MECHANISM OF LIPOLYSIS IN INSECTS:

ROLE OF SUBSTRATE

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Master of Science

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2000

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2004

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ACKNOWLEDGEMENTS

This thesis is the result of the cooperation, support, and guidance of many individuals. At this time, I would like to express my gratitude to those who have helped me in this accomplishment.

First and foremost, I would like to thank Drs. Jose L. Soulages and Estela L. Arrese for providing me an opportunity to work in this project and to pursue my Master's degree in their lab. I am grateful for their encouragement, kindness and their guidance.

I sincerely thank Dr. Richard C. Essenberg for his guidance, supervision, and expertise. Were it not his unwavering support and patience, I could not have become the scientist I am today. I am eternally grateful for his patience and invaluable suggestions.

I would also like to thank Dr. Robert L. Matts for being in my committee, for his suggestions and help in completing my thesis.

I owe my sanity to my colleagues Rajesh Patel, Philip Benjamin Verghese and Palaniappan Sevugan Chetty for being a great research partners and friends. I would like to thank my friends Alisha Smith, Ashok Rajendran and Satish Nagaraj for their friendship and insight, which was most critical to the successful completion of this study.

Moreover, I wish to express my sincere gratitude to the Department of Biochemistry & Molecular Biology for supporting me during my study.

Most of all, I wish to thank my mother and father to whom I am forever in debt for their constant love and support. When I thought I could go no further, their faith gave me the strength to continue. None of this would have been possible without their support. They are the best and I love them dearly.

I would also extend my heartfelt thanks to my brothers and sister, for constantly motivating and encouraging me during my study.

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ABBREVIATIONS

1-D	1-Dimensional
2-D	2-Dimensional
ADRP	Adipose Differentiation-Related Protein
AKH	Adipokinetic Hormone
BLAST	Basic Local Alignment Search Tool
cAMP	cyclic 3', 5'-adenosine monophosphate
DAG	Diacylglycerol
DEAE	Di Ethyl Amino Ethyl
DMPC	1,2-DiMyristoyl-sn-glycero-3-PhosphoCholine
FFA	Free Fatty Acid
GnRH	Gonadotropin-Releasing Hormone
HSL	Hormone sensitive lipase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LD	Lipid droplet
pI	Isoelectric point
LSD-1	Lipid storage droplet protein-1
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

pH — power of Hydrogen

PKA — Protein Kinase A

Q-sepharose — Quarternary ammonium sepharose

rpm — rotations per minute

SDS-PAGE — Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

TAG — Triacylglycerol

TB — Tris Buffer

UeqB — Urea equilibration Buffer

UexB — Urea extraction Buffer

CHAPTER 1

INTRODUCTION

Energy metabolism is a remarkable dynamic process, which is required to sustain all forms of life. The energy metabolism involves accumulation of energy reserves when energy is in excess and mobilization of energy stores when the system is in need of energy. There must be an efficient cooperativity between the metabolic pathways of various tissues for an animal to survive. Though the details and the regulation of most of the metabolic pathways within a single cell or tissue have been elucidated, there is much less understanding of the way in which the metabolism of various tissues is regulated in a multi-organ animal. Furthermore, there are some differences in the metabolic pathways or in the metabolic regulation of the pathways between organisms. One such major metabolic pathway is the mobilization of lipids from the insects.

1.1 Role of phosphorylation in controlling metabolism:

Phosphorylation plays an important role in most of the metabolic reactions and it plays a pivotal role in energy metabolism. When the system is in need of energy, phosphorylation activates specific enzymes, which eventually initiate the mobilization of the stored molecules whereas when energy supplies are low, it inactivates certain enzymes that are involved in energy storage. For proper energy metabolism to occur, there must be a regulation at the level of phosphorylation/de-phosphorylation by kinases

and phosphatases respectively. In the lipid mobilization from the adipocytes or in lipolysis, adipokinetic hormone (AKH) activated protein kinase A (PKA) phosphorylates hormone sensitive lipase (HSL), which acts as a lipid-mobilizing enzyme when the system is in need of energy. When the system is in fed state, protein phosphatase hydrolyzes the phosphoryl group from HSL, rendering it inactive, thus disallowing the mobilization of lipid stores.

CHAPTER 2

LIPOLYSIS

Lipolysis is the process whereby the triacylglycerol (TAG) is being broken-down into free fatty acids (FFAs) and glycerol. Lipolysis occurs intracellularly as well as extracellularly in a variety of tissues and it is a main event in both lipid storage and lipid mobilization.

2.1 Occurrence:

Lipolysis occurs in adipocytes of adipose tissue, liver cells, muscle cells and in the intravascular space. In general, lipolysis occurs in all triglyceride-storing tissues hence the lipid can be mobilized and the energy released during lipid mobilization can be used for the energy requirement process. Though it takes place in many places it mainly takes place in adipocytes, which is the primary focus for many scientists. Hence, the term lipolysis refers to the several related processes taking place in adipocytes. In adipocytes, lipolysis ends up in releasing FFA into the circulation, which is regulated by HSL. In liver and muscle cells, lipolysis occurs mainly to provide FFA for local oxidation.

Though lipid mobilization has become one of the important areas in the field of research, the information regarding the molecular mechanism of deposition of neutral lipids in the

adipocytes of vertebrates or fat body of insects as well as the molecular mechanism of lipolysis or lipid mobilization from adipose tissue and fat body remains unclear.

2.2 Mechanism of lipolysis:

Adipocytes are the major reservoir of energy stored in the form of TAG in the body. TAG is stored within intracellular lipid droplets covered by a monolayer of phospholipids, free cholesterol and proteins. Lipolysis in adipocytes is a HSL mediated pathway, which catalyzes the rate-limiting step in the breakdown of TAG into FFA and glycerol.

When the system is in demand of energy as in starvation, catecholamine activates β-adrenergic receptors residing on the adipocyte plasma membrane, which in turn triggers a signaling cascade that activates adenyl cyclase (Garcia et al., 2004). The subsequent reactions result in the activation of cAMP-dependent protein kinase (PKA) (Greenberg et al., 1993) and HSL in order to perform lipolysis.

2.3 Difference between vertebrate and invertebrate lipolysis:

The lipolysis and the lipolytic mechanisms are more or less similar in both vertebrates as well as invertebrates with some notable difference. One such major difference is that the end products of lipolysis in vertebrates are glycerol and FFAs whereas the end products are FFA and diacylglycerol (DAG) in invertebrates. Since insects are invertebrates, the end products are FFA and DAG, which will be carried away by lipophorin complex. In this review we are interested in the mobilization of lipid in insects.

2.4 Lipolysis in vertebrates:

In vertebrates, the vast majority (>95%) of body's TAG is found in adipose tissue stores (Clifford et al., 2000; Egan et al., 1992; Lu et al., 2001). Hence adipose tissue lipolysis is the major regulator of the body's supply of lipid energy because it controls the release of FFA into plasma, where they circulate as FFA complexed to albumin. A small amount of TAG is found in liver and muscle.

2.5 Lipolysis in insects:

TAGs are stored in the fat body; hence the principal location of lipolysis in insects is in fat body. The lipid mobilization from the fat body of insects acts as the major regulator of the body's supply of lipid energy, which will be utilized for flying and reproduction. A fat body TAG-lipase, presumably the first enzyme involved in the lipolytic process, was purified and characterized (Arrese and Wells, 1994). Some properties of the TAG-lipase are paralleled with the vertebrate adipose tissue lipase, HSL. But unlike adipose tissue, the insect fat body secretes DG into the hemolymph. The principal difference in the final product represents an important variation because FFA can be channeled out of adipocytes so efficiently.

2.6 Secondary messengers in lipolysis of insects:

During the non-feeding stages such as pupae and adult stages of *Manduca sexta*, the concentrated TAG stored in the insect fat body acts as a stored energy (Ryan, 1990). In most insects, the mobilization of stored TAG is mediated by an active TAG-lipase (Arrese and Wells, 1994), which is stimulated by the secondary messengers such as Ca^{2+}

and cAMP of AKH (Arrese et al., 1999; Pannabecker and Orchard, 1987). The end products of lipolysis in insects are FFA and sn-1,2 DAG (Arrese and Wells, 1997), which is then transported by lipophorin, the lipid transfer particle.

2.7 Translocation of protein in lipolysis:

Under basal conditions, the inactive non-phosphorylated HSL is principally a cytosolic protein. Whereas, under lipolytic stimulation, the active phosphorylated HSL undergoes a rapid and dramatic redistribution, which results in the translocation of the active HSL to the surface of the lipid droplet (Egan et al., 1992; Morimoto et al., 2001). In contrast to HSL, perilipin, the major phosphoprotein in the lipid droplet is associated principally with the lipid droplet. Upon lipolytic stimulation, perilipin becomes hyper phosphorylated and dissociates from the large droplet, allowing HSL to access the neutral lipid in lipid droplet (Clifford et al., 2000).

CHAPTER 3

LIPID STORAGE ORGANS

Most of the organisms in both animal and plant kingdom possess fat, which fulfils multiple functions in our bodies and the major function of fat is that it acts as a fuel (Gerritsen, 2001).

3.1 Adipose tissue and fat body:

In animals, adipose tissue of vertebrates and fat body of invertebrates act as the major lipid storage organs. Fat is stored in the intracellular neutral lipid droplets of these tissues in the form of TAG. In vertebrates, fat body is the principal site for the storage of both glycogen and lipids, of which TAGs constitute the main lipid storage form (Beccari et al., 2002). Because fat body cells as well as adipocyte cells accumulate TAG in intracellular lipid droplets, it suggests that energy storage as well as the machinery and the control of lipid mobilization may have developed before the divergence of vertebrates and invertebrates.

Since fats are stored with very little water and furthermore upon hydrolysis it gives 9 Kcal/gm of fat, it is an efficient way to store the excess energy and hence it serves as a buffer for energy balances. Accumulation of lipids or lipid storage as well as the lipid

mobilization (lipolysis) in adipocytes of vertebrates as well as fat body of an insects are subjected to acute control (Lu et al., 2001).

3.2 Adipose tissue:

Adipose tissue is a loose specialized connective tissue, which functions as a storage depot for glycogen as well as lipids. It also cushions and insulates the body. It is primarily located beneath the skin, but is also found around internal organs. There are two types of adipose tissue in mammals, namely white and brown adipose tissue (Albright and Stern, 1998). The presence, amount, and distribution of each adipose tissue vary depending upon the species. Most adipose tissue is white, which functions in heat insulation, mechanical cushion, and is primarily involved in the passive storage of fat and plays an important role in energy balance. In humans, fat is spread throughout the body in specialized fat-producing cells called adipocytes, which accounts for roughly half of the adipose tissue, scientifically known as lipid droplets (Albright and Stern, 1998).

3.3 Fat body:

The insect fat body combines many of the properties and functions of vertebrate liver and adipose tissue and plays a fundamental role in energy metabolism. Similar to vertebrate adipose tissue, fat body is involved in many homeostatic mechanisms and is an active center of metabolic activity in insects.

3.3.1 Physiology and biochemistry of the insect fat body:

In most insects, the fat body serves as a storage depot for food reserves. Lipid reserves are often accumulated in massive quantities in this organ, so that it looks like a body, or organ, of fat. The term fat body was coined during early days of studying insect morphology because of its fatty appearance.

In addition to its important role as a storage depot, the fat body of insects functions as a key center of metabolism and biochemistry. As a metabolic and biochemical center, the biological significance of fat body is its ability to maintain a balance between resources and requirements during the many phases of an insect's life. On one side of the balance, during times of feeding fat bodies biosynthesize and accumulate carbohydrates, proteins, amino acids and mainly lipids. Many of the responses to physiological needs occur on a relatively large scale, and they can have substantial impact on insect biology. Very often fat body metabolic functions are regulated by various endocrinological mechanisms.

3.3.2 Structure of fat body:

The morphology of fat bodies varies considerably in various insect groups. In many species it is a loose aggregation of cells that may be unevenly distributed among all body segments, including the brain case. In adult *M. sexta,* the masses and sheets of adipose tissue are distributed throughout the moth and are collectively called the fat body. Fat body cells often surround the major organ systems in insects, including reproductive tissues, alimentary canals, thoracic muscles, and elements of central nervous systems. Fat body also protects organs from physical damage by providing packing. In some species

fat bodies are compact cell masses invested in a membranous covering such that they can be easily removed from the insect intact.

Structures of fat bodies may vary according to life stage, sex and reproductive status. Fat bodies can have different functions between sexes, and functions also can differ among life stages. Fat bodies of all insects are in intimate contact with circulating hemolymph, which is consistent with movements of molecules between the two compartments.

CHAPTER 4

INSECTS

Insects are the most abundant form of animal life on earth. Most of the metabolic pathways are similar in both insects and vertebrates. For many biochemical processes and their regulation, insects provide a fascinating model system (Arrese et al., 2001; Canavoso et al., 2001). Furthermore insects are less complex systems and much easier to handle compared to vertebrates; hence insects are useful models in many areas of research in order to understand the molecular biology and biochemical pathway.

4.1 Fat metabolism in insects

Recent evidence indicates that the basic process of lipolysis is similar in vertebrates as well as in invertebrates, like insects (Van der Horst, 2003). Hence we can effectively use the less complex insect system as a useful tool to unravel the mystery behind the molecular mechanism of lipid mobilization, which may be useful to understand much more complex systems like the vertebrate system.

For our experiments, we used *M. sexta*, the tobacco hornworm, as our model system and furthermore we used biochemical and physiological approaches in order to shed some light on the molecular mechanism of lipid mobilization in insects. This may allow us to

gain insight into the fundamental process with respect to molecular mechanism of lipolysis.

4.2 TAG in insects:

Though the cells of most tissues have the ability to synthesize TAG, only a few tissues can store TAG (Gibbons et al., 2000). Insects store majority of the lipids as TAG in the fat body, which is analogous to the vertebrate adipose tissue and liver.

4.3 Advantages of *M. sexta*

The salient feature of *M. sexta* is that when it is in larvae stage, it feeds constantly and stores TAG in its fat body. This behavior changes only when the insect reaches its molting stage. Once it attains the molting stage it no longer can eat. Hence, in *M. sexta*, the maximum accumulation of TAG occurs at the end of 5th instar larva, as a consequence of the accumulation of reserves during larval feeding (Arrese et al., 1999). During pupal and adult stage, the stored TAG reserves are mobilized to sustain energy requirement process such as flying and reproduction, hence the TAG stores start to decline (Arrese et al., 2001). Due to the above-mentioned character, *M. sexta* remains one of the best model systems to study the mobilization of lipid.

Due to the above-mentioned advantages of insects over vertebrate systems, the study of lipid mobilization in insects, particularly *M. sexta*, has received considerable attention over the years. Though it has received attention recently, the mechanism by which the insects mobilize the lipid reserve is not completely understood.

4.4 Lipid mobilizing enzyme in insects:

TAGs are stored in the fat body. Recently fat body TAG-lipase, the first enzyme involved in the lipolytic process was purified and characterized (Arrese and Wells, 1994). It has been shown that TAG lipase of *M. sexta* and HSL of vertebrate adipose tissue have common function (Ziegler et al., 1998). But unlike adipose tissue, the insect fat body secretes DG into the hemolymph.

4.5 Lipid mobilizing hormones in insects:

As mentioned earlier, in response to lipid mobilizing hormones, the stored TAG are mobilized from the fat body into the hemolymph as sn-1, 2-DAGs in *M. sexta* in contrast to FFA in vertebrates. Lipolytic stimuli induce the release of energy mobilizing hormones such as AKH and octopamine. AKH is the major lipid-mobilizing hormone in insects and it is released from the glandular lobes of corpus cardiacum, a neuroendocrine gland located caudally to the insect brain and physiologically equivalent to the pituitary of mammal (Van der Horst, 2003).

CHAPTER 5

LIPID DROPLET: SUBSTRATE FOR LIPOLYSIS

Whenever we deal with lipolysis, people tend to give more importance to HSL, the lipid-mobilizing enzyme responsible for lipolysis. The popularity of HSL prevails over lipid droplet (LD), the substrate for lipolysis, hence there was only little information available about the lipid droplets in the field of composition of neutral lipids in lipid droplet, composition of polar lipids in lipid droplet, lipid droplet associated proteins and in particular the role of substrate in lipolysis.

5.1 Lipid droplet:

Most of the eukaryotic cells including mammals, insects, plants, algae and yeast possess intracellular lipid droplets, which are otherwise called lipid particles or lipid bodies. So far only a few prokaryotes are known to possess the ability of accumulating lipids in the form of lipid droplets as their energy source (Murphy and Vance, 1999; Tauchi-Sato et al., 2002; Yamaguchi et al., 2004).

The lipid droplets in the adipose tissue of animals and fat body of insects are the largest and most easily observed (DiDonato and Brasaemle, 2003). In adipocytes, the major components of lipid droplets are the fatty acyl moieties of TAG. Due to the high caloric

density and hydrophobic character, TAG provides highly efficient packaging of reserves. Recently, these specialized structures have been found across the biological kingdom and are now characterized as ubiquitous sub cellular organelles of most types of cells (Fujimoto et al., 2001; Zweytick et al., 2000). Though LDs are present in most of the eukaryotic cells, we have just started to understand the structure and dynamics of these ubiquitous structures.

5.3 Structure of Lipid droplet:

Lipid droplet is a highly organized little entity and globular in shape (Tauchi-Sato et al., 2002). The structure of lipid droplets is quite simple consisting of a highly hydrophobic core of neutral lipids (TAG) surrounded by a monolayer of amphipathic phospholipid where the proteins are embedded in the lipid droplet. Perilipin, ADRP and TIP47 are found as the lipid droplet associated proteins in vertebrates, whereas LSD-1 protein and LSD-2 are the lipid droplet associated proteins found in invertebrates. It is believed that the proteins associated with LD play an important role in metabolism (Zweytick et al., 2000).

5.4 The need to know the composition of Lipid droplets

Though lipid droplets are found in fat-related tissues, they are present in most of the cell types (Teixeira et al., 2003). In order to understand the lipid metabolism, which includes the anabolism and catabolism of lipids, we must know the composition of lipid droplets.

Though the lipid particles of all species are structurally related (Zweytick et al., 2000), the number of lipid droplets, the relative mass of stored TAG and the composition of lipid

droplet associated proteins vary depending on the cell type. Adipose tissue, similar to fat body, can store TAG in LD up to 100 µm in size (Garcia et al., 2003).

5.6 Conclusion:

In conclusion, we can be certain that the intracellular neutral lipid droplets are not merely passive repositories for storing lipids. The experimental results of (Zweytick et al., 2000) suggest that LD functions as distinct functional entities, perhaps “organelles”. Lipid droplets, the substrate of lipolysis, together with lipid droplet associated proteins play an important role in lipolysis.

CHAPTER 6

LIPID MOBILIZING HORMONES IN INSECTS

Hormones play an important role in the regulation of lipid concentration in hemolymph of insects. AKH and octopamine are the two major lipid-mobilizing hormones that have been discovered from insects till date. AKH of *Manduca* (M-AKH) is primarily considered as a neurohormone controlling energy metabolism (Ziegler, 1990), whereas another major lipid mobilizing hormone, octopamine, is regarded as the invertebrate counterpart of nor-adrenaline (Milde et al., 1995).

6.1 Octopamine:

Octopamine is the other major lipid-mobilizing hormone in insects such as *Locusta migratoria, Drosophila melanogaster, Acheta domesticus* and *M.sexta*. This neurohormone is a functional analog of vertebrate catecholamine nor-adrenaline. The primary function of this hormone is to mobilize the stored lipid energy from the fat body of insect (Fields and Woodring, 1981; Orchard, 1987; Orchard and Loughton, 1985). The salient feature of this hormone is that it has the ability to modulate the release of AKH from the carpus cardiacum (Passier et al., 1995).

6.2 AKH:

The primary hormone responsible for the lipid mobilization is AKH. As the name indicates AKH is a lipid-mobilizing hormone. In 1976 an English group succeeded in purification of the hormone, then later they found out that hormone is a peptide hormone composed of 10 amino acids. AKH was the first complete identification of an insect peptide hormone. Though it suggests that, there may be three possible physiological roles for AKH such as mobilization of fat body lipid reserves, a shift in flight muscle oxidative balance and change in lipophorin pattern, mobilization of fat body lipid reserves by AKH appears to be its primary function.

6.2.1 AKH receptors:

Binding of AKHs to their G-protein coupled plasma membrane receptors at the fat body cells is the first step in triggering the signal transduction events responsible for lipid mobilization (Van Marrewijk et al., 1996; Vroemen et al., 1998). The following steps results in the activation of key enzymes, which results in the phosphorylation of HSL and lipid droplet associated proteins. Recently, insect AKH receptors have been identified from *Drosophila melanogaster* and silkworm *Bombyx mori* (Staubli et al., 2002), which has a structural relationship with mammalian gonadotropin releasing hormone (GnRH) receptors.

6.2.2 Occurrence of AKH in insects:

While most of the information we know about AKH comes from the work performed on locusts, there is also evidence for similar hormones in other insect species like *D.melanogaster, M.sexta* and *B.mori*. It is clear that most insects have AKH (Gade, 1990), but details of the broader physiology remain rather fuzzy. The tobacco hornworm is one of the best model systems to study about AKH and it was reported AKH mediated lipid mobilization takes place in *M.sexta* (Goldsworthy et al., 1997; O'Shea and Rayne, 1992; Orchard, 1987).

Insect AKHs are peptide hormones composed of 8–11 amino acid residues. So far, over 35 different AKHs have been identified, which were blocked by pyroglutamate (pGlu) at their N-termini and all but one are amidated at their C-termini. Though the AKH from different species vary considerably, they share a common structural relationship (Oudejans and van der Horst, 2003; Van der Horst et al., 2001).

6.2.3 *M. sexta*AKH :

It appears that each insect species has one to three peptides of this family. In locusts and in some Lepidoptera, like *M. sexta*, AKH plays an important in the lipid mobilization from fat body under prolonged starvation as in the non-feeding *M. sexta* (Beenakkers et al., 1984; Ziegler and Schulz, 1986) and mobilizes lipids (Ziegler et al., 1995) as well as fat body glycogen in starving larvae of *M. sexta* by activating glycogen phosphorylase (Gies et al., 1988; Siegert and Ziegler, 1983).

6.2.4 Secondary messengers of AKH in insects:

The experimental results of (Ziegler et al., 1998) shows that AKH mediated lipid mobilization in insects involve stimulation of cAMP production, which depends on extra cellular Ca^{2+}(Arrese et al., 1999; Arrese and Wells, 1997). Furthermore, it was shown that the AKHs enhance the production of inositol 1,4,5-trisphosphate (IP_3), which in turn mobilizes Ca^{2+} from intracellular stores.

CHAPTER 7

HSL: LIPID MOBILIZING ENZYME

Lipid metabolism plays an important role in energy metabolism in both normal as well as pathological conditions (Mulder et al., 2003). HSL is the key enzyme in lipid mobilization in many cell types. HSL is subjected to acute hormonal control as the name indicates. As mentioned earlier, HSL mobilizes the stored lipid reserves into FFA and glycerol in vertebrates in contrast to DAG and FFA in invertebrates.

7.1 HSL-history:

HSL was regarded as an adipose tissue specific enzyme with the only function of catalyzing hormone-stimulated lipolysis in that tissue till 1980 (Yeaman, 2004). The later findings suggest that HSL is a serine hydrolase and it is the third reported substrate of cAMP dependent protein kinase. It is one of the enzymes regulated by phosphorylation and dephosphorylation reactions.

7.2 TAG lipase and HSL lipase:

Though the lipase in vertebrates is known as HSL and the lipase in insects is known as TAG lipase, there is a clear functional similarity between vertebrate adipose tissue HSL and insect fat body TAG lipase (Ryan and van der Horst, 2000; Van der Horst et al., 2001).

7.3 Role of phosphorylation in translocation of HSL:

HSL exists in two types namely phosphorylated and non-phosphorylated form in which the former is the active form whereas the later is the inactive form. A number of coordinated cAMP dependent intracellular processes involve the activation of protein kinase A, which in turn phosphorylates HSL. Phosphorylation of HSL results in its activation as well as its translocation (Brasaemle et al., 2000a; Clifford et al., 2000; Egan et al., 1992) to the surface of the lipid droplet, where it interacts with the lipid droplet at the water-lipid interface (Degerman et al., 1997; Dodson et al., 1992; Yeaman et al., 1994).

7.4 Interacting partner(s) of HSL:

Hormone-sensitive lipase (HSL) is a well-characterized partner of the major known PAT domain protein, perilipin. PAT proteins associated with the lipid droplets seem to lack catalytic domains. It is expected that partners of PAT proteins may help the PAT domain proteins to perform their functions in lipolysis (Yamaguchi et al., 2004). Phosphorylation of HSL by hormone induced PKA results in the translocation of the lipase from cytosol to the surfaces of lipid droplets (Brasaemle et al., 2000b; Tansey et al., 2003), where lipolysis then occurs. Recent mutagenesis studies have demonstrated that phosphorylation of both HSL and perilipin (Sztalryd et al., 2003; Tansey et al., 2003) is necessary for translocation of HSL to the lipid droplet. Hence, it is suggested that the perilipin likely includes a sequence or domain that binds lipases, as well as sequences or domains that shield stored TAGs from lipases (Garcia et al., 2004). Hence it is clear that the perilipin can be one of the interacting partners of HSL.

7.5 Role of HSL in identifying the mystery of lipolysis:

The major breakthrough in the molecular mechanism of lipolysis came from the findings that the purified HSL is activated only by approx. 2–3-fold *in vitro* following stoichiometric phosphorylation by cAMP-dependent protein kinase (Cook et al., 1982; Fredrikson et al., 1981; Stralfors and Belfrage, 1983), whereas lipolytic rates can increase up to 100-fold in fat cells in response to lipolytic hormones (Londos et al., 1985; Nilsson et al., 1980). Due to the above-mentioned difference between the *in vivo* and *in vitro* it was believed that the activation of substrate rather than the activation of the lipase is the primary step in lipolysis. Furthermore it was suggested that the solution for the difference in the lipolytic rates *in vitro* and in *vivo* might be due to a lipid droplet associated protein, perilipin. It was thought that the lipid droplet associated proteins activate the substrate and plays a key role in mediating the association of HSL with its lipid substrate in the insect adipocyte (Londos et al., 1999b).

CHAPTER 8

LIPID STORAGE DROPLET PROTEINS

Lipid droplets are mainly composed of neutral lipids (TAG) surrounded by a monolayer of amphipathic phospholipid with a few lipid droplet associated proteins at the surface of the lipid droplets. The notable feature of lipid droplets in most organisms is the proteins intrinsic to the interface between the aqueous cytoplasm and the lipid droplets. A set of proteins known as PAT proteins (perilipin, ADRP, TIP47, and S3–12) have been identified as lipid droplet associated proteins (Miura et al., 2002), which share a conserved region at the N terminus. It is expected that, similar to other organelles, the protein components associated with the membrane surface would regulate the dynamics and the molecular mechanism of associated with LDs.

Once the proteinaceous layer surrounding the lipid droplets was considered to perform the sole function of preventing the lipid droplet fusion with any adjacent lipophilic surface (Nakamura et al., 2004). Though the view of the sole function of the lipid droplet associated proteins has changed considerably, analysis of the properties of surface proteins has received only limited attention. In order to reveal the molecular mechanism of lipid mobilization we need to perform more analysis on lipid droplet associated proteins.

The information about the lipid storage droplet associated proteins is scarce and the study of lipid droplet-associated proteins is an emerging field of inquiry. Until now, only a few of the lipid storage droplets protein have been identified and the information about their structural properties, interacting partners, how proteins are integrated into the phospholipids monolayer and their exact role in lipid mobilization are not known in detail (Garcia et al., 2004).

Understanding the role of the lipid storage droplet proteins in lipid mobilization will increase our knowledge of molecular mechanism of lipolysis, which may stimulate us to use insects as an analogous system to vertebrates to study the metabolic pathways.

8.1.1 Perilipin and its occurrence:

Perilipins can be loosely grouped as part of a small protein family that shares a common N-terminal motif (Lu et al., 2001). Perilipins belong to a family of polypeptides(Londos et al., 1995; Londos et al., 1999a), which are localized to the periphery of lipid storage droplets in adipocytes and steroidogenic cells of adrenal cortex, testes and ovary(Brasaemle et al., 1997b; Greenberg et al., 1991). In these tissues perilipins are found at the surface of lipid droplet, where they become hyperphosphorylated under lipolytic conditions. The selective expression of perilipin in tissues is also confirmed (Blanchette-Mackie et al., 1995; Brasaemle et al., 1997a; Servetnick et al., 1995).

8.1.2 Identification of perilipin:

Perilipin, the lipid droplet associated hydrophobic protein, was first identified as a ~60-kDa protein of rat primary adipocytes. The degree of phosphorylation of perilipin will increase dramatically due to the action of lipolytic hormones such as AKH and octopamine (Egan et al., 1990; Greenberg et al., 1991). Initially, perilipins were considered as lipid droplet associated proteins but later it was hypothesized that they may act as regulators of lipolysis (Londos et al., 1999a; Londos et al., 1999b).

8.1.3 Identification of Perilipin role:

Though perilipin appears to be restricted to adipocytes and steroidogenic cells, it is predominantly found in adipocytes, which are specialized for lipid storage. Since perilipin is always associated with lipid storage cells, it is suggested that perilipin may play an important role in lipid droplet dynamics. The findings of Brasaemle et al., (2000b) Tauchi-Sato et al., (2002) suggest that the phosphorylation of perilipin disrupts the lipid droplet structure, adds a strong notion to the role of perilipin in lipid metabolism. The concurrent phosphorylation of perilipin along with HSL by protein PKA suggests a role for this protein in lipid mobilization.

8.1.4 Possible roles of perilipin *in vivo*

Though the functions of perilipin are not exactly known, the profound researches in this field fetched us few hypotheses namely barrier hypothesis, docking hypothesis, role of perilipin in translocation of HSL and substrate activation hypothesis (Londos et al., 1999a; Londos et al., 1999b).

8.1.5 Barrier hypothesis:

Though perilipin is not an inhibitor in vitro, it does reduce the lipolysis rate *in vivo* by preventing the HSL to the lipid droplet. Hence, it is suggested that perilipin sterically blocks access of hormone-sensitive lipase *in vivo* (Ostermeyer et al., 2001). Under basal conditions, when the body is in the fed state, perilipin of adipocytes is minimally phosphorylated and functions to reduce the access of cytosolic lipases (acts as a barrier) to store TAGs by an as yet uncharacterized mechanism and thus promotes TAG storage. Recently, convincing evidence was shown that the role of perilipin is indeed to prevent lipolysis under basal conditions (Brasaemle et al., 2000a), which leads to increased deposition of lipid. The experimental results of perilipin expression in pre-adipose tissue culture increasing lipid storage by reducing TAG hydrolysis, adds additional strength to the barrier hypothesis (Souza et al., 2002).

Previously, evidences for the function of perilipin *in vivo* using perilipin-deficient mice were shown (Martinez-Botas et al., 2000; Tansey et al., 2001). The experimental results showed that the perilipin-deficient mice are viable and fertile and have reduced adipose tissue mass and more muscle mass than controls. Furthermore, the results of other authors suggest perilipin has a protective role against lipases (Brasaemle et al., 2000b; Souza et al., 1998).

8.1.6 Docking hypothesis:

According to docking hypothesis, perilipin is a lipid droplet associated protein in fat cells and it was originally proposed to be a docking protein for HSL, which assists HSL to access its TAG substrate (Greenberg et al., 1991). Though perilipin acts as a barrier to

prevent lipolysis under basal conditions, it acts as a stimulator of lipolysis under lipolytic conditions in which perilipin is multi phosphorylated by cAMP-dependent protein kinase (PKA), which results in its redistribution. Due to the above-mentioned reason perilipin barrier is removed, giving HSL access to LDs, thereby acts as a docking protein, which promotes lipolysis (Sztalryd et al., 2003; Zhang et al., 2003).

8.1.7 Antagonist role of perilipin in lipid dynamics:

In summary it is believed that perilipin acts as both inhibitor for lipolysis under basal conditions as well as a stimulator under lipolytic conditions. Thus, it performs an antagonist role in lipid dynamics (Blanchette Mackie et al., 1995; Greenberg et al., 1991).

8.1.8 Perilipin in translocation of lipase:

Brasaemle et al. 2000, proposed an intriguing model, which says that phosphorylation of perilipin facilitates access of hormone-sensitive lipase to the droplet surface, thus stimulating lipolysis in a hormone-dependent manner. It is evident that the main role of phosphorylation of HSL is to translocate the enzyme from cytosol to lipid droplet (Tauchi-Sato et al., 2002). Recent mutagenesis studies have demonstrated that phosphorylation of both HSL and perilipin is necessary for translocation of HSL to the lipid droplet (Su et al., 2003; Sztalryd et al., 2003).

8.1.9 Substrate activation:

Under basal conditions, the non-phosphorylated perilipin covers a large proportion of the surface area of the TAG droplet (Murphy and Vance, 1999), hence it protects the LD from the lipolytic action of HSL. Due to the action of lipid mobilizing hormones as in lipolytic conditions, cAMP mediated phosphorylation of perilipin results in its activation as well as its redistribution which in turn fosters the interaction of HSL with the TAG in the lipid droplet by promoting the translocation of HSL to the lipid droplet while translocating itself from the lipid droplet surface. Translocation of perilipin results in the change in the lipid droplet structure. This is consistent with studies reporting that the adipocyte lipid becomes 'activated' following lipolytic stimulation. It is clear that when the perilipin coats the lipid droplet, the substrate is inactive and when the barrier is being removed by phosphorylation, the substrate becomes active. This process is collectively called as "substrate activation", the most emerging field in the lipolytic studies.

8.1.10 Conclusion:

Proteins with a PAT domain have been found in a wide variety of species, including *D.melanogaster* (Blanchette-Mackie et al., 1995; Brasaemle et al., 1997a; Lu et al., 2001). In summary, it is clear that lipid storage proteins, in particular perilipin, present in higher eukaryotes have an ancestral relationship, which plays a fundamental role in lipid homeostasis by acting as a docking, barrier, translocating lipase and activating substrate for lipolysis.

8.2.1 Perilipin like Lipid storage droplet protein in invertebrates:

The lack of information regarding lipid storage droplet (LSD) protein, gives us a notion that the study of LSD protein is still in its infancy stage. Though the preliminary data about the lipid storage droplets occurrence and functions are emerging for the vertebrate system, the factors mediating the corresponding processes in invertebrates are still unknown (Gronke et al., 2003). Recent experimental results indicate that perilipin plays an important role in the dynamics of lipid metabolism in vertebrates and since invertebrates lack perilipin there should be another protein, which can perform perilipin like function in invertebrates such as insects. It is recently shown that the lipid storage droplet protein is the perilipin like protein in invertebrates (Martinez-Botas et al., 2000; Miura et al., 2002; Tansey et al., 2001). LSD-2, one of the two PAT domain-encoding proteins in *D. melaogaster* acts in a perilipin-like manner, suggesting that LSD-2 could play the role of perilipin in invertebrates (Gronke et al., 2003). Hence, it is necessary to identify the lipid storage droplet proteins, their cofactors and their interactions in order to elucidate the regulatory mechanism of lipolysis.

8.2.2 Identification of LSD protein in insects:

Analogous to perilipin in invertebrates, two *Drosophila melanogaster* members of the PAT family, LSD-1 and LSD-2, have been discovered in BLAST searches by (Lu et al., 2001; Miura et al., 2002). Furthermore, they also identified a putative LSD protein in *D. melanogaster* (dmLSD-1) and *B. mori* (bmLSD). Moreover using the PAT-1 sequence of dmLSD1 in a similarly restricted search the authors identified another LSD protein of

Drosophila (dmLSD2) (Canavoso et al., 2001). It is considered that LSD-1 has a perilipin-like function in insects like *Drosophila.*

8.2.3 Role of LSD-1 in invertebrate lipolysis:

The experimental results by Teixeira et. al. 2003, show that neutral lipid droplet accumulation and LSD-1 accumulation coincide after mid-oogenesis. Furthermore, the experimental results show that the level of TAG was 27% lower in the perilipin null mutant than in the wild type. It is evident that the improper LSD-1 or lack of LSD-1 affects the neutral lipid storage (Teixeira et al., 2003). Hence we can conclude that LSD-1, similar to perilipin in the mouse, is required for normal storage of lipids in the insects.
A number of other proteins may likely to play a role in this phenomenon. It is important to emphasize that the perilipin-like lipid storage droplet proteins (LSD-1) plays an important role in the lipolysis (Yeaman, 2004).

8.2.4 Future study for LSD proteins:

Since lipid storage droplet proteins are associated with lipid droplets, we need to know whether these proteins act as structural proteins, perhaps to maintain droplet integrity. Since these proteins are involved in lipid storage, we have to find out whether these proteins act as docking proteins for lipogenic or lipolytic enzymes. Furthermore we also have to find out the presence and role of other proteins at the lipid droplet surface.

CHAPTER 9

MATERIALS AND METHODS

9.1 Materials:

($H_3{}^{32}PO_4$) orthophosphate (carrier free) was purchased from ICN (Irvine, CA). Phosphatase inhibitor and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO), AKH was purchased from Peninsula lab (Belmont, CA), DEAE and Q-sepharose resins were purchased from Amersham Pharmacia (Piscataway, NJ). All other chemicals were of analytical grade.

9.2 Insects:

M. sexta eggs were purchased from Carolina Biological Supplies (NC) and the insect colony was maintained in our lab. Adult *M. sexta* insects were kept at 23 ^{0}C without food. We used 2-3 day old adult male insects for our experimental conditions.

9.3 Decapitation:

To avoid the endogenous hormone contamination, we used a novel procedure namely decapitation of insects. We decapitate the insects and seal the injury with petroleum jelly to avoid the loss of hemolymph. Insects were decapitated 24 hours prior to the experiment and subjected to trehalose injection.

9.4 Injection of trehalose:

Similar to glucose in vertebrates, trehalose, the disaccharide, acts as a major sugar in *M. sexta*. To avoid the lipid mobilization as well as to provide energy to the decapitated insects, we injected 13 mgs of trehalose in 20 µl of water using 50 µl Hamilton syringe. Furthermore the insects were injected with another 13 mgs of trehalose in 20 µl of water 2 hrs prior to the experiment.

9.5 *In vivo* phosphorylation:

The experiment was started with injection of 500 µCi of radiolabeled phosphoric acid ($H_3{}^{32}PO_4$) using 50 µl Hamilton syringe. Following the injection of radiolabeled phosphoric acid, insects were subjected to 90 minutes incubation at 23º C.

9.6 AKH treatment:

Arrese et al., (1999) have proved that 100 pico moles of AKH is sufficient to mobilize the stored lipids from the fat body of *M. sexta*. As soon as the incubation period of *in vivo* phosphorylation is over, we injected 100 pmoles of AKH in to the experimental insects, whereas insect saline was injected in to the control insects. AKH was dissolved in AKH reconstitution buffer (5 mM H_2KPO_4 pH 6.5 containing 0.1 M KCl, 18 mM $MgCl_2$ and 4 mM NaCl). Since the amount (100 pmoles) of AKH is important in this experiment, one wing was temporarily immobilized and the other was allowed to move freely to ensure that AKH has been taken up by the insects. Following the injection of AKH, the insects were subjected to 20 minutes incubation at 23º C.

9.7 Dissection and obtaining fat body:

The control and experimental insects were dissected. We used insect saline (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) to wash the insects while dissecting. Furthermore after the dissection, the fat body was rinsed in insect saline to wash off the hemolymph contamination.

9.8 Homogenization:

The fat bodies obtained from the control and experimental insects were placed in the homogenization buffer (20 mM Tris pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/L leupeptine, 1 mg/L aprotonin and 0.1% β mercaptoethanol). To avoid the action of proteases and phosphatases, we used 1X protease inhibitor cocktail and 1X phosphatase inhibitor cocktail. 100 X protease inhibitor cocktail consists of benzamidine 100 mM, aprotonin 100 mg/L and PMSF 100 mM, whereas the 100 X phosphatase inhibitor cocktail consists of imidazole 200 mM, sodium fluoride 200 mM, sodium molybdate 150 mM, sodium ortho vanadate 100mM and sodium tartarate 400 mM. We maintained the ratio of 3 ml of homogenization buffer per insect fat body and the fat body was homogenized using a Potter-Elvehjem homogenizer with a Teflon pestle.

9.9 Ultra-centrifugation:

The samples were centrifuged in ultra centrifuge at 4º C. The homogenate was subjected to ultracentrifugation using Ti 70 rotor at 100,000g (40,000 rpm) for 70 minutes, which

yielded the fat cake, cytosol and membranes along with cell debris. To ensure that we get a uniform layer of fat cake, cytosol and intact debris we used low acceleration and low deceleration with no brake.

9.10 Obtaining pure fat cake:

The fat cake obtained from the first ultra-centrifugation was mixed with lysis buffer having 50 mM Tris pH 7.4, 1 mM EDTA and subjected to another centrifugation in order to avoid the cytosolic contamination and to get an intact fat cake (lipid droplet). The second ultra-centrifugation was performed in a SW 40 rotor at 100,000g for 70 minutes. The fat cake obtained from the second ultra-centrifugation was used for our further experiments.

9.11 Obtaining pellet sample:

The pellet sample obtained from the first ultra-centrifugation was resuspended in lysis buffer and centrifuged at 5000 g for 10 minutes in order to remove the cell debris. The supernatant obtained after the 5000 g centrifugation was further subjected to a second ultra-centrifugation at 100,000 g in SW 40 for 70 minutes. The pellet obtained from the second ultra-centrifugation was used for the analysis in SDS-PAGE.

Purification of LD from *M. sexta* fat body

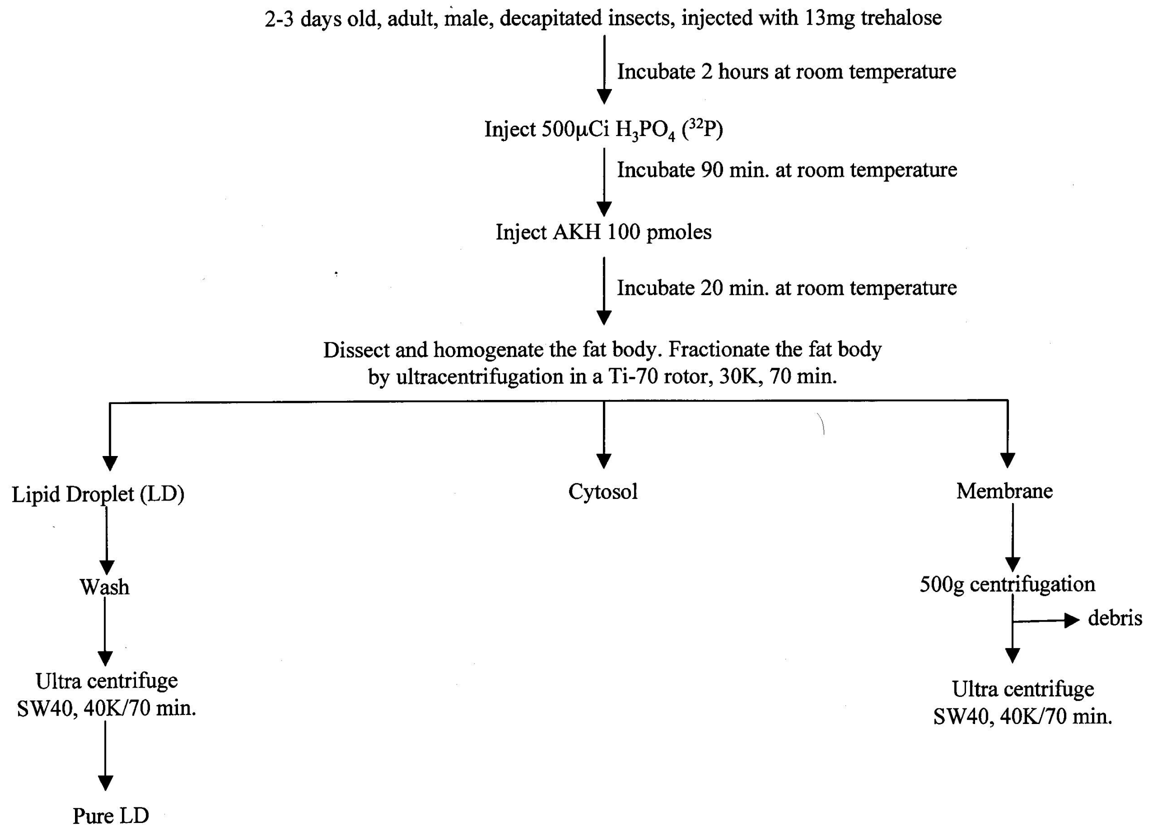

Figure-1: Flow chart for purification of LD from *M.sexta* fat body.

9.12 *In vitro* phosphorylation:

Lipid droplet sample was collected from the *Manduca sexta* insects as mentioned earlier. The obtained lipid droplet sample was subjected to *in vitro* phosphorylation by using MOPS buffer (MOPS 50 mM and 10 mM magnesium acetate having pH 7.0), DTT 1 mM, 0.3 mM cold ATP, 250 μCi of hot ATP (^{32}P) and commercial kinase. To perform the *in vitro* phosphorylation, the substrate lipid droplet, MOPS buffer, DTT, cold ATP, 250 μCi of hot ATP (^{32}P) and commercial kinase were incubated at 23º C for 20 minutes. After the incubation, the *in vitro* phosphorylation reaction was terminated by placing the mixture on ice.

9.13 SDS-PAGE and autoradiography:

SDS-PAGE was performed as mentioned in Laemmli (1970). Proteins were separated in 4-20% and 10% SDS-PAGE based on the molecular weight and were visualized either by coomassie brilliant blue R 250 or by silver staining. The gel was dried by using gel dryer and the dried gel was then subjected to autoradiography using Kodak films. The film was then scanned in densitometer (Bio-Rad model GS-700) and the analysis was performed by using Multi- Analyst Macintosh software .

9.14 MALDI-Tof mass spectrometry:

The endogenous lipid droplet samples obtained from the 2-3 days old *M. sexta* were subjected to 10% SDS-PAGE. The highly phosphorylated protein band was excised from the gel, cut into small pieces and destained using 100% acetonitrile, followed by four washes in water. The gel pieces were then equilibrated for 20 min in 500 µl of 100 mM

ammonium bicarbonate and subjected to incubation in 500 µl of 50% acetonitrile and 50 mM ammonium bicarbonate for 20 min (Patel et al., 2004). Gel pieces were dried for 20 min in a vacuum centrifuge and rehydrated for digestion with 50ng/µl trypsin (Promega sequencing grade) in 25 mM ammonium bicarbonate. The processed sample was then sealed with parafilm and incubated overnight at 4º C. The digested peptide was then extracted and analyzed by MALDI-Tof mass spectrometry in the core facility of Biochemistry and Molecular Biology department by Palaniappan Sevugan Chetty. We used α-cyano-4-hydroxycinnamic acid as matrix and external standards as calibrants in order to perform mass characterizations.

9.15 Purification of lipid storage droplet-1 (LSD-1) protein from *M. sexta* fat body:

9.15.1 Extraction of LSD-1 protein from *M. sexta* fat body:

Endogenous lipid droplets were obtained from 10 experimental insects and the volume of the lipid droplets was adjusted to 500 µl using TEB (10 mM Tris pH 7.2, 2 mM EDTA and 0.1% β mercaptoethanol). Lipid droplet samples were diluted six times using urea extraction buffer (UexB) which consists of 20 mM NaH_2PO_4 pH 7.2, 8 M urea, 2 mM EDTA and 250 mM NaCl in order to extract the protein from the lipid droplet. The extraction was performed at 23º C for 150 min with constant stirring using magnetic stir bar. After the incubation period, it was subjected to a brief centrifugation in the tabletop centrifuge at 5000 rpm for 5 minutes. The above-mentioned centrifugation yielded a top layer mainly of lipids and an infranatant, which consists of protein in the extraction buffer.

Extraction of LSD-1 protein from endogenous LD obtained from *M. sexta* fat body

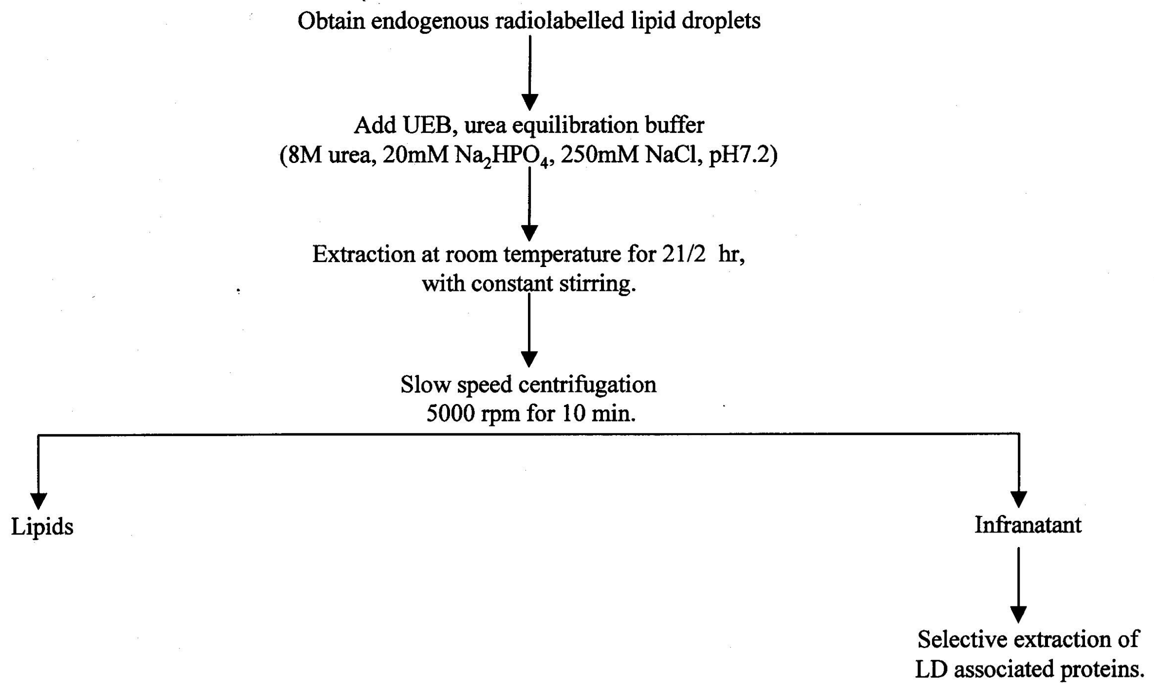

Figure-2: Flow chart for extraction of LSD-1 protein from endogenous LD obtained from M. sexta fat body.

9.15.2 DEAE column chromatography:

3 ml of DEAE sepharose resin was equilibrated with urea equilibration buffer (UeqB), which consists of 20 mM sodium dihydrogen phosphate pH 7.2, 8 M urea, 2 mM EDTA. The infranatant sample obtained from the extraction was diluted 10 times with (UeqB) in order to reduce the salt concentration to 25 mM, which was eventually passed through the column. The column was then washed with (UeqB) to remove the unbound components. The bound proteins were eluted from the column using sodium chloride steps of 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 600 and 1000 mM in Tris buffer (TB) pH 7.2. After the elution of proteins using sodium chloride step gradient the column was then washed with urea elution buffer (UeqB) and further eluted with (UeqB)-350 mM salt. (UeqB)-1M salt and (UeqB)-0.2% reduced Triton X-100.

The eluted fractions, TB-sodium chloride eluted fractions, UeqB eluted fractions, UeqB-sodium chloride fractions and UeqB-0.2% reduced Triton X-100 eluted fractions, were tested for radioactivity as well as for the protein content. We measured the radioactivity of the fractions by using liquid scintillation counter whereas we measured the protein by measuring the absorbance at 280 nm. The analysis of the eluted fractions using liquid scintillation counter, measuring the Abs @ 280 nm and 10% SDS-PAGE revealed that LSD-1 protein is present in UeqB-0.2% reduced Triton X-100 eluted fractions.

9.15.3 Q-sepharose column chromatography:

The peaks (fractions 3-6) obtained from the UeqB-0.2% reduced Triton X-100 fractions were pooled and were subjected to Q-sepharose column chromatography. 1 ml of Q-

sepharose resin was equilibrated with (UeqB) and the samples were diluted 4 times to reduce the concentration of Triton X-100 to 0.05%. The diluted sample was then allowed to pass through the column for 3 times to ensure that all proteins bound to the resin. The column was then washed with UeqB and eluted with 20 mM, 40 mM and 80 mM of N-octyl β-D glucopyranoside, which is 0.5%, 1% and 2% respectively.

9.15.4 Characterization of LSD-1 protein:

The analysis of fractions by absorbance and liquid scintillation counter revealed that the fractions eluted with 80mM N-octyl β-D glucopyranoside have LSD-1 protein. The detergent N-octyl β-D glucopyranoside has to be removed in order to assess the lipid-binding activity of LSD-1 protein. N-octyl β-D glucopyranoside was effectively removed by incubating the protein-detergent sample with bio-beads SM2. The protein sample was then incubated with 100 μg of 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposome, while diluting 8M urea with 20mM Na_2HPO_4 buffer (pH 7.2), having 50 mM NaCl. Upon the incubation the sample mixture was then subjected to bench top centrifugation @ 5000 rpm for 5 min. The incubation at room temperature for 2 hours with constant shaking allowed the liposomes to settle down at the bottom as pellet and the analysis of radioactivity revealed that LSD-1 is present along with liposomes.

CHAPTER 10

RESULTS AND DISCUSSION

10.1 Role of phosphorylation in lipid mobilization in insects:

Phosphorylation plays an important role in most of the metabolic reactions and it plays a pivotal role in maintaining the energy metabolism like lipid mobilization in insects. The insect fat body acts as an excellent reservoir to store the lipid molecules as TAG. For proper energy metabolism to occur, there must be a regulation at the level of phosphorylation/de-phosphorylation by kinases and phosphatases respectively. Hence, it is essential to analyze the role of phosphorylation in lipid mobilization of insects. Furthermore in view of the fact that the lipid droplet contains a large number of proteins embedded in the lipid surface, we were interested in investigation of the phosphorylation state of the lipid droplet associated proteins. For our experimental approaches we used AKH to stimulate the lipolytic condition in our model system *M. sexta*.

Though *in vivo* studies with intact insects are considered as the most relevant approach, the *in vivo* experimental results may have a few disadvantages. One such major disadvantage of *in vivo* experiment is the effect of endogenous hormones. The lipid mobilizing hormones such as AKH and octopamine may be released due to stress and starvation, the condition in which adult *M. sexta* were kept in the lab. However, Arrese et al., (1996) have shown that decapitated insects injected with 13 mg of trehalose, the

major sugar in hemolymph of insects, are better model system to study the lipid mobilization in *M. sexta*. Hence we decapitated the *M. sexta* insects in order to avoid the endogenous secretion of hormones and to overcome the effects of starvation. In female *M. sexta*, the bulk of the lipids are used in the eggs during oogenesis and the regulation of lipid dynamics in eggs may be different from the regulation of lipid dynamics in the fat body of *M. sexta*. In order to avoid the complexity, we used adult male insects for our experiments.

In this study, we are interested in finding out the molecular mechanism of lipolysis in insects in response to lipid mobilizing hormone (AKH). Hence it is necessary to use the optimum age group of insects for the experiments to accomplish our goal. In order to analyze the optimum age group of insects to study the phosphorylation states of lipid droplet associated proteins under AKH stimulated lipolytic conditions in insects, we used young (1-2 days old) and old (5-6 days old) insects.

10.2 *In vitro* phosphorylation of young and old insect lipid droplets:

The experimental results of Patel et al., (2004) have shown that *in vitro* phosphorylation is similar to *in vivo* phosphorylation. In order to analyze the optimum age group of insects, we performed *in vitro* phosphorylation in the lipid droplet of control and AKH treated young and old insects.

The autoradiography results of young and old insects show that the phosphorylation state of proteins around 42-44 kDa region of young insects is higher compared to the old insects and furthermore it indicates that the phosphorylation state of proteins decreases as the age increases. Since we did not use 1X phosphatase inhibitor cocktail in this experiment, the endogenous phosphatase might have dephosphorylated the phosphorylated proteins. Hence, upon *in vitro* phosphorylation, we could see the difference in the phosphorylation state of proteins between control and AKH treated lipid droplets of young and old insects.

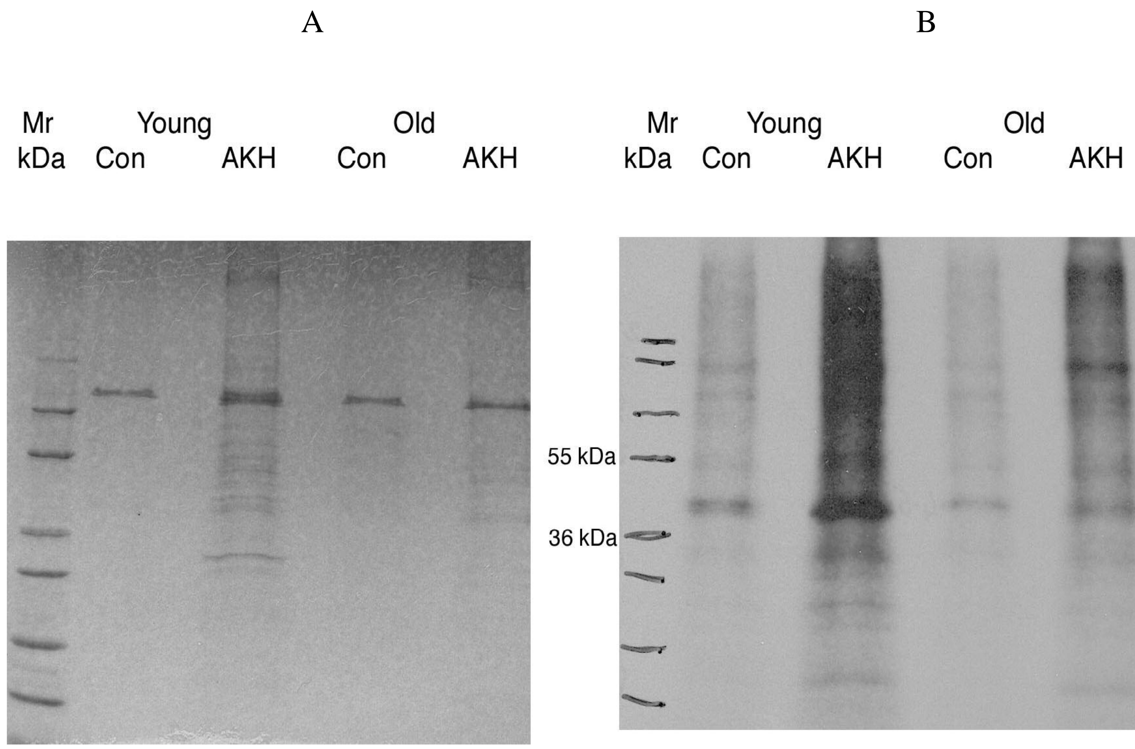

Figure-3: *In vitro* phosphorylation of young and old insect lipid droplets. Analysis of young and old insects lipid droplet in 4-20% coomassie stained gel (A). Autoradiography of young and old (B).

The densitometric analysis of autoradiography of young and old insect lipid droplet reveals that difference in the phosphorylation state of proteins around 42-44 kDa between control and AKH treated young insects is 5.2 fold, whereas the difference between the phosphorylation state of proteins around 42-44 kDa is only 2.5 fold in control and AKH treated young and old insects. This may be due to the presence of less number of receptors in old insects or it may be due to the degradation of receptors by proteases as the age increases or it may be due to the affinity of hormone towards its receptor. Though the incorporation of phosphorylation is higher in young insects, we cannot use the newly hatched insects, because the AKH receptor may not be developed properly. Arrese et al., (1995) have shown that the response of AKH in adult *M. sexta* develops only 8 hours after the imaginal molt. Furthermore, Ziegler et al., (1984) have shown that the response of AKH in adult *M. sexta* is greater in 2nd day of adult life. Hence we decided to use 2-3 days old, male, decapitated insects injected with trehalose as our model system to study the molecular mechanism of lipid mobilization in insects.

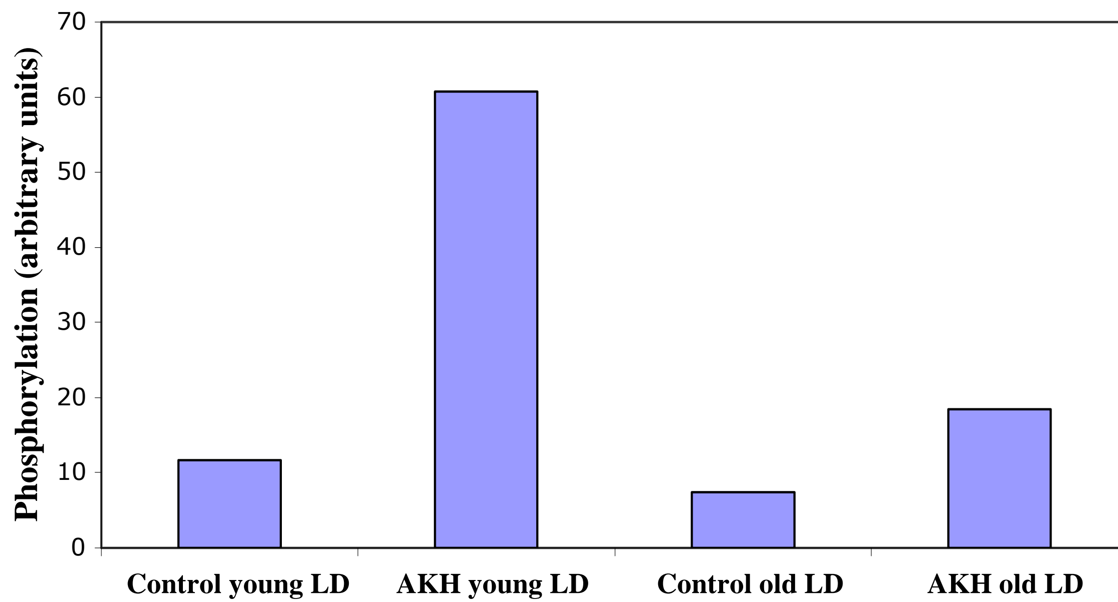

Figure 4: Densitometric analysis of autoradiography of young and old insect LD

10.3 *In vivo* phosphorylation:

The *M. sexta* insects were divided into two major groups namely control and experimental (AKH treated insects) insects. *In vivo* phosphorylation and the collection of endogenous radiolabeled lipid droplets were performed as mentioned in materials and methods. Upon the injection of ^{32}P (H_3PO_4) to the control and experimental insects, the experimental insects were furthermore injected with 100 pico moles of AKH and subjected to 20 minutes incubation, whereas the control insects were injected with insect saline. Since the only difference between the control and experimental insects is the injection of AKH, if there is any difference in the incorporation of radiolabeled phosphate between the control and the experimental insects, then it should be due to the action of AKH. Since we were interested in finding out the difference in the phosphorylation state of proteins between control and AKH treated insects, we performed our experiments using 4-20% gradient gels (data not shown).

AKH induces lipolysis in insects by activating cAMP dependent PKA, that in turn phosphorylates proteins involved in insect lipolysis. Those proteins that play a role in the lipolysis of insects will likely get phosphorylated due to the action of AKH. Since AKH is a lipid-mobilizing hormone, we believe that those proteins
phosphorylated under AKH conditions rather than control plays an important role in the lipid mobilization in insects.

Similar to *in vitro* phosphorylation, there is a major difference in the phosphorylation state of a protein in around 42-44 kDa between control and AKH treated insects, which is

the major phosphorylated protein in the AKH treated insects. The difference in the phosphorylation state of a protein in around 42-44 kDa indicates that this protein may play an important role in the lipolysis of insects. Hence we focused our attention on finding out the role of the highly phosphorylated protein under AKH stimulated lipolytic conditions of insects.

As a first step to find more about the role of the highly phosphorylated protein in the lipid mobilization of insects, we obtained the endogenous radiolabeled lipid droplets from control and experimental insects, which were subjected to 10 % SDS-PAGE to resolute the proteins of our interest.

The experimental results of 10% SDS-PAGE indicate that there were two protein bands lying close to each other in gel with approximate molecular weight of 42 and 44 kDa respectively. The autoradiography result (5 B) shows that there was a single major highly phosphorylated band. Based on the autoradiography, it was hard to say whether the protein in the region of 42-44 kDa was a single or a doublet protein. It could be due to the overlapping of two phosphorylated bands. To ensure that there were two proteins highly phosphorylated under AKH stimulated lipolytic conditions in *M. sexta*, we performed 2-D gel electrophoresis using Invitrogen ZOOM IPG Runner system.

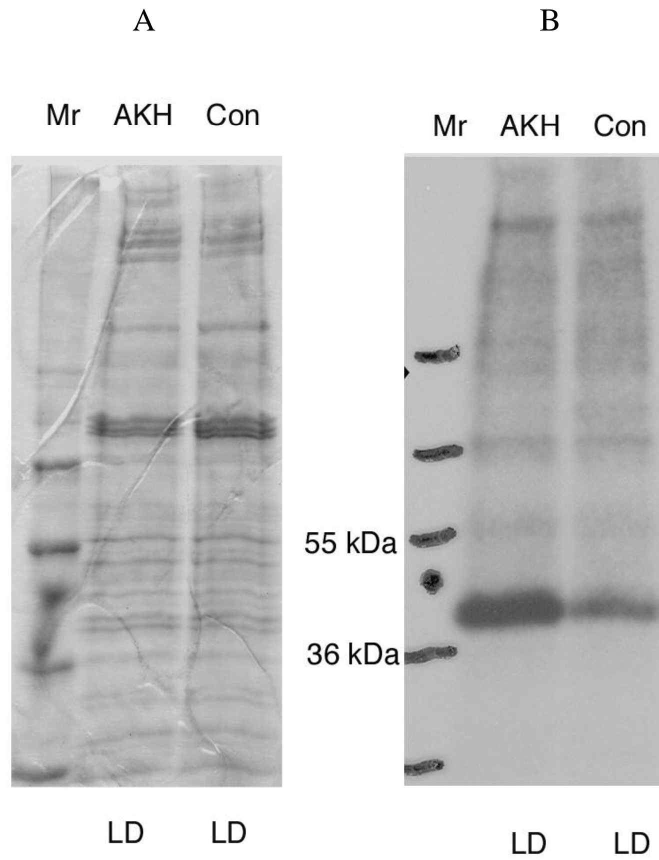

Figure-5: *In vivo* phosphorylation of control and AKH treated LD in 10% SDS-PAGE. Analysis of control and AKH treated lipid droplets in 10% coomassie stained gel (A). Autoradiography of control and AKH treated lipid droplets (B).

10.4 2-D gel electrophoresis:

Proteins were loaded to IPG ZOOM strips containing a pH gradient of 3-10. First dimension involves the separation of proteins based on their isoelectric point (pI), whereas the second dimension involves the separation of proteins based on their molecular weight using denaturing 10% SDS-PAGE. The resolution of 2-D gel electrophoresis due to the IEF and 10% SDS-PAGE revealed that the highly phosphorylated protein under our experimental condition in around 42-44 kDa was a doublet protein rather than a single protein and the pI of highly phosphorylated protein is 8.8. In order to identify the highly phosphorylated proteins that were heavily phosphorylated under AKH conditions, we performed MALDI-Tof experiment.

The experimental results of 2-D SDS-PAGE showed that there were two major phosphorylated bands in the gel, which is similar to the results we obtained from the 1-D gel electrophoresis.

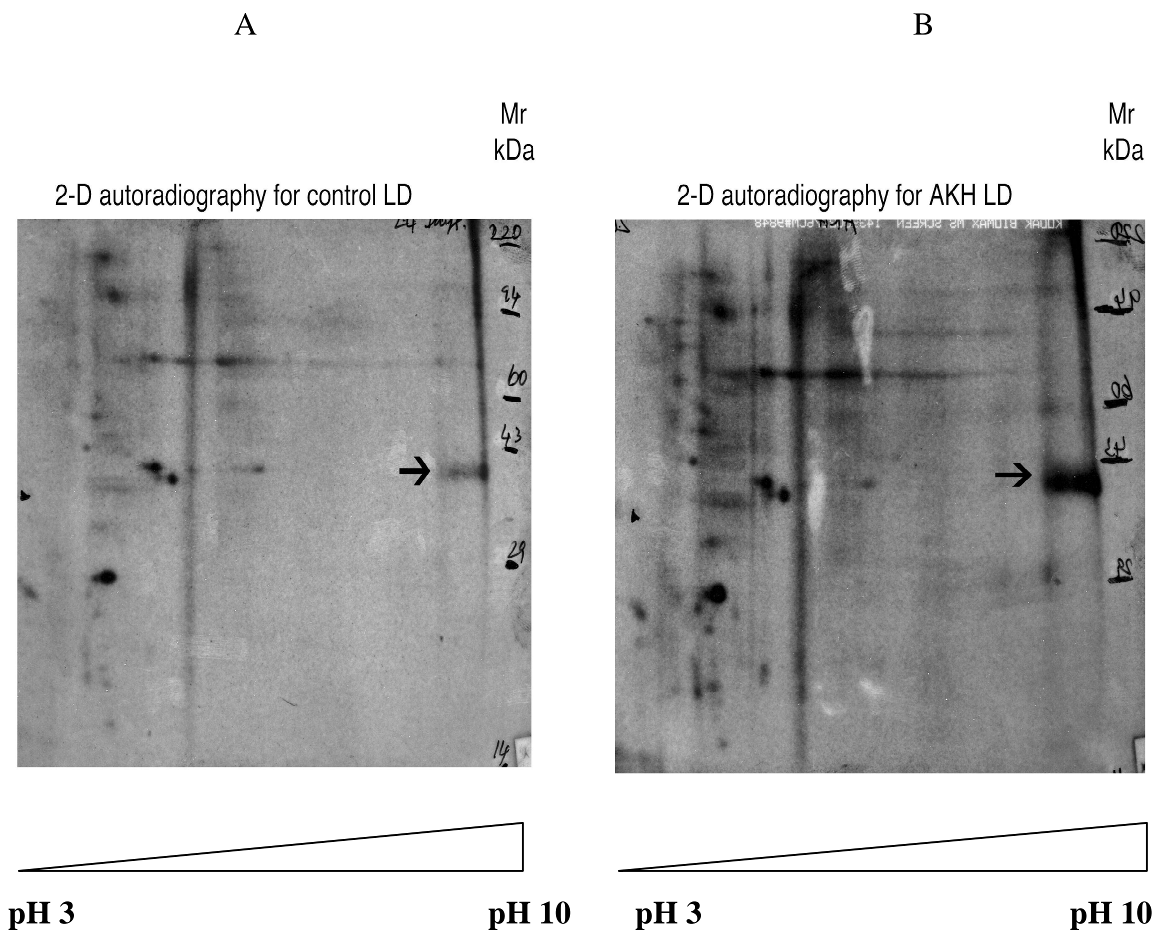

Figure-6: Analysis of control and AKH treated lipid droplet in 2-D gel. 2-D autoradiography for control LD sample (A). 2-D autoradiography for AKH LD sample (B).

Since we obtained similar MALDI-Tof spectra for both protein bands, we concluded that these two are isoforms of the same protein. Our experimental results of a doublet protein is similar to the experimental results of Gronke *et al*., (2003), who found a doublet protein having molecular weights of 44 kDa and 46 kDa in their experiment. Gronke *et al*., (2003) named the doublet proteins as LSD-2H and LSD-2L, where H and L stands for heavy and light respectively.

Furthermore, to study the mechanism of lipid mobilization in insects, we are interested in finding out the role of 42-44 kDa highly phosphorylated protein in insect lipolysis and purification of 42-44 kDa highly phosphorylated proteins. Hence our next logical step was to obtain the sequence of the protein. The poorly resolved doublet protein spot corresponding to the highly phosphorylated band having an approximate molecular weight of 42 kDa was cut from the 2-D gel. The excised protein band was then cleaved with trypsin and then sequenced by microcapillary reverse-phase HPLC nano-spray tandem mass spectrometry (μLC/MC/MS) on a Finnigan LCQ DECA XP plus quadruple ion trap mass spectrometer (Harvard Microchemistry lab).

The sequence obtained from Harvard Microchemistry lab (Figure-5) had an exact match (100%) with the sequence found in cDNA of *B. mori* (AU001457) and furthermore the sequence of our protein had significant sequence similarity with the lipid storage droplet (LSD-1) protein of *D. melanogaster*. Since the *M. sexta* sequences share high sequence similarity with LSD-1 protein of insects, the protein was identified as LSD-1 protein. LSD-1 protein is a perilipin like protein and hence it may be involved in the TAG

mobilization to ensure extended survival when food supply is limiting. Therefore we believe that our protein performs a more or less similar role of LSD-1 protein Drosophila.

Experiments performed on the insect species such as *Anopheles gambiae, D. melanogaster, B. mori* and slime mold *Dictyostelium discoideum,* revealed that the insect genome encodes only two PAT domain proteins namely LSD1 and LSD2 (Miura et al., 2002),. Though LSD-1 and LSD-2 are associated with intracellular lipid droplets they cannot be directly compared to any of the vertebrate family members. Even though LSD-1 protein and LSD-2 act in a perilipin-like manner in insects, it is very important to remember that the insect proteins LSD-1 protein and LSD-2 are not perilipin, but rather distant relatives that have evolved separately from the mammalian proteins. The *D. melanogaster* proteins will be much more similar to the *M. sexta* proteins than any of the mammalian proteins.

For the first time, we have shown that a highly phosphorylated protein from the fat body of *M. sexta* has sequence similarity to the LSD-1 protein of Drosophila.

10.5 Sequence of highly phosphorylated protein under AKH stimulated lipolytic condition:

	Peptide Sequences		Mass (kDa)	Accesion number
Manduca sexta[A]	**KVVHLVNYTHTDLPR**	**TYLEHLAIFLAGNEEREK**	42.8[C]	-
Bombyx mori[B]	**KVVHLVNYTHTDLPR**	**TYLEHLAIFLAGNEEREK**	-	AU001457
D.melanogaster[B]-*Lsd1*	280RVVHLVNFSAHVAANIPR	544AYLERVASFLSGRMEAEK	45.8	AY051436
Anopheles gambiae[B] *Lsd1*	331KMVHLINFVTGAVTRVP	406LALERLAVFLSGRLEAEK		EAA07592
Apis mellifera	273RIVHLVNGTAALAAKTPR	310TLNEQFAAFLAGRPSVSK		gi:48104779

[A] Sequence analysis of *M. sexta* protein was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-spray tandem mass spectrometry (μLC/MC/MS) on a Finnigan LCQ DECA XP Plus quadruple ion trap mass spectrometer

[B] Predicted sequence from the translated nucleotide sequence

[C] Determined by electrophoretic migration on 10% SDS-PAGE

Figure-7: Sequence of 42-44 kDa highly phosphorylated protein under AKH stimulated lipolytic condition in insects.

Our MALDI-Tof experiment and the sequence analysis of highly phosphorylated protein suggest that LSD-1 protein is a perilipin-like protein. Perilipin plays an important role in the lipolysis of vertebrates. Since few hypotheses suggest that perilipin or perilipin-like protein translocates from the lipid droplet to cytosol and membrane, we were interested in finding out whether LSD-1 protein involves in translocation phenomenon. Among the three major sub cellular fractions (lipid droplet, cytosol and membrane), we have analyzed the lipid droplet fraction (figure 3). In order to find whether LSD-1 protein undergoes translocation phenomenon, it is necessary to analyze all the three major sub cellular fractions. In order to analyze the translocation phenomenon and to validate our data, we performed a time course AKH treatment for the major sub cellular fractions.

10.6 AKH time course experimental analysis:

The insects were divided into 3 groups, which were labeled as control (0 minutes AKH treatment), 5 minutes AKH treatment and 20 minutes AKH treatment. The endogenous radioactive lipid droplets were collected from the insects separately and subjected to SDS-PAGE in 4-20% gradient gel.

Since we do not know the concentration of protein in the above-mentioned fractions, we have to rely on the autoradiography in order to find out the distribution of LSD-1 protein in the above-mentioned fractions during the AKH time course experiment.

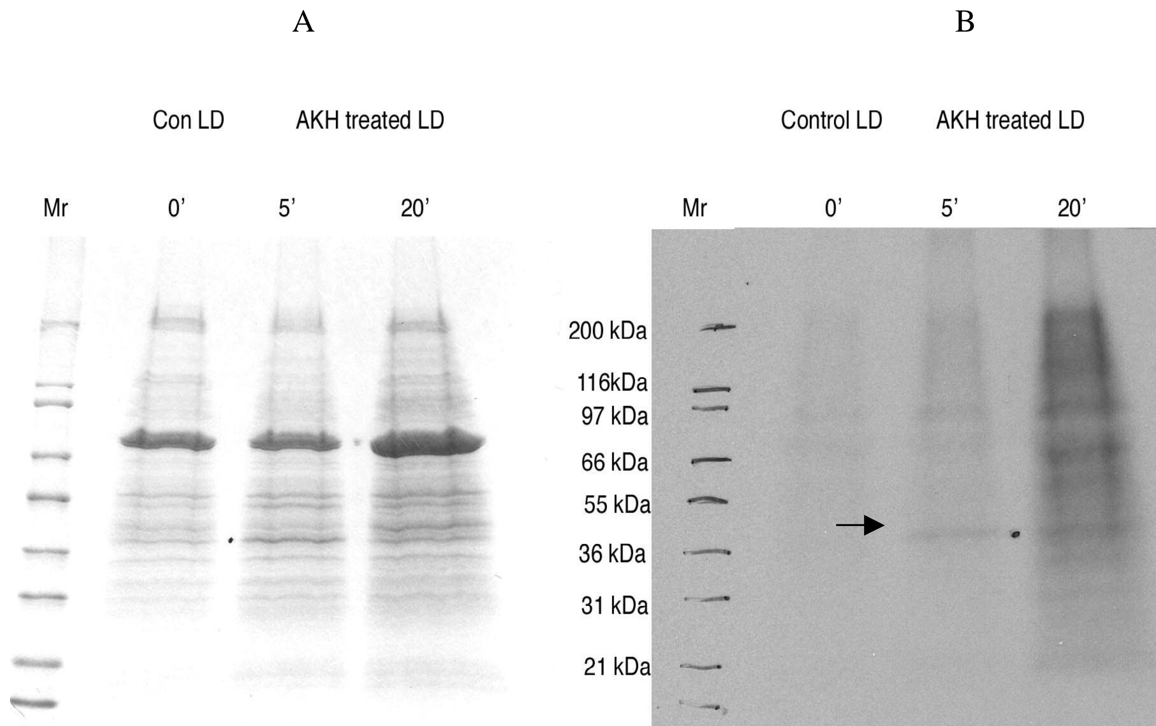

Figure-8: Time course experimental analysis of control and AKH treated lipid droplets. Time course experimental analysis of control, 5 minutes and 20 minutes AKH treated lipid droplet in 4-20% coomassie stained gel (A). Autoradiography for time course experimental analysis (B).

The analysis of proteins in SDS-PAGE and the subsequent autoradiographic analysis revealed that the incorporation of radioactive phosphate in LSD-1 protein is less in control, approximately 3.2 folds more compared to control in 5 minutes AKH treated insects and highly phosphorylated (nearly 13 folds compared to control) in 20 minutes AKH treated insects (Figure-7). This indicates that AKH induces phosphorylation of LSD-1 protein. Furthermore, the experimental results show that LSD-1 protein is the major phosphorylated band in 5 minutes AKH treatment. It indicates that LSD-1 protein may be the first protein to be phosphorylated under AKH stimulated conditions. Moreover, the LSD-1 protein was the highly phosphorylated even in 20 minutes AKH treated lipid droplet samples. It implies that LDS-1 may be the first member of the crewmembers involved in lipid mobilization in insects and furthermore AKH-mediated phosphorylation of this protein plays an important role in the lipid mobilization of *M. sexta*. The densitometric analysis indicates that the phosphorylation of LSD-1 protein is mild in control and the phosphorylation of LSD-1 protein increases as the time course of AKH experiment increases. In summary, the autoradiography and the densitometric analysis clearly shows that the LSD-1 protein is the first phosphorylated protein under AKH stimulated conditions and it remains as the highly phosphorylated protein band even at the end of 20 minutes AKH treatment. It clearly suggests that LSD-1 protein plays an important role in the lipolysis of insects.

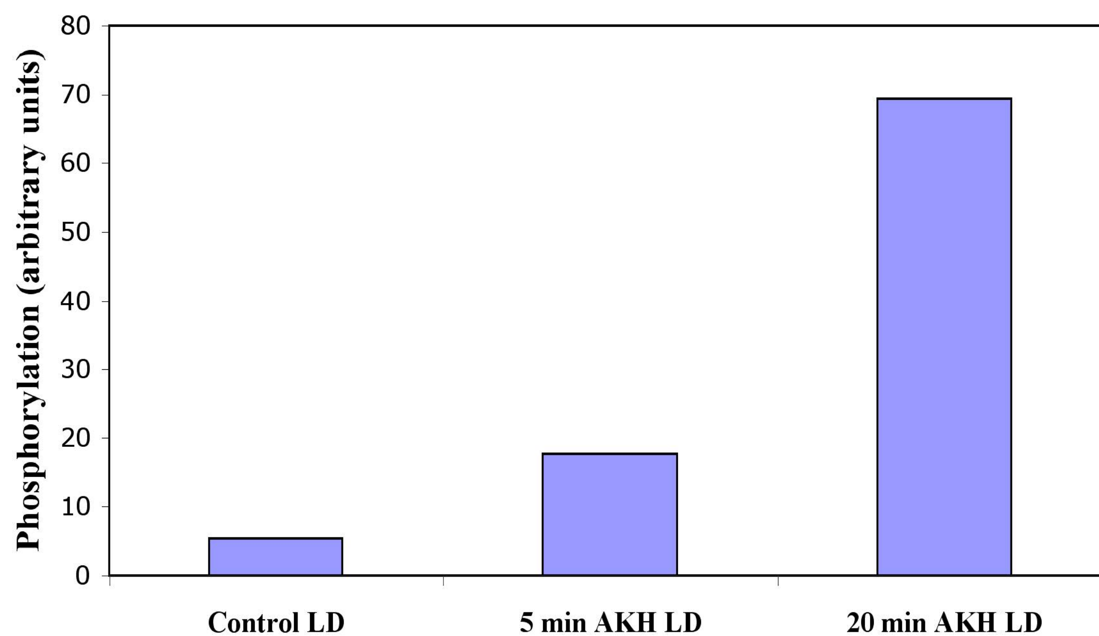

Figure-9: Densitometric analysis of autoradiography of time course experimental analysis of control and AKH treated lipid droplets.

Since the autoradiography result of cytosol shows that there was a faint band in the cytosol in the time course experiment, it may be due to the translocation of LSD-1 protein from the lipid droplet to another sub cellular organelle through cytosol. Since LSD-1 protein is a hydrophobic protein, it cannot reside in the cytosol rather it has to remain with membrane fractions. If the above-mentioned translocation hypothesis is true, it needs a carrier molecule in order to reach the membrane fractions. The presence of a faint band in the cytosol may be due to the contamination of protein from the lipid droplet.

Comparing the levels of LSD-1 protein in the membrane fractions (pellet sample), it is evident that the level of phosphorylation of LSD-1 protein increases as the incubation time period of AKH increases. Similar to LD, at the end of 20 minutes, LSD-1 protein is the most intense phosphorylated band in the membrane fractions. The intensity of LSD-1 protein in the membrane fractions is much less compared to the lipid droplet samples. It adds additional strength to the fact that the localization of LSD-1 protein is associated with lipid droplets and further more it indicates that LSD-1 protein is a membrane-associated protein.

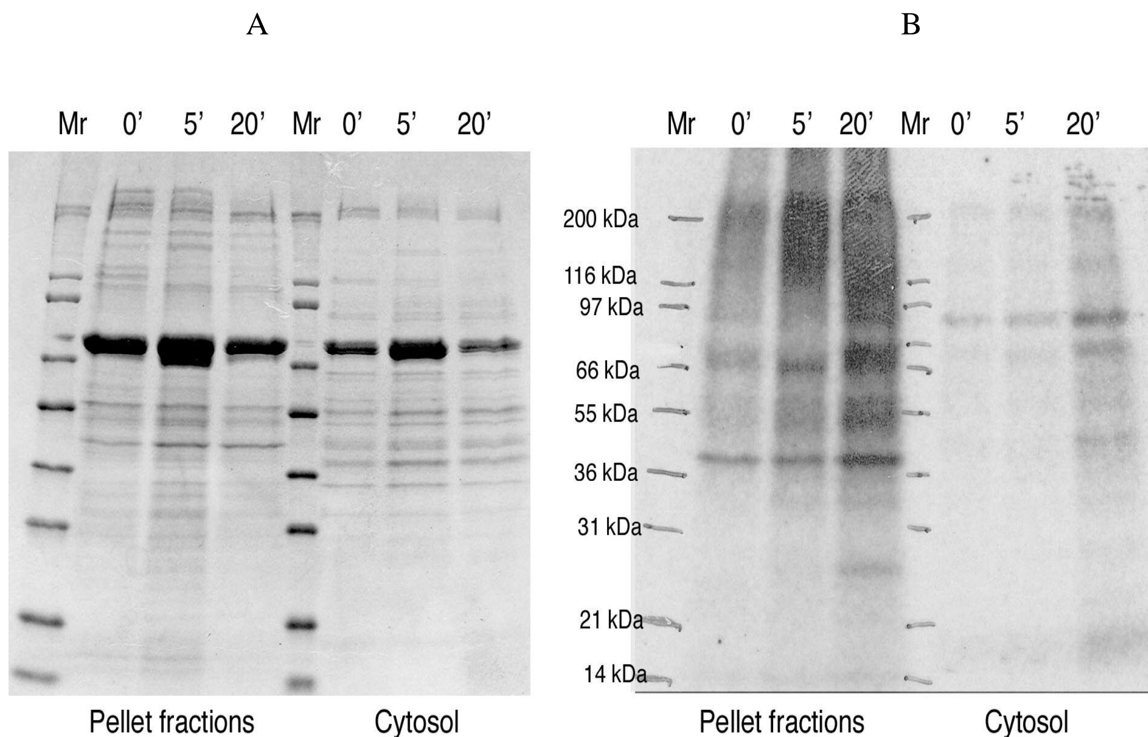

Figure-10: Ti me course experimental analysis of control and AKH treated membrane and cytosol fractions. Time course experimental analysis of control and AKH treated membrane and cytosol fractions in 4-20% SDS-PAGE (A). Autoradiography of time course experimental analysis of control and AKH treated membrane and cytosol (B).

10.7 Extraction of LSD-1 protein from lipid droplets:

In order to know more about LSD-1 protein, like characterization and role of LSD-1 protein in the lipid mobilization, it is essential to purify LSD-1 protein from the *M. sexta* fat body.

Analyzing the autoradiography of *in vitro* phosphorylation and *in vivo* phosphorylation (figure 1-B and figure 3-B) revealed that the phosphorylation state of LSD-1 protein is higher *in vivo* phosphorylation than *in vitro* phosphorylation and furthermore it was difficult to extract LSD-1 protein from the *in vitro* phosphorylated lipid droplets. Hence we used *in vivo* labeled lipid droplets as our sample in order to extract and purify LSD-1 protein from *M. sexta* fat body. The initial step in purification of LSD-1 protein from the *M. sexta* fat body is the extraction of LSD-1 protein from the lipid droplet. Since LSD-1 protein is a strong hydrophobic protein, we used 8 M urea and 250 mM NaCl in the extraction buffer in order to extract the protein from the lipid droplets as well as to keep the protein in the solution. The extraction of LSD-1 protein from the lipid droplets was done as mentioned in materials and methods. The presence of 8M urea and 250 mM NaCl facilitated the extraction of the protein from the lipid droplet. The analysis of infranatant in 10% SDS-PAGE revealed that most of the LSD-1 protein proteins were extracted from the lipid droplet (data not shown). Furthermore the analysis revealed that infranatant has only few other proteins compared to the original lipid droplets. Hence we conclude that extraction of LSD-1 protein from the lipid droplets using UexB is effective and furthermore it suggests that using UexB, LSD-1 protein can be purified partly from the lipid droplet.

10.8 Purification of LSD-1 protein:

In order to purify LSD-1 protein from *M. sexta* fat body, we used ion exchange chromatography. The infranatant obtained from the extraction was passed through the column and the proteins were eluted with sodium chloride step gradient. Analysis of the sodium chloride eluted fractions by SDS-PAGE and the presence of radioactivity in the column indicates that LSD-1 protein was still in the column even after passing 1M sodium chloride, which suggest that LSD-1 protein is a strongly hydrophobic protein.

In order to elute the protein we used 0.2% reduced Triton X-100, which will elute the hydrophobic proteins. Since LSD-1 protein does not have an enzymatic activity, the only way to track LSD-1 protein throughout our experimental procedures was to measure the radioactivity and the absorbance at 280 nm. The measurement of protein using A_{280} and the Cpm from the liquid scintillation counter indicates that our protein was in 0.2 % reduced Triton X-100 eluted fractions.

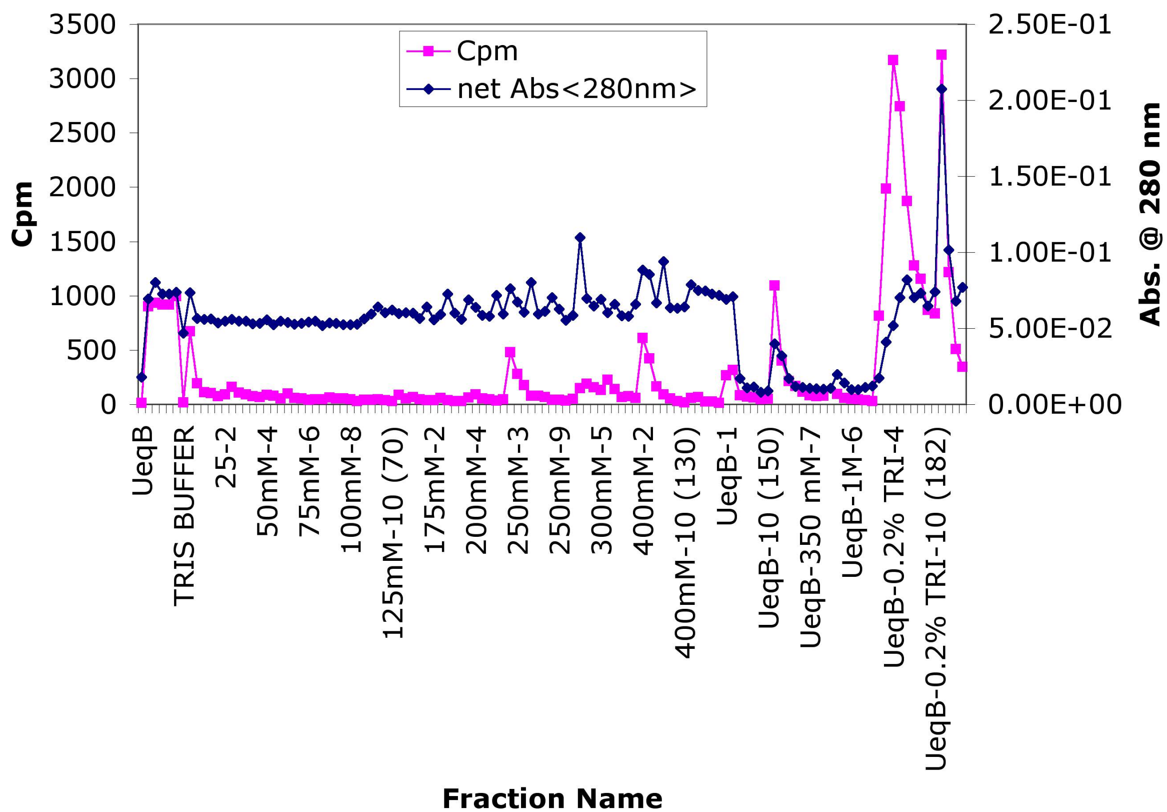

Figure-11: Analysis of fractions eluted from DEAE column by measuring the Abs @ 280 nm and measuring the Cpm by liquid scintillation counter

Though we do not know much about the LSD-1 protein, we do know that LSD-1 protein is a highly phosphorylated protein and the concentration of LSD-1 protein is less in *M. sexta*. Hence we analyzed the fractions containing more radioactivity and less protein. The analysis of the 0.2 % reduced Triton X-100 eluted fractions (fractions 4 and 5) in 10% SDS-PAGE clearly showed the presence of our protein in those fractions.

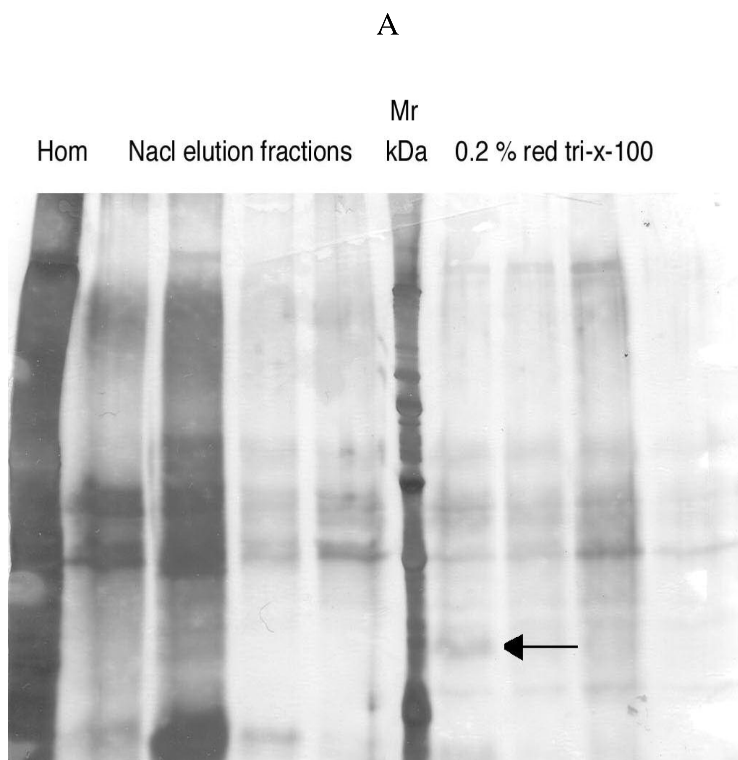

Figure-12: Analysis of sodium chloride and 0.2% reduced Triton X -100 fractions. Analysis of sodium chloride and 0.2% reduced Triton X-100 fractions in 10% SDS-PAGE silver stained gel (A). The arrow indicates the presence of LSD-1 protein in 0.2 % reduced Trition X-100 fractions.

In order to concentrate and to remove the reduced Triton X-100, we passed the 0.2% reduced Triton X-100 eluted fractions through Q-sepharose and the protein was eluted with increasing concentrations of (20 to 80 mM) N-octyl β-D glucopyranoside, an excellent detergent to elute the strong hydrophobic proteins. The samples having high A_{280} and the Cpm were then pooled and the measurement of A_{280} gave us the approximate concentration of protein in the sample, which is 0.05 mg/ml with 23.3 mg/ml of N-octyl β-D glucopyranoside.

10.9 Characterization of LSD-1 protein:

Since the concentration of LSD-1 protein is low, we decided to characterize the protein while we purify the protein from the fat body of *M. sexta*. Since LSD-1 protein is a lipid storage droplet protein, it should have lipid-binding activity. Hence we are interested to test whether LSD-1 protein has the lipid binding activity by incubating the protein with DMPC liposomes. In order to assess its lipid binding activity, we must remove the detergent from the sample, hence we incubated the protein sample having detergent with bio-beads. Incubation of bio-beads with the detergent allowed the detergent to bind with bio-beads, which was eventually removed by centrifugation. To ensure that our protein has lipid-binding activity, we incubated the protein (devoid of detergent) with liposomes, while diluting 8M urea with 20 mM Na_2HPO_4 buffer (pH 7.2), having 50 mM NaCl. Incubation at room temperature for 2 hours with constant shaking allowed the liposomes to settle down at the bottom as a pellet and the analysis of the radioactivity revealed that our protein is present in the bottom along with liposomes. It is evident that our protein has lipid binding activity and the sample was further subjected to 10% SDS-PAGE and

the proteins were visualized by Coomassie R-250 stain. Since the concentration of our protein is less, we could not see our protein band in the gel, but the autoradiographic analysis reveals that LSD-1 protein, which has the molecular weight of 42 kDa, is present in the gel.

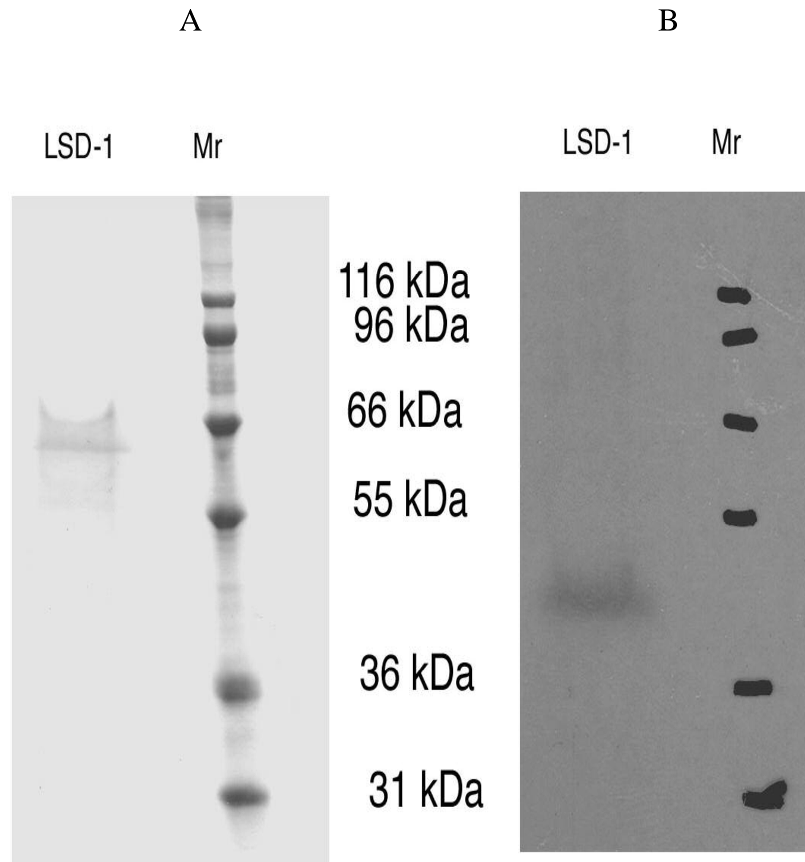

Figure-13 Purification of LSD-1 protein from *M. sexta* fat body. 10% SDS-PAGE gel to show the purification of LSD-1 protein from *M. sexta* (A). Autoradiography for the purification of LSD-1 protein from *M. sexta* fat body (B).

10.10 Conclusion:

Our experimental results of investigation of the phosphorylation states of lipid droplet associated proteins under AKH stimulated lipolytic conditions revealed that 42-44kDa doublet protein is the main target of the phosphorylation cascade triggered by AKH, mediated by PKA. 2-D gel electrophoresis, MALDI-Tof and subsequent sequence analysis revealed that the highly phosphorylated 42-44 kDa protein in *M. sexta* fat body is LSD-1 protein. Though LSD-1 protein shares a PAT domain structural motif similar to perilipin A of vertebrates, unlike perilipin, LSD-1 protein is not abundant in the lipid droplet of adult insect. However, in similar to perilipin A, LSD-1 protein may be a hyper phosphorylated protein having multiple phosphorylation sites. In this study for the first time, we have purified and characterized the lipid binding activity of LSD-1 protein from *M. sexta* fat body. Since the amount of the purified LSD-1 protein is less, further purification of LSD-1 protein with high yield is essential. Purifying the LSD-1 protein with high yield will be useful to obtain antibodies and to perform reconstitution experiment in the lipid droplet. The reconstitution experiment will allow us to analyze whether LSD-1 protein acts as a barrier to HSL, docking protein, to find the interacting partners of LSD-1 protein, to find the role of LSD-1 protein in activation of lipid droplet and the exact role of LSD-1 protein in the molecular mechanism of lipolysis in insects.

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VITA

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Title of Study: MECHANISM OF LIPOLYSIS IN INSECTS: ROLE OF SUBSTRATE

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Scope and Method of Study: The purpose of this study was to investigate the role of substrate in lipolysis in insects. In insects, lipolysis is the process whereby the neutral lipid triacylglycerol (TAG) is broken down into FFA (free fatty acid) and DAG (diacylglycerol). Lipid droplet (LD), the substrate of lipolysis, is composed of neutral lipids (>95% is TAG), surrounded by a monolayer of amphipathic phospholipids. LD associated proteins are embedded on to the phospholipid monolayer. In order to study the role of substrate in mechanism of lipolysis in insects, we need to analyze the role of lipid droplet associated proteins. Hence we investigated the phosphorylated states lipid droplet associated proteins under AKH stimulated lipolytic conditions. Upon *in vivo* phosphorylation, the highly phosphorylated protein with an apparent molecular weight of 42-44 kDa protein was subjected to 2-D gel electrophoresis, MALDI-Tof and sequence analysis. The highly phosphorylated protein under AKH stimulated lipolytic conditions was then purified and characterized.

Findings and Conclusions: Investigation of phosphorylated states of lipid droplet associated proteins under adipokinetic hormone (AKH) stimulated conditions revealed that 42-44kDa doublet protein is the main target of the phosphorylation cascade triggered by AKH, mediated by protein kinase A (PKA). 2-D gel electrophoresis and MALDI-Tof results revealed that the doublet proteins are the isoforms of the same protein. The subsequent sequence analysis revealed that the sequence of the highly phosphorylated protein has a sequence similarity with the lipid storage droplet -1 (LSD-1) protein of *Drosophila melanogaster.*

Advisor’s approval: ____