PURIFICATION AND CHARACTERIZATION OF LIPOPROTEIN LIPASE FROM RAT BROWN FAT

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1. Introduction

The lipoprotein lipase (EC 3.1.1.34) is a major factor in the uptake of chylomicrons and very low density lipoprotein triglycerides by extrahepatic tissues. It has been purified from various sources [1–7] by affinity chromatography on heparin–Sepharose.

Brown adipose tissue present in hibernating mammals, but also in homeotherm mammals, including Man, where it is particularly developed in the new-born, plays a very important role in non-shivering thermogenesis. The latter is particularly active at birth, in animals exposed to the cold and during the waking period following hibernation.

Lipoprotein lipase, by enabling the energy substrates (free fatty acids) necessary for heat production to enter the brown fat cell, appears as a key enzyme controlling for a large part the calorigenic possibilities of this tissue. However, little work [8–11] has been devoted to the study of brown fat LPL and no purification assay has been made.

In this work, we describe for the first time a highly purified preparation of LPL obtained from rat brown fat. We have studied the extraction and stability of the enzyme as compared with rat heart and in relation to the presence of NaCl (in the extraction buffer), purification on heparin–Sepharose and finally the properties of the purified enzyme.

Abbreviations: LPL, lipoprotein lipase (EC 3.1.1.34); barbital buffer, 0.025 M sodium barbital buffer, 20% glycerol (pH 7.4); FFA, free fatty acids

2. Materials and methods

2.1. *Enzyme preparation*

Heart and interscapular brown fat were removed from rats of the OFA breed, initially Sprague-Dawley (IFFA-CREDO, 69 St Germain sur l'Arbresle France). We used max. 4–6 week old animals. With older animals the brown fat shows a progressive tissue degeneration with respect to white fat. Studies of extraction and stability have been carried out with brown fat from normal animals and from animals stressed by the cold at 4°C for 3 h. The comparative study with rat heart was carried out solely with the hearts of normal animals. We carried out the purification of brown fat LPL from animals stressed by the cold in the conditions described.

Rats were killed by decapitation. Brown fat and heart (stocked at –20°C) are homogenized at 4°C in acetone, filtered on Whatman no. 1, washed with cold acetone, then with ether. For brown fat, the resulting powder was extracted with 0.025 M ammonium buffer (pH 8.6) at 4°C during 30 min with a magnetic stirrer (50 mg powder/2 ml buffer). The same procedure was used with different barbital buffers for the LPL extractibility study of powders from brown fat and heart. After centrifugation (3000 × *g*) at 4°C for 30 min to remove insoluble material, the supernatant was immediately used as crude enzyme preparation or purified on heparin–Sepharose.

2.2. *Enzyme purification*

Sepharose 4B (Pharmacia Fine Chemicals, Uppsala) was activated by cyanogen bromide [12]. Heparin

(Inolex Corp. 128 USP/mg) was covalently bound to gel as [13]. The crude enzyme in ammonium buffer is slowly precipitated at pH 5.2 (4°C) with 0.1 M acetic acid for 10 min. After centrifugation, the precipitate was dissolved in barbital buffer and applied on an heparin–Sepharose column (11 × 0.9 cm) at 4°C. Elution was with a discontinuous gradient 0.5 M, 0.75 M, 1.2 M NaCl in barbital buffer. Fractions (2.5 ml) were collected after A_{280} recording. In some experiments, the purified LPL in 1.2 M NaCl barbital buffer was immediately dialyzed against barbital buffer (3 changes) at 4°C for 3 h. According to [14] we added heparin (10 μg/ml) during dialysis to prevent the enzyme precipitating at <0.5 M NaCl.

2.3. *Disc gel electrophoresis*

Fractions obtained after heparin–Sepharose chromatography were dialyzed extensively against water (16 h) and lyophilized. The preparation homogeneity was studied by disc-gel electrophoresis in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel. The lyophilized protein samples were incubated for 1 h at 90°C (instead of the usual 37°C) in 10% SDS and 5% β-mercaptoethanol. Bovine serum albumin, ovalbumin, chymotrypsin and cytochrome *c* were used as standards for molecular weight determination. The gels were stained with Coomassie blue.

2.4. *Enzyme assay*

For the LPL assay, the triolein emulsion was prepared as follows: 300 mg triolein (Sigma Grade) and 18 mg egg yolk lecithin in chloroform were weighed in an 8 ml bowl. The solvant was dried under N_2 and 5 ml (6g25) glycerol (Sigma) added. This mixture was then homogenized with a Virtis 45 homogenizer for 5 min in an ice-bath. The opaque emulsion became clear after some hours at room temperature. This emulsion remained stable for 6 weeks [15]. Incubations were for 1 h at 27°C. The assay system contained in 560 μl total vol.: 4.43 μmol triolein/ml; bovine albumin (Sigma fraction V poor in FFA) 3% (w/v); rat fresh serum 10% (v/v); heparin (128 USP/mg) 16 μg/ml; NaCl 0.15 M; Tris–HCl (pH 8.6) 0.094 M. The optimum pH was 8.6 and the assay linear for 2 h. Released FFA were estimated in 0.2 ml aliquot at time 0 and 60 min. After extraction with Dole mixture [16], the solvant was washed with sulfuric acid 1/2000 (v/v). Acid oleic standards, obtained from a 8 mM stock solution in Dole mixture, were made throughout the assay. Finally FFA were assayed using the microcolorimetric method [17]. Protein was determined by the method in [18] with bovine albumin as standard. The enzyme unit was defined as the amount of enzyme which releases 1 μmol FFA/h under the described conditions.

Table 1
Extraction of rat brown fat and heart LPL from acetone–ether powders with different buffers

Tissue	Buffer	Protein (mg/g tissue)	Spec. act. (U/mg protein)
Normal brown fat	1	39 ± 6	0.32 ± 0.05
	2	34 ± 4	0.33 ± 0.07
	3	37 ± 4	0.17 ± 0.06
	4	31 ± 7	0.25 ± 0.05
Stressed brown fat	1	35 ± 7	2.0 ± 0.6
	2	33 ± 4	1.8 ± 0.6
	3	35 ± 4	1.0 ± 0.2
	4	37 ± 5	1.0 ± 0.4
Normal heart	1	31 ± 7	0.17 ± 0.02
	2	30 ± 7	0.17 ± 0.02
	3	32 ± 7	0.43 ± 0.06
	4	37 ± 9	0.79 ± 0.17

Mean of 3 assays: (1) ammonium buffer (pH 8.6); (2) barbital buffer (pH 7.4); (3) barbital buffer, 0.5 M NaCl (pH 7.4); (4) barbital buffer, 1.5 M NaCl (pH 7.4)

3. Results

Table 1 summarizes the comparative LPL extraction of brown fat and heart acetone–ether powders with different buffers. In all tissues, there was no more protein extraction with NaCl barbital buffer. Nevertheless with brown fat (stressed or not), the LPL activity extraction was greater with ammonium and barbital buffers than with NaCl barbital buffer. Opposite results were obtained with rat heart: NaCl had a positive effect on LPL activity extractibility. In brown fat, exposure to the cold (3 h at 4°C) rapidly increased the rate of triglyceride hydrolysis by LPL (under these conditions no increase in protein content was shown).

LPL from brown fat and heart was particularly unstable (table 2). Approximately half the activity in ammonium buffer and barbital buffer was lost within 1 day at 4°C. In all cases, NaCl presence notably increases this instability, even with heart LPL where NaCl increases LPL extraction. Also ammonium buffer is commonly used for LPL purification from stressed brown fat.

The results of a representative purification carried out on 3.7 g (20 rats) of stressed brown fat are presented in table 3. Precipitation at pH 5.2 of LPL in ammonium buffer allows a maximal recovery of LPL activity but decreases the protein amount applies to the column of >60%. 40% of LPL activity did not bind to heparin–Sepharose and LPL was eluded in 1.2 M barbital buffer in a single peak. Only 20% of the LPL activity applied was found (15 ml). The specific activity of the LPL eluted in this peak was ~900-times that in the crude extract. 50% of the purified LPL are eluted in two major fractions (5 ml).

Table 2
Stability at 4°C of rat brown fat and heart LPL in different buffers

Tissue	Buffer	Remaining activity (%) after		
		1h	3 h	20 h
Normal brown fat	1	98	81	38
	2	92	62	48
	3	88	56	20
	4	66	24	12
Stressed brown fat	1	88	67	43
	2	89	74	46
	3	79	44	22
	4	52	13	10
Normal heart	1	90	61	55
	2	78	58	48
	3	73	46	14
	4	84	50	25

Mean of 3 assays: (1) ammonium buffer (pH 8.6); (2) barbital buffer (pH 7.4); (3) barbital buffer, 0.5 M NaCl (pH 7.4); (4) barbital buffer, 1.50 M NaCl (pH 7.4)

After purification, brown fat LPL was still very unstable. After 7 days storage at −20°C in the elution buffer, barbital buffer 1.2 M NaCl, the residual enzyme activity was 10%. After adding glycol 1 M, this residual activity was 7%, and if the glycerol was increased to 50%, it remained at 11%. Adding bovine

Table 3
Steps of purification of stressed rat brown fat LPL

	Volume (ml)	Protein (mg)	Enzyme act. (U)	Spec. act. (U/mg protein)	Recovery	Purification
Ammonium buffer[a] (pH 8.6)	20	172	339	2.0	100	
Barbital buffer	18	47	305	6.5	96	3.25
Applied on heparin–Sepharose	17.5	46	297	6.5	100	
Not fixed	21	37	106	2.9	36	
Eluted in barbital buffer 1.2 M NaCl	15	0.032	59	1843	20	921

[a] 3.7 g of stressed rat brown fat were used

Fig.1. Disc gel electrophoresis of rat brown fat LPL purified on heparin–Sepharose in 7.5% acrylamide.

albumin at 1 mg/ml enables better conservation: 38%.

Under the conditions of very high denaturation used (1 h at 90°C in the presence of SDS and β-mercaptoethanol) practically all the protein deposited on the polyacrylamide gel penetrated the latter. A major strip of ~67 000 mol. wt and 2 annexe strips of high molecular weight (~190 000 and 300 000) became visible after coloration by Coomassie blue (fig.1).

With a triolein glycerol emulsion, purified LPL had a maximum activity with substrate at 4.4 μmol triolein/ml and pH 8.6 (fig.2).

Serum was necessary to obtain maximum activity (10–15%). In its absence the residual LPL activity was low (10%). For bovine albumin, optimum conditions are 1–3% (fig.3).

Heparin was an important activator of LPL activity up to 40 μg/ml. For stronger concentrations there was a small but constant inhibition. In the presence of NaCl, LPL activity was maximum at 0.10–0.15 M. Larger concentrations caused a progressive inhibition of LPL activity: at 1 M NaCl, the remaining activity only represents 8% of that obtained at 0.15 M (fig.4).

Finally, inhibition by protamine sulfate (without enzyme preincubation) was very strong. The residual activity was 32% in the presence of 0.1 mg/ml and 17% with 1 mg/ml.

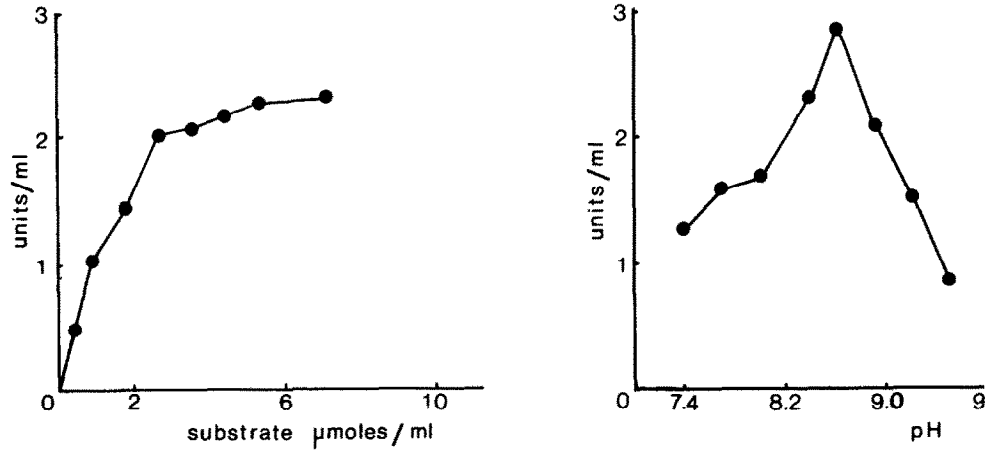

Fig.2. Properties of rat brown fat LPL purified by heparin–Sepharose chromatography with respect to pH and substrate concentration of assay.

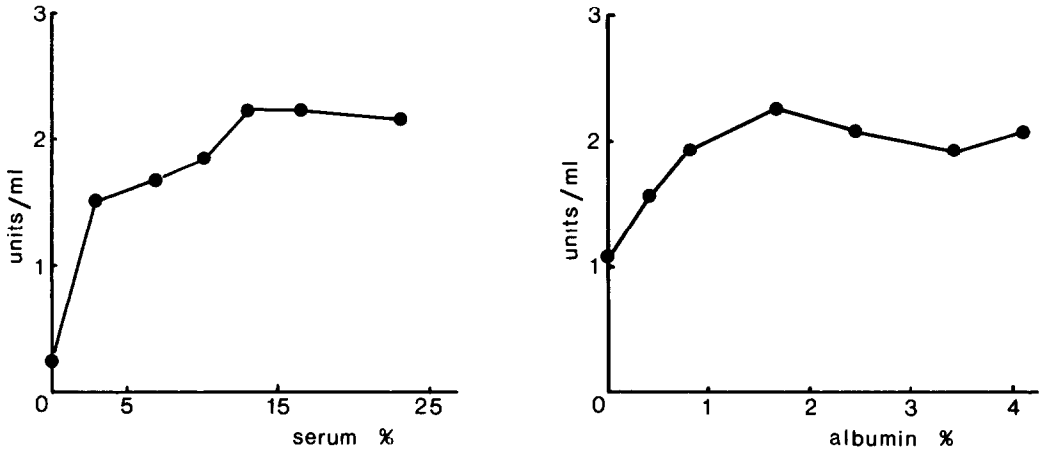

Fig.3. Properties of rat brown fat LPL purified by heparin–Sepharose chromatography with respect to serum and albumin concentration of assay.

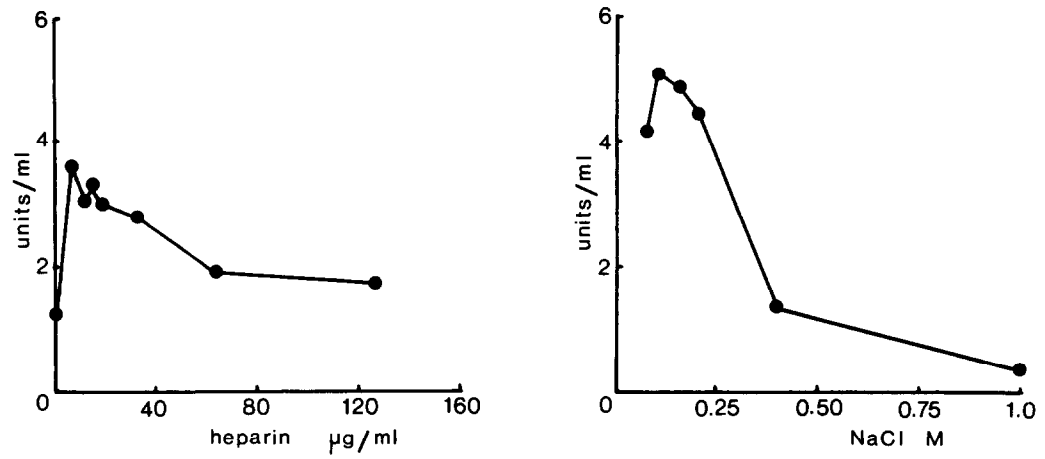

Fig.4. Properties of rat brown fat LPL purified by heparin–Sepharose chromatography with respect to heparin and NaCl concentration of assay.

4. Discussion

Ammonium buffer is the most commonly used for extracting LPL from acetone ether powders. However Bensadoun et al. [4] have shown with white pig fat powders, that extraction is 6-times greater in 1.5 M NaCl buffer than in ammonium buffer. The behaviour of the LPL of brown rat fat still remained to be defined, and that is what we have done in this work. The presence of NaCl in the extraction buffer brings no additional LPL solubilisation either with brown fat removed from normal rats or those stressed by the cold. As described [7], we check that rat heart LPL, for its part, presents a better extraction in the presence of NaCl. In all cases, enzyme instability is high: moreover, it is increased in the presence of NaCl, even for rat heart LPL. One can finally note that the presence of 20% glycerol in barbital buffer does not, in comparison with ammonium buffer, increase enzymatic stability.

Under our conditions, specific activity of purified LPL varied from 1000–2200 U/mg protein and

depends on the specific activity of the crude enzymatic preparation. This variability of the crude preparation is explained by strong variations in the response to the cold recorder from one rat to another, and more particularly by a decrease in response with the animals age: this is why we systematically used 4–6 week old animals. Precipitation at pH 5.2 and chromatography on heparin–Sepharose supply a purification of 800–900-times the crude preparation.

The minimum molecular size determined for 67 000 mol. wt brown rat fat LPL is comparable to that obtained with cow milk LPL 64 000 mol. wt [2], with adipose pig tissue 62 000 mol. wt [4], with rat heart 60 000 mol. wt [5], but varies with the results in [7] where 34 000 mol. wt for rat heart LPL was determined.

The characteristics of LPL purified from the brown fat of rats stressed by the cold for 3 h at 4°C are identical to those normally described: pH 8.6; the need of serum; the activating effect of heparin; maximal activity at 0.10–0.15 M NaCl; inhibition by protamine sulfate.

The lack of work on the purification of brown fat LPL can perhaps be explained by the small amount of tissue which can be removed from animals, and in particular from rat (150–200 mg). The use of animals stressed at 4°C for 3 h has enabled us to carry out this work and begin the study of brown adipose tissue LPL.

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