Purification and properties of a phosphorylatable triacylglycerol lipase from the fat body of an insect, *Manduca sexta*

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Abstract A triacylglycerol lipase, presumably the first enzyme involved in the mobilization of lipid from the insect fat body, has been purified to homogeneity from the fat body of *Manduca sexta*. The purification procedure involved polyethyleneglycol precipitation, and chromatography on DEAE-cellulose, phenyl-Sepharose, Q-Sepharose and hydroxylapatite. The final product, a protein with an M_r = 76,000 by SDS-PAGE, was purified nearly 8000-fold from the original homogenate in a yield of about 11%. The enzyme catalyzed the hydrolysis of tri-, di-, and mono-oleoylglycerols, but showed highest affinity for tri- or di-oleoylglycerol. Thus, under initial reaction conditions, the end products of trioleoylglycerol hydrolysis were: free fatty acids (66%), *sn*-2-monooleoylglycerol (24%), *sn*-1,2(2,3)-dioleoylglycerol (7%), and glycerol (3%). The fat body lipase exhibited a preference for hydrolyzing the primary ester bonds of acylglycerols, and did not show stereoselectivity toward either the *sn*-1 or *sn*-3 position of trioleoylglycerol. The enzyme had a pH optimum of 7.9, and was inhibited by diisopropylfluorophosphate, ATP, ADP, Mg^{2+}, and NaF. The enzyme showed a strong tendency to aggregate, but was stable in detergent solutions at high concentration of glycerol. The polypeptide was phosphorylated by the cAMP-dependent protein kinase from bovine heart; however, phosphorylation did not cause activation of the enzyme. It is suggested that this fat body lipase could be analogous to the "hormone-sensitive lipase" of vertebrate adipose tissue.—**Arrese, E. L., and M. A. Wells.** Purification and properties of a phosphorylatable triacylglycerol lipase from the fat body of an insect, *Manduca sexta*. *J. Lipid Res.* 1994. **35:** 1652–1660.

Supplementary key words *Manduca sexta* • lipase • lipid mobilization • fat body

The insect fat body, which combines many of the properties and functions of vertebrate liver and adipose tissue, plays a fundamental role in energy metabolism. It is the principal site for storage of both glycogen and lipids (1). Lipids serve as an important substrate to meet energy demands during migratory flight and starvation. Triacylglycerols (TG) constitute the main lipid storage form (2). The content of TG in the fat body is influenced by several factors, including development stage, nutritional state, sex, and migratory flight (3). In the tobacco hornworm, *Manduca sexta,* which is widely used as a model insect, the maximum content of fat body TG occurs at the end of larval development, as a consequence of the accumulation of reserves during larval feeding (4). Afterwards, as a result of lipolysis and the fatty acid oxidation required to sustain energy metabolism during the subsequent non-feeding periods (pupal and adult), the TG stores start to decline (4, 5). Unlike vertebrates, where the stored fatty acids are mobilized as free fatty acids, in insects, most fatty acids are released from the fat body as *sn*-1,2-diacylglycerols (DG). The DG is carried from the fat body to the sites of utilization, e.g., flight muscle (6, 7) and ovary (8, 9), by lipophorin, the major lipoprotein present in the hemolymph of most insects (10).

Insect adipokinetic hormone (AKH), a peptide that is released from the corpora cardiaca into the hemolymph during flight (11), seems to be the major hormone that controls lipid mobilization from the fat body, but little is known about the mechanism of action of AKH. AKH has been shown to elevate the hemolymph DG levels in many insect species (3), and it has been reported that AKH stimulates the uptake of Ca^{2+} by fat body cells (12–14) and an increase in the concentration of cAMP in fat body (12, 14). On this basis, it has been suggested that the primary action of AKH in lipid mobilization is to activate a protein kinase A, which in turn activates, by phosphorylation, a fat body lipase that converts TG into DG (11, 13, 14). However, the lack of a pure preparation of fat body TG-lipase has made it impossible to obtain conclusive results about the activation of the lipase by AKH. Simi-

Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid; DFP, diisofluorophosphate; PEG, polyethyleneglycol; AKH, adipokinetic hormone.

larly, the mechanism of stereospecific synthesis of *sn*-1,2-DG that takes place in the fat body remains to be elucidated. DG production could involve several steps rather than the direct hydrolysis of TG, as has been proposed by some investigators (15–17). In order to understand the mechanism of lipolysis which takes place in the insect's fat body, as well as the mode of action of AKH, it is necessary to characterize the fat body TG-lipase. Clearly, an understanding of these metabolic pathways is of fundamental importance in insect biochemistry and might elucidate potential specific targets for insect control. In the present paper, we report the purification and some of the properties of a TG-lipase from *M. sexta* fat body.

MATERIALS AND METHODS

Materials

Two- and 3-day-old *M. sexta* adults from a colony maintained in this laboratory were used (18). Labeled trioleoylglycerol ([tri-[9,10[^{3}H(N)]oleoylglycerol) was obtained from New England Nuclear and trioleoyl[1,2,3-^{3}H]glycerol was purchased from American Radiolabeled Chemicals Inc. DEAE-cellulose (DE-52) was from Whatman; hydroxylapatite was from Bio-Rad; Q-Sepharose was from Pharmacia-LKB Biotechnology; and Triton X-100, diisopropylfluorophosphate (DFP), phenyldichlorophosphate, benzamidine, and polyethyleneglycol 8000 (PEG) were from Aldrich. Phenyl-Sepharose, leupeptin, aprotinin, and gel electrophoresis molecular weight markers were purchased from Sigma. Silica gel G plates were obtained from Baker. All other chemicals were of analytical grade.

Assay of TG-lipase

Lipase activity was assayed using a micellar TG substrate according to Hirayama and Chino (19) with minor modifications.

Substrate preparation. Radiolabeled trioleoylglycerol was purified by TLC on silica gel prior to use (20). The band corresponding to monooleoylglycerol was also recovered from the plate and used for the preparation of the *sn*-1(3)-monooleoylglycerol substrate. The composition of the monooleoylglycerol fraction was assessed by TLC on silica gel G impregnated with 1.2% boric acid (21) and found to be approximately 90% the *sn*-1(3) isomer and 10% the *sn*-2 isomer. Radiolabeled *sn*-1,2(2,3)-dioleoylglycerol and *sn*-2-monooleoylglycerol were obtained from radiolabeled trioleoylglycerol using either pancreatic (22) or *Rhizopus delemar* (23) lipases and used within 48 h of preparation.

Assay procedure. The enzyme activity for samples at the beginning of the purification procedure was assayed using the following substrate solution: unlabeled trioleoylglycerol (final concentration 2 mM) and Triton X-100 (final concentration 10 mM) were dissolved in toluene–ethanol 1:1 (v/v), and purified; radiolabeled trioleoylglycerol was added to give a specific activity of about 7×10^{12} dpm/mol. After the polyethyleneglycol precipitation, the ratio of trioleoylglycerol: Triton X-100 was changed to 1:3. Dioleoylglycerol and monooleoylglycerol substrates were prepared in the same manner, using a substrate: Triton X-100 ratio of 1:3. Substrate solution (50 μl) was transferred to a large glass centrifuge tube and solvent was removed under a stream of nitrogen. After addition of 300 μl of reaction buffer (20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.02% (w/v) defatted BSA), the lipids were dispersed by intense vortexing for 30 sec. The reaction was initiated by adding enzyme. The final volume of the mixture reaction was 500 μl and incubations were carried out at 37°C with constant shaking for 30 min. The reaction was terminated by the addition of 2.5 ml of an extraction mixture consisting of chloroform–methanol–benzene 2:2.4:1 (v/v/v), containing 0.5 μmol of unlabeled oleic acid as a carrier. Then, 100 μl of 1 N NaOH was added, the mixture was vortexed for 20 sec, and then centrifuged at 2,000 *g* for 5 min at room temperature. Aliquots (150 μl) of the upper aqueous phase were transferred to scintillation vials for counting. The recovery of FFA in the procedure was determined to be 77% using [1-^{14}C]oleic acid. Blank reactions did not contain enzyme. Enzyme activity is expressed as μmol of FFA produced/min.

Extraction, separation, and analysis of hydrolysis products

The substrate solution was prepared as described above except that [1,2,3-^{3}H]glycerol-labeled trioleoylglycerol (11×10^{12} dpm/mol) was used. The lipase reaction was terminated by addition of the extraction mixture, as described above. The upper methanol-aqueous phase was concentrated to dryness and redissolved in scintillation liquid to determine free [^{3}H]glycerol (24). The organic phase was transferred to a glass tube and evaporated to dryness. The lipid extract was redissolved in 50 μl of chloroform–methanol 2:1 (v/v) and applied to a silica gel G TLC plate impregnated with 1.2% boric acid and activated at 100°C for 15 min (21). The lipids were separated in a solvent system containing chloroform–acetone 96:4 (v/v). The lipids, *sn*-1-MG, *sn*-2-MG, *sn*-1,2(2,3)-DG, *sn*-1,3 DG, and TG, were visualized using iodine vapor and radioactivity in scraped bands was determined by liquid scintillation counting; the results are expressed as molar percentage. The stereochemistry of the *sn*-1,2(2,3)-DGs was determined by the method of Brockerhoff (25). The monoacyl- and diacylphosphatidylphenols were separated on TLC silica gel plates developed in a mixture of chloroform–methanol–3% aqueous ammonia 63:30:7 (v/v/v). The lipid spots were visualized by iodine vapor and the radioactivity of the scraped spots was determined.

Purification of TG-lipase

All steps were carried out on ice or at 4°C. Immediately after decapitation, fat body tissue from 600 adults was collected in homogenization buffer (solution A:20 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM NaEDTA, 0.1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 10 mg/l leupeptin, and 1 mg/l aprotinin). The tissue was homogenized, at a ratio of 3 ml of solution A per fat body, using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min. The floating fat cake was removed by vacuum aspiration and the infranatant was filtered through glass wool in order to remove as much remaining fat as possible. The infranatant was then subjected to ultracentrifugation in a 60Ti rotor at 100,000 *g* for 60 min. The supernatant was collected and solid PEG was slowly added, with constant stirring, to a final concentration of 20% (w/v). After 2 h of stirring, the suspension was centrifuged (20,000 *g*, 20 min) and the supernatant was discarded. The pellet was resuspended in solution A (200 μl/fat body). The suspension was stored at −20°C until used; under these conditions the enzyme activity was stable for at least 3 months. NaCl and Triton X-100 were added to the suspension to achieve final concentrations of 10 mM and 0.5% (v/v), respectively; the resulting suspension was centrifuged (12,000 *g*, 20 min). The supernatant was collected and the pellet was resuspended in the same solution (100 μl/fat body), centrifuged as described above, and the supernatant was combined with the first supernatant.

The solution was loaded onto a chromatography column (3 × 30 cm) containing DEAE-cellulose equilibrated with solution B (10 mM sodium phosphate, pH 7.4, containing 1 mM NaEDTA, 0.1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 10 mg/l leupeptin, 1 mg/l aprotinin, and 0.02% Triton X-100). The unbound protein was removed by washing the column with 5 column volumes of solution B. Then the column was developed with a linear salt gradient between 50–500 mM NaCl in buffer B. Fractions containing significant lipase activity (eluted between 120 and 250 mM NaCl) were pooled, dialyzed against solution B, and submitted to a second DEAE-cellulose chromatography on a smaller column (2 × 15 cm), following the protocol described above. The lipase-activity peak was pooled and loaded onto a phenyl-Sepharose column (1.5 × 15 cm) equilibrated with solution B. After washing the column with 10 column volumes of solution B, followed by 20% ethylene glycol in the same buffer, the lipase activity was eluted with a linear gradient of ethylene glycol between 20–70% (v/v) in solution B. Fractions containing lipase activity (eluted between 35–55% ethylene glycol) were pooled, diluted with solution B to 20% ethylene glycol, and applied onto a Q-Sepharose anion exchange column (1.5 × 15 cm) equilibrated with solution B. The lipase activity was eluted with a linear (50–350 mM) sodium chloride gradient. The pooled active fractions (eluted between 180 and 320 mM NaCl) were diluted to 5 mM sodium phosphate and loaded onto a hydroxylapatite column (1.5 × 6 cm) equilibrated with solution C (same composition as solution B except for the molarity of sodium phosphate, which was decreased to 5 mM). After elution of the unbound protein with solution C, the column was washed with 5 column volumes of solution C containing 50 mM NaCl followed by one column volume of solution C. Then the lipase activity was eluted with a linear sodium phosphate gradient (5–100 mM) in solution C. Active fractions (eluted between 20–35 mM) were pooled and stored at −20°C in 50% (v/v) glycerol for up to 2 months with no loss in activity. A short column of hydroxylapatite was also used in order to concentrate the sample when necessary.

Phosphorylation of TG-lipase

TG-lipase was phosphorylated by incubation with the catalytic subunit of the cAMP-dependent protein kinase from bovine heart (10 units) in 100 μl of 0.1 M Tis-HCl (pH 7.4), containing 0.2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM [γ-^{32}P]ATP (10^4 dpm/pmol), for 30 min at room temperature. Incubations were stopped by addition of electrophoresis sample buffer. After SDS-PAGE, the stained and dried polyacrylamide gel was autoradiographed. Unlabeled phosphorylated enzyme was made using the same conditions, except that unlabeled ATP was used and the reaction was stopped by addition of 5 mM NaEDTA.

Other Methods

Protein concentrations were determined by the Bradford (26) dye-binding assay using bovine serum albumin as a standard. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on vertical slab gels according to Laemmli (27), using 3.0% and 10.5% polyacrylamide for the stacking and resolving gels, respectively. Proteins were visualized with Coomassie brilliant blue R250 (0.5%, w/v) in methanol–water–acetic acid 50:40:10.

RESULTS

Purification of the fat body TG-lipase

Because lipids represent a high proportion of insect fat body (approximately 50% of its dry weight (2)) and because it was possible that the lipase might be associated with the fat droplets, we carried out preliminary studies to determine whether delipidation of the crude homogenate might increase the yield of lipase in the extract. However, either preparation of an acetone powder

or extraction of the crude homogenate with butanol (10% v/v) leads to complete enzyme inactivation. In the final procedure, the crude homogenate was centrifuged at 20,000 *g* for 20 min, the fat cake and pellet were discarded, and the infranatant was subjected to ultracentrifugation. Comparison of lipase activity in the initial infranatant with that in the supernatant after the ultracentrifugation indicated that the fat body lipase was a cytosolic enzyme.

The results of a representative purification are summarized in **Table 1.** The initial purification step involved the use of PEG precipitation. Isoelectric precipitation at pH 5.2 was also effective in precipitating the lipase activity, but the recovery on subsequent resuspension was very low, thus PEG precipitation was preferred. The PEG precipitate could be stored at −20°C for several months without loss of enzyme activity. The enzyme was extracted from the PEG precipitate with 0.5% Triton X-100 (v/v) and 10 mM NaCl. In order to avoid loss of the enzyme due to aggregation, Triton X-100 (0.02% v/v) was included in all solutions in subsequent purification steps. The elution patterns of enzymatic activity obtained in the steps of chromatography are depicted in **Fig. 1** (the protein profile is not shown because benzamidine, which was present in the buffers to inhibit proteolysis, absorbs strongly at 280 nm). Two peaks of activity were eluted from the DEAE-cellulose column, at 150 mM and 400 mM, respectively (Fig. 1,A). The first peak, representing approximately 75% of the total eluted activity, was rechromatographed on DEAE-cellulose. The pooled active fractions from the second DEAE-cellulose column were directly applied onto a phenyl-Sepharose column. The enzyme was tightly bound by this hydrophobic resin. The lipase activity was not released from the column at 0 mM NaCl, but Triton X-100 or ethylene glycol proved to be effective in displacing the lipase activity from the column. Because a high detergent concentration (0.3% v/v) was required, which partially inhibits the lipase activity, the enzyme was eluted with ethylene glycol. Approximately 10–15% of the total eluted lipase activity was eluted with the first wash of 20% ethylene glycol, and afterwards an activity peak (75–80% of total eluted activity) was eluted between 35–55% ethylene glycol (Fig. 1,B). The pooled main peak was then chromatographed on Q-Sepharose. One peak of lipase activity appeared between 150–250 mM NaCl (Fig. 1,C), which was pooled, diluted, and loaded onto a hydroxylapatite column. The enzyme was eluted by a phosphate gradient and a major peak, representing 70% of the eluted activity, was recovered at 20–35 mM phosphate (Fig. 1,D). The overall yield of activity was about 12% with a purification of about 8,000-fold. The material in the second peak from the initial DEAE-cellulose column was also carried through the purification scheme (data not shown) and yielded an enzyme with properties that are identical to those of the enzyme purified from the first peak of activity eluted from the initial DEAE-cellulose column. At present we have no explanation for the appearance of two peaks of enzyme activity on the initial DEAE-cellulose column.

The purified enzyme showed a single band on SDS-PAGE from which its molecular weight was estimated to be 76 ± 3kDa (**Fig. 2**). A band migrating at 76 kDa was also visible in the preparation after phenyl-Sepharose chromatography (Fig. 2).

Substrate specificity of the TG-lipase

Lipase activities towards tri-, di-, monooleoylglycerol, and cholesteryl oleate, all solubilized in Triton X-100 micelles, were assayed under identical experimental conditions. The enzyme hydrolyzed all three acylglycerols, but was inactive in cholesteryl ester hydrolysis. The concentration-dependence of lipase activity with TG and DG substrates showed saturation kinetics with apparent K_ms of 60 and 90 μM, respectively (**Fig. 3**). DG was hydrolyzed at a slightly higher rate than TG, with the ratio of V_m for DG to TG being approximately 2:1. The activity against MG did not show a true substrate saturation curve (Fig. 2). The hydrolysis of *sn*-1(3)-MG was at least 2.7 times faster than *sn*-2-MG, which was the poorest acylglycerol substrate assayed.

TABLE 1. Summary of fat body TG-lipase purification

Step	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	*μmol FFA/min*	*activity/mg protein*	*%*	*-fold*
Homogenate	12,900	2.06	0.00016	100	1
PEG suspension	4,000	1.44	0.00037	70	2.3
DE-52 I	1,080	0.59	0.00055	29	3.4
DE-52 II	16.0	0.15	0.0094	7.3	59
Phenyl-Sepharose	1.58	0.24	0.15	11.7	939
Q-Sepharose	0.41	0.24	0.60	11.7	3.757
Hydroxyapatite	0.18	0.23	1.26	11.2	8,016

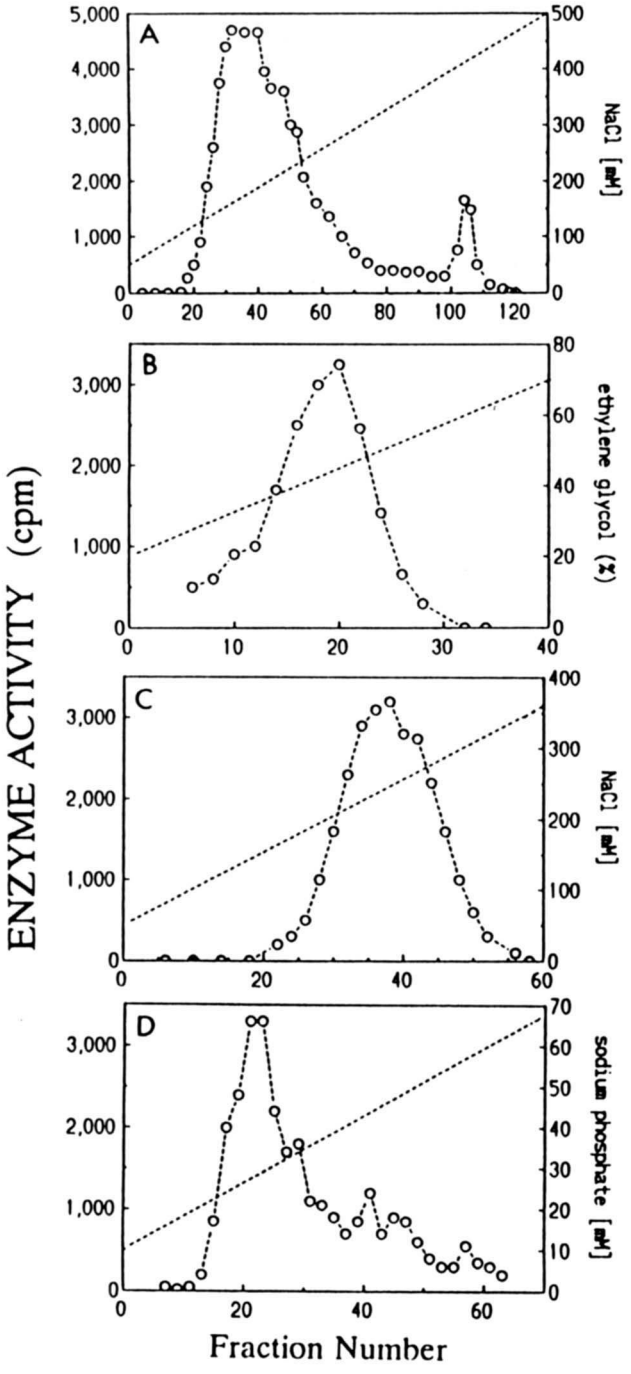

Fig. 1. Purification of the triacylglycerol-lipase. Panels A–D correspond to the patterns of enzymatic activity elution profiles from the first DEAE-cellulose, phenyl-Sepharose, Q-Sepharose, and hydroxyapatite columns, respectively. Fractions of 7 ml were collected, and aliquots of 100 μl were assayed for TG-lipase activity.

TG hydrolysis products

Under initial hydrolysis conditions (< 5% hydrolysis), the hydrolysis of trioleoylglycerol led to the accumulation of MGs, which accounted for 76% of total acylglycerol products (**Fig. 4**). The *sn*-2 isomer represented 86% of the MG products, while the *sn*-1(3) isomers represented 14% of the MGs. Because the equilibrium mixture of MGs contains 90% of the *sn*-1(3) isomers, it is very likely that the *sn*-1(3)-MG detected in the hydrolysis mixture was the result of acyl migration of the *sn*-2-MG, which occurred during incubation. In the case of the DGs, which represented 24% of total acylglycerol products, the *sn*-1,2(2,3) isomers accounted for 95%, whereas the *sn*-,1,3 isomer only represented 5%. When the mixture *sn*-1,2(2,3)-DGs was subjected to stereospecific analysis, the *sn*-1,2 isomer was found to represent 56% of the mixture and the *sn*-2,3 isomer represented 44%.

Other poperties of the enzyme

DFP, a serine protease inhibitor, which is known to inhibit several lipases (28–32), was an effective inhibitor of the fat body lipase with 150 μM DFP causing 50% inhibition (**Fig. 5**). Figure 5 also shows the inhibitory effects of ATP and ADP, with 50% inhibition occurring at 0.8 and 1.5 mM, respectively. GTP at the same concentrations was also inhibitory (data not shown). Magnesium ions were also inhibitory (Fig. 5), as well as NaF, which caused 50% inhibition at a concentration of 25 mM.

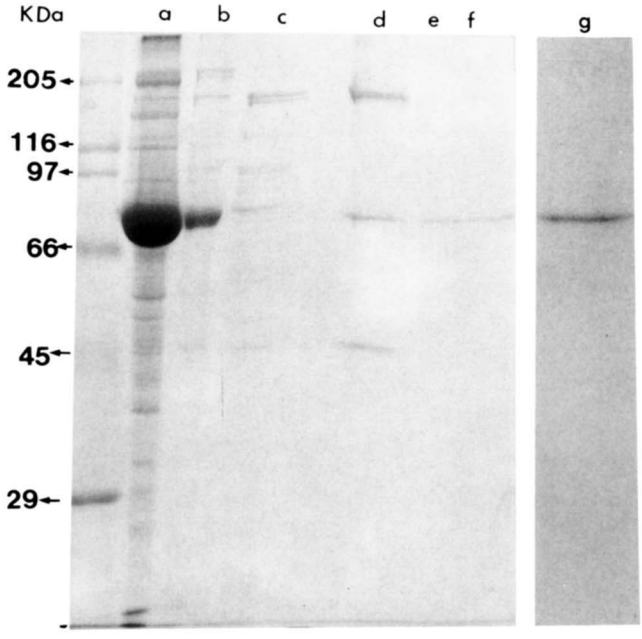

Fig. 2. SDS-polyacrylamide gel electrophoresis of *M. sexta* fat body triacylglycerol-lipase. a) PEG resuspension (approximately 75 μg of protein); b) first DEAE-cellulose column (approximately 30 μg of protein); c) second DEAE-cellulose column (approximately 20 μg of protein); d) phenyl-Sepharose fraction (approximately 15 μg of protein); e) Q-Sepharose fraction (approximately 5 μg of protein); f) hydroxyapatite fraction (approximately 3 μg of protein); g) hydroxyapatite fraction (approximately 20 μg of protein). Molecular weight marker values are indicated on the left side. The gel contained 10.5% acrylamide and proteins were stained with Coomassie Brilliant Blue.

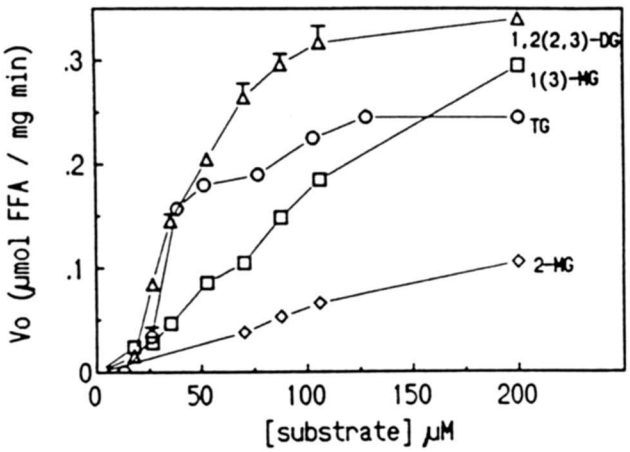

Fig. 3. Dependence of triacylglycerol-lipase activity on substrate concentration using different acylglycerol substrates. (△) *sn*-1,2(2,3)-DG; (□) *sn*-1(3)-MG; (○) TG; (◇) *sn*-2-MG. The activity was assayed with 2 μg of TG-lipase after the phenyl-Sepharose step. K_m values were estimated by a nonlinear regression fit of the data to the Michaelis-Menten equation. Values are the mean of two determinations.

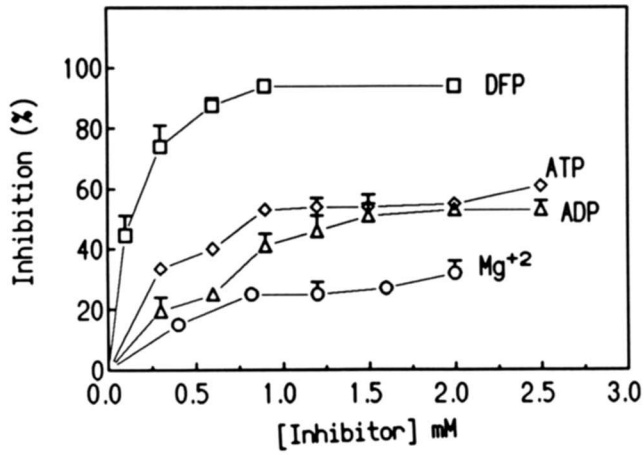

Fig. 5. Inhibition of TG-lipase by DFP, ATP, ADP, and Mg^{2+}. The purified enzyme was incubated at room temperature for 20 min with the indicated concentrations of each compound. After incubation, enzyme activity was assayed using 41 μM trioleoylglycerol at 38°C for 30 min. Results are expressed as % inhibition relative to the activity of the enzyme incubated in the absence of inhibitor.

The enzymatic hydrolysis of TG exhibited an optimum pH of 7.9 (data not shown). Maximal activity was obtained in the presence of 0.25 M NaCl and a Triton X-100 concentration between 0.005 and 0.05% (v/v). High detergent concentration, greater than 0.5%, caused severe inhibition of the lipase activity, but this inhibition could be reversed by diluting the detergent.

When the purified TG-lipase was incubated with catalytic subunit of the cAMP-dependent protein kinase from bovine heart and [γ-^{32}P]ATP-Mg the 76 kDa polypeptide was phosphorylated and phosphorylation did not change the apparent subunit molecular weight of the enzyme (**Fig. 6**). After phosphorylation, the activity of the lipase toward TG was unchanged (data not shown).

The purified lipase did not bind to Sepharose-concanavalin A or Sepharose-heparin columns. The purified enzyme showed a strong tendency to aggregate, and certain procedures with the native enzyme, such as concentration by ultrafiltration or electrophoresis under nondenaturing conditions were unsuccessful. High concentration of glycerol (50% v/v) proved useful in preventing protein aggregation and inactivation.

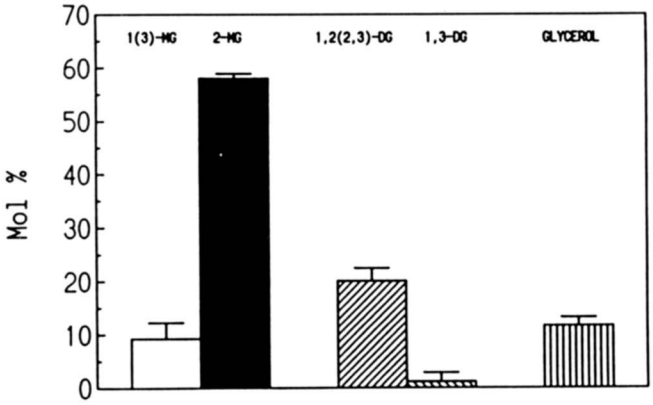

Fig. 4. Molar composition of the reaction products after incubation of trioleoylglycerol with purified TG-lipase. [^{3}H]glycerol-labeled trioleoylglycerol (41 μM) was incubated for 20 min with 2 μg of enzyme, which resulted in 4% hydrolysis of the trioleoylglycerol. The values are means ± standard deviations for five determinations.

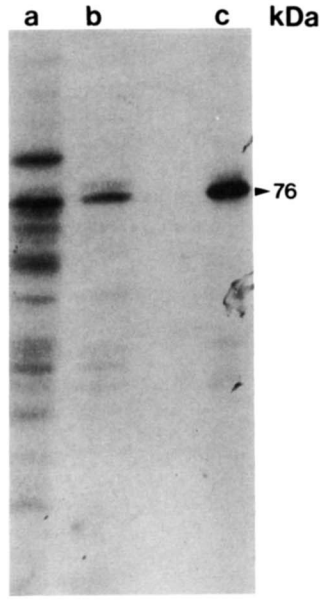

Fig. 6. Autoradiography of an SDS-polyacrylamide gel after phosphorylation of TG-lipase. The samples were incubated with the catalytic subunit of the cAMP-dependent protein kinase from bovine heart and [γ-^{32}P]ATP. a) Fraction after first DEAE-cellulose column; b) fraction after phenyl-Sepharose column; c) fraction after hydroxyapatite column.

DISCUSSION

We have purified a TG-lipase from *M. sexta* fat body. The final product, the result of an 8,000-fold purification, is composed of a single polypeptide with an M_r of 76,000, and represents the first lipase purified from an insect. Recently, some properties of a partially purified preparation of lipoprotein lipase (LpL) from *M. Sexta* flight muscle were reported (31). Although the fat body TG-lipase's alkaline pH optimum and inability to hydrolyze cholesterol esters are similar to mammalian lipoprotein lipase, the majority of its properties, such as stability to acetone-ether treatment, isoelectric precipitation, affinity for heparin- or concanavalin A-Sepharose, affinity for phenyl-Sepharose, insensitivity to 0.5 M NaCl, and the size of the subunit, are most similar to the hormone-sensitive lipase from mammalian adipose tissue (28, 33–35). Because the insect fat body combines many of the functions of vertebrate liver and adipose tissue, it is also reasonable, on physiological grounds, that the fat body lipase should be similar to hormone-sensitive lipase. Both the fat body and hormone-sensitive lipases are phosphorylated by a cAMP-dependent protein kinase (34). Unlike hormone-sensitive lipase, the fat body lipase did not increase its activity after phosphorylation. At least two possibilities could explain this result. *1)* For the phosphorylation reaction we used a commercial preparation of the catalytic subunit of the cAMP-dependent protein kinase from bovine heart, which raises the question of whether the fat body lipase might require a specific fat body kinase in order to be phosphorylated on the correct residue. *2)* It has been demonstrated that phosphorylation of hormone-sensitive lipase does not greatly increase the activity of the enzyme in vitro, but rather produces a protein that translocates to its substrate, the fat droplet, and that this translocation probably accounts for the activation of the enzyme in vivo (35). If phosphorylation of the fat body lipase also activates it by causing it to translocate from the cytoplasm to the fat droplet, we might not see an effect of phosphorylation on enzyme activity using a TG-detergent micelle. Other experiments are necessary in order to clarify this point.

DFP inhibition of the fat body lipase indicates that a serine residue is essential for its catalytic function. Because several lipases are susceptible to serine-directed reagents such as DFP, a similarity between their respective active sites has been proposed. A putative lipase consensus sequence (GlyXSerXGly) has been identified in all known mammalian and prokaryotic lipase sequences (36), and the presence of a Ser has been shown in the active site of two lipases (37, 38).

The fat body lipase can hydrolyze all acylglycerols, but unlike hormone-sensitive lipase, it did not hydrolyze cholesterol esters. Amongst acylglycerols, the fat body lipase hydrolyzes DG faster than TG, and *sn*-2-MG is the poorest substrate. These data suggest that the enzyme has a strong preference for hydrolyzing the primary ester bonds of acylglycerols. The results obtained from TG hydrolysis end products analysis are in accord with the enzyme activity exhibited toward different acylglycerol substrates. The hydrolysis proceeds with the accumulation of *sn*-2-MG, which is the poorest substrate. Lesser amounts of DG are found in the reaction products, which is in agreement with the fact that the enzyme has a higher activity against DG than against MG. The DGs are almost entirely *sn*-1,2(2,3)-DG, which is also consistent with the suggestion that the enzyme shows a preference for hydrolyzing primary ester bonds. This property is common among several lipases including pancreatic lipase (39), lipoprotein lipase (40), and hormone-sensitive lipase (39).

When the stereochemistry of the DG produced during TG hydrolysis was analyzed, almost equal yields of *sn*-1,2- and *sn*-2,3-DGs were detected. Therefore, under our experimental conditions, the fat body lipase did not exhibit a stereopreference for hydrolyzing either the *sn*-1 or *sn*-3 position. A lack of stereospecificity has also been reported for pancreatic (40, 41) and lipoprotein lipases (42, 43). However, it is known that several factors, such as the type of detergent, the presence of organic solvents, temperature, and length of the acyl chain in the TG, can affect the apparent stereospecifity of some lipase (41, 43–45). Therefore, a definitive conclusion about the stereospecificty of the fat body TG-lipase will require more extensive analysis.

Under conditions of energy demand, the fat body releases *sn*-1,2-DG into the hemolymph. The pathway for its formation is unknown and three alternatives have been considered: *1)* the stereospecific hydrolysis of TG into *sn*-1,2-DG (11, 13, 14); *2)* the hydrolysis of TG to *sn*-2-MG followed by stereospecific acylation of *sn*-2-MG (15, 16); and *3)* de novo synthesis of DG from glycerol-3-phosphate via phosphatidic acid using the fatty acids produced by TG hydrolysis. The direct conversion of stored TG to *sn*-1,2-DG in *M. sexta* fat body seems unlikely due to the following observations. *1)* The endproducts of TG hydrolysis produce in vitro under initial reaction conditions were free fatty acids (66%), *sn*-2-monooleoylglycerol (24%), *sn*-1,2(2,3)-dioleoylglycerol (7%), and glycerol (3%); and *2)* the lack of stereospecificity of the purified lipase. Assuming that TG hydrolysis in vitro proceeds in a manner similar to that observed in these in vitro experiments and that there is not an MG-lipase present in fat body, which would convert *sn*-MG to free glycerol, all the properties of the purified lipase are consistent with the suggestion that the acylation of *sn*-2-MG could be the most important pathway for *sn*-1,2-DG synthesis in *M. sexta* fat body.

During studies on phosphorylation of the enzyme we noted that the enzyme was inhibited by ATP and other nucleotides at concentrations comparable to those found

in fat body (46); however, the physiological importance of this observation is unknown at present. Because the TG-lipase was assayed in the presence of EDTA, it seems unlikely that the role of Ca^{2+} in activating lipolysis is a direct effect on the TG-lipase. Rather, the Ca^{2+} most likely plays some second messenger role.

The availability of the pure TG-lipase, presumably the first enzyme involved in lipid mobilization from the fat body, will permit additional studies on the mechanism of the hormonal regulation of this important metabolic pathway in insects, including an investigation of the role of phosphorylation of the enzyme and the possible regulatory importance of enzyme inhibition by ATP and other nucleotides.

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