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Invited Mini Review

Microorganism lipid droplets and biofuel development

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Lipid droplet (LD) is a cellular organelle that stores neutral lipids as a source of energy and carbon. However, recent research has emerged that the organelle is involved in lipid synthesis, transportation, and metabolism, as well as mediating cellular protein storage and degradation. With the exception of multi-cellular organisms, some unicellular microorganisms have been observed to contain LDs. The organelle has been isolated and characterized from numerous organisms. Triacylglycerol (TAG) accumulation in LDs can be in excess of 50% of the dry weight in some microorganisms, and a maximum of 87% in some instances. These microorganisms include eukaryotes such as yeast and green algae as well as prokaryotes such as bacteria. Some organisms obtain carbon from CO_2 via photosynthesis, while the majority utilizes carbon from various types of biomass. Therefore, high TAG content generated by utilizing waste or cheap biomass, coupled with an efficient conversion rate, present these organisms as bio-tech 'factories' to produce biodiesel. This review summarizes LD research in these organisms and provides useful information for further LD biological research and microorganism biodiesel development. [BMB Reports 2013; 46(12): 575-581]

INTRODUCTION

Lipid droplets (LDs) are a spherical cellular structure that consists of a neutral lipid core, a monolayer phospholipid membrane, and numerous proteins (1-4). LDs have been found in almost all organisms, from mammals to bacteria (1, 5). LD biology research in mammals has developed rapidly due to the drastic development of human metabolic syndromes, such as obesity, fatty liver, atherosclerosis, and type 2 diabetes. Since perilipin was identified in adipocytes in 1991 (6), another four LD proteins that are expressed in other tissues have also been uncovered. These LD proteins, including perilipin, adipocyte differentiation related protein (ADRP) (7, 8), tail interacting protein (Tip47) (9), S3-12 (10), and OXPAT (11) contain a PAT (Perilipin, ADRP and Tip47) domain, thus termed PAT family proteins initially (12) and later the name changed to perilipin family proteins, with a recent simplification as PLIN 1-5 (13). Unfortunately, PLINs are only expressed in mammals and *Drosophila* (12). Recent proteomic analyses of isolated LDs identified several groups of functional proteins, including LD resident proteins, lipid synthetic enzymes, membrane trafficking proteins, signaling proteins, and lipases (5). Based on current studies, LDs are proposed to be generated on endoplasmic reticulum (ER) and found to migrate onto microtubules (14), and also are observed to interact with other cellular organelles via Rab proteins (15-18), and fuse each other using SNAREs (19) and Fsp27 (20). At least three types of neutral lipids, such as triacylglycerol, ether lipids, and cholesterol ester, were identified as major components of LDs using lipidomic analysis (21). In culmination, these findings lead to a conclusion that LDs are a cellular organelle (15).

LDs are also observed in plant seeds and some plant cells, and often termed lipid bodies within this field. Plant LDs have also been successfully isolated and analyzed (22-25). Their LD resident proteins were identified, including oleosins (26) and caleosins (27). Interestingly, both types of plant LD resident proteins do not contain PAT domain that are common with all 5 mammalian PLINs. Further, a neutral lipid insertion sequence plays an important role in the targeting of oleosin to LDs, which is also different with PLINs. Early works also found that oleosins can be recognized by anti-apolipoprotein antibodies (28). An apolipoprotein motif has recently been found to be common in most LD resident proteins (5).

Moreover, LDs in microorganisms have also been studied, although in similarity to plants and seed embryos, that they do not contain PLINs either (29). At least LDs present in three types of microorganisms, such as yeast (30-32), green algae (33, 34), and bacteria (35-37), have been well analyzed and characterized. This is primarily due to their importance as useful models to study cellular organelle biology as well as of biofuel development. Many LD-associated proteins have been

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identified, especially several LD resident proteins, which have been found to be involved in LD dynamic regulation. Among these LD proteins, lipid synthetic enzymes have also drawn attention because of their ability for triacylglycerol (TAG) production.

The accepted consensus is that fossil oil deposits are limited and non-renewable. The extensive use of fossil fuels has lead to climatic and subsequent social problems such as the "greenhouse" effect and the air pollution (38) in addition to potential energy exhaustion. Therefore, it is imperative to develop renewable biomass that can quickly accumulated carbon source such as crops, grass, and microorganisms (39, 40). Due to the lower sulphur and nitrogen pollution, the rapid accumulation and the high applicability, biofuel especially biodiesel is becoming more and more popular as a potential replacement for fossil fuel derived diesel (41).

TAG stored in the LDs of plants and microorganisms can be converted to biodiesel. Using oil crops (i.e. canola) to produce biodiesel not only in some cases competes human food requirement but also has very low efficiency of TAG production when compared with microorganisms. For example, green algae can produce nearly 100 fold more TAG than what the best oil plant, soybean can make (42). In addition, one type of bacteria, *Rhodococcus opacus* PD630 is able to store TAG in LDs nearly 87% of its dry weight, making it potentially the ideal microorganism for biodiesel development (43, 44). Interestingly, this bacterium can also be used as a model organism to study LD biology since it does not contain any other cellular organelles (45).

Therefore, it is necessary to review existing researches that have been done on these organisms so far, in particular to review proteins that have been identified by proteomics using isolated LDs from these organisms. The information accumulated will not only facilitate these organisms as LD biology model systems but also promote biofuel development using these organisms.

YEAST

As a good genetic model organism, yeast has been utilized in biological research field well and many important molecular mechanisms of biological processes have been discovered using the organism. Since nearly all yeasts contain LDs, yeast is a useful organism for LD research and in fact, many studies have been done and some important pathways that govern LD biogenesis and dynamics have been revealed. Yeast is also a good organism to convert biomass to neutral lipids such as TAG in LDs that can be used produce biodiesel.

Using *Saccharomyces cerevisiae* (*S. cerevisiae*), Dr. Daum's research group has gained many progresses in LD biology of the organism, especially in the understanding of LD lipids and proteins. LDs had been designated as lipid particles in *S. cerevisiae* until recently (46). To study LDs in detail, LDs were isolated from *S. cerevisiae* (47). Lipid composition of the isolated LDs was determined, with more than 90% of lipids comprising as TAG and steryl esters (47). In 1999, the same group isolated LDs again and conducted a proteomic analysis on LD proteins (48). Among these proteins, lipid synthetic enzymes were identified on LDs for the first time, such as Erg1p, Erg6p, and Erg7p that are involved in ergosterol biosynthesis, and Faa1p and Faa4p that are involved in long-chain fatty acid acyl-CoA synthesis. Moreover, detailed lipidomics and proteomics were carried out recently on isolated LDs from *S. cerevisiae* (46). The comparative studies of LDs from cells cultured in glucose and oleate uncovered dynamic changes of LD lipids and proteins. Based on these findings, it is proposed that LDs are a cellular organelle that is not only involved in lipid metabolism but also contributes to lipid synthesis.

In *S. cerevisiae*, LDs/lipid bodies are found to be in contact with peroxisomes and also a part of peroxisome is often observed in LDs, which may accumulate free fatty acids. It is termed "gnarl", indicating that this physical contact between LDs and peroxisome facilitates lipolysis within LDs and fatty acid oxidation in peroxisomes (49). The paper also identifies many other proteins in isolated LDs, further verifying previous finding, suggesting the interaction between LDs and other cellular organelles, such as endoplasmic reticulum (ER) and mitochondria (49). Moreover, the lipid synthetic enzymes identified by Dr. Daum are also verified by this study.

In an applied field, *S. cerevisiae* has also been utilized as a biological model to study metabolic disorders. For example, a lipodystrophy protein seipin was found to alter LD morphology by two research groups, Drs. Goodman and Yang, respectively (32, 50). By screening *S. cerevisiae* mutants, 59 genes were revealed to be associated with LD morphological alternation, with seipin being one of those identified (50). Another mutant screening of *S. cerevisiae* identified that the dysfunction of seipin, which regulates LD morphology and often results in super-sized LD formation (31). Although *S. cerevisiae* is a good model to study LD biology and related metabolic disorders, it may not be a suitable organism to use for biodiesel production due to the high steryl esters (SE) content and low neutral lipid storage capacity. So, genetic engineering optimization of *S. cerevisiae* is required to utilize it for biodiesel development.

Other types of yeast species have also been studied and manipulated for neutral lipid storage and biodiesel development, i.e. *Yarrowia lipolytica* (*Y. lipolytica*) (51). *Y. lipolytica* is considered as an oleaginous yeast, and has a pronounced ability to digest hydrophobic substances, and also convert the metabolites to lipids, and further store the lipids in LDs. Further, LDs/lipid particles of *Y. lipolytica* were isolated and their proteins and lipids analyzed (52). In comparison with *S. cerevisiae*, *Y. lipolytica* yeast contains a much higher ratio of TAG/SE, such as 1.2 in *S. cerevisiae* (47) as opposed to 10.8 in *Y. lipolytica* (52), suggesting that *Y. lipolytica* is suitable for biodiesel production. Moreover, another yeast, *Pichia pastoris* (*P. pastoris*) has also been found to have higher ratio of

TAG/SE as recently by analyzing isolated LDs using lipidomics (53). The proteomic study of isolated LDs determined that LD-associated proteins of *P. pastoris* are less than the LD proteins of *S. cerevisiae*. Together, these finding suggests that yeasts *Y. lipolytica* and *P. pastoris* are better utilized than *S. cerevisiae* for TAG production and TAG accumulation, therefore more suitable for biodiesel development.

Lastly, another oleaginous yeast *Rhodosporidium toruloides* (*R. toruloides*) has also been established by Dr. Zhao's group recently. The whole genome and transcriptome of the organism are sequenced and characterized (54). Furthermore, the genes involved in lipid synthesis and metabolism are identified, providing a useful database for further developing the organism for biodiesel production.

GREEN ALGAE

The growing crisis of world energy and food shortages initiates the use of green algae to produce vegetable oil, essential fatty acids, as well as biofuel. As a photosynthetic microorganism, algae convert CO_2 and H_2O to TAG using sunlight. Usage of CO_2 from the atmosphere reduces greenhouse gas accumulation and slows down global warming. As opposed with agricultural oil crops, algaculture can produce oil 10 to 100 fold more per unit of land (42). Using urban building design such as skyscrapers for algaculture, this yield can be multiplied as a function of level number and utilize the vertical aspect of the building which would be perpendicular to the sun's angle throughout the day and thus energy needed for growth and biodiesel production. In addition, genetic engineering algaculture can produce high value vegetable oil such as omega-3 oil and food supplemental oil such as arachidonic acid containing TAG. Furthermore, genetic engineering can modify algae to synthesize many other types of biofuels. Therefore, alga LD biology is rapidly gained more attention.

Similar as plants, LDs are not only found in the cytoplasm of green algae but also in chloroplast (55). Based on lipid analysis of isolated chloroplasts from *Chlamydomonas reinhardtii* (*C. reinhardtii*), it appears to be that TAG synthesis is located in chloroplasts, with LDs being distributed to both cytosol and chloroplasts (56). This is in agreement with the hypothesis of LD biogenesis in mammals in which LDs are proposed to be formed on surface of endoplasmic reticulum (2).

Proteomic studies have been performed using isolated LDs from several alga species and alga LD protein databases generated, which promote the development of utilization of green algae in lipid storage for biodiesel. In common with LD proteomes, alga LD-associated proteins contain lipid synthetic enzymes and membrane trafficking proteins. The difference between mammalian LDs and green alga LDs is the perilipin family proteins, with alga LDs lacking of these proteins. Therefore, the important discovery of alga LD proteomic studies is the identification of a 28 kDa alga LD protein that has been named major lipid droplet protein (MLDP) in *C. reinhardtii* (57). Reduction of MLDP expression has been found to increase the size of LDs without changing TAG content, suggesting its function to regulate LD morphology (57). MLDP has also been found in other green algae by sharing a conserved motif with 21 identical amino acids (58). No homology of the MLDP protein sequence has been observed in other organisms, which indicates that MLDP is a unique protein in the green algal lineage of photosynthetic organisms (57, 58). These characteristics of MLDP make it an alga LD resident/structural protein, analogous to the perilipin 1 and ADRP for LDs in mammals.

In extreme conditions, for instance, nitrogen deprivation or highlight exposure, the growth of many green algae can be limited, and in addition neutral lipids will be accumulated in LDs. Using this treatment, several studies of LD isolation from *C. reinhardtii* have been conducted recently (33, 57, 59). Wang *et al*. obtained relative pure LDs that are absence of chloroplast specific neutral lipids (galactolipids) (60). They analyzed the lipid compositions of the LDs but did not examine the proteome. Since perilipin family proteins are only expressed in mammals and Drosophila, to identify alga LD resident proteins, Moellering and Benning isolated LDs from *C. reinhardtii* and conducted the proteomic studies (57). 259 proteins were found to be associated with the isolated LDs, including proteins involved in lipid metabolism, vesicular trafficking, translation, mitochondrial activity, and photosynthesis (57). Many of these proteins have been identified previously in the isolated LDs of other organisms, particularly in mammals, such as acyl-CoA synthetases, acyl-CoA transferases, Rab proteins, and ARF-related GTPase. A primary protein band about 28 kDa was identified to be MLDP. James *et al*. then isolated LDs from *C. reinhardtii* and conducted another proteomic analysis (61). 28 kDa MLDP is also the most abundant band in LDs proteins. Nguyen *et al*. further verified the proteomic study of LDs that was carried out by Moellering and Benning, and analyzed LD proteins in detail for lipid metabolism (33).

More proteomic studies of isolated LDs from green algae have been conducted recently, including LDs from *Dunaliella salina* (*D. salina*) and *Haematococcus pluvialis* (*H. pluvialis*). The protein profile of LDs isolated from *H. pluvialis* significantly differs when compared with other LD proteins. A protein that shares partial homology with *C. reinhardtii* MLDP is observed in isolated LDs with a molecular weight 33 kDa (34). The protein is then termed the *Haematococcus* Oil Globule Protein (HOGP) (34). In isolated LDs from *D. salina*, MLDP is identified as the most abundant LD protein with molecular weight of approximately 28 kDa (58). Based on a sequence alignment, MLDP was found in six species of green algae including *C. reinhardtii*, *D. salina*, *D. bardawil*, *D. parva*, *H. pluvialis*, *Volvox carteri* (*V. carteri*) (58). This analysis also identified a 21 amino acids conserved domain in all six species and a 4-proline signature near the C-terminus of the protein (58). In addition, expression of MLDP is positively associated with TAG accumulation in nitrogen deficient culture

condition (58). Moreover, by reducing MLDP expression using RNAi, the LD size is observed to be increased without altering TAG content and metabolism rate (58). MLDP is also localized on alga LDs by immunogold labeling (59).

The accumulated data from proteomic and lipidomic studies of isolated LDs have facilitated LD biology of green algae, which stimulates the development of green algae to become a better model organism to photosynthesize TAG for biodiesel production.

BACTERIA

Except almost all eukaryotic organisms contain LDs, with some prokaryotic cells have also been observed to accumulate large amount of lipids (62). The LD studies in bacteria have recently been motivated by the search to understand the factors that govern infective bacterial action in humans as well as by developing prokaryotic organisms to produce biodiesel more efficiently. The actual size of bacterial LDs is relative small, which presents difficulty in their isolation and also analysis. Several studies of isolated LDs from bacteria have been conducted using an infective bacterium named *Mycobacterium bovis bacillus* Calmette-Guérin (*M. bovis* BCG) (63) and also in two oleaginous bacteria, *Rhodococcus opacus* PD630 (PD630) (45, 64) and *Rhodococcus sp.* RHA1 (RHA1) (37).

LDs isolated from the infective bacterium *M. bovis* BCG were analyzed by Dr. Wenk's group (63). The LD-associated protein composition significantly differs with the total cell lysate, encouraging the group further to subject these unique protein bands to MS analysis. Their discoveries are similar to Yeast in terms of identification of proteins on the isolated LDs that are involved in lipid synthetic and hydrolysis (63).

The genome of RHA1 was obtained in 2006, which allows the researchers to study this organism in many aspects (65). For biodiesel development, Dr. Liu's research group isolated LDs from RHA1 and also conducted comprehensive proteomic analyses (37). They identified a LD-associated protein and determined its LD targeting sequence. Deletion of this gene in RHA1 causes larger LD formation without increasing cellular TAG, suggesting that it functions to protect LD fusion (37). They designated the protein as the microorganism lipid droplet small (MLDS) in order to distinguish it from MLDP in green algae (37).

Strikingly, PD630 is able to accumulate 87% TAG of the bacterial dry weight (43, 44), which proposes it as an ideal organism for the study of the regulation of lipid biosynthesis and storage of the organism. Dr. Steinbüchel's research group has investigated this bacterium since 1995 and generated many useful data that are the basis of recent studies (44). His laboratory isolated lipid inclusions from both PD630 and *Rhodococcus ruber* for the first time and identified a series of granule-associated (GA) proteins (64). The genes that may govern lipid metabolism in PD630 were then analyzed and the genome was partially sequenced (66). In order to obtain a complete genomic regulation roadmap that controls lipid metabolism as well as storage in PD630, Dr. Liu's group has conducted a whole genome sequencing, comparative transcriptomes, and LD isolation and proteomic study since 2009, and eventually published them this year (45). Together, these works established not only a model organism for LD biology but also developed a good microorganism for applied biodiesel production.

CONCLUDING REMARKS

We herein summarized three microorganisms that have been well established in LD biology including isolation of their LDs as well as the proteomic studies of the isolated LDs (Table 1). Commonality of these findings is that the lipid synthetic path-

Table 1. Lipid droplets of microorganisms

Species	Name	TAG	Reference	LD proteomics	Whole cell proteomics
Bacteria	RHA1	Major	(37)	√	
	PD630	Major	(45, 64)	√	
	R. opacus MR22	Major	(64)	√	
	R. ruber	Major	(64)	√	
	M. bovis BCG	Major	(63)	√	
	M. xanthus	Major	(67)	√	
Algae	*C. reinhardtii*	Major	(33, 57, 59, 61)	√	
	Dunaliella	Major	(58)	√	
	JPCC DA0580	Major	(68)	√	
	Nannochloropsis	Major	(69)	√	
	H. pluvialis	Major	(34)	√	
	C. vulgaris	Major	(70, 71)		√
Yeast	*S. cerevisiae*	TAG : SE = 1 : 1	(46-50, 72-74)	√	√
	P. pastoris	Major	(53)	√	
	Y. lipolytica	Major	(52)	√	
	Schizosaccharomyces		(75)	√	
	R. toruloides		(54)		√

way is conserved throughout these organisms. The studies on LDs from the bacteria RHA1 and PD630 develop a new path for micro-diesel production using waste biomass.

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