. In view of the evidence implicating this enzyme in the hydrolysis *in vivo* of plasma triglycerides the properties of the pure protein are of interest, especially in so far as they reveal those features of the molecule responsible for its affinity for lipoprotein[2].

It has been previously shown that low concentrations of long-chain unesterified fatty acid stabilise the enzyme in partially purified preparations against the denaturation promoted by inorganic ions[6]. Further investigation has shown that in the presence of fatty acid and heparin, common techniques can be successfully used to prepare the enzyme in a highly purified state, provided that these do not involve exposure to very high ionic strengths. In the present publication, a purification procedure is presented, together with an account of some of the properties of the enzyme molecule.

MATERIALS AND METHODS

Male Wistar strain laboratory rats, weight 350–400 g, were used in all experiments. Sodium heparin (grade 1, lot 87B 1450, 155 I.U./mg) was purchased from Sigma Biochemicals, London. The following purified proteins were obtained from the same source: bovine liver catalase, 2 × crystallised; bovine serum albumin, crystallised and lyophilised; bovine α-chymotrypsinogen A, 6 × crystallised; yeast alcohol dehydrogenase, crystalline. Intralipid 20% triglyceride emulsion* was obtained from Vitrum, Stockholm, Sweden.

Oleic acid (99%) was obtained from CalBiochem, London and its composition confirmed as the methyl ester by gas–liquid chromatography. Potassium oleate was prepared as previously described[6].

All other chemicals were of the best available commercial grade, used without further purification except where otherwise stated. Calcium phosphate gel (0.2 M) was prepared according to KEILIN AND HARTREE[7]. Protein was assayed by the LOWRY[8] method using whole rat plasma as standard. Lipoprotein lipase was assayed at pH 8.5, otherwise as previously described, using activated triglyceride emulsion as substrate[6]. Other analytical techniques are described under RESULTS.

RESULTS

Purification of the enzyme

The method of purification of the enzyme is summarised in Table I. The procedure can be conveniently divided into four steps.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE OF LIPOPROTEIN LIPASE

Step	*Volume (ml)*	*Total units**	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Purification (fold)*
Crude plasma	140	8960	8780	1.02		
Enzyme–substrate complex	4.7	4560	8.15	560	50.9	499
Soluble enzyme extract**	4.2	3740	2.80	1310	41.8	1285
Calcium phosphate eluate	2.1	2690	1.13	2390	30.0	2345

* 1 unit catalyses the release of 1 μmole unesterified fatty acid/h from the lipoprotein assay medium[6] at 37°.

** For a single extraction only of the enzyme–substrate complex.

Preparation of post-heparin plasma

A 1 mg/ml solution of heparin in 0.145 M NaCl was prepared and 0.4 ml was injected into each animal *via* the femoral vein. After 5 min the dorsal aorta was cannulated and the blood drawn off into polythene tubes cooled in ice. Preliminary experiments were carried out in which the post-heparin plasma was withdrawn

* Formula: Fractionated soybean oil 20% (w/v), fractionated egg lecithin 1.2% (w/v), glycerol 2.5% (w/v), in distilled water.

between 3 and 20 min after injection of heparin. Maximal recovery of enzyme was obtained after 5 min.

All further steps were carried out at 0–2°. 1/20 vol. of 0.25 M sodium citrate was added to the blood, which was then centrifuged at 2500 × *g* for 30 min. The supernatant plasma was collected.

Preparation of enzyme–substrate complex

In the presence of plasma lipoprotein an enzyme–substrate complex is formed which can be purified by flotation[2]. The cold post-heparin plasma from the previous step was mixed with 1/40 vol. of intralipid emulsion. The flask containing the mixture was gently shaken in a water bath at 37° for 7 min to activate the emulsion[1] and allow the formation of the enzyme–substrate complex, then plunged into an ice-bath and gently shaken until the temperature of the plasma fell below 2°. It was loaded into cooled cellulose tubes of the 30-rotor of a Spinco Model L ultracentrifuge, and the machine run at 30 000 rev./min for 1 h. The tubes were then pierced and the clear lower layer drawn off. The opaque lipid-containing layer, comprising about 2 ml of the 38 ml of contents, was transferred to another tube, and homogenised with a close-fitting ground-glass plunger in about 20 ml of 0.05 M NH_4OH–NH_4Cl buffer (pH 8.5), containing 10% w/v sucrose and 1.0 μg/ml heparin. The tube was then filled with the same buffer and the centrifugation repeated as before. The lower layer was again removed, and the floating enzyme–substrate layer washed several times more until the protein concentration in the lower layer after centrifugation fell below 10 μg/ml. Four or five washings in all were required. The addition of sucrose to the washing solution, by increasing its specific gravity, stabilised the floating lipid layer and permitted its easier isolation.

The product from this procedure contained approx. 55% of the enzyme activity, purified about 500-fold (Table I). In the absence of heparin in the washing solution the recovery fell to about 40%.

Solubilisation of the enzyme–substrate complex

The purified complex was mixed with an equal volume of 0.05 M NH_4OH–NH_4Cl buffer (pH 8.5), containing 0.5% w/v sodium deoxycholate, 0.5 mM potassium oleate, 5.0 μg/ml heparin (standard buffer medium). The mixture was agitated without foaming in an ice bath for 1–2 h to disperse the packed lipid. It was then centrifuged in the SW-39 rotor of the ultracentrifuge at 35 000 rev./min for 1 h. Over 80% of the added enzyme activity was recovered in the aqueous lower layer, in a final concentration of deoxycholate 0.3% w/v, potassium oleate 0.3 mM, heparin 4 μg/ml. 15% could be recovered by washing the supernatant lipid a second time with the same solvent. The precipitate of insoluble protein was without activity and was discarded. A further 2-fold purification was obtained (Table I).

Adsorption on calcium phosphate gel

Lipoprotein lipase in unpurified post-heparin plasma was shown by NIKKILA[4] to be strongly absorbed to calcium phosphate gel. This technique was therefore used with the partially purified preparation obtained by the previous step in case a further purification could be obtained.

The enzyme solution from the previous step (pH 8.5), was mixed with 1/4 vol.

of 0.2 M calcium phosphate gel. After 30 min the mixture was centrifuged and the supernatant of unadsorbed protein removed. This was without measurable enzyme activity. The pH of enzyme adsorption to the gel was not critical over the range 7.5–8.7. The gel was then washed with 2 vol. of 0.05 M NH_4OH–NH_4Cl buffer (pH 8.5), containing 0.5% w/v sodium deoxycholate, 0.5 mM potassium oleate, 0.1 M sodium oxalate. After 30 min the mixture was again centrifuged, and the supernatant removed. It contained less than 5% enzyme activity. The gel was finally washed with 1/4 vol. of the standard buffer medium, containing 0.05 M sodium citrate. About 65% of added enzyme activity was recovered. A further 2-fold purification was obtained (Table I). When the concentration of sodium oxalate in the washing solution was raised from 0.1 M to 0.2 M, 25% of added enzyme activity was released along with the non-enzyme protein. When 0.5 M oxalate was used, 50% activity was lost in the washing solution.

Acetone fractionation

The citrate eluate from the previous step, containing approx. 0.5 mg protein/ml, was transferred to a polythene centrifuge tube, and acetone at 0° was slowly added from a micrometer syringe. Over 80% protein was precipitated between 33% and 50% acetone v/v. The precipitate was washed with 2 vol. of acetone, and then re-dissolved in the standard buffer medium. Enzyme was recovered in a yield of about 80%, without increase in specific activity.

Analysis of the purified enzyme

The purified preparation contained about 2400 enzyme units/mg protein as measured by the enzyme assay system previously described[6]. It was further analysed by molecular exclusion chromatography and by acrylamide gel electrophoresis, to determine the degree of purity.

Molecular exclusion chromatography

10 g of dry Sephadex G-150 beads, 40–120 μ diameter (Pharmacia, Uppsala, Sweden) was allowed to swell in the cold for 14 days in the standard buffer medium. After deaeration the gel was packed into a 60 cm × 3 cm chromatography column, to a final height of 55 cm. After stabilisation by passage of buffer medium for 2 days, the packing was tested and the void volume determined by passage of 2 ml of 0.02% Pharmacia Blue Dextran 2000.

2 ml of purified enzyme solution (0.2–1.0 mg/ml, 2400 units/mg) was then passed into the gel and the column eluted with the standard buffer medium. 5-ml fractions were collected. These were assayed for protein and for enzyme activity. Since the protein content of the fractions was in general too low for measurement by the standard LOWRY[8] procedure, 1/10 vol. of 10-fold concentrated Lowry C reagent was used, with consequent reduction in assay volume and intensification of the colour developed. A single enzyme peak was obtained which contained about 85% of the added protein, without change in specific activity. A further 10–15% protein was eluted at the void volume, and was without enzyme activity (Fig. 1). This may represent aggregated denatured enzyme protein. No variation in the elution volume was observed over the experimental range of protein concentrations. In the absence of heparin in the standard buffer medium, enzyme recovery was 20–30% only.

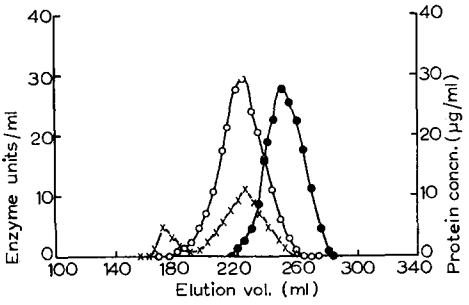

Fig. 1. Determination of Stokes radius of lipoprotein lipase. The column of Sephadex G-150 gel (column dimensions 3 cm × 55 cm) was equilibrated with the standard buffer medium, (pH 8.5). ○—○, lipoprotein lipase activity (left hand scale). ×—×, enzyme protein; ●—●, bovine serum albumen protein concentration (right hand scale). Void volume of the column was 166 ml.

An estimate of the Stokes radius a of lipoprotein lipase and therefore of the diffusion coefficient D was obtained by running through the same column standard proteins of known diffusion coefficient (Fig. 1). The following standard values were used in these experiments: ($D_{20,w} \cdot 10^{-7}$ cm$^2 \cdot$sec^{-1}): bovine serum albumen 6.1 (ref. 10); bovine α-chymotrypsinogen A 10.2 (ref. 11); yeast alcohol dehydrogenase 4.7 (ref. 12). The elution volume was determined from the principal peak of protein (more than 95% total protein for each standard). By the method of ACKERS[9] the Stokes radius of lipoprotein lipase was found to be 37.05 Å (3 determinations, range 36.5–37.4 Å).

Using the relation:

$$D = \frac{kT}{6\pi\eta a}$$

where k is Boltzmann's constant, and T the absolute temperature, η the solvent viscosity, the diffusion coefficient of the enzyme could be estimated. A value of $5.8 \cdot 10^{-7}$ cm$^2 \cdot$sec^{-1} was obtained.

Determination of the sedimentation coefficient

Combination of the diffusion coefficient with the sedimentation coefficient obtained by the sucrose gradient method[13] would permit an estimate of the molecular weight of the enzyme by substitution in the Svedberg equation.

Linear gradients of 5–25% sucrose w/v in the standard buffer medium were prepared in the cellulose tubes of the Spinco SW-39 rotor. The volume of each gradient was 4.9 ml. 0.1 ml of purified enzyme solution (0.5–1.0 mg/ml, 2400 enzyme units/ml) was added or the same volume of a standard protein solution at the same protein concentration. The following standard values were used ($s^\circ_{20,w} \cdot 10^{-13}$ sec): bovine serum albumin 4.3 (ref. 10); yeast alcohol dehydrogenase 7.4 (ref. 13); bovine α-chymotrypsinogen A 2.5 (ref. 11); bovine liver catalase 11.3 (ref. 13). The gradients were run for between 10 and 20 h at 38 000 rev./min. 3-drop fractions (about 0.12 ml) were collected from the bottom of the gradients. Each fraction was assayed for protein, and in the case of lipoprotein lipase for enzyme activity also.

Comparison of the distance travelled by this enzyme with that by the standard proteins under the same conditions allowed an estimate of the sedimentation coefficient

($s^{\circ}_{20,w}$) of lipoprotein lipase. Comparison with each of the standard proteins provided a value consistent within experimental error ($s_{20,2} = 4.85 \pm 0.05 \cdot 1^{-13}$ sec, 8 experiments, range 4.82–4.91). A recovery of 90% of enzyme activity was obtained after a 20 h run, without increase of specific activity.

Calculation of molecular weight using enzyme assay data

The sedimentation and diffusion coefficients obtained permit the calculation of a molecular weight value by substitution in the Svedberg equation, provided that a value for the partial specific volume $\bar{v}$ is known or can be assumed. Insufficient material has been as yet available for the determination of $\bar{v}$. The majority of proteins have a $\bar{v}$ of between 0.70 and 0.75 (see ref. 13) and in these preliminary studies a value of 0.72 has been used. In this case, an apparent molecular weight of 72 600 was obtained for lipoprotein lipase.

Acrylamide gel electrophoresis of the purified enzyme

The purity of the enzyme preparation was also examined by acrylamide gel electrophoresis. The analyses were kindly carried out by Dr. D. G. Wild. 50 μl of enzyme solution (1.0 mg/ml, 2390 units/mg) was subjected to electrophoresis for 2.5 h in a slab of polyacrylamide gel, at an initial current of 90 mA (145 V). Gels were made from cyanogum 41 (5%), *N,N,N',N'*-tetramethyl ethylene diamine (0.1%) in Tris (25 mM)–glycine (190 mM) buffer (pH 8.3), containing 1.0% sodium dodecyl sulphate,

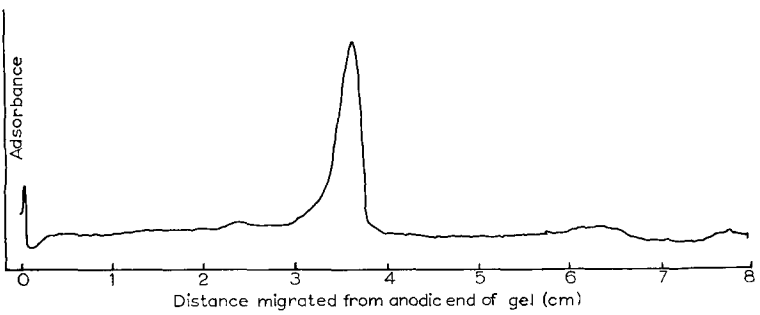

Fig. 2. Acrylamide gel electrophoresis of purified lipoprotein lipase. Densitometric trace after Amido Black staining. The origin is at the left of the trace.

heparin 10 μg/ml, and were polymerised with ammonium persulphate 0.15%. The same buffer was contained in the electrode vessels. Proteins were stained with Amido Black (Fig. 2). The gels were scanned with a Joyce–Loebl Chromoscanner. More than 96% of protein was contained in a single band.

In the absence of sodium dodecyl sulphate the enzyme protein did not migrate to any extent into the gel, apparently due to formation of an aggregate under the influence of the applied current.

Temperature optimum of lipoprotein lipase

Purified enzyme (2400 units/mg protein) was incubated in the standard assay medium[6] at temperatures between 0° and 60° and the amount of unesterified fatty

acid liberated during 1 h was measured (Fig. 3). An optimum release occurred at 37°. Activity rapidly fell off above 40°. At 0°, significant enzyme activity, approx. 10% maximal, was observed.

pH optimum of lipoprotein lipase

Assay media were prepared as in the previous experiments, but with pH values between 6.0 and 10.0. Incubations were carried out at 37°. A maximum of enzyme activity was obtained at pH 8.5 (Fig. 4).

Stability of the purified enzyme preparation

The enzyme activity of the purified enzyme protein (2400 units/mg protein, 0.5–2.0 mg/ml) was moderately stable when stored in the standard buffer medium.

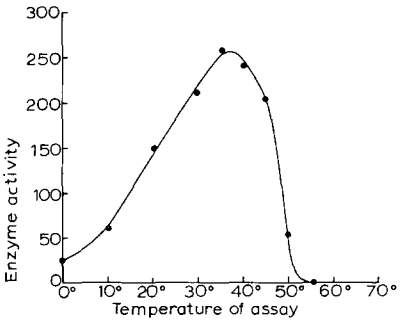

Fig. 3. Effect of temperature on enzyme activity. 0.05 ml of purified enzyme (0.1 mg/ml, 2390 units/mg) added to 3.95 ml of assay medium equilibrated at each temperature. Incubation period 1 h. Enzyme activity expressed as μmoles free fatty acid /h per ml of enzyme solution.

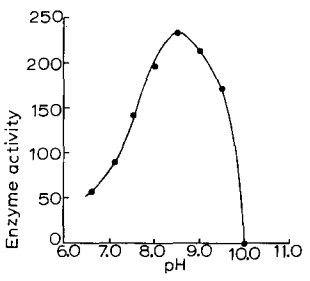

Fig. 4. Effect of pH on enzyme activity. 0.05 ml of purified enzyme (0.1 mg/ml, 2390 units/mg) in the standard buffer medium added to 3.95 ml of assay medium prepared at each pH. Incubation period 1 h at 37°. Enzyme activity expressed as μmoles free fatty acid /h per ml of enzyme solution.

During incubation at 37°, loss of activity was found to follow first-order kinetics, with a first-order constant of 0.003 min^{-1} (3 experiments, range 0.0028–0.0034). At 0° 10–15% activity was lost per 24 h. At −20° about 5% activity was lost during the same time. It has therefore been possible to store enzyme preparations for only a few days without marked decrease in specific activity.

DISCUSSION

The procedure described has permitted the routine preparation on a small scale of lipoprotein lipase homogeneous by chromatography and by electrophoresis, for a study of the structure of this enzyme (Table I). The activity of the preparation during purification has been largely maintained by the presence of heparin and of unesterified fatty acid in the buffers used. The stabilising role of fatty acid in this system has been previously discussed[6] and an excess of this factor has been used in all buffers. Nevertheless the use of solutions of high ionic strength in techniques such as ion-exchange chromatography and step-wise elution of hydroxyl apatite columns with phosphate buffer, caused very severe loss of enzyme activity (C. J. FIELDING, unpublished

experiments). A continued requirement for heparin in the buffers used also seems indicated by the lower yield produced at each step when this compound was omitted. Heparin has been implicated on several occasions as a component of the enzyme molecule in post-heparin plasma[1], and the present results appear to support this proposal. A particularly marked loss of activity in the absence of heparin was found during molecular exclusion chromatography, under conditions where the dissociation of such a protein–heparin complex would be favoured. As has been previously pointed out[4] the strong adsorption of the enzyme to calcium phosphate gel and its elution by citrate, is consistent with the existence of an enzyme–heparin complex.

The results of the molecular exclusion and electrophoresis experiments suggest that the enzyme as prepared is greater than 95% pure. Since solubilisation of the enzyme–substrate complex by deoxycholate presumably involved delipidation of the complex, it is possible that the molecule isolated consists of a tightly-bound enzyme–apolipoprotein complex. Nevertheless, the lack of effect of high sodium dodecyl sulphate concentrations suggest that the preparation is free of non-enzyme protein.

The data obtained for the sedimentation and diffusion coefficients of lipoprotein lipase suggest a molecular weight of about 73 000, assuming a partial specific volume of 0.72. The determination of the amino-acid composition of this enzyme which is in progress is expected to provide a more exact value of this parameter. The sedimentation coefficient was found to be 4.85-S units.

Recent evidence has suggested that in the case of human lipoprotein lipase, a temperature of below 30° is required to maintain zero-order kinetics of enzymatic hydrolysis over a 1 h incubation period[14]. This effect has not been observed over the same time interval in the case of the rat enzyme (Fig. 3) perhaps due to a greater stability of the enzyme–substrate complex of this species at 37°. The pH optimum of 8.5 is identical to that found by these authors for the human enzyme.

The value of the first-order constant at 37° found for the pure plasma enzyme is consistent with that found for partially purified tissue enzyme in the presence of fatty acid[6] and this evidence suggests the identity of the enzyme molecule from these different sources.

It is hoped that the availability of highly purified lipoprotein lipase will assist in the investigation of the structural basis of the action of this enzyme. This may in turn lead to a fuller understanding of the factors regulating the hydrolysis of plasma triglyceride for utilisation in the tissues.

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