Partial purification of monoglyceride lipase from adipose tissue

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ABSTRACT A monoglyceride lipase was partly purified from extracts of rat adipose tissue by ammonium sulfate fractionation, alcohol precipitation, and lyophilization, or by ammonium sulfate fractionation, sodium deoxycholate treatment, and a second ammonium sulfate fractionation.

Partial purification and heat denaturation showed the lipase to be different from tributyrinase and from an enzyme(s) which hydrolyzes diglycerides and triglycerides. Although the best preparations hydrolyzed monobutyrin this activity decreased with purification, indicating that the enzyme acts on insoluble substrates and is therefore a lipase and not an esterase. Furthermore, classification of the enzyme as a lipase is consistent also with its behavior with inhibitors, since low concentrations of esterase inhibitors, e.g., fluoride, sodium deoxycholate, and physostigmine did not inhibit lipolytic activity. Inhibition studies with EDTA, sodium pyrophosphate, protamine, and fluoride showed that the enzyme differs from clearing factor lipase. The enzyme catalyzed hydrolysis of monostearin in the pH range 6.3–9.0, with a maximum at 7.4–7.6.

KEY WORDS monoglyceride lipase · rat · adipose tissue · partial purification · esterase activity

Lipolytic activity in adipose tissue has been studied in several laboratories. Lynn and Perryman (1) reported a 900-fold purification of a lipase from pig adipose tissue which hydrolyzed mono-, di-, and tributyrin as well as several naphthyl esters and esters of monohydric alcohols. Rat epididymal fat pads have been shown to contain a monoglyceride (2) and triglyceride lipase (2, 3), a clearing factor lipase (4, 5), an epinephrine-sensitive lipase activity (6, 7), and a Tween (polyoxyethylene sorbitan monoester) hydrolyzing activity (8). It is difficult to compare the results in these different reports, mainly because of variability in experimental design and in types of substrates used. However, it seems quite likely that these activities are attributable to at least three different lipases: clearing factor lipase, triglyceride lipase, and monoglyceride lipase.

This report is concerned with the partial purification and characterization of a monoglyceride lipase from rat epididymal adipose tissue.

MATERIALS AND METHODS

Monoolein, monostearin (Nutritional Biochemicals Corp., Cleveland, Ohio), monomyristin, monolaurin (C. P. Hall Co., Chicago, Ill.), and trilaurin (Calbiochem, Los Angeles, Calif.) were recrystallized from 95% alcohol before use. Thin-layer chromatography showed them to contain only traces of impurities. Tributyrin (Eastman Kodak, Rochester, N. Y.) and triolein (Nutritional Biochemicals Corp.) were extracted twice with a solution of $NaHCO_3$ to remove free fatty acids (FFA). Samples of >99% purity of monopalmitin, monolinolein, distearin, and tristearin were purchased from the Hormel Institute, Austin, Minn.

Bovine albumin, Fraction V, was obtained from Nutritional Biochemicals Corp. and extracted as described by Dole (9) or was purchased "free from fatty acids" from Gallard Schlesinger Chemical Mfg. Corp., Carle Place, N. Y., and used without further treatment.

Epididymal fat pads were obtained from 250–350 g Sprague-Dawley rats which had been fed ad lib. and killed by decapitation.

Suspensions of substrates were prepared by homogenizing a suitable amount of mono-, di-, or tristearin in 12.5 ml of water in a Virtis-45 homogenizer at full speed for 3 min. The suspensions were then diluted with an equal volume of a solution containing albumin and buffer and were homogenized for 1 min. The final monostearin substrate contained 0.04 M Tris, pH 7.9, 6% albumin, and 16 mg/ml monostearin. Distearin and tristearin substrates contained 0.02 M sodium phosphate pH 7.4, 6% albumin, and 20 or 30 mg/ml diglyceride or triglyceride respectively. All other monoglycerides and esters of monohydric alcohols were prepared by the

Abbreviation: FFA, free fatty acid.

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monostearin procedure with appropriate changes for the difference in molecular weight.

Preparation of Enzyme

Two methods gave about the same extent of purification. The procedures differed only after Step II. All steps were carried out at 0–5°C unless otherwise stated.

Step I: Extract. Thirty rats were killed by decapitation, the epididymal fat pads removed, chilled in ice, and subsequently rinsed with water and homogenized with 200 ml of water for 3 min in a Waring Blendor. The homogenate was centrifuged at 35,000 × *g* for 15 min and the floating fat layer and sediment were homogenized again with 150 ml of water. The combined supernatant fractions, designated "extract" in Table 1, were used for further purification.

TABLE 1 Procedure for Partial Purification of Monoglyceride Lipase with Monostearin as Substrate

	Preparation	Protein	Specific Activity*	Recovery
		mg	*units/mg*	*%*
I.	Extract	2490	2.0	100
II.	$(NH_4)_2SO_4$ Fraction I	640	4.3	43
III.	Ethanol fraction	330	4.7	31
IV.	Lyophilized precipitate	144	7.5	21
I.	Extract	1890	2.5	100
II.	$(NH_4)_2SO_4$ Fraction I	640	6.3	85
III-A.	$(NH_4)_2SO_4$ Fraction II	39	12.1	10

Each tube contained 0.25 ml of monostearin substrate and enzyme in a final volume of 0.50 ml. The incubation time was 20 min in a 12-ml centrifuge tube.

* Defined as micromoles of stearic acid liberated per milligram of protein per hour.

Step II: Ammonium Sulfate Fraction I. To 315 ml of the combined extract, 36 g of solid $(NH_4)_2SO_4$ was added (0.20 saturation) during 15 min with mechanical stirring. Stirring was continued for 20 min, then the solution was centrifuged at 35,000 × *g* for 15 min. The sediment was discarded and the supernatant fluid was brought to 0.45 saturation by adding 49 g of $(NH_4)_2SO_4$ as described above. After centrifugation the precipitate was dissolved in water to give a solution with a final volume of 50 ml. This is designated "$(NH_4)_2SO_4$ Fraction I" in Table 1.

Step III: Alcohol Precipitate. The solution from Step II was made 0.002 M with respect to $MgCl_2$ and 4.2 ml of alcohol was added with stirring at 0 to −3°C during 10 min. After 15 min of additional stirring, the solution was centrifuged at 35,000 × *g*, the precipitate was discarded, and the supernatant solution was raised to an alcohol concentration of 20% by adding 7.7 ml of alcohol as before. After centrifugation the precipitate was dissolved in 14 ml of 0.05 M phosphate buffer, pH 7.4. This is "ethanol fraction" in Table 1.

Step IV: Lyophilized Precipitate. Thirteen ml of the ethanol fraction was lyophilized and the powder was taken up in 13 ml of water and centrifuged at 35,000 × *g* for 15 min. Most of the activity remained in the precipitate which was suspended in 0.05 M phosphate, pH 7.4, in a Potter-Elvehjem all-glass homogenizer.

Step III-A: Ammonium Sulfate Precipitate II. A sufficient volume of a 5% sodium deoxycholate solution was added to ammonium sulfate Fraction I to give a concentration of 0.2 mg sodium deoxycholate per mg protein. After 10 min the volume was adjusted with 0.05 M sodium phosphate, pH 7.4, to give a protein concentration of 10 mg/ml. Solid ammonium sulfate was added with stirring during 15 min to bring the concentration to 0.10 saturation. After additional stirring for 20 min the solution was centrifuged at 35,000 × *g* for 15 min, the sediment was discarded, and the supernatant solution was raised to 0.20 saturation with ammonium sulfate as described above. The resulting precipitate was dissolved in 0.05 M sodium phosphate, pH 7.4.

In other experiments, an enzyme extract was prepared as described by Rizack (6). In this case epididymal pads were removed from anesthetized rats and homogenized in a Potter-Elvehjem homogenizer in 3 volumes of 0.25 M sucrose. The homogenate was centrifuged at 12,000 × *g* for 10 min and the supernatant layer, without the fat, was used.

Lipase Assays

Incubations were carried out in 12-ml centrifuge tubes at 37° for 20 min. The rate of FFA production was constant for at least 40 min. The incubation mixtures contained enzyme and 0.25 ml of substrate mixture (described above) in a final volume of 0.50 ml. The reaction was stopped by adding 0.02 ml of 10 N sulfuric acid when FFA were measured by titration, or by adding 1.0 ml of the copper reagent when they were measured colorimetrically.

Three different methods were used for measuring FFA. In method I acids were measured as copper salts by the method of Duncombe (10) as modified by Vaughan, Berger, and Steinberg (2). Method II was that described by Dole (9). Method III is a modified Dole procedure developed in these laboratories by Dr. H. Ko and M. E. Royer (personal communication).

RESULTS

Purification of Enzyme

As shown in Fig. 1, the rate of FFA production was proportional to the concentration of enzyme when mono-

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TABLE 2 Temperature Sensitivity of Lipolysis of Monostearin, Triolein, and Tributyrin

Substrate	Specific Activity Control	% of Control Activity			Ratio 50°/60°C
		50°C	56°C	62°C	
	µmoles FFA/mg protein/hr				
Monostearin	4.44	102(96–110)*	57(40–75)	13(11–14)	7.8
Triolein	0.51	88(83–93)	53(46–59)	11(8–14)	8.0
Tributyrin	4.77	80(70–89)	74(57–85)	66(57–74)	1.2

Samples (5 ml) of extract were placed in 12-ml glass centrifuge tubes and stirred with a thermometer. Samples were held at the indicated temperature for 5 min. Assays were conducted as described in the text with 0.40 mg of protein for monostearin and tributyrin and 2.0 mg for triolein.

* Mean and range of 3 assays.

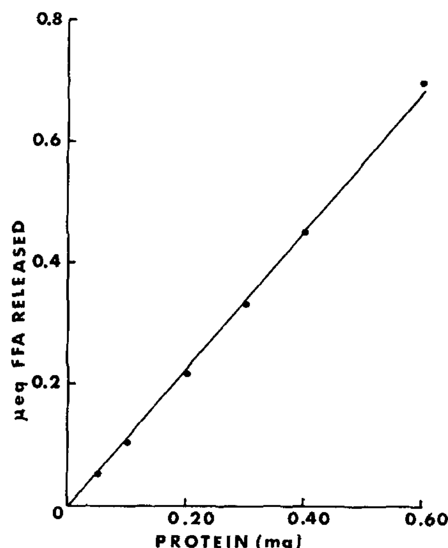

Fig. 1. FFA production as a function of enzyme concentration with monostearin as substrate. Incubated for 20 min with indicated amount of ammonium sulfate Fraction I.

stearin was used as the substrate. Table 1 shows the procedures which gave optimum purification. The lipase was precipitated from an aqueous extract with ammonium sulfate between 0.20 and 0.45 of saturation. This was followed by precipitation between 8 and 20% ethanol and finally by lyophilization of a phosphate buffer solution of the alcohol precipitate. Most of the activity remained in that part of the lyophilized powder which was insoluble in phosphate buffer. No further useful purification was obtained from the insoluble preparation. In the alternative method ammonium sulfate Fraction I was treated with sodium deoxycholate and fractionated again with ammonium sulfate. The enzyme was obtained in low yield and proved to be quite unstable at this point. More than half of the activity was lost on storage in the frozen state at −20°C for a week. Both methods produce large losses of enzyme due to inactivation, since the enzyme is not present in other fractions. Other procedures gave at least a twofold purification from the extract. These were precipitation at pH 5.6, Sephadex G-100 gel filtration, and alcohol precipitation. However, no useful subsequent purification was obtained by any procedure following these steps.

Substrate Specificity

Evidence indicating that at least two different lipases are present is provided by the data in Table 2. When the enzyme extract was heated for 5 min at the indicated temperatures, the tributyrinase activity proved to be less labile than the others. At 62°C only 34% of the tributyrinase activity was lost whereas almost 90% of the monostearin and triolein lipase activity was destroyed. From the results obtained at the three temperatures with the long-chain substrates, it is not possible to say whether the low triglyceride lipase activity is due to the nonspecificity of the monoglyceride lipase or to contamination by a triglyceride lipase with almost identical heat sensitivity.

The results in Table 3 show that monoglycerides and long-chain triglycerides are hydrolyzed by different enzymes, since lipolytic activity with the two monoglycerides, monoolein and monostearin, in contrast to the activity with the triglycerides, is increased to the same extent during the purification procedure. However, the activity toward the triglycerides is either unchanged during purification (tristearin and triolein) or reduced (tributyrin). It thus appears that at least three different enzymes are involved: a lipase for long-chain monoglyceride, a lipase for long-chain triglyceride, and an enzyme which cleaves tributyrin.

TABLE 3 Substrate Specificity of Enzyme Fractions

Substrate	Extract	20–45% $(NH_4)_2SO_4$	8–20% Alcohol	Lyophilized Precipitate
		µmoles fatty acid liberated/hr		
Monostearin	2.25	5.04	4.44	6.21
Distearin	0.15	0.27	—	0.24
Tristearin	0.24	0.18	0.27	0.24
Monoolein	3.15	7.38	6.69	11.25
Triolein	0.57	0.66	0.78	0.60
Tributyrin	12.87	2.76	0.75	1.38

Each tube contained 0.25 ml of substrate and enzyme in a final volume of 0.50 ml. Incubation time was 20 min.

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TABLE 4 Relative Rates of Hydrolysis of Various Substrates*

Substrate	Relative Hydrolysis Rate†	Substrate	Relative Hydrolysis Rate†
Monoglycerides		*Triglycerides*	
Monobutyrin	2.51	Tristearin	0.19
Monolaurin	0.96	Triolein	0.10
Monomyristin	0.95		
Monopalmitin	0.88	*Miscellaneous*	
Monostearin	1.00		
Monoolein	1.80	Methyl butyrate	0.25
Monolinolein	0.76	Methyl stearate	0.08
		Cholesteryl acetate	2.53
Diglycerides		Cholesteryl stearate	0.03
		Lecithin	0.00
Distearin	0.17		

* The enzyme preparation was ammonium sulfate fraction II.
† The rates of hydrolysis are relative to monostearin taken as unity.

A comparison of action upon several monoglycerides, triglycerides, diglycerides, and miscellaneous substrates is made in Table 4. The results show that there is little difference in activity toward monoglycerides of different chain length, except for monobutyrin. In experiments not shown, the activity with monobutyrin decreased significantly during purification, indicating that this substrate might be being hydrolyzed by a tributyrinase.

A final comparison of substrates was made using an enzyme preparation prepared as described by Rizack (6) (see Methods). This enzyme is reported to be sensitive to epinephrine when Ediol is used as the substrate (6). Ediol is a commercial emulsion of 50% coconut oil containing 1.5% added monostearin. The activity toward this substrate was compared with activity toward monostearin, tristearin, and trilaurin. The results in Table 5 show that the enzyme liberates as much FFA from monostearin as from Ediol, while it shows much less activity with the pure triglycerides. It is particularly noteworthy that trilaurin, which is the main component of coconut oil, is hydrolyzed very poorly.

Effect of Inhibitors

The effects of various inhibitors on monoglyceride lipase

TABLE 5 Comparison of Lipolytic Activity of Adipose Tissue Extract on Ediol and Pure Substrates

Substrate	Activity
	μeq/hr/100 mg tissue
Ediol (3.1 mg)	7.92
Ediol (6.2 mg)	9.84
Monostearin	10.80
Tristearin	2.16
Trilaurin	0.48

The assay was conducted according to the method of Rizack (6) as described in the text.

are listed in Table 6. Sodium chloride, an effective inhibitor of clearing factor lipase (11) but not of the epinephrine-sensitive lipase (6), inhibits strongly. EDTA, sodium pyrophosphate (12), and protamine (11) inhibit clearing factor lipase but were without effect on monoglyceride lipase in the present study. Conversely, 0.20 M sodium fluoride inhibits monoglyceride lipase but not clearing factor lipase (3, 11). Both 0.20 M sodium fluoride and sodium pyrophosphate inhibit the epinephrine-sensitive lipase (6). Sodium fluoride (10^{-3} M) completely inhibits esterases (13) but was not an inhibitor in the present study. Physostigmine, an inhibitor of choline esterase (14), and sodium deoxycholate, also an esterase inhibitor (15), had no effect on the monoglyceride lipase. Three sulfhydryl group inhibitors, *p*-chloromercuribenzoate, iodoacetate, and *N*-ethyl maleimide, were strong inhibitors of lipolysis at low concentrations. Epinephrine, prostaglandin E_1, and heparin at 5, 1, and 20 μg/ml respectively had no effect on monostearin lipolysis (unpublished experiments).

pH Optimum

A typical curve showing the effect of pH on lipolytic activity is presented in Fig. 2. The optimal pH lies be-

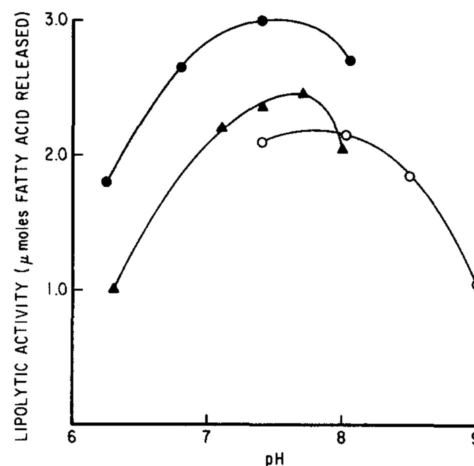

Fig. 2. Effect of pH on lipolytic activity. ●, phosphate buffer with monostearin; ○, Tris buffer with monostearin; △, phosphate buffer with Ediol.

tween 7.4 and 7.6 and appears to be similar in phosphate and Tris buffers. The latter buffer, however, gives a lower rate of lipolysis at the two points where both were tested. The third curve shows that lipolysis with Ediol as substrate has about the same optimum as lipolysis with monostearin.

Enzyme Stability

All enzyme preparations except ammonium sulfate Fraction II retained full activity when stored overnight

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TABLE 6 INHIBITION OF MONOSTEARIN LIPOLYTIC ACTIVITY BY VARIOUS INHIBITORS

Inhibitor	Concentration	% Inhibition
NaCl	0.50 M	46
	0.25 M	31
NaF	0.20 M	45
	0.05 M	8
	0.001 M	0
EDTA	5×10^{-3} M	0
	5×10^{-4} M	0
Protamine sulfate	300 μg/ml	0
	150 μg/ml	0
Na pyrophosphate	1.6×10^{-2} M	0
	0.8×10^{-3} M	0
Na deoxycholate	1.0 mg/ml	0
Physostigmine	10^{-4} M	0
	10^{-5} M	0
Diisopropyl phosphofluoridate	10^{-5} M	39
	10^{-6} M	26
Na *p*-chloromercuribenzoate	10^{-4} M	79
	10^{-5} M	15
Iodoacetic acid	10^{-2} M	23
	10^{-3} M	0
N-Ethyl maleimide	10^{-4} M	33
	10^{-5} M	0
Na azide	10^{-2} M	0
	10^{-3} M	0

Each tube contained 0.25 ml of monostearin substrate, inhibitor as indicated, and enzyme (0.30 mg ammonium sulfate Fraction I) in a final volume of 0.50 ml.

at 0°C or for a week at −20°C. Ammonium sulfate Fraction II lost more than half of its activity on overnight storage at 0°C, but retained almost full activity when stored for a week at −20°C. Mercaptoethanol (0.01 M), 20 μg/ml epinephrine, or 0.014 M glycerol did not affect stability of this fraction at 0°C.

DISCUSSION

The purification reported here demonstrates that the enzyme differs from tributyrinase and triglyceride lipase. The relative stability to heat of the activity toward tributyrin further serves to differentiate that enzyme from monoglyceride and triglyceride lipases. It appears possible that tributyrin is acted upon by two different enzymes, since about 20% of the activity is lost consistently when the enzyme is heated at 50°C, but very little of the remaining activity is destroyed by heat at 56°C and 62°C. That the monoglyceride lipase is different from the triglyceride lipase is shown by partial purification; the most purified fraction was not different from the first extract in activity toward long-chain triglycerides. The loss of tributyrinase activity on purification agrees with the behavior toward heating and shows that this is a different enzyme. The similarity of diglyceride and triglyceride lipolytic activities is demonstrated by the fact that each fraction shows about the same lipolytic activity toward these substrates, and the activity does not increase when the monoglyceride lipase is purified. This similarity agrees with the results of Gorin and Shafrir (3) who found that fluoride strongly inhibited activity toward diolein and tripalmitin but not toward monoolein. They also found that the activity toward di- and triglycerides, but not toward monoglycerides, was lower upon storage. Addition of epinephrine prevented this lowering of activity.

Difference in experimental design of other investigations adds to the difficulty of making comparisons with adipose tissue lipases described previously. However, in some cases comments on other lipases are justified. The results with monoglyceride lipase confirm and extend the work of Vaughan et al. (2), who have shown that monoglycerides are hydrolyzed by whole homogenates and by aqueous acetone powder extracts of rat adipose tissue. We have also confirmed their finding that this hydrolysis is not affected by epinephrine as is the hydrolysis of endogenous triglycerides. All attempts to demonstrate an epinephrine effect on the hydrolysis of monoglycerides, triglycerides, and Ediol by extracts prepared according to Rizack (6) and Björntorp and Furman (7) have been unsuccessful. This failure may be related to differences in animal preparations as regards age, anesthesia, and ganglionic blocking. Furthermore, extracts prepared in this way released FFA from Ediol and monostearin at a much faster rate than from tristearin and trilaurin. Since trilaurin is the main component of coconut oil and is hydrolyzed so slowly, it appears that when the hydrolysis of Ediol is used to follow lipolytic activity, the results can be misleading; the added monostearin which serves as an emulsifier in Ediol can account for a large part of the FFA released by this substrate. Further evidence indicating that this may be the case was furnished by Vaughan et al. (2) and Hollett (16), who have shown that most of the triglyceride lipase activity of the homogenate is associated with the supernatant fat layer and not with the extract after centrifugation.

The behavior of the monoglyceride lipase towards various inhibitors shows that it differs from clearing factor lipase and is not an esterase but a lipase.

The role of monoglyceride lipase in adipose tissue metabolism is at present unknown. As pointed out by Vaughan et al. (2), it is unlikely to be rate-limiting in lipid mobilization. However, it is conceivable that this may not be so in different physiological and pathological states.

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