CHARACTERIZATION OF INSECT

LIPID STORAGE DROPLET PROTEIN I

By

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ABBREVIATIONS

A-Kinase	cAMP dependent protein kinase A
ADRP	Adipose Differentiation-Related Protein
AKH	Adipokinetic Hormone
ATP	Adenosine triphosphate
cAMP	cyclic 3', 5'-adenosine monophosphate
DAG	Diacylglycerol
DEAE	Di Ethyl Amino Ethyl
DMPG	1,2-DiMyristoyl-sn-glycero-3-PhosphoGlycerol
DTT	dithiothretiol
EDTA	Ethylenediamietetraacetic acid
FFA	Free Fatty Acid
FT	Flow thorough
GnRH	Gonadotropin-Releasing Hormone
HDLp	High density lipophorin
HSL	Hormone sensitive lipase
LD	Lipid droplet
LDLp	Low density lipophorin
Lp	Lipophorin
Lsdp-1	Lipid storage droplet protein-1

LTP	Lipid transfer protein
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-flight
μLC/MC/MS	Microcapillary reverse phase HPLC nano-spray tandem Mass Spectrometry
mM	milli Molar
MOPS	3-(*N*-morpholino) propane-sulfonic acid
MG	Monoglyceride
MS	Mass Spectrometry
Peri A	Perilipin A
pI	Isoelectric point
PKA	cAMP dependent protein kinase A
rpm	revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TAG	Triacylglycerol
TIP47	Tail interacting 47-kDa protein
TLC	Thin layer chromatography

CHAPTER I

INTRODUCTION

Triacylglycerols (TGs) constitute more than 90% of lipids stored in insect fat body (Law 1989). They are stored in fat body adipocytes as cytosolic lipid droplets (Willott 1988). A lipid droplet is a macromolecular lipid assembly surrounded by a monolayer of amphipathic phospholipids, glycolipids, sterols and proteins. The fat body is the principal site for storage of both glycogen and lipids, and plays a fundamental role in energy metabolism. It stores triglycerides (TG) and synthesizes most of the proteins found in plasma. Therefore, this tissue accomplishes many of the roles that in vertebrates are carried out by both liver and adipose tissue (Law 1989). Stored lipids serve as the most compact energy form providing more energy than carbohydrates and proteins. TG contains three fatty acids, which are used as substrates during energy requiring processes. Mobilization of stored lipids in the form of energy is extremely important for insects to support life. Insects rely on lipid reserves to survive during physiological non-feeding periods or to meet the energy requirements of developing eggs, flight, and starvation (Patel et al. 2005; Ryan 1990; Law 1989). To understand how lipids are mobilized is of scientific importance because we can address many issues involved in diseases that are caused by defects in lipid storage and mobilization in insects or vertebrates. The primary diseases caused by errors in lipid metabolism are hyperlipidemia and obesity. The secondary

diseases include atherosclerosis and diabetics. To study this question in insects is very important as well since many insects are considered pests and serve as disease vectors.

We have utilized tobacco hornworm, *Manduca sexta*, as our model system since the metabolic features of *M. sexta* represent an excellent model for studying the fundamental mechanisms involved in the deposition of energy in larvae and the mobilization of the energy reserves in adults (Arrese et al., 1999). The life cycle of *M. sexta* is illustrated in figure 1. *M. sexta* larvae feed constantly until the content of TG in the fat body increases from a few micrograms to approximately 80 mg (Fernando-Warnakulasuriya et al., 1988; Ziegler 1991; Arrese et al., 2001; Canavoso et al., 2001). As a result of constant feeding, the TG stores reach their maximum at the end of larval development. During subsequent development, the lipid reserves in *M sexta* are utilized to sustain life (Fernando-Warnakulasuriya et al., 1988). There are several advantages to work with this insect model: First, it is very easy to rear and is harmless to human. Second, this is a simple model in which the processes are less complex to study than in vertebrates. Moreover, insects have economical impact and significance in our society. They can be extremely beneficial as pollinators or harmful as pests. Studying the mechanism by which insects utilize energy may uncover potential target to control insect pests.

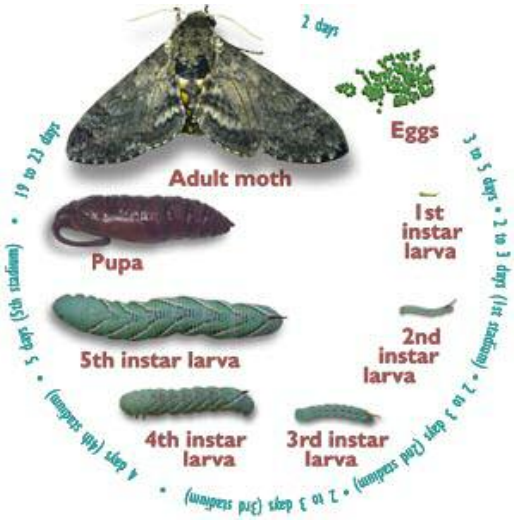


Figure 1. Life Cycle of *Manduca sexta*. The three different life stages in *M. sexta* are larva, pupa, and adult. On average, the complete metamorphosis from an egg to an adult takes about forty to fifty days. Each instar is marked by a headcap, representing the developmental stages. The stadium is the period of time between larval molts (Source: http://www.manducaproject.com/).

Lipolysis is a process of hydrolyzing TG stores to provide energy. The known process of lipolysis in insects is as follows: when the neuropeptide adipokinetic hormone (AKH) is released from the cephalic region of the moth to the adipocytes containing lipid droplets the lipids are mobilized from the fat body as diacylglyceride (DG) unlike vertebrates, in which stored fatty acids are mobilized as free fatty acid (FFA) (Beenakkers 1985; Orchard 1987; Arrese et al., 1996; Gade 1997). The sn-1, 2-diacylglycerol (DG) released into the hemolymph are loaded into the lipophorin, a hemolymph lipoprotein (Law 1989; Van Heusden 1989). Lipophorin transports DG to the sites of utilization such as the flight muscle and ovaries, where it is hydrolyzed to fatty acids by a lipophorin-lipase (Arrese et al., 1997). Resent studies have shown the mechanism that occurs in the fat body which causes the lipids to be mobilized in *Manduca sexta*. In the fat body AKH promotes a rapid activation of cAMP-dependent protein kinase A (PKA) (Patel et al. 2006). The targets of PKA mediated protein phosphorylation were identified as the TG-lipase

and lipid droplet proteins. It has been shown that phosphorylation of TG-lipase does not increases the enzymatic activity and therefore does not seem to be the rate limiting step in lipolysis. On the other hand, the phosphorylation of the lipid droplet associated proteins represents a major factor in the activation of the lipolytic cascade (Patel et al. 2005). The time dependent changes in phosphoproteins of the lipid droplet studies have revealed a 42-44 kDa protein, localized in the surface of lipid droplets as the main target of PKA (Patel et al. 2005). Based on the partial peptide sequence obtained by mass spectrometry of 42-44 kDa band, this protein was identified as homolog of Lsdp1 from *Drosophila melanogester*. The level of phosphorylation of Lsdp1 correlates with the activity of purified TG-lipase hydrolyzing TG contained in the lipid droplets (Patel et al. 2005). A schematic overview for the mechanism of AKH induced lipolysis is provided in figure 2.

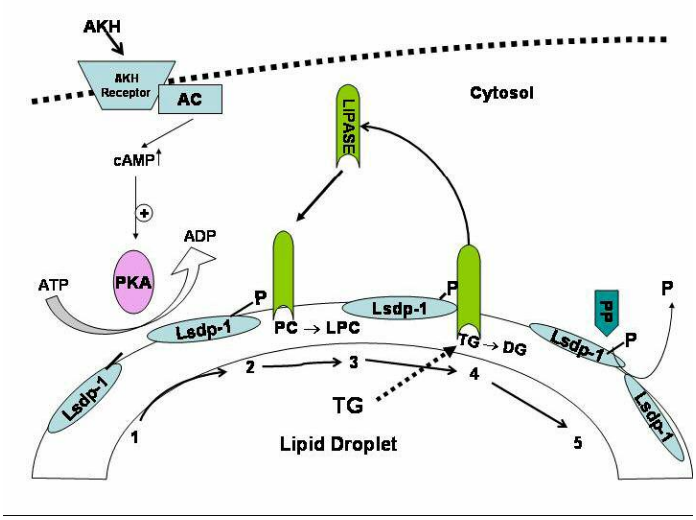


Figure 2. Current model for the mechanism of AKH induced lipolysis. AKH binding triggers activation of the adenylate cyclase (AC) and concomitant increase in the cAMP concentration followed by PKA activation. PKA phosphorylates Lsdp-1 (1). Phosphorylation of Lsdp-1 enhances binding of the lipase to the surface of the lipid droplet and/or its catalytic activity (Step 2). Lipid droplet bound lipase catalyzes the hydrolysis of phospholipid (Step 3) allowing the access of TG to the lipid surface (step 4) and its subsequent hydrolysis also catalyzed by the lipase. The lipolytic process ends by release of the lipase form the lipid surface. The insect TG-lipase does not bind tightly to the lipid droplets, even under conditions of high lipolysis. Additional binding of the lipase to the lipid droplet would be prevented by dephosphorylation of Lsdp-1 mediated by a protein phosphatase (PP). Source: Arrese et al. 2006.

This project was designed to investigate further the hypothesis that Lsdp-1 is involved in activation of lipolysis. The specific aims were: 1) Purification of Lsdp-1 from the fat body of adult *M. sexta*; 2) Determining the role of Lsdp-1 in TG-lipase activity using Lsdp-1 purified from *M.sexta*; 3) Determining the role of Lsdp-1 in TG-lipase activity using *D. melanogester* recombinant protein (dLsdp-1); 4) Investigate the protein expression levels of Lsdp-1 in different developmental stages and tissues of *M. sexta*.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials:

[$^{32}PO_4$] orthophosphate (carrier free) was purchased from MP Biochemicals (Irvine, CA). Phosphatase inhibitors and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). Electrophoresis items were from Invitrogen (Carlsbad, CA). Gels and autoradiograms were scanned on a ScanMaker i900 (Microtek). Polyclonal antibodies against lipid storage droplet protein peptides were raised in rabbit at Cocalico Biologicals (Reamstown, PA). DEAE resin was purchased from Amersham Pharmacia (Piscataway, NJ). Silica gel G plates were purchased from J.T. Baker (Phillipsburg, NJ). Labeled trioleoylglycerol ([tri-9,10-^{3}H(N)]oleoylglycerol) was purchased from PerkinElmer Life Sciences (Boston, MA). *M. sexta* AKH was obtained from Peninsula Laboratories (Belmont, CA). All of the other chemicals were of analytical grade.

Experimental Insects:

Manduca sexta eggs were purchased from Carolina Biological supplies, and larvae were reared on artificial diet in our laboratory. Adult insects were maintained at room temperature without food. Experiments were carried out using 2–3 days old adult male insects. To achieve a consistent basal level of lipolysis the insects were decapitated 24 hours prior to the experiment.

Decapitation was achieved by removing the head of the experimental insects using scissors and sealing the injury with petroleum jelly to avoid the loss of hemolymph. Two hours before the experiments the insects were injected with 13 mg of trehalose dissolved in 20 µl of water (Arrese et al. 1996).

***In vivo* phosphorylation:**

Experimental insects were injected with 200 µCi of radiolabeled phosphoric acid [$^{32}PO_4$]. After 60 min insects were injected with trehalose. After two hours insects were used for experiments.

AKH treatment:

Experimental insects were injected with 100 pmoles of AKH to induce lipolysis (Arrese et al. 1996). Control insects were injected with water. AKH was dissolved in reconstitution buffer (5 mM H_2KPO_4 pH 6.5 containing 0.1 M KCl, 18 mM $MgCl_2$ and 4 mM NaCl). Fat body tissue was dissected 10 min after hormone injection.

Fat body homogenate:

Fat body tissue was collected after washing away the hemolymph using insect saline containing sodium bicarbonate 10 mM, HEPES 10 mM, sucrose 100 mM, potassium chloride 40 mM, sodium chloride 10 mM, calcium chloride 8 mM and magnesium chloride 30 mM at pH 6.5. Fat body tissue was homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle using 1ml of homogenization buffer (HB) consisting of 20 mM Tris, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/L leupeptine, 1 mg/L aprotonin, 0.1% β-mercaptoethanol, 1 mM benzamidine, 1 mg/L aprotonin, 2 mM imidazole, 2 mM sodium

fluoride, 1.5 mM sodium molybdate, 1 mM sodium orthovanadate and 4 mM sodium potassium tartarate. All steps were carried out on ice.

Subcellular fractionation of fat body homogenates:

The homogenate's sucrose concentration was adjusted to 15% (w/v), and a layer of 2-ml homogenization buffer without sucrose was laid on top. Preparation was subjected to ultracentrifugation using Ti 70 rotor at 100,000 X *g* (40,000 rpm) for 60 minutes at 4˚C. Three distinct fractions consisting of fat cake, cytosol, and pellet were clearly visualized and separated after ultracentrifugation.

Purification of lipid droplets:

To purify lipid droplets, the fat cake was resuspended in homogenization buffer and gently vortexed for 10 min at 4˚C. The sucrose concentration was adjusted to 15% (w/v), and a layer of 2-ml buffer without sucrose was laid on top. Sample was centrifuged in a SW 40 rotor at 100,000 x *g* (30,000 rpm) for 30 min. Purified lipid droplets were collected from the top and used for further experiments.

Isolation of membranes from pellet:

The pellet sample obtained from the first ultracentrifugation was resuspended in homogenization buffer and centrifuged at 800 x *g* for 15min in order to remove the cell debris and nuclei. The resulting supernatant was used as membranes fraction.

Extraction of Lsdp-1 from lipid droplets:

Purified lipid droplets were combined with one volume of buffer containing 8 M urea, 50 mM Tris pH 7.5, and 0.1% β-mercaptoethanol. The mixture was incubated at 37°C for 1 hour

with vigourous vortexing. After the incubation period, the mixture was placed on ice for 15 min and layered on top with homogenizing buffer. Sample was subjected to centrifugation in the tabletop centrifuge at 5000 rpm for 20 minutes. The centrifugation yielded a top layer and an infranatant. Aliquot of both fractions were analyzed by SDS-PAGE to determine the efficiency of the Lsdp-1 extraction.

***In vitro* phosphorylation:**

Urea extracted Lsdp-1 was extensively dialyzed against 50 mM MOPS, pH 7.0, and 1 mM DTT to prepare the protein for *in vitro* phosphorylation. Sample was phosphorylated by incubation with the catalytic subunit of protein kinase A in the presence of 5 mM Mg acetate, 2.02 mM ATP, and ~800 μCi ^{32}P ATP. Reaction was carried out at RT for 20 min and terminated by placing sample on ice. An aliquot was run on a gel to examine the phosphorylation of Lsdp-1 by autoradiography. The gel was stained with Coomassie and autoradiogram were then scanned on a ScanMaker i900 (Microtek). The analysis was performed by using Alpha-Inotech stand alone software.

DEAE column chromatography:

DEAE Sepharose resin was equilibrated with pH 7.4, 50 mM Tris-HCl, 2 mM EDTA, and 0.1 % β-mercaptoethanol. After *in vitro* phosphorylation the sample containing ^{32}P-Lsdp-1 was dialyzed against the equilibration buffer to remove excess radioactive ATP. Sample was mixed gently with the equilibrated resin at 4˚C for 30 min. The slurry was applied to a column and flow through was collected. The column was then washed with 10 volumes of equilibration buffer. Proteins were eluted from the column using sodium a chloride step gradient of 50, 100, 140, 180, 200, 250, 300, 500, and 1000 mM in equilibration buffer pH 7.4, followed by 1M

sodium chloride, 7M urea and 2% *N*-octyl β-D glucopyranoside. Six to seven fractions were collected with approximately 9 ml in each fraction. Aliquots of 5 µl were taken from each fraction and the radioactivity was measured using liquid scintillation counter. The fractions containing highest amount of radioactivity were analyzed by SDS-PAGE and autoradiography to monitor Lsdp-1.

Ni-column chromatography:

Fractions from the DEAE column containing Lsdp-1 were combined and dialyzed against 20 mM Tris-HCl, 500 mM NaCl, and 6 M urea, pH 7.4. The resin was charged with 100 mM of nickel sulfate and equilibrated with 10 volumes of the loading buffer. Dialyzed sample was applied to a column and flow through was collected. The column was then washed with loading buffer. Proteins were eluted from the column using an imidazole step gradient of 10, 15, 20, 25, 30, 35, 40, 45, 50, and 100 mM in the loading buffer. Imidazole eluted fractions were collected in ~500 µl aliquots in centrifuge tubes. The radioactivity of all the fractions was measured using the Cerenkov method. The fractions containing highest amount of radioactivity were run on 10% SDS-PAGE to assay the elution profile of proteins from Ni-affinity column.

Generation of Lsdp-1 polyclonal antibody

MALDI-TOF and subsequent sequence analysis provided a sequence for two peptides (KVVHLVNYTHTDLPCR and TYLEHLAIFLAGNEEREKC) corresponding to *M. sexta* Lsdp-1 (Patel et al. 2005). Peptides were synthesized and conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) by Sigma-Aldrich. Peptide-protein conjugate was used for antibody generation in rabbit at Cocalico Biologicals. Western blot analysis was carried out using lipid

droplet proteins separated by SDS-PAGE, transferred to nitrocellulose, and probed with the antiserum against Lsdp-1 antibody.

SDS-PAGE and Western blotting:

Proteins were separated by 10% SDS-PAGE based on the molecular weight and were visualized by Coomassie Brilliant Blue R 250. The gel was dried on a gel dryer and subjected to autoradiography using Kodak films. The gel and autoradiogram were then scanned on a ScanMaker i900 (Microtek). The analysis was performed by using Alpha-Inotech stand alone software. For Western blotting, proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Immunodetection was performed using anti-Lsdp-1 antiserum (1:8000) diluted in superblocker. After incubation of the blot with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:30,000), peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences). X-ray films were scanned using ScanMaker i900 (Microtek).

Lipase assay:

The activity of purified *M. sexta* TG-lipase was measured using artificial TG substrate and Lsdp-1. The final assay volume of 0.1 ml contained 50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.02 % (w/v) BSA, 0.5 mM EDTA, 2 mM dithiothreitol, 0.22 mM triolein [9,10-^{3}H, 0.043 µCi] and 1 mM Triton X-100. The reaction was initiated by adding enzyme, vortexed gently for 20 s and incubated at 37ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of stop solution (chloroform: methanol: benzene, 2: 2.4: 1 and oleic acid) and 8µl of 6N HCl. The samples were vortexed gently and centrifuged at 2000 x g for 2min. The organic phase was collected, dried, and lipids were separated by TLC using (hexane: ethyl

ether: formic acid, 70: 30: 3) as the developing solvent. MG, DG, FFA, and TG fractions were visualized using iodine vapors and scraped and cpm counted using liquid scintillation counter.

CHAPTER III

RESULTS AND DISCUSSION

Investigation of the phosphorylation states of lipid droplet associated proteins under AKH stimulated lipolytic conditions revealed that a doublet with an apparent mass of 42-44 kDa is the main target of the phosphorylation cascade triggered by AKH. MALDI-TOF and subsequent sequence analysis revealed that the highly phosphorylated 42-44 kDa protein in *M. sexta* fat body is Lsdp-1. Previous studies from our lab suggested that Lsdp-1 could be a prominent player in lipolysis since, the phosphorylation of Lsdp-1 correlated with augmented TG-lipase activity. In order to examine the function of Lsdp-1 in lipid mobilization, we attempted to purify this protein from the adult *M. sexta* fat body. Purified Lsdp-1 will be useful to investigate its function using *in vitro* reconstitution experiment with TG-lipase. The purification steps involved the isolation of lipid droplets, extraction of Lsdp-1 from the lipid droplets, DEAE chromatography, and Ni- column chromatography as discussed in the subsequent paragraphs.

Lipid droplet preparation

Pilot experiments using fat body from a few insects indicated that to obtain pure lipid droplets we need to wash lipid droplets at least two times. The extensive washing of lipid droplets removes contaminants from cytosol and in particular reducing the storage proteins. In order to obtain Lsdp-1 in large quantity we started the purification with approximately 200 adult

insects. Fat body tissue was collected, homogenized, and subjected to ultracentrifugation using a sucrose density gradient. Purified lipid droplets were collected from the top fraction as described in materials and methods. Purification steps are summarized in the following scheme (figure 3).

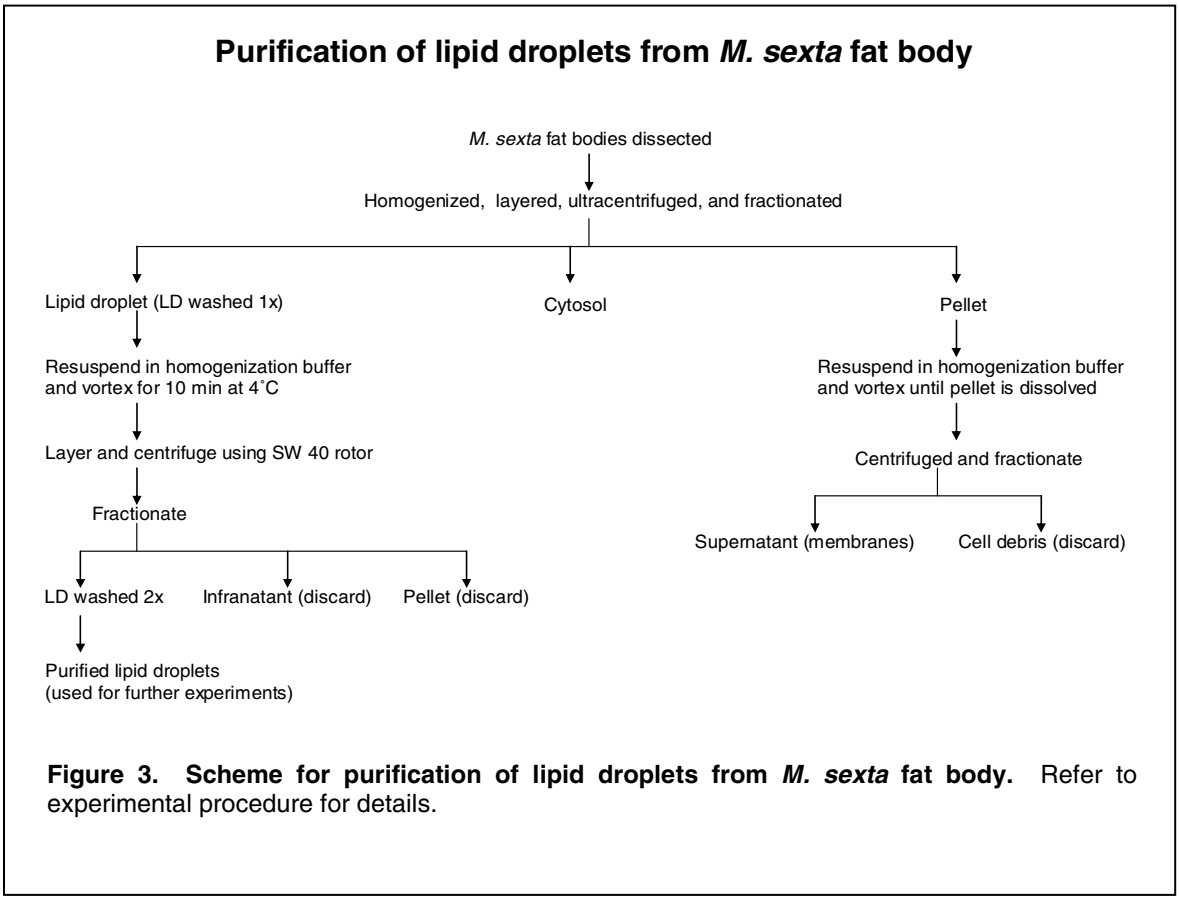


Figure 3. Scheme for purification of lipid droplets from *M. sexta* fat body. Refer to experimental procedure for details.

Extraction of Lsdp-1 from lipid droplets:

In our initial studies we tested various buffer compositions to extract Lsdp-1 from the purified lipid droplets. The extraction procedure was monitored by SDS-PAGE. The optimizing process included varying pH, temperature, detergent, and urea concentration. As a result of these experiments we concluded that a significant amount of Lsdp-1 can be extracted from the lipid

droplets by incubation with 8 M urea, 50 mM Tris-HCl, pH 7.5, and 0.1% β-mercaptoethanol and vigourous shaking for 1 hour at 37°C.

The majority of Lsdp-1 was found in the urea extracted infranatant. Since Lsdp-1 has been discovered by its increase phosphorylation during the activation of lipolysis and the phosphorylation is catalyzed by cAMP dependent protein kinase. Phosphorylation was used to monitor Lsdp-1 using radioactive ATP and commercial catalytic subunit of protein kinase A. Gel and autoradiography was conducted to observe the ^{32}P incorporation in the urea extracted lipid droplet proteins. Figure 4 shows the phosphorylated Lsdp-1.

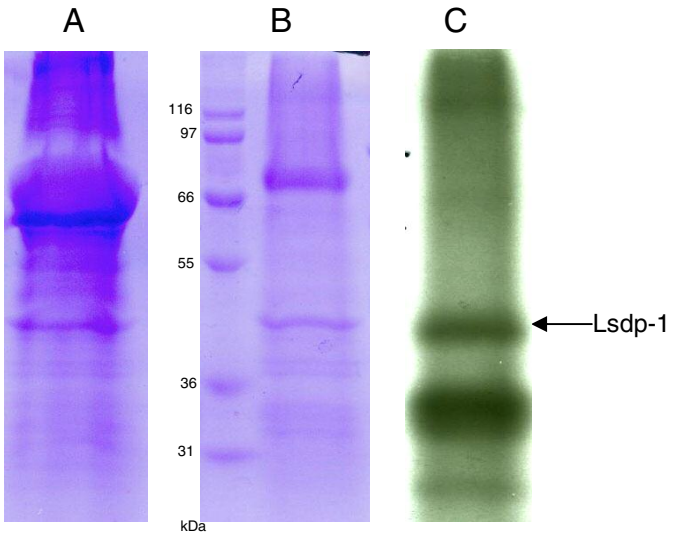


Figure 4. Lipid droplets and *in vitro* phosphorylation of urea extracted lipid droplets. (A) Lipid droplets (B) Lipid droplets extract was incubated with [γ-^{32}P] ATP and catalytic subunit of PKA from bovine serum. After 20min incubation at room temperature, the reaction was terminated by placing sample on ice and an aliquot was analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. (C) The gel shown in B was exposed for autoradiography to visualize ^{32}P incorporation.

DEAE chromatography

Infranatant sample obtained from urea extraction was phosphorylated, dialyzed, and passed through DEAE, an ion exchange chromatography. Previous studies conducted by Patel et al. 2005 showed that Lsdp-1 has an isoelectric point of 9 therefore, we were expecting

contaminants in the infranatant to bind in the DEAE column and Lsdp-1 to elute in the flow through. The proteins were eluted using a NaCl step gradient and the eluted fractions having high amount of radioactivity were subjected to SDS-PAGE. As expected the flow through had radioactivity but, Lsdp-1 band was not detected in the gel. Passing 1M NaCl through the column removes all bound proteins but, in our case even after passing 1M NaCl the Lsdp-1 was retained in the column. This observation led us to conclude that binding of Lsdp-1 to DEAE is through hydrophobic interactions. To elute Lsdp-1 from the column we used denaturing eluting conditions containing 7 M urea and 2% N-octyl β-D glucopyranoside (figure 5A). N-octyl β-D glucopyranoside was used since it is comparatively easier to dialyze compared to Tween 20 and Triton X-100. The fraction eluted using urea and detergent had the highest cpm count and analysis of SDS-PAGE showed that we were successful in eluting Lsdp-1 (figure 5B). Although, we see a very light band in the gel, the autoradiography indicates that Lsdp-1, which has the molecular weight of 42-44 kDa, is present in the gel (figure 5C, lane 4). There are two other unknown proteins at approximately 31-36 kDa. The autoradiography shows that these proteins are also highly phosphorylated (figure 5C, lane 4).

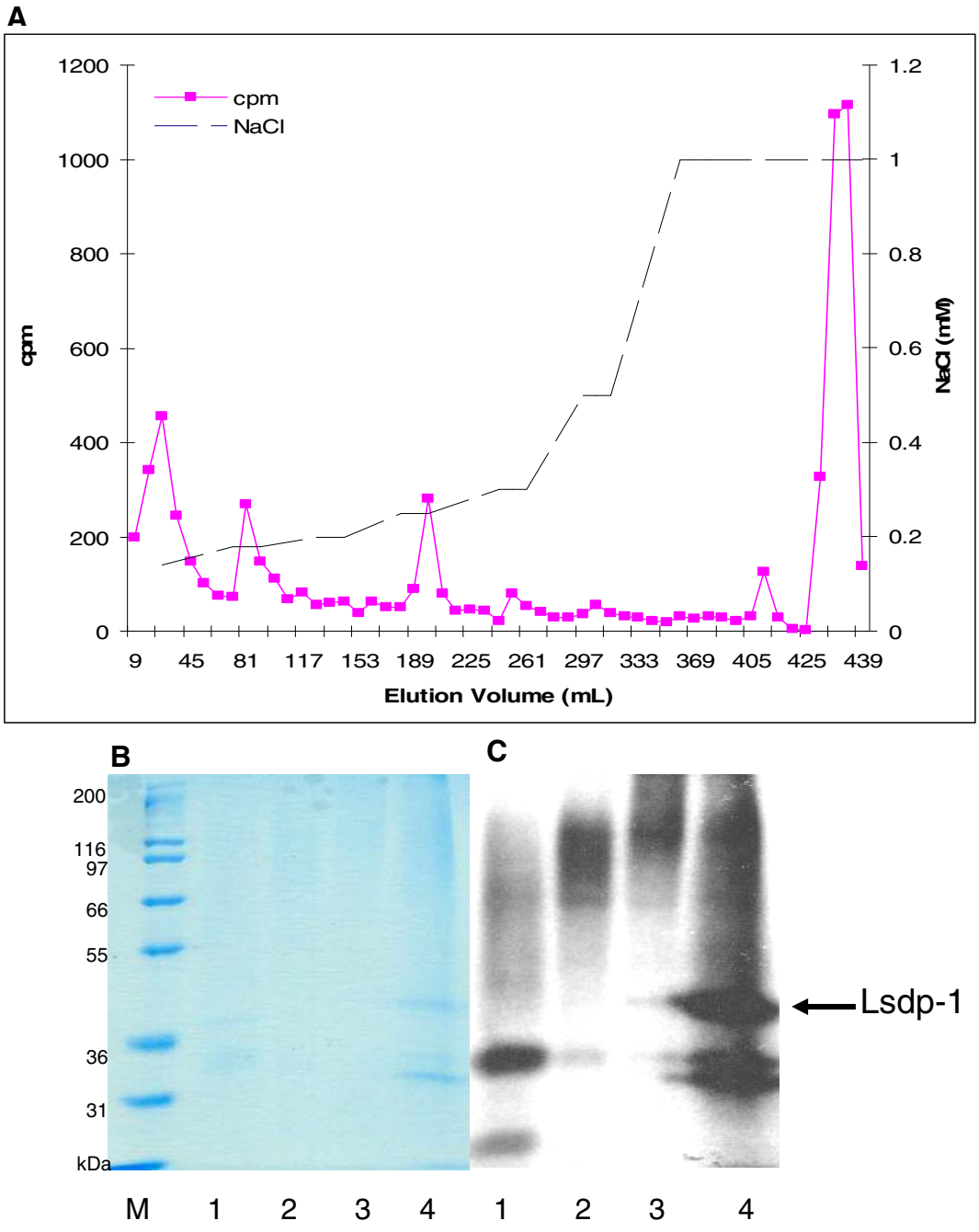


Figure 5. Partial purification of lipid storage droplet protein 1. (A) Elution profile from DEAE column. Fractions of 9 mL were collected, and radioactivity was measured in 5 µl aliquots by liquid scintillation counter (LSC). The fractions at ~430-439 mL were eluted by using 1 M NaCl, 7 M urea, and 2% N-octyl β-D glucopyranoside in equilibration buffer. A peak was observed at 435-437 mL having the highest cpm count. (B) Fractions containing highest cpm counts were run on 10% SDS-PAGE. Lane 1-4 corresponds to 27, 81, 198, and 437 mL (respectively). Lane 4 had the highest cpm and contained Lsdp-1 plus two other proteins at ~31-36 kDa (C) corresponds to the autoradiography of gel shown in B.

Lipase assay:

Previous studies from our lab indicated that the phosphorylation states of Lsdp-1 correlated with augmented TG-lipase activity. We are interested in examining *in vitro* the function of Lsdp-1 in lipolysis. For this purpose, partially purified and phosphorylated Lsdp-1 sample obtained from DEAE column was dialyzed to remove urea and detergent and an aliquot was used for lipase activity assay. The partially purified Lsdp-1 was incubated with TG-lipase purified from the cytosolic fraction of *M. sexta* and artificial substrate (as described in Material and Methods). Simultaneously the experimental controls were carried out where only lipase was incubated with the artificial substrate or only Lsdp-1 was incubated with the artificial substrate. Figure 6 shows a 2.88 fold increase in lipase activity with the addition of DEAE eluted Lsdp-1 fraction. However, we can not conclude that the increase in lipase activity is solely due to Lsdp-1 since two other protein bands are present in our sample that may be playing a contributing factor for this observation. For further studies we need to remove these contaminants and conduct additional lipase assays to elucidate the function of Lsdp-1.

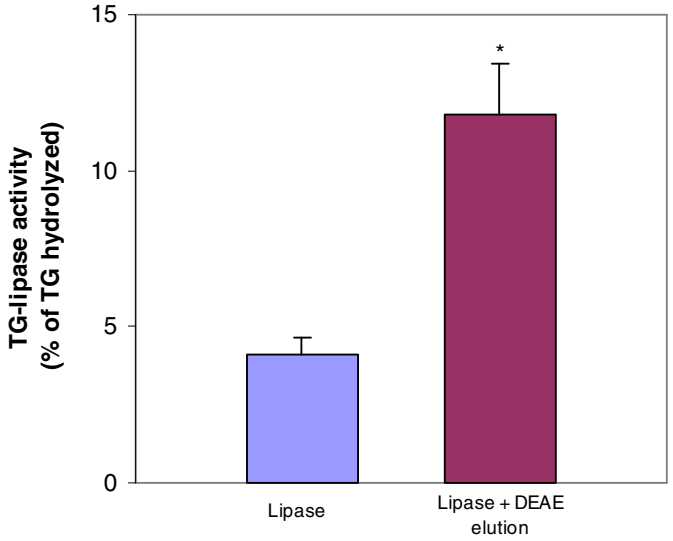


Figure 6. TG-lipase activity using the partially purified phosphorylated Lsdp-1 eluted from DEAE column. The activity of purified TG-lipase was measured using artificial TG substrate and Lsdp-1. The reaction was initiated by adding enzyme, followed by gently vortexing for 20 s and incubation at 37 ºC with constant shaking. After 30 min, the reaction was terminated and lipids were separated by TLC using (hexane: ethyl ether: formic acid, 70: 30: 3) as the developing solvent. Fractions were visualized using I_2 vapors and scraped and cpm counted. Data are expressed in % of TG hydrolyzed and represent the mean ± SEM (n=4). *P<0.05 versus control (lipase) the sum of classes of lipids (MG, DG, FFA, and TG) represent 100%. To calculate the % of TG hydrolyzed the values of the blank (incubation of the substrate in the absence of TG-lipase) were substracted to the corresponding amounts of TG.

Ni-column chromatography:

To further purify Lsdp1, fractions containing Lsdp1 proteins after DEAE column were pooled, dialyzed and loaded in Ni- affinity column. This chromatography was used since it is good for purifying phosphorylated proteins. We were hoping that most of the proteins will bind to the column including the contaminants and will elute in different fraction than Lsdp-1. Dialyzed sample was added to the column and flow through was collected which had some radioactivity. The column was washed with loading buffer to remove the unbound components. The bound proteins were eluted from the column using an imidazole step gradient. Imidazole eluted fractions were collected in approximately 500 µl aliquots and radioactivity was measured using the Cerenkov method. Two fractions containing highest cpm counts from FT, 15mM, 20mM, and 30mM imidazole samples were pooled dialyzed, concentrated and resolved by 10% SDS-PAGE. The gel was stained with Coomassie Blue but, the protein bands were not visualized. After drying the gel the autoradiographic analysis reveals that Lsdp-1 is present in the gel as seen in figure 7.

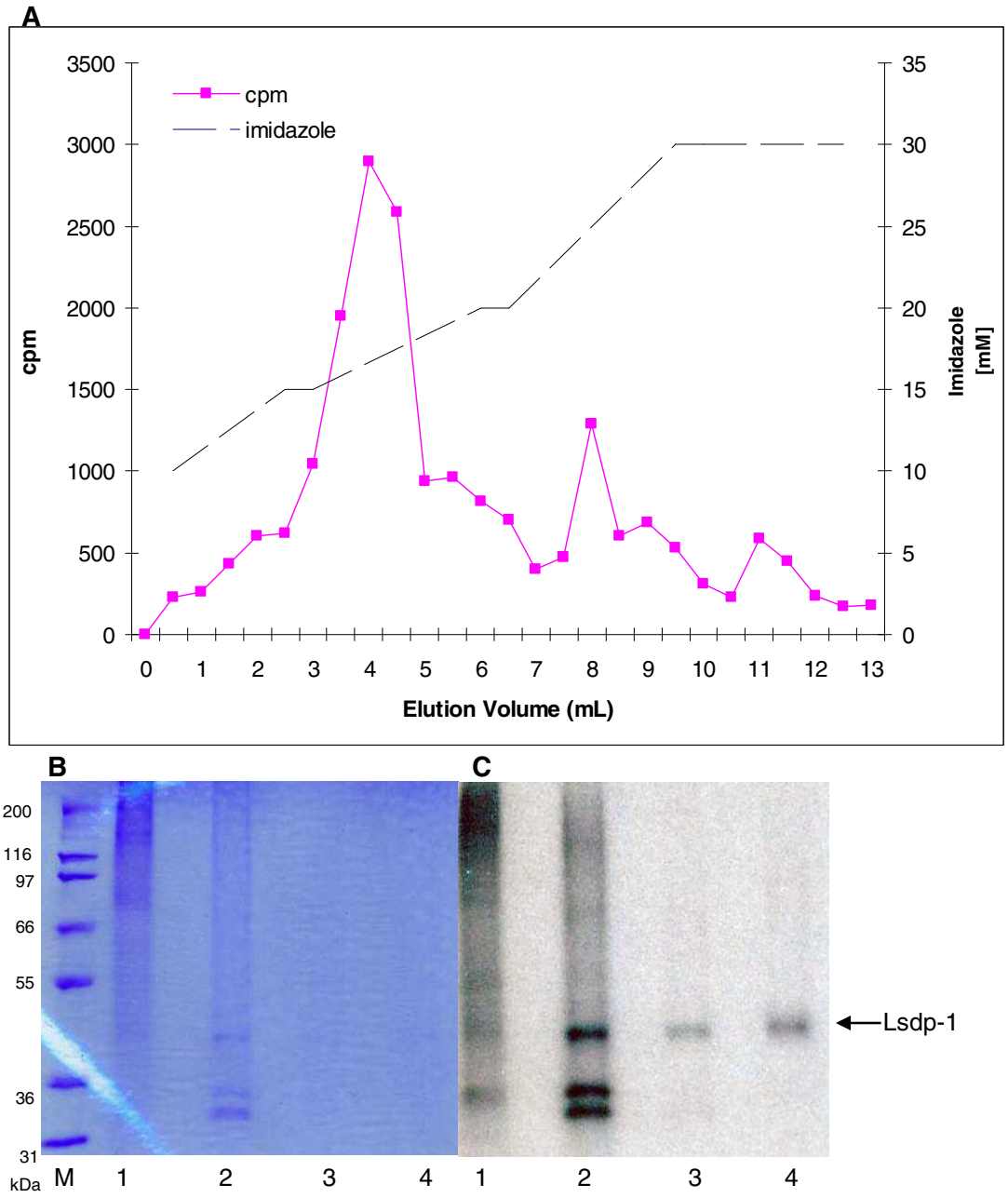


Figure 7. Elution profile from Ni-affinity column. Partially purified Lsdp-1 from DEAE column was dialyzed against loading buffer and placed on Ni-Column. (A) Elution profile from Ni-affinity column. Fractions of 500 µl were collected and radioactivity was counted by Cerenkov counting. (B) Fractions containing highest cpm counts were pooled, dialyzed, and run on 10% SDS-PAGE. Lane 1 corresponds to FT not shown in the elution profile. Lane 2-4 represents pooled fraction volumes of 4 & 4.5, 8.5 & 9, 11 & 11.5 mL (respectively). (C) Autoradiography of gel shown in B. 3rd and 4th lanes contained only Lsdp-1.

We were able to recover extremely low amount of Lsdp-1 after the Ni-affinity column. Similar fractions that were run on the gel in figure 7B were used for lipase activity assay. The results showed that an unknown factor in the FT was enhancing lipase activity much more than Lsdp-1 alone (results not shown, not enough repetitions). We did not repeat the lipase assays to preserve the limited sample instead we ran a SDS-PAGE since we wanted to know which proteins in the FT are involved in activating lipase activity. The Ni-column elutions were concentrated and we placed the entire sample on gel and the bands in the gel were still not visualized using silver staining.

In this study, we had somewhat high yield of partially purified Lsdp-1 protein from *M. sexta* fat body that was used for SDS-PAGE and lipase assays. There is indeed a necessity for purifying Lsdp-1 exclusively with high yield to perform reconstitution experiment in the lipid droplet that will enable us to know the function of Lsdp-1 in the molecular mechanism of lipolysis in insects.

Purification and characterization of Recombinant Lsdp-1 from fruit fly, *Drosophila melanogaster*

Since purification of Lsdp-1 protein was very complicated and time-consuming a recombinant protein was generated in our laboratory by colleagues. An overview of how this recombinant protein was prepared is as follows: Firstly, a DNA sequence extension in a vector consisting of thioredoxin, His-Tag, and S-tag was merged to the N-terminal region of Lsdp-1 sequence from *Drosophila melanogaster* (dLsdp-1) see figure 8. Secondly, the recombinant vector was expressed and purified from E. coli using Ni-affinity chromatography. The additional N-terminal sequence was cleaved using thrombin to provide a recombinant dLsdp-1 protein. Due to instability of dLsdp-1 protein S-tag was not cleaved.

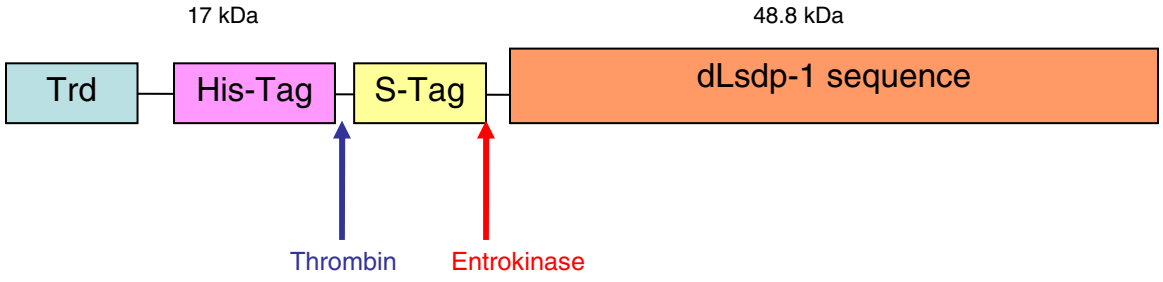
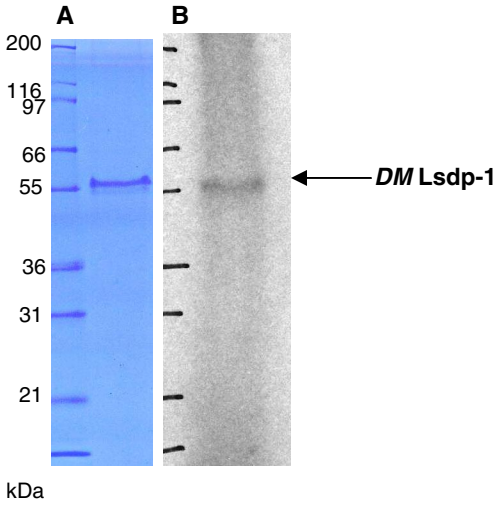


Figure 8. Construction model of recombinant Lsdp-1 from *Drosophila melanogaster*. Addition of peptide sequence to the N-terminal region of Lsdp-1 to form a fusion protein. The cleaveage sites are pointed out with arrows corresponding to the appropriate kinases. The approximate sizes are listed above in kDa.

Subsequently, recombinant dLsdp-1 was analyzed using a 10% SDS-PAGE gel. The data consistently provided us with a single band where recombinant dLsdp-1 band was expected. We then tested if the dLsdp-1 was capable of being phosphorylated and how it influences the lipase activity since these functional properties were characterized in the partial preparation of Lsdp-1

from *M. sexta*. In order to test whether dLsdp-1 can be phosphorylated we used the same *in vitro* phosphorylation method that was used to phosphorylate Lsdp-1 from *M. sexta* (refer to experimental procedure). As seen in figure 9, dLsdp-1 is phosphorylated in a reaction catalyzed by the catalytic subunit of protein kinase A. The phosphorylation site was identified by mass spectrometry. dLsdp-1 was separated on 10% SDS-PAGE and the bands visualized by Coomassie staining were excised and minced. Peptides were extracted and analyzed by MS/MS ESI. The underlined sequence corresponds to the S-tag sequence and the bold letter "**S**" in dLsdp-1 amino acid sequence represents serine as the site for phosphorylation as illustrated in figure 9C.



C

GSGMLETAAKFEKQHMDSPDLGTDDDDKMATATSGSGLHLE
AIDRIGSIPLVESSVKRVETIYDKVKNNNRLFSWYFETAEATISA
AYETIQPAVKLFEPSIQRLDNVMCKSLDILEQRIPLVYLPPEMMY
WNTKEYMSDHLVRPVLKRADSVKQIGNAVLESPLTTYAAERID
GAFTVGDKFVDKYLVPIQTDQDQTDGPQEDDNEAVPDERGAI
KAIHHGQRFSRKLKRRLTQRTIAEARALKKQSKEAIHVLFYAAE
LIATDPKQAVQKAKELWVYLSADEPENQARPATLEQLIVLLTRE
SARRVVHLVNFSAHVAANIPRNLAHTTTEVAHHIIYINHRIITISRL
DKVKTISKEEAESLFKRMLAFYGSLQGLTNAYLERVASFLSGRM
EAEKVTGSDGGNSNHRSSRRRQDPNHYSATHNNINGVY

Figure 9. *Drosophila melanogaster* recombinant Lsdp-1. (A) dLsdp-1 was incubated with [γ-^{32}P] ATP and catalytic subunit of PKA from bovine serum. After 20min incubation at room temperature, the reaction was terminated by addition of electrophoresis sample buffer and protein was subjected to analyze by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. (B) The gel shown in A was dried and exposed for autoradiography to visualize ^{32}P incorporation. Approximately 3μg of recombinant protein was loaded. (C) Recombinant dLsdp-1 was phosphorylated using non radioactive ATP and few micrograms were sent for the identification of the phosphorylation site in dLsdp-1.

Furthermore, dLsdp-1 was investigated for its influence on lipid mobilization. We wanted to use dLsdp1 to confirm the TG-lipase activity results seen in *M*. sexta since the increase in TG-lipase activity could be due to contamination in the DEAE preparation. The contaminants could be responsible for the increase in the lipase activity and not *M. sexta* Lsdp1. Lipase activity experiments were conducted in similar manner as mentioned in experimental

procedure with minor modifications. These modifications include use of dLsdp-1 bound DMPG liposomes, termination of reaction by 40µl of 1N NaOH, and counting cpm from an aliquot collected from the aqueous phase. The cpm counts only provide the number of free fatty acids (FFA) released. The lipase assay was carried out by utilizing purified dLsdp-1 bound to DMPG at a 1:50 protein to lipid molar ratio, artificial TG substrate, and purified TG-lipase from *M. sexta*. Concurrently the experimental controls were carried out where only lipase was incubated with DMPG liposomes. Our experimental result in figure 10 indicates that DMPG liposomes also act as an activator for lipase. This observation might be due to the negative surface charge of DMPG liposomes. Our results provided evidence that dLsdp-1 bound to DMPG liposomes enhances lipase activity but not to the extent seen in the Lsdp-1 DEAE elution from *M. sexta*. However, the purified dLsdp1 proved that the increase in TG-lipase activity was because of Lsdp1 and not because of other proteins.

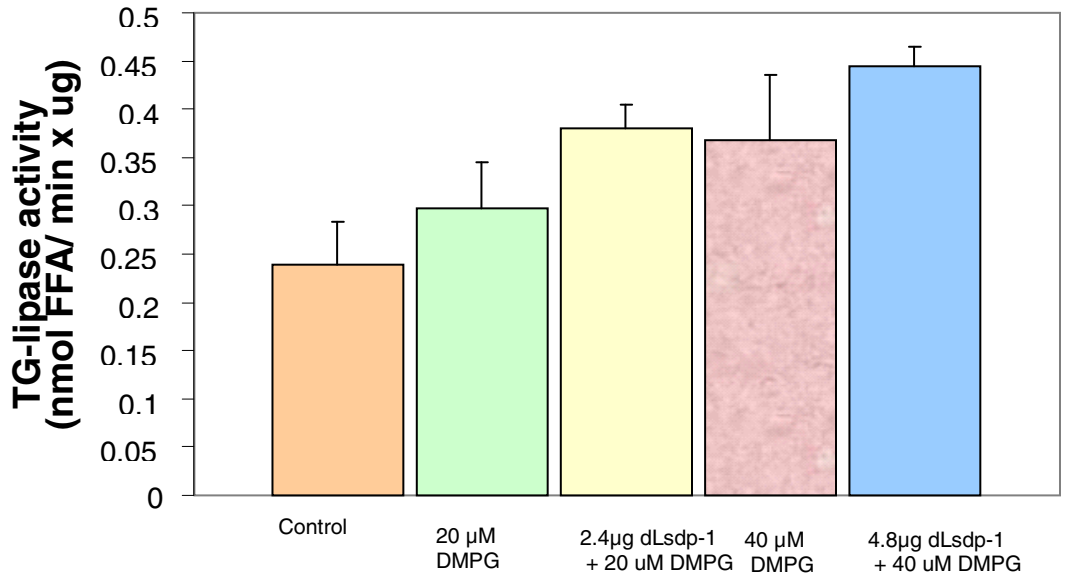


Figure 10. TG-lipase activity using dLsdp-1 and DMPG liposomes. TG-lipase activity was measured using purified TG-lipase, artificial substrate, and dLsdp-1 bound DMPG liposomes. The reaction was initiated by adding TG-lipase purified from *M. sexta* followed by gently vortexing for 20 s and incubation at 37 ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of stop solution (chloroform: methanol: benzene, 2: 2.4: 1 and oleic acid) and 40µl of 1N NaOH. Approximately 363 µl of aqueous phase was collected. An aliquot of 100 µl of aqueous phase was used for LSC and cpm counted. Data are expressed in nmol FFA/min*µg and represent the mean ± SEM (n=4).

Additional experiments were conducted using phosphorylated and unphosphorylated dLsdp-1 incorporated into DMPG liposomes to elucidate the role of phosphorylated dLsdp-1 in TG lipase activity. The data obtained from these experiments suggested that phosphorylation of Lsdp-1 enhances TG hydrolysis (figure 11).

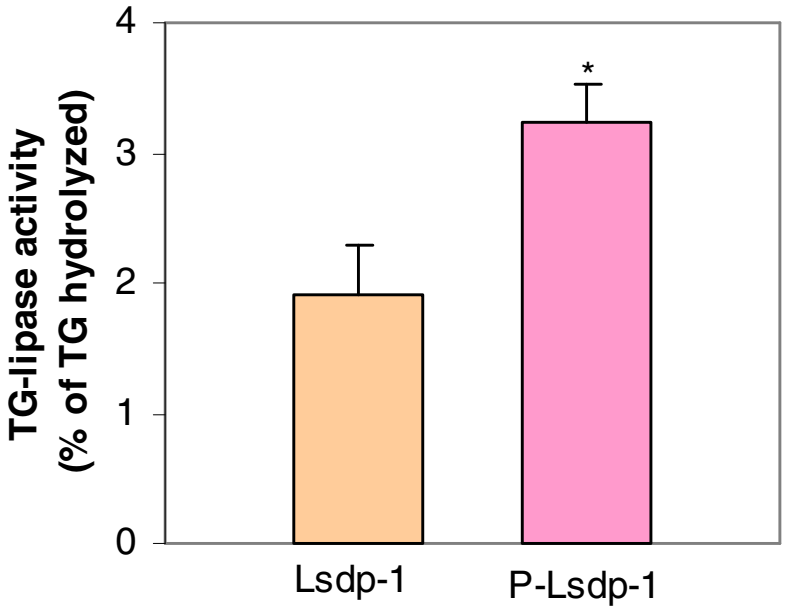


Figure 11. TG-lipase activity using Lsdp-1 from *Drosophila melanogaster*. The activity of purified TG-lipase was measured using substrate consisting of DMPG liposomes bound phosphorylated and unphosphorylated dLsdp-1. The reaction was initiated by adding TG-lipase purified from *M. sexta* and vortexing gently for 20 s and incubating at 37ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of stop solution (chloroform: methanol: benzene, 2: 2.4: 1 and oleic acid) and 8 µl of 6 N HCl. Vortex tubes gently and centrifuge at 2000 x *g* for 2 min. The organic phase was collected, dried, and lipids were separated by TLC using (hexane: ethyl ether: formic acid, 70: 30: 3) as the developing solvent. Fractions were visualized using I_2 vapors and scraped and cpm counted. Data are expressed in % of TG hydrolyzed and represent the mean ± SEM (n =10). *P<0.05 versus control (lipase) the sum of classes of lipids (MG, DG, FFA, and TG) represent 100%. To calculate the % of TG hydrolyzed the values of the blank (incubation of the substrate in the absence of TG-lipase) were substracted to the corresponding amounts of TG.

Investigating the sub-cellular distribution of Lsdp-1

The relative sub-cellular distribution of Lsdp-1 in cytosolic, lipid droplet, and membrane fractions of fat body homogenates during basal and lipolytic conditions was determined. Immunoblotting was used to estimate the abundance of Lsdp-1 in the cytosolic, lipid droplet, and membrane fractions. A comparison between the fractions obtained from control insects, and insects treated with AKH for 10min indicated that activation of lipolysis dramatically changes the phosphorylation level of Lsdp-1 in the lipid droplets and has a minor effect on phosphorylation level of Lsdp-1 in the membranes. Moreover, we were not able to detect the presence of Lsdp-1 in the cytosol suggesting that this protein does not translocate to other sub cellular organelle. Regardless of the lipolytic condition, the fat body Lsdp-1 was mainly found in the lipid droplets (figure 12).

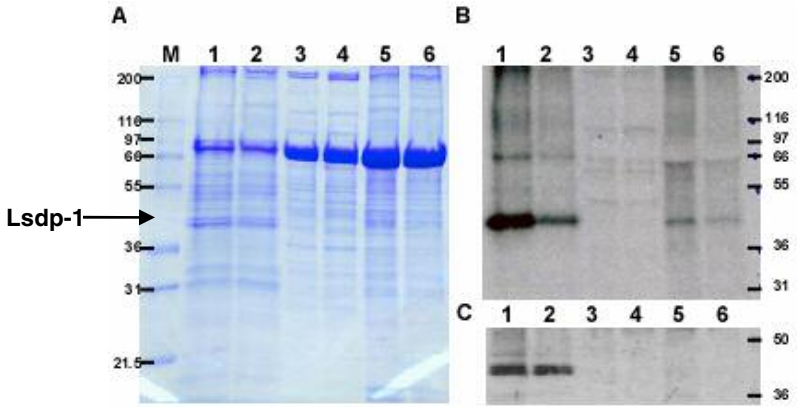


Figure 12. Subcellular localization of lipid storage droplet protein 1 (Lsdp-1) in adult *Manduca sexta*. [^{32}P] Fat body homogenates were collected at 0 and 10 min of hormone treatment. Lipid droplets, cytosol and membranes were isolated and subjected to SDS-PAGE in 10% acrylamide gel. A, Coomassie Brilliant Blue- stained gel. B, autoradiogram of the gel shown in A. C, Western blot hybridization with Lsdp1 antibody. Lanes 2, 4, and 6 represent the control (0 min AKH) lipid droplets, cytosol and membranes (respectively). The lipid droplets, cytosol and membranes isolated after 10 min of AKH injection are represented in lane 1, 3, and 5 (correspondingly). Approximately 30-35 μg of total protein was loaded into each lane.

Lsdp-1 protein level in the fat body of *M. sexta* during development

M. sexta larvae feeds constantly until it maximizes the TG stores at the end of the larval development and during pupae and adult stage it uses the lipid reserves to sustain life. We are interested in finding the insect developmental stage in which Lsdp-1 protein is formed and the quantity of protein during the various insect developmental stages. We are interested in this because of two reasons: First, identification of the insect's stage at which Lsdp-1 is formed will allow us to understand the controlled lipolytic process that is needed to use the lipid stores efficiently. Second, the ability to find the maximum amount of Lsdp-1 in its respective stage might aid in obtaining and purifying Lsdp-1 with high yield from *M. sexta*. The Lsdp-1 protein levels during the development of *M. sexta* fat body were studied in lipid droplet fractions using antibodies raised against synthetic Lsdp-1 peptides from *M. sexta*. Insects at various stages were dissected, fat bodies were collected, centrifuged, and lipid droplet fractions were run on 10% SDS PAGE gel and stained with Coomassie Blue. Western blot of the identical fat cake (~15ug/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the antiserum against Lsdp-1. Immunoreactive bands were visualized by probing the blot with anti-rabbit IgG horseradish peroxidase conjugate followed by reaction with ECL reagents and exposure to film. Our results identified that Lsdp-1 is only present in the fat cakes of wanderer, pupae, and adult insects based on the sensitivity of Lsdp-1 antibody as shown in Figure 13. Based on these results we can conclude that the appearance of Lsdp-1 in pupae and adult stages is indicative of controlled lipolytic process that is needed to use the lipid stores efficiently. Unphosphorylated Lsdp-1 might be playing a protective role in storage of lipid droplets in fat body.

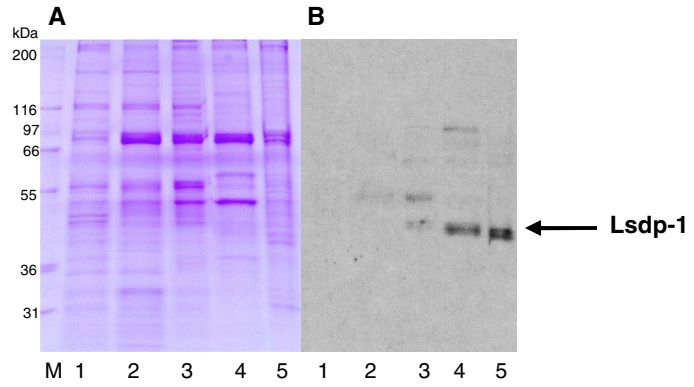


Figure 13. Variation in Lsdp-1 protein levels in the fat body of *M. sexta* during development. (A) 10% SDS-PAGE of lipid droplets and (B) Western blot analysis of the lipid droplets collected from different developmental stages of *Manduca sexta*. M- standard Lane 1) 5th instar (2nd day), 2) 5th instar (5th day), 3) Wanderer (2nd day), 4) Pupa (17th day), and 5) Adult (2nd day) accordingly. Data represents means ± SE (n=3).

Tissue specific protein expression of Lsdp-1 in *M. sexta*

The two tissues known to accumulate lipid stores are the fat body and ovaries since female *Manduca sexta* use part of the lipids stored in fat body during generation of ovaries. The Lsdp-1 protein levels in fat body and ovaries of *M. sexta* were studied in lipid droplet fractions using western blot analysis. Our results indicated that Lsdp-1 is only present in the fat body of an adult insect as shown in figure 14. However, we were not able to detect any LSDP-1 expressed in the lipid droplets of the early oocyte development in ovaries or the latter hatched eggs. Lipid droplets obtained from female fat body (lane 1) contains massive amount of storage proteins at ~64-70 kDa.

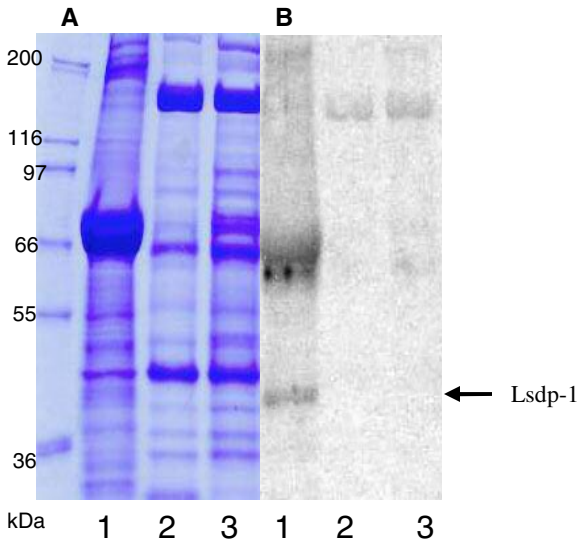


Figure 14. Tissue specificity of Lsdp-1 expression in lipid droplets of fat body and ovary. (A) 10% SDS PAGE gel stained with Coomasie Blue. Lane 1 & 2 corresponds to the lipid droplets from fat body and ovaries (respectively) samples were obtained from adult 3-4 day old female insects. Lane 3 represents lipid droplets from eggs purchased from Carolina Biological supplies. (B) Western blot of the identical lipid droplets (~30ug/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiserum against Lsdp-1. Immunoreactive bands were visualized by probing the blot with anti-rabbit IgG horseradish peroxidase conjugate followed by reaction with ECL reagents and exposure to film. Lsdp-1 was only present in the lipid droplets of adult female.

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Master of Science

Thesis: CHARACTERIZATION OF INSECT LIPID STORAGE DROPLET PROTEIN I

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Title of Study: CHARACTERIZATION OF INSECT LIPID STORAGE DROPLET PROTEIN I

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Major Field: Biochemistry and Molecular Biology

Scope and Method of Study: Insects use stored fat as energy during flight, stress, and reproduction. These lipid stores need to be mobilized in order to provide energy for essential functions. Recent development in the study of lipolysis has revealed that it is mainly controlled by substrate (lipid droplet) activation. The substrate activation is mediated via protein kinase A phosphorylation of lipid droplet associated proteins on the surface of lipid droplets as well as TG-lipase in the cytosol. Analysis of the phosphorylation state of LD-associated protein showed that *in vivo* stimulation of lipolysis promotes a rapid phosphorylation of a 42-44 kDa protein. This protein was identified by mass spectrometry as Lipid storage droplet protein-1 (Lsdp-1). Phosphorylation of Lsdp-1 correlates with augmented TG-lipase activity suggesting that Lsdp-1 is one of the prominent contributors of lipolysis. The purpose of this study was to investigate further the hypothesis that Lsdp-1 is involved in activation of lipolysis. The specific aims were: 1) Purification of Lsdp-1 from the fat body of adult *M. sexta*; 2) Determining the role of Lsdp-1 in TG-lipase activity using Lsdp-1 purified from *M.sexta*; 3) Determining the role of Lsdp-1 in TG-lipase activity using *D. melanogester* recombinant protein (dLsdp-1); 4) Investigate the protein expression levels of Lsdp-1 in different developmental stages and tissues of *M. sexta*.

Findings and Conclusions: The partially purified Lsdp-1 from *M. sexta* increased the lipase activity by 2.88 fold. The use of recombinant dLsdp-1 and DMPG liposomes complex provided evidence that the increase observed in TG-lipase activity was due to Lsdp-1. Our results also showed that DMPG liposomes enhance lipase activity. However, little is understood about the structure and dynamics of the DMPG liposomes and their interactions with lipase, therefore these areas need further investigation. Additionally, the recombinant dLsdp-1 was phosphorylated *in vitro* and the phosphorylation site was identified by MS/MS. Results showed that the phosphorylation of dLsdp-1 enhanced lipase activity by 59%. Furthermore, it was determined that Lsdp-1 is localized in the LD under stimulated and basal conditions. Finally, Lsdp-1 protein was expressed in wanderer, pupae, adult stage, and was not expressed in early oocytes or eggs.

ADVISER'S APPROVAL: Jose L. Soulages