ANALYTICAL-SCALE ISOLATION OF TRIACYLGLYCEROL LIPASE FROM HUMAN ADIPOSE TISSUE

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1. Introduction

The purification of human adipose tissue triacylglycerol lipase (EC 3.1.1.3.) by conventional methods [1,2] has been so far severely impeded by the limited availability of human fat, its very low content in lipase activity and the unusual susceptibility of the enzyme to most physicochemical agents used for protein purification. We have shown that dioleoylglycerol, a substrate molecule [3], can be covalently attached to CH-Sepharose and serve as a ligand for the lipase. By this method and via a one-step procedure, we obtained a 10-fold purified enzyme [4]. The analytical-scale experiments reported below allowed a 53-fold purification of the lipase and its subsequent visualization as a discrete band upon disc gel electrophoresis. During purification, protein kinase was found to behave in a manner close to lipase.

2. Materials and methods

2.1. *Preparation of the extracts*

Specimens of adipose tissue from patients undergoing surgery were immediately homogenized (4 ml/g of tissue) in 0.05 M sodium phosphate buffer (pH 7.4) containing 10^{-4} M EDTA and 20% glycerol as stabilizing agent [5]. After centrifugation at 12 000 *g* for 30 min, the lipase present in the supernatant fluid was precipitated at pH 5.4 as previously reported [2]. Prior to chromatography, the pH 5.4-precipitated extract was thoroughly dialyzed against 0.01 M sodium phosphate buffer (pH 7.4) without glycerol.

2.2. *Enzymatic assays*

Emulsification of the substrate glycerol [9,10-^{3}H] oleate (Radiochem. Centre, Amersham) was achieved by sonication in 0.5% defatted serum albumin (Calbiochem) in a Branson sonifier model B-12 (microtip, setting 4) for 30 s at 4°C. Triacylglycerol lipase activity was assayed as recently published [2] in a final volume of 1 ml at 37°C containing: 10 mM sodium phosphate buffer, 1% defatted albumin, 1 mM [^{3}H]triolein (10^6 cpm) and the enzyme at the optimum pH of 7.4. The release of [^{3}H]oleic acid was linear during 10-min incubation and served to monitor the rate of the lipolytic process. For each value of activity, duplicate assays were reproducible within 10% of [^{3}H]oleic acid released. One unit of activity corresponds to the release of 1 μmol of acid per min.

Protein kinase activity was assayed at pH 6.0 in a 0.3 ml-reaction mixture at 30°C containing: 50 mM magnesium acetate, 10 mM NaF, 3 mM EGTA, 2 mM theophylline, 10 μM cyclic AMP, 0.7 mg protamine sulfate per ml and the enzyme. After addition of 0.6 mM [γ-^{32}P]ATP (50–100 cpm per pmole), incubations were carried out for 5 min terminated by pipetting 50 μl aliquots of the reaction mixture onto filter paper discs and washing according to Wastela et al. [6]. Controls contained the complete system without enzyme. One unit of protein kinase activity catalyzes the incorporation of 1 pmol of ^{32}P into protamine sulfate per min.

2.3. *Other analytical procedures*

Agarose-6-aminohexanoyl dioleoylglycerol ester (diolein-agarose) was prepared by esterification of dioleoylglycerol to the hexanoyl group of CH-Sepha-

Table 1
Purification of triacylglycerol lipase from human adipose tissue

Enzyme fraction	Volume (ml)	Total proteins (mg)	Total lipase activity (munits)	Specific activity (munits/mg of proteins)	Purification (-fold)	Yield (%)
Crude extract	188	377	113	0.3	–	100
pH-5.4 precipitate	14	67	81	1.2	4	72
Pooled fractions 15–16	4.6	0.46	7.6	16	53	6.7

rose in the presence of *N*-ethyl, *N'*-(dimethylaminopropyl)carbodiimide as previously described [4]. Protein concentrations were determined by the method of Lowry et al. [7] with crystalline bovine serum albumin as standard. ^{3}H radioactivity was measured with a yield of 82%.

3. Results and discussion

The results of typical purification procedure (Purification I) are summarized in table 1. About 5 g of wet adsorbent was gently stirred for 4 h with 14 ml of a pH 5.4-precipitated extract (81 munits of lipase, 67 mg of proteins). Unretained material was partly removed with supernatant I obtained by centrifuging the mixture at 1000 *g* for 10 min. The sedimented adsorbent was washed twice with 10 ml of glycerol-free buffer (supernatants II and III) and finally loaded on a column. Upon elution (fig.1,a) 43 munits of activity (53% of the amount submitted to chromatography) emerged as a single peak, slightly after a main protein peak. The total amount of unretained proteins (supernatants I, II and III) was 63 mg. Frac-

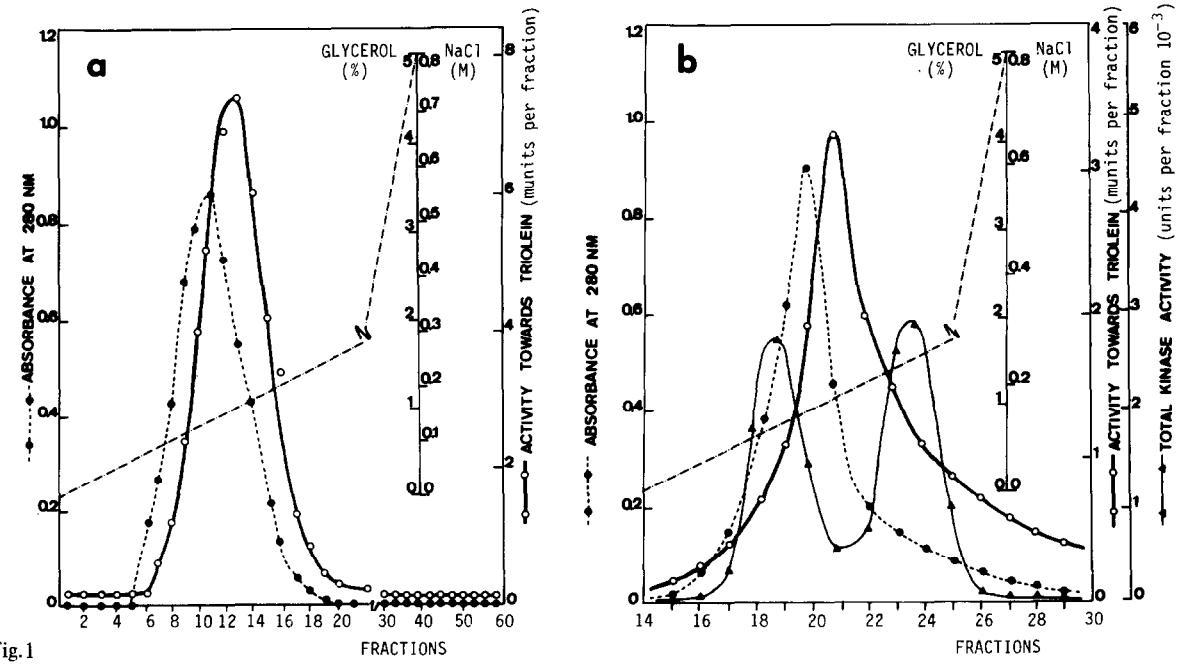

Fig.1

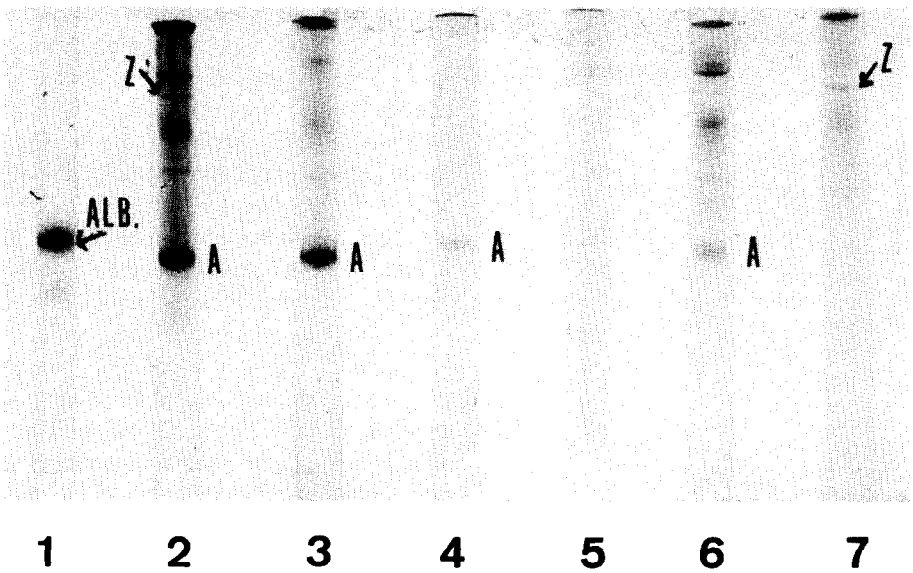

Fig.2. Polyacrylamide gel electrophoresis of samples taken at successive stages during purification. The gel contained 4.16% acrylamide monomer (T) with a 3.8% crosslinking (C). Electrophoresis were performed in 0.05 M Tris-glycine buffer (pH 8.5) with serum albumin as internal standard. A current of 1.5 mA for 5 min, then 4 mA for 30 min was applied per tube (75 × 5 mm). (1) albumin (ALB); (2) pH 5.4-precipitated extract; (3) supernatant I; (4) supernatnat II; (5) supernatant III; (6) pooled fractions 15–16. Band Z: see the text. Stain, Coomassie blue. Origin at top (cathode).

tions 6–18 contained 5.6 mg, i.e. less than 1.5% of the total tissue proteins, as evaluated in the crude extract. The specific activity of the pooled fractions 15–16 was 16 munits/mg of proteins, indicating a 53-fold purification.

Polyacrylamide gel electrophoresis performed at successive stages of purification is shown in fig.2. Band A, which migrated identically to standard albumin (ALB) most probably originated from blood in the tissue samples. A discrete band seen in the extract before chromatography (sample 2, Band Z) with a R_f of 0.30 versus albumin, appeared to be of interest. This material was readily adsorbed on diolein-agarose, as shown by its complete disappearance from all the subsequent washings (samples 3,4,5). Band Z was unequivocally identified again in sample 7, deriving from the active fractions. These observations suggested that Band Z might correspond to lipase. Confirmation of this point required the preparation of an additional amount of purified enzyme (Purification II) obtained under conditions identical to those used for purification I. In both procedures, lipase behaved identically (compare fig.1,a and b). The presence of catalytic activity in Band Z was tested by subjecting to electrophoresis, 2.2 munits of purified lipase (spec. act. 9 munits/mg of proteins) immediately

Fig.1. Elution pattern of human lipase from diolein-agarose. (a) 81 munits of lipase (14 ml of a pH 5.4-precipitated extract, 67 mg of proteins) were adsorbed on diolein-agarose as a batch procedure. The enzyme-adsorbent complex was loaded in a glass column (4 × 1 cm) and eluted at a flow rate of about 10 ml/h with a 120-ml linear gradient of increasing NaCl and glycerol concentrations (–·–·–) in 0.01 M sodium phosphate (pH 7.4). Fractions (2 ml) were collected in tubes containing 0.3 ml of 60% glycerol in water. The entire chromatography was carried out at 4°C and was completed in 16 h. One to three enzymatic assays contributed to each point. The 280 to 260 nm absorbance ratio was 1.4 ± 0.3 from fraction 10 to 15. (b) 99 munits of lipase (10 ml of a pH 5.4-precipitated extract, 36 mg of proteins) were adsorbed and eluted as above. The peak fraction 21 served for electrophoresis immediately after collection (see legend to fig.3). All protein kinase assays were performed in the presence of 10 μM cyclic AMP and are referred to as 'Total protein kinase activity'.

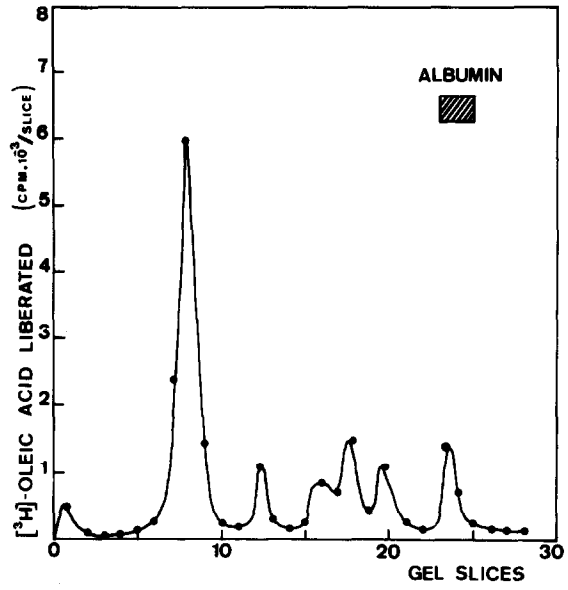

Fig.3. Pattern of migration of purified lipase activity upon polyacrylamide gel electrophoresis. Material from fraction 21 (fig.1,b) was subjected to electrophoresis under conditions identical to those indicated with fig.2. After migration, the gel column was immediately sliced into 1.5-mm segments and each segment was assayed for its content in lipase activity after homogenization into the lipolytic medium. Assays were otherwise performed under standard conditions. Activity is expressed in arbitrary units because the small amount of gel present in the assay medium may cause modifications of the substrate interface susceptible to affect the V of the reactions (see [2]). Origin at left.

after elution. The migration pattern in the gel showed a major peak of lipase activity with a R_f of 0.31 versus albumin (fig.3), compared to 0.30 for Band Z in the gel stained for proteins.

The lipase activity eluted from diolein-agarose in highly diluted solution was unstable. Comparative assays performed in all fractions within a few hours after elution showed that diacylglycerol lipase activity coeluted in strict coincidence with triacylglycerol lipase (data not shown). This is in agreement with earlier results [3] indicating that both activities are referable to a single protein. Surprisingly, protein kinase activity was found to elute from the gel at NaCl and glycerol concentrations very close to those eluting lipase (fig.1,b). Protein kinase activity was resolved in two peaks, which might reflect the type I and II protein kinases described recently by Corbin et al. in adipose tissue [8]. The two peaks contained altogether about 50% of the amount of protein kinase activity assayed in the extract subjected to chromatography (28 000 units). Their differential cyclic-AMP dependence is under investigation. These findings show that enzymes other than lipase bind to and elute from the affinity gel. Whether this apparent parallelism is governed by the ligand dioleoylglycerol, the uncoupled hexanoic groups (40 to 80%), the agarose matrix itself or a combination of these factors remains to be established.

There is at present no easy way to explore by physical method the hormono-sensitive system which controls, through cyclic AMP-dependent protein kinase(s) [9,10], the rate of triglyceride hydrolysis in adipocytes. The present results show that by using the affinity gel diolein-agarose, it has been possible to visualize, within numerous technical limitations, the target enzyme triacylglycerol lipase as (or as a part of) a single electrophoretic band*. This technique should be amenable to the purification of this enzyme on a preparative scale.

Acknowledgements

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* The inactivation of triacylglycerol lipase by detergents precluded the use of sodium dodecylsulfate as dissociating agent during electrophoresis.

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