Purification of rat pancreatic lipase

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ABSTRACT A procedure for the isolation of lipase (glycerol-ester hydrolase, EC 3.1.1.3) from rat pancreas is described. The purification scheme includes homogenization of the pancreas, centrifugation at 3,000 rpm, centrifugation at 40,000 rpm, DEAE-cellulose chromatography, precipitation of amylase as the amylase–glycogen complex, gel filtration of the amylase-free proteins on Sephadex G-100, and chromatography on carboxymethyl-Sephadex C-50. The enzyme showed only one band on polyacrylamide gel electrophoresis and had a specific activity of 5330 ± 80 units/mg of protein.

SUPPLEMENTARY KEY WORDS amylase · DEAE-cellulose · Sephadex G-100 · CM-Sephadex C-50

During the past several years many investigations of factors influencing the biosynthesis of pancreatic enzymes in rats have been published. It has been demonstrated that the levels and rates of biosynthesis of amylase (1, 2) and chymotrypsinogen (1, 3) are altered by diet; more recently, the effect of actinomycin D on the biosynthesis of amylase, ribonuclease, and the proteolytic enzymes of rat pancreas has been examined (4). These studies have been possible because of the development of methods for the isolation of the pure enzymes in amounts sufficient for the determination of the specific radioactivity after administration of labeled amino acids to the intact rat. If similar studies are to be carried out on pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) in the rat, one must be able to isolate and purify the enzyme. This paper describes a method of doing so. The procedures are suited for the small number of rats that one might normally use in a study involving the extent of incorporation of a labeled amino acids into a purified protein.

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Abbreviations: DFP, diisopropyl fluorophosphate; V_o, void volume; V_e, elution volume.

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METHODS

Lipase activity was determined according to the method of Marchis-Mouren, Sarda, and Desnuelle (5). The volume of 0.1 N NaOH necessary to maintain the pH at 9.0 (37°C) after addition of the enzyme to an olive oil–gum arabic emulsion in the presence of bile salts was measured by means of a Radiometer Titrigraph (1).

Amylase was assayed by measurement of the reducing groups (with 3,5-dinitrosalicylic acid) formed as a result of the action of the enzyme on starch (1, 6).

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (7), with crystalline bovine serum albumin as reference standard. DNA was measured according to Schneider (8).

Enzyme Purification

The initial steps in the purification are essentially those used by Marchis-Mouren, Pasero, and Desnuelle (2) for the purification of rat pancreatic amylase.

Pancreata, obtained from five to eight rats, were homogenized (Teflon pestle) individually in 0.05 M or 0.1 M phosphate buffer, pH 8.2 (9 ml of buffer/g of pancreas), and 0.4 M diisopropyl fluorophosphate (DFP) was added to a final concentration of 10^{-3} M. The combined homogenates were centrifuged at 40,000 rpm (Spinco model L ultracentrifuge; the type 40 rotor was used throughout except where otherwise specified) for 45 min. In some preparations an initial centrifugation for 15 min at 3,000 or 15,000 rpm (Sorvall RC-2 centrifuge, SS-34 rotor) was carried out; the residue was homogenized again with an amount of buffer equal to 0.4 times the volume of the original homogenate, and recentrifuged. The combined supernatant fractions were then centrifuged at 40,000 rpm. It was found that prior

centrifugation at 3000 rpm (but not at 15,000 rpm) improved the yield.

The clear supernatant solution (S-1) obtained after centrifugation at 40,000 rpm was dialyzed against water for 2 hr and then dialyzed overnight against 0.005 M phosphate buffer, pH 8.2. These and all subsequent dialyses were carried out at 4°C. DFP was added to both the water and phosphate buffer to a final concentration of 10^{-4} M. There was little or no loss of lipase activity during the second dialysis. After adjustment of the pH to 8.2, the dialyzed solution of the enzyme was passed through a column of DEAE-cellulose (15 × 0.9 cm) previously equilibrated with 0.005 M phosphate buffer, pH 8.2. The breakthrough peak, which contained cationic proteins (e.g., amylase, lipase, ribonuclease, etc.), was collected (D-2).

The solution of cationic proteins was concentrated to about $^1/_4$ of its volume by pressure dialysis against 0.02 M phosphate buffer containing 0.006 M NaCl, pH 6.9. After an estimation of the amylase concentration, glycogen was added (2.5 mg/mg of amylase) at 0°C to form the amylase–glycogen complex (9). This complex was precipitated by addition of ethanol (at 0°C) to a concentration of 12%, and removed by centrifugation.

The amylase-free protein solution (A-3) was dialyzed for 4 hr or longer against 0.005 M Tris–HCl buffer, pH 7.0, to remove the ethanol. The proteins were concentrated to a volume of about 1 ml by pressure dialysis against the same buffer. The concentrated protein solution was put on a 54 × 2 cm column of Sephadex G-100 (cross-linked dextran, Pharmacia, Uppsala, Sweden) previously equilibrated and subsequently eluted with the above buffer. Lipase activity appeared after about 1.57 column volumes (V_e/V_o) (50–55 ml). Similar results were obtained with 0.005 M phosphate buffer, pH 8.0, but the Tris–HCl buffer was used in most preparations. The three or four fractions containing most of the enzymatic activity were combined (G-4) and concentrated by pressure dialysis against 0.04 M phosphate buffer, pH 6.1, to a volume of about 0.5 ml. This solution was applied to a 15 × 0.9 cm column of the anion exchanger CM-Sephadex C-50 (carboxymethyl-Sephadex, Pharmacia) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of phosphate buffer (0.04 to 0.4 M), pH 6.1, with 80 ml in each reservoir. Lipase (CM-5) appeared when the conductivity of the eluate reached 3.46–3.58 millimhos (4°C). This position on the chromatogram corresponded to about 5.2 column volumes (about 56 ml). (The conductivity of 0.04 M phosphate buffer, pH 6.1, was 1.70 millimhos at 4°C.)

Concentration of Enzyme Solutions

In the studies reported in this paper, the protein solutions were concentrated by pressure dialysis, i.e. ultrafiltration under suction, through a collodion membrane immersed in an appropriate buffer. In more recent studies, high pressure ultrafiltration through Diaflo membranes (Amicon Corp., Lexington, Mass.) has been used to concentrate the protein solutions. The rapidity of this method enables one to carry out the entire isolation procedure in 3–4 days.

RESULTS AND DISCUSSION

Table 1 summarizes the yields of activity at each step and the specific activities of the enzyme. The values for the complete purification were obtained from four to seven different preparations. The wide range of specific activities of the first two fractions (homogenate and S-1) is due to the fact that we used rats on three different diets. The low yields of activity in S-1 and the relatively poor degree of purification in the first step appear to be due to an interaction of lipase with insoluble protein. The poor recovery could not be attributed to denaturation of the enzyme since it was shown in a separate study that nearly all the activity of the homogenate was distributed between the clear supernatant solution and the residue after centrifugation. Another series of studies showed that most of the "insoluble lipase" was sedimented after 1 hr of centrifugation at 15,000 rpm and was thus associated with unbroken cells, cellular debris, etc. Both reextraction of the residues (after centrifugation at 15,000 or 40,000 rpm) with buffer, with or without

TABLE 1 PURIFICATION OF RAT PANCREATIC LIPASE

Fraction	Designation	Activity Recovered*	Mean Specific Activity†
		%	
Homogenate		100	126‡ (80–182)
40,000 rpm Supernate	S-1	59 (48–73)	166 (86–370)
DEAE eluate	D-2	93	326
Amylase supernate	A-3		1010
G-100 eluate	G-4	52	3280
CM-Sephadex eluate	CM-5	60	5330
Homogenate§		100	
3000 rpm combined supernate		86 (76–100)	
40,000 rpm supernate		81 (68–100)	
Over-all yield		70 (59–83)	

* Refers to average activity recovered relative to previous step.

† Units of lipase/mg of protein. 1 unit is the amount of enzyme that catalyzes the liberation of 1 μeq of fatty acid/min.

‡ The units of enzyme per μg of DNA phosphorus ranged from 35 to 125. For comparison, the specific activity of lipase in homogenates prepared from pancreata of rats fed a normal pellet diet was 102 ± 6 units/mg of protein and 46 ± 2 units/μg of DNA phosphorus.

§ These data are the means of results from six experiments (seven or eight rats per experiment) in which the purification was carried through the ultracentrifugation step.

sonication, and treatment of an aqueous suspension of the residue with ether and ethanol at −12°C and subsequent reextraction of the protein with buffer failed to improve the yield appreciably. The procedure, including ether–ethanol extraction, was carried out to determine whether additional enzyme could be extracted after the removal of lipid from the residues. Furthermore, it was found that when the pancreata were homogenized more vigorously (Sorvall Omni-Mixer) the yield was only 28% from homogenate to the 40,000 rpm supernatant solution (as compared to 59% with Teflon pestle homogenization) although an additional 20% of enzyme could be recovered by lipid extraction, etc., as outlined above.

The second section of Table 1 demonstrates the increased yield of lipase that one obtains by carrying out a preliminary centrifugation of the homogenate at 3000 rpm, as outlined previously.

Fig. 1 shows a Sephadex G-100 chromatogram. The identity of the second large peak was not determined; had amylase not been removed prior to the Sephadex G-100 chromatography, it would have appeared after the second peak. Recent studies have shown that amylase is eluted after about 3 column volumes. In the sample shown in Fig. 1 the lipase in the two peak tubes had a specific activity of 5600 units/mg of protein; in most other preparations, the specific activities of the enzyme in peak tubes ranged from 2700 to 4140.

In some preparations the lipase peak was slightly colored (pink). Measurement of optical density at 415 mμ revealed the presence of a material (V_e/V_o = 1.46) which was always eluted before the lipase (V_e/V_o = 1.52). This absorption at 415 mμ might have been due to cytochromes extracted from the pancreas. Chromatography on CM-Sephadex served to remove the contaminating protein, and in all preparations so treated, the lipase eluted from CM-Sephadex contained no material that absorbed at 415 mμ.

Fig. 2 shows curves of absorption at 280 mμ, total lipase activity, and total protein after CM-Sephadex C-50 chromatography of the protein fractions from the Sephadex G-100 column (Fig. 1) that contained most of the lipase activity. The three curves are virtually superimposable. The amount of protein recovered after CM-Sephadex chromatography was sufficient for the subsequent enzymatic, chemical, electrophoretic, and radioactive assays.

In many of the experiments valine-U-^{14}C was administered intravenously to the rats 10 min before they were killed. Table 2 shows the specific enzyme activity and specific radioactivity of samples of protein obtained from four different preparations of lipase. In each case only the samples with maximal lipase activity (usually two to four tubes) were used for analysis, e.g. samples

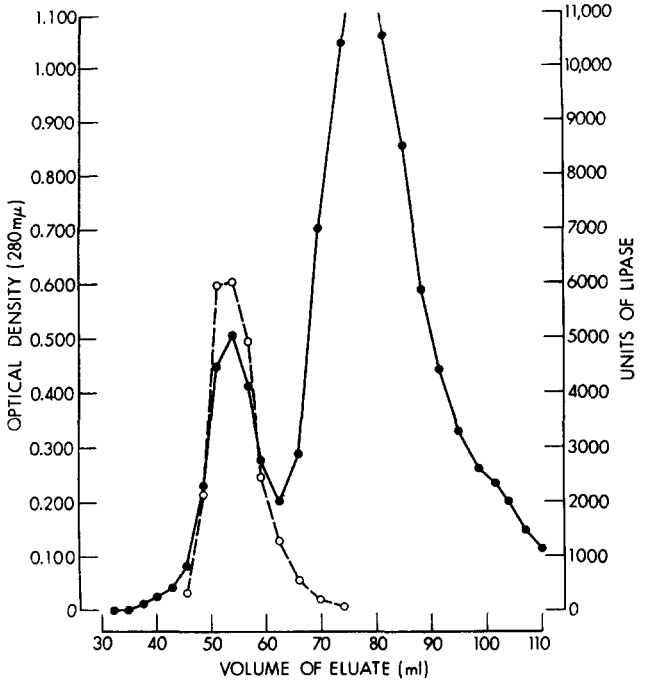

FIG. 1. Chromatography of fraction A-3 (Table 1) on Sephadex G-100. ●——●, optical density at 280 mμ; O- - -O, total units of lipase in each fraction.

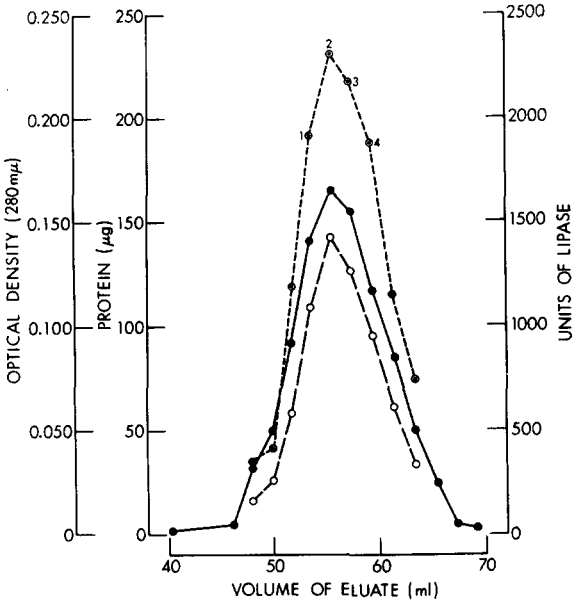

FIG. 2. Chromatography of the two peak fractions of S-4 (fractions at 51.2 and 53.9 ml, Fig. 1) on CM-Sephadex C-50. ●——●, optical density at 280 mμ; O- - -O, total units of lipase in each fraction; ⊙···⊙, total protein in each fraction. The numbers 1–4 on this last curve refer to data in Expt. P_7 of Table 2.

1–4 of Fig. 2. The constancy of the specific enzymatic activity for all the samples analyzed and the constancy of the specific radioactivity of samples of each preparation, as well as the curves in Fig. 2, suggest that the enzyme is pure unless a contaminating protein is present

TABLE 2 SPECIFIC ENZYMATIC ACTIVITY AND SPECIFIC RADIOACTIVITY* OF PURIFIED RAT PANCREATIC LIPASE

Expt.	Sample	Enzymatic Activity	Radioactivity†
		units/mg protein	*cpm/mg protein*
P_7	1	5680	678
	2	5350	646
	3	5770	748
	4	5030	649
A_4	1	5040	2270
	2	5720	2015
	3	5260	2130
P_2	4	5390	785
	5	5150	739
	6	5430	976
A_0	8	5175	3040
	9	4940	2880

* Valine-U-^{14}C was injected into rats 10 min before they were killed.

† 0.4 ml of enzyme solution was counted in 10 ml of Bray's solution.

whose chromatographic characteristics on the three columns used are identical with those of lipase.

After CM-Sephadex chromatography the enzyme had a mean specific activity of 5330 units/mg of protein, which represents approximately a 40- to 50-fold purification. On the basis of this value, it can be estimated that lipase comprises 1–2% of the total pancreatic proteins. By way of comparison, Sarda, Maylié, Roger, and Desnuelle (10) found the specific activity of hog pancreatic lipase to be 7000 units/mg. The molecular weight of rat lipase was kindly determined by Dr. L. Sarda by means of gel filtration on a column of Sephadex G-100 (10); it was about 32,000 as compared to 38,000 for the hog pancreatic lipase (10).

Fig. 3 shows the results of polyacrylamide gel electrophoresis at pH 5.0 of five samples of lipase obtained after CM-Sephadex C-50 chromatography. Although there were two colored bands on each gel, only the upper one (blue) represented a protein component. The lower, faster-moving band at the front (blue-green to gray) was an artifact possibly derived from interaction of the bromophenol blue (added to the sample gel with the protein in order to mark the extent of electrophoretic migration) with the Aniline blue-black used to stain the protein. That it contained no radioactivity was established by slicing and counting the gel according to the procedure of Maizel (11); only the single protein band was radioactive.

Recently, Vandermeers and Christophe (12) have reported the isolation of rat pancreatic lipase by chromatographic techniques similar to those in the present study. Their lipase had a specific enzymatic activity of about 4500 units/mg at 37°C (2500 at 25°C) and a molecular weight of 43,000 (37,000 after extraction of

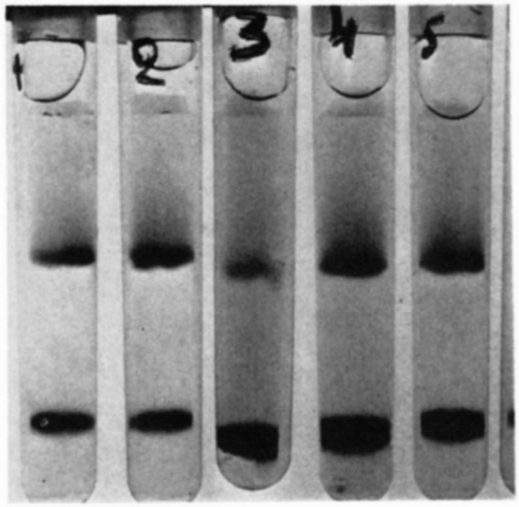

FIG. 3. Electrophoresis of the purified samples in 7.5% acrylamide gel at pH 5.0. Tubes 1–5 correspond to the following samples in Table 2: tube 1, P_2 4, 5, and 6; tube 2, P_2 7 and 8 (not in Table 2); tube 3, P_7 2 and 3; tube 4, P_7 1 and 4; tube 5, A_4 1, 2, and 3. These analyses were kindly performed by Mr. Maurice Charles of the Institut de Chimie Biologique.

about 14% of lipid from the purified enzyme). These values are to be compared to our specific enzymatic activity of 5330 units/mg at 37°C (Table 2) and a molecular weight of about 32,000. No effort was made to determine whether lipid was present in our purified lipase, but the low molecular weight suggests that it was not. This molecular weight was estimated on a sample of lipase after removal of amylase by glycogen precipitation. It is possible that any lipid originally bound to the impure lipase might have been removed during treatment of the cationic protein solution with ethanol to precipitate the amylase–glycogen complex.

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