The main triglyceride-lipase from the insect fat body is an active phospholipase A₁: identification and characterization

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Abstract  The main triglyceride-lipase (TG-lipase) from the fat body of *Manduca sexta* has been identified as the homolog of *Drosophila melanogaster* CG8532. This protein is conserved among insects and also shares significant sequence similarity with vertebrate phospholipases (PLs) from the phosphatidic acid preferring-phospholipase A1 (PA-PLA₁) family. It is shown here that the TG-lipase is also a PL. TG-lipase and PL activities copurify and are inhibited by, or resistant to, the same lipase inhibitors, indicating that both activities are catalyzed by the same enzyme and active site. The PL activity of TG-lipase corresponded to PL type A₁. The concentration dependence of lipase activity with TG and PL micellar substrates showed saturation kinetics, with apparent *Kₐₐₐ* values of 152 ± 11 and 7.8 ± 1.1 M, respectively. TG-lipase was able to hydrolyze the major phospholipid components of the lipid droplets, phosphatidylcholine and phosphatidylethanolamine. The enzyme hydrolyzes 77 molecules of TG for every molecule of PL contained in the lipid droplets. It was observed that the activation of lipolysis in vivo is accompanied by activation of the hydrolysis of phospholipids of the lipid droplets. These results suggest that the PL activity of the insect TG-lipase could be required to allow access of the lipase to TG molecules contained in the core of the lipid droplets.

Supplementary key words  adipokinetic hormone • lipolysis • *Manduca sexta*

The fat body is the principal organ for the storage of lipids in insects. Triglycerides (TGs) constitute the main lipid form, representing ~90% of the total fat body lipids (1, 2). The content of TG in the fat body is influenced by several factors, including development stage, nutritional state, sex, and migratory flight. In the tobacco hornworm, *Manduca sexta*, which is widely used as a model insect, the content of fat body TG increases continuously until the end of the larval (feeding) period, from a few micrograms to ~80 mg (3). During subsequent development, lipid reserves are mobilized to sustain the life of the adult insect (moth), which feeds occasionally (4, 5). TG is stored in fat body adipocytes as lipid droplets within the cytoplasm (6). Unlike vertebrates, in which stored fatty acids are mobilized as free fatty acids, most fatty acids are released from the fat body to hemolymph as sn-1,2-diacylglycerol (DG) (2, 7). In the circulation, DG is carried by lipophorin, the insect lipoprotein, for delivery to tissues (e.g., the flight muscle) and ovaries, where it is hydrolyzed to fatty acids by a membrane-bound lipophorin-lipase (8).

TG lipolysis is under hormonal regulation by the neuropeptide adipokinetic hormone (AKH) (9), which elicits a glucagon-like action mediated by a G protein-coupled receptor that activates both inositol phosphate and cAMP signaling responses (10, 11). In *M. sexta*, the effect of AKH on the mobilization of energy reserves is dependent on the developmental stage. During the larval stage, AKH mobilizes glycogen through the activation of glycogen-phosphorylase, whereas it promotes a massive lipolytic response in the adult stage (12). The lipolytic response induced by AKH is associated with a rapid activation of fat body cAMP-dependent protein kinase A (PKA) and a sustained increase in calcium influx (13). The mobilization of TG is mediated by lipolytic enzymes. The major TG-lipase of the fat body has been purified from adult *M. sexta* (14). This is a cytosolic enzyme with a molecular mass of 76 kDa that can be phosphorylated in vitro by PKA. Contrary to expectations, phosphorylation of the lipase induces only minor changes in the lipase activity (15, 16). TG-lipase is constitutively phosphorylated, and the level of phosphorylation remains constant when lipolysis is stimulated (17). Recent in vivo and in vitro experiments have shown that the enzyme does not bind tightly to the lipid droplets and that its activity is highly correlated with the phosphorylation level of a lipid droplet-associated protein, Lsdp1 (16). AKH-induced lipolysis pro-
vokes a rapid phosphorylation of Lsdp1, a protein that shares a small region of sequence identity with perilipin A from mammalian lipid droplets (18), and this event accounts for the majority of the lipolytic response induced by AKH (16, 17).

Although significant details of the mechanism of lipolysis are emerging, these studies and many studies carried out in adipocytes of vertebrates clearly show that the activation of lipolysis is a complex process that involves cytosolic proteins and lipid droplet-associated proteins (19). The details of the interactions and signals that ultimately lead to an increase in the rate of TG hydrolysis remain to be elucidated. Here, we report the identification of the TG-lipase from *M. sexta* as the homolog of CG8552 (FlyBase annotation) from the fruit fly, *Drosophila melanogaster* (Dme). This study shows that the enzyme is conserved among insects and shares significant sequence similarity with vertebrate phospholipases (PLs) from phosphatidic acid preferring-phospholipase A1 (PA-PLA1). The identification of the lipase prompted us to study its possible PL activity. Our data demonstrate that fat body TG-lipase has PLA₁ activity, which allows the hydrolysis of the phospholipid monolayer of the lipid droplets. This finding suggests that the PL activity of the insect lipase is sufficient to allow access of the lipase to TG, which for the most part is contained in the core of the lipid droplets.

### MATERIALS AND METHODS

**Materials**

[^32P]orthophosphate was purchased from MP Biochemicals (Irvine, CA). L-α-l-Palmitoyl-2-[1-14C]oleoyl-sn-glycerol-3-phosphocholine was purchased from American Radiolabeled Chemicals (St. Louis, MO). [Tri-9,10-3H(N)]oleoylglycerol was purchased from American Radiolabeled Chemicals (St. Louis, MO). [32P]orthophosphate was purchased from MP Biochemicals (Irvine, CA). DNA sequencing was performed by the Core Facility of our laboratory (22). Protein sequence analysis by Edman degradation dem mass spectrometry (MS/MS) spectra were correlated with a Finnigan LCQ DECA XP Plus quadrupole ion trap tandem mass spectrometer. The tandem mass spectrometry (MS/MS) spectra were correlated with a known algorithm and the Sequest program developed in that laboratory (22). Protein sequence analysis by Edman degradation of some peptides separated by HPLC after trypsin digestion was also performed at the same facility.

**Experimental insects**

*M. sexta* eggs were purchased from Carolina Biological Supplies, and larvae were reared on artificial diet (20). Adult insects were maintained at room temperature without food. To achieve consistent basal levels of lipolysis, the insects were decapitated 24 h before the experiments and injected with 15 mg of trehalose 2 h before the experiments (21).

**Purification of TG-lipase**

TG-lipase was purified from the cytosolic fraction of *M. sexta* fat body homogenates as reported previously with minor modifications (14). Fat body tissue from 200 insects was collected in ice-cold homogenization buffer (buffer H: 20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l leupeptin, 1 mg/l aprotinin, and 0.1% 2-mercaptoethanol). The tissue was homogenized with a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle and centrifuged at 100,000 g for 1 h. The cytosolic fraction was used to purify TG-lipase using a combination of anion-exchange (DEAE Sepharose and Q Sepharose), hydroxyapatite, and hydrophobic interaction (Phenyl Sepharose) chromatography.

**Protein identification by mass spectrometry**

Purified TG-lipase was separated by SDS-PAGE on an 8% gel. The gel was stained with Coomassie blue, and the band was excised from the gel. The protein was cleaved with trypsin and then sequenced by microcapillary reverse-phase high pressure liquid chromatography-nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer at the Harvard Microchemistry Facility. The tandem mass spectrometry (MS/MS) spectra were correlated with a known algorithm and the Sequest program developed in that laboratory (22). Protein sequence analysis by Edman degradation of some peptides separated by HPLC after trypsin digestion was also performed at the same facility.

**Expression and partial purification of recombinant CG8552**

Total mRNA from adult Dme was reverse-transcribed using oligo(dt)₁₅ primer. The resulting cDNA was used to amplify the coding region of CG8552 (accession number NP_509185) from position 4,203, corresponding to the second methionine, amino acid residue 1,354, to the C-terminal amino acid residue, 2,016, by PCR. The primers were 5’-GAGGAGCACAGATGGCAGTGCCAGCGGAAAAGGGCACA-C and 5’-GAGGAGAACCGCGTTCAATGCGGACACAGGAGTGGCGTACG. The 5’ end of the primers incorporated the ligation-independent cloning (LIC) sequences (underlined). The amplified product was ligated into the vector pIEx-1Ek/LIC after being treated with LIC-qualified T4 DNA polymerase. pIEx vector contains N-terminal His tag and S tag coding sequences and is designed for transient transfection and protein expression in Sf9 cells. The generated plasmid (pIEx-CG8552) was then transformed into *E. coli* strain NovaBlue, and the positive clones were confirmed by DNA sequencing. Sf9 (Spodoptera frugiperda) cells that were cultured in SF9-900 II SFM medium were transfected with pIEx-CG8552 dissolved in Insect Gene Juice (Novagen, EMD Biosciences) according to the manufacturer’s instructions. Suspension cultures were grown at 28°C with shaking at 140 rpm. Cells contained in 30 ml of culture were harvested 48 h after transfection and sedimented by centrifugation. Cells were resuspended in 5 volumes of cold homogenization buffer (20 mM phosphate, pH 7.8, 20% glycerol, 5 mM DTT, 1 mM EDTA, and 1 mg/l aprotinin) and lysed by sonication on ice in two steps of 30 s each. The crude lysate was centrifuged first at 300 g for 20 min and then at 100,000 g for 1 h at 4°C to separate cytosolic and membrane fractions. The 100,000 g pellet contained most of the recombinant protein and was used for purification. The pellet that was resuspended in a volume of homogenization buffer equivalent to the volume of the cytosol fraction was combined with an equal volume of a solution containing 20 mM Tris, pH 7.9, 500 mM NaCl, and 2 M urea and incubated for 2 h at 4°C with orbital shaking. The solution was adjusted to 40 mM imidazole and combined with 2 ml of resin preequilibrated with
the same buffer. The slurry was incubated for 4 h. The resin was washed with 10 bed volumes of wash buffer I (20 mM Tris, 500 mM NaCl, 40 mM imidazole, and 0.5 mM DTT, pH 7.9) followed by 5 bed volumes of wash buffer II (20 mM Tris, 500 mM NaCl, 75 mM imidazole, and 0.5 mM DTT, pH 7.9). The flow was stopped, and the column was incubated for 4 h with 1 volume of elution buffer (20 mM Tris, 500 mM NaCl, 75 mM imidazole, 50 mM EDTA, and 0.5 mM DTT, pH 7.9). Fractions were assayed for TG-lipase activity as described below.

Assay for TG-lipase activity

The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02% (w/v) defatted BSA, 0.5 mM EDTA, 2 mM DTT, 0.44 mM \[9,10-\text{3H}\]triolein (1.9 mCi/mm, mol), 2 mM Triton X-100, and 0.5 μg of purified TG-lipase. The mixture was incubated at 37°C with constant shaking. After 30 min, the reaction was terminated by the addition of 500 μl of a mixture of chloroform-methanol-benzene (2:2:1, v/v/v) and 40 μl of 1 N NaOH. Aliquots of 150 μl from the upper aqueous phase were transferred to scintillation vials for counting. Blank reactions did not contain enzyme. Enzyme activity was expressed as nmol FFA/min/mg.

Assays for TG-lipase activity of recombinant protein were performed under the same conditions described above using \[9,10-\text{3H}\]triolein (7.6 mCi/mm) as substrate. The reaction was stopped by the addition of 500 μl of chloroform-methanol (2:1, v/v) and 40 μl of 1 N HCl. Radiolabeled lipids from the organic phase were separated by TLC using hexanes:ethyl ether:formic acid (70:30:3, v/v/v) as the developing solvent. Regions of the plate corresponding to TG, DG, monoacylglycerol (MG), and FFA were scraped and quantified by liquid scintillation counting.

Assay of PL activity

The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02% (w/v) defatted BSA, 0.5 mM EDTA, 2 mM DTT, 2 nmol of 1-palmitoyl-2-[\(1\text{-14C}\)]oleoyl-sn-glycero-3-phosphocholine (18 mCi/mm mol), 2 mM Triton X-100, and 0.5 μg of purified TG-lipase. The mixture was incubated at 37°C with constant shaking. After 30 min, the reaction was terminated by the addition of 500 μl of chloroform-methanol (2:1) and 5 μl of 6 N HCl. The organic phase was collected and dried. The lipids were separated by TLC on Si Gel K6 plates using chloroform-methanol-water (50:25:4) as the developing solvent. Spots corresponding to phosphatidylcholine (PC), lysophosphatidylcholine (LPC; PLA1 activity), and FFA (PLA2 activity) were scraped, and their radioactivity was determined by liquid scintillation counting. Blank reactions did not contain enzyme. Enzyme activity was expressed as nmol LPC and FFA/min/mg. The activity against phosphatidic acid (PA) was assayed under the same experimental conditions. PA was prepared by incubating 1-palmitoyl-2-[\(1\text{-14C}\)]oleoyl-sn-glycerol-3-phosphocholine with PLD (type IV from cabbage). PA was purified by TLC and eluted from silica using a mixture of chloroform-methanol-acetic acid-water (50:30:1:10).

In inhibition studies, the purified enzyme was preincubated for 10 min at room temperature with inhibitor before the measurement of activity.

Preparation of endogenously \([^{32}\text{P}]\)phospholipid-labeled lipid droplets

Insects were injected with 200 μCi of \([^{32}\text{PO}_4]\)orthophosphate. After 36 h, insects were decapitated and injected with trehalose as indicated above. Tissue from two insects was pooled and homogenized in 6 ml of buffer H. Lipid droplets were purified as described previously (16). Typically, lipid droplets of two insect fat bodies were resuspended in 0.5 ml of buffer. For lipid droplets under high lipolysis conditions, lipolysis was stimulated by injection of 100 pmol of AKH, whereas injection of buffer provided the basal lipolysis. The adipokinetic effect was confirmed by the phosphorylation of Lsdpl, the major phosphoprotein of the lipid droplet, whose phosphorylation is induced by AKH, which was monitored by SDS-PAGE and autoradiography (data not shown).

Lipid composition of lipid droplets

Total lipids were extracted by adding 5 volumes of chloroform-methanol (v/v) (23). The lipids in the extracts were separated by TLC using hexane-ethyl ether-formic acid (70:30:3, v/v/v) as the developing solvent (24). Plates were sprayed with 3% cupric acetate in 8% orthophosphoric acid (v/v) and heated at 200°C for charring (25). Plates were scanned on an imaging densitometer (Bio-Rad model GS-700). The intensity of spots was quantified using the Multi Analyst Macintosh software. Results were expressed as percentage of total lipids (PL, MG, DG, TG, FFA, cholesterol, and cholesteryl ester). Neutral glycerides were determined in the lipid extracts using the Infinity TG reagent kit as described by the manufacturer (ThermoTrace, Ltd., Melbourne, Australia). Phospholipids were determined by measuring inorganic phosphorous after digestion in deionized water and perchloric acid for 1 h at 180°C followed by the addition of ammonium molybdate and ascorbic acid (26). The sample was further heated for 5 min in a boiling water bath and cooled, and the absorbance was read at 800 nm to quantify total phosphorous. The mole ratio of neutral glycerides to PL was 102 ± 9.4:1.

Polar lipids from lipid droplet lipid extract were separated by TLC using chloroform-methanol-28% ammonia (65:25:5) or chloroform-methanol-acetic acid-water (50:30:8:4) as the developing solvent. Individual phospholipids of the lipid droplets were made visible by charring as described above. Phospholipids were identified by comparison with standards run on the same plates. When analyzing \([^{32}\text{P}]\)phospholipid-labeled lipid droplets, \([^{32}\text{P}]\)phospholipid forms were visualized by autoradiography. The intensity of phospholipid spots from the plate and autoradiogram scans was quantified as indicated above. Results were expressed as percentage of total phospholipids and total radioactive activity, respectively.

PL and TG-lipase activity against \([^{32}\text{P}]\)labeled lipid droplets

An aliquot of lipid droplet preparation containing 1 nmol of PL and 100 nmol of TG was transferred to a glass tube containing TG-lipase reaction buffer. The reaction was initiated by adding TG-lipase (3 μg) in a final volume of 150 μl. Final reaction conditions were 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02% (w/v) BSA, 2 mM DTT, 0.60 mM TG, and 0.17 mM Triton X-100 (16). The mixture was gently vortexed for 20 s and incubated at 37°C with constant shaking. After 30–45 min, the reaction was terminated by the addition of 750 μl of chloroform-methanol (2:1) and 5 μl of 6 N HCl. The organic phase was collected and dried. The lipids were separated by TLC using chloroform-methanol-ammonia (65:25:5) as the developing solvent. \([^{32}\text{P}]\)labeled phospholipids were visualized by autoradiography. Autoradiograms were scanned and analyzed as described above. Blank reactions in which TG-lipase was omitted were used to obtain a basal level of distribution of radioactivity. For TG-lipase activity, the spots corresponding to FFA (visualized by I₂ vapors) were scraped from the plate, and fatty acids were eluted from silica using chloroform. The amount of FFA was estimated using the nonesterified fatty acid detection kit as described by the manufacturer (Wako Chemicals USA, Inc.).
Other methods

Protein concentrations were determined by the Bradford (27) dye-binding assay using BSA as a standard. SDS-PAGE was performed according to Laemmli (28), and proteins were visualized by Coomassie Brilliant Blue R staining. Two different anti-lipase antibodies were generated in chickens at Cocalico Biologicals (Reamstown, PA). The first antibody was produced using purified M. sexta TG-lipase as antigen. A second antibody was obtained using a mixture of two peptides (ERPVSHESSVSHSL and VGRVEVLPISWHGHLHSEE) that were coupled to Keyhole limpet hemocyanin (KLH). Anti-S tag monoclonal antibody was purchased from Novagen. Immunodetection was performed using the corresponding horseradish peroxidase-conjugated secondary antibody and ECL chemiluminescence reagents (Amersham Biosciences).

Statistical comparisons were made using Student’s t-test. P < 0.05 was considered significant.

RESULTS

Identification of fat body TG-lipase

To identify the TG-lipase from the insect fat body, TG-lipase from M. sexta was purified to homogeneity according to a previously reported procedure (14) in which the enzyme activity is monitored using [3H]triolein as substrate. The final preparation showed a single band in two-dimensional PAGE with a relative mass of 74–76 kDa and an isoelectric point of 5.8–6.0. The protein band obtained after separation by SDS-PAGE on 8% acrylamide was excised and subjected to identification by LC/MS/MS as indicated in Materials and Methods. LC/MS/MS revealed the presence of three peptide sequences that matched DmeCG8552-PA: CSWFYK, SVEEVVDDFR, and MHLELK. Figure 1 shows the region of CG8552-PA (accession num-

![Fig. 1. Amino acid sequence alignment of the partial deduced sequences from transcript CG8552 from Drosophila melanogaster (Dme; fruit fly) and corresponding homologs from Anopheles gambiae (Aga; mosquito) and Apis mellifera (Ame; honey bee). The amino acid microsequence obtained from Manduca sexta is underlined. The numbering at the beginning and end of the sequences indicates the position of that amino acid in the deduced amino acid sequence. Lines in the sequences indicate gaps introduced by the program (ClustalW) to optimize the alignment. Identical residues in all of the sequences are denoted by asterisks; conservative substitutions are denoted by dots. The putative lipase catalytic site is boxed. Conserved domains are boxed in gray.](image-url)
ber NP_609185) in which these peptides are found. This region is 662 amino acids long and extends from the second methionine (amino acid residue 1,354) to the C terminus (amino acid residue 2,016). In addition, internal sequence of M. sexta TG-lipase was obtained by Edman degradation. The following peptide sequences were obtained: VEVLPLIS, ATSLQLVQSHYK, and YHWFYSVDVEDK. The sequence VEVLPLIS is found in CG8552-PA, whereas the other two peptides shared a high degree of identity with two regions also present in CG8552-PA (Fig. 1).

Partial transcripts from Anopheles gambiae (accession number XP_312576) and Apis mellifera (accession number XP_392149) similar to CG8552 have been reported. Both translates also contain similar sequences to those found in M. sexta TG-lipase (Fig. 1). The complete coding sequence of CG8552 from Dme encodes a product of 2,016 amino acid residues. The alignment of the deduced amino acid sequences from different insects shows a region of high conservation (53–58% identity) localized between amino acids 1,354 and 2,016 of the Dme product (Fig. 1). This region includes the lipase consensus sequence (GXSXG), containing the active site serine essential for catalysis (29) (Fig. 1).

In addition, two conserved domains, a DDHD domain (accession number PF02862) and a WWE domain (accession number PF02825) are also found in the same region. The DDHD is a long domain (180 residues) toward the C terminus named for these four residues, which may form a metal binding site (Fig. 1). The WWE domain, named for three of its conserved residues, is localized between amino acid residues 1,368 and 1,446 of the Dme protein (Fig. 1). The WWE domain has been identified in diverse proteins with predicted ubiquitin- and ADP-ribosylation-related functions, and it is predicted to mediate specific protein-protein interactions (30).

To confirm the identification of the M. sexta TG-lipase as the homolog of CG8552 from the fruit fly, the region localized between the second methionine (amino acid 1,354) and the C terminus (amino acid 2,016) of the Dme product shown in Fig. 1 was cloned and expressed in the insect cell line Sf9 as indicated in Materials and Methods. Samples from all chromographic steps were analyzed by Western blot using the S tag antibody. A single band of the predicted size was displayed in homogenates of transfected cells and was absent in the corresponding control cells (Fig. 2A). In addition, Western blot analysis using two antibodies that react with M. sexta lipase was also performed. The first antilipase antibody was generated against purified M. sexta TG-lipase, whereas the second antilipase antibody was produced using a mixture of two peptides found in the CG8552-PA sequence from Dme. Figure 2B shows the Western blots of purified M. sexta TG-lipase (76 kDa) and recombinant CG8552 (80.6 kDa) probed with different antibodies. The antibody against M. sexta TG-lipase recognized the recombinant protein from CG8552 (Fig. 2B, panel 1). On the other hand, the antibody against the peptides recognized M. sexta TG-lipase (Fig. 2B, panel 2). As expected, the antibody against the S tag recognized only the fusion protein and did not react with purified M. sexta TG-lipase (Fig. 2B, panel 3). The immuno cross-reactivities of the proteins and the presence of the lipase consensus sequence in CG8552-PA are consistent with the identification of M. sexta fat body TG-lipase based on the MS/MS study and the internal peptide sequences obtained by Edman degradation.

For further characterization, the recombinant protein was affinity-purified and its ability to hydrolyze TG was determined. The majority (82.1 ± 5.7%) of the recombinant CG8552 protein was associated with the membrane fraction (Fig. 3A). The pellet was incubated in the presence of 1 M urea as described in Materials and Methods. The mixture was adjusted to 40 mM imidazole and incubated with Ni Sepharose resin. Under these conditions, most of the fusion protein (87 ± 13%) bound to the resin. After extensive washes with 40 and 75 mM imidazole, the fusion protein was eluted with 50 mM EDTA in the presence of 75 mM imidazole. Elution of the fusion protein can be done with 200 mM imidazole. However, imidazole was avoided because at high concentration it inhibited M. sexta TG-lipase activity. The fractions were analyzed by
Western blot and TG-lipase activity. The fraction eluted with 50 mM EDTA contained recombinant protein and also exhibited TG-lipase activity (130 ± 4 nmol TG hydrolyzed/min/mg) (Fig. 3B).

A Basic Local Alignment Search Tool search revealed two proteins with high sequence similarity in two regions of CG8552: a PLA1 (KIAA0725p) and p125, a Sec23-interacting protein. CG8552 showed 40.9% and 34.3% identity in a 696 and 1,000 amino acid overlap with KIAA0725 and p125, respectively. Likewise, a region of CG8552 (321 amino acids) containing the lipase conserved sequence exhibited 30.5% identity with the phosphatidic acid-prefering PLA1 (PA-PLA1; accession number Q8NEL9) (data not shown). Of note, the lipase consensus sequence in PA-PLA1 has a serine residue instead of glycine (SHSLG) (31). In mammals, these three proteins (KIAA0725p, p125, and PA-PLA1) are referred as the PA-PLA1 family (32).

**PL and TG-lipase activities copurify and are inhibited by or resistant to identical lipase inhibitors**

Preliminary experiments with *M. sexta* purified lipase indicated that the enzyme had PL activity. To confirm that this activity was inherent to the TG-lipase rather than to a contaminant, fat body TG-lipase was purified by monitoring the lipase activity against [3H]triolein and determining contaminant, fat body TG-lipase was purified by monitoring this activity was inherent to the TG-lipase rather than to a contaminant. Table 1 shows TG-lipase and PL activities during the purification of TG-lipase from *M. sexta* fat body. The preparations showed a significantly higher activity hydrolyzing TG than PL, and both specific activities were increased with the purification. The final preparation represented a 4,000-fold purification of TG-lipase and showed a major protein band in SDS-PAGE of molecular mass 76 kDa (Fig. 4A). Western blot analysis confirmed that the band corresponds to TG-lipase (Fig. 4B). The proportion of TG versus PL hydrolyzed was used to assess the progress of the lipase purification. This ratio increased during the first steps of the purification, indicating the presence of other PLs. However, the ratio remained relatively constant through the last three chromatography steps. At the same time, the degree of purification of the TG-lipase was increased, as judged by the specific activity and protein composition observed on the SDS-PAGE gel. Together, the data indicate that the PL activity associated with the TG-lipase is an intrinsic activity of the enzyme. Moreover, Ni affinity-purified recombinant CG8552 protein was also assayed for PL activity. The recombinant protein hydrolyzed [14C]PC at a rate of 0.35 ± 0.01 nmol/min/mg. This result provides further support to the finding that *M. sexta* TG-lipase also possesses PL activity.

The specificity of the PL activity of the insect lipase was determined using 1-palmitoyl-2[1-14C]oleoyl-sn-glycerol-3-phosphocholine solubilized in Triton X-100 micelles. TLC analysis of the radiolabeled products of PL hydrolysis showed that 70% was [14C]LPC and 30% was 14C-labeled fatty acid, indicating a lipase preference for the sn-1 position. Moreover, as shown previously for the TG-lipase activity, the PL activity was calcium-independent.

Given the homology found between *M. sexta* lipase and vertebrate PLs from the PA-PLA1 family, it was interesting to know whether the *M. sexta* TG-lipase hydrolyzes PA. The enzyme hydrolyzed PA at a lower rate than PC under the same assay conditions, 1.07 ± 0.04 and 2.85 ± 0.04 nmol/ min/mg, respectively. Likewise, TG-lipase showed a strong preference for the sn-1 position of PA.

PL inhibitors were evaluated for their abilities to discriminate between PL and TG-lipase activities. 7,7-Dimethyleicosadienoic acid and bromoenoil-lactone, which are PLA2 inhibitors, were unable to inhibit PL or

### Table 1. TG-lipase and PL activity during the purification of TG-lipase from *M. sexta* fat body

<table>
<thead>
<tr>
<th>Variable</th>
<th>TG-Lipase</th>
<th>PL</th>
<th>Ratio of TG to PL Hydrolyzed</th>
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<tbody>
<tr>
<td>Cytosol</td>
<td>0.27 ± 0.04</td>
<td>0.007 ± 0.001</td>
<td>0.12 ± 0.001</td>
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<tr>
<td>DEAE I</td>
<td>8.5 ± 0.4</td>
<td>0.04 ± 0.004</td>
<td>0.064 ± 0.003</td>
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<tr>
<td>DEAE II</td>
<td>17.2 ± 0.6</td>
<td>0.08 ± 0.001</td>
<td>0.098 ± 0.005</td>
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<tr>
<td>Phenyl Sepharose I</td>
<td>38.0 ± 1.8</td>
<td>0.13 ± 0.002</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>565 ± 4</td>
<td>1.00 ± 0.01</td>
<td>0.58 ± 0.06</td>
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<tr>
<td>Q Sepharose</td>
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<td>1.50 ± 0.02</td>
<td>0.80 ± 0.04</td>
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<tr>
<td>Phenyl Sepharose II</td>
<td>1086 ± 8</td>
<td>2.21 ± 0.07</td>
<td>0.89 ± 0.11</td>
</tr>
</tbody>
</table>

PL, phospholipase; TG-lipase, triglyceride-lipase. Lipase activities hydrolyzing [3H]triolein and 1-palmitoyl-2-[1-14C]oleoyl-sn-glycerol-3-phosphocholine were determined after each purification step as described in Materials and Methods. Data represent means ± SEM (n = 3).

**Fig. 4. M. sexta** fat body TG-lipase purification. A: SDS-PAGE. Lanes a, b, first and second DEAE (20 and 10 µg, respectively); lane c, first Phenyl Sepharose (8 µg); lane d, hydroxyapatite (4.5 µg); lane e, Q Sepharose (2.5 µg); lane f, second Phenyl Sepharose (1 µg). B: Western blot of the same fractions. Ten micrograms of protein was loaded in lanes a–c; 2 µg was loaded in lane d; and 1.4 and 1 µg was loaded in lanes e and f, respectively. The faint band at ~92 kDa in lane f was identified by MALDI-TOF as glycogen phosphorylase.
TG-lipase activities at the expected concentrations. On the other hand, the PL activity was sensitive to MAAP, a methyl arachidonyl fluorophosphonate analog of arachidonic acid, which binds irreversibly to serine residues and is a potent PL inhibitor (IC50 = 0.5–5 μM) (33). Preincubation of the enzyme with 5 μM MAAP inhibited both lipase activities to a similar extent (83.5 ± 8.2% and 84.5 ± 2.0% inhibition of PL and TG-lipase, respectively), providing further support to the notion that a single active site is involved in the hydrolysis of PL and TG.

The concentration dependence of lipase activity with TG and PL substrates determined in micellar substrates of Triton X-100 micelles at pH 7.9, 0.5 M NaCl, 0.5 mM EDTA, and 2 mM DTT showed saturation kinetics with apparent Km values of 152 ± 11 and 7.8 ± 1.1 μM, respectively, and Vmax values of 560 ± 10 and 5.5 ± 0.3 nmol/min/mg, respectively. These values indicate that the enzyme has a strong (5-fold) preference for acylglycerides over phospholipids.

**PL activity against the native substrate (lipid droplets)**

The fact that TG-lipase has PL activity against the micellar substrate suggested that the enzyme may catalyze the hydrolysis of native phospholipids present on the surface of the lipid droplets. As shown in Table 2, lipids of lipid droplets isolated from fat tissue of *M. sexta* consist of TG with small amounts of PL, DG, FFA, and cholesterol ester. This lipid composition is consistent with the lipid compositions of lipid droplets isolated from other sources (34, 35). PC and phosphatidylethanolamine (PE) were the main phospholipid forms, representing 60% of total phospholipids (Table 2). Although most lipid droplets isolated from other systems also exhibited PC as the major phospholipid component, a higher content of PE and lysoPL was observed in *M. sexta* lipid droplets than in lipid droplets from vertebrate adipose tissue or plants.

To assay PL activity of TG-lipase against the lipid droplets, cellular phospholipids were radiolabeled in vivo and lipid droplets were isolated. The distribution of radioactivity among radiolabeled phospholipids of lipid droplets was as follows: PC and PE represented 39.8 ± 1.9%, whereas radioactive LPC and lysoPE represented 14.9 ± 0.6% and 20.2 ± 0.3% of total radioactive phospholipids, whereas radioactive LPC and lysoPE represented 14.9 ± 0.5% and 9.6 ± 1.2%, respectively. Phosphatidylserine and phosphatidylinositol combined represented 14.6 ± 0.14%, and sphingomyelin represented 3.0 ± 0.3% of total radioactive phospholipids. The distribution of radioactivity among phospholipids was very close to the distribution of mass (Table 2), indicating that the radiolabeling procedure yielded homogenously radiolabeled phospholipids. 32P-labeled lipid droplets were incubated with purified TG-lipase under the conditions identified previously to measure TG-lipase activity against the native substrate (16). After reaction, the main PL classes were separated by TLC and quantified by autoradiography. The decrease of radioactivity associated with PC and PE, and the concomitant increase of radioactivity in LPC and lysoPE, proved that the enzyme has the ability to hydrolyze both PC and PE (Fig. 5A, B).

A simultaneous analysis of the TG-lipase activity hydrolyzing PL and TG contained in the lipid droplets showed that the enzyme hydrolyzes 77 molecules of TG for every molecule of PL contained in the lipid droplets (Table 3). The ratio of hydrolysis of TG to PL determined with lipid droplets was much lower than the corresponding ratio obtained with separate micellar substrates under similar conditions. Using the Km and Vmax values obtained with micelles, and the concentrations of TG and PL found in the lipid droplets, one would expect a ratio of activities of 750. The fact that the ratio of hydrolysis of TG to PL...

**TABLE 2. Lipid composition of the lipid droplet isolated from *M. sexta* fat body**

<table>
<thead>
<tr>
<th>Total Lipids</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>TG</td>
<td>87.8 ± 0.4 PC</td>
</tr>
<tr>
<td>1,2-Diacylglycerol</td>
<td>3.5 ± 0.4 PE</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>3.4 ± 0.2 Lysophos</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>3.2 ± 0.5 LysoPE</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.6 ± 0.2 Phosphatidylserine + phophatidylinositol</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>0.8 ± 0.1 Sphingomyelin</td>
</tr>
</tbody>
</table>

PC, phosphatidylcholine; PE, phosphatidylethanolamine. Values of total lipid composition are expressed as percentage of total lipids. Values of phospholipid composition are expressed as percentage of total phospholipids. Data represent means ± SEM (n = 3).

**Fig. 5.** Phospholipase (PL) activity of TG-lipase against endogenously [32P]PL-labeled lipid droplets. A: TG-lipase was incubated with [32P]PL-radiolabeled lipid droplets that were isolated from insects with basal lipolytic activity. PL classes were separated by TLC and quantified by autoradiography and densitometry. The figure shows the autoradiogram of a representative TLC plate obtained with control lipid droplets (no lipase; lane a), lipid droplets incubated with 3 μg of purified TG-lipase (lane b), and lipid droplets incubated with 6 μg of purified TG-lipase (lane c). All reactions contained 1 nmol of total phospholipids (100 nmol of TG) and were incubated for 45 min. PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPE, lysoPE; LPC, lysoPC. B: PL activity expressed as nmol PC and PE hydrolyzed/min by 3 and 6 μg of TG-lipase. To calculate the amount of PC and PE hydrolyzed, the values of the blank (incubation of the substrate with 0 μg of TG-lipase) were subtracted from the corresponding amounts of PC and PE. Data represent means ± SEM (n = 4).
determined with lipid droplets was much lower than the ratio estimated from the data obtained with micelles is indicative of the lower accessibility of TG in the lipid droplets. On the other hand, consistent with a surface location of PC in the lipid droplets, similar PL activities were observed in micelles and lipid droplets (Table 3).

To evaluate a possible role of the PL activity in vivo, the phospholipid compositions of the lipid droplets isolated from control and lipolytically stimulated insects (5, 10, 20, and 30 min after hormonal injection) were analyzed. This study showed that the relative contents of PE and PC decreased slightly in the lipid droplets isolated from stimulated tissue. Significant differences in the contents of the major phospholipids of the lipid droplets were found 10 and 20 min after hormonal stimulation (Fig. 6A). The reduction of PL content was accompanied by a greater reduction in the content of lipid droplet TG (Fig. 6B).

**DISCUSSION**

Lipolysis of TG is extremely active during long-term flight in insects such as *M. sexta*, and this process can be mimicked by the injection of AKH in the hemolymph (7). Mobilization of TG stores from lipid droplets is catalyzed by TG-lipases. The fat body of *M. sexta* has a major cytosolic TG-lipase that is the only insect TG-lipase purified and characterized (14). This study shows that TG-lipase is the homolog of Dme CG8552. Based on homology searches, the predicted functions for this gene are PLA₁ activity and metal ion binding. This work shows that a major function of CG8552 is the hydrolysis of TG in insect adipocytes and confirms that, as predicted, the enzyme has PL activity. Furthermore, this gene appears to be conserved among insects (Fig. 1).

CG8552 has a 6.5 kb transcript that encodes a 214 kDa putative protein. This transcript is larger than the *M. sexta* TG-lipase. However, analysis of the coding sequence using GeneMark.SPL identified the presence of alternative start codons. Although the consensus sequence for *Drosophila* (36) is not present at either of these start codons, according to Kozak (37), the sequence context in the proximity of the second start codon (at position 4,203) seems more favorable for the initiation of translation than the first start codon. This raises the possibility that the functional initiation site could be located at the second start codon. Initiation of translation at the second start codon would result in a 662 amino acid long protein with a calculated molecular mass of 75 kDa and a theoretical isoelectric point of 5.62. These calculated parameters coincide with...

**TABLE 3. Lipase activity of TG-lipase against native and artificial substrates**

<table>
<thead>
<tr>
<th>Substrate Assay Conditions</th>
<th>Lipase Activity</th>
<th>TG</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid droplets 600 μM TG, 2.8 μM PC, 1.4 μM PE, 0.17 mM Triton X-100</td>
<td>77.2 ± 1.2</td>
<td>1.07 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>[³H]triolein in micelles 660 μM TG, 2.7 mM Triton X-100</td>
<td>455 ± 12</td>
<td>1.63 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>[¹⁴C]PC in micelles 4 μM PC, 0.68 mM Triton X-100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TG-lipase and PL activities of the enzyme were determined with the native substrate (lipid droplets) and the artificial substrate (micelles of Triton X-100) as described in Materials and Methods under the conditions specified here. Data represent means ± SEM (n = 3). All reactions were performed in 50 mM Tris, 500 mM NaCl, 0.5 mM EDTA, and 2 mM DTT, pH 7.9.

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Effect of stimulation of lipolysis on the content of the major phospholipids of the lipid droplets. A: [³²P]PL-radiolabeled lipid droplets were isolated from insects with basal and stimulated [5, 10, 20, and 30 min after adipokinetic hormone (AKH) treatment] lipolysis and analyzed by TLC and autoradiography. The intensity of phospholipid spots from autoradiogram scans was quantified as indicated in Materials and Methods. Values are expressed as percentage of total phospholipids. B: The contents of TG and PE plus PC of the lipid droplets are expressed as nmol/µg total lipid droplet protein. Data represent means ± SEM (n = 4–8). * P < 0.05 versus control.
the experimental values obtained with the purified TG-lipase. All of the peptide sequences obtained from M. sexta, as well as the sequence GSHSLG identified as the active site corresponding to the consensus sequence GXSXG characteristic for lipases, are present in that region of 662 amino acids depicted in Fig. 1. The fact that the recombinant protein generated from the second initiation codon of CG8552 to the stop codon that was expressed in Sf9 cells showed both TG-lipase and PL activities supports this possibility. Alternatively, initiation of translation at the first start codon implies that the lipase is the result of posttranslational modification of a much larger precursor. In that case, an unusual protein of ~139 kDa that lacks internal methionine residues would also be produced in addition to TG-lipase.

Comparison between the insect lipase and other proteins

Drosophila CG8552 has significant sequence similarities with the proteins p125, KIAA00725, and PA-PLA1. These proteins share a central and C-terminal region and were considered to form the PA-PLA1 mammalian family (32, 38). KIAA00725 is a cytosolic PLA1 ubiquitously expressed in human tissues whose physiological function is unknown (38), whereas PA-PLA1 is a cytosolic PL highly expressed in human testis that preferentially hydrolyzes PA (31). The expression of these enzymes in adipose tissue is unknown. p125 localizes in endoplasmic reticulum (ER) and has high sequence similarity with KIAA0725. It also contains an N-terminal proline-rich region responsible for the interaction with Sec23p (32), and it seems to be involved in the organization of ER exit sites (39).

At least three distinct regions can be recognized in Drosophila CG8552: the lipase consensus sequence and the conserved domains WWE and DDHD. KIAA00725 is the only member of the PA-PLA1 mammalian family that has all of the regions present in CG8552. Moreover, PA-PLA1 possesses a coiled-coil-forming region and a potential transmembrane domain (31). p125, KIAA00725, and CG8552 also exhibit a potential transmembrane region that is localized in a region that contains the lipase active site. On the other hand, there is no indication of a potential coiled-coil region in CG8552 or KIAA00725, as is seen in PA-PLA1 and p125. Together, this information suggests a closer functional relationship between CG8552 and KIAA00725 than between CG8552 and the other two proteins.

Genes of the PA-PLA1 family were found in yeast (YORO22c), Caenorhabditis elegans (MO3A16), and plants (SGR2) (40). These genes encode proteins that share less similarity with TG-lipase. The proteins of this family have in common a sequence containing a lipase active site and a DDHD domain. However, PL activity is exhibited by PA-PLA1 (41), KIAA00725 (38), and CG8552 (this study) but not by p125 (38) and SGR2 (40). The biological function of proteins of the PA-PLA1 family is unknown or not well defined. This study identifies CG8552 as a major player in the hydrolysis of TG in insects. This information could be beneficial for the characterization of other proteins of this family.

The lipase identified in this study is unrelated to the known TG-lipases identified to date from vertebrate adipocytes. The fat body TG-lipase shares several functional similarities with hormone-sensitive lipase (HSL) (42), such as its preference for the primary ester bonds of TG, faster hydrolysis of DG than TG (2- and 10-fold for TG-lipase and HSL, respectively), phosphorylation by PKA, and reducing conditions for activity. On the other hand, HSL has no detectable PL activity (43), and it does not have significant sequence similarity. In addition to the extensively studied HSL, four new TG-lipases from vertebrate adipocytes have been disclosed recently (44–46). Interestingly, three of them, adiponutrin, TTS2.2, and GS2, are members of the calcium-independent PLA2 family. These proteins have high TG-lipase and acyl-transacylase activity and much lower PL activity (46). TTS2.2, also designated desnutrin or ATGL, might act coordinately in the catabolism of TG (45), particularly under basal conditions (47). The ATGL homolog in Drosophila has been reported, and a functional role of this lipase on lipid homeostasis in Drosophila has been indicated, because the loss of ATGL activity causes obese flies, whereas its overexpression depletes the fat stores (48). The insect TG-lipase identified in this study is unrelated to the ATGL homolog or any other protein from Drosophila, including the previously reported lipases lip1, lip2, and lip3, that have been identified through homology searches (49).

LPL, HL, pancreatic lipase, and endothelial lipase are enzymes of the lipase gene family that hydrolyze TGs and phospholipids to different extents (50, 51). The ratio of TG-lipase to PL activity for the human lipases is 833, 139.9, 24.1, and 0.65 for pancreatic lipase, LPL, HL, and endothelial lipase, respectively (51, 52). This ratio for M. sexta fat body TG-lipase activities measured with the artificial and natural substrates is 350 and 72, respectively. Within the limitation of this comparison attributable to the difficulties of comparing lipase activity determined under different conditions, these ratios indicate that the lipase activity of M. sexta TG-lipase on phospholipids is intermediate between LPL and PL.

Role of PL activity in the activity of TG-lipase

The action of the lipase on TG contained in the lipid droplets requires its intimate association with the lipid substrate. All types of lipid droplets identified to date are characterized by a hydrophobic core coated by a monolayer of phospholipids embedded with proteins (35). The process by which the lipase gains access to TG is unknown. Given the low solubility of TG in both water and phospholipids, the surface of the lipid droplets is likely to have a very low concentration of TG. Experimental estimations of the solubility of TG in PL bilayers and monolayers indicate that between 1% and 2% of TG could be solubilized on the phospholipid surface (53, 54). A comparison of the kinetic data obtained in this study also suggests that the surface concentration of TG in the lipid droplet is very low. This low surface TG concentration is expected to limit the accessibility of the lipase to the TG substrate and thus the rate of lipolysis. However, in this context, one could envis-
age that the intrinsic PL activity of the lipase could generate a higher local concentration of TG, resulting in a higher rate of lipolysis. As shown in this study, the fat body TG-lipase was able to hydrolyze the main phospholipids of the insect lipid droplets, PC and PE, in vitro. On the other hand, analysis of the phospholipid composition of the lipid droplets isolated from insects under basal and stimulated lipolysis revealed that the lipolytic process stimulated by AKH involves partial hydrolysis of the main phospholipids of the lipid droplets. Together, these results support the notion that the dual PL/TG-lipase activity of the lipase could actually take place in vivo. Based on this information, it is proposed that the PL activity of the insect TG-lipase is sufficient to allow access of the lipase to TG molecules contained in the core of the lipid particles. The fact that several TG-lipases from vertebrate adipocytes that have TG lipase activity also are capable of hydrolyzing phospholipids supports the idea that the dual lipase activity could be a requirement of lipases hydrolyzing TG in lipid droplets. On the other hand, TG-lipases lacking PL activity, such as HSL, could require additional steps of activation to allow the access of enzyme to the core of the lipid droplets, as has been speculated previously (35).

Mechanism of AKH-induced lipolysis of TG

The mechanisms of basal and stimulated lipolysis are complex processes whose details are not fully understood in any system. The complexity of the process derives from the low solubility of the substrate and its products in aqueous medium, which imposes specialized mechanisms, presumably involving multiple steps and proteins. The current information on the mechanism of AKH-induced lipolysis in *M. sexta* anticipates the course of events depicted in Fig. 7. Several aspects of the mechanism remain to be elucidated. Once DGs are formed, they are likely to be transferred to a carrier. TG-lipase could interact with such a carrier, which would bind DG, preventing its hydrolysis and transporting it to the membranes for export to the hemolymph. TG-lipase could interact with other proteins through the WWE domain, which is involved in specific protein-protein interactions (30). The role of this domain in the function of the lipase is unknown. The enzyme could associate with other partners, perhaps with some implication in the regulation of its activity.

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