PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 683–687 (1975)

Studies on Catalytic Properties of Purified High Molecular Weight Pancreatic Lipase[1] (38879)

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The molecular weight of lipase prepared by using acetone or other organic solvents is less than 3×10^4 (1, 2). By using phosphate buffer instead of organic solvents we isolated a high-molecular-weight (3×10^6) lipase from rat and porcine pancreas (3). Our enzyme preparation differed from those in the literature in that its activity was inhibited by phenoxybenzamine (4) and activated by the addition of cAMP, Ca^{2+}, and protein kinase (5). Okuda and Fujii (6) have shown that the substrate specificity of rat liver and epididymal adipose tissue lipase was totally altered when the enzyme was treated with acetone, in the sense that the lipase was converted into an esterase. The chromatographic properties were also altered by acetone treatment. In an effort to understand the basis of the difference between the high- and low-molecular-weight enzymes, we purified the high-molecular-weight enzyme 500-fold and wish to report here its physical and catalytic properties.

Experimental Procedure. Materials. Rat pancreatic tissue was obtained from male albino rats of the Wistar strain (120–150 g). Sodium deoxycholate, triglycerides, diglycerides, monoclycerides, chromatographic lipid standard, bovine serum albumin (Fraction V), gum arabic, and Tween 20 were obtained from Sigma Chemical Company, St. Louis, MO. Methyl esters were obtained from Nutritional Biochemical Corporation. Metal salts were of analytic grade. All lipid substrates had a purity greater than 99% by thin-layer chromatography.

Methods. Isolation of the high-molecular weight pancreatic lipase. Rat pancreas was homogenized in ice-cold 0.01 *M* potassium phosphate buffer, pH 7.2, containing 0.1% sodium deoxycholate (10 ml buffer/g tissue) with the use of a VirTis 23 homogenizer at full speed for 1 min. The homogenate was filtered through a few layers of cheesecloth, and the filtrate was centrifuged at 105,000*g* for 1 hr at 4° in a Spinco Model L preparative ultracentrifuge. The supernatant obtained was freeze-dried immediately. The freeze-dried crude extract was purified by successive filtration through Sephadex G-50, G-200 and Sepharose 4B gel columns as described previously (3).

Assay of lipase. Lipase activity was assayed in 3.0 ml of assay medium containing 5 m*M* Tris, 5 m*M* potassium phosphate, pH 7.2, 1% (w/v) bovine serum albumin (Fraction V), and 30 μmoles substrate. The substrates were suspended by sonication with a Sonifier Cell Disrupter, Model W 185 D (Heat Systems-Ultrasonics, Inc.), at 70 W for 1 min. Enzyme, at a concentration of approximately 5–10 μg was suspended in 0.1 ml water and added to the assay medium. The incubation was conducted at 40° for 1 hr. The reaction was stopped by the addition of 6.0 ml of isopropanol–heptane-*N*-sulfuric acid (80:20:2, v/v/v). The rate of hydrolysis was determined by titrating the liberated free fatty acids (FFA) by the Dole method (7). The results are expressed as μmoles free fatty acid released $\times$ mg protein^{-1} $\times$ hr^{-1}. Protein concentration was determined by the method of Lowry *et al.* (8).

Identification of products of lipolysis. Tributyrin, tricaprin, tripalmitin, and triolein were individually subjected to hydrolysis by rat pancreatic lipase as described above. At

[1] This investigation was supported by USPHS Grant 04790.

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TABLE I. SUBSTRATE SPECIFICITY OF RAT PANCREATIC LIPASE[a]

Substrare	Fatty acyl chain lenght	No. of double bonds	μmoles FFA released/mg protein/hr
Triglycerides			
Triacetin	2	—	0 ± 0.03
Tributyrin	4	—	78.4 ± 0.8
Tricaprylin	8	—	77.0 ± 1.0
Tricaprin	10	—	60.3 ± 0.6
Trilaurin	12	—	44.8 ± 0.7
Trimyristin	14	—	15.6 ± 0.4
Tripalmitin	16	—	10.2 ± 0.5
Tristearin	18	—	1.4 ± 0.02
Triolein	18	1	37.4 ± 0.8
Trilinolein	18	2	51.6 ± 1.4
Trilinolenin	18	3	75.6 ± 1.4
Partial glycerides			
Dipalmitin	16	—	29.3 ± 0.8
Monopalmitin	16	—	0.0 ± 0.04
Diolein	18	—	69.5 ± 1.7
Monoolein	18	1	0 ± 0.02
Methyl esters			
Caproic-Me[b]	6	—	35.6 ± 0.8
Caprylic-Me	8	—	59.2 ± 1.2
Capric-Me	10	—	75.4 ± 1.7
Lauric-Me	12	—	67.0 ± 2.1
Palmitic-Me	16	—	69.5 ± 1.9
Oleate-Me	18	1	70.4 ± 1.8
Phospholipid			
Lecithin			0 ± 0.07
Cephalin			0 ± 0.06

[a] Reaction mixture contained approximately 10 μg of purified lipase protein with 30 μmoles of each of the substrates in phosphate buffer containing 1% albumin.

[b] -Me = methyl ester.

the end of the incubation period 6 ml of Dole–acid mixture were added to the incubation medium. The triglycerides and FFA, which were extracted into the heptane phase, were identified by thin-layer chromatography on Silica gel G (Merck) using the solvent system petroleum ether (30–60°), diethylether and acetic acid (84:15:1). 2′,7′-Dichlorofluoresceine in isopropanol (0.1%) was used to develop the plates and viewed under ultraviolet light.

Results and Discussion. Effect of fatty acid chain length. Rates of hydrolysis of triglycerides of various fatty acyl chain length are given in Table I. The enzyme was not active against triacetin which is soluble in the assay medium at the given concentration and by definition, lipase is active on water-insoluble emulsions only. Rates of hydrolysis of the triglycerides were found to decrease with increasing fatty acid chain length. This may be due to the fact that up to trilaurin, the substrates tend to be liquids at the temperature of incubation (40°) and a better dispersion of the substrates is available. After trilaurin, the substrates are all solids and the activity declines. The decreasing rate of hydrolysis with increasing fatty acid chain length has been reported by many workers (9–12).

TABLE II. HYDROLYSIS OF COMBINED SUBSTRATES BY RAT PANCREATIC LIPASE[a]

Substrate	μmoles/3 ml	μmoles FFA/mg protein/hr
Tributyrin	15	12.6 ± 0.8
Tripalmitin	15	7.8 ± 0.2
Trilinolein	15	27.8 ± 0.7
Tributyrin + Tripalmitin	30[b]	11.0 ± 0.7
Tributyrin + Trilinolein	30[b]	23.5 ± 0.5

[a] Reaction mixture contained 10 μg of purified lipase protein.

[b] Fifteen μmoles of each substrate were combined and suspended in a total volume of 3 ml.

The question of whether separate lipases are responsible for hydrolysis of triglycerides of certain chain lengths has also been raised by Desnuelle (13). To investigate the coexistence of separate lipases, equimolar quantities of tributyrin and trilaurin and tributyrin and triolein were combined in the same assay medium and subjected to hydrolysis by lipase. If separate lipases indeed exist for the hydrolysis of triglycerides of specific fatty acid chain lengths, then the amount of FFA released should be the sum of the hydrolysis of the two triglycerides. It is evident from the results summarized in Table II that such is not the case. The amount of FFA released appeared to be an average of, rather than the sum of, the two combined substrates.

Effect of unsaturation in fatty acyl moiety.

As summarized in Table I, the hydrolytic activity was found to increase with increase in number of double bonds in the fatty acid residues of the same chain length. But Clement and Clement-Champaugny (14) observed that fats with a low degree of unsaturation were more readily hydrolyzed than highly unsaturated fats by lipase. Savary and Desnuelle (15) and Mattson and Volpenheim (16) found that all of the long-chain fatty acids, namely, the saturated chains from C_{12} to C_{18} and the C_{18} unsaturated chains were removed at about the same rate by lipase.

Lipolytic activity against partial glycerides. As shown in Table I, diglycerides were hydrolyzed at more than twice the rate found for the corresponding triglycerides, while the monoglycerides were not hydrolyzed. The high specific activity against diglycerides is not consistent with the observation reported in the literature (17–19) as it has been shown that large amounts of diglycerides and monoglycerides accumulate during lipolysis by pancreatic lipase *in vitro* and *in vivo*.

Hydrolytic activity against methyl esters. Table I indicated that methyl esters, with chain lengths above 8, are hydrolyzed at a rate higher than those for the corresponding triglycerides and that the rate increases with increasing fatty acid chain length up to a length of 10. This may be due to the fact that the methyl esters used are all liquids at the temperature of assay and that better emulsification occurs with increasing numbers of carbon atoms.

Identification of products of lipolysis. Qualitative identification of the products of lipolysis of tributyrin, tricaprin, tripalmitin, and triolein by purified lipase was carried out by thin-layer chromatography. Spots corresponding to triglycerides, the isomers of diglycerides and monoglycerides, and FFA were found in the hydrolysates, and the results are similar to those reported by Mattson *et al.* (17), Blankenhorn and Ahrens (20), and Borgstrom (21).

Effect of various emulsifiers. Emulsifying agents such as albumin, gum arabic, Tween 20, and sodium deoxycholate have been shown to increase the rate of hydrolysis by lipase. Table III summarizes the effect of these agents on the rate of hydrolysis. Only the substrates which are liquid at the temperature of incubation were hydrolyzed in the absence of an emulsifying agent. Suspension of the triglyceride in 1% (w/v) solutions of the emulsifying agents resulted in increased rates of hydrolysis. The order of stimulation of lipolytic activity was sodium deoxycholate > Tween 20 > gum arabic > albumin.

Effect of metal ions and sulfhydryl reagents. As shown in Table IV, all the metals tested showed varying degrees of inhibitory action. Ca^{2+} had no stimulatory action. Mg^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} strongly inhibited

TABLE III. EFFECT OF EMULSIFYING AGENTS ON RAT PANCREATIC LIPASE ACTIVITY[a]

Buffers (μmoles FFA released per mg protein/hr)	Substrates			
	Tributyrin	Tricaprylin	Tripalmitin	Triolein
PO_4 buffer alone	81.6 ± 2.1	3.51 ± 0.08	1.2 ± 0.03	28.3 ± 1.2
PO_4 buffer with albumin (1%)	84.0 ± 1.5	61.7 ± 1.7	9.4 ± 0.4	37.2 ± 1.9
PO_4 buffer with gum arabic (1%)	83.2 ± 1.9	64.3 ± 2.0	14.9 ± 0.2	50.2 ± 2.1
PO_4 buffer with Tween 20[b] (1%)	130.0 ± 2.4	72.7 ± 1.7	17.3 ± 0.7	61.4 ± 1.5
PO_4 buffer with NaDOC (0.1%)[c]	124.0 ± 0.7	—	34.0 ± 1.1	—

[a] Reaction mixture contained approximately 10 μg of purified lipase with 30 μmoles of each of the substrates.

[b] Tween 20 = Polyoxyethylene sorbitan monolaurate.

[c] NaDOC = Sodium doexycholate.

TABLE IV. EFFECT OF METAL IONS ON RAT PANCREATIC LIPASE ACTIVITY.[a]

Compound[b]	Tributyrin		Tripalmitin		Triolein	
	Specific activity[c]	Inhibition (%)	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)
None	87.2 ± 0.9	—	9.8 ± 0.4	—	39.7 ± 0.5	—
$BaCl_2$	73.6 ± 0.2	15.5	6.7 ± 0.1	31.6	36.9 ± 0.4	17.0
$CaCl_2$	82.5 ± 0.6	5.5	8.2 ± 0.2	16.4	41.5 ± 0.7	0
$CuCl_2$	14.8 ± 0.1	63.0	2.7 ± 0.8	72.4	0.9 ± 0.02	97.7
$FeCl_3$	8.8 ± 0.06	89.9	1.4 ± 0.05	85.7	0.9 ± 0.01	97.7
$MgCl_2$	78.8 ± 0.5	9.6	6.7 ± 0.2	31.6	37.0 ± 0.8	6.8
$MnCl_2$	75.2 ± 0.7	13.8	5.1 ± 0.1	48.0	36.7 ± 0.8	7.5
$HgCl_2$	44.8 ± 0.2	48.6	4.3 ± 0.1	56.0	21.8 ± 0.3	45.5
NaCl	86.0 ± 0.4	1.4	9.8 ± 0.3	0	40.8 ± 1.0	0
$ZnCl_2$	26.8 ± 0.2	70.0	2.7 ± 0.06	72.4	0.9 ± 0.01	97.8
NaF	58.8 ± 0.5	32.5	7.1 ± 0.2	27.5	26.2 ± 0.4	34.0
PCMB[d]	30.6 ± 0.6	59	2.8 ± 0.08	70	14.6	71.2
Iodoacetic acid	84.8 ± 0.3	2	9.7 ± 0.5	0	38.8	0

[a] The reaction mixture contained approximately 10 μg of purified lipase protein with 30 μmoles of each of the substrates. All the compounds were added at the beginning of the incubation.

[b] Amount of metal ions was added to give a final concentration of 1×10^{-3} *M*.

[c] Specific activity is expressed as μmoles FFA released/mg protein/hr. The values given are means ± SEM for three observations.

[d] Parachloromercurybenzoate.

lipase activity, an observation similar to those reported by Wills (22) and Parfentijev *et al.* (23). The sulfhydryl reagents *p*-chloromercurybenzoate inhibited the lipolysis as reported by many workers. However, Wills (24) has shown that *p*-CMB has a strong affinity for glycerides and proposed that the partition of *p*-CMB at the glyceride/water interface is an important factor in its inhibition.

Schoor and Melius (25) have suggested that lipid–protein interactions play a vital role in preserving the enzyme during isolation and purification involving organic solvents. On the other hand, our studies indicate that by employing a different method of isolation and purification involving no organic solvents, we get the enzyme in a more native state. The specificity of this native lipase against partial glycerides and triglycerides having unsaturated fatty acyl moieties is altered when treated with acetone.

These studies with various substrates and inhibitors indicate that the high molecular weight pancreatic lipase is similar in some aspects to adipose tissue (28) and liver mitochondria lipase (27) but in other respects differs greatly from low-molecular-weight lipases. Its properties are markedly different from those of lysosomal lipase, microsomal esterase, and lipoprotein lipase. Schnatz and Cortener (28) and also Huttunnen and Steinberg (26) have demonstrated that purified adipose tissue lipase exists as subunits in a lipid protein complex. Okuda and Fujii (29) have indicated that liver lipase is complexed with a lipid moiety. The high molecular weight of the pancreatic lipase and the observation that it could always be associated with the turbidity in the collection tubes, suggest that it may also be aggregated with lipid. It is possible that the high-molecular-weight enzyme is composed of a number of protein molecules attached to a lipid core, presumably by hydrophobic or electrostatic interaction. The lipid core may account for the high molecular weight of this enzyme preparation since an individual lipoprotein complex may contain as many as 1000–2000 lipid molecules (30) and the molecular weight of such an aggregate has been reported to be 4×10^6 (31). Variations in the molecular weight of lipases isolated by solvent extraction methods may indicate that

the high-molecular-weight enzyme has been broken down to different extents.

Summary. The properties of the 500-fold purified high-molecular-weight lipase have been studied. The rate of hydrolysis of the triglycerides decreases with increasing fatty acid chain length. The lipolytic activity also increases with increase in unsaturation in the fatty acyl moiety. Diglycerides are hydrolyzed at more than twice the rate for triglycerides while monoglycerides are not hydrolyzed. Methyl esters are generally hydrolyzed at a higher rate which increases with increasing chain length of the fatty acid but the enzyme does not act on phospholipids. Emulsifying agents such as Tween 20, gum arabic, and albumin increase the rate of hydrolysis. Metal ions such as Hg^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} strongly inhibit the lipolytic activity of the high-molecular-weight lipase while Ca^{2+} or Mg^{2+} by themselves show no stimulating effect. Treatment of the high-molecular-weight lipase with *P*-chloromercurybenzoate inhibits hydrolytic activity by 70% while iodoacetic acid has no effect.

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Received Feb. 25, 1975. P.S.E.B.M., 1975, Vol. 149.