Mag. Vasyl' A. Ivashov

Non-polar lipids in *Pichia pastoris*

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* is frequently used as host for heterologous protein expression in industry. Surprisingly, fundamental knowledge of the cell biology of this microorganism including membrane and organelle structure and lipid metabolism is largely missing. This Thesis provides first insight into the non-polar lipid storage and mobilization machinery of *P. pastoris* and structural features of the lipid storage compartment, the lipid droplets. A method for the isolation of highly pure lipid droplets is described. Isolated lipid droplets were analyzed for their lipidome and proteome. Major components of lipid droplets from *P. pastoris* are triacylglycerols (TG) and steryl esters (SE). The fatty acid compositions as well as the phospholipid pattern of lipid droplets largely reflect the total cell extracts. Proteome analysis of lipid droplets identified a small but distinct set of proteins with many of them being involved in lipid metabolism. To study lipid storage in *P. pastoris*, genes encoding for the major enzymes of TG and SE synthesis were identified. The two gene products of *LRO1* and *DGA1* were characterized as TG synthases; and Are2p was identified as the only SE synthase. Experiments with single and multiple mutants deficient in these genes showed that Dga1p and Are2p have certain substrate specificities towards saturated fatty acids. Moreover, the ability of *P. pastoris* to form lipid droplets was shown to depend completely on TG formation. Interestingly, the absence of non-polar lipids dramatically changes the pool of total cellular fatty acid to higher amounts of polyunsaturated species. In addition, as a preliminary result of this Thesis identification of TG lipases and SE hydrolases from *P. pastoris* is presented. Moreover, a protocol for isolation of intact vacuoles is described which fulfills requirements for fundamental lipidome and proteome analysis.

KURZFASSUNG

Die methylotrophe Hefe *Pichia pastoris* wird häufig in der Industrie als Wirtsorganismus für die Expression heterologer Proteine verwendet. Überraschenderweise ist das Wissen über Grundlagen der Zellbiologie einschließlich der Membran- und Organellenstruktur dieses Mikroorganismus sehr begrenzt. Diese Dissertation beschreibt Studien zur Depotbildung von Neutrallipiden und zu deren Mobilisierung in *Pichia pastoris* sowie eine Charakterisierung der Lipidpartikel, dem Organell der Lipidspeicherung. Isolierte Lipidpartikel wurden hinsichtlich ihres Lipidoms und Proteoms untersucht. Die wichtigsten Komponenten der Lipidpartikel von *Pichia pastoris* sind Triacylglycerole (TG) und Sterolester (SE). Die Zusammensetzung der Fettsäuren und der Phospholipide der Lipidpartikel entspricht weitgehend dem Gesamtzellextrakt. Die Proteomanalyse der Lipidpartikel identifizierte eine kleine Gruppe von Proteinen, wobei einige dieser Proteine im Lipidmetabolismus eine Rolle spielen. Um Lipidspeicherung in *Pichia pastoris* genauer zu untersuchen wurden die Gene, welche für Enzyme der TG und SE Synthese kodieren, identifiziert. Die beiden Genprodukte von *LRO1* und *DGA1* wurden als TG Synthasen charakterisiert, während Are2p als einzige SE Synthase identifiziert wurde. Experimente mit Einzelmutanten und multiplen Mutationen zeigten bestimmte Substratspezifitäten von Dga1p und Are2p in Richtung gesättigter Fettsäuren. Außerdem wurde gezeigt, dass die Bildung der Lipidpartikel vollständig von der TG Synthese abhängt. Interessanter Weise hat die Abwesenheit der unpolaren Lipide dramatische Auswirkungen auf das Fettsäuremuster im Gesamtzellextrakt, wobei mehrfach ungesättigte Fettsäuren bevorzugt werden. Zusätzlich zu den oben genannten Aspekten wurden TG Lipasen und SE Hydrolasen von *Pichia pastoris* in dieser Dissertation identifiziert. Schließlich wurde ein Protokoll für die Isolierung intakter Vakuolen etabliert, das als Grundlage für Lipidom und Proteom-Analysen dienen kann.

AIMS AND OUTLINE OF THIS THESIS

Pichia pastoris is a well characterized microorganism regarding its application as host for heterologous protein expression. Interestingly, fundamental knowledge about its cell biology is confined to a few areas dealing with peroxisome biogenesis and function, autophagy and secretion. Although some basic research has been performed recently, a comprehensive knowledge about *P. pastoris* organelles is still largely missing. Particularly, the field of *P. pastoris* lipid metabolism and organelle lipidomes and proteomes requires more detailed investigations. The present study is aimed to shed light on the lipid and protein composition of two *P. pastoris* organelles, the lipid droplets and the vacuole, and to characterize non-polar lipid storage in this yeast by means of cell and molecular biology, lipidomics and proteomics. The first main topic of this thesis is the design of reliable procedures for the isolation of lipid droplets and vacuoles as a prerequisite for profound lipid and protein analysis. The second aim of the work was molecular characterization of genes and gene products of non-polar lipid synthesis and storage *P. pastoris*.

Chapter I is an introductory to the development and utilization of *P. pastoris* in biotechnology and basic research. Peculiarities and advantages of this system for heterologous protein expression are presented along with advances of using *P. pastoris* as a model organism for fundamental science.

Chapter II covers all methodological approaches employed for detailed lipid analysis of *P. pastoris*. Optimized conventional lipid analysis and lipidomics techniques are rendered in the form of detailed protocols and as guidance for lipidome analysis.

Chapter III summarizes techniques of subcellular fractionation which were specifically designated for isolation of different *P. pastoris* organelles and biomembranes. This section is organized as stepwise protocols to obtain highly enriched and intact organelles and membrane compartments suitable for structural and functional analysis.

Chapter IV is dedicated to structural analysis of the non-polar lipid storage compartment, the lipid droplets. Data presented here describe the lipidome and the proteome of this organelle to broaden our current knowledge of lipid storage in yeast.

Chapter V describes non-polar lipid synthesis in *P. pastoris* at the molecular level. In this section, identification of genes responsible for non-polar lipid synthesis and the role of the respective gene products in lipid droplet formation and cellular lipid homeostasis are reported.

Appendix 1 describes preliminary results on the identification of *P. pastoris* lipases and their possible role in degradation of storage lipids;

Appendix 2 describes development of a protocol to isolate *P. pastoris* vacuoles as a prerequisite for membrane lipid analysis.

Chapter VI is a general discussion of the work done throughout this study, the obtained results and concluding remarks.

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CHAPTER 1

INTRODUCTION

Abstract

Yeast has been successfully used for the production of recombinant proteins over last two decades. During this time the methylotrophic yeast *Pichia pastoris* has developed from being a source of single cell protein to the most frequently used yeast protein expression system. *P. pastoris* has several advantages compared to other expression systems such as *S. cerevisiae*, *E. coli* and mammalian cell cultures. Recently, the system underwent serious developments in the number of available strains and vectors facilitating production of a broad spectrum of new recombinant proteins for industrial, medical and research applications. Moreover, fermentation processes were optimized much to improve the production yields. Nowadays, more than 500 proteins are successfully expressed in this host serving needs of science and industry. Intensive proliferation of peroxisomes in *P. pastoris* consuming methanol made this yeast also a perfect model organism for fundamental research in peroxisome biogenesis and turnover and as a model for human cell biology and disease. Moreover, *P. pastoris* is also used as a model system for studying fundamental aspects of secretion and organization of endomembrane system organelles such as the endoplasmic reticulum and Golgi and the evolutionary conserved autophagy processes. The field of *P. pastoris* lipid research is still in its infancy, but gains more interest nowadays as fundamental knowledge of lipid metabolism may aid industrial strain engineering and understanding certain aspects of eukaryotic lipid metabolism.

Chapter 1

1.1. Highlights of *Pichia pastoris* as an expression system

1.1.1. History of the *Pichia pastoris* system development

Eukaryotic methylotrophs which can utilize methanol as a sole carbon source are represented by several yeast genera including *Pichia pastoris*, *Hansenula*, *Candida*, *Torulopsis* [1] and *Ogataea, Kuraishia* and *Komagataella* recently segregated from *Pichia pastoris* genera [2]. When isolated for the first time in 1969 [3], methylotrophic yeasts and particularly *Pichia pastoris* gained much interest in fundamental research and potential industrial applications. Low cost of methanol production allowed using this alcohol as a carbon source for industrial fermentation, and *Pichia pastoris* found its first application as a source of single cell derived high-protein animal food additive [4]. In 1970s the Philips Petroleum Company was the first company which developed technology for growing *Pichia pastoris* to high cell density [5] and later in 1980s participated in elaboration of the *Pichia pastoris* expression system. The investigation of the methanol metabolic pathways in *Candida* and *Hansenula* led to identification and characterization of relevant enzymes [6]. Subsequently, a number of enzymes participating in methanol metabolism and methanol induced protein expression were cloned and studied in more detail [7]. At the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA La Jolla, CA, USA) alcohol oxidase gene and its promoter were isolated and vectors and strains were constructed for recombinant protein expression in *Pichia pastoris* [8]. In 1993, Phillips Petroleum and the next patent holder, Research Corporation Technologies (Tucson, AZ, USA), released the *Pichia pastoris* expression system to academic research resulting in fast accumulation of knowledge required for biotechnology and fundamental cell biology research [5]. The "*Pichia pastoris* expression kit" is available from Invitrogen Corporation (Carlsbad, CA, USA).

1.1.2. Advantages of the *Pichia pastoris* expression system

The methylotrophic yeast *Pichia pastoris* became a popular host for heterologous protein expression. The choice of *P. pastoris* system was successfully applied for expression of hundreds of proteins due to its intrinsic features which make this system advantageous in many cases over other conventional systems such as *E. coli*, *S. cerevisiae* and mammalian and insect cell cultures [9]. Main benefits of this system are: (i) the ability of *P. pastoris* to grow

to high cell densities using methanol as a non-fermentable carbon source; (ii) availability of strongly inducible alcohol oxidase (AOX1) promoter as well as a number of other inducible and constitutive promoters which allow large-scale production of functional target proteins at reasonably low costs; (iii) the ease of the system to manipulate genetically; (iv) proper folding and post-translational modification capacity required for protein functionality; (v) the possibility for direct genomic integration of expression vectors by homologous recombination which allows generation of stable cell lines, increased copy number of protein coding sequences, expression of several subunits of original protein complexes; and (vi) less intensive glycosylation of secreted proteins.

P. pastoris versus *E. coli*.

In recent times, the *P. pastoris* expression system became more popular than bacterial systems like *E. coli*. The majority of large size proteins are preferentially expressed in eukaryotic systems, whereas bacterial expression systems can be the choice for the expression of small proteins [10]. *P. pastoris* as a eukaryote is capable of producing soluble properly folded and herein active recombinant proteins. Being fast, easy in handling and inexpensive it is often advantageous over other expression systems [11]. The first advantage is posttranslational modification and folding of foreign proteins. Unlike eukaryotes, *E. coli* is not capable of performing posttranslational modifications essential for eukaryotic proteins. Therefore, proteins with require such modifications are often fold incorrectly when expressed in *E. coli*. This feature limits significantly the number of protein types which can be expressed in bacteria. The proteins which require disulfide bond formation, glycosylation, isomerization, phosphorylation or the absence of an amino-terminal methionine are often not properly expressed, misfolded, unstable and insoluble which raised a need for time consuming, cost inefficient and low yield procedures for further solubilizing, re-folding and retrieval from bacterial inclusion bodies [12,13]. Another advantage of *P. pastoris* over bacterial systems is the availability of several powerful promoter systems. Different strong inducible and constitutive promoters promote high yield expression. The methylotrophic yeast *P. pastoris* possesses the strong and tightly regulated AOX1 promoter which allows simple manipulation of the expression by changing culture conditions [14]. The strong constitutive GAP promoter enables constant expression of high protein levels. If high amounts of expressed protein may become toxic fine-tuning of the expression by using a number of recently engineered promoters with various strengths is possible [15–17]. The next advantage of *P. pastoris* system is the possibility for secretion of expressed proteins along with low levels of

endogenous secretion which makes purification of the host protein much easier than in *E. coli* [18].

P. pastoris versus *S. cerevisiae*.

There are several major advantages to use *P. pastoris* instead of other yeast systems, particularly *S. cerevisiae*. The preference of *P. pastoris* for respiratory growth when most of the carbon source is converted to biomass is the basis for obtaining high cell density cultures (more than 100 g dry cell weight per liter) [19] and hence more protein amounts compared to the fermentative yeast. The other advantage is the ability of *P. pastoris* to grow on methanol as sole carbon and energy source. There are two copies of alcohol oxidase gene in the *P. pastoris* genome. These genes named AOX1 and AOX2 enable cells to use methanol and have tightly regulated promoters. The AOX1 promoter is the stronger one and induces 85% of alcohol oxidase production, whereas the AOX2 promoter is weaker. Depending on the functionality of the AOX1 gene two strains with different functional phenotypes can be easily obtained. One phenotype is designated methanol utilization plus (Mut^+), and the second is called methanol utilization slow (Mut^s). Mut^+ strain contains both AOX genes, whereas Mut^s has only the AOX2 gene [20,21]. The AOX promoter is heavily repressed in cells grown on glucose and a number of other carbon sources, but induced tremendously (over 1000 fold) when cultures are shifted to methanol containing media. This feature facilitates cell mass production prior to intensive protein expression which is essential if foreign proteins at large amounts are toxic. Another advantage of *P. pastoris* is the ability to perform post-translational protein processing similarly to higher eukaryotes. Particularly, there is a crucial difference in the glycosylation patterns of secreted proteins between *P. pastoris* and *S. cerevisiae*. *P. pastoris* is distinguished from other yeasts by the absence of hyperglycosylation and alpha-1,3-linked mannosylation [22], which is detrimental for medical applications as the secreted protein becomes immunogenic or has impaired therapeutic effect [23,24]. Oligosaccharide chains attached to proteins secreted in *P. pastoris* are short [25]. Nevertheless, glycosylation in *P. pastoris* wild type strains lacks typical “human” saccharides such as galactose and sialic acid. Therefore, many efforts have been made to engineer *P. pastoris* the glycosylation pathway to overcome this obstacles [26,27]. Engineering technologies such as GlycoSwitch and GlycoFi led to new strains with humanized glycosylation properties [23,24,28–31].

P. pastoris versus mammalian and insect cell cultures.

In many cases *P. pastoris* is preferential because this system is faster, easier, less expensive, and much more productive than higher eukaryotic cell cultures [9]. The comparison of features from *P. pastoris* and other systems is shown in Table 1.

1.1.3. Strains and vectors for protein expression in *Pichia pastoris*

Several *P. pastoris* strains with various genotypes are now available for protein expression. The choice of a strain is an important factor for successful expression and depends on the required application. The most common wild type strains are X33, CBS7435 and GS115. The number of auxotrophic strains is increasing which allows positive transformation selection based on vector supplementation of missing genes required for amino acid synthesis such as *HIS4*, *ARG4*, *ADE1*, *URA3*, *CYS4*, methanol dissimilation pathway, such as *FLD1* [32–36], and many other metabolic related genes [34,37,38]. Protease deficient strains lacking genes like *PEP4*, *PRB1*, *KEX1*, *KEX2* and their combinations are provided to reduce proteolysis of the expressed proteins [39,40]. Another set of strains was generated to improve folding, processing, glycosylation and secretion of heterologous proteins, e.g. strains overexpressing the protein disulfide isomerase *PDI* and the secretion helpers *ERO1*, *SSO2*, *KAR2* [41–43]; or strains with reduced hyperglycosylation and humanized glycosylation [29,30]. All *P. pastoris* strains nowadays applied for heterologous protein expression are derivatives of the wild type strain Y-11430 from Northern Regional Research Laboratories (NRRL, Peoria, IL, USA) [44]. An increasing number of vectors have become available to facilitate specific needs of protein expression and fundamental research with *P. pastoris*. Basically all of them have common features such as presence of bacterial and yeast selection markers, different inducible or constitutive promoters, multiple cloning sites and optional secretion signal sequences. The vector must be incorporated to the chromosome to a specific locus by homologous recombination. Most commonly used expression vectors contain as selection markers the antibiotic resistance genes kan^r, Zeo^r, Bsd^r [45] or amino acid biosynthesis genes. The level of drug resistance is directly related to the integrated vector copy number and can be manipulated to achieve high expression levels. Some vectors are designed to bear multiple head-to-tail expression cassettes to achieve higher expression. Complete expression systems are commercially available from Invitrogen Corporation (http://www.lifetechnologies.com).

1.2. *Pichia pastoris* as a model eukaryote

Methylotrophic yeasts became popular experimental systems for studying genetics and physiology of peroxisome biogenesis and function [46]. Peroxisomes are eukaryotic organelles coated with a single membrane. In methylotrophic yeasts, peroxisomes harbor the methanol metabolism enzymes, such as alcohol oxidase and catalase, which detoxify the peroxide and share enzymes of acyl-CoA β-oxidation [47]. Dependent on the carbon source availability, *P. pastoris* can rapidly proliferate or degrade its peroxisomes. Therefore, these processes can be easily studied in *P. pastoris* as an easy to handle eukaryote. The processes are fast and controlled, the peroxisomes are easy to isolate and that metabolic pathways can be localized to peroxisomes at different stages of induction [48]. Using *P. pastoris* much progress was made in the investigation of matrix and membrane protein import into peroxisomes [49]. Hence, it was shown that translocation through the peroxisomal membrane requires peroxisomal targeting signals (PTS). Three PTS types conserved in eukaryotes have been characterized: PTS1 and PTS2 are responsible for luminal peroxisome proteins targeting, while mPTS mediates translocation of peroxisomal membrane proteins [50]. Upon switch of the carbon source to glucose, peroxisomes can be degraded fast and efficiently as their function is not required any more. This process involves engulfment of peroxisomes by vacuoles and subsequent degradation called pexophagy. Two types of this process designated macro- and micropexophagy were well characterized, and pexophagy-related genes were identified in *P. pastoris* [47,51–54].

The knowledge from *P. pastoris* peroxisome biogenesis, and identification of *P. pastoris* peroxins and their functions helped to reveal molecular mechanisms of several peroxisomal associated human disorders [55]. Peroxisome function, autophagy and fusion of autophagosome-like vesicles to the plasma membrane explored in *P. pastoris* system shed light on conserved mechanisms governing unconventional secretion of an acyl-CoA binding protein (human ACBP and yeast Abp1), which plays an important role in the regulation of neural processes, steroid synthesis and insulin secretion in humans [56]. Moreover, *P. pastoris* is used as a model system for studying the secretory pathway. In *P. pastoris* ER and Golgi structures differ from the conventional yeast model *S. cerevisiae*, but resemble those of higher eukaryotes [57]. Similarly to mammalian cells, *P. pastoris* Golgi cisternae are more discrete, form visible cis- and trans-Golgi counterparts and localize specifically close to ER transitional sites. This properties makes *P. pastoris* feasible to investigate ER-Golgi relationships [58]. It was shown in *P. pastoris* that individual cisternae undergo maturation via anterograde intra-Golgi transport [59,60]. Recently, COPII assembly phenomena similar to mammalian cells were observed in *P. pastoris* [61]. Novel components of ER-to-Golgi

transport were identified which were not found in *S. cerevisiae* [62]. Interestingly, the unfolded protein response transcription activation mechanism also resembles mammalian cells more than *S. cerevisiae* [63]. It was observed that upon UPR induction *P. pastoris* exhibited reduction of the ER lumen redox state and reduction of the cytosolic redox state is a response to oxidative protein folding in the ER [64]. Many age-related human diseases like atherosclerosis, diabetes mellitus, and neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease have been associated with protein misfolding or aggregation in the ER and the loss of redox control [65,66]. Therefore, *P. pastoris* may serve as a valuable model system to study the interrelation between ER stress and cellular redox homeostasis, and contribute to a better understanding of the development of aging-related diseases [67].

1.3. Lipid research in *Pichia pastoris*

So far most intensive industrial and basic research in *P. pastoris* cell biology was dedicated to understanding the mechanisms of protein synthesis, modifications and secretion with the goal to improve these processes for production of heterologous proteins [63,68–71]. The largely missing fundamental knowledge in *P. pastoris* lipid and membrane functions and properties, however, may also be helpful for applied research with *P. pastoris* by providing knowledge for engineering the lipid composition of secretory membranes. Moreover, *P. pastoris* has also the potential to become an alternative non-conventional yeast model for lipid research, especially for studying synthesis and physiological functions of glycolipids and sphingolipids in eukaryotic cell. In contrast to *S. cerevisiae* such higher eukaryote related lipids such as cerebrosides and sterol glycosides are highly abundant in *P. pastoris* especially under stress conditions [72]. Moreover, *P. pastoris* in contrast to *S. cerevisiae* possesses two classes of sphingolipids, namely inositolphosphoceramides (IPC) or glycosylated IPC and glucosylceramides with glucose or galactose moieties (GlcCer and GalCer). It was shown that in *P. pastoris* these two classes of sphingolipids are formed by separated pathways, and gene products involved in the synthesis were identified [73,74]. Another interesting aspect of *P. pastoris* lipid research is that unsaturated fatty acids including oleic acid (OA, C18:1), linoleic acid (LA, C18:2) and α-linolenic acid (ALA, C18:3) are major components of membrane lipids. Therefore molecular studies of fatty acid biosynthesis in this yeast became an intriguing research topic. Recently, several genes responsible for fatty acid desaturation were identified and their transcriptional regulation and their role in fatty acid biosynthesis, cell

survival and stress adaptation were shown [75,76]. Fad12 and Fad15 genes encode Δ(12)- and Δ(15)-desaturases responsible for the formation of LA and ALA. Mutants deficient in Fad12 and hence linoleic acid showed retarded growth under standard conditions. Fad9A and Fad9B encode Δ(9)-desaturases which convert stearic acid to oleic acid. Simultaneous deletion of Fad9A and Fad9B is lethal, but supplementation of oleic acid restores growth, indicating that OA is indispensable for cell viability. It was shown that at low temperature the transcription of desaturase genes is rapidly enhanced and suppressed by exogenous OA. The transcriptional activator gene *SPT23* was reported to be an essential factor regulating OA synthesis [75,76].

Systematic investigations of *P. pastoris* organelle lipidomes and proteomes were initiated by our laboratory. Peroxisomes and mitochondria outer and inner membranes were isolated at high purity and studied regarding their lipid and protein composition under different growth conditions [77,78]. In general, the phospholipid and sterol composition of peroxisomal membranes reflected *S. cerevisiae* although with a few distinct differences. The main phospholipid classes of peroxisomal membrane are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and main sterol is ergosterol. The low amount of phosphatidylinositol (PI) and substantial amounts of cardiolipin (CL) are characteristic features of *P. pastoris* peroxisomes [78]. Inner and outer mitochondrial membranes of *P. pastoris* show pronounced differences in their lipid composition [77]. The outer membrane has a much higher phospholipid to protein ratio and a significantly lower ergosterol to phospholipid ratio compared to the inner membrane. PC and PE are major phospholipids of mitochondrial membranes, although the phospholipid patterns differ between the two membranes. CL is highly enriched in the inner membrane, whereas the outer membrane has more of PI [77,78]. Two *P. pastoris* phosphatidylserine decarboxylases encoded by *PSD1* and *PSD2* were recently identified [79]. The *PSD1* gene product is responsible for the majority of phosphatidylserine decarboxylase activity in the cell and particularly acts in providing mitochondria with PE. In contrast, the *PSD2* gene product localizes to the endomembrane system and has minor activity. Psd1p considerably contributes to the amount of total cellular PE and is essential for normal growth on minimal media. Both Psd1p and Psd2p show selectivity for the synthesis of unsaturated PE species. These findings underline the potential to engineer the *P. pastoris* membrane lipid composition for biotechnological purposes [79]. As prominent example of *P. pastoris* lipid engineering the sterol biosynthetic pathway directed towards the synthesis of cholesterol instead of ergosterol has to be mentioned [80]. This attempt was made to improve the functional expression of human membrane proteins. All aforementioned facts show the high demand in further, more detailed and comprehensive

investigation of *P. pastoris* lipid metabolic pathways, membrane structural and functional characterization which will contribute to develop a fundamental knowledge platform required for improving *P. pastoris* as an expression host and as model eukaryote.

References

[1] K.N. Faber, W. Harder, G. Ab, M. Veenhuis, Review: methylotrophic yeasts as factories for the production of foreign proteins, Yeast Chichester Engl. 11 (1995) 1331–1344.

[2] S. Limtong, N. Srisuk, W. Yongmanitchai, H. Yurimoto, T. Nakase, Ogataea chonburiensis sp. nov. and Ogataea nakhonphanomensis sp. nov., thermotolerant, methylotrophic yeast species isolated in Thailand, and transfer of Pichia siamensis and Pichia thermomethanolica to the genus Ogataea, Int. J. Syst. Evol. Microbiol. 58 (2008) 302–307.

[3] K. Ogata, H. Nishikawa, M. Ohsugi, A yeast capable of utilizing methanol, Agric. Biol. Chem. 33 (1969) 1519–1520.

[4] P.J. Senior, J. Windass, The ICI single cell protein process, Biotechnol. Lett. 2 (1980) 205–210.

[5] J.L. Cereghino, J.M. Cregg, Heterologous protein expression in the methylotrophic yeast Pichia pastoris, FEMS Microbiol. Rev. 24 (2000) 45–66.

[6] M. Veenhuis, J.P.V. Dijken, W. Harder, The Significance of Peroxisomes in the Metabolism of One-Carbon Compounds in Yeasts, in: J.G.M. and D.W.T. A.H. Rose (Ed.), Adv. Microb. Physiol., Academic Press, 1983: pp. 1–82.

[7] F.S. Hartner, A. Glieder, Regulation of methanol utilisation pathway genes in yeasts, Microb. Cell Factories. 5 (2006) 39.

[8] S. Macauley-Patrick, M.L. Fazenda, B. McNeil, L.M. Harvey, Heterologous protein production using the Pichia pastoris expression system, Yeast Chichester Engl. 22 (2005) 249–270.

[9] J.M. Cregg, Introduction: distinctions between Pichia pastoris and other expression systems, Methods Mol. Biol. Clifton NJ. 389 (2007) 1–10.

[10] A.M. Gonçalves, A.Q. Pedro, C. Maia, F. Sousa, J.A. Queiroz, L.A. Passarinha, Pichia pastoris: a recombinant microfactory for antibodies and human membrane proteins, J. Microbiol. Biotechnol. 23 (2013) 587–601.

[11] A.L. Demain, P. Vaishnav, Production of recombinant proteins by microbes and higher organisms, Biotechnol. Adv. 27 (2009) 297–306.

[12] R. Daly, M.T.W. Hearn, Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production, J. Mol. Recognit. JMR. 18 (2005) 119–138.

[13] A. Lueking, C. Holz, C. Gotthold, H. Lehrach, D. Cahill, A system for dual protein expression in Pichia pastoris and Escherichia coli, Protein Expr. Purif. 20 (2000) 372–378.

[14] G.P. Cereghino, J.M. Cregg, Applications of yeast in biotechnology: protein production and genetic analysis, Curr. Opin. Biotechnol. 10 (1999) 422–427.

[15] X. Qin, J. Qian, G. Yao, Y. Zhuang, S. Zhang, J. Chu, GAP promoter library for fine-tuning of gene expression in Pichia pastoris, Appl. Environ. Microbiol. 77 (2011) 3600–3608.

[16] G. Stadlmayr, A. Mecklenbräuker, M. Rothmüller, M. Maurer, M. Sauer, D. Mattanovich, et al., Identification and characterisation of novel Pichia pastoris promoters for heterologous protein production, J. Biotechnol. 150 (2010) 519–529.

[17] H. Hohenblum, B. Gasser, M. Maurer, N. Borth, D. Mattanovich, Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris, Biotechnol. Bioeng. 85 (2004) 367–375.

[18] M. Weidner, M. Taupp, S.J. Hallam, Expression of recombinant proteins in the methylotrophic yeast Pichia pastoris, J. Vis. Exp. JoVE. (2010).

[19] J.M. Cregg, T.S. Vedvick, W.C. Raschke, Recent advances in the expression of foreign genes in Pichia pastoris, Biotechnol. Nat. Publ. Co. 11 (1993) 905–910.

[20] J.M. Cregg, K.R. Madden, K.J. Barringer, G.P. Thill, C.A. Stillman, Functional characterization of the two alcohol oxidase genes from the yeast Pichia pastoris., Mol. Cell. Biol. 9 (1989) 1316–1323.

[21] J.M. Cregg, J.L. Cereghino, J. Shi, D.R. Higgins, Recombinant protein expression in Pichia pastoris, Mol. Biotechnol. 16 (2000) 23–52.

[22] R. Montesino, R. García, O. Quintero, J.A. Cremata, Variation in N-linked oligosaccharide structures on heterologous proteins secreted by the methylotrophic yeast Pichia pastoris, Protein Expr. Purif. 14 (1998) 197–207.

[23] S. Ha, Y. Wang, R.R. Rustandi, Biochemical and biophysical characterization of humanized IgG1 produced in Pichia pastoris, mAbs. 3 (2011) 453–460.

[24] S.R. Hamilton, T.U. Gerngross, Glycosylation engineering in yeast: the advent of fully humanized yeast, Curr. Opin. Biotechnol. 18 (2007) 387–392.

[25] P. Li, A. Anumanthan, X.-G. Gao, K. Ilangovan, V.V. Suzara, N. Düzgüneş, et al., Expression of recombinant proteins in Pichia pastoris, Appl. Biochem. Biotechnol. 142 (2007) 105–124.

[26] M.M. Wim Martinet, Modification of the protein glycosylation pathway in the methylotrophic yeast Pichia pastoris, 20 (1998) 1171–1177.

[27] R.K. Bretthauer, Genetic engineering of Pichia pastoris to humanize N-glycosylation of proteins, Trends Biotechnol. 21 (2003) 459–462.

[28] T.I. Potgieter, M. Cukan, J.E. Drummond, N.R. Houston-Cummings, Y. Jiang, F. Li, et al., Production of monoclonal antibodies by glycoengineered Pichia pastoris, J. Biotechnol. 139 (2009) 318–325.

[29] P.P. Jacobs, S. Geysens, W. Vervecken, R. Contreras, N. Callewaert, Engineering complex-type N-glycosylation in Pichia pastoris using GlycoSwitch technology, Nat. Protoc. 4 (2009) 58–70.

[30] A. Beck, O. Cochet, T. Wurch, GlycoFi's technology to control the glycosylation of recombinant therapeutic proteins, Expert Opin. Drug Discov. 5 (2010) 95–111.

[31] D. Zha, Glycoengineered Pichia-based expression of monoclonal antibodies, Methods Mol. Biol. Clifton NJ. 988 (2013) 31–43.

[32] D.I. Crane, S.J. Gould, The Pichia pastoris HIS4 gene: nucleotide sequence, creation of a non-reverting his4 deletion mutant, and development of HIS4-based replicating and integrating plasmids, Curr. Genet. 26 (1994) 443–450.

[33] G.P. Lin Cereghino, J. Lin Cereghino, A.J. Sunga, M.A. Johnson, M. Lim, M.A. Gleeson, et al., New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of Pichia pastoris, Gene. 263 (2001) 159–169.

[34] J.H. Nett, T.U. Gerngross, Cloning and disruption of the PpURA5 gene and construction of a set of integration vectors for the stable genetic modification of Pichia pastoris, Yeast Chichester Engl. 20 (2003) 1279–1290.

[35] D.Y. Li, X.S. Ji, J. Yu, M.J. Chen, Z.Y. Yuan, PCR Based Cloning and Sequence Analysis of the Pichia pastoris Cystathionine beta-Synthase Gene, Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao Acta Biochim. Biophys. Sin. 33 (2001) 600–606.

[36] A.J. Sunga, J.M. Cregg, The Pichia pastoris formaldehyde dehydrogenase gene (FLD1) as a marker for selection of multicopy expression strains of P. pastoris, Gene. 330 (2004) 39–47.

[37] M.M. Whittaker, J.W. Whittaker, Construction and characterization of Pichia pastoris strains for labeling aromatic amino acids in recombinant proteins, Protein Expr. Purif. 41 (2005) 266–274.

[38] J.H. Nett, N. Hodel, S. Rausch, S. Wildt, Cloning and disruption of the Pichia pastoris ARG1, ARG2, ARG3, HIS1, HIS2, HIS5, HIS6 genes and their use as auxotrophic markers, Yeast Chichester Engl. 22 (2005) 295–304.

[39] T. Boehm, S. Pirie-Shepherd, L.B. Trinh, J. Shiloach, J. Folkman, Disruption of the KEX1 gene in Pichia pastoris allows expression of full-length murine and human endostatin, Yeast Chichester Engl. 15 (1999) 563–572.

[40] M.A. Gleeson, C.E. White, D.P. Meininger, E.A. Komives, Generation of protease-deficient strains and their use in heterologous protein expression, Methods Mol. Biol. Clifton NJ. 103 (1998) 81–94.

[41] B. Gasser, M. Sauer, M. Maurer, G. Stadlmayr, D. Mattanovich, Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts, Appl. Environ. Microbiol. 73 (2007) 6499–6507.

[42] A. Conesa, P.J. Punt, N. van Luijk, C.A. van den Hondel, The secretion pathway in filamentous fungi: a biotechnological view, Fungal Genet. Biol. FG B. 33 (2001) 155–171.

[43] B. Gasser, D. Mattanovich, Antibody production with yeasts and filamentous fungi: on the road to large scale?, Biotechnol. Lett. 29 (2007) 201–212.

[44] D.R. Higgins, J.M. Cregg, Introduction to Pichia pastoris, Methods Mol. Biol. Clifton NJ. 103 (1998) 1–15.

[45] J. Lin-Cereghino, M.D. Hashimoto, A. Moy, J. Castelo, C.C. Orazem, P. Kuo, et al., Direct selection of Pichia pastoris expression strains using new G418 resistance vectors, Yeast Chichester Engl. 25 (2008) 293–299.

[46] S. Subramani, Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement, Physiol. Rev. 78 (1998) 171–188.

[47] A. Till, R. Lakhani, S.F. Burnett, S. Subramani, Pexophagy: the selective degradation of peroxisomes, Int. J. Cell Biol. 2012 (2012) 512721.

[48] V.I. Titorenko, R.A. Rachubinski, The life cycle of the peroxisome, Nat. Rev. Mol. Cell Biol. 2 (2001) 357–368.

[49] C.S. Collins, J.E. Kalish, J.C. Morrell, J.M. McCaffery, S.J. Gould, The peroxisome biogenesis factors pex4p, pex22p, pex1p, and pex6p act in the terminal steps of peroxisomal matrix protein import, Mol. Cell. Biol. 20 (2000) 7516–7526.

[50] I. van der Klei, M. Veenhuis, Protein targeting to yeast peroxisomes, Methods Mol. Biol. Clifton NJ. 390 (2007) 373–391.

[51] Y. Sakai, M. Oku, I.J. van der Klei, J.A.K.W. Kiel, Pexophagy: autophagic degradation of peroxisomes, Biochim. Biophys. Acta. 1763 (2006) 1767–1775.

[52] M. Oku, Y. Sakai, Pexophagy in Pichia pastoris, Methods Enzymol. 451 (2008) 217–228.

[53] J.-C. Farré, S. Subramani, Peroxisome turnover by micropexophagy: an autophagy-related process, Trends Cell Biol. 14 (2004) 515–523.

[54] L.A. Schroder, B.S. Glick, W.A. Dunn, Identification of pexophagy genes by restriction enzyme-mediated integration, Methods Mol. Biol. Clifton NJ. 389 (2007) 203–218.

[55] I.J. van der Klei, M. Veenhuis, Yeast and filamentous fungi as model organisms in microbody research, Biochim. Biophys. Acta. 1763 (2006) 1364–1373.

[56] R. Manjithaya, C. Anjard, W.F. Loomis, S. Subramani, Unconventional secretion of Pichia pastoris Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation, J. Cell Biol. 188 (2010) 537–546.

[57] E. Papanikou, B.S. Glick, The yeast Golgi apparatus: insights and mysteries, FEBS Lett. 583 (2009) 3746–3751.

[58] O.W. Rossanese, J. Soderholm, B.J. Bevis, I.B. Sears, J. O'Connor, E.K. Williamson, et al., Golgi structure correlates with transitional endoplasmic reticulum organization in Pichia pastoris and Saccharomyces cerevisiae, J. Cell Biol. 145 (1999) 69–81.

[59] S. Mogelsvang, N. Gomez-Ospina, J. Soderholm, B.S. Glick, L.A. Staehelin, Tomographic evidence for continuous turnover of Golgi cisternae in Pichia pastoris, Mol. Biol. Cell. 14 (2003) 2277–2291.

[60] E. Losev, C.A. Reinke, J. Jellen, D.E. Strongin, B.J. Bevis, B.S. Glick, Golgi maturation visualized in living yeast, Nature. 441 (2006) 1002–1006.

[61] B.S. Glick, A. Nakano, Membrane traffic within the Golgi apparatus, Annu. Rev. Cell Dev. Biol. 25 (2009) 113–132.

[62] M. Esaki, Y. Liu, B.S. Glick, The budding yeast Pichia pastoris has a novel Sec23p homolog, FEBS Lett. 580 (2006) 5215–5221.

[63] A. Graf, B. Gasser, M. Dragosits, M. Sauer, G.G. Leparc, T. Tüchler, et al., Novel insights into the unfolded protein response using Pichia pastoris specific DNA microarrays, BMC Genomics. 9 (2008) 390.

[64] M. Delic, C. Rebnegger, F. Wanka, V. Puxbaum, C. Haberhauer-Troyer, S. Hann, et al., Oxidative protein folding and unfolded protein response elicit differing redox regulation in endoplasmic reticulum and cytosol of yeast, Free Radic. Biol. Med. 52 (2012) 2000–2012.

[65] H. Yoshida, ER stress and diseases, FEBS J. 274 (2007) 630–658.

[66] N. Naidoo, ER and aging-Protein folding and the ER stress response, Ageing Res. Rev. 8 (2009) 150–159.

[67] B. Gasser, R. Prielhofer, H. Marx, M. Maurer, J. Nocon, M. Steiger, et al., Pichia pastoris: protein production host and model organism for biomedical research, Future Microbiol. 8 (2013) 191–208.

[68] M. Delic, M. Valli, A.B. Graf, M. Pfeffer, D. Mattanovich, B. Gasser, The secretory pathway: exploring yeast diversity, FEMS Microbiol. Rev. 37 (2013) 872–914.

[69] K.R. Love, T.J. Politano, V. Panagiotou, B. Jiang, T.A. Stadheim, J.C. Love, Systematic single-cell analysis of Pichia pastoris reveals secretory capacity limits productivity, PloS One. 7 (2012) e37915.

[70] G. Stadlmayr, K. Benakovitsch, B. Gasser, D. Mattanovich, M. Sauer, Genome-scale analysis of library sorting (GALibSo): Isolation of secretion enhancing factors for recombinant protein production in Pichia pastoris, Biotechnol. Bioeng. 105 (2010) 543–555.

[71] B. Gasser, M. Maurer, J. Rautio, M. Sauer, A. Bhattacharyya, M. Saloheimo, et al., Monitoring of transcriptional regulation in Pichia pastoris under protein production conditions, BMC Genomics. 8 (2007) 179.

[72] T. Sakaki, U. Zähringer, D.C. Warnecke, A. Fahl, W. Knogge, E. Heinz, Sterol glycosides and cerebrosides accumulate in Pichia pastoris, Rhynchosporium secalis and other fungi under normal conditions or under heat shock and ethanol stress, Yeast Chichester Engl. 18 (2001) 679–695.

[73] D. Warnecke, E. Heinz, Recently discovered functions of glucosylceramides in plants and fungi, Cell. Mol. Life Sci. CMLS. 60 (2003) 919–941.

[74] P. Ternes, T. Wobbe, M. Schwarz, S. Albrecht, K. Feussner, I. Riezman, et al., Two pathways of sphingolipid biosynthesis are separated in the yeast Pichia pastoris, J. Biol. Chem. 286 (2011) 11401–11414.

[75] A.-Q. Yu, J.-C. Zhu, B. Zhang, L.-J. Xing, M.-C. Li, Knockout of fatty acid desaturase genes in Pichia pastoris GS115 and its effect on the fatty acid biosynthesis and physiological consequences, Arch. Microbiol. 194 (2012) 1023–1032.

[76] A.-Q. Yu, T.-L. Shi, B. Zhang, L.-J. Xing, M.-C. Li, Transcriptional regulation of desaturase genes in Pichia pastoris GS115, Lipids. 47 (2012) 1099–1108.

[77] T. Wriessnegger, E. Leitner, M.R. Belegratis, E. Ingolic, G. Daum, Lipid analysis of mitochondrial membranes from the yeast Pichia pastoris, Biochim. Biophys. Acta. 1791 (2009) 166–172.

[78] T. Wriessnegger, G. Gübitz, E. Leitner, E. Ingolic, J. Cregg, B.J. de la Cruz, et al., Lipid composition of peroxisomes from the yeast Pichia pastoris grown on different carbon sources, Biochim. Biophys. Acta. 1771 (2007) 455–461.

[79] T. Wriessnegger, A.J. Sunga, J.M. Cregg, G. Daum, Identification of phosphatidylserine decarboxylases 1 and 2 from Pichia pastoris, FEMS Yeast Res. 9 (2009) 911–922.

[80] M. Hirz, G. Richter, E. Leitner, T. Wriessnegger, H. Pichler, A novel cholesterol-producing Pichia pastoris strain is an ideal host for functional expression of human Na,K-ATPase α3β1 isoform, Appl. Microbiol. Biotechnol. 97 (2013) 9465–9478.

Tables

Table 1. Comparison of *P. pastoris* expression system to other systems.

Features	*P. pastoris*	*E. coli*	*S. cerevisiae*	Mammalian cell culture
Protein yield	high	high	average	low
Cost	very low	very low	low	high
Secretion capacity	high	low	medium	medium/high
Glycosylation	medium	–	high	medium

Chapter 2

Pichia Lipidomics

Lisa Klug[1*], Vasyl Ivashov[1*], Karlheinz Grillitsch[2] and Günther Daum[1]

[1]Institute of Biochemistry, Graz University of Technology, Austria; [2]Austrian Centre of Industrial Biotechnology, Graz, Austria

*Contributed equally to this paper and should be considered as co-first-authors

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Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology; Petersgasse 12/2, A-8010 Graz, Austria; Tel.: +43 316 873 6462; Fax: +43 316 873 6952; E-mail: guenther.daum@tugraz.at

Summary

The yeast *Pichia pastoris* has become a valuable eukaryotic model organism which is frequently used as a tool for industrial protein production. Despite these important applications, cell biological aspects of *Pichia pastoris* including problems of biomembrane and lipid research have not been adequately addressed. As lipids play an important role in various cell biological processes such as compartmentation, transport processes and signaling, our laboratory has started a systematic approach to study this class of biomolecules in some detail. In this chapter we describe materials and protocols for lipid analysis with *P. pastoris*. Standard methods for fatty acid, phospholipid and sterol analyses are presented.

Key words

Lipids, fatty acids, phospholipids, sterols, triacylglycerols

1. Introduction

Lipids are a major group of biomolecules characterized by their hydrophobic properties. They are essential components of all biological membranes but also serve as storage molecules to provide a source of energy and building blocks for membrane biogenesis. Moreover, lipids are signaling molecules and involved in many cellular processes like membrane fusion or induction of apoptosis *(1–3)*. Lipids can be classified in eight distinct groups based on their structure and function. These classes are fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterols and sterol derivatives, prenol lipids, glycolipids and polyketides *(4, 5)*. Biosynthesis of lipids occurs mainly in the endoplasmic reticulum, the Golgi apparatus and mitochondria. Consequently, newly synthesized lipids have to be transported to the other organelles which are devoid of lipid synthesizing enzymes *(6, 7)*.

Yeast organelles are characterized by their specific lipid composition *(8, 9)*. Such studies have been extensively performed with *Saccharomyces cerevisiae,* whereas lipids of the biotechnologically important yeast *P. pastoris* have not been studied in much detail. In the present chapter, different techniques for the analysis of lipids from *P. pastoris* are described. Most of these methods are adopted from protocols used for *S. cerevisiae*. The first step in lipid analysis is the extraction from cellular material. Different chromatographic methods such as thin layer chromatography (TLC) and gas lipid chromatography (GLC) are employed to separate and quantify individual lipid classes and species *(1)*. More detailed lipidome studies are performed by mass spectrometry techniques *(10, 11)*. Here, we provide basic lab protocols for lipid analysis of *P. pastoris* with a focus on the most common lipid classes.

2. Materials

Use deionized water for preparation of all solutions and organic solvents of analytical grade purity. Use preferably glassware when working with organic solvents. Prepare and store all reagents at room temperature unless mentioned otherwise. Waste disposal regulations should be considered.

2.1. Lipid extraction

2.1.1. Equipment

1. 12 ml glass tubes with Teflon liner caps.
2. Vibrax orbital shaker with glass tube attachment.
3. Table top centrifuge.
4. Water-jet or vacuum pump.
5. Glass Pasteur pipettes.
6. Glass pipettes.
7. Compressed nitrogen supply.

2.1.2. Reagents

1. $CHCl_3$/Methanol (2:1; v/v).
2. 0.034% $MgCl_2$ in water.
3. 2M KCl in water/methanol (4:1; v/v).
4. Methanol/H_2O/$CHCl_3$ (48:47:3; per vol.).

2.2. Fatty acid analysis by gas liquid chromatography

2.2.1. Equipment

1. 12 ml glass tubes with Teflon liner caps.
2. Oven (100°C).
3. Vibrax orbital shaker with glass tube attachment.
4. Table top centrifuge.

5. Glass Pasteur pipettes.
6. Glass pipettes.
7. GLC-sample vials with caps.
8. Microsyringe.
9. Gas liquid chromatograph with autosampler.

2.2.2. Reagents

1. 2.5 % H_2SO_4 (v/v) in methanol.
2. Light petroleum.
3. Deionized water.
4. Fatty acid methyl ester standards.

2.3. Phospholipid analysis

2.3.1. Equipment

1. TLC glass chambers.
2. Silica gel 60 TLC plates.
3. Glass pipettes.
4. Microsyringe.
5. Hairdryer.
6. Glass tubes with tight-fitting glass lids.
7. Oven (100°C).
8. Heating block (180°C) with inlets for glass tubes fixed at 45° angle.
9. Iodine vapor chamber.
10. Glassware cleaned with phosphate free detergent: pipettes, graduated cylinders, bottles etc.

2.3.2. Reagents

1. $CHCl_3$/Methanol (2:1; v/v).
2. Double distilled water.
3. TLC solvent for the first direction: $CHCl_3$/methanol/25 % NH_3 (65:35:5; per vol.).

4. TLC solvent for the second direction: $CHCl_3$/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.).
5. Acid mixture: 90 % H_2SO_4 (conc.), 10 % $HClO_4$ (72 %).
6. 0.26 % Ammonium heptamolybdate in water.
7. ANSA solution: 40 g $K_2S_2O_5$, 1.25 g Na_2SO_3, 0.63 g 1-anilinonaphthalene-8-sulfonic acid (ANS-acid) dissolved in 250 ml double distilled water.
8. Phosphate standard: 1 mg P/ml (73 mg $K_2HPO_4 \times 3H_2O$ in 10 ml double distilled water).

2.4. Sphingolipid analysis by UPLC-nano ESI-MS

2.4.1. Equipment

1. 12 ml Pyrex glass tubes with Teflon liner caps.
2. Glass vials with caps.
3. Water bath.
4. Vortex.
5. Sonicator.
6. Table top centrifuge.
7. Ultra Performance Liquid Chromatograph followed by nano-electrospray ionization mass spectrometer (UPLC-nano ESI-MS/MS).

2.4.2. Reagents

1. Internal standard mix: 0.15 nmol *N*-(dodecanoyl)-sphing-4-enine, 0.15 nmol *N*-(dodecanoyl)-1-β-glucosyl-sphing-4-enine, 4.5 nmol C17 sphinganine (Avanti Polar Lipids, Inc., Alabaster, AL, USA).
2. Propan-2-ol/hexane/water (60:26:14; per vol.).
3. Tetrahydrofuran/methanol/water (4:4:1; per vol.).

2.5. Non-polar lipid analysis

2.5.1. Equipment

1. TLC glass chambers.

2. Silica gel 60 TLC plates.
3. Microsyringe.
4. Oven (100°C).
5. Hairdryer.
6. TLC scanner.

2.5.2. Reagents

1. TLC solvent 1: light petroleum/diethyl ether/acetic acid (70:30:2; per vol.).
2. TLC solvent 2: light petroleum/diethyl ether (49:1; v/v).
3. Charring solution: 0.63 g $MnCl_2 \times 4\ H_2O$, 60 ml water, 60 ml methanol and 4 ml H_2SO_4 (conc.).
4. Lipid standards (1 mg/ml): ergosterol, cholesterol oleate, squalene, fatty acids, triolein, 1,2-diolein, monoolein.
5. Chloroform/acetone/acetic acid (90:8:1; per vol.) for the analysis of diacylglycerols.

2.6. Sterol analysis

2.6.1. Equipment

1. 12 ml glass tubes with Teflon liner caps.
2. Water bath.
3. Vibrax orbital shaker with glass tube attachment.
4. Table top centrifuge.
5. Compressed nitrogen supply.
6. Gas Liquid Chromatograph coupled with mass selective detector.

2.6.2. Reagents

1. Methanol.
2. 0.5% (w/v) pyrogallol in methanol.
3. 60% (w/v) aqueous KOH.
4. Internal cholesterol standard (2 mg/ml) in ethanol.
5. *n*-Heptane.
6. Pyridine.

7. N'O'-bis(trimethylsilyl)-trifluoracetamide.
8. Ethyl acetate.

2.7. Mass spectrometry of non-polar lipids and phospholipids

2.7.1. Equipment

1. Glass HPLC acquisition vials.
2. HPLC coupled with FT-ICR-MS hybrid mass spectrometer.

2.7.2. Reagents

1. $CHCl_3$/methanol (1:1; v/v).
2. Solvent A: 1 % ammonium acetate, 0.1 % formic acid in water.
3. Solvent B: 1 % ammonium acetate, 0.1 % formic acid in acetonitrile/2-propanol (5:2; v/v).
4. Internal standards: triacylglycerol (TG) species 51:0; and phosphatidylcholine species (PC) 24:0.

3. Methods

3.1. Lipid extraction

Lipids with the exception of sphingolipids are extracted as described by Folch et al. *(12)*. The amount of biological material to be used for extraction depends on the type of analysis and the sample. In principle, the amounts of lipids can be referred to the protein content, cell dry weight or OD_{600}. Here, we provide guidelines referring lipid to protein amounts. For analyzing lipids from total cell extracts we recommend the following amounts of protein from the cell homogenate. For phospholipid analysis, lipids are extracted from samples containing 1-2 mg protein; for diacylglycerol analysis samples containing 0.4 mg protein are used; triacylglycerols are analyzed from samples containing 0.2 mg protein; and analysis of sterols and fatty acids as well as species analysis of phospholipids, diacylglycerols, triacylglycerols and sphingolipids are performed with samples containing 0.3 mg protein. Extracted lipids are dried under a stream of nitrogen and stored at -20°C.

3.1.1. Extraction of fatty acids, phospholipids, sterols and non-polar lipids

1. Transfer appropriate amount of sample to a glass tube (see Note 1) and add 3 ml $CHCl_3$/methanol (2:1; v/v). Lipids are extracted by vigorous shaking on a Vibrax for 30 min.
2. After addition of 1 ml 0.034 % $MgCl_2$, extraction is continued for another 30 min with vigorous shaking.
3. Phase separation is accomplished by centrifugation in a table top centrifuge at 250 x g for 3 min.
4. Remove upper aqueous phase and as much as possible of the white intermediate layer of precipitated proteins without disturbing the lower organic phase (see Note 2).
5. Add 2 ml 2 N KCl/methanol (4:1; v/v), shake on a Vibrax for 10 min and centrifuge as described above.
6. Remove the aqueous phase and proteins without disturbing the lower organic phase.
7. Add 2 ml of methanol/H_2O/ $CHCl_3$ (48:47:3; per vol.). Shake for 10 min on a Vibrax and centrifuge as described above.
8. Remove the aqueous phase with the remaining proteins.
9. Dry the organic phase completely under the stream of nitrogen (see Note 3).
10. Dissolve dry lipids in an appropriate volume of $CHCl_3$/methanol (2:1 or 1:1; v/v) prior to analysis.

3.1.2. Extraction of sphingolipids for mass spectrometry

1. Cell fractions containing 0.3 mg protein are spiked with an internal standard mixture of 0.15 nmol *N*-(dodecanoyl)-sphing-4-enine; 0.15 nmol *N*-(dodecanoyl)-1-β-glucosyl-sphing-4-enine; and 4.5 nmol C17 sphinganine as an extraction control.
2. Samples are suspended in 6 ml propan-2-ol/hexane/water (60:26:14; per vol.) and incubated at 60°C for 30 min slightly modifying a protocol described previously *(13)*.
3. During incubation, samples are shortly vortexed and sonicated after 0, 10, 20 and 30 min.
4. Extracts are cleared from cell debris by centrifugation, transferred to new tubes and dried under a stream of nitrogen.

5. Extracted sphingolipids are re-dissolved in 800 µl tetrahydrofuran/methanol/water (4:4:1; per vol.) *(14)* and stored under argon at -20°C.

3.2. Fatty acid analysis by gas-liquid chromatography

1. Dissolve dried lipid extracts in 1 ml of a 2.5 % (v/v) H_2SO_4 in methanol and close tubes tightly.
2. Heat samples in the oven at 85°C for 90 min (see Note 1).
3. Let the samples cool down and add 1 ml H_2O and 3 ml light petroleum.
4. Extract the formed fatty acid methyl esters by shaking the tubes on a Vibrax for 30 min.
5. Centrifuge the samples for phase separation for 5 min at 250 x g.
6. Transfer the upper organic phase to a new tube.
7. Repeat the extraction step by adding 3 ml of light petroleum to the remaining aqueous phase for 30 min.
8. Centrifuge as described above. Combine the organic phases of the two extraction steps.
9. Remove solvent under a stream of nitrogen.
10. Dissolve the samples in 100 µl light petroleum for GLC-analysis and transfer to glass acquisition vials.
11. Fatty acid methyl esters are separated by GLC using a capillary column (e.g., 15 m x 0.25 mm i.d. x 0.50 µm film thickness) with helium as carrier gas.
12. Fatty acids are identified by comparison of retention times with a mix of commercially available fatty acid methyl ester standards which is analyzed separately.

3.3. Phospholipid analysis

3.3.1. Analysis of individual phospholipids

1. Phospholipids are extracted (see. Section 3.1.1) from cell homogenates or specific subcellular fractions containing 1-3 mg protein and dried under a stream of nitrogen.
2. Dried lipids are dissolved in 50 µl $CHCl_3$/methanol (2:1; v/v) and applied as a spot on a Silica gel 60 TLC plate (see Note 4).
3. After evaporation of the solvent, the plate is developed in the first dimension using a TLC chamber saturated with first developing solvent for ~50 min (see Note 5).

4. Withdraw TLC plate from the chamber and dry with a hairdryer until the smell of ammonia has disappeared (see Note 6).
5. Develop the plate in the second direction using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) for 50 min (Fig. 1) (see Note 5).
6. Withdraw TLC plate from the chamber and dry with a hairdryer (see Note 6).
7. Phospholipids are visualized by staining with iodine vapor, and spots are marked with a pencil (Fig. 1B) (see Note 7).
8. Phospholipids are quantified by the procedure of Broekhuyse *et al. (15)*. Silica gel with the identified lipid spots are scrapped off and transferred to phosphate-free glass tubes with the aid of a phosphate-free razor blade, scalpel or spatula (see Note 8). In addition, unstained areas of the TLC plate are analyzed as blanks for correction of the background.
9. Remove residual moisture by drying samples in an oven for 10 min at 100°C.
10. After cooling down the samples, add 0.2 ml conc. H_2SO_4/72 % $HClO_4$ (9:1, v/v) to each tube and mix briefly.
11. Incubate the tubes in a heating block at 180°C for 30 min to hydrolyze samples (see Note 9).
12. Cool samples down to room temperature and add 4.8 ml of a freshly prepared solution containing 500 vol. of 0.26 % ammonium molybdate and 22 vol. of ANSA.
13. Close the tubes tightly with a lid and incubate them in an oven at 100°C for 30 min. Phosphate containing samples turn blue.
14. Cool the sample down and sediment silica gel by centrifugation at 250 x g for 3 min in a table top centrifuge.
15. Quantification of phospholipids is achieved by measuring the absorption at a wavelength of 830 nm. Values have to be corrected for the blank. For calculation of the absolute amounts of individual phospholipids a series of phosphorus standards is required.

3.3.2. Quantification of total phospholipids

1. Lipids are extracted as described in section 3.1.1, dissolved in chloroform/methanol (2:1; v/v) and transferred to phosphate free glass tubes. Samples are brought to complete dryness either by evaporation in an oven or under a stream of nitrogen.

2. Samples and phosphorus standards are directly subjected to hydrolysis. The following steps are performed as described in section 3.3.1 starting at step 10. Centrifugation at step 15 is omitted.
3. Amounts of total phospholipids are calculated from a standard curve using inorganic phosphate at known amounts (0.5, 1, 2, 4, and 8 µg phosphorus per tube are recommended). Obtained values correspond to µg phosphorus per mg protein. Values are multiplied by the factor of 25, the approximate ratio of molar masses of phosphorus and an average phospholipid, to yield µg phospholipid/mg protein.

3.4. Sphingolipid analysis

Analysis is performed by Ultra Performance Liquid Chromatography followed by nano-electrospray ionization mass spectrometry (UPLC-nano ESI-MS/MS) *(16, 17)*.

1. For analysis, sphingolipids extracted as described in section 3.1.2 are solubilized by gentle heating and sonication.
2. Sphingolipid analysis is initiated by Ultra Performance Liquid Chromatography (UPLC) (see Note 10).

   Aliquots of 2 µl are injected in the partial loop with needle overfill mode. The flow rate is set at 0.12 ml/min, and the separation temperature at 35°C. Inositol containing sphingolipids are separated by elution with a linear gradient of solvents A and B as follows: 65 % solvent B held for 2 min, linear increase to 100 % solvent B for 8 min, 100 % solvent B held for 2 min, and equilibration to 65 % solvent B in 2 min. Ceramides (Cer) and hexosylceramides (HexCer) are separated as follows: 80 % solvent B held for 2 min, linear increase to 100 % solvent B for 8 min, 100 % solvent B held for 2 min and equilibration to 80 % solvent B in 2 min. Solvent B is tetrahydrofuran/methanol/20 mM ammonium acetate containing 0.1 % (v/v) acetic acid; and solvent A is methanol/20 mM ammonium acetate containing 0.1 % (v/v) acetic acid.
3. Chip-based nano-electrospray ionization is achieved in the positive ion mode with 5 µm internal diameter nozzles, a flow rate of 209 nl/ min, and a voltage of 1.5 kV.
4. Detection of sphingolipid molecular species is carried out with a tandem mass spectrometer by monitoring (i) the transition from $[M+H]^+$ molecular ions to dehydrated long chain base (LCB) fragments for Cer, HexCer and LCB; and (ii) the

loss of phosphoinositol containing head groups for inositol containing sphingolipids. Dwell time is 30 ms (see Note 11).

3.5. Non-polar lipid analysis by thin layer chromatography

The profile of the major *P. pastoris* non polar lipids has been reported previously *(18)*. Here we provide a protocol suitable for quantitative analysis of non-polar lipids from *P. pastoris* total cell extracts or subcellular fractions. The sample size used for lipid extraction varies. For total cell extracts we recommend to use samples containing 0.3 mg protein. If isolated lipid droplets are used for analysis, samples containing 0.5-1 µg protein are sufficient. Dissolve dried lipids in 15-30 µl (or any appropriate volume) of $CHCl_3$/methanol (2:1; v/v) prior to loading on TLC plates.

3.5.1. Separation of non-polar lipids by a two-step procedure

This procedure allows proper separation of sterols, free fatty acids, steryl esters and triacylglycerols.

1. Prepare two glass TLC chambers by filling one chamber with 50-100 ml of the first running solvent solution and the second chamber with 50-100 ml of the second running solvent solution (see Note 5).
2. Load TLC plates with samples either manually (see Note 12) or with a sample loading device. Leave 1.5 cm space from the bottom and side margins of TLC plates. Load each sample as a narrow 0.8-1 cm wide band leaving 1-1.5 cm space in between. Load each TLC plate with at least three lanes containing standards with increasing amounts of the respective component (see Note 13).
3. First step separation: Develop the TLC in an ascending manner until the solvent front reaches half of the plate (~5 min, see Note 14). Remove plate and dry with the hairdryer (see Note 6).
4. Second step separation: Develop the TLC using the second solvent system in the same direction as in the first step until the solvent front reaches the top of the plate (~20 min) (see Note 14). Remove plate and dry with hairdryer (see Note 6). Separated lipids are now ready for visualization and quantification.

3.5.2. Separation of diacylglycerols

For separation of diacylglycerols a one-step solvent system is applied *(19)*. Prepare lipid extracts (Section 3.1.1) and dissolve lipids in an appropriate volume of $CHCl_3$/methanol (2:1; v/v). Load lipid extracts on the TLC plate and develop the TLC with chloroform/acetone/acetate (90:8:1, per vol.) for ~20 min. Dry the TLC plate with a hairdryer. Separated lipids can be visualized and quantified (see Notes 6-7 and 12-14).

3.5.3. Visualizing separated lipids and quantification.

To quantify sterols and steryl esters, TLC plate can be directly scanned at a wavelength of 275 nm. Peaks of standards with known amounts loaded are used for calibration. The peak areas of individual samples are set in relation to standards.

To quantify other lipids visualization is needed as follows (Fig. 2):

1. Put the TLC plate into a charring solution and keep it there for 15-20 sec (see Note 15).
2. Put TLC plate into an oven and heat for 30 min at 100°C.
3. After heating separated lipids and standards become visible as bands and can be scanned at 400 nm wavelengths using a TLC scanner. Amounts of individual lipids are calculated relative to standards as described above (see Note 16).

3.6. Sterol analysis by Gas Liquid Chromatography- Mass Spectrometry

The analysis of sterols from yeast was described previously *(9, 20)*. Here we summarize the method for the analysis of *P. pastoris* sterols by GLC-MS.

3.6.1. Sample preparation

The size of biological samples required for sterol analysis depends on the content of sterols. For analyzing the sterol composition from total cell extracts the amount of 0.3 mg protein is sufficient. For subcellular fractions the sample size can differ strongly and has to be estimated empirically. As examples, mitochondrial fractions containing 0.5 mg protein, plasma

membrane fractions containing 1 mg protein, peroxisomal fractions containing 0.25 mg protein, and lipid droplet fractions containing 0.02-0.03 mg protein are sufficient.

1. Alkaline hydrolysis. Put the following reagents into a 15 ml glass tube: 0.6 ml methanol; 0.4 ml 0.5% pyrogallol in methanol; 0.4 ml 60% KOH; 5 µl of cholesterol solution (2 mg/ml) as an internal standard; and an appropriate amount of the specimen to be analyzed.
2. Close tubes tightly, wrap in aluminum foil to protect the sample from light, and put the tube into a water bath for 2 h at 90°C (see Note 17).
3. Extract lipids by adding 1 ml of *n*-heptane and mix the sample vigorously for 30 sec. Centrifuge tubes for 3 min at 250 x g. Transfer the upper phase into a new tube. Repeat the extraction twice with 1 ml of *n*-heptane and combine upper phases.
4. Dry extracts under a stream of nitrogen and store at -20°C.
5. For analysis, dissolve extracts in 10 µl pyridine, add 10 µl of N'O'-bis(trimethylsilyl)-trifluoracetamide, incubate for 10 min and dilute with 50 µl ethyl acetate (see Note 18). Transfer samples to glass acquisition vials.

3.6.2. GLC-MS analysis

GLC-MS analysis can be performed on a gas chromatograph equipped with a mass selective detector and a MS capillary column (crosslinked 5% Phenyl Methyl Siloxane) with 30 m×0.25 mm×0.25 µm film thickness.

1. Aliquots of 1 µl are injected at 270°C injection temperature in the splitless mode with helium as a carrier gas at a constant flow rate of 0.9 ml/min. The temperature program is as follows: 1 min at 100°C and 10°C/min to 250°C and 3°C/min to 310°C.
2. Mass spectra are obtained in scan mode with 3.27 scans/sec using a scan range of 200-500 amu. Individual sterols are identified according to their retention times (Fig. 3A) and mass fragmentation pattern (Fig. 3B, see Note 19) using appropriate chromatography software. For quantification of individual sterols, integrated peak areas are related to the internal standard cholesterol peak area.

3.7. Analysis of non-polar lipid and phospholipid species by HPLC coupled mass spectrometry

The procedure for lipid analysis by HPLC/MS has been described previously in much detail *(21, 22)*. Here we provide a protocol for the analysis of molecular species of triacylglycerols and phospholipids.

1. Prepare lipid extracts as described in section 3.1.1. The amount of extracted lipids and downstream dilution factors depend on the sample and lipid class being analyzed and should be adjusted empirically (see Note 20).
2. Dissolve lipids in 500 µl $CHCl_3$/methanol (1:1; v/v) and add 5 µM TG (species 51:0) and 5 µM PC (species 24:0) as internal standards. Transfer 30 µl to the acquisition vial and use 3 µl injection volume for HPLC-MS.
3. Use a reversed-phase C18 column (reversed-phase C18; 100 × 1 mm i.d., 1.9 µm particle size) employing solvent A and solvent B for chromatographic separation of lipid species. The gradient changes from 35 % to 70 % solvent B within 4 min and further to 100 % solvent B within 16 min. These conditions are held constant for 10 min at a flow rate of 250 µl/min. Mass spectrometry is performed by HPLC directly coupled to a FT-ICR-MS hybrid mass spectrometer equipped with an ionMax ESI source. The mass spectrometer is operated at a mass accuracy of <2 ppm with external calibration and resolution of 200,000 full width at half height at 400 *m/z*. The spray voltage is set at 5,000 V, the capillary voltage at 35 V, the tube lens at 120 V and the capillary temperature at 250°C. Peak areas are calculated by QuanBrowser for all lipid species identified according to their exact mass and retention time.

4. Notes

1. Glass tubes need to be closed tightly with organic solvent resistant seals. Make sure that no evaporation of solvent occurs by using intact glass tubes and caps with gaskets.
2. Removal of the protein containing interphase without losing substantial amounts of the organic phase is critical, especially when absolute quantifications are required. Therefore, it is recommended to leave some residual proteins in the interphase, which still can be removed during further steps of phase separation. Alternatively, protein interphases can be left intact during the entire extraction process, and the organic phase can be collected by penetrating the protein layer with a small glass pipette.
3. Dried lipid extracts can be stored in -20° till needed.
4. Extracted phospholipids should be applied as a spot of about 3-5 mm in diameter ~2 cm from the left and the lower margins of a Silica gel 60 TLC plate.
5. TLC chambers have to be saturated with solvents for about 30 min before usage.
6. Do not overheat the Silica gel 60 TLC plate.
7. At this stage, the plates can be stored overnight in the dark. Iodine should be completely removed by spraying the plate with distilled water and heating at ~50°C in the oven, or by evaporating on air in a hood.
8. Moistening the TLC with phosphate-free distilled water facilitates scrapping off the respective spots.
9. The tube should be put into the heating block only to half of the height thus allowing circulation and condensation of acid fumes. It is important that this step is performed in a hood because the hot acids may give rise to hazardous fumes. After 30 min the liquid in the tubes should be colorless or slightly yellow. If it is dark yellow or brownish, it can be bleached by addition of 5-10 µl 30 % H_2O_2.
10. Use an appropriate UPLC column, e.g. with dimensions 100 mm × 1 mm, 1 µm.
11. Optimize MS parameters to maximize detector response.
12. For loading TLC plates manually, glass syringes with 25-100 µl volume are suitable. Try to load bands as thin as possible.
13. The amounts of standards to load should be estimated empirically and depend on the samples to be analyzed.
14. TLC plates should not get in contact with front or side walls of the chamber during development.
15. Avoid keeping TLC plates in charring solution for more than 1 min.

16. Alternatively to densitometric scanning procedures, bands visualized on TLC plates can be digitalized at high resolution, and any graphic analyzing software (e.g., ImageJ, CorelDRAW) can be used for calculating amounts of standards and individual lipids.
17. After heating samples in the water bath, the color of the mixture should turn yellow.
18. This step should be performed directly before GLC-MS analysis.
19. Retention times of sterols can differ slightly depending on the samples, but the overall pattern is similar.
20. As an example, 0.3 mg protein from a cell homogenate is sufficient for non-polar lipid and phospholipid species analysis.

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References

1. G. Daum, N.D. Lees, M. Bard, et al. (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*, Yeast 14, 1471–1510.

2. J.R. Van Brocklyn and J.B. Williams (2012) The control of the balance between ceramide and sphingosine-1-phosphate by sphingosine kinase: oxidative stress and the seesaw of cell survival and death, Comp. Biochem. Phys. Part B, Biochem. Mol. Biol. 163, 26–36.

3. A.X.S. Santos and H. Riezman (2012) Yeast as a model system for studying lipid homeostasis and function, FEBS Lett. 586, 2858–2867.

4. M. Sud, E. Fahy, D. Cotter, et al. (2007) LMSD: LIPID MAPS structure database, Nucl. Acids Res. 35, D527–532.

5. E. Fahy, S. Subramaniam, R.C. Murphy, et al. (2009) Update of the LIPID MAPS comprehensive classification system for lipids, J. Lipid Res. 50 Suppl, S9–14.

6. D. Peretti, N. Dahan, E. Shimoni, et al. (2008) Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport, Mol. Biol. Cell. 19, 3871–3884.

7. M. Schnabl, G. Daum, and H. Pichler (2005) Multiple lipid transport pathways to the plasma membrane in yeast, Biochim. Biophys. Acta. 1687, 130–140.

8. E. Zinser, C.D. Sperka-Gottlieb, E.V. Fasch, et al. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*, J. Bacteriol. 173, 2026–2034.

9. G. Tuller, T. Nemec, C. Hrastnik, et al. (1999) Lipid composition of subcellular membranes of an FY1679-derived haploid yeast wild-type strain grown on different carbon sources, Yeast 15, 1555–1564.

10. M. Ståhlman, C.S. Ejsing, K. Tarasov, et al. (2009) High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry, J. Chromatography 877, 2664–2672.

11. C. Klose, M.A. Surma, and K. Simons (2013) Organellar lipidomics--background and perspectives, Curr. Op. Cell Biol. 25, 406–413.

12. J. Folch, M. Lees, and G.H. Sloane Stanley (1957) A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226, 497–509.

13. J.E. Markham, J. Li, E.B. Cahoon, et al. (2006) Separation and identification of major plant sphingolipid classes from leaves, J. Biol. Chem. 281, 22684–22694.

14. C. Buré, J.-L. Cacas, F. Wang, et al. (2011) Fast screening of highly glycosylated plant sphingolipids by tandem mass spectrometry, Rapid Comm. Mass Spectr. 25, 3131–3145.

15. R.M. Broekhuyse (1968) Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids, Biochim. Biophys. Acta. 152, 307–315.

16. J.E. Markham and J.G. Jaworski (2007) Rapid measurement of sphingolipids from Arabidopsis thaliana by reversed-phase high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry, Rapid Comm. Mass Spectr. 21, 1304–1314.

17. C.S. Ejsing, T. Moehring, U. Bahr, et al. (2006) Collision-induced dissociation pathways of yeast sphingolipids and their molecular profiling in total lipid extracts: a study by quadrupole TOF and linear ion trap-orbitrap mass spectrometry, J. Mass Spectr. 41, 372–389.

18. V.A. Ivashov, K. Grillitsch, H. Koefeler, et al. (2013) Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*, Biochim. Biophys. Acta. 1831, 282–290.

19. C. Heier, U. Taschler, S. Rengachari, et al. (2010) Identification of Yju3p as functional orthologue of mammalian monoglyceride lipase in the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta. 1801, 1063–1071.

20. M.A. Quail and S.L. Kelly (1996) The extraction and analysis of sterols from yeast, Meth. Mol. Biol. 53, 123–131.

21. J. Hartler, M. Trötzmüller, C. Chitraju, et al. (2011) Lipid Data Analyzer: unattended identification and quantitation of lipids in LC-MS data, Bioinformatics 27, 572–577.

22. A. Fauland, H. Köfeler, M. Trötzmüller, et al. (2011) A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry, J. Lipid Res. 52, 2314–2322.

Figures

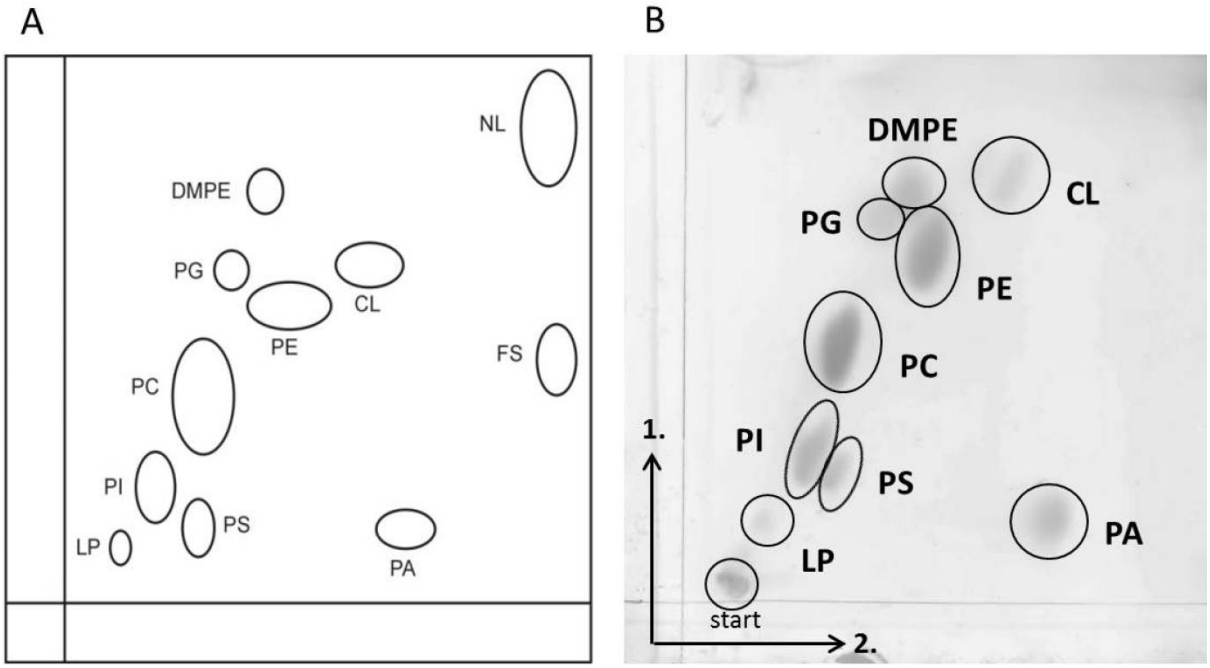

Fig.1 Phospholipid separation by two-dimensional thin layer chromatography

Phospholipids are extracted from samples containing 1-2 mg protein and applied to two-dimensional thin layer chromatography (2D-TLC) as described in section 3.3.1. Figure 1A indicates how phospholipids should typically separate in a 2D-TLC. Figure 1B shows the phospholipid profile of a *P. pastoris* microsomal fraction after 2D-TLC separation and iodine vapor staining. Numbers indicate running directions and the solvent system. CL: cardiolipin; DMPE: dimethylphosphatidylethanolamine; FS: fatty acids; LP: lysophospholipids; NL: neutral lipids; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; start: lipids are applied to the TLC plate at this point.

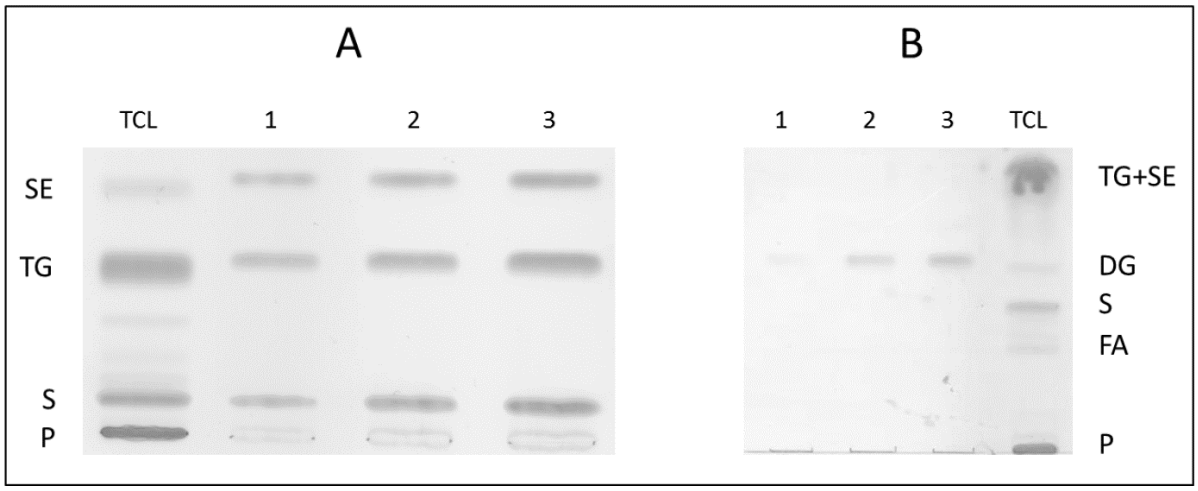

Fig. 2 Non-polar lipids separated by TLC and visualized

Pichia pastoris cells were grown on YPD to the stationary phase (26 h). Total cellular lipids (TCL) were extracted from cell homogenates containing 0.3 mg protein and applied onto TLC plates. Lipids were separated by a two-step (A) or one-step procedure (B) and visualized by charring and heating. P- phospholipids, S- sterols, TG- triacylglycerols, SE- steryl esters, FA- fatty acids, DG- diacylglycerols. Increasing amounts of the following standards were loaded in lanes 1-3 of plate A: 3, 6, and 10 µg ergosterol; 10, 20, 40 µg triolein; 2, 4, 6 µg cholesterol oleate. TLC plate B was loaded with 0.7, 3.3, and 5 µg of 1,2-diolein standard in lanes 1-3.

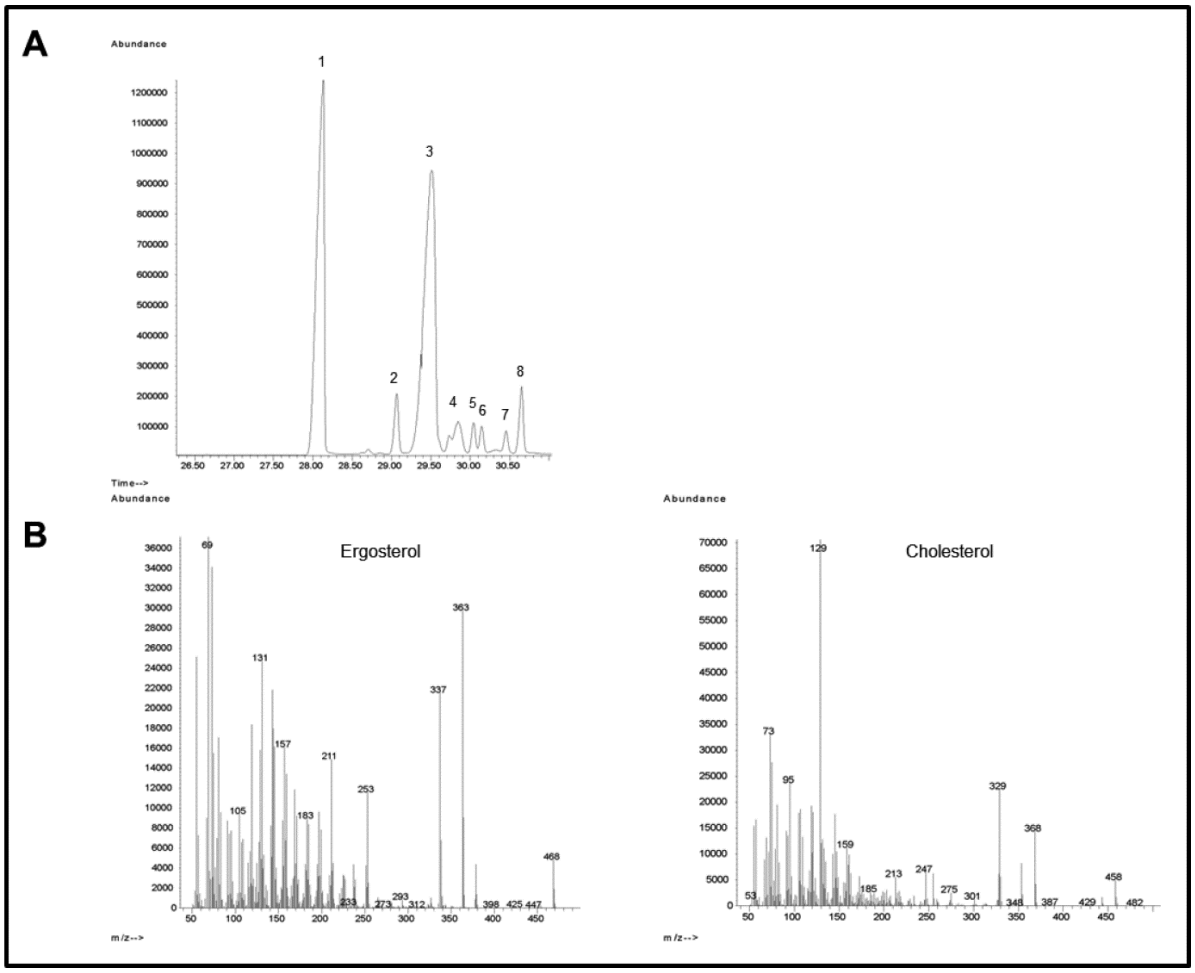

Fig.3 Example of GLC chromatogram and mass fragmentation patterns of *Pichia pastoris* total cell sterols

Cells were grown on YPD to the stationary phase (26 h). Sterols were extracted from homogenates containing 0.3 mg protein and analyzed by gas liquid chromatography/mass spectrometry. A- GLC pattern. Numbered peaks represent individual sterols as follows: 1- cholesterol as internal standard, 2- zymosterol, 3- ergosterol, 4- 4-methylzymosterol, 5- fecosterol, 6- episterol, 7- lanosterol, 8- 4,14-dimethylcholesta-8,24-dienol. B- mass fragmentation pattern of ergosterol and cholesterol.

CHAPTER 3

Isolation of organelles from *Pichia pastoris*

Vasyl Ivashov[1*], Lisa Klug[1*], Karlheinz Grillitsch[2] and Günther Daum[1]

[1]Institute of Biochemistry, Graz University of Technology, Austria; [2]Austrian Centre of Industrial Biotechnology, Graz, Austria

*Contributed equally to this paper and should be considered as co-first-authors

Running title: Organelles from *Pichia pastoris*

Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology; Petersgasse 12/2, A-8010 Graz, Austria; Tel.: +43 316 873 6462; Fax: +43 316 873 6952; E-mail: guenther.daum@tugraz.at

Summary

Pichia pastoris is widely used for heterologous protein expression but fundamental knowledge about its cell biology is largely missing. Since eukaryotic cells are compartmentalized and have an internal endomembrane system, techniques which allow isolation of these compartments (organelles) or subcellular membranes are required for studying various aspects of cell biology such as organelle biogenesis, membrane traffic, segregation of metabolic pathways and metabolite transport inside the cell. Many reliable protocols for cell fractionation were designed for the model yeast *Saccharomyces cerevisiae.* We adopted and modified these methods to study organelles from *Pichia pastoris* which is frequently used for applied biotechnology and industrial purposes. In this chapter we will describe optimized protocols for the isolation of the most important organelles from *Pichia pastoris* and briefly address applications of these techniques for cell biological investigations.

Key words

Organelles, endoplasmic reticulum, plasma membrane, vacuole, Golgi, lipid droplets, peroxisomes, mitochondria

1. Introduction

Eukaryotic cells are structurally and functionally organized in compartments called organelles. These structures are surrounded by lipid membranes with specific membrane proteins embedded. Membranes segregate the interior of organelles from the environment and therefore allow spatial separation of metabolic pathways inside the cell. Each organelle has its distinct subset of membrane and luminal proteins which fulfill structural, metabolic or regulatory functions. Such proteins can be localized exclusively to one organelle but may also be present in several compartments with related functions. To address the various questions of cell biology, molecular biology and biochemistry, isolation of organelles and cellular membranes is required. These methods are also employed to study cell biology of the yeast *Pichia pastoris* in fundamental and applied research. As examples, isolated organelles can be used to investigate their biogenesis, structural or functional properties, their components by -omics studies, and the role of individual enzyme, metabolic pathways or transport processes ***(1–3)***. Recombinant proteins which are expressed heterologously in *Pichia pastoris* move in many cases gradually from their site of synthesis to the cell periphery. The process of protein secretion which includes protein folding and protein modifications is linked to vesicle flux from the endoplasmic reticulum via Golgi to the plasma membrane. Production and secretion of proteins may lead to their accumulation in a certain organelle or in the cytoplasm ***(4, 5)***. Also in this case, organelle isolation techniques are useful tools to follow the traffic routes of such recombinant proteins.

Here we describe optimized protocols designed for isolation and quality control of organelles from *Pichia pastoris*. Techniques for yeast organelle isolation with a focus on *Saccharomyces cerevisiae* were established and described previously ***(6)***. Most of these methods are based on cell disruption and a combination of differential and density gradient centrifugation steps followed by quality control of the obtained fractions ***(7)***. Although some general principles and strategies can be applied for cell fractionation of different yeast species, each isolation procedure should be optimized for each cell type and for different culture conditions. In this chapter, we provide guidelines for the isolation of subcellular fractions from *Pichia pastoris*. Moreover, we describe the quality control of organelles which is essential for the evaluation of cell fractionation and for subsequent cell biological investigations.

2. Materials

2.1. Equipment

The basic equipment required is similar for most organelle preparation procedures.

1. 2 L shaking flasks with baffles.
2. High speed centrifuge, fixed angle rotors (e.g., SLC-3000, F21S 8x50) and centrifugation tubes (500 and 50 ml).
3. Ultracentrifuge, swing-out rotors (e.g., AH629, TH641), fixed angle rotor (e.g., T865) and centrifuge tubes.
4. Dounce homogenizer with large clearance pestle (L-pestle).
5. Cell disruptor with cooling device.
6. Glass beads, diameter 0.3 mm.

2.2. Reagents

2.2.1. Spheroplast preparation

1. Buffer SP-A: 0.1 M Tris/SO_4, pH 9.4.
2. Buffer SP-B: 1.2 M sorbitol in 20 mM KH_2PO_4/KOH, pH 7.4.
3. Dithiothreitol (DTT).
4. Zymolyase (see Note 1).

2.2.2 Isolation of peroxisomes

1. Growth media containing methanol or oleic acid (see Note 2)
2. Buffers and chemicals for spheroplast preparation (see section 2.2.1.).
3. Breaking buffer: 5 mM MES, pH 6.0, 1 M sorbitol, 1 mM KCl, 1 mM Na_2EDTA, 0.1 % ethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF).

4. Gradients: 50 %, 35 %, 30 % and 24 % Accudenz (w/v) in 5 mM MES, pH 6.0, 1 mM KCl, 0.24 M sucrose.
5. 10 mM Tris/HCl, pH 7.4.

2.2.3. Isolation of mitochondria and microsomes

1. Growth media have to be chosen according to experimental requirements (see Note 3).
2. Buffers and chemicals for spheroplast preparation (see section 2.2.1.).
3. Buffer C: 0.6 M mannitol, 10 mM Tris/HCl, pH 7.4.
4. Buffer D: 10 mM Tris/HCl, pH 7.4.
5. 1 M PMSF stock in dimethylsulfoxid (DMSO).

2.2.4 Isolation of Golgi

1. Growth media should be chosen according to experimental requirements.
2. Buffers and chemicals for spheroplast preparation (see section 2.2.1.).
3. Lysis buffer: 0.6 M sorbitol, 10 mM triethanolamine hydrochloride/NaOH, pH 7.2, 1 mM EDTA.
4. MES buffer: 10 mM Mes/Tris, pH 7.2.
5. Tris buffer: 10 mM Tris/HCl, pH 7.4.
6. 40 % sucrose/MES: 40 % sucrose, 60 % MES buffer.
7. 35 % sucrose/MES: 35 % sucrose, 65 % MES buffer.
8. 30 % sucrose/MES: 30 % sucrose, 70 % MES buffer.
9. 25 % sucrose/MES: 25 % sucrose, 75 % MES buffer.
10. 5 M sorbitol.
11. 1 M PMSF stock in DMSO.

2.2.5. Isolation of vacuoles

1. Any rich growth media.
2. Buffers and chemicals for spheroplast preparation (see section 2.2.1.).
3. Stock solution: 10 mM MES/Tris, pH 6.9, 0.2 mM EDTA, 2 mM PMSF.
4. Breaking buffer: 12 % Ficoll (w/w) in stock solution.

5. 6 % Ficoll (w/w) in stock solution.
6. 3.5 % Ficoll (w/w) in stock solution.
7. 1.5 % Ficoll (w/w) in stock solution.

2.2.6. Isolation of lipid droplets

1. Any rich growth media.
2. Buffers and chemicals for spheroplast preparation (see section 2.2.1.).
3. Stock solution: 10 mM MES/Tris, pH 6.9, 0.2 mM EDTA, 2 mM PMSF.
4. Buffer LD-A: 12 % Ficoll (w/w) in stock solution.
5. Buffer LD-B: 4 % Ficoll (w/w) in stock solution.
6. Buffer LD-D: 0.25 M Sorbitol in stock solution.

2.2.7. Isolation of plasma membrane

1. Any rich growth media.
2. Solution 1: 0.5 M Tris/HCl, pH 8.5.
3. Solution 2: 0.5 M EDTA/NaOH, pH 8.0.
4. TE buffer: 10 mM Tris/HCl, 0.2 mM EDTA, pH 7.5.
5. TED: 10 mM Tris/HCl, 0.2 mM EDTA, pH 7.5, 0.2 mM DTT (see Note 4).
6. TEDG buffer: 80 % TE buffer, 20 % glycerol, 0.2 mM DTT, pH 7.5. (see Note 4).
7. 53 % sucrose/TED: 53 % sucrose, 47 % TE buffer, 0.2 mM DTT (see Note 4).
8. 43 % sucrose/TED: 43 % sucrose, 57 % TE buffer, 0.2 mM DTT (see Note 4).
9. MES buffer: 5 mM MES, 0.2 mM EDTA, pH 6.0.
10. 53 % sucrose/MES: 53 % sucrose, 47 % MES buffer.
11. 43 % sucrose/MES: 43 % sucrose, 57 % MES buffer.
12. 38 % sucrose/MES: 38 % sucrose, 62 % MES buffer.
13. 1 M PMSF stock in DMSO.
14. Dithiothreitol (DTT).

3. Methods

3.1 Spheroplast preparation

Yeast spheroplast preparation based on the treatment with Zymolyase® 20T has been described previously ***(8)***. This procedure is prerequisite for most isolation protocols described here and allows digestion/removal of the cell wall making cells susceptible for gentle mechanical and osmotic disintegration.

1. When the culture has reached the desired growth phase, cells are harvested by centrifugation at 2,500 x g for 4 min.
2. Cell pellets are washed once with deionized water and the cell wet weight (CWW) is estimated.
3. Suspend cells in pre-warmed (30°C) buffer SP-A: Use 0.5 g CWW/ml buffer and add 0.66 mg/ml DTT per ml SP-A. Incubate with shaking at 30°C for 10 min.
4. Centrifuge cells at 2,500 x g for 4 min. Remove supernatant, wash cell pellet with 1-2 volumes buffer SP-B and centrifuge at 2,500 x g for 4 min.
5. Resuspend cell pellet in pre-warmed (30°C) buffer SP-B. Use 0.15 g CWW/ml, add Zymolyase 20T (2 mg/g CWW) and incubate with shaking at 30°C.
6. Conversion of whole cells to spheroplasts takes approximately 30-60 minutes (see Note 5).
7. Harvest spheroplasts by centrifugation at 2,500 x g for 4 min. Remove supernatant carefully without suspending the pellet.
8. Wash spheroplasts with ice-cold SP-B and centrifuge as described above (see Note 6). After this step, spheroplasts are ready for disintegration.

3.2. Isolation of peroxisomes

The protocol for the isolation of peroxisomes from *Pichia pastoris* was adapted from procedures described previously ***(1, 9)***.

1. Inoculate YPM or YPO media with an aliquot of a YPD grown preculture to an OD_{600} of 0.1. Grow cells to the late logarithmic phase in shaking flasks at the desired temperature. The time of cultivation depends on the growth conditions. Typically, 3-5

L main culture will yield peroxisome samples with a total amount of 1.5–2 mg protein.

2. Harvest cells and prepare spheroplasts (see section 3.1.).
3. Preparation of homogenate: Add an equal volume of breaking buffer and disintegrate spheroplasts with 10 strokes in a Dounce Homogenizer using a loose (L) fitting pestle. Save an aliquot of the homogenate if needed and store at -80°C.
4. Centrifuge the homogenate for 5 min at 3,000 x g to remove cell debris and high density components. Collect supernatant in a fresh tube. Resuspend the pellet in an equal volume of breaking buffer and repeat homogenization and centrifugation. Discard pellet and combine cell-free supernatants.
5. Centrifuge supernatant at 30,000 x g for 30 min.
6. Discard supernatant, add 2-3 volumes of breaking buffer to the pellet and resuspend in a Dounce homogenizer by 3-5 strokes.
7. Load suspension on top of a step gradient composed of 5 ml 50 %, 10 ml 35 %, 10 ml 30 %, 7 ml 24 % of Accudenz (w/v) in 5 mM MES, pH 6.0, l mM KCl, 0.24 M sucrose (see Note 7).
8. Centrifuge gradient at 122,000 x g for 2 h. After centrifugation, peroxisomes form a band at the interface of 35 % and 30 % Accudenz. Collect the peroxisomal fraction carefully with a syringe by inserting the needle from the side of the tube. As a byproduct of this centrifugation step, a fraction enriched in mitochondria can be collected from the 30 % - 24 % Accudenz interphase.
9. Wash peroxisomes with 4 volumes of breaking buffer and centrifugation at 30,000 x g for 30 min. Remove supernatant. The pellet containing purified peroxisomes can be suspended in a small volume of any buffer for further analysis.

3.3. Isolation of mitochondria

The isolation of mitochondria from *Pichia pastoris* has been reported previously ***(2)***.

1. Pre-cultures grown to the stationary phase are used for inoculating main cultures in Erlenmeyer flasks with baffles. Cells are grown at the desired cultivation conditions (temperature, growth media and carbon source, aeration). Typically, cells are harvested when they have reached the late exponential growth phase (see Note 8).
2. Harvest cells by centrifugation at room temperature (RT) at 2,500 x g for 5 min.

3. Wash cells with deionized water and prepare spheroplasts (see section 3.1.).
4. Suspend spheroplast pellet in buffer C (1 ml/g CWW) and add 1 M PMSF (2 µl/g CWW).
5. Transfer cells into a Dounce homogenizer and disintegrate them with 15 strokes on ice using an L-pestle.
6. Centrifuge for 5 min at 5,100 x g at 4°C. Rinse the homogenizer with 10 ml buffer C to collect residual material.
7. Collect supernatant (homogenate) and store on ice.
8. Repeat steps 7 to 9 twice. Add PMSF each time. Combine all three supernatants, withdraw 1-3 ml homogenate samples and store them at -80°C for further analysis. The pellet can be discarded after the third round of cell disintegration.
9. Sediment the homogenate (combined supernatants) for 10 min at 12,000 x g at 4°C.
10. Remove the supernatant and clean the inside of the centrifugation tube carefully with a paper towel to avoid contamination with other organelles without suspending the pellet. The supernatant contains the cytosol and microsomes (see section 3.4.).
11. The pellet is suspended in 30 ml buffer C and transferred to a new centrifugation tube.
12. Centrifuge for 5 min at 5,100 x g at 4°C to remove all cell debris.
13. Transfer supernatant into a new centrifugation tube and centrifuge for 10 min at 12,000 x g at 4°C. Discard pellet.
14. Remove the supernatant and clean the inside of the tube carefully with a paper towel to reduce the risk of contamination with other subcellular fractions. Mitochondria (pellet) are suspended in 1-2 ml buffer D depending on the size of the pellet. Samples can be stored at -80°C for further analysis.

3.4. Isolation of microsomes

The isolation of microsomes from *Pichia pastoris* as described recently ***(10)*** can be performed as a continuation of the isolation protocol of mitochondria (see section 3.3.). Typically 30-40 g of cells will yield a microsomal fraction containing 1-2 mg protein.

1.-11. These steps are identical to section 3.3.

12. After centrifugation at 12,000 x g for 10 min at 4°C, transfer supernatant to a new centrifuge tube. The supernatant contains the cytosol and microsomes, the pellet contains the mitochondria.

13. Centrifuge supernatant for 30 min at 20,000 x g at 4°C using a fixed angle rotor.
14. Discard pellet which contains an intermediate/mixed fraction. Transfer supernatant to a new centrifuge tube and centrifuge for 30 min at 30,000 x g at 4°C.
15. The pellet contains the 30,000 x g microsomal fraction. Transfer supernatant to a new centrifuge tube and centrifuge supernatant for 30 min at 40,000 x g at 4°C. Suspend the 30,000 x g pellet in 1-2 ml buffer D depending on the size of the pellet. The resuspended pellet can be stored at -80°C till further analysis.
16. After centrifugation at 40,000 x g, the resulting pellet contains the 40,000 x g microsomal fraction. Transfer the supernatant to ultracentrifugation tubes and centrifuge for 45 min at 100,000 x g at 4°C using a fixed angle rotor. Suspend the 40,000 x g pellet in 1-2 ml buffer D depending on the size of the pellet and store the sample at -80°C until further analysis.
17. After ultracentrifugation at 100,000 x g, the supernatant (cytosol) is removed; the 100,000 x g microsomal pellet is suspended in 1-2 ml buffer D and stored at -80°C for further analysis. Cytosol samples can also be collected and stored at -80°C.

3.5. Isolation of the Golgi

The isolation of the Golgi follows a procedure developed recently in our lab (Grillitsch *et al.*, manuscript in preparation).

1. Pre-cultures grown to the stationary phase are used for inoculating main cultures in baffled Erlenmeyer flasks.
2. Cells are incubated until they have reached the mid exponential to late exponential growth phase (see Note 9).
3. Harvest cells by centrifugation at room temperature at 2,500 x g for 5 min using a fixed angle rotor.
4. Wash cells with deionized water and prepare spheroplasts as described in section 3.1.
5. Suspend sedimented spheroplasts in lysis buffer (1 ml/1.5 g CWW) and add PMSF (2 µl/g CWW). Disintegration of spheroplasts for the isolation of Golgi is critical and requires gentle handling and moderate mechanical force.
6. Homogenize spheroplasts in suspension using a Dounce homogenizer by disintegrating with 3 strokes on ice using an L-pestle.

7. Transfer homogenized suspension into a beaker and fill up with approximately 2-3 volumes of lysis buffer.
8. Incubate spheroplasts under slight agitation on a magnetic stirrer for 20 min at 4°C.
9. Add sorbitol from a 5 M stock to a final concentration of 1 M. Take into account that the suspension already contains 0.6 M sorbitol from the lysis buffer.
10. Sediment intact spheroplasts and cell debris for 5 min at 3,600 x g at 4°C using a fixed angle rotor.
11. Discard pellet, withdraw 1-2 ml from the supernatant (homogenate) and store at -80°C for further analysis.
12. To obtain loading material for density gradient ultracentrifugation the cleared homogenate is subjected to several steps of differential centrifugation (Figure 1). First, high speed centrifugation is performed at 12,000 x g at 4°C for 10 min (pellet 1, P1).
13. To remove residual mitochondria the supernatant of the previous centrifugation step is centrifuged for 20 min at 20,000 x g at 4°C (pellet 2, P2).
14. The supernatant resulting from step 14 is centrifuged at 30,000 x g for 30 min at 4°C. In the obtained pellet fraction (pellet 3, P3) cis-Golgi marker proteins are enriched. This sample is loaded onto the final sucrose density gradient to obtain enriched and purified cis-Golgi fractions (see step 19). The sample should be stored on ice.
15. The resulting supernatant of step 15 is centrifuged at 40,000 x g for 30 min at 4°C (pellet 4, P4).
16. Transfer supernatant to an ultracentrifuge tube and separate soluble cytosol from membranes (mostly vesicle populations) by centrifugation at 200,000 x g at 4°C using a fixed angle rotor. The pellet (pellet 5, P5) is enriched in trans-Golgi marker proteins and loaded onto the final sucrose density gradient to obtain enriched and purified trans-Golgi (see step 19). The sample should be stored on ice.
17. Preparation of sucrose density gradients: The gradient consists of 2.5 ml, each, of sucrose/MES with concentrations of 40 %, 35 %, 30 % and 25 % sucrose. High quality of gradients is critical for successful separation of subcellular fractions.
18. Pellet fractions obtained after 30,000 x g (P3) and 200,000 x g (P5) centrifugation are resuspended in MES buffer (10 mM, pH 7.2). Samples are well homogenized in a Dounce homogenizer before loading onto a sucrose density gradient for separate isolation of cis- and trans-Golgi fractions. Ultracentrifugation using a swing out rotor is performed for at least 5 h at 200,000 x g at 4°C (see Note 10).

19. After ultracentrifugation, samples are taken from the four phase separation zones (cis F1-F4, trans F1-F4) with the aid of a syringe (approximately 1.5 ml per sample). Samples can be either frozen directly in sucrose solution or sedimented in a final centrifugation step. For sedimentation samples should be diluted 5-fold with 10 mM Tris/HCl, pH 7.4, and centrifuged at 200,000 x g for 45 min at 4°C using a fixed angle rotor.
20. Samples should be critically evaluated for the enrichment of Golgi markers, e.g. Emp47p, and the absence of other subcellular fractions. Depending on the strain, cultivation conditions, and experimental handling the best enrichment of highly purified fractions of cis- or trans-Golgi, respectively, may vary in cis F1-F4 and trans F1-F4.
21. Pellet fractions generated throughout this isolation procedure can be suspended in appropriate volumes of 10 mM Tris/HCl, pH 7.4, and used as loading controls when testing the quality of the final Golgi samples. Samples should be kept at -80°C.

3.6. Isolation of vacuoles

A number of protocols have been published for the isolation of vacuoles from yeast. Most of these methods are based on gradient centrifugation and floatation ***(11, 12)***. Here we present a modified protocol for the isolation of *Pichia pastoris* vacuoles which yields highly enriched samples (Ivashov *et al.*, manuscript in preparation).

1. Inoculate YPD media or any other rich media with a preculture grown on YPD to a final OD_{600} of 0.1. Grow cells to the mid stationary phase (16-17 h) in shaking flasks at 30°C. The cultivation time depends on growth conditions. Typically, 50 g cells will yield a vacuolar fraction containing 2-3 mg protein.
2. Harvest cells by centrifugation at 2,500 x g for 4 min.
3. Remove supernatant, wash cells with deionized water and sediment them at 2,500 x g for 4 min.
4. Spheroplasts preparation has been described in section 3.1. As the whole procedure is time consuming, steps after spheroplasts preparation may be continued on the next day. Spheroplasts can be frozen in liquid nitrogen and then thawed quickly on a 30°C water bath. Noteworthy, storage of spheroplasts overnight reduces the yield of vacuoles to ~50%.

5. For the preparation of the homogenate, suspend spheroplasts in 1.5 to 2 volumes of ice-cold breaking buffer. Disintegrate spheroplasts with 30 strokes in a Dounce Homogenizer using a loose fitting pestle. Utilization of complex protease inhibitors is strongly recommended (see Note11). Save homogenate sample if needed for further analysis and store at -80°C.
6. Load 25 ml of homogenate, each, to the bottom of ultracentrifuge tubes and overlay with 6% Ficoll solution to the top of the tube. Centrifuge at 140,000 x g for 90 min using a swing-out rotor, e.g. AH629. Collect the floating layers from each tube.
7. Add an equal volume of 6 % Ficoll solution to the combined floating layers and mix with 6 strokes in a Dounce homogenizer. Load suspension to the bottom of ultracentrifuge tube and overlay carefully with stock solution to the top of the tube. Centrifuge at 140,000 x g for 2 h.
8. Withdraw the band from the interphase of 6 % Ficoll layer and stock solution with the aid of a syringe. This fraction contains crude vacuoles.
9. Add 2 volumes of 6 % Ficoll to crude vacuoles and load to the bottom of an ultracentrifuge tube. Overlay the suspension with 3.5 % Ficoll solution to the top of the tube. Centrifuge at 140,000 x g for 3 h. Collect the floating layer and, if present, any dispersed white fraction beneath.
10. Dilute the collected fraction with 5-6 volumes of 1.5 % Ficoll. Centrifuge suspension at 100,000 x g for 30 min. Discard the supernatant, but avoid dispersing the pellet which contains purified vacuoles. Suspend vacuoles in 2-3 ml of stock solution or any buffer required for further experiments and store at -80°C.

3.7. Isolation of lipid droplets

A protocol for the isolation of highly purified lipid droplets from *Pichia pastoris* has been described previously ***(3)***.

1. Inoculate YPD media or any other rich media with a preculture grown on YPD to a final OD_{600} of 0.1. Grow cells to the early stationary phase (26 h) in shaking flasks at 30°C. The cultivation time depends on growth conditions. Lipid droplets are formed during growth on any media, but the highest yield can be obtained from cells grown on YPO. Typically, 100 g of cells grown on YPD will yield lipid droplets containing

250-350 µg protein. Please note that the amount of proteins in lipid droplets compared to lipid components is very low.

2. Harvest cells by centrifugation at 2,500 x g for 4 min.
3. Remove supernatant, wash cells with deionized water and sediment them at 2,500 x g for 4 min.
4. Prepare spheroplasts as described in section 3.1.
5. To prepare the homogenate suspend spheroplasts in an equal volume of ice-cold buffer LD-A. Disintegrate spheroplasts with 30 strokes in a Dounce Homogenizer using a loose fitting pestle.
6. Centrifuge the homogenate at 6,000 x g for 5 min and save supernatant. Add an equal volume of buffer LD-A to the remaining pellet, repeat disintegration in a Dounce Homogenizer by 15 strokes and centrifuge again at 6,000 x g for 5 min. Discard pellet and combine supernatants.
7. Centrifuge combined supernatants at 12,000 x g for 15 min. Save supernatant and discard pellet.
8. Load 25 ml of the supernatant, each, to the bottom of ultracentrifuge tubes and overlay with buffer LD-B to the top of the tube. Centrifuge at 140,000 x g for 1 h. Collect floating layers from the top of tubes and combine.
9. Suspend floating layer in 10 volumes of buffer LD-B and mix by 5-7 strokes in a Dounce homogenizer. Fill samples into ultracentrifuge tube and centrifuge at 140,000 x g for 1 h. Collect the floating layer.
10. Suspend the floating layer in an equal volume of buffer LD-B and load to the bottom of ultracentrifuge tube. Overlay suspension with buffer LD-D. Centrifuge at 140,000 x g for 90 min. The resulting floatate contains highly purified lipid droplets. Collect lipid droplets from the top of the tube using a sampler and store at -80°C.

3.8. Isolation of Plasma Membrane

A procedure for the isolation of plasma membrane from *Pichia pastoris* was recently developed in our lab (Grillitsch *et al*., manuscript submitted). Typically, 30 g of cells yield plasma membrane samples containing 2-3 mg protein.

1. Stationary grown pre-cultures are used to inoculate main cultures in baffled Erlenmeyer flasks.

2. Incubation conditions can vary depending on experimental requirements. However, cells should be incubated until they have reached the late exponential growth phase.
3. Harvest cells by centrifugation at room temperature at 2,500 x g for 5 min using a fixed angle rotor.
4. Wash cells once with deionized water and estimate cell wet weight (CWW).
5. Yeast cells are suspended in breaking buffer (20 g CWW in 84 deionized water; 5 ml of 0.5 M TrisCl, pH 8.5; 1 ml of 0.5 M EDTA, pH 8.0; 0.25 ml of 0.2 M PMSF).
6. Disintegrate cells mechanically by using glass beads under constant cooling and vigorous shaking for 3 min in any tissue/cell disintegrator. Let the glass beads sediment and collect supernatant on ice. After disintegration of the entire cell pellet, glass beads are washed once with TEDG buffer.
7. Cell extracts are cleared of unbroken cells and residual glass beads by centrifugation at 1,000 x g for 10 min using a fixed angle rotor. Discard pellet.
8. The resulting supernatant (homogenate) is centrifuged at 35,000 x g for 30 min to sediment bulk membranes using a fixed angle rotor. Suspend the pellet in TEDG-buffer and store on ice until loading the sucrose density gradient. Samples should be homogenized with 10 strokes in a Dounce homogenizer using a loose-fitting pestle.
9. Preparation of sucrose density gradients: 1 volume of 53 % sucrose in TED-buffer and 2 volumes of 43 % sucrose in TED-buffer. Do not load samples from more than 20 g CWW per gradient (total volume ~40ml).
10. Centrifuge for at least 3.5 h at 100,000 x g at 4°C using a swing out rotor.
11. After ultracentrifugation harvest the crude plasma membrane fraction at the 43/53 % sucrose interface with the aid of a syringe.
12. Dilute the sample 3- to 4-fold with deionized water and centrifuge for 20 min at 45,000 x g at 4°C using a fixed angle rotor to sediment membranes. Suspend the pellet in MES-buffer and store on ice until loading the sucrose density gradient. Samples should be homogenized with 10 strokes in a Dounce homogenizer using a loose-fitting pestle.
13. Preparation of the second sucrose density gradient: 1 volume of 53 %, 43 % and 38 % sucrose, each, in MES-buffer. Do not load samples from more than 20 g CWW per gradient.
14. Centrifuge for at least 2.5 h at 100,000 x g at 4°C using a swing out rotor (see Note 12).

15. After ultracentrifugation harvest the plasma membrane fraction at the interface between 53 % and 43 % sucrose.
16. Dilute the plasma membrane fraction 5-fold with Tris/HCl buffer (10 mM, pH 7.4) and centrifuge for 20 min at 45,000 x g at 4°C using a fixed angle rotor.
17. Discard supernatant without dispersing the pellet (PM2) which is not very stable.
18. Suspend pellet in Tris/HCl buffer (10 mM, pH 7.4) and store the plasma membrane fraction at -80°C for further analysis.

3.9. Quality Control of Isolated Organelles

Quality control of organelle fractions obtained by procedures described above is essential to ensure enrichment and purity. These tests are routinely carried out by Western blot analysis. For this purpose, 5-15 µg of precipitated protein from organelle fractions of interest and homogenate sample as a control are subjected to SDS polyacrylamide electrophoresis (SDS-PAGE), transferred to nitrocellulose or PVDF membrane and probed with primary antibodies recognizing typical marker proteins of particular organelles. By comparing intensities of bands from organelle samples and homogenate the enrichment factor can be estimated, provided that the amount of protein loaded onto each lane is exactly the same. Typical examples of yeast organelle marker proteins are: mitochondrial porin Por1p; plasma membrane ATPase Pma1p; sterol 24-C-methyltransferase Erg6p from lipid droplets, vacuolar carboxypeptidase Y (CPY; Prc1p), Pex3p from peroxisomes; beta-subunit of the endoplasmic reticulum resident oligosaccharyl transferase glycoprotein complex Wbp1p; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the cytosol. Specific antibodies raised against *Pichia pastoris* proteins are rare. In many cases, antibodies raised against the *Saccharomyces cerevisiae* orthologs can be used, although sometimes immunoreactions are weak. Another possibility to test for organelle markers is the use of hybrid proteins containing GFP, HIS, HA or any other tag. When these hybrids are expressed in *Pichia pastoris* under the native promoter, they can be detected with commercially available primary antibodies specific for the respective tag. Alternatively to Western Blot analysis marker enzyme assays can be performed to assess the quality of organelle preparations ***(6, 13)***.

High enrichment of organelles over the homogenate is desirable for cell fractionation experiments (Fig. 2A). In most cases, however, organelle preparations still contain impurities of other subcellular compartments. These contaminations can be due to naturally occurring

tight association of organelles (e.g. ER and Golgi, ER and lipid droplets, mitochondria and peroxisomes) ***(14–17)*** or to artifacts created during the cell fractionation procedure. Therefore, it is important to check organelle purity, e.g. by Western Blot analysis as shown in Fig. 2B. If signals of "foreign" markers are present in homogenate but largely absent from the organelle fraction of interest, the purity can be regarded as high. The presence of markers from other organelles in the sample at the amounts comparable to homogenate would, however, indicate low quality of the respective cell fraction.

4. Notes

1. Instead of zymolyase, lyticase can be used for spheroplasts preparation following the manufacturer's instructions.
2. For the isolation of most organelles rich growth media like YPD (1 % yeast extract, 2 % peptone, 2 % glucose) are suitable. For isolation of peroxisomes, media containing methanol (0.5 -2 %; YPM) or oleic acid (0.2 %; YPO) as carbon sources are required to induce the proliferation of peroxisomes. In principle, however, different growth media (minimal or complex media) can be used depending on the scientific problem.
3. Repression of mitochondria proliferation by high glucose concentrations has to be taken into account. Utilization of non-fermentable carbon sources such as glycerol or lactate may be advantageous for increasing the yield of mitochondria.
4. DTT must be added directly before use of the buffer. Buffers containing DTT are not considered for long term storage.
5. Withdraw samples of 10 µl, each, prior to the addition of zymolyase and at different time points of incubation with zymolyase during the spheroplasting procedure. Samples are diluted 1000-fold in deionized water. To monitor the efficiency of zymolyase treatment compare untreated to treated samples. When the solution of zymolyase treated samples turns clear, spheroplasting is complete. In case of inefficient conversion of intact cells to spheroplasts, the incubation time can be extended, or amounts of the enzyme may be increased.
6. After spheroplasts were obtained, all further steps should be performed on ice using ice-cold buffers. All centrifugation steps should be performed at 4°C.

7. Step gradients should be loaded carefully to avoid mixing of layers. Correct handling results in sharp border lines of interphases which are clearly visible. If no border lines are visible, gradients should be discarded.
8. Cells grown on glucose (30-40 g CWW) will yield mitochondria fractions containing 5-6 mg protein. Please note that the amount of mitochondria obtained highly depends on the carbon source utilized. Growth of cells on-fermentable carbon sources will increase the yield markedly.
9. For the isolation of Golgi fractions cells should be harvested at latest when they have reached the late exponential phase. Depletion of nutrients or starvation of cell should be strictly avoided, because all conditions provoking autophagy might negatively affect the yield of Golgi.
10. In case no clear phase separation has been achieved after 5 h of ultracentrifugation, the centrifugation period can be extended. Another reason for unsuccessful separation may be overloading of gradients. The maximum load of gradients has to be defined empirically. As a rule of thumb, single gradients for the isolation of trans-Golgi can be loaded with the double amount of material compared to single gradients used for the isolation of cis-Golgi.
11. If inhibition of proteolysis is crucial for further experiments, commercially available protease inhibitor cocktails can be added to the breaking buffer and all other buffers at amounts recommended by the manufacturer. Since vacuoles harbor many highly active proteases, the use of complex protease inhibitor cocktails and not only PMSF is strongly recommended.
12. Ultracentrifugation of the second density gradient can be continued overnight.

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References

1. T. Wriessnegger, G. Gübitz, E. Leitner, et al. (2007) Lipid composition of peroxisomes from the yeast *Pichia pastoris* grown on different carbon sources, Biochim. Biophys. Acta, 1771, 455–461.
2. T. Wriessnegger, E. Leitner, M.R. Belegratis, et al. (2009) Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*, Biochim. Biophys. Acta, 1791, 166–172.
3. V.A. Ivashov, K. Grillitsch, H. Koefeler, et al. (2013) Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*, Biochim. Biophys. Acta, 1831, 282–290.
4. A. Lenassi Zupan, S. Trobec, V. Gaberc-Porekar, et al. (2004) High expression of green fluorescent protein in *Pichia pastoris* leads to formation of fluorescent particles, J. Biotechnol., 109, 115–122.
5. G. Yang, T. Liu, W. Peng, et al. (2006) Expression and localization of recombinant human EDG-1 receptors in *Pichia pastoris*, Biotechnol. Letters, 28, 1581–1586.
6. E. Zinser and G. Daum (1995) Isolation and biochemical characterization of organelles from the yeast *Saccharomyces cerevisiae*, Yeast, 11, 493–536.
7. S.E. Rieder and S.D. Emr (2001) Overview of subcellular fractionation procedures for the yeast *Saccharomyces cerevisiae*, Curr. Protoc. Cell Biol., Chapter 3, Unit 3.7.
8. G. Daum, P.C. Böhni, and G. Schatz (1982) Import of proteins into mitochondria. Cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria, J. Biol. Chem., 257, 13028–13033.
9. K.N. Faber, J.A. Heyman, and S. Subramani (1998) Two AAA family peroxins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes, Mol. Cell. Biol., 18, 936–943.
10. L. Klug, P. Tarazona, C. Gruber, et al. (2014) The lipidome and proteome of microsomes from the methylotrophic yeast *Pichia pastoris*, Biochim. Biophys. Acta, 1841, 215-226.

11. J. Sinha, B.A. Plantz, M. Inan, et al. (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-tau, Biotechnol. Bioeng., 89, 102–112.
12. E. Wiederhold, T. Gandhi, H.P. Permentier, et al. (2009) The yeast vacuolar membrane proteome, Mol. Cell. Prot., 8, 380–392.
13. B. Gaigg, R. Simbeni, C. Hrastnik, et al. (1995) Characterization of a microsomal subfraction associated with mitochondria of the yeast, *Saccharomyces cerevisiae*. Involvement in synthesis and import of phospholipids into mitochondria, Biochim. Biophys. Acta, 1234, 214–220.
14. A. Toulmay and W.A. Prinz (2011) Lipid transfer and signaling at organelle contact sites: the tip of the iceberg, Curr. Op. Cell Biol., 23, 458–463.
15. A.A. Rowland and G.K. Voeltz (2012) Endoplasmic reticulum-mitochondria contacts: function of the junction, Nat. Rev. Mol. Cell Biol., 13, 607–625.
16. T. Levine and C. Loewen (2006) Inter-organelle membrane contact sites: through a glass, darkly, Curr. Opin. Cell Biol., 18, 371–378.
17. D. Binns, T. Januszewski, Y. Chen, et al. (2006) An intimate collaboration between peroxisomes and lipid bodies, J. Cell Biol., 173, 719–731.

Figures

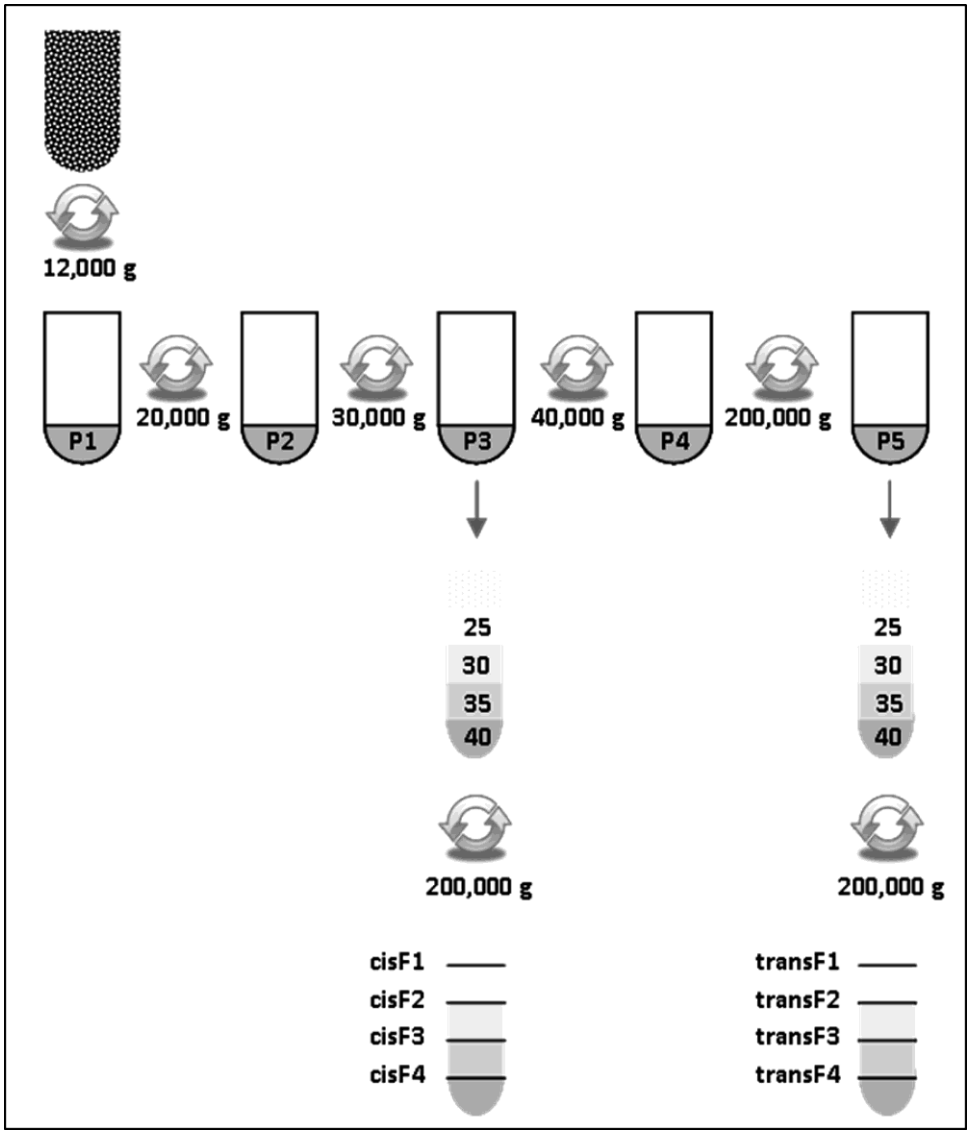

Figure 1. Chart flow of differential and density gradient ultracentrifugation steps for the isolation of Golgi fractions.

P1-5 pellet fractions are sedimented after centrifugation as indicated. Pellet fractions P3 and P5 are used for loading sucrose density gradients (40/35/30/25 % sucrose). Fractions cisF1-F4 and transF1-F4 are harvested from gradients and tested for the enrichment of Golgi marker proteins.

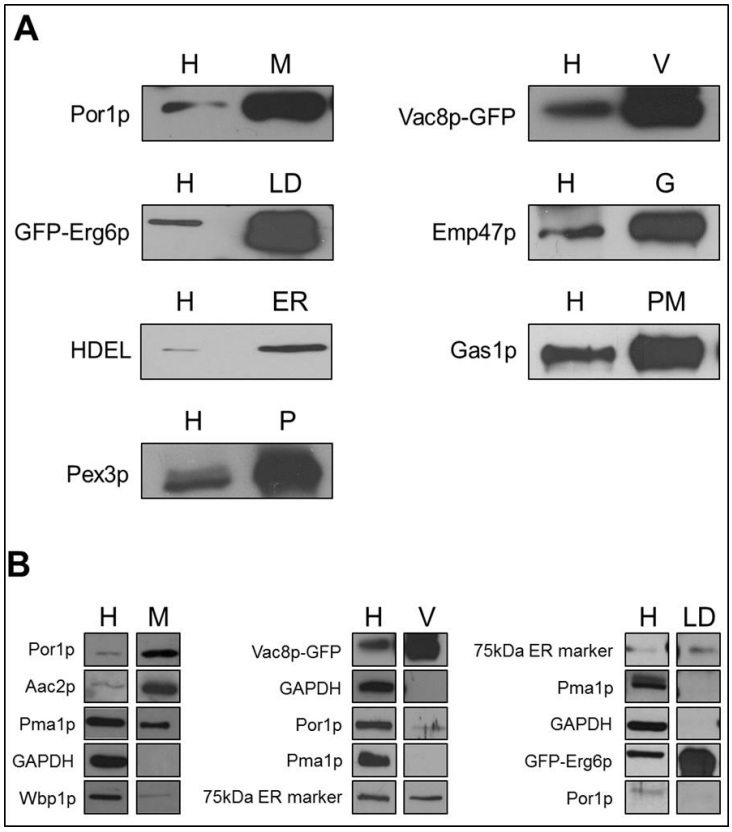

Fig.2 Enrichment and quality control of isolated organelles

A - Western Blot analysis showing the enrichment of organelle fractions over cell homogenate. Antibodies raised against organelle marker proteins were Por1p, Aac2p, Pex3p, Emp47p, and Gas1p. Commercially available GFP-reactive antibodies were applied for detecting fusion constructs of Erg6p and Vac8p, respectively. Commercially available HDEL-antibody recognizes the retention signal motif HDEL of the endoplasmic reticulum resident protein BiP. Ten µg of organelle protein and homogenate were applied. H- homogenate, M- mitochondria, LD- lipid droplets, ER- endoplasmic reticulum 40,000 x g microsomes, P- peroxisomes, V- vacuoles, G- trans-Golgi fraction, PM- plasma membrane.

B – Evaluation of cross-contamination by Western Blot analysis. Ten µg of organelle proteins and homogenate were applied. H- homogenate, M- mitochondria, V- vacuoles, LD- lipid droplets. Organelle marker proteins were mitochondrial Por1p and Aac2p; plasma membrane ATPase Pma1p; cytosolic GAPDH; endoplasmic reticulum 75 kDa marker protein and Wbp1p; vacuolar Vac8p-GFP fusion protein; and lipid droplet GFP-Erg6p fusion protein.

CHAPTER 4

Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*

Vasyl A. Ivashov,[a] Karlheinz Grillitsch,[b] Harald Koefeler,[c] Erich Leitner,[d] Dominic Baeumlisberger,[e] Michael Karas,[e] and Günther Daum[a,]*

[a] Institute of Biochemistry, Graz University of Technology, Austria; [b] Austrian Centre of Industrial Biotechnology, Graz, Austria; [c] Core Facility for Mass Spectrometry/Lipidomics, Center for Medical Research, Medical University of Graz, Austria; [d] Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology, Austria; [e] Cluster of Excellence "Macromolecular Complexes", Institute of Pharmaceutical Chemistry, Goethe University Frankfurt/Main, Germany;

* Corresponding author at: Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria. Tel.: + 43 316 873 6462; fax: + 43 316 873 6952. Email: guenther.daum@tugraz.at

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Abstract

Lipid droplets (LD) are the main depot of non-polar lipids in all eukaryotic cells. In the present study we describe isolation and characterization of LD from the industrial yeast *Pichia pastoris*. We designed and adapted an isolation procedure which allowed us to obtain this subcellular fraction at high purity as judged by quality control using appropriate marker proteins. Components of *P. pastoris* LD were characterized by conventional biochemical methods of lipid and protein analysis, but also by a lipidome and proteome approach. Our results show several distinct features of LD from *P. pastoris* especially in comparison to *Saccharomyces cerevisiae*. *P. pastoris* LD are characterized by their high preponderance of triacylglycerols over steryl esters in the core of the organelle, the high degree of fatty acid (poly)unsaturation and the high amount of ergosterol precursors. The high phosphatidylinositol to phosphatidylserine of ~ 7.5 ratio on the surface membrane of LD is noteworthy. Proteome analysis revealed equipment of the organelle with a small but typical set of proteins which includes enzymes of sterol biosynthesis, fatty acid activation, phosphatidic acid synthesis and non-polar lipid hydrolysis. These results are the basis for a better understanding of *P. pastoris* lipid metabolism and lipid storage and may be helpful for manipulating cell biological and/or biotechnological processes in this yeast.

Abbreviations: TG, triacylglycerols; SE, steryl esters; LD, lipid droplets; PA, phosphatidic acid; LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimethyl-PE; CL, cardiolipin; DMCD, 4,14-dimethyl-cholesta-8,24-dienol; MS, mass spectrometry; GFP, green fluorescent protein; WT, wild type

Keywords: Lipid droplet, Triacylglycerol, Steryl ester, Lipidome, Proteome, *Pichia pastoris*

Highlights

► We isolated and characterized lipid droplets from *P. pastoris* for the first time. ► Lipidome and proteome analysis of *P. pastoris* lipid droplets were performed. ► Lipid droplets from *P. pastoris* are different from *S. cerevisiae* lipid droplets. ► *P. pastoris* lipid droplets contain much triacylglycerols but little steryl esters. ► A large number of *P. pastoris* lipid droplet proteins are involved in lipid metabolism.

1. Introduction

The methylotrophic yeast *Pichia pastoris* is widely used for heterologous protein expression [1–4]. Despite its extensive commercial use the cell biological characterization of this yeast is lacking behind. For this reason our laboratory initiated a systematic approach to investigate *P. pastoris* organelles with the emphasis on the characterization of biomembranes and lipids [5,6]. Despite the progress which we made a number of subcellular compartments remained uncharacterized so far. As an example, isolation and characterization of lipid droplets (LD) from *P. pastoris* have not yet been reported. LD, also named lipid particles or oil bodies are specific subcellular compartments which gained much interest recently regarding their emerging role in health and disease [7]. They mainly function as depots of excess lipids (sterols and fatty acids) in the biological inert form of triacylglycerols (TG) and steryl esters (SE), but also contribute to non-polar lipid synthesis and mobilization [8]. LD are supposed to originate from the ER by a budding process, although steps and mechanism(s) leading to the biogenesis of this organelle are still a matter of dispute. Alternative models for LD formation have also been proposed (for reviews, see Refs. [9–14]). Recent studies in *Saccharomyces cerevisiae* advocated structural and functional connection between ER and LD and proposed the possibility of protein exchange between these two compartments [15].

The general structure of LD is similar in all eukaryotic cells (for reviews, see [8,9]). Yeast LD consist of a hydrophobic core formed by TG and SE encompassed by a phospholipid monolayer with a small number of proteins embedded [16,17]. Most recently, more than 90 proteins were allocated to LD from *S. cerevisiae* [18]. Many of these polypeptides participate in lipid metabolism, such as phosphatidate and sterol synthesis [19,20], fatty acid activation [21–23], and TG and SE synthesis/lipolysis [24–30]. Besides lipid metabolic functions several other functions unrelated to lipid metabolism were assigned to LD, such as storage and sequestration of protein aggregates and incorrectly folded proteins [9,31].

TG and SE comprise the highly hydrophobic core of LD. In *S. cerevisiae*, these two major non-polar lipids are synthesized by four enzymes [32]. TG are formed via acylation of diacylglycerols (DAG) with the fatty acid moiety derived from different sources. The diacylglycerol:phospholipid acyltransferase Lro1p possesses phospholipase A_2 (B) and acyltransferase activities and catalyzes TG formation in an acyl-CoA-independent manner utilizing phospholipids, especially phosphatidylethanolamine as acyl donor [33,34]. The

second TG synthesizing enzyme, Dga1p, esterifies DAG in an acyl-CoA-dependent way and requires activated fatty acids as co-substrates. Lro1p is found exclusively in the ER whereas Dga1p is dually localized to LD and ER [35]. Are1p and Are2p from *S. cerevisiae* are two homologous SE synthases [36,37]. They are mainly present in the ER and esterify sterols with fatty acids using acyl-CoA as fatty acid donor [20]. Are2p has the major acyl CoA:sterol acyltransferase activity in *S. cerevisiae* and predominantly forms esters of ergosterol, the final product of the sterol biosynthetic pathway in yeast. Are1p esterifies ergosterol precursors as well as ergosterol and has elevated activity under hypoxic conditions [36,38–40]. Noteworthy, only one acyl CoA:sterol acyltransferase, Are2p, has been annotated in the *P. pastoris* genome database [41].

In the present report we extend our knowledge about LD biochemistry and cell biology to *P. pastoris* and compare these data to the well-established model yeast *S. cerevisiae*. The strategy to characterize LD from *P. pastoris* cells included (i) isolation of highly pure organelles; (ii) conventional biochemical analysis of lipid components; and (iii) mass spectrometric (MS) analysis of lipids and proteins. Especially the lipidome and proteome studies allowed us to investigate LD from *P. pastoris* at the molecular level which may become highly relevant for biotechnological applications.

2. Experimental procedures

2.1. Strains and culture conditions

P. pastoris X33 (MATa, Mut^+, His^-) and *P. pastoris* X33_*GFP-ERG6* (MATa, Mut^+, His^+) strains were used throughout this study. Cells were grown under aerobic conditions to the early stationary phase (26 h) at 30 °C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck). Media were inoculated to a starting OD_{600} of 0.1 from precultures grown aerobically for 48 h in YPD medium at 30 °C.

2.2. Construction of *GFP-PpErg6p* expression vector

The primer pair (GFP-fwd CGCGGATCCGCGTTTTTGTAGAAATGTCTTG GTGTCCTCGTCCAATCAGGTAGCCATCTCTG and GFP-rev ATAGTTTAGCGGCC GCCTCGAGCCCGGGATTTAAATACTTGTACAATTCATCCATGCCATGTGTAATCC CAGCAGCAGT) was used for amplifying a GAP promoter fused to a Cy3-GFP open reading frame lacking the stop codon. The PCR product was inserted into BamHI and NotI restriction sites at the multiple cloning site of the pPIC3.5 plasmid. Primer GFP-rev also contained additional recognition sites enabling N-terminal fusion of GFP to genes of interest. The *PpERG6* open reading frame was amplified from genomic DNA using the forward primer ERG6-fwd (5′-GCGCGATTTAAATATGACTACCT CTACAACTGAACAAG-3′) which contained a SmiI site and the reverse primer ERG6-rev (5′-CAATGCGGCCGCTTATTTGGCATCCAATGGTTTTC-3′) with a NotI site. *PpERG6* was inserted within the corresponding sites of the aforementioned vector behind the GFP gene. The quality of the final construct was confirmed by sequencing.

2.3. Yeast cell transformation

For transformation experiments, the expression vector described above was linearized by cutting within the 5′ AOX1 promoter fragment with SacI restriction endonuclease (Fermentas). The DNA was introduced into *P. pastoris* competent cells by electroporation as described by Lin-Cereghino et al. [42] with the aid of a MicroPulser™ Electroporator (Bio-Rad). Transformed cells were transferred to plates containing 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.4 mg/l biotin and 2% agar and incubated at 30 °C for 2–3 days until colonies appeared. Transformants were checked by PCR for the presence of the GFP-*PpERG6* open reading frame. Successful expression of the GFP-PpErg6p fusion product was confirmed by fluorescence microscopy and Western blot analysis (see below).

2.4. Fluorescent microscopy

P. pastoris cells were grown on YPD medium to the early stationary phase (26 h). Cells from 1 ml culture were harvested by centrifugation, washed once with deionized water, stained for

10 min with 10 µg/ml Nile Red (Sigma) and analyzed using a Zeiss Axiovert 35 microscope with a 100-fold oil immersion objective. Detection ranges of 450–490 nm for Nile Red and 510–520 nm for GFP were used. Images were taken with a CCD camera.

2.5. Subcellular fractionation

Lipid droplets (LD) from *P. pastoris* were obtained at high purity from cells grown to the early stationary phase as described previously [16,43] with minor modifications. Briefly, cells were grown aerobically on YPD to the early stationary phase (26 h), harvested by centrifugation and washed with deionized water. Cells were converted to spheroplasts using Zymolyase 20T (Seikagaku Corporation, Japan). Spheroplasts (~ 90 g) were suspended in buffer A (10 mM MES/Tris, pH 6.9, 12% Ficoll 400, 0.2 mM EDTA, 1 mM PMSF) and disintegrated using a Dounce Homogenizer (30 strokes) on ice. After centrifugation at 6,000*g* for 5 min the supernatant was removed and the pellet was homogenized and centrifuged again as described above. Combined supernatants (homogenate) in buffer A were centrifuged at 12,000*g* for 15 min. The resulting supernatant was put into an Ultra-Clear Centrifuge Tube (Beckman), overlaid with buffer A and centrifuged at 141,000*g* for 45 min using a swing out rotor AH-629 (Sorvall). The resulting floating layer was collected, overlaid with buffer B (10 mM MES/Tris, pH 6.9, 8% Ficoll 400, 0.2 mM EDTA, 1 mM PMSF) and ultracentrifuged at 141,000*g* for 30 min. The floating layer was collected again, homogenized, transferred to the bottom of the ultracentrifuge tube filled with buffer C (0.25 M sorbitol, 10 mM MES/Tris, pH 6.9, 0.2 mM EDTA) and centrifuged at 141,000*g* for 90 min. The resulting top layer represents highly pure lipid droplets.

Isolation of other subcellular fractions used in this study was described before [6,7,43]. For the isolation of mitochondria, microsomes and cytosol, spheroplasts (~ 25 g) were suspended in 10 mM Tris–HCl, pH 7.4, containing 0.6 M mannitol, homogenized with a Dounce Homogenizer (15 strokes) and centrifuged at 4,000*g* for 5 min. The supernatant was collected and the pellet was homogenized and centrifuged again. Combined supernatants were centrifuged at 12,000*g* for 15 min. The supernatant was used for microsomal and cytosol preparations (see below). The pellet was re-suspended in the same buffer and centrifuged at 4,000*g* for 5 min. The resulting supernatant was centrifuged at 12,000*g* for 10 min. The final pellet represents the mitochondria fraction. Combined supernatants from the previous steps

were centrifuged at 20,000*g*, 30,000*g* and 40,000*g* for 30 min each. Pellet fractions of 30,000*g* and 40,000*g* centrifugation steps correspond to 30,000 g and 40,000 g microsomes. The final supernatant is cytosol including 100,000 g microsomes. The quality of subcellular fractions was routinely tested by Western blot analysis (see below).

2.6. Protein analysis

Proteins were quantified by the method of Lowry et al. [44] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification with the exception of LP proteins which were precipitated and de-lipidated according to the method of Wessel and Flügge [45].

SDS–PAGE was performed by the method of Laemmli [46] using 12.5% SDS gels. Samples were denaturated at 37 °C to avoid aggregation of membrane proteins. Proteins were visualized by staining with Coomassie Blue. Western blot analysis was performed according to Haid and Suissa [47]. Primary rabbit antibodies used in this study were directed against Por1p, Pma1p, Erg6p and GAPDH from *S. cerevisiae*, and against the 75-kDa microsomal marker protein (75-ER marker) from *P. pastoris*. The 75 kDa microsomal protein appears as a typical band of microsomal fractions on SDS–polyacrylamide gels. The function of this protein is unknown. For immunization the respective band was excised from a preparative SDS–polyacrylamide gel, and the protein was electro-eluted and injected into rabbits using standard procedures. Mouse antiserum against GFP was purchased from Roche. Peroxidase-conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce) were used to visualize immunoreactive bands.

2.7. Mass spectrometry of proteins

Protein (20 μg) from the LD fraction was precipitated following the protocol of Wessel and Fluegge [45]. Dried samples were dissolved in digestion buffer (25 mM triethylammonium bicarbonate). After addition of 0.375 μg porcine trypsin to each sample, the digestion was performed for 18 h at 37 °C with gentle agitation. The reaction was stopped by adding 1 μl

TFA (trifluoroacetic acid) to each sample. Prior to sample analysis volumes were decreased to 10 µl by vacuum centrifugation.

Peptide separation was performed on a Proxeon Biosystems EASY-nLC™ system coupled with a SunCollect MALDI Spotting device (SunChrom, Germany). The MALDI Spotting was done by mixing the LC-eluent with matrix solution containing 3 mg/ml alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) dissolved in 70% MeCN and 0.1% TFA. The final mixture was spotted every 20 s on a blank LC-MALDI insert metal target. All MS and MS/MS were acquired on a 4800 TOF/TOF™ Analyzer (ABSciex, Darmstadt, Germany). For protein and peptide identification an in-house Mascot database search engine v2.2.03 (Matrix Science Ltd.) and the Swissprot Protein Database were used.

2.8. Blast analysis of lipid droplet protein sequences

Amino acid sequences of *P. pastoris* LD proteins were obtained from Universal Protein Resource Knowledgebase—UniProtKB (http://www.uniprot.org/) and used for blast analysis. For the identification of orthologs from *S. cerevisiae*, the online blast tool of the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/cgi-bin/blast-sgd.pl) was applied. Candidate orthologs with functions similar to those annotated for previously detected LD proteins and homologies/similarities in amino acid sequences were selected.

2.9. Lipid extraction and analysis

Lipids from yeast cells were extracted as described by Folch et al. [48]. For quantification of non-polar lipids, extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by a two-step developing system. First, light petroleum/diethyl ether/acetic acid (25/25/1; per vol.) was used as mobile phase, and chromatograms were developed to half-distance of the plate. Then, plates were dried briefly and chromatograms were further developed to the top of the plate using light petroleum/diethyl ether (49/1; v/v) as the second mobile phase. To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2 \times 4\ H_2O$, 60 ml water, 60 ml methanol and 4 ml of concentrated sulfuric acid, briefly dried, and heated at

105 °C for 40 min. Visualized lipids together with ergosterol, triolein and cholesteryl ester as standards were quantified by densitometric scanning at 400 and 600 nm using a Shimadzu scanner CS-930.

For phospholipid analysis, lipids from homogenate and LD were loaded on silica gel 60 plates (Merck, Darmstadt, Germany), separated by two dimensional TLC (2D-TLC) using chloroform/methanol/25%NH_3 (65/35/5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5; per vol.) as second developing solvent system. Phospholipids were stained with iodine vapor, scraped off and quantified by the method of Broekhyuse [49]. The same method was applied for quantification of total phospholipids obtained as bands from one dimensional TLC using light petroleum/diethyl ether/acetic acid (25/25/1; per volume) as developing system.

Fatty acids were analyzed by gas–liquid chromatography (GLC). Lipid extracts prepared as described above were subjected to methanolysis using 2.5% H_2SO_4 in methanol and converted to methyl esters. Fatty acid methyl esters were separated using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-INNOWax capillary column (15 m × 0.25 mm inner diameter × 0.50 μm film thickness) and helium as carrier gas. Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN).

Individual sterols from total cell extracts (homogenates) or LD were identified and quantified by GLC/MS after alkaline hydrolysis of lipid extracts [50]. GLC/MS was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a mass selective detector (HP 5972), using an HP5-MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness). Aliquots of 1 μl were injected in the splitless mode at 270 °C injection temperature with helium as a carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100 °C, 10 °C/min to 250 °C, and 3 °C/min to 310 °C. Mass spectra were acquired in scan mode (scan range 200–550 atomic mass units) with 3.27 scans/s. Sterols were identified based on their mass fragmentation pattern.

2.10. Lipidome analysis by LC–MS

Lipids were extracted as described above and dissolved in chloroform/methanol (1/1; v/v) spiked with a set of 28 internal standards. Internal standardization and data acquisition by HPLC coupled to a FT-ICR-MS hybrid mass spectrometer (LTQ-FT, Thermo Scientific) was described previously in much detail by Fauland et al. [51]. Data processing takes into account exact mass and retention time and was performed by Lipid Data Analyzer according to Hartler et al. [52].

3. Results

3.1. Isolation of lipid droplets from *P. pastoris* and quality control

Lipid droplets (LD) are lipid storage compartments present in all types of eukaryotic cells including various yeast species. Here we describe isolation and characterization of LD from the industrial yeast *P. pastoris* to broaden our fundamental knowledge of the cell biology and the lipid metabolism of this important microorganism. For the isolation of LD from *P. pastoris* cells we adapted and optimized a protocol which had been established previously for *S. cerevisiae* [16]. The procedure is described in detail in Experimental procedures section.

For the quality control of isolated LD from *P. pastoris* cellular fractions were separated by SDS–polyacrylamide electrophoresis (Fig. 1A) and tested by Western blot analysis (Fig. 1B). As marker protein for *P. pastoris* LD we used Erg6p and Erg6-GFP. Localization of the Erg6-GFP-hybrid protein to LD was confirmed by fluorescence microscopy and co-staining with Nile Red (Fig. 1C). Western blot analysis (see Fig. 1B) revealed high enrichment of Erg6p and GFP-Erg6p in isolated LD fractions. Not surprisingly, the overexpressed GFP-Erg6 hybrid was also found in microsomal fractions at lower concentration as had been shown previously for Erg6p in *S. cerevisiae* [24]. Cross-contamination of *P. pastoris* LD with other subcellular fractions was only marginal. Hence, the isolation protocol was efficient and could be used as the basis for biochemical characterization of *P. pastoris* LD.

3.2. Non-polar storage lipids of lipid droplets from *P. pastoris*

Non-polar lipids are the major components of LD. In yeast, non-polar lipids accumulate at substantial amounts in the stationary growth phase. For this reason, *P. pastoris* cells grown on glucose were harvested at the early stationary phase (26 h) and LD were analyzed. TG (~ 55–65 mg/mg LD protein) comprised more than 90% of all non-polar lipids from LD. The amount of SE (~ 3–5 mg/mg LD protein) was low resulting in an approximate TG to SE ratio of ~ 15. Only traces of free sterols were detected in LD. This finding is in sharp contrast to LD from *S. cerevisiae* where SE and TG are present at equal amounts [16]. However, the high TG to SE ratio in *P. pastoris* is reminiscent to LD from the yeast *Yarrowia lipolytica* [53] but also to mammalian adipocytes [54].

Whereas in *S. cerevisiae* the excess of sterols formed is converted to substantial amounts of SE stored in LD [36,37] the obviously small amount of sterols formed in *P. pastoris* appears to be the reason for moderate occurrence of SE. However, accumulation of ergosterol precursors in LD from *P. pastoris* is similar to *S. cerevisiae*. As can be seen from Fig. 2, the sterol composition of *P. pastoris* LD mainly derived from the esterified form as SE is completely different from total cell extracts. In homogenates, the final product of the sterol biosynthetic pathway, ergosterol, is strongly enriched and comprises up to 80% of total sterols. Only small amounts of ergosterol precursors were found in total cell extracts. In contrast, LD contain ergosterol only at 30% of total sterol, but sterol intermediates are present at large quantities. In LD, the concentration of zymosterol is comparable to ergosterol (~ 26%), and also episterol, 4-methylzymosterol, fecosterol, episterol, lanosterol and 4,14-dimethylcholesta-8,24-dienol are strongly enriched over the homogenate.

3.3. Phospholipid analysis

A phospholipid monolayer forms the surface membrane of LD and shields the highly hydrophobic particle from the aqueous environment. This surface membrane is assumed to be very important for the maintenance and the structure of LD [55]. Fig. 3 shows a comparison of phospholipid compositions from *P. pastoris* cell homogenate and LD. First, the ratio of phospholipids to proteins was found to be very high in LD which is, however, mainly result of the low abundance of proteins in LD (Fig. 3A). The ratio of TG to total phospholipids was

~ 60 indicating the low abundance of the latter lipid class in LD and at the same time confirming the high purity of isolated LD fractions. The pattern of major phospholipids in LD roughly reflected total cell extracts, although certain differences are noteworthy (Fig. 3B). Whereas phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major phospholipids of both LD and homogenate, the ratio of phosphatidylinositol (PI) to phosphatidylserine (PS) differs strongly. This PI to PS ratio is ~ 1 in the homogenate and ~ 7.5 in LD. Other phospholipid species such as cardiolipin (CL), phosphatidic acid (PA), lysophosphatidic acid (LPA) and dimethyl-phosphatidylethanolamine (DMPE) were only present at trace amounts in LD.

Besides conventional phospholipid analysis we extended our studies to mass spectrometric analysis of PC, PE, PI and PS (Fig. 4). This analysis revealed that there were only minor differences in the phospholipid species patterns of homogenate and LD. The vast majority of all phospholipid species in both samples comprised C36 and C34 species. The fact that the entire phospholipid species profile of LD follows the homogenate supports the notion postulated in a study with *S. cerevisiae* [18] that there is no species selective transfer of phospholipids to LD. This result also suggests that proteins of the LD surface membrane do not require a specific phospholipid species milieu. However, the difference in the PI/PS ratio as mentioned above has to be kept in mind.

When examining individual phospholipids of *P. pastoris* homogenate and LD fractions we observed peculiar properties of the different phospholipid classes. PC is the phospholipid with the largest variety of species in both homogenates and LD. Noteworthy, PC occurs in the form of C36:2 and several polyunsaturated species such as C36:3, C36:4 and C36:5. The species pattern of PE is markedly different from PC. The most prominent PE species are C36:2, C34:1 and C34:2. Interestingly, in both homogenate and LD PI is presented mostly as C34:1 and C34:2 species. The simplest species pattern was observed with PS being restricted to C34:1 and C34:2. Similar results were obtained with *S. cerevisiae* LD where C34:1 was the major PI species and PS was mostly present in its C34:1 and C34:2 form [18].

3.4. Fatty acids analysis of lipid droplets

The vast majority of fatty acids in LD are stored in the form of TG and SE and only a minor part is present in the surface phospholipid monolayer. To address the specificity of the lipid storage machinery in *P. pastoris* we analyzed the fatty acid composition of LD. As can be seen from Fig. 5, oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and palmitic (C16.0) acids are dominant in both LD and homogenate. Other fatty acids are present only at minor amounts in both samples. This finding indicates that in *P. pastoris* the distribution of fatty acid species is not specific for LD and reflects the total cell fatty acid pattern. We can exclude from these data that *P. pastoris* enzymes synthesizing non-polar lipids have strong fatty acid substrate specificity. This result is in contrast to the two TG synthases from *S. cerevisiae*, Dga1p and Lro1p, which have a clear preference for unsaturated fatty acids, especially C16:1 and C18:1 [18,56]. With that respect, *P. pastoris* LD are different from other yeasts such as *S. cerevisiae* [18] and *Y. lipolytica* [53], but similar to plant oil bodies which also contain marked amounts of polyunsaturated fatty acids [57].

3.5. *P. pastoris* lipid droplet proteome

To complete the characterization of *P. pastoris* LD we performed a proteome analysis of this compartment. For this investigation we utilized direct MS as outlined in detail in Experimental procedures section. Proteins were identified through annotation in the Universal Protein Resource Knowledgebase (http://www.uniprot.org/) and assigned to putative functions according to blast and motif searches. Additionally, we identified *S. cerevisiae* orthologs with their cellular localization and biological functions. Data are summarized in Table 1.

The function of most *P. pastoris* LD proteins identified here is hypothetical and derived from bioinformatic analysis (http://www.uniprot.org/20121203). However, detected proteins can be divided in two groups depending on association with lipid metabolism or being involved in other cellular processes. The first group includes the most abundant proteins of the *P. pastoris* LD proteome such as enzymes involved in ergosterol, phospholipid and sphingolipid biosynthesis as well as fatty acid metabolism, fatty acid transport and activation, and lipolysis. These findings are in line with proteomic studies of *S. cerevisiae* LD performed

recently in our laboratory [18]. The second group of *P. pastoris* LD proteins identified here is not associated with lipid metabolism, but with ribosomal translation, energy metabolism and mitochondria function. Although our LD preparations from *P. pastoris* were shown to be of high quality, contamination with other subcellular fractions cannot be completely excluded. However, recent studies showed structural and functional interactions between LD and other organelles [58–62] which are of physiological relevance. The possible involvement of proteins found in this study in such processes will be subject to individual investigations.

4. Discussion

During the investigation presented here we established a reliable technique for the isolation of highly pure LD from the industrial yeast *P. pastoris* which enabled us to analyze lipidome and proteome of this organelle. Our results confirm in general our current knowledge of yeast LD biochemistry, although several peculiarities in lipid and protein composition of LD from *P. pastoris* were observed. A specific and remarkable feature of *P. pastoris* LD is the high prevalence for TG over SE in its hydrophobic core. This result is in strong contrast to *S. cerevisiae* LD which contain equivalent amounts of TG and SE [16]. The other difference between *P. pastoris* and *S. cerevisiae* LD is the unselective occurrence of poly/unsaturated fatty acids in the former microorganism. This finding is in line with our studies of other *P. pastoris* organelles, e.g. with peroxisomes and mitochondria [5,6], which did not show any specific fatty acid targeting either. Thus, the high degree of fatty acid unsaturation appears to apply to most *P. pastoris* organelles.

Proteome analysis of *P. pastoris* LD revealed a distinct set of LD associated proteins. Based on homologies to *S. cerevisiae* a number of these proteins were identified. However, functions of some of these newly identified proteins remained unassigned. Interestingly, the total number of *P. pastoris* LD proteins found in this study was rather low compared to investigations with *S. cerevisiae* [18], mammalian cells [59,63,64] and plant cells [65–67]. We can speculate that *P. pastoris* LD require only a minimal set of proteins to maintain structure and function of this organelle, and that functions of LD from this yeast are not diverse from other organisms [68–70]. Our results also demonstrate that LD from *P. pastoris* contribute to lipid metabolism (see Table 1) as has been shown before for LD from other cell

types. Individual features or functions of LD from different species may be explained by differences in the proteome. From this view point "unusual" or unidentified proteins in the LP proteome from *P. pastoris* may become important. The functional relevance of such proteins will have to be tested rather on an individual than on a global basis. Proteome analysis as presented here may set the stage for such studies.

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References

1. Cregg J.M., Cereghino J.L., Shi J., Higgins D.R. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 2000;16:23–52.

2. Daly R., Hearn M.T. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. 2005;18:119–138.

3. Macauley-Patrick S., Fazenda M.L., McNeil B., Harvey L.M. Heterologous protein production using the *Pichia pastoris* expression system. Yeast. 2005;22:249–270.

4. Li P., Anumanthan A., Gao X.G., Ilangovan K., Suzara V.V., Duzgunes N., Renugopalakrishnan V. Expression of recombinant proteins in *Pichia pastoris*. Appl. Biochem. Biotechnol. 2007;142:105–124.

5. Wriessnegger T., Gübitz G., Leitner E., Ingolic E., Cregg J., de la Cruz B.J., Daum G. Lipid composition of peroxisomes from the yeast *Pichia pastoris* grown on different carbon sources. Biochim. Biophys. Acta. 2007;1771:455–461.

6. Wriessnegger T., Leitner E., Belegratis M.R., Ingolic E., Daum G. Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*. Biochim. Biophys. Acta. 2009;1791:166–172.

7. Greenberg A.S., Coleman R.A., Kraemer F.B., McManaman J.L., Obin M.S., Puri V., Yan Q.W., Miyoshi H., Mashek D.G. The role of lipid droplets in metabolic disease in rodents and humans. J. Clin. Invest. 2011;121:2102–2110.

8. Olofsson S.O., Bostrom P., Andersson L., Rutberg M., Perman J., Boren J. Lipid droplets as dynamic organelles connecting storage and efflux of lipids, Biochim. Biophys. Acta. 2009;1791:448–458.

9. Cheng J., Suzuki M., Shinohara Y. Lipid droplets: a classic organelle with new outfit. Histochem. Cell Biol. 2008;130:263–279.

10. Kalantari F., Bergeron J.J., Nilsson T. Biogenesis of lipid droplets—how cells get fatter. Mol. Membr. Biol. 2010;27:462–468.

11. Murphy D.J., Vance J. Mechanisms of lipid-body formation. Trends Biochem. Sci. 1999;24:109–115.

12. Ploegh H.L. A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. Nature. 2007;448:435–438.

13. Robenek H., Buers I., Hofnagel O., Robenek M.J., Troyer D., Severs N.J. Compartmentalization of proteins in lipid droplet biogenesis. Biochim. Biophys. Acta. 2009;1791:408–418.

14. Waltermann M., Hinz A., Robenek H., Troyer D., Reichelt R., Malkus U., Galla H.J., Kalscheuer R., Stoveken T., von Landenberg P., Steinbuchel A. Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. Mol. Microbiol. 2005;55:750–763.

15. Jacquier N., Choudhary V., Mari M., Toulmay A., Reggiori F., Schneiter R. Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. J. Cell Sci. 2011;124:2424–2437.

16. Leber R., Zinser E., Zellnig G., Paltauf F., Daum G. Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae*. Yeast. 1994;10:1421–1428.

17. Athenstaedt K., Zweytick D., Jandrositz A., Kohlwein S.D., Daum G. Identification and characterization of major lipid particle proteins of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 1999;181:6441–6448.

18. Grillitsch K., Connerth M., Köfeler H., Arrey T.N., Rietschel B., Wagner B., Karas M., Daum G. Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta. 2011;1811:1165–1176.

19. Athenstaedt K., Daum G. Phosphatidic acid, a key intermediate in lipid metabolism. Eur. J. Biochem. 1999;266:1–16.

20. Zinser E., Paltauf F., Daum G. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. J. Bacteriol. 1993;175:2853–2858.

21. Johnson D.R., Knoll L.J., Levin D.E., Gordon J.I. *Saccharomyces cerevisiae* contains four fatty acid activation (FAA) genes: an assessment of their role in regulating protein N-myristoylation and cellular lipid metabolism. J. Cell Biol. 1994;127:751–762.

22. Duronio R.J., Knoll L.J., Gordon J.I. Isolation of a *Saccharomyces cerevisiae* long chain fatty acyl:CoA synthetase gene (FAA1) and assessment of its role in protein N-myristoylation. J. Cell Biol. 1992;117:515–529.

23. Watkins P.A., Lu J.F., Steinberg S.J., Gould S.J., Smith K.D., Braiterman L.T. Disruption of the *Saccharomyces cerevisiae FAT1* gene decreases very long-chain fatty acyl-CoA synthetase activity and elevates intracellular very long-chain fatty acid concentrations. J. Biol. Chem. 1998;273:18210–18219.

24. Sorger D., Athenstaedt K., Hrastnik C., Daum G. A yeast strain lacking lipid particles bears a defect in ergosterol formation. J. Biol. Chem. 2004;279:31190–31196.

25. Oelkers P., Cromley D., Padamsee M., Billheimer J.T., Sturley S.L. The *DGA1* gene determines a second triglyceride synthetic pathway in yeast. J. Biol. Chem. 2002;277:8877–8881.

26. Athenstaedt K., Daum G. YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of *Saccharomyces cerevisiae*. J. Biol. Chem. 2003;278:23317–23323. 27. Athenstaedt K., Daum G. Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles. J. Biol. Chem. 2005;280:37301–37309.

28. Mullner H., Deutsch G., Leitner E., Ingolic E., Daum G. *YEH2/YLR020c* encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 2005;280:13321–13328.

29. Koffel R., Tiwari R., Falquet L., Schneiter R. The *Saccharomyces cerevisiae YLL012*/*YEH1*, *YLR020*/*YEH2*, and *TGL1* genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. Mol. Cell. Biol. 2005;25:1655–1668.

30. Jandrositz A., Petschnigg J., Zimmermann R., Natter K., Scholze H., Hermetter A., Kohlwein S.D., Leber R. The lipid droplet enzyme Tgl1p hydrolyzes both steryl esters and triglycerides in the yeast, *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. 2005;1735:50–58.

31. Fei W., Wang H., Fu X., Bielby C., Yang H. Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in *Saccharomyces cerevisiae*. Biochem. J. 2009;424:61–67.

32. Czabany T., Athenstaedt K., Daum G. Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta. 2007;1771:299–309.

33. Oelkers P., Tinkelenberg A., Erdeniz N., Cromley D., Billheimer J.T., Sturley S.L. A Lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. J. Biol. Chem. 2000;275:15609–15612.

34. Dahlqvist A., Stahl U., Lenman M., Banas A., Lee M., Sandager L., Ronne H., Stymne S. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. U. S. A. 2000;97:6487–6492.

35. Sorger D., Daum G. Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 2002;184:519–524.

36. Yang H., Bard M., Bruner D.A., Gleeson A., Deckelbaum R.J., Aljinovic G., Pohl T.M., Rothstein R., Sturley S.L. Sterol esterification in yeast: a two-gene process. Science. 1996;272:1353–1356.

37. Yu C., Kennedy N.J., Chang C.Y., Rothblatt J.A. Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. J. Biol. Chem. 1996;271:24157–24163.

38. Zweytick D., Leitner E., Kohlwein S.D., Yu C., Rothblatt J., Daum G. Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 2000;267:1075–1082.

39. Jensen-Pergakes K., Guo Z., Giattina M., Sturley S.L., Bard M. Transcriptional regulation of the two sterol esterification genes in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 2001;183:4950–4957.

40. Valachovic M., Klobucnikova V., Griac P., Hapala I. Heme-regulated expression of two yeast acyl-CoA:sterol acyltransferases is involved in the specific response of sterol esterification to anaerobiosis. FEMS Microbiol. Lett. 2002;206:121–125.

41. Kuberl A., Schneider J., Thallinger G.G., Anderl I., Wibberg D., Hajek T., Jaenicke S., Brinkrolf K., Goesmann A., Szczepanowski R., Puhler A., Schwab H., Glieder A., Pichler H. High-quality genome sequence of *Pichia pastoris* CBS7435. J. Biotechnol. 2011;154:312–320.

42. Lin-Cereghino J., Wong W.W., Xiong S., Giang W., Luong L.T., Vu J., Johnson S.D., Lin-Cereghino G.P. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. Biotechniques. 2005;38:44–48.

43. Zinser E., Daum G. Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. Yeast. 1995;11:493–536.

44. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951;193:265–275.

45. Wessel D., Flügge U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 1984;138:141–143.

46. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–685.

47. Haid A., Suissa M. Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Methods Enzymol. 1983;96:192–205.

48. Folch J., Lees M., Sloane Stanley G.H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 1957;226:497–509.

49. Broekhuyse R.M. Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids. Biochim. Biophys. Acta. 1968;152:307–315.

50. Quail M.A., Kelly S.L. The extraction and analysis of sterols from yeast. Methods Mol. Biol. 1996;53:123–131.

51. Fauland A., Koefeler H., Troetzmueller M., Knopf A., Hartler J., Eberl A., Chitraju C., Lankmayr E., Spener F. A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry. J. Lipid Res. 2011;52:2314–2322.

52. Hartler J., Trötzmüller M., Chitraju C., Spener F., Köfeler H.C., Thallinger G.G. Lipid Data Analyzer: unattended identification and quantitation of lipids in LC–MS data. Bioinformatics. 2011;27:572–577.

53. Athenstaedt K., Jolivet P., Boulard C., Zivy M., Negroni L., Nicaud J.M., Chardot T. Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. Proteomics. 2006;6:1450–1459.

54. Suzuki M., Shinohara Y., Ohsaki Y., Fujimoto T. Lipid droplets: size matters. J. Electron Microsc. (Tokyo) 2011;60:101–116.

55. Tauchi-Sato K., Ozeki S., Houjou T., Taguchi R., Fujimoto T. The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition. J. Biol. Chem. 2002;277:44507–44512.

56. Czabany T., Wagner A., Zweytick D., Lohner K., Leitner E., Ingolic E., Daum G. Structural and biochemical properties of lipid particles from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 2008;283:17065–17074.

57. Horn P.J., Ledbetter N.R., James C.N., Hoffman W.D., Case C.R., Verbeck G.F., Chapman K.D. Visualization of lipid droplet composition by direct organelle mass spectrometry. J. Biol. Chem. 2011;286:3298–3306.

58. Pu J., Ha C.W., Zhang S., Jung J.P., Huh W.K., Liu P. Interactomic study on interaction between lipid droplets and mitochondria. Protein Cell. 2011;2:487–496.

59. Zhang H., Wang Y., Li J., Yu J., Pu J., Li L., Zhang H., Zhang S., Peng G., Yang F., Liu P. Proteome of skeletal muscle lipid droplet reveals association with mitochondria and apolipoprotein A-I. J. Proteome Res. 2011;10:4757–4768.

60. Wolinski H., Kolb D., Hermann S., Koning R.I., Kohlwein S.D. A role for seipin in lipid droplet dynamics and inheritance in yeast. J. Cell Sci. 2011;124:3894–3904.

61. Binns D., Januszewski T., Chen Y., Hill J., Markin V.S., Zhao Y., Gilpin C., Chapman K.D., Anderson R.G., Goodman J.M. An intimate collaboration between peroxisomes and lipid bodies. J. Cell Biol. 2006;173:719–731.

62. Liu P., Bartz R., Zehmer J.K., Ying Y.S., Zhu M., Serrero G., Anders R.G. Rab-regulated interaction of early endosomes with lipid droplets. Biochim. Biophys. Acta. 2007;1773:784–793.

63. Larsson S., Resjö S., Gomez M.F., James P., Holm C. Characterization of the lipid droplet proteome of a clonal insulin-producing β-cell line (INS-1 832/13) J. Proteome Res. 2012;11:1264–1273.

64. Ding Y., Wu Y., Zeng R., Liao K. Proteomic profiling of lipid droplet-associated proteins in primary adipocytes of normal and obese mouse. Acta Biochim. Biophys. Sin. (Shanghai) 2012;44:394–406.

65. Jolivet P., Roux E., d'Andrea S., Davanture M., Negroni L., Zivy M., Chardot T. Protein composition of oil bodies in *Arabidopsis thaliana* ecotype WS. Plant Physiol. Biochem. 2004;42:501–509.

66. Katavic V., Agrawal G.K., Hajduch M., Harris S.L., Thelen J.J. Protein and lipid composition analysis of oil bodies from two *Brassica napus* cultivars. Proteomics. 2006;6:4586–4598.

67. Moellering E.R., Benning C. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryot. Cell. 2010;9:97–106.

68. Fujimoto T., Parton R.G. Not just fat: the structure and function of the lipid droplet. Cold Spring Harb. Perspect. Biol. 2011;3:a004838.

69. Chapman K.D., Dyer J.M., Mullen R.T. Biogenesis and functions of lipid droplets in plants: thematic review series: lipid droplet synthesis and metabolism: from yeast to man. J. Lipid Res. 2012;53:215–226.

70. Buers I., Hofnagel O., Ruebel A., Severs N.J., Robenek H. Lipid droplet associated proteins: an emerging role in atherogenesis. Histol. Histopathol. 2011;26:631–642.

Tables

Table 1. Proteome of *P. pastoris* lipid droplets.

LD were isolated from cells grown on glucose to stationary phase (26 h, 30 °C). Proteins were subjected to MS analysis (see Experimental procedures). Blast analysis of amino acid sequences revealed *S. cerevisiae* orthologs and the degree of homology. Localization and biological processes inferred from homology are shown. Databases used were Universal Protein Resource Knowledgebase—UniProtKB (http://www.uniprot.org/20121203) and Saccharomyces Genome Database—SGD (http://www.yeastgenome.org/20121203). C, cytosol; M, mitochondria; PM, plasma membrane; ER, endoplasmic reticulum; LD, lipid droplets; E, endosomes; G, Golgi, N, nucleus; R, ribosome.

UniProtKB ID	Submitted name (UniProtKB)	*S. cerevisiae* ortholog (homology %)	*S. cerevisiae* ortholog localization (SGD)	Biological process inferred from homology (SGD)
C4R4C9	Delta(24)-sterol C-methyltransferase	Erg6p (67%)	ER/LD/M	Ergosterol biosynthetic process
C4R1R9	Long chain fatty acyl-CoA synthetase with a preference for C12:0-C16:0 fatty acids	Faa1p (59%)	LD/M/PM	Long-chain fatty acid transport, long-chain fatty-acyl-CoA metabolic process
C4R3L8	Squalene epoxidase, catalyzes the epoxidation of squalene to 2,3-oxidosqualene	Erg1p (53%)	ER/LD	Sterol metabolism
C4QXK6	3-ketosphinganine reductase, catalyzes the second step in phytosphingosine synthesis	Tsc10p (38%)	C/ER/M	3-Keto-sphinganine metabolic process, sphingolipid biosynthetic process
C4QX24	Acyl-coenzymeA:ethanol O-acyltransferase	Eht1p (37%)	LD/M	Medium-chain fatty acid biosynthetic/catabolic process

UniProtKB ID	Submitted name (UniProtKB)	*S. cerevisiae* ortholog (homology %)	*S. cerevisiae* ortholog localization (SGD)	Biological process inferred from homology (SGD)
C4R403	Steryl ester hydrolase	Tgl1p (42%)	LD	Cellular lipid metabolic process, sterol metabolic process
C4R0I8	Lanosterol synthase, an essential enzyme that catalyzes the cyclization of squalene 2,3-epoxide	Erg7p (60%)	ER/LD/PM	Ergosterol biosynthetic process
C4R6T8	Putative acyltransferase with similarity to Eeb1p and Eht1p	Eeb1p (35%), Eht1p (35%)	LD/M	Medium-chain fatty acid biosynthetic/catabolic process
C4QVA2	Protein component of the small (40S) ribosomal subunit	Rps3p (82%)	R	Translation (RNA binding)
F2QTD3	Uncharacterized membrane protein YLR326W	Ylr326wp (28%)	Unknown	Unknown
C4QV50	Conserved ribosomal protein P0 similar to rat P0, human P0, and E. coli L10e	Rpp0p (71%)	R	Cytoplasmic translation, ribosomal large subunit assembly, translational elongation
C4R1Z2	Mitochondrial porin (Voltage-dependent anion channel), outer membrane protein	Por1p (47%)	M	Apoptotic process, cell redox homeostasis, transport, ion transport, mitochondrion organization
C4QVF8	Putative uncharacterized protein	Not found	Unknown	Unknown
C4QVE4	1-Acyl-sn-glycerol-3-phosphate acyltransferase	Slc1p (50%)	LD	Glycerophospholipid biosynthetic process
C4R196	3-Keto sterol reductase	Erg27p (63%)	ER/M	Ergosterol biosynthetic process
C4R2N5	ATP synthase subunit beta	Atp2p (87%)	M	ATP synthesis coupled proton transport
C4QZB0	Elongation factor 1-alpha	Tef1p (89%)	R/C	Translational elongation, tRNA export from nucleus
C4R4Y8	ATP synthase subunit alpha	Atp1p (88%)	M	ATP synthesis coupled proton transport

UniProtKB ID	Submitted name (UniProtKB)	*S. cerevisiae* ortholog (homology %)	*S. cerevisiae* ortholog localization (SGD)	Biological process inferred from homology (SGD)
C4QXD6	Fatty acid transporter and very long-chain fatty acyl-CoA synthetase	Fat1p (49%)	ER/LD/PM	Long-chain fatty acid transport, very long chain fatty acid metabolic process
C4QXC1	Putative fatty aldehyde dehydrogenase	Hfd1p (43%)	E/LD/M	Cellular aldehyde metabolic process
C4R2Z6	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase	Ayr1p (52%)	ER/LD/C/M	Phosphatidic acid biosynthetic process
C4QW07	Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tgl1p)	Tgl1p (39%)	LD	Cellular lipid metabolic process, sterol metabolic process
C4R760	Major ADP/ATP carrier of the mitochondrial inner membrane	Pet9p (85%)	M	Respiration
F2QNQ8	Transcriptional repressor OPI1	Opi1p (27%)	ER/N	Unfolded protein response, regulation of transcription, phospholipid biosynthetic process
C4QYN4	40S ribosomal protein subunit	Rps29ap (88%)	R	Cytoplasmic translation
C4QW21	Protein involved in ER-associated protein degradation	Ubx2p (25%)	ER/M	ER-associated protein catabolic process, proteasomal protein degradations, protein secretion
C4R7R7	Protein with similarity to oxidoreductases, found in lipid particles	Env9p (48%)	LD	Vacuolar protein processing, vacuole organization
C4QYK0	40S ribosomal protein S0 nearly identical to Rps0Bp	Rps0ap (84%)	R	Structural constituent of ribosome
C4R7L9	Putative uncharacterized protein	Tom5p (39%)	M	Protein targeting to mitochondrion
C4QVS9	Plasma membrane H^+-ATPase, pumps protons out of the cell	Pma1p (86%)	PM	Proton transport, regulation of pH

UniProtKB ID	Submitted name (UniProtKB)	*S. cerevisiae* ortholog (homology %)	*S. cerevisiae* ortholog localization (SGD)	Biological process inferred from homology (SGD)
F2QT41	Alcohol dehydrogenase class-3	Yim1p (30%)	LD/C/M	Response to DNA damage stimulus
F2QYU4	AN1-type zinc finger protein YNL155W	Ynl155wp (30%)	C/N	Unknown
C4R4C3	Mitochondrial matrix ATPase	Ssc1p (82%)	M	Protein import into mitochondrial matrix, protein folding
C4QXL9	GTPase, similar to Ypt51p and Ypt53p and to mammalian rab5	Vps21p (54%)	E	Endocytosis, protein targeting to vacuole
C4QWQ5	Prenyltransferase, required for cell viability	Nus1p (47%)	LD/ER	Protein glycosylation
C4R3N7	Secretory vesicle-associated Rab GTPase essential for exocytosis	Ypt1p (49%)	ER/G/M	ER to Golgi vesicle-mediated transport

Figures

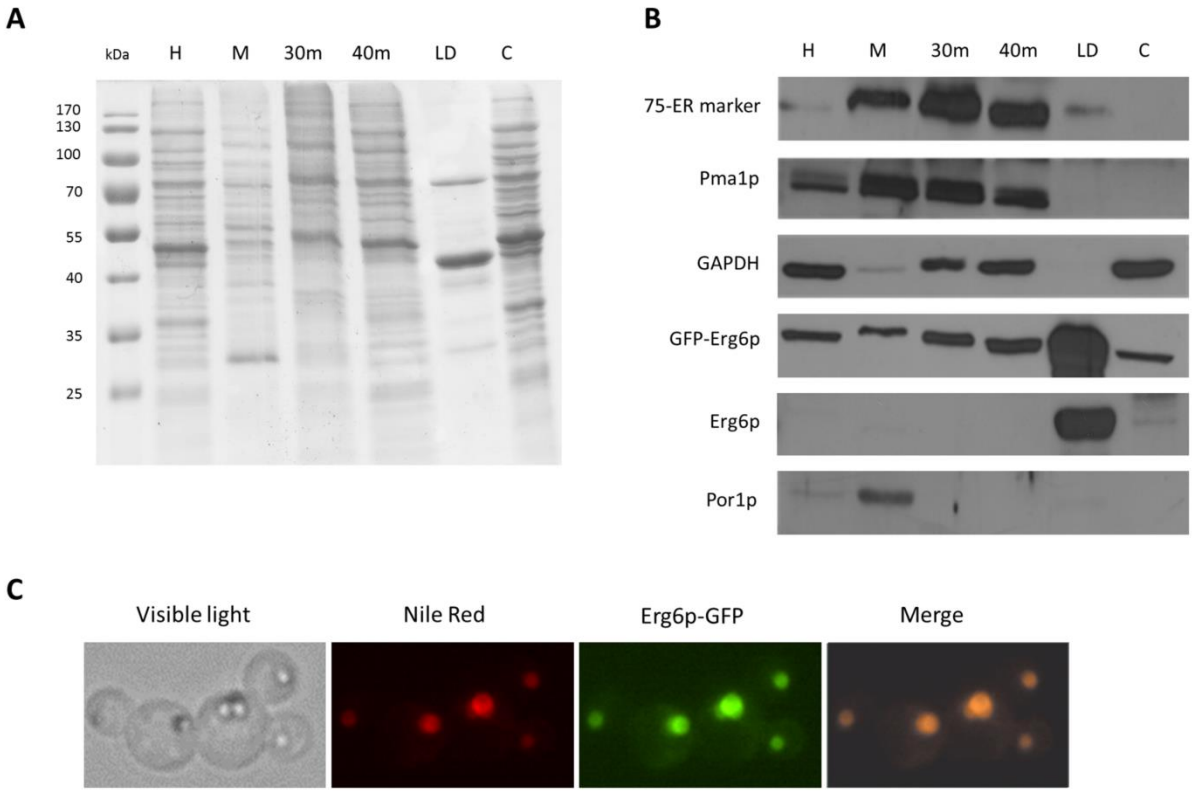

Fig.1 Quality control of isolated lipid droplets.

A–Protein pattern of lipid droplet fraction and control fractions (10 µg protein). Homogenate (H), lipid droplets (LD), 30,000 g microsomes (30 m), 40,000 g microsomes (40 m), cytosol (C) and mitochondria (M) fractions were loaded onto an SDS–polyacrylamide gel. B–Western blot analysis. Antisera against *Pichia pastoris* 75-ER marker protein (microsomal marker); Pma1p, plasma membrane H^+-ATPase (plasma membrane marker); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (cytosolic marker); GFP-Erg6p, green fluorescent protein fused to Erg6p (LD marker); Erg6p, Δ(24)-sterol C-methyltransferase (LD marker); and Por1p, mitochondrial porin (mitochondrial marker) were applied. C–Fluorescent imaging. Cells were grown on glucose at 30 °C for 26 h, stained with Nile Red and subjected to fluorescent microscopy. The Erg6p-GFP-signal coincides with Nile Red stained LD.

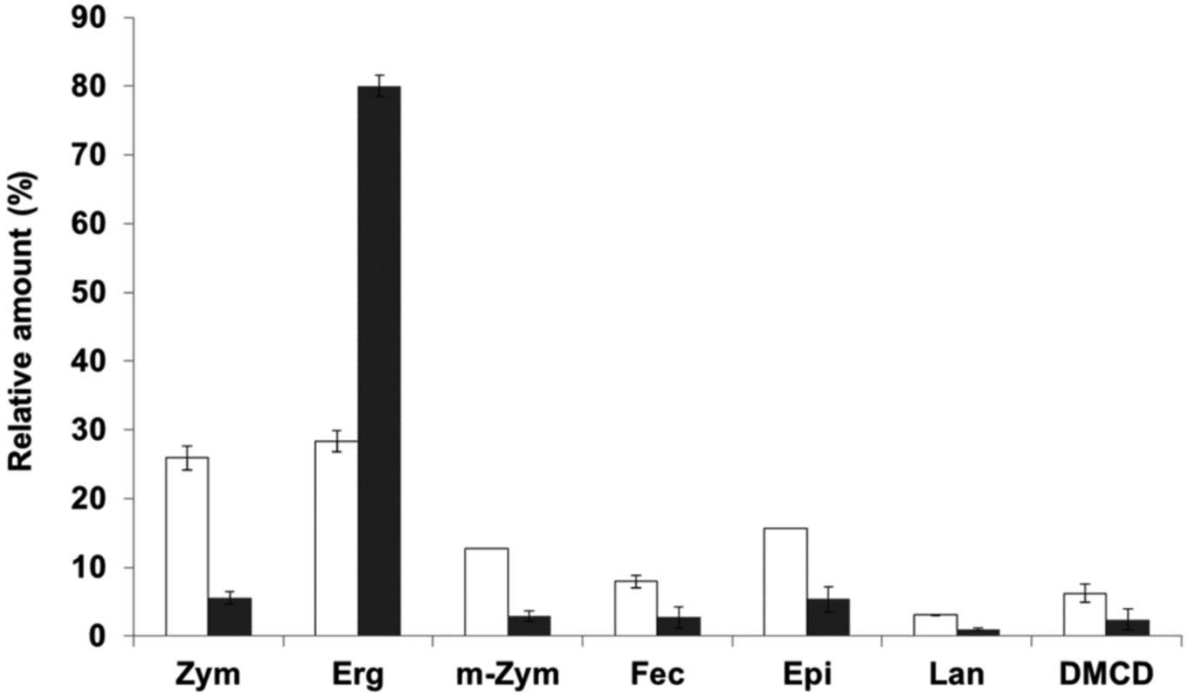

Fig.2 Sterol composition of lipid droplets.

Cells were grown on glucose at 30 °C to the stationary phase (26 h). Lipid extracts from LD (white bars) and homogenates (grey bars) were subjected to MS analysis of sterols. Amounts of individual sterols are shown as percentage of total sterols. Zym, Zymosterol; Erg, ergosterol; m-Zym, 4-methylzymosterol; Fec, fecosterol; Epi, episterol; Lan, lanosterol; DMCD, 4,14-dimethylcholesta-8,24-dienol. Data are mean values of three independent experiments. Error bars indicate standard deviation.

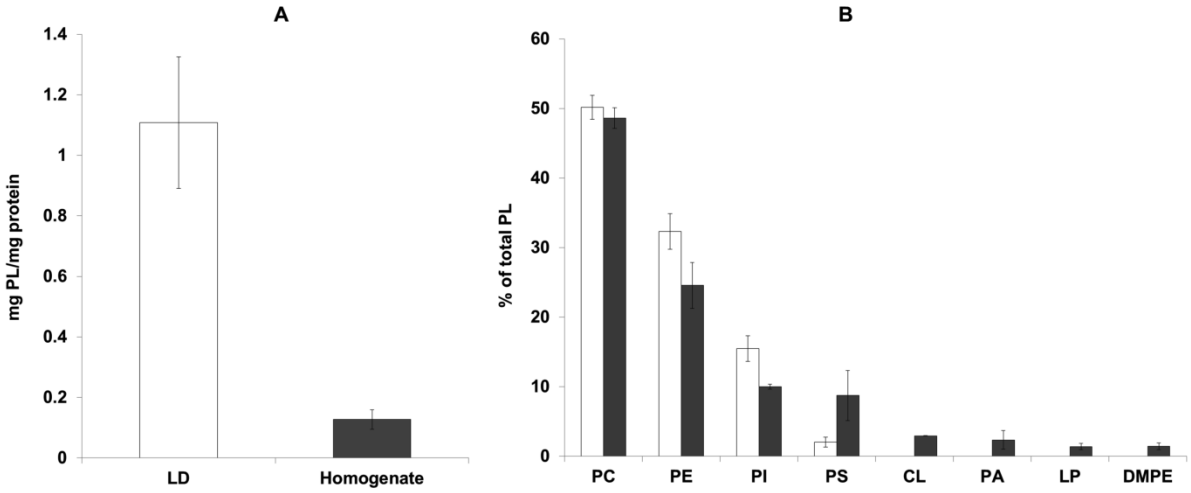

Fig.3 Phospholipid pattern of lipid droplets.

Cells were grown on glucose at 30 °C to stationary phase (26 h). Lipid extracts were analyzed for phospholipids as described in the experimental section. A–Total phospholipids in LD and homogenate. B–Relative distribution of individual phospholipids in LD (white bars) and homogenates (grey bars). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), phosphatidic acid (PA), lysophospholipids (LP), dimethylphosphatidylethanolamine (DMPE), are shown as percentage of total phospholipids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

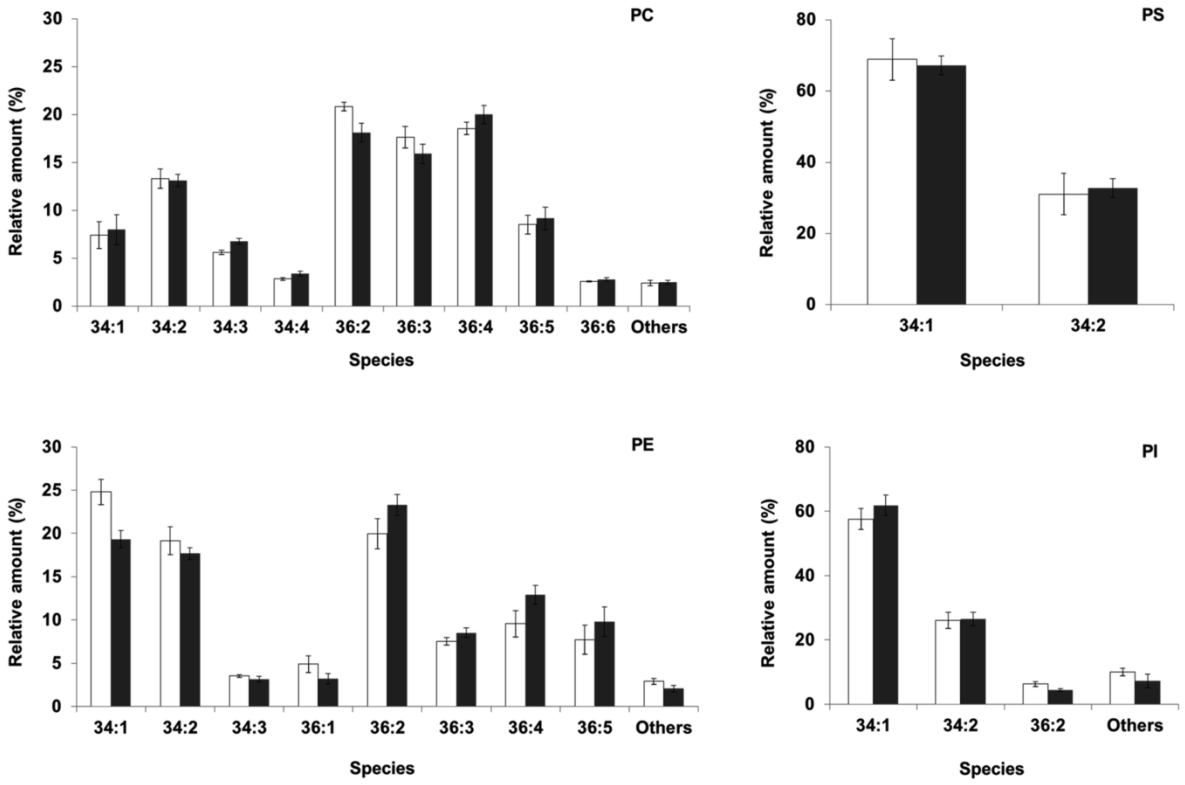

Fig.4 Phospholipid species composition.

Lipid extracts from homogenate (grey bars) and LD (white bars) were analyzed by LC–MS for phospholipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

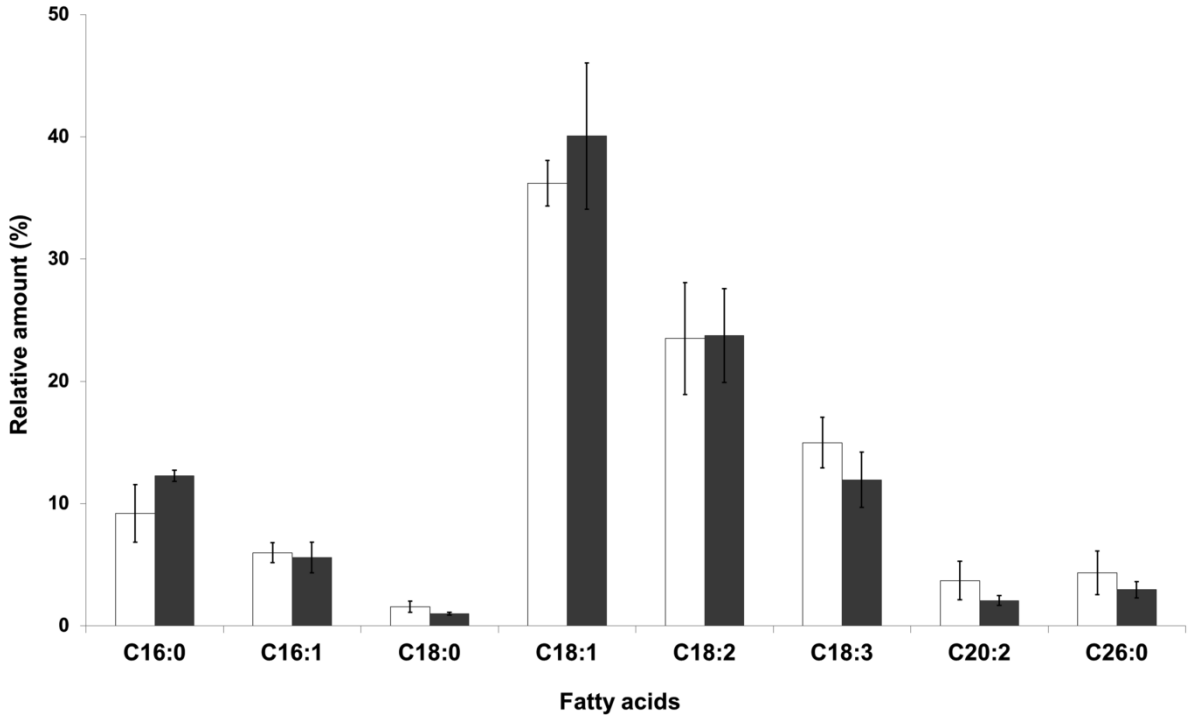

Fig.5 Fatty acid pattern of lipid droplets.

Lipid extracts from LD (white bars) and homogenate (grey bars) were analyzed by GC-FID for fatty acid composition. The amounts of individual fatty acids in homogenate and LD are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

CHAPTER 5

Identification of triacylglycerol and steryl ester synthases of the methylotrophic yeast *Pichia pastoris*

Vasyl A. Ivashov[a], Guenther Zellnig[b], Karlheinz Grillitsch[c] and Guenther Daum[a]*

[a] Institute of Biochemistry, Graz University of Technology, Austria; [b] Institute of Plant Sciences, Karl Franzens University Graz, Austria; [c] Austrian Centre of Industrial Biotechnology

Keywords: Acyltransferase, triacylglycerol, steryl ester, lipid droplet, *Pichia pastoris*

* Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology; Petersgasse 12/2, A-8010 Graz, Austria; Tel.: + 43 316 873 6462; Fax: + 43 316 873 6952; E-mail: guenther.daum@tugraz.at

Abstract

In yeast like in many other eukaryotes, fatty acids are stored in the biologically inert form of triacylglycerols (TG) and steryl esters (SE) as energy reserve and/or as membrane building blocks. In the present study, we identified gene products catalyzing formation of TG and SE in the methylotrophic yeast *Pichia pastoris*. Based on sequence homologies to *Saccharomyces cerevisiae*, the two diacylglycerol acyltransferases Dga1p and Lro1p and one acyl CoA:sterol acyltransferase Are2p from *P. pastoris* were identified. Mutants bearing single and multiple deletions of the respective genes were analyzed for their growth phenotype, lipid composition and the ability to form lipid droplets. Our results indicate that the above mentioned gene products are most likely responsible for the entire TG and SE synthesis in *P. pastoris*. Lro1p which has low fatty acid substrate specificity *in vivo* is the major TG synthase in this yeast, whereas Dga1p contributes less to TG synthesis although with some preference to utilize polyunsaturated fatty acids as substrates. In contrast to *S. cerevisiae*, Are2p is the only SE synthase in *P. pastoris.* Also this enzyme exhibits some preference for certain fatty acids as judged from the fatty acid profile of SE compared to bulk lipids. Most interestingly, TG formation in *P. pastoris* is indispensable for lipid droplet biogenesis. The small amount of SE synthesized by Are2p in a *dga1*Δ*lro1*Δ double deletion mutant is insufficient to initiate the formation of the storage organelle. In summary, our data provide a first insight into the molecular machinery of non-polar lipid synthesis and storage in *P. pastoris* and demonstrate specific features of this machinery in comparison to other eukaryotic cells, especially *S. cerevisiae*.

Keywords: Acyltransferase, Triacylglycerol, Steryl ester, Lipid droplet, *Pichia pastoris*

1. Introduction

The methylotrophic yeast *Pichia pastoris* is widely used in industry for the production of heterologously expressed proteins [1–4]. The intense commercial interest in this yeast has raised various questions about its cell biology. A deeper knowledge of biomembranes, organelle physiology and lipid metabolism of this microorganism can obviously contribute to a better understanding of protein processing and secretion routes through the endomembrane system in the host cells. For this reason, we started a systematic approach to investigate organelles from *P. pastoris* with emphasis on their membrane lipid and protein composition [5–7].

Most recently, we characterized for the first time the lipidome and proteome of lipid droplets (LD) from *P. pastoris*, the storage organelle for the non-polar lipids triacylglycerols (TG) and steryl esters (SE) [5]. In the present study we extended these investigations to the identification of genes and gene products performing synthesis of non-polar lipids in this yeast and characterized their contribution to lipid storage in LD. Enzymes which synthesize non-polar lipids in yeast were so far best characterized in *Saccharomyces cerevisiae* [8–13] and *Yarrowia lipolytica* [14,15]. In *S. cerevisiae*, the two TG synthases Lro1p and Dga1p and the two SE synthases Are1p and Are2p are typical representatives of these enzyme classes.

TG are typically formed by acylation of diacylglycerols (DAG) with fatty acids. In *S. cerevisiae*, the diacylglycerol:phospholipid acyltransferase Lro1p with its phospholipase A_2/B and acyltransferase activities forms TG in an acyl-CoA-independent manner utilizing phospholipids as acyl donors [8,9]. In contrast, the diacylglycerol acyltransferase Dga1p utilizes activated fatty acids as co-substrates to esterify DAG in an acyl-CoA-dependent reaction [10,11]. Lro1p is a component of the endoplasmic reticulum (ER) whereas Dga1p was found to be dually located to LD and the ER [11]. It has been reported that Dga1p and Lro1p from baker's yeast have a certain preference for unsaturated fatty acids, especially C16:1 and C18:1 [16,17]. We showed recently that the fatty acid composition of TG from *P. pastoris* reflects rather the total cellular pool of fatty acids [5] suggesting moderate fatty acid specificity of the enzymes involved in synthesis.

The two homologous SE synthesizing enzymes from *S. cerevisiae*, Are1p and Are2p, are present in the ER [12,13,18,19]. Both enzymes esterify sterols using acyl-CoAs as co-substrates. Are2p is the major acyl CoA:sterol acyltransferase in *S. cerevisiae* and

preferentially forms esters of ergosterol, the final product of the sterol biosynthetic pathway in this yeast. Are1p esterifies sterol precursors with similar efficiency as ergosterol and is more active under hypoxic conditions [13,20,21]. In our previous study [5] we showed that SE in LD from *P. pastoris* are present as esters of ergosterol and ergosterol precursors in comparable amounts.

TG and SE are synthesized in the ER and stored in the core of specialized organelles, the LD [22]. The basic structure of LD is similar in different cell types and consists of a non-polar lipid core surrounded by a phospholipid monolayer with a small but distinct set of proteins embedded. Most recently, proteome and lipidome analyses of LD from *P. pastoris* identified the major components of this compartment [5]. In contrast to *S. cerevisiae*, LD from *P. pastoris* contain much TG but only a small amount of SE. It was hypothesized that the low amount of SE was due to the limited capacity of *P. pastoris* to form sterols. The lipid composition of LD was reminiscent of the yeast *Y. lipolytica* [23] but also of mammalian adipocytes [24].

Interestingly, in *P. pastoris* the presence of the two diacylglycerol acyltransferases Dga1p and Lro1p but only of one acyl CoA:sterol acyltransferase, Are2p, has been predicted by homologies [25]. The aim of the present study was to identify and characterize these non-polar lipid synthesizing enzymes from *P. pastoris* at the molecular and biochemical level, to investigate their contribution to TG and SE synthesis and study their influence on LD biogenesis. For this purpose, we constructed strains lacking the putative *P. pastoris* diacylglycerol acyltransferases, Dga1p and Lro1p and the acyl CoA:sterol acyltransferase, Are2p. Single, double and triple deletion mutants were subjected to growth phenotype, lipid and organelle analysis. The results presented here provide molecular information about the network of non-polar lipid synthesis and storage in *P. pastoris* and further contribute to our understanding of cell and molecular biology of this microorganism.

2. Experimental procedures

2.1. Strains and culture conditions

P. pastoris strain CBS7435*ku70his4* (MATa, Mut$^+$, His$^-$) (kindly provided by A. Glieder) [26] considered as wild type and its derivative strains *lro1*Δ (MATa, Mut$^+$, His$^-$), *dga1*Δ (MATa, Mut$^+$, His$^-$), *are2*Δ (MATa, Mut$^+$, His$^+$), *dga1*Δ*lro1*Δ (MATa, Mut$^+$, His$^-$), *lro1*Δ *are2*Δ (MATa, Mut$^+$, His$^+$), and *dga1*Δ*lro1*Δ*are2*Δ (MATa, Mut$^+$, His$^+$) were used throughout this study. Cells were grown under aerobic conditions to the early stationary phase (26 h) at 30 °C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck); and in YPO medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid), 0.2% oleic acid, and 0.02% TWEEN® 40 (Sigma-Aldrich). Media were inoculated to a starting OD_{600} of 0.1 from precultures grown aerobically for 48 h in YPD at 30 °C.

2.2. Strain constructions

For the analysis of protein sequences of *P. pastoris* Dga1p (C4R3W3), Lro1p (C4R1G7) and Are2p (F2QWB7) we made use of annotations in UniProtKB/TrEMBL (protein entries shown in brackets). Alignment with *S. cerevisiae* and *Y. lipolytica* orthologs with Clustal Omega algorithm [27] and common domain search in the Pfam protein families databases [28] was performed with tools available at KEGG (Kyoto Encyclopedia of Genes and Genomes) database resource (http://www.genome.jp/kegg/).

DNA from wild type *P. pastoris* CBS7435Δ*Ku70* strain was isolated employing the Yeast DNA Kit (Omega bio-tek) and used for applications described below. The construction of acyltransferase deletion mutants was based on the gene deletion strategy described by Wach [29]. Deletion cassettes either contained antibiotic resistance markers or auxotrophy genes as core selection markers flanked by 5′ and 3′ adjacent regions of target genes. The homologous recombination strategy replaced target genes by selection marker genes. All primers used in this study are listed in Table 1.

The cassette for deletion of *LRO1* was produced using overlap extension PCR strategy. Long 5′ (593 bp) and 3′ (600 bp) flanking regions of *LRO1* gene were amplified from genomic DNA using primers 1, 2 and 3, 4, respectively. Then, these flanking regions were fused with the Kanamycin resistance gene together with promoter and terminator parts from pFA6a-kanMX6 plasmid in one overlap extension (OE) PCR with the outermost primers 1 and 4, resulting in the *LRO1* deletion cassette (2673 bp). After column spin purification the cassette was ready for yeast transformation.

For construction of the *DGA1* gene deletion cassette 5′ (540 bp) and 3′ (426 bp) flanking regions of the *DGA1* gene were amplified from genomic DNA using primers 5, 6 and 7, 8, respectively. Then flanking regions were fused with the Zeocin resistance gene together with promoter and terminator parts (1194 bp) from pPICz/B plasmid (Invitrogen) in one OE PCR with the outermost primers 5 and 8, resulting in the *DGA1* deletion cassette (2160 bp). After column spin purification the cassette was ready to use.

To create an *ARE2* gene deletion cassette, long 5′ (551 bp) and 3′ (551 bp) flanking regions of *ARE2* gene were amplified from genomic DNA using primers 9, 10 and 11, 12, respectively. The *HIS4* open reading frame (2536 bp) was amplified from pPIC3.5 K plasmid (Invitrogen) using primers 13 and 14. PCR products were column purified with NucleoSpin® Extract II kit (genXpress) and fused in a sequential overlap extension resulting in the *ARE2* deletion cassette (3885 bp). After column spin purification the cassette was ready to use.

All deletion cassettes obtained as described above were utilized for transformation of the *P. pastoris* wild type strain CBS7435Δ*Ku70*. Transformations were performed following the protocol of Lin-Cereghino et al. [30]. In brief, 50–70 µl electro-competent cells mixed with 1–2 µg of the respective purified deletion cassette were subjected to electroporation with a MicroPulser™ (BIO-RAD), incubated for 2 h in 1 ml 1 M sorbitol/YPD (1:1; v:v), centrifuged, resuspended in 100 µl of supernatant and incubated on appropriate selection plates for 48–60 h at 30 °C until colonies appeared.

Gene deletions were verified by PCR and sequencing. For this purposes, two sets of primers for each gene deletion were designed. The first set was complementary to the ends of the full or almost full length ORFs. Primers 15 and 16 correspond to *LRO1*, 17 and 18 to *DGA1*, and 19 and 20 to *ARE2*. The second set of primers was designed as follows. One primer binds 50–100 bases outside the putative integration place. The other primer binds inside the cassette to

its selection marker part. The presence and the correct size of the resulting PCR product confirmed the correct locus of integration of the cassette. Primers 21 and 22 were designed to confirm the presence of the *LRO1* deletion cassette, primers 23 and 24 for the *DGA1* deletion cassette, and primers 25 and 14 for the *ARE2* deletion cassette. DNA from non-transformed wild type cells was used as a negative control.

Clones which did not show a signal for target ORFs but the correct signal for the integration event were subjected to sequencing. Sequencing primer 26 was used for proving *LRO1* deletion, 27 for *DGA1* deletion, and 10 for *ARE2* deletion. Confirmed single mutants were used for generating double and triple deletion mutants by sequential deletions using the same procedure described above for each single deletion.

2.3. Analytical methods

Proteins were quantified by the method of Lowry et al. [31] using bovine serum albumin as a standard. Prior to quantification, proteins from yeast cell homogenates were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH.

Lipids from homogenates of yeast cells grown to the early stationary phase (26 h) were extracted as described by Folch et al. [32]. For quantification of non-polar lipids, extracts were loaded onto Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by a two-step developing system. First, light petroleum/diethyl ether/acetic acid (25/25/1; per vol.) was used as mobile phase and chromatograms were developed to half-distance of the plate. Then, plates were dried briefly and chromatograms were further developed to the top of the plate using light petroleum/diethyl ether (49/1; v/v) as the second mobile phase. To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2$ x · 4 H_2O, 60 ml water, 60 ml methanol and 4 ml of concentrated sulfuric acid, briefly dried and heated to 105 °C for 40 min. Visualized bands were scanned at 400 nm and quantified using triolein and cholesteryl ester as standards. The amounts of non-polar lipids were related to 1 mg total cell protein.

Fatty acids were analyzed by gas liquid chromatography (GLC). Total cell lipid extracts were prepared as described above. For the analysis of the fatty acid composition of individual non-polar lipids, TLC-separated TG and SE were scrapped off the plate, re-extracted and dried. Then, samples were subjected to methanolysis using 2.5% H_2SO_4 in methanol and fatty acids were converted to methyl esters. Fatty acid methyl esters were separated using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-INNOWax capillary column (15 m × 0.25 mm inner diameter × 0.50 μm film thickness) and helium as carrier gas. Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN).

2.4. Microscopy

For fluorescence microscopy, *P. pastoris* cells were grown on YPD medium to the early stationary phase (26 h). Cells from 1 ml culture were harvested by centrifugation, washed once with deionized water, stained for 10 min with 5 μl (10 μg/ml) of the lipid droplet specific dye AC-201 [2-(2,6-diisopropylphenyl)-4-(ethylamino)-5,6,7-trifluoroisoindole-1,3-dione], a structural analog of trifluoroaminophthalimide [33] and analyzed using a LEICA DM LB2 microscope with a 100-fold oil immersion objective and excitation filter BP 515–560 nm. Images were taken with Leica DFC 350 FX camera.

For electron microscopy, cells were harvested in the early stationary phase by centrifugation and washed 3 times with double distilled water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with double distilled water and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again. Fixed cells were washed four times in distilled water and incubated in 0.5 % aqueous uranyl acetate overnight at 4 °C. Samples were then dehydrated for 20 min each in a graded series of 50%, 70%, 90%, and 100% ethanol. Pure ethanol was then changed to propylene oxide, and specimen were gradually infiltrated with increasing concentrations (30%, 50%, 70% and 100%) of Agar 100 epoxy resin (Agar Scientific Ltd., Stansted, England) mixed with propylene oxide for a minimum of 3 h per step. Samples were embedded in pure, fresh Agar 100 epoxy resin and polymerized at 60 °C for 48 h. Ultrathin sections of 80 nm were stained for 3 min with lead citrate and viewed with a Philips CM 10 transmission electron microscope.

3. Results

3.1. Identification of non-polar lipid forming acyltransferases from *Pichia pastoris*

Protein sequences of *P. pastoris* Dga1p, Lro1p and Are2p were aligned with orthologs from *S. cerevisiae* and *Y. lipolytica* [8–15] and analyzed for their conserved domain architecture (Fig. 1) as described in the method section. UniProtKB entries are shown in brackets.

Diacylglycerol acyltransferase, Dga1p from *P. pastoris* (C4R3W3) has a total amino acid sequence identity of 40% with Dga1p from *S. cerevisiae* (Q08650) and 33% identity with Dga1p from *Y. lipolytica* (Q6C3R2). Similar to its orthologs, Dga1p from *P. pastoris* has two diacylglycerol acyltransferase (DAGAT) domains at amino acid positions 115–175 and 199–450 and the highly conserved DAGAT motifs ^{161}YFPI^{164}and ^{222}FGYHPHG^{228} [34] (Fig. 1A).

The whole amino acid sequence of the phospholipid:diacylglycerol acyltransferase Lro1p from *P. pastoris* (C4R1G7) has 49% identity with *S. cerevisiae* (P40345) and 52% with *Y. lipolytica* (Q6C5M4) orthologs. As its orthologs, it contains a large lecithin:cholesterol acyltransferase (LCAT) domain at amino acids 176–615 and an α/β hydrolase-fold at amino acid positions 292–369 (Fig. 1B). Lro1p from *P. pastoris* has also a predicted transmembrane domain at amino acid positions 74–91, which has similarity to the transmembrane domains of *S. cerevisiae* [35] and *Y. lipolytica* orthologs. Noteworthy, the amino acids of *P. pastoris* Lro1p in positions Ser^{313}, Asp^{558}, and His^{609} are conserved between orthologs and correspond to the active site of *S. cerevisiae* Lro1p. Ser^{324} is the potential site of acyl ester intermediate formation, and Asp^{567} and His^{618} are predicted to be involved in the charge relay system (UniProtKB).

The acyl-CoA:sterol acyltransferase Are2p (F2QWB7) from *P. pastoris* has 40% identity with the *S. cerevisiae* (P53629) and 42.5% identity with the *Y. lipolytica* (Q6C2M6) ortholog (Fig. 1C). As its homologs, it has two membrane associated O-acyl transferase family domains between amino acids 163–525 and 409–481. The conserved amino acid motifs of *P. pastoris* Are2p at positions 224–237 and 406–412 are similar to two sites of *S. cerevisiae* Are2p providing affinity for oleoyl-CoA [36]. Additionally, the *P. pastoris* enzyme has a conserved motif at positions 457–461 where His^{459} corresponds to His^{579} of the potential active site of the *S. cerevisiae* ortholog.

3.2. Growth phenotype of *P. pastoris* strains deficient in non-polar lipid synthetic enzymes

To address possible general physiological effects caused by the lack of non-polar lipid biosynthetic enzymes in *P. pastoris* we analyzed the growth phenotype of *lro1*Δ, *dga1*Δ and *are2*Δ single deletion strains; *dga1*Δ*lro1*Δ and *lro1*Δ*are2*Δ double deletion mutants; and the *dga1*Δ*lro1*Δ*are2*Δ (*TM*) triple deletion mutant when grown on glucose and oleic acid, respectively (Fig. 2). Growth of all mutants was similar to wild type when glucose was used as a carbon source. When cells were grown on oleic acid, the *dga1*Δ*lro1*Δ double mutant lacking both diacylglycerol acyltransferases Dga1p and Lro1p and the *dga1*Δ*lro1*Δ*are2*Δ triple mutant showed slight growth retardation, whereas single deletion mutants grew like wild type.

3.3. Non-polar lipid analysis of deletion mutants

To confirm the predicted function of gene products encoded by *LRO1*, *DGA1* and *ARE2* from *P. pastoris* we measured the amounts of TG and SE in total cell extracts of deletion mutants and wild type strain grown on glucose or oleic acid (Fig. 3). In strains grown on glucose, deletion of *LRO1* led to a 46% decrease of TG compared to wild type, whereas in a *dga1*Δ mutant only a moderate decrease of TG was observed (Fig. 3A). Interestingly, the TG level was also slightly decreased in a strain deleted of *ARE2*. The *dga1*Δ*lro1*Δ double mutant lacking both predicted TG synthases was practically devoid of TG similar to the TM where all three genes encoding for non-polar lipid synthesizing enzymes were missing. Essentially the same results were obtained with cells grown on oleate. Also on this carbon source deletion of *LRO1* led to highest reduction of the TG amount, and strains deleted of both *DGA1* and *LRO1* as well as the TM were practically devoid of TG.

Surprisingly, cellular SE levels were also decreased by deletion of *DGA1* and *LRO1* (Fig. 3B). Also in this case, the effect was stronger in the *lro1*Δ mutant than in *dga1*Δ. In a *dga1*Δ*lro1*Δ double mutant the SE level was ~ 40 % of wild type grown on glucose, and only ~ 10% of wild type when oleate was used as a carbon source. Not unexpectedly, deletion of *ARE2* alone or in combination with *DGA1* and *LRO1* (TM) led to a complete loss of SE in cells grown on both carbon sources.

Based on these results, Lro1p appears to be the major and Dga1p the minor TG synthase in *P. pastoris* under conditions tested. It is very unlikely that these two gene products catalyze SE formation, because the single deletion of *ARE2* led to a more or less complete loss of SE. The decreased amounts of SE in *lro1*Δ and *dga1*Δ strains as well as the slightly reduced level of TG in the *are2*Δ mutant can rather be explained by a negative feedback control to fatty acid synthesis caused by the lack of non-polar lipid producing enzymes. The occurrence of trace amounts of TG and SE in deletion mutants described above may serve as a hint that further enzymes capable of synthesizing non-polar lipids although with minor capacity exist in *P. pastoris*.

The major fatty acids found in *P. pastoris* lipids from cells grown under standard conditions are palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3) [5–7]. In *dga1*Δ and *are2*Δ single mutants the fatty acid pattern was similar to wild type and only in *lro1*Δ a slight increase in C18:3 and a decrease of C18:1 was observed (data not shown). In the *dga1*Δ*lro1*Δ double mutant and in the *dga1*Δ*lro1*Δ*are2*Δ TM, however, fatty acid patterns were markedly changed (Fig. 4). Growth of both mutants on YPD led to elevation of polyunsaturated fatty acids C18:2 and C18:3 accompanied by a strong decrease in oleic acid (C18:1). At the same time, the amount of palmitic acid (C16:0) increased in both mutants although more pronounced in *dga1*Δ*lro1*Δ than in TM. When cells were grown on YPO, oleic acid was the major fatty acid found in lipid extracts of all strains for obvious reasons. Also under these conditions, in *dga1*Δ*lro1*Δ and TM the relative amounts of polyunsaturated fatty acids increased in comparison to wild type. In particular, a 1.6-fold increase in C18:2 and a 2-fold increase in C18:3 were observed. In both strains, the amounts of C16:0 increased, whereas reduced levels of C18:1 were detected. Thus, it appears that the capability of fatty acid storage in the form of SE and TG has a strong impact on the fatty acid pattern in *P. pastoris*.

To address the substrate specificity of *LRO1* and *DGA1* gene products toward fatty acids we performed fatty acid analysis of TG isolated from *lro1*Δ and *dga1*Δ single deletion mutants and compared the patterns to wild type (Fig. 5). This analysis showed that in *dga1*Δ where only Lro1p is active the fatty acid pattern of TG was identical to wild type indicating low substrate selectivity of Lro1p. In the *lro1*Δ mutant bearing Dga1p as the only TG synthesizing enzyme the fatty acid pattern of TG was changed toward more saturated species. A 1.5-fold increase in C16:0 and a more than 2 fold increase in the relative amount of C18:0

were observed at the expense of C18:1 and C18:2. Although Dga1p appears to be the minor contributor to total TG synthesis in *P. pastoris* (see Fig. 3) and only marginally affects the total cellular fatty acid composition (see Fig. 4) this enzyme appears to be more specific for acyl CoA substrates than Lro1p.

As described above the *ARE2* gene product appears to be the only SE synthase in *P. pastoris*. To test for the substrate specificity of Are2p *in vivo* we analyzed the fatty acid pattern of SE and compared it to total cell lipid extracts from wild type. Fig. 6 shows that in SE the relative amount of stearic acid (C18:0) was 8-fold higher than in bulk lipids and the amount of C16:0 was slightly increased. Surprisingly, C16:1 was completely missing in SE and the amounts of polyunsaturated fatty acids C18:2 and C18:3 were markedly lowered. These data suggest that Are2p has a rather strong preference for certain fatty acid substrates. The specificity of Are2p from *P. pastoris* for sterols used as the second substrate for the formation of SE can be deduced from the sterol pattern of SE present in LD [5]. These results showed that similar to *S. cerevisiae* the Are2p from *P. pastoris* can utilize both ergosterol and its precursors as substrates.

3.4. Microscopic inspection of mutants compromised in non-polar lipid synthesis

To test the ability of *P. pastoris* mutants bearing defects in non-polar lipid synthesizing enzymes to store lipids in the form of LD microscopic inspections were performed. For this purpose, wild type and mutants were grown on glucose or oleate, respectively, to the stationary phase and stained with the LD specific lipophilic dye AC-201 [2-(2,6-diisopropylphenyl)-4-(ethylamino)-5,6,7-trifluoroisoindole-1,3-dione] [33]. Fluorescence microscopy (Fig. 7) revealed that single deletion mutants formed LD similar to wild type independently of the carbon source. However, the *dga1*Δ*lro1*Δ double mutant lacking both TG synthases and the *dga1*Δ*lro1*Δ*are2*Δ TM deleted of all three genes encoding non-polar lipid synthesizing enzymes did not form LD at all. These observations suggested that TG are indispensable for LD formation in *P. pastoris* and the small amount of SE which is present in *dga1*Δ*lro1*Δ is not sufficient for LD biogenesis.

To unambiguously prove the role of *LRO1* and *DGA1* gene products in the process of LD formation and to investigate in addition the general cellular structure of non-polar lipid

synthesis deficient strains, we performed electron microscopic studies of mutants grown on YPD and YPO (Fig. 8). The appearance of LD in all strains tested perfectly matched data obtained with fluorescence microscopy. Also these experiments showed that *dga1*Δ*lro1*Δ and TM strains are unable to form LD. Furthermore, no major differences in the cell structure of mutants and wild type were observed at least under growth conditions used in this study.

4. Discussion

In the present study we identified gene products catalyzing formation of non-polar lipids in the methylotrophic yeast *P. pastoris*. Bioinformatic analysis identified two diacylglycerol acyltransferases Dga1p and Lro1p and one acyl-CoA:sterol acyltransferase Are2p. The proposed catalytic functions of these proteins were tested by using single, double and triple deletion mutants and analyzing growth phenotype, lipid composition and cell structure.

Under standard growth conditions with glucose as the sole carbon source the growth behavior of all generated mutants was similar to wild type. This result is in agreement with data obtained with *S. cerevisiae* before [8,11,12]. However, a marked difference between these two yeast species was observed when oleate was used as a carbon source. Whereas in *S. cerevisiae* strains lacking the ability to form non-polar lipids [37,38] growth on oleate led to a lipotoxic effect, growth retardation of *P. pastoris dga1*Δ*lro1*Δ and *dga1*Δ*lro1*Δ*are2*Δ on this carbon sources was only moderate. Most likely this effect is due to efficient induction of peroxisome proliferation and fatty acid β-oxidation upon growth of *P. pastoris* on fatty acids [6] which is much less pronounced in *S. cerevisiae*.

Another marked difference to *S. cerevisiae* is the contribution of Dga1p and Lro1p from *P. pastoris* to TG synthesis. Whereas in *P. pastoris* Lro1p is the more potent diacylglycerol acyltransferase, Dga1p is the major contributor to TG synthesis in *S. cerevisiae* [16]. *P. pastoris* mutants *dga1*Δ*lro1*Δ and *dga1*Δ*lro1*Δ*are2*Δ bearing deletions of both diacylglycerol acyltransferases contained only traces of TG indicating that these two gene products are the major or even the only TG synthase in *P. pastoris*. Our molecular biological and biochemical analyses also suggested that *P. pastoris* in contrast to *S. cerevisiae* harbors only one acyl-CoA:sterol acyltransferase, the gene product of *ARE2*. *P. pastoris* is a low sterol producing

yeast [6,7] which also contains only small amounts of SE. Lack of SE in *P. pastoris are2*Δ strongly suggests that the respective gene product is the only SE biosynthetic enzyme in this yeast. Notably, however, deletion of *LRO1* gene alone or in combination with *DGA1* also resulted in a marked decrease of SE. We assume that in the absence of TG synthetic enzymes fatty acid synthesis may be reduced resulting in an indirect negative effect on SE production. Similar observations were made with *S. cerevisiae* [39]. Interestingly, growth of *P. pastoris* on oleate did not considerably affect SE synthesis as has been shown in *S. cerevisiae* [39] where SE biosynthetic enzymes are inhibited in the presence of oleate.

Since non-polar lipids are the core components of LD it was not surprising that deletion of the respective biosynthetic genes from *P. pastoris* affected the formation of this lipid storage organelle. Our microscopic inspections showed that single deletions of acyltransferases changed neither number nor size of LD significantly. However, a *dga1*Δ*lro1*Δ double deletion strain completely lacked LD. Obviously, the low amount of SE produced in this strain was not sufficient for LD biogenesis. In contrast, *S. cerevisiae* strains grown on glucose form LD even if only one SE biosynthetic enzyme is active [16]. Electron microscopy of *P. pastoris* deletion mutants grown on YPD or YPO did not show altered organelle structure. Thus, a putative lipotoxic stress caused by the presence of oleate in the medium does not harm *P. pastoris* cells. Previous studies had demonstrated that the endoplasmic reticulum in *S. cerevisiae* was highly proliferated when an excess of fatty acids was present in a *dga1*Δ *lro1*Δ*are1*Δ*are2*Δ mutant grown on oleate [38]. This is not the case in *P. pastoris.* The more robust utilization of fatty acids as a source of energy in *P. pastoris* may explain these findings. This view was supported by the finding that the levels of total and individual phospholipids from *P. pastoris* mutants compromised in non-polar lipid biosynthesis grown either on YPD or YPO remained unaffected (our own unpublished results).

The absence of TG in *P. pastoris dga1*Δ*lro1*Δ and in *dga1*Δ*lro1*Δ*are2*Δ had a marked effect on the bulk membrane fatty acid composition (see Fig. 4). In both mutants the amount of polyunsaturated fatty acids was enhanced. At present, we can only speculate about a possible feedback regulation of non-polar lipid formation on fatty acid synthesis and modification. The slight preferences of TG and SE biosynthetic enzymes for certain fatty acids cannot explain the observed effects with bulk fatty acids.

In conclusion, our results presented here describe for the first time molecular components of the non-polar lipid biosynthetic machinery of *P. pastoris*. Interestingly, some of these results

parallel data obtained with *S. cerevisiae*, whereas other findings unveil marked differences in the synthesis of TG and SE in the two yeast species. One striking example is the robust utilization of fatty acids in *P. pastoris* even in the absence of non-polar biosynthetic enzymes. Thus, results reported here expand our current knowledge of *P. pastoris* lipid metabolism and shed some light on lipid storage and LD formation in this yeast, but also provide more general evidence for the variety of lipid metabolic processes in different types of cells.

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References

1. Cregg J.M., Cereghino J.L., Shi J., Higgins D.R. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 2000;16:23–52.

2. Daly R., Hearn M.T. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. 2005;18:119–138.

3. Macauley-Patrick S., Fazenda M.L., McNeil B., Harvey L.M. Heterologous protein production using the *Pichia pastoris* expression system. Yeast. 2005;22:249–270.

4. Li P., Anumanthan A., Gao X.G., Ilangovan K., Suzara V.V., Duzgunes N., Renugopalakrishnan V. Expression of recombinant proteins in *Pichia pastoris*. Appl. Biochem. Biotechnol. 2007;142:105–124.

5. Ivashov V.A., Grillitsch K., Koefeler H., Leitner E., Baeumlisberger D., Karas M., Daum G. Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*. Biochim. Biophys. Acta. 2013;1831:282–290.

6. Wriessnegger T., Gübitz G., Leitner E., Ingolic E., Cregg J., de la Cruz B.J., Daum G. Lipid composition of peroxisomes from the yeast *Pichia pastoris* grown on different carbon sources. Biochim. Biophys. Acta. 2007;1771:455–461.

7. Wriessnegger T., Leitner E., Belegratis M.R., Ingolic E., Daum G. Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*. Biochim. Biophys. Acta. 2009;1791:166–172.

8. Oelkers P., Tinkelenberg A., Erdeniz N., Cromley D., Billheimer J.T., Sturley S.L. A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. J. Biol. Chem. 2000;275:15609–15612.

9. Dahlqvist A., Stahl U., Lenman M., Banas A., Lee M., Sandager L., Ronne H., Stymne S. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. U. S. A. 2000;97:6487–6492.

10. Oelkers P., Cromley D., Padamsee M., Billheimer J.T., Sturley S.L. The DGA1 gene determines a second triglyceride synthetic pathway in yeast. J. Biol. Chem. 2002;277:8877–8881.

11. Sorger D., Daum G. Synthesis of triacylglycerols by the acyl-coenzyme A:diacylglycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 2002;184:519–524.

12. Yu C., Kennedy N.J., Chang C.Y., Rothblatt J.A. Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. J. Biol. Chem. 1996;271:24157–24163.

13. Zweytick D., Leitner E., Kohlwein S.D., Yu C., Rothblatt J., Daum G. Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 2000;267:1075–1082.

14. Athenstaedt K. YALI0E32769g (DGA1) and YALI0E16797g (LRO1) encode major triacylglycerol synthases of the oleaginous yeast *Yarrowia lipolytica*. Biochim. Biophys. Acta. 2011;1811:587–596.

15. Beopoulos A., Haddouche R., Kabran P., Dulermo T., Chardot T., Nicaud J.M. Identification and characterization of DGA2, an acyltransferase of the DGAT1 acyl-CoA:diacylglycerol acyltransferase family in the oleaginous yeast *Yarrowia lipolytica*. New insights into the storage lipid metabolism of oleaginous yeasts. Appl. Microbiol. Biotechnol. 2012;93:1523–1537.

16. Czabany T., Wagner A., Zweytick D., Lohner K., Leitner E., Ingolic E., Daum G. Structural and biochemical properties of lipid particles from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 2008;283:17065–17074.

17. Grillitsch K., Connerth M., Köfeler H., Arrey T.N., Rietschel B., Wagner B., Karas M., Daum G. Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta. 2011;1811:1165–1176.

18. Yang H., Bard M., Bruner D.A., Gleeson A., Deckelbaum R.J., Aljinovic G., Pohl T.M., Rothstein R., Sturley S.L. Sterol esterification in yeast: a two-gene process. Science. 1996;272:1353–1356.

19. Zinser E., Paltauf F., Daum G. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. J. Bacteriol. 1993;175:2853–2858.

20. Jensen-Pergakes K., Guo Z., Giattina M., Sturley S.L., Bard M. Transcriptional regulation of the two sterol esterification genes in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 2001;183:4950–4957.

21. Valachovic M., Klobucnikova V., Griac P., Hapala I. Heme-regulated expression of two yeast acyl-CoA:sterol acyltransferases is involved in the specific response of sterol esterification to anaerobiosis. FEMS Microbiol. Lett. 2002;206:121–125.

22. Olofsson S.O., Bostrom P., Andersson L., Rutberg M., Perman J., Boren J. Lipid droplets as dynamic organelles connecting storage and efflux of lipids. Biochim. Biophys. Acta. 2009;1791:448–458.

23. Athenstaedt K., Jolivet P., Boulard C., Zivy M., Negroni L., Nicaud J.M., Chardot T. Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. Proteomics. 2006;6:1450–1459.

24. Suzuki M., Shinohara Y., Ohsaki Y., Fujimoto T. Lipid droplets: size matters. J. Electron Microsc. (Tokyo) 2011;60:101–116.

25. Kuberl A., Schneider J., Thallinger G.G., Anderl I., Wibberg D., Hajek T., Jaenicke S., Brinkrolf K., Goesmann A., Szczepanowski R., Puhler A., Schwab H., Glieder A., Pichler H. High-quality genome sequence of *Pichia pastoris* CBS7435. J. Biotechnol. 2011;154:312–320.

26. Näätsaari L., Mistlberger B., Ruth C., Hajek T., Hartner F.S., Glieder A. Deletion of the *Pichia pastoris KU70* homologue facilitates platform strain generation for gene expression and synthetic biology. PLoS One. 2012;7:e39720.

27. Sievers F., Wilm A., Dineen D.G., Gibson T.J., Karplus K., Li W., Lopez R., McWilliam H., Remmert M., Söding J., Thompson J.D., Higgins D.G. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 2011;7:539.

28. Punta M., Coggill P.C., Eberhardt R.Y., Mistry J., Tate J., Boursnell C., Pang N., Forslund K., Ceric G., Clements J., Heger A., Holm L., Sonnhammer E.L., Eddy S.R., Bateman A., Finn R.D. The Pfam protein families database. Nucleic Acids Res. 2012;40:D290–D301.

29. Wach A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast. 1996;12:259–265.

30. Lin-Cereghino J., Wong W.W., Xiong S., Giang W., Luong L.T., Vu J., Johnson S.D., Lin-Cereghino G.P. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. Biotechniques. 2005;38:44–48.

31. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951;193:265–275.

32. Folch J., Lees M., Sloane Stanley G.H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 1957;226:497–509.

33. Puskás L.G., Fehér L.Z., Vizler C., Ayaydin F., Rásó E., Molnár E., Magyary I., Kanizsai I., Gyuris M., Madácsi R., Fábián G., Farkas K., Hegyi P., Baska F., Ozsvári B., Kitajka K. Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells. Lipids Health Dis. 2010;9:56.

34. Liu Q., Siloto R.M., Snyder C.L., Weselake R.J. Functional and topological analysis of yeast acyl-CoA:diacylglycerol acyltransferase 2, an endoplasmic reticulum enzyme essential for triacylglycerol biosynthesis. J. Biol. Chem. 2011;286:13115–13126.

35. Ghosal A., Banas A., Ståhl U., Dahlqvist A., Lindqvist Y., Stymne S. *Saccharomyces cerevisiae* phospholipid:diacylglycerol acyl transferase (PDAT) devoid of its membrane anchor region is a soluble and active enzyme retaining its substrate specificities. Biochim. Biophys. Acta. 2007;1771:1457–1463.

36. Guo Z., Cromley D., Billheimer J.T., Sturley S.L. Identification of potential substrate-binding sites in yeast and human acyl-CoA sterol acyltransferases by mutagenesis of conserved sequences. J. Lipid Res. 2001;42:1282–1291.

37. Kohlwein S.D., Petschnigg J. Lipid-induced cell dysfunction and cell death: lessons from yeast. Curr. Hypertens. Rep. 2007;9:455–461.

38. Petschnigg J., Wolinski H., Kolb D., Zellnig G., Kurat C.F., Natter K., Kohlwein S.D. Good fat, essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J. Biol. Chem. 2009;284:30981–30993.

39. Connerth M., Czabany T., Wagner A., Zellnig G., Leitner E., Steyrer E., Daum G. Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J. Biol. Chem. 2010;285:26832–26841.

Tables

Table 1. List of primers used in this study.

Nr	Sequence from 5′ to 3′ end
1	CTAGTGATATAGCAACAGATGTTGTTG
2	CGCCTTAATTAACCCGGGGATCCGAGGTGAAAGGCTGACGGCTCAAG
3	CCATCCAGTTTAAACGAGCTCGAATTCTGTTTACATCTGTGAGTTGTAAAC
4	GCTGATCAGGATGGTCAGC
5	AGGTTTGCCGTTATTCTGGG
6	GTGTGTGGGGGATCCGCACAAACGAAGGTTTTATCGCCAGTTTGCGGA
7	CGCTCGAAGGCTTTAATTTGCAAGCTGGAGTTGAGCCAGTATCTTTTTTA
8	AGATGTCAACTGAAACAGAAGATGG
9	CGTATGCAGGTAGCAAGGGAAATGTCATCATTAAATGGAGGTGAAAGTTTG
10	TACTTGTATCGGCATTACACAGCC
11	CAGAGACTGGATGTTGCGGTATTC
12	CGTATGGAGAAACTGGGACTTATTTAAGTGTTTTAAATAGGGATATAC
13	ATGACATTTCCCTTGCTACCTGCATACG
14	TTAAATAAGTCCCAGTTTCTCCATACG
15	ATGCAACTACGGAAAAGAGG
16	GTTTAACTCTGCAGATCCCAG
17	ATGCCTGAAAAGAAGAACAGTCG
18	AGGATCCAGCATTAAACCTCTC

Nr	Sequence from 5’ to 3’ end
19	ATGCCGATCCCAGTGGCGAG
20	CTAAGTATAGAGAGCACAAGAGGC
21	CAAACGGGGTTACTATTTCC
22	AAAATCACTCGCATCAACCA
23	GAGAATTAAAAGGTAGATACGC
24	TCAGTCCTGCTCCTCGGCCACGAAGTGCACGC
25	CATTCATCGGATAAAACTGTGCTGC
26	CTGTGGCTTCCTTATCGACCC
27	CATTCCATCCATGGATAACAGG

Figures

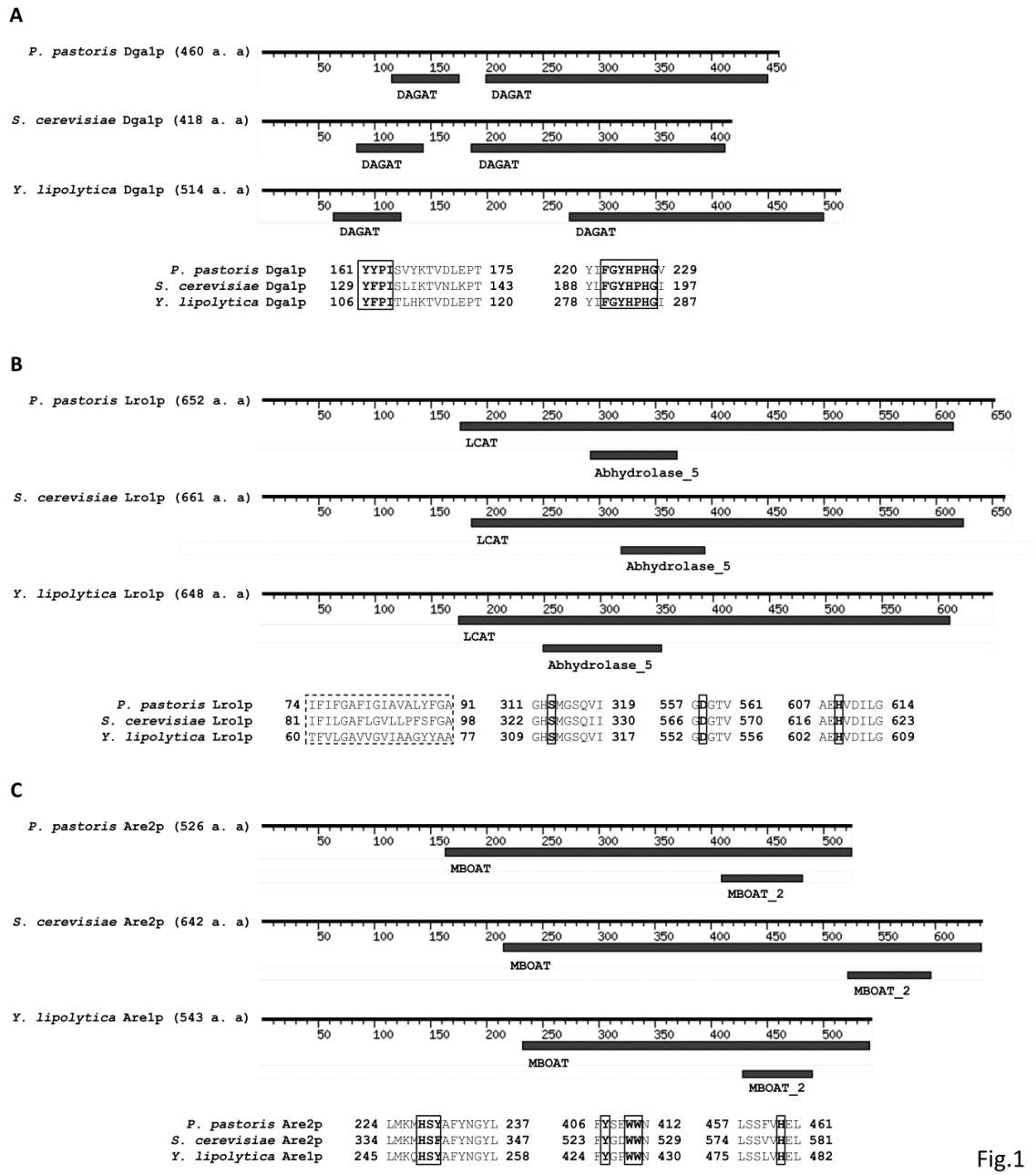

Fig.1

Fig.1 Sequence alignment of non-polar lipid synthesizing enzymes from *P. pastoris*.

A: Domain architecture of Dga1p from *P. pastoris*, *S. cerevisiae* and *Y. lipolytica*. Common diacylglycerol acyltransferase domains (DAGAT) are retrieved from Pfam database. Multiple protein sequence alignments show two highly conserved yeast Dga1p amino acid motifs (in bold, marked with squares). B: Domain architecture of Lro1p from *P. pastoris*, *S. cerevisiae*

and *Y. lipolytica*. Common lecithin:cholesterol acyltransferase (LCAT) and α/β hydrolase (Abhydrolase_5) domains are retrieved from Pfam database. Aligned amino acid sequences of transmembrane domains are marked with dashed squares and active site vicinity motifs with potential active site catalytic triade (in bold) are shown within squares. C: Domain architecture of Are2p from *P. pastoris*, *S. cerevisiae* and *Y. lipolytica*. Common membrane bound O-acyl transferase family domains (MBOAT) are retrieved from Pfam database. Protein sequence alignment shows conserved active site motifs with crucial amino acids (in bold, marked with squares). Numbers indicate amino acid positions.

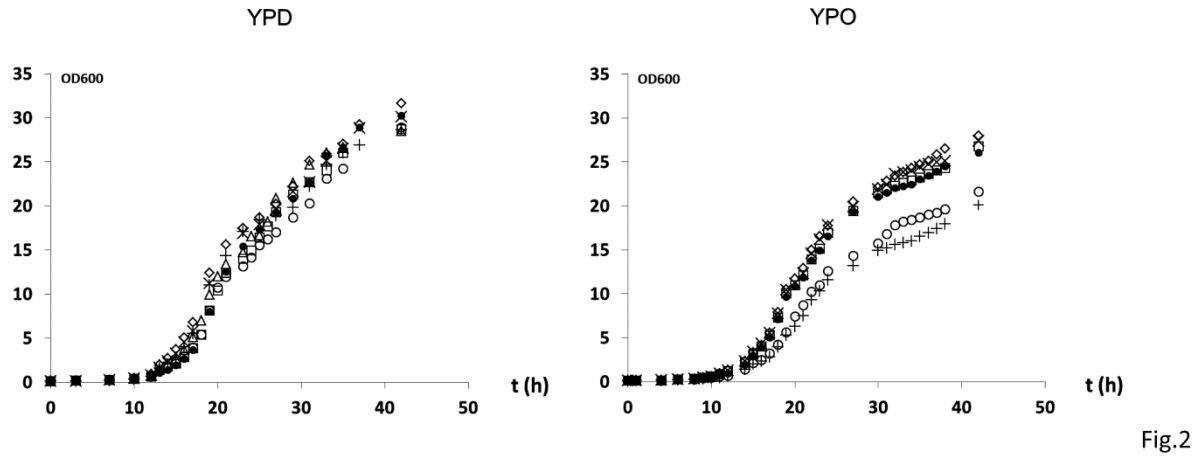

Fig.2 Growth of *P. pastoris* mutants bearing defects in non-polar lipid synthesis.

A yeast pre-culture grown in YPD for 48 h was used to inoculate fresh YPD or YPO media at a starting OD_{600} of 0.1. Cells were incubated at 30 °C with vigorous shaking. At time points indicated aliquots were withdrawn, cells were washed in 0.5% fatty acid-free BSA, and absorbance was measured. ◇, WT; □, *lro1*Δ; ∆, *dga1*Δ; ×, *are2*Δ; ●, *lro1*Δ*are2*Δ; ○, *dga1*Δ*lro1*Δ; +, *TM (dga1*Δ*lro1*Δ*are2*Δ*).*

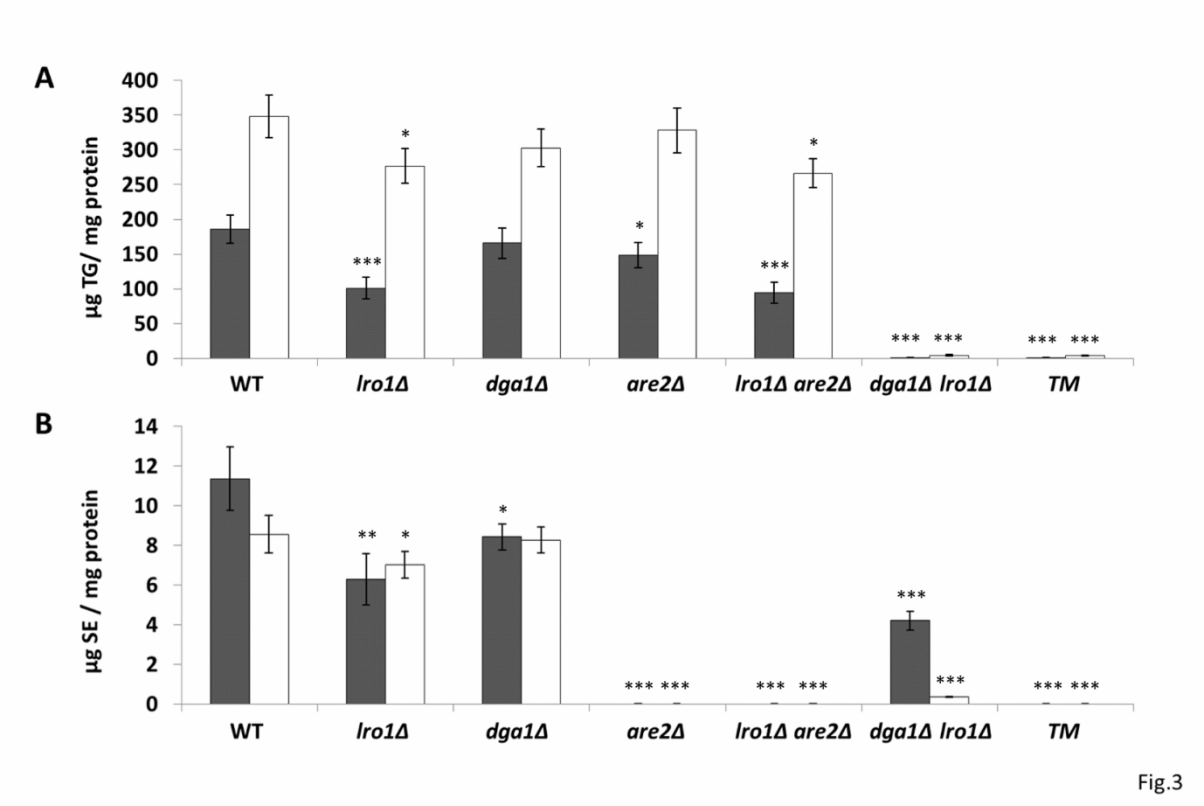

Fig.3 Non-polar lipid composition in *P. pastoris* strains deleted of *DGA1*, *LRO* and *ARE2*.

Cells were grown at 30°C on YPD or YPO media, respectively, to the early stationary phase. Total cell lipids were extracted and analyzed by TLC as described in the methods section. A: TG content in deletion strains grown on glucose (grey bars) and oleate (white bars). B: SE content in deletion strains grown on glucose (grey bars) and oleate (white bars). Data are mean values of at least three independent experiments. Error bars indicate the standard deviation.

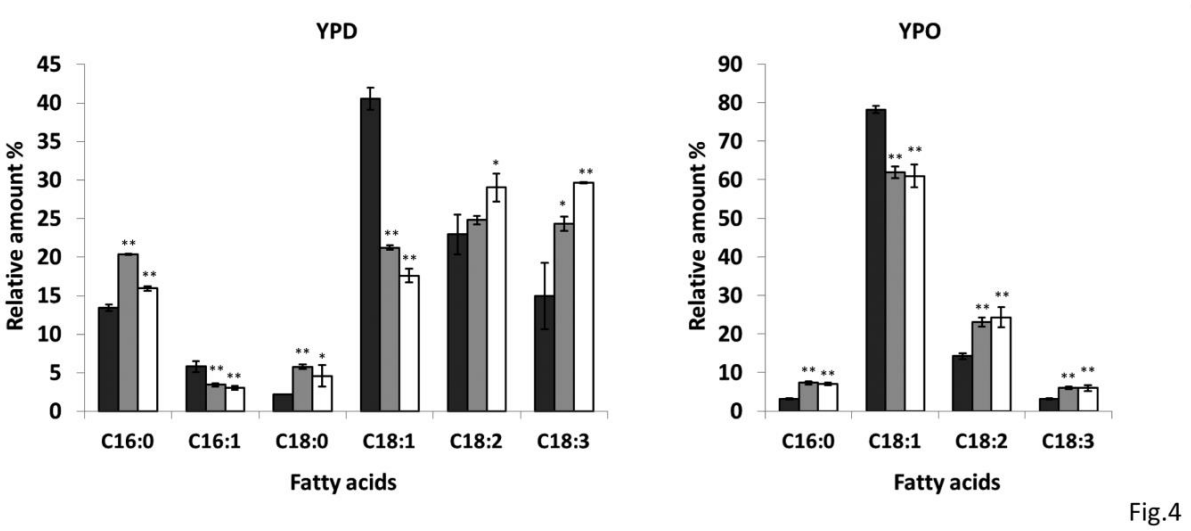

Fig. 4 Fatty acid profile of *P. pastoris dga1*Δ*lro1*Δ and *dga1*Δ*lro1*Δ*are2*Δ strains.

Total cell lipid extracts from wild type (black bars), *dga1*Δ*lro1*Δ (grey bars) and *dga1*Δ*lro1*Δ*are2*Δ TM (white bars) strains grown on YPD or YPO to the early stationary phase were analyzed by GLC for fatty acid composition. The amounts of individual fatty acids are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate the standard deviation.

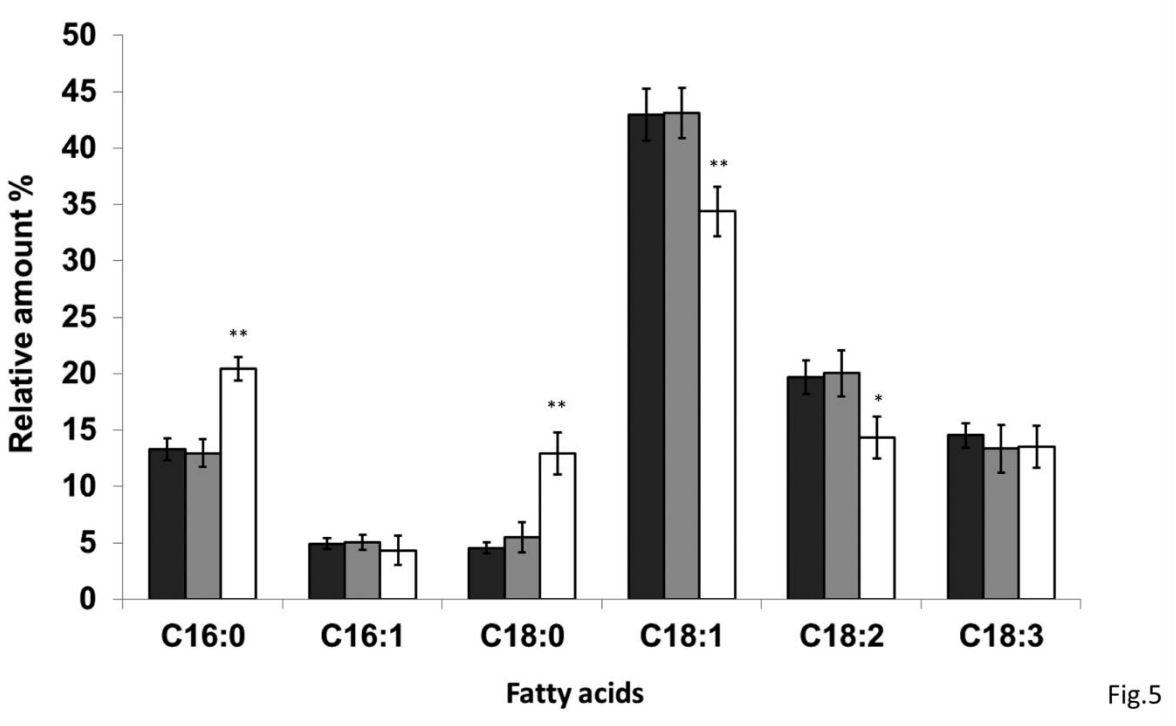

Fig. 5 Fatty acid composition of triacylglycerols from *dga1*Δ and *lro1*Δ.

TG from wild type (black bars), *dga1*Δ (grey bars) and *lro1*Δ (white bars) strains grown on YPD to the early stationary phase were analyzed for their fatty acid composition as described in the methods section. The amounts of individual fatty acids are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate the standard deviation.

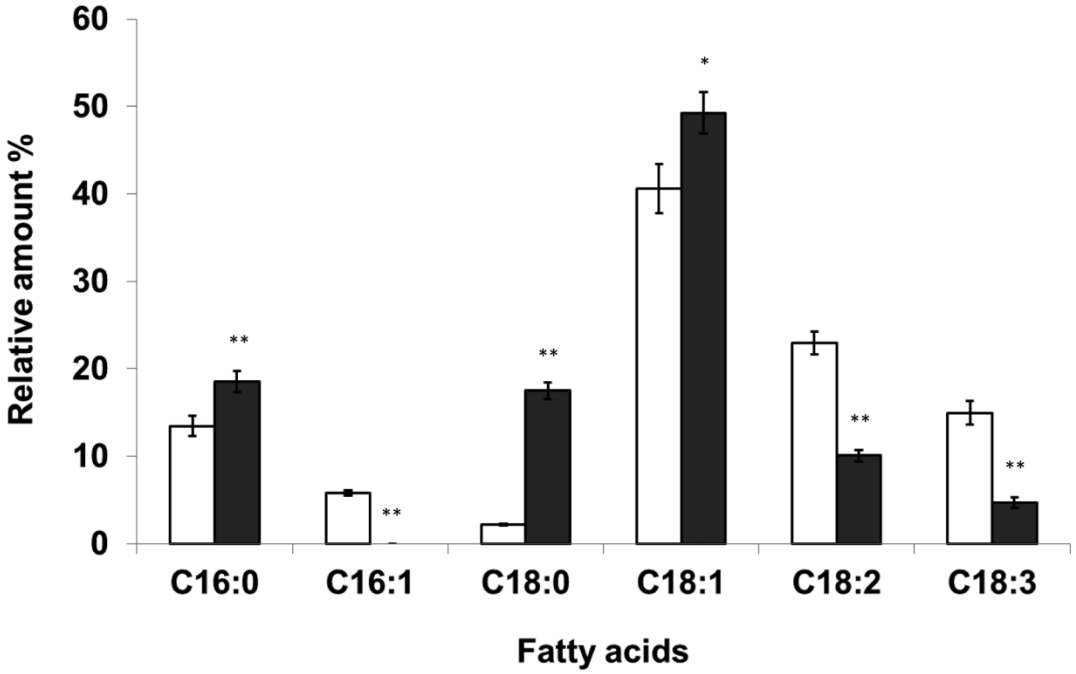

Fig.6

Fig. 6 Fatty acid composition of steryl esters from wild type.

P. pastoris wild type was grown on YPD to the early stationary phase. Total cellular lipids (white bars) and SE (black bars) were analyzed for their fatty acid composition. Amounts of individual fatty acids are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate the standard deviation.

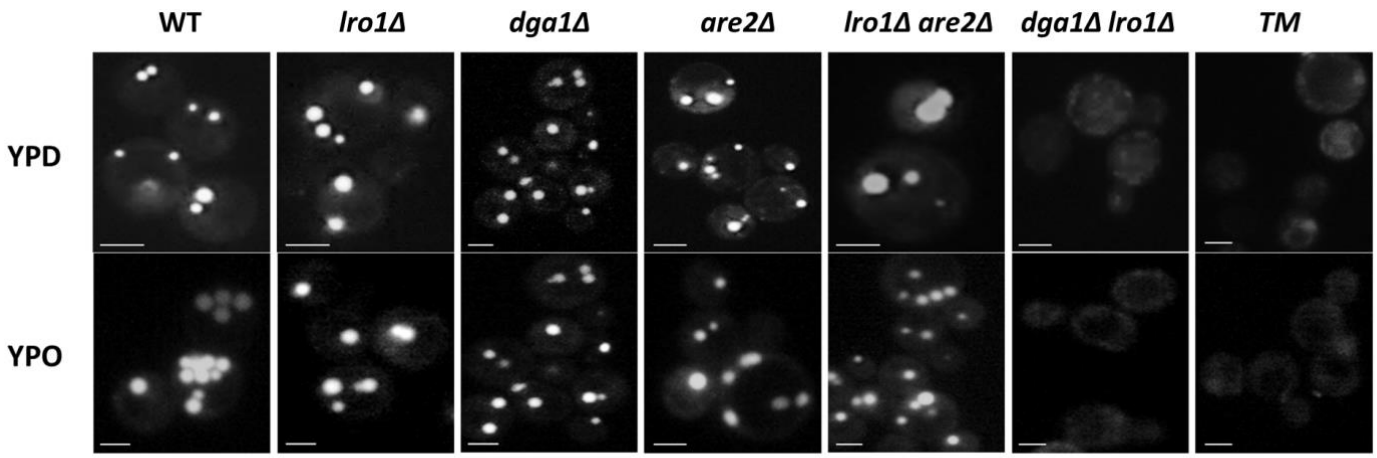

Fig.7

Fig. 7 Fluorescence microscopy of *P. pastoris* strains compromised in non-polar lipid synthesis.

Wild type (WT), single mutants (*lro1*Δ*, dga1*Δ*, are2*Δ), double mutants (*lro1*Δ*are2*Δ and *dga1*Δ*lro1*Δ) and triple mutant (TM, *dga1*Δ*lro1*Δ*are2*Δ) were grown on YPD or YPO at 30° for 26 hours, stained with AC-201 thalidamide analog and subjected to fluorescent microscopy as described in the methods section. Green fluorescence highlights lipid droplets. Scale bars, 2.5 µm.

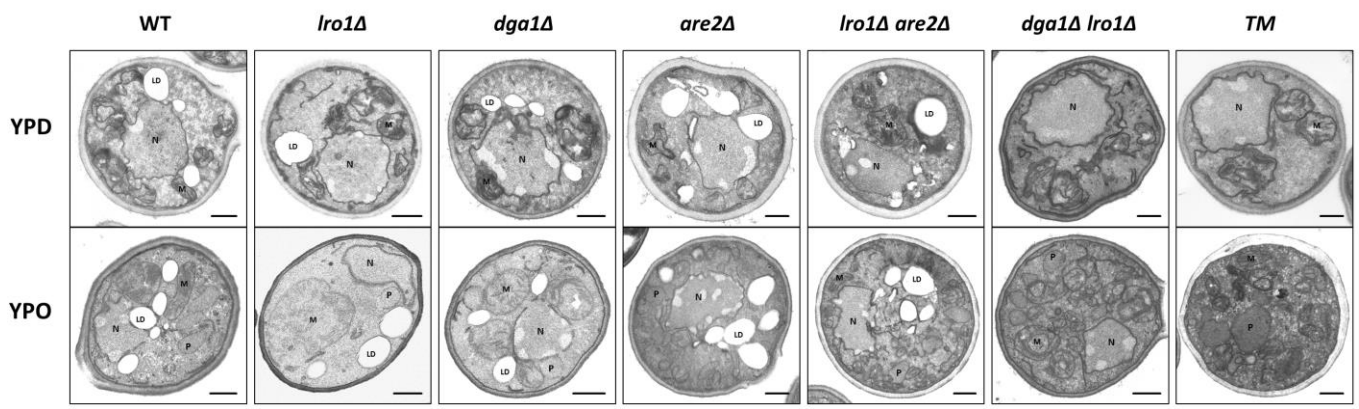

Fig. 8 Electron microscopy of *P. pastoris* strains compromised in non-polar lipid synthesis.

Pictures show electron micrographs of $KMnO_4$-fixed wild type (WT), single mutants (*lro1*Δ*, dga1*Δ*, are2*Δ), double mutants (*lro1*Δ*are2*Δ and *dga1*Δ*lro1*Δ) and the triple mutant (TM, *dga1*Δ*lro1*Δ*are2*Δ) grown on YPD or YPO, respectively, at 30° to the early stationary phase. Details of the experimental procedures are described in the methods section. *Abbreviations indicate specific organelles: LD- lipid droplets, M- mitochondria, P- peroxisomes, N- nucleus.* Scale bars, 0.5 µm.

Appendix 1

Non-polar lipid hydrolytic enzymes in *Pichia pastoris*

(A preliminary investigation)

1. Introduction

The major storage lipids in yeast and particularly in *P. pastoris* are non-polar triacylglycerols (TGs) and steryl esters (SEs). Under normal conditions, TG and SE are stored in specific compartments called lipid droplets (LD). The ability to convert an excess of sterols and free fatty acids to their biological inert forms of TG and SE is essential for preventing detrimental effects of their accumulation termed lipotoxicity. Non-polar lipids can be mobilized upon demand to provide energy or constituents required for the formation of new membranes. Products of TG mobilization, namely fatty acids, diacylglycerols (DG) can be as well used for the synthesis of membrane phospholipids and other complex lipids. Fatty acids, diacylglycerols and sterols formed upon non-polar lipid degradation and their metabolic derivatives are also important signaling molecules [1–3].

The initial step of such a mobilization is hydrolysis of TG and SE which is catalyzed by conserved family of enzymes called lipases and hydrolases [4]. Cleavage of TG ester bonds is performed by TG lipases in sequential steps resulting in the production of free fatty acids, DG, monoacylglycerol (MG) and finally glycerol. Mammalian lipases have distinct substrate specificities. TG are mainly hydrolyzed by adipose triglyceride lipase [5], DG by hormone sensitive lipase [6], and MG by monoglyceride lipase [7]. Yeast lipases are less specific, but the mechanism of lipolysis is evolutionarily conserved in eukaryotes [4]. Almost all known lipases from different organisms share the highly conserved motif GXSXG where the serine residue is essential for the catalytic activity [8,9]. Many TG lipases also contain a so-called patatin domain [10]. In the model yeast *S. cerevisiae* degradation of TG is mainly catalyzed by the three homologous gene products of *TGL3*, *TGL4* and *TGL5*. These lipases are located to LD [11,12] and perform TG hydrolysis at the aqueous/non-aqueous interface [13,14]. Tgl3p has a broad substrate specificity hydrolyzing TG and DG with different acyl moieties; Tgl4p hydrolyzes TG and has a preference for palmitic and myristic acid containing species; and Tgl5p preferentially uses TG containing C26:0 as a substrate [12,15]. Efficient mobilization of TG was reported to be linked to the cell cycle onset [16]. Tgl3p and Tgl5p also harbor lysophospholipid acyltransferase activity which is required for sporulation. Tgl4p additionally possesses SE hydrolase and phospholipases A2 (PLA2) activities and therefore participates as a multifunctional enzyme in different aspects of lipid metabolism [17]. Steryl ester hydrolysis in *S. cerevisiae* is performed by three SE hydrolases, namely Yeh1p, Yeh2p and Tgl1p. Tgl1p and Yeh1p are located to LD, and Yeh2p is found in the plasma membrane

[18–20]. Tgl1p has also low TG lipase activity *in vitro* [21]. Yeh1p is essential for mobilization of SE under anaerobic conditions [22].

In *Pichia pastoris* the non-polar lipid mobilization was not studied so far. There are several genes annotated in the *Pichia* genome database [23] predicted to encode lipase genes. Genes and their products annotated as *TGL1* (UniProtKB/TrEMBL entry: F2QWY8, Triglyceride lipase-cholesterol esterase), *TGL3* (two annotations depending on the strain database; UniProtKB/TrEMBL entry: F2QTH7 Patatin-like phospholipase domain-containing protein NFIA_019760 or alternatively UniProtKB/TrEMBL entry: C4R069, Triacylglycerol lipase of the lipid particle, responsible for all the TAG lipase activity of the lip) and Esterase (UniProtKB/TrEMBL entry: F2QSY4) which is the closest *Pichia* homolog of *S. cerevisiae TGL4* are predicted to have TG lipase and/or hydrolase activity. The only gene annotated in the GS115 strain genome sequence coding for a predicted SE hydrolase (UniProtKB/TrEMBL entry: C4QW07, Steryl ester hydrolase one of three gene products (Yeh1p, Yeh2p, Tgl1p)) is the closest *Pichia* homolog of *S. cerevisiae YEH1* and *YEH2*. Interestingly, this gene product is annotated as well as Lipf (UniProtKB/TrEMBL entry: F2QM82, Gastric triacylglycerol lipase) in the CBS7435 strain genome sequence, but has exactly the same chromosomal location, identical sequence and predicted TG lipase activity. This fact raises an ambiguity of having two different predicted annotated proteins originating from the same DNA sequence. Moreover, all these genes have similar levels of homology between each other and to *S. cerevisiae* lipases and hydrolases.

Noteworthy the proteomic study of *Pichia* lipid droplets (chapter 4 of this thesis) revealed only two proteins with predicted lipase/hydrolase activity localized to this compartment, namely Tgl1p (UniProtKB/TrEMBL entry: F2QWY8, Triglyceride lipase-cholesterol esterase) and Steryl ester hydrolase (C4QW07), one of the three gene products (Yeh1p, Yeh2p, Tgl1p) which will be further named YEH1 in this report. Taking into account all aforementioned facts it is difficult to affiliate each gene to a certain function based on bioinformatics annotation only.

Thus, the current study was aimed to identify genes encoding major non-polar lipid hydrolyses in *Pichia pastoris*. To identify the in vivo function of predicted TG lipases or SE hydrolases we generated a set of mutant strains bearing deletions of one or more of the annotated lipases. The obtained single mutants *tgl1*Δ*, tgl3*Δ and *yeh1*Δ*,* and double mutants *tgl1*Δ*yeh1*Δ and *tgl3*Δ*yeh1*Δ were analyzed for their non-polar lipid composition. The

obtained results are preliminary and a more detailed study of the role of these genes products in non-polar lipid turnover and general cellular lipid metabolism will be a task for further investigations.

2. Experimental procedures

2.1. Strains and culture conditions

The *P. pastoris* strain CBS7435*ku70his4* (MATa, Mut^+, His^-) (kindly provided by A. Glieder) [24] considered as wild type and its derivative strains *tgl1*Δ (MATa, Mut^+, His^-), *tgl3*Δ (MATa, Mut^+, His^-), *yeh1*Δ (MATa, Mut^+, His^-), *tgl1*Δ*yeh1*Δ (MATa, Mut^+, His^-), *tgl3*Δ*yeh1*Δ (MATa, Mut^+, His^-) were used throughout this study. YPD media (1% yeast extract, 2% peptone and 2% glucose) were inoculated to a starting OD_{600} of 0.1 from precultures grown aerobically for 48 h in YPD at 30 °C. Cells were grown under aerobic conditions to the early stationary phase (26 h) at 30 °C.

2.2. Strain construction

Genomic DNA isolation procedure: DNA from wild type *Pichia pastoris* CBS7435*ku70his4* was isolated using the Yeast DNA Kit (Omega bio-tek) and was used for applications described below. Construction of non-polar lipid hydrolases deletion mutant strains was based on the gene deletion strategy described by A. Wach [25]; and the *Pichia* knockout strategy developed by Ahmad et al. (manuscript in preparation). All primers designed are listed in the Table 1.

Construction of the tgl1Δ *strain:* The *TGL1* gene was deleted using the *Pichia* knockout strategy developed by Ahmad et al. (manuscript in preparation). The 5' prime upstream flanking region DNA sequence was chosen to start with a GGG repeat at the position 829 bp upstream the start codon and finish at 31 bp upstream the *TGL1* start codon, and was

amplified from the isolated genomic DNA using primers P1_TGL1_F and P2_TGL1_R. The 3' prime downstream flanking region (764 bp) was amplified starting directly after the stop codon and finishing with a CCC repeat using primers P3_TGL1_F and P4_TGL1_R. Primers P2_TGL1_R and P3_TGL1_F were designed to have specific 13 nucleotide overlaps containing the SfiI cleavage sequence on their 5' prime ends, each, of those identical to corresponding sites of the pPpKC3 vector plasmid. The primer P1_TGL1_F was designed to have a 5' prime overlap identical to the last 18 bases of 3' prime flanking region and P4_TGL1_R to have a 5' prime overlap identical to the first 15 bases of the 5' flanking region. The ends of the resulting flanking regions had 33 base pairs overlap which allowed their fusion with overlap extension PCR using outermost primers P2_TGL1_R and P3_TGL1_F and Phusion high fidelity polymerase (Thermo Scientific). The 3'-5' flanking region fusion product (1593 bp) was gel purified (NucleoSpin kit, Macherey-Nagel) and cut with SfiI (Thermo Scientific). The cut fusion product was cloned within identical SfiI sites of the vector plasmid pPpKC3 which has the *HIS4* gene under *ARG4* promoter as a selection marker for the histidine auxotrophy and FLP recombinase (flippase) [26,27] system under *AOX1* promoter control. The construct was transformed into the *E. coli* Top10 strain, isolated from the bacterial culture at high amount and confirmed by sequencing using two sequencing primers PucSeqF and PAox1SeqR. The final construct was linearized by SmaI (Thermo Scientific) which recognizes GGGCCC set up sequence and cut exactly between two flanking regions. 500 ng of the linearized plasmid were used for transformation of 50 µl of *P. pastoris* strain CBS7435*ku70his4* [24] electrocompetent cells prepared as described previously [28], plated on the MMD media agar plates (1,34% yeast nitrogen base, 4 x 10-5% biotin, 2% glucose, 2% agar, without amino acids) and incubated for 2 days at 30°C. Single colony forming units were inoculated to 2 ml fresh MMD media in 24 deep-well plates and grown overnight shaking at 30°C. The genomic DNA was isolated from each transformant's overnight culture and checked for the presence of the *TGL1* gene sequence with the primer pair TGL1_Fwd, TGL1_Rev. Transformants whose DNA preparations did not show the presence of *TGL1* were checked further for the vector integration place. For the 5' prime integration control primer binding upstream of 5' prime flanking region 5f-1075_TGL1_Fwd, and second primer PAox1SeqR which binds inside the *AOX1* promoter region of the vector backbone was used. For 3' prime integration control the primer binding downstream of the 3' flanking region 3f+994_TGL1_Rev and the second primer PucSeqF which binds inside the Puc origin of the vector backbone were used. The transformants which showed the correct product size corresponding to both 5' and 3' prime integration events were considered as

confirmed *tgl1*Δ mutant strain. The pPpKC3 vector was recycled from the chromosome by growing the obtained strain in YPM media (1% yeast extract, 2% peptone and 2% methanol). Methanol induced expression of the FLP recombinase (flippase) system led to the excision of the whole construct from the chromosomal DNA by flippase, leaving short fragment containing SfiI sites and flippase recognition target sequence between used flanking regions of the *TGL1* gene. The methanol induced culture was plated on the fresh YPD agar plate and colonies appearing were plated to MMD (and YPD as a backup) agar plates to check for the inability to grow on histidine deficient media. Clones which did not grow were considered as histidine negative transformants with recycled pPpKC3 vector and used for further deletions based on *HIS4* supplementation and selection. The final strain with recycled marker was considered confirmed CBS7435*ku70his4tgl1* (MATa, Mut^{+}, His^{-}).

Construction of the tgl3Δ *strain:* The *TGL3* gene deletion cassette was constructed using the overlap extension (OE) PCR strategy for the deletion of most of the *TGL3* gene (nucleotides 16-1953 of the interpreted gene open reading frame at the *Pichia pastoris* GS115 strain genome sequence: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=full_report&list_uids=8198828). Long 5' (1047 bp) and 3' (1265 bp) flanking regions of *TGL3* gene were amplified from genomic DNA using primers 5'f_TGL3_F-1032, 5'f_Tgl3_R+15 and 3'f_Tgl3_Fwd, 3'f_TGL3_R+1157, respectively. Primers 3'f_Tgl3_Fwd and 5'f_Tgl3_R+15 had 28 base pairs added to produce overlaps with the core selection marker element. Then, flanking regions were fused with the selection marker element (Zeocin resistance gene together with its promoter and terminator parts, 1194 bp) from pPICz/B plasmid (Invitrogen) in one OE PCR with the outermost primers 5'f_TGL3_F-1032 and 3'f_TGL3_R+1157, resulting in the *TGL3* deletion cassette (3506 bp). After column spin purification, 1 µg of the cassette was used for transformation of 50 µl of *P. pastoris* CBS7435*ku70his4* electrocompetent cells, plated on YPD media agar plates containing 100 µg/ml zeocin and incubated for 2 days at 30°C. Single colony forming units were inoculated into 2 ml fresh YPD media with 100 µg/ml zeocin in 24 deep-well plates and grown overnight shaking at 30°C. The genomic DNA was isolated from each transformant's overnight culture and checked for the presence of the *TGL3* partial gene sequence with the primer pair: TGL3_F+19 and TGL3_R+1949. Transformants whose DNA preparations did not show the presence of the corresponding part of the *TGL3* gene were further checked for the cassette integration place. For the 5' prime integration, a control primer binding upstream of the 5' prime flanking region 5'f_TGL3-1156, and the second primer Zeo_Rev which is

binding in the end of the zeocin resistance gene were used. Transformants which showed the correct product size corresponding to the predicted 5' integration event were considered as confirmed CBS7435*ku70his4tgl3*Δ (MATa, Mut^{+}, His^{-}).

Construction of yeh1Δ *strain:* For deletion of the full *YEH1* gene the cassette was constructed based on the OE PCR strategy. Long 5' upstream (871 bp) and 3' downstream (768 bp) flanking regions of *YEH1* gene were amplified from genomic DNA using primers 5f-904YEH1_Fwd, 5f-32YEH1_Rev+ and 3fYEH1+1_Fwd+, 3f+768YEH1_Rev, respectively. Primers 5f-32YEH1_Rev+ and 3fYEH1+1_Fwd+ had 28 base pairs added to produce overlaps with core selection marker element. Then, flanking regions were fused with a selection marker element (Zeocin resistance gene together with its promoter and terminator parts, 1194 bp) from pPICz/B plasmid (Invitrogen) in one OE PCR with the outermost primers 5f-904YEH1_Fwd and 3f+768YEH1_Rev, resulting in the *YEH1* deletion cassette (2833 bp). After column spin purification, 1 µg of the cassette was used for transformation of 50 µl of *P. pastoris* CBS7435*ku70his4* electrocompetent cells, plated on the YPD media agar plates containing 100 µg/ml zeocin and incubated for 2 days at 30°C. Single colony forming units were inoculated into 2 ml fresh YPD media with 100 µg/ml zeocin in 24 deep-well plates and grown overnight shaking at 30°C. The genomic DNA was isolated from each transformant's overnight culture and checked for the presence of the *YEH1* gene sequence with the primer pair YEH1_Fwd, YEH1_Rev. The transformants whose DNA preparations did not show the presence of the *YEH1* gene were further checked for the cassette integration place. For the 5' prime integration, a control primer binding upstream of the 5' prime flanking region 5f-1001-YEH1_Fwd, and a second primer Zeo_Rev binding in the end of the zeocin resistance gene were employed. For the 3' prime integration event a primer binding at the beginning of zeocin resistance gene - Zeo_Fwd and a primer binding downstream of the 3' prime flanking region - 3f+885-YEH1_Rev were used. The transformants which showed correct product size corresponding to the predicted 5' and 3' prime integration events were considered as confirmed CBS7435*ku70his4yeh1*Δ (MATa, Mut^{+}, His^{-}).

Construction of double deletion strains: Double deletion strains *tgl1*Δ *yeh1*Δ and *tgl3*Δ*yeh1*Δ were constructed as follows. The single deletion strains *tgl1*Δ and *tgl3*Δ were used as background strains for the deletion of the *YEH1* gene. The *yeh1* deletion strategy was alternative to one used for the *yeh1* single deletion mutant construction. Here, the *Pichia* knockout strategy based on pPpKC3 plasmid vector was employed (see *TGL1* deletion paragraph) following the same designing strategy. Primers P1-YEH1_F, P2_YEH1_R were

used to amplify the 5' prime upstream flanking region (848 bp) starting with GGG motif and spanning from 848 bp upstream of the *YEH1* open reading frame to the start codon. Primers P3-YEH1-F, P4-YEH1-R were used to amplify the 3' prime downstream flanking region (753 bp) starting directly after the stop codon of the *YEH1* gene and ending with a CCC motif. Primers P2_YEH1_R , P3-YEH1-F were designed to have specific 13 nucleotide overlaps containing an SfiI cleavage sequence on their 5' prime ends, each, of those identical to corresponding sites of the pPpKC3 vector plasmid. The primers P1-YEH1_F and P4-YEH1-R were designed to have correspondingly 16 and 15 bases 5' prime overlaps which together made 31 bases overlap allowing further fusion of both flanking regions by OE PCR using the outermost primers P2_YEH1_R , P3-YEH1-F. The 3'-5' flanking regions fusion product (1593 bp) was gel purified (NucleoSpin kit, Macherey-Nagel) and cut with SfiI (Thermo Scientific). The cut fusion product was cloned within identical SfiI sites of the vector plasmid pPpKC3. The construct was amplified in the *E. coli* Top10 strain and confirmed by sequencing using two sequencing primers PucSeqF and PAox1SeqR. The final construct was linearized by SmaI (Thermo Scientific). 500 ng of linearized plasmid was used for transformation of 50 µl of electrocompetent cells of *P. pastoris tgl1*Δ and *tgl3*Δ strains, each, obtained as described above. Transformants were selected on MMD media agar plates by the ability to grow without histidine supplementation. Selected transformants were inoculated into 2 ml fresh MMD media in 24 deep-well plates and grown overnight shaking at 30°C. The genomic DNA was isolated from each transformant's overnight culture and checked for the presence of the *YEH1* gene sequence by PCR with the primer pair YEH1_Fwd, YEH1_Rev. *YEH1* negative transformants were further checked for the proper construct integration. The 5' prime integration event was confirmed by PCR with primer 5f-1001-YEH1_Fwd which binds upstream of the used 5' prime flanking region and the primer PAox1SeqR which binds inside the *AOX1* promoter region of the vector backbone. The 3' prime integration event was confirmed by PCR with primer 3f+885-YEH1_Rev which binds downstream of the used 3' prime flanking region, and the primer PucSeqF which binds inside the Puc origin of the vector backbone. The PCR reactions yielding the anticipated fragment sizes for both reactions served as a confirmation of the deletion of *YEH1* open reading frame in corresponding transformants. The vector was then recycled by methanol induction, and *HIS4* negative transformants were selected as described above. The final double deletion strains obtained were as follows: CBS7435*ku70his4_tgl1*Δ*yeh1*Δ (MATa, Mut^+, His^-) and CBS7435*ku70his4_tgl3*Δ*yeh1*Δ (MATa, Mut^+, His^-).

2.3. Non-polar lipid analysis

Lipids were extracted as described by Folch et al. [29]. Dried lipids were dissolved in $CHCl_3$/Methanol, separated by TLC and quantified by scanning as described in Chapter 2.

3. Results and discussion

3.1. Deletion of putative non-polar lipid hydrolases

Here, the approach is presented which was used for the identification of genes and gene products involved in non-polar lipid hydrolysis. The bioinformatics analysis revealed four candidate genes homologous to *S. cerevisiae TGL1, TGL3, TGL4* and *YEH1*. To identify *in-vivo* function of these gene products we aimed to generate a full set of deletion mutants and analyze their non-polar lipids under different growth conditions. Currently, three single deletion mutant strains *tgl1*Δ*, tgl3*Δ and *yeh1*Δ and two double mutants *tgl1*Δ*yeh1*Δ and *tgl3*Δ*yeh1*Δ are available. Deletion of the *TGL4* gene was not achieved applying procedures described here and other methods, even by screening more than 500 transformants. The work on generation of other multiple deletion mutants is currently in progress.

3.2. Analysis of non-polar lipids

A preliminary analysis of non-polar lipids from the generated mutant strains grown under standard conditions (Fig. 1) was performed. All analyzed mutants had similar amounts of TG as a wild type strain. Only single and double deletion mutants lacking *TGL1* showed a significant increase in the amount of SE. Thus, Tgl1p is a good candidate for as a SE hydrolase from *P. pastoris*. The other deletion strains had similar amounts of SE as wild type. The analysis of non-polar lipids from deletion mutants cultivated under different growth conditions is in progress.

3.3. Perspectives

For the assessment of the in vivo function of the aforementioned gene products a more rational experimental setup should be designed where cells are forced to mobilize storage lipids. Such conditions can be generated by manipulating growth conditions inducing cell starvation or by using drugs inhibiting fatty acid synthesis. Lipids from deletion strains grown under such conditions can be further analyzed. However, we should not exclude the possibility that one or more of the annotated gene products are not non-polar lipid hydrolases, whereas other genes with unknown or incorrectly annotated function might be indeed encode for lipases or SE hydrolases in *P. pastoris*.

Alternatively, and taking into account difficulties of gene deletions in *P. pastoris*, the overexpression of identified *Pichia* genes in lipases/hydrolases deficient *S. cerevisiae* strains and measuring non-polar lipid amounts and lipase/hydrolase activity can be helpful for the identification of their function. Overexpression of the aforementioned genes in *P. pastoris* and subsequent analysis of non-polar lipid amounts can be another way to identify their function. Moreover, overexpression and purification of the putative *Pichia* non-polar lipid hydrolases and their in vitro activity analysis can be an alternative approach.

Conclusively, work on the identification of *P. pastoris* non-plar lipid hydrolases has been initiated. Objectives are defined and the experimental set-up was designed. Preliminary indicate, however, that homologies as deduced from *S. cerevisiae* may not be correct for *P. pastoris*. Thus, biochemical experiments will be important to prove or disprove the identity of the gene products under investigation.

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References

[1] K. Athenstaedt, G. Daum, The life cycle of neutral lipids: synthesis, storage and degradation, Cell. Mol. Life Sci. CMLS. 63 (2006) 1355–1369.

[2] K. Grillitsch, G. Daum, Triacylglycerol lipases of the yeast, Front. Biol. 6 (2011) 219–230.

[3] S.D. Kohlwein, Triacylglycerol Homeostasis: Insights from Yeast, J. Biol. Chem. 285 (2010) 15663–15667.

[4] S. Rajakumari, K. Grillitsch, G. Daum, Synthesis and turnover of non-polar lipids in yeast, Prog. Lipid Res. 47 (2008) 157–171.

[5] R. Zimmermann, J.G. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-Gruenberger, M. Riederer, et al., Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase, Science. 306 (2004) 1383–1386.

[6] J. Osuga, S. Ishibashi, T. Oka, H. Yagyu, R. Tozawa, A. Fujimoto, et al., Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 787–792.

[7] R. Zimmermann, A. Lass, G. Haemmerle, R. Zechner, Fate of fat: the role of adipose triglyceride lipase in lipolysis, Biochim. Biophys. Acta. 1791 (2009) 494–500.

[8] L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, et al., A serine protease triad forms the catalytic centre of a triacylglycerol lipase, Nature. 343 (1990) 767–770.

[9] J.D. Schrag, M. Cygler, Lipases and alpha/beta hydrolase fold, Methods Enzymol. 284 (1997) 85–107.

[10] G.A. Mignery, C.S. Pikaard, W.D. Park, Molecular characterization of the patatin multigene family of potato, Gene. 62 (1988) 27–44.

[11] K. Athenstaedt, G. Daum, YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of Saccharomyces cerevisiae, J. Biol. Chem. 278 (2003) 23317–23323.

[12] K. Athenstaedt, G. Daum, Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast Saccharomyces cerevisiae are localized to lipid particles, J. Biol. Chem. 280 (2005) 37301–37309.

[13] R.D. Schmid, R. Verger, Lipases: Interfacial Enzymes with Attractive Applications, Angew. Chem. Int. Ed. 37 (1998) 1608–1633.

[14] R. Verger, "Interfacial activation" of lipases: facts and artifacts, Trends Biotechnol. 15 (1997) 32–38.

[15] C.F. Kurat, K. Natter, J. Petschnigg, H. Wolinski, K. Scheuringer, H. Scholz, et al., Obese yeast: triglyceride lipolysis is functionally conserved from mammals to yeast, J. Biol. Chem. 281 (2006) 491–500.

[16] C.F. Kurat, H. Wolinski, J. Petschnigg, S. Kaluarachchi, B. Andrews, K. Natter, et al., Cdk1/Cdc28-dependent activation of the major triacylglycerol lipase Tgl4 in yeast links lipolysis to cell-cycle progression, Mol. Cell. 33 (2009) 53–63.

[17] S. Rajakumari, G. Daum, Multiple functions as lipase, steryl ester hydrolase, phospholipase, and acyltransferase of Tgl4p from the yeast Saccharomyces cerevisiae, J. Biol. Chem. 285 (2010) 15769–15776.

[18] E. Zinser, F. Paltauf, G. Daum, Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism, J. Bacteriol. 175 (1993) 2853–2858.

[19] S. Taketani, T. Osumi, H. Katsuki, Characterization of sterol-ester hydrolase in Saccharomyces cerevisiae, Biochim. Biophys. Acta. 525 (1978) 87–92.

[20] H. Müllner, G. Deutsch, E. Leitner, E. Ingolic, G. Daum, YEH2/YLR020c encodes a novel steryl ester hydrolase of the yeast Saccharomyces cerevisiae, J. Biol. Chem. 280 (2005) 13321–13328.

[21] A. Jandrositz, J. Petschnigg, R. Zimmermann, K. Natter, H. Scholze, A. Hermetter, et al., The lipid droplet enzyme Tgl1p hydrolyzes both steryl esters and triglycerides in the yeast, Saccharomyces cerevisiae, Biochim. Biophys. Acta. 1735 (2005) 50–58.

[22] R. Köffel, R. Schneiter, Yeh1 constitutes the major steryl ester hydrolase under heme-deficient conditions in Saccharomyces cerevisiae, Eukaryot. Cell. 5 (2006) 1018–1025.

[23] A. Küberl, J. Schneider, G.G. Thallinger, I. Anderl, D. Wibberg, T. Hajek, et al., High-quality genome sequence of Pichia pastoris CBS7435, J. Biotechnol. 154 (2011) 312–320.

[24] L. Näätsaari, B. Mistlberger, C. Ruth, T. Hajek, F.S. Hartner, A. Glieder, Deletion of the Pichia pastoris KU70 homologue facilitates platform strain generation for gene expression and synthetic biology, PloS One. 7 (2012) e39720.

[25] A. Wach, PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae, Yeast. 12 (1996) 259–265.

[26] J.R. Broach, V.R. Guarascio, M. Jayaram, Recombination within the yeast plasmid 2μ circle is site-specific, Cell. 29 (1982) 227–234.

[27] O. Reuss, A. Vik, R. Kolter, J. Morschhäuser, The SAT1 flipper, an optimized tool for gene disruption in Candida albicans, Gene. 341 (2004) 119–127.

[28] J. Lin-Cereghino, W.W. Wong, S. Xiong, W. Giang, L.T. Luong, J. Vu, et al., Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris, BioTechniques. 38 (2005) 44, 46, 48.

[29] J. FOLCH, M. LEES, G.H. SLOANE STANLEY, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.

Tables

Table 1. List of primers used in this study.

Primer name	Sequence from 5′ to 3′ end	Features
P1_TGL1_F	GAGCCCGACAGTGTTCCCGGGACACATACTCTCACAA GTTGTGTC	Overlap with 3' flanking region, SmaI site
P2_TGL1_R	ATGGCCCTAGTGGCCGAATATATTTGCATCTCTACAT TCC	SfiI site
P3_TGL1_F	AAGGCCGATCAGGCCATAAAGAGTGCTTGATACCAG TC	SfiI site
P4_TGL1_R	GAGAGTATGTGTCCCGGGAACACTGTCGGGCTCTAGC TCTG	Overlap with 5' flanking region, SmaI site
5f-1075_TGL1_Fwd	TCTTGGAGCATGAAGTTAAGGCTGG	5' integration check primer
PAox1SeqR	GGTTTCATTCAACCTTTCGTCTTTGGATG	Sequencing and 5' integration check primer
PucSeqF	CTTTTTACGGTTCCTGGCCTTTTGC	Sequencing and 3' integration check primer
3f+994_TGL1_Rev	GAGATGGAAAATGCAAAGAGTGGC	3' integration check primer
TGL1_Fwd	ATGTCATCTCCCCATAGGAAAAGAAG	*TGL1* ORF check primer
TGL1_Rev	TTATGTATTTATTATAATGGATTTTCCGC	*TGL1* ORF check primer
5'f_TGL3_F-1032	CATTCTCTCATAGACCCAGTAGTCATCGG	–

Primer name	Sequence from 5′ to 3′ end	Features
5'f_Tgl3_R+15	GTGTGTGGGGGATCCGCACAAACGAAGGCCAACACG TCTTCATCTTCTCCCCA	Overlap with TEF promoter start
3'f_Tgl3_Fwd	CGCTCGAAGGCTTTAATTTGCAAGCTGGCTGGTGGGA GAACGCAGTGTCTGGCC	Overlap with *CYC* TT end
3'f_TGL3_R+1157	TACGGGTGTGGCCGAGACCATAAACC	–
5'f_TGL3-1156	TGGCGGTTTCAAGGGGTAAATACACG	5' integration check primer
Zeo_Rev	TCAGTCCTGCTCCTCGGCCACGAAGTGCACGC	Binds in the end of zeocin resistance gene
TGL3_F+19	ACCGGGCCATCTCAAGGTCTATGG	*TGL3* ORF check primer
TGL3_R+1949	CAGTATGGAATATTCTCGTTGGACC	*TGL3* ORF check primer
5f-904YEH1_Fwd	GCTTTTCTTCTTCTCTCAATACCAG	–
5f-32YEH1_Rev+	GTGTGTGGGGGATCCGCACAAACGAAGGCGGCTGAG ACCACAGATTCAGATTGGG	Overlap with TEF promoter start
3fYEH1+1_Fwd+	CGCTCGAAGGCTTTAATTTGCAAGCTGGTGACAGATT ATAATAGAAGTTCTG	Overlap with *CYC* TT end
3f+768YEH1_Rev	TTGATTCCTCCTCCTGGGACTGG	–
YEH1_Fwd	GATAATACAACAAATGACTAAAGCC	*YEH1* ORF check primer
YEH1_Rev	CTGTATCGTTGCATATCTTTGAGG	*YEH1* ORF check primer
5f-1001-YEH1_Fwd	CTGGCCGTGAGACGATTCCACGG	5' integration check primer
3f+885-YEH1_Rev	GGTCAAAGAGAAGGATAAGCATTGCC	3' integration check primer

Appendix 1

Primer name	Sequence from 5′ to 3′ end	Features
Zeo_Fwd	ATGGCCAAGTTGACCAGTGCCGTTCC	Binds at the beginning of zeocin resistance gene
P1-YEH1_F	GACCAATCCAGTCCCGGGTTCCTCCAAAGGCTCCATACAATCC	Overlap with 3' flanking region, SmaI site
P2_YEH1_R	TTGGCCCTAGTGGCCGTGTTTACGCTGAACTGTAGGCAG	SfiI site
P3-YEH1-F	AAGGCCGATCAGGCCTGACAGATTATAATAGAAGTTCTGTAAC	SfiI site
P4-YEH1-R	GCCTTTGGAGGAACCCGGGACTGGATTGGTCTTGGGAGAG	Overlap with 5' flanking region, SmaI site

Figures

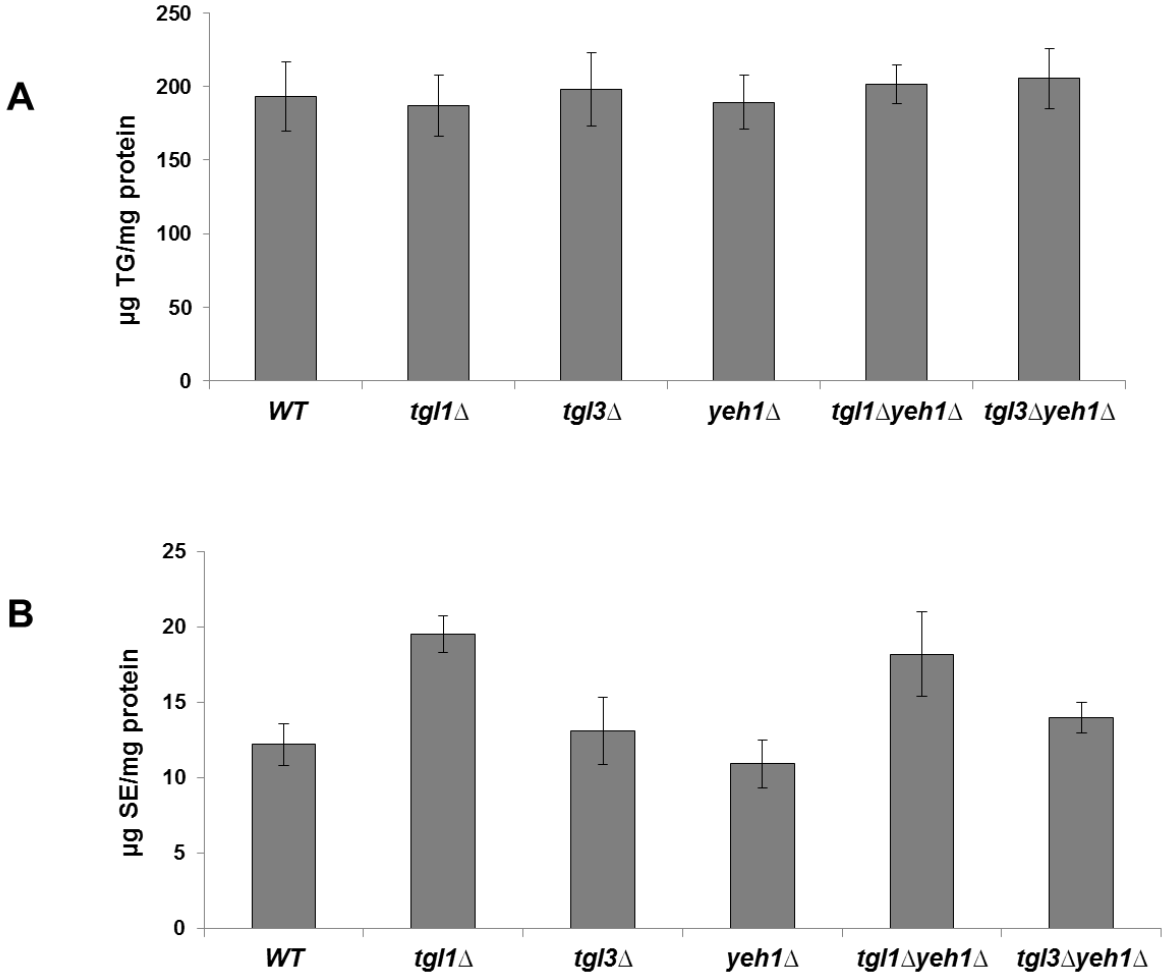

Fig.1 Non-polar lipid composition of strains lacking putative non-polar lipid hydrolases.

Cells were grown to the early stationary phase in YPD. Lipids were extracted from cell homogenates and analyzed as described in experimental procedures. A–Triacylglycerol and B–Steryl ester (SE) amounts in wild type (WT) and deletion mutants. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

Appendix 2

Isolation and lipid analysis of vacuoles from *Pichia pastoris*

(A preliminary investigation)

1. Introduction

Yeast vacuoles are the largest cell organelles with acidic interior and have many similarities to mammalian lysosomes and plant vacuoles [1]. The vacuole is surrounded by a single membrane, where V-ATPase1 complex resides which acidifies the interior of the vacuole resulting in a difference of 1.7 pH units between the vacuolar lumen and the cytosol. This difference renders the driving force for a substrate-proton antiport systems located to the vacuolar membrane [2]. Transport across the vacuolar membrane allows storage of organic molecules such as polyphosphates and carbohydrates [3,4]; detoxification from heavy metals and drugs; and proton and ion homeostasis [5]. The other major function of vacuoles is the proteolysis of cellular proteins [6,7] and turnover of organelles [8–12] implemented by the presence of proteases, lipases, phosphatases and nucleases in this compartment [5].

The yeast vacuole harbors a set of resident proteins, a specific ionic medium, and a distinct membrane lipid composition [1]. Two hundred of the 6,000 yeast ORFs (www.yeastgenome.org) are characterized by the gene ontology (GO) term "vacuolar localization" [13]. 27 % of the GO annotated vacuolar proteins have transporter functions participating in import/export of amino acids [14], metals [15], and glutathione conjugates [16] underlining the importance of this compartment in general cellular homeostasis. Vacuoles are enriched in proteases and other hydrolases [17], along with autophagy-related proteins, and proteins participating in vacuolar protein targeting and vacuole fusion [18,19].

The vacuolar membrane has its specific lipid profile. The yeast vacuolar membrane has a remarkably low ergosterol to phospholipid ratio and a low amount of sphingolipids. [20–22] making vacuolar membrane proteins detergent soluble [23]. Homotypic vacuole fusion is facilitated by lipids regulating membrane properties such as ergosterol, diacylglycerol, phosphatidylinositol 3-phosphate (PI_3P) and phosphatidylinositol 4-phosphate (PI_4P) [24,25]. These lipids interdependently converge at the copes of fusing membranes and promote enrichment of other fusion factors such as SNAREs, Ypt7p [26] and HOPS [27]. The autophagy and cytosol to vacuole targeting pathways require presence of phosphatidyl-ethanolamine that is covalently bound to the autophagy factor Atg8p [28]. Phosphorylated inositol species represent a minority of cellular lipids [29], but are indispensable signaling molecules for appropriate vesicle trafficking. The major phosphoinositide species of vacuolar membranes is phosphatidylinositol 3,5-bisphosphate ($PtdIns[3,5]P_2$), which is generated from PI_3P by the Fab1 kinase [30,31]. Cells deficient in this lipid have severe defects in vacuolar

morphology impotent in fission [32–34]. PtdIns[3,5]P_2 effectors are required for protein sorting from multivesicular bodies to the vacuole and in the opposite direction [30]. Though the mechanism is not defined, PtdIns[3,5]P2 is essential for vacuolar acidification and convolution in plants [35].

P. pastoris became an interesting model for studying processes related to vacuolar functioning such as cytoplasm to vacuole protein targeting [36], autophagy and pexophagy [37] as well as role of vacuolar proteases in proteolytic degradation of recombinant proteins [38] Nevertheless, comprehensive structural knowledge on *Pichia* vacuoles is missing. Due to this demand the goal of the current study was to characterize the major molecular components of isolated vacuoles from this yeast, i.e. their lipid and protein composition. Here we describe a procedure for the isolation of highly enriched intact vacuoles from *P. pastoris*. Additionally, we report our preliminary results on the analysis of vacuolar phospholipids and fatty acids.

2. Experimental procedures

2.1. Strains and culture conditions

The *P. pastoris* strain CBS7435*ku70-PpVAC8-GFP* (MATa, Mut^+, His^+) was constructed on the basis of the *P. pastoris* strain CBS7435*ku70his4* (MATa, Mut^+, His^-) (kindly provided by A. Glieder) [39] and was considered as wild type throughout this study. Cells were grown under aerobic conditions to the mid logarithmic phase (16 h) at 30 °C in YPD medium (1% yeast extract, 2% peptone and 2% glucose). Media were inoculated to a starting OD_{600} of 0.1 from precultures grown aerobically for 48 h in YPD at 30 °C.

2.2. Construction of the CBS7435*ku70-PpVAC8-GFP strain*

DNA from the wild type *Pichia pastoris* CBS7435*ku70his4* strain was isolated using the Yeast DNA Kit (Omega bio-tek) and used for applications described below. The open

reading frame of e-GFP was amplified from the pGAPzA-eGFP plasmid (provided by A. Glieder) using the primers e-GFP_Fwd and e-GFP_Rev containing SacI and NotI sites respectively and cloned into the pPIC3.5 plasmid (Invitrogen-Life Technologies) within corresponding sites resulting in the pPIC3.5_e-GFP plasmid. The *Pichia pastoris PpVAC8* gene together with its upstream genomic region (considered as promoter (P) region) was amplified from genomic DNA using the primer pair AliI bV Fw and Vac8_Rev containing AliI and SacI sites, respectively, and further cloned into the pPIC3.5_e-GFP plasmid resulting in the final vector pPIC3.5_PpPVAC8-e-GFP. This plasmid was linearized within the *PpVAC8* region by BamHI and used for transformation of *P. pastoris* CBS7435*ku70his4* strain [40]. All used primers are listed in Table 1.

2.3. Fluorescence microscopy

P. pastoris cells were grown on YPD medium to the mid logarithmic phase (16 h) at 30 °C. Cells from 1 ml culture were harvested by centrifugation, washed once with deionized water, and analyzed using a Zeiss Axiovert 35 microscope with a 100-fold oil immersion objective. Isolated vacuoles were directly subjected to microscopy. The detection ranges of 510–520 nm for GFP was used. Images were taken with a CCD camera.

2.4. Isolation of intact vacuoles

Vacuoles were isolated as described in much detail in Chapter 3 of this Thesis (Section 4.6.) from cells grown on YPD or YPM (1% yeast extract, 2% peptone, 1% methanol) to the mid logarithmic phase (16 h) at 30 °C or 25°C.

2.4. Protein analysis

Proteins were quantified by the method of Lowry et al. [41] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification. SDS–PAGE was performed by the method of Laemmli

[42] using 10% SDS gels. Samples were denaturated at 37 °C to avoid aggregation of membrane proteins. Proteins were visualized by staining with Coomassie Blue. Western Blot (WB) analysis was performed according to Haid and Suissa [43]. Primary rabbit antibodies used in this study were directed against Por1p, Pma1p, GAPDH from *S. cerevisiae*, and against the 75-kDa microsomal marker protein (75-ER marker) from *P. pastoris*. The 75 kDa microsomal protein appears as a typical band of microsomal fractions on SDS–polyacrylamide gels. The function of this protein is unknown. Mouse antiserum against GFP was purchased from Roche. Peroxidase-conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce) were used to visualize immunoreactive bands.

2.5. Lipid analysis

Lipid extraction procedure was performed as described in in Chapter 2 of this Thesis (Section 3.1.1.). For the quantification of total phospholipids from homogenized cells and vacuoles samples containing 1 mg and 0.5 mg protein, respectively, were extracted and analyzed as described in Chapter 2 of this thesis (Section 3.3.2.). Individual phospholipids were analyzed from cell homogenates and vacuoles containing 3 mg and 1 mg protein, respectively, and their relative amounts were quantified as described in Chapter 2 of this Thesis (Section 3.3.1.). For the identification of the vacuolar fatty acid composition, lipids were extracted from cell homogenates and vacuoles containing 1 mg and 0.3 mg protein, respectively, and relative amounts of fatty acids were analyzed as described in Chapter 2 (Section 3.2.).

3. Results and discussion

3.1. Development of vacuole isolation procedure

A protocol for the isolation of intact vacuoles from *P. pastoris* was adopted and modified from standard procedures described for *S. cerevisiae* [5,44]. The step-wise procedure

described in Chapter 3 (Section 3.6.) was optimized to avoid contamination of the vacuolar fraction with other cellular compartments, especially mitochondria, endoplasmic reticulum and lipid droplets. The crucial step of the aforementioned procedure (step 9) allows separation of vacuoles from most of the ER and mitochondrial membranes making use of the specific buoyant behavior of these organelles in a 3.5 % buffered Ficoll PM400 gradient. Standard procedures were employed to evaluate the quality of obtained fractions (Fig.1) First, the Vac8p-e-GFP fusion protein was localized to vacuolar membrane (Fig. 1A). Hence the high enrichment of the obtained vacuolar fractions over the cell homogenate was confirmed by microscopic observations (Fig. 1B) and Western blot analysis using primary mouse antiserum against GFP (Fig. 1C). The plasma membrane marker protein Pma1p and cytosolic GAPDH were not detected in vacuolar fractions, although minor amounts of mitochondrial Por1p was still present. Noteworthy, similar signal intensities of the 75-ER marker were observed in vacuolar fractions and in cell homogenates. Taking into account the physiological relevance of contacts between yeast vacuoles and mitochondria during selective and non-selective mitophagy [9,45,46], the presence of low amounts of outer mitochondrial membrane protein Por1p in obtained vacuolar fractions is presumably inevitable. The presence of the 75-ER marker protein in vacuolar fractions may indicate incomplete depletion of ER membranes through the isolation procedure. However, this result should be interpreted with caution due to the fact that the antibody was raised against the mixture of proteins of the same size enriched in crude *P. pastoris* ER fractions (our unpublished data) which turned out to be rather unspecific and not well suited for cell fractionation quality control. On the other hand, intimate contact between vacuole and ER and crosstalk of the two compartments are known in yeast and appear to be physiologically relevant [47,48]. Therefore, the presence of ER marker proteins in intact vacuolar fractions may be difficult to avoid. However, the quality control of vacuole fractions needs further investigations applying a more reliable set of antibodies reactive against *Pichia* native proteins. Nevertheless, for the current study the obtained vacuolar fraction was considered appropriate for preliminary lipid analysis and for mass spectrometry analysis of the vacuolar proteome.

3.2. Preliminary results of vacuolar lipid analysis

Phospholipids (PL) and fatty acids from vacuolar fractions were analyzed as described in experimental procedures. Analysis of total phospholipids (Fig. 2A) in vacuoles revealed that

this organelle has a much higher PL to protein mass ratio (~0.91) than total cell extracts (~0.14). Taking into account that PL are vacuolar membrane constituents whereas proteins locate dually to the membrane and the lumen of the compartment, we can presume that phospholipids are highly enriched in the vacuolar membrane. Interestingly, the PL to protein ratio of *Pichia* vacuoles is also much higher than reported for *S.* cerevisiae vacuoles (0.51) [21]. The other distinct feature of *P. pastoris* vacuoles is the high sterol to protein ratio (Table 2.). This finding can be explained by the low abundance of proteins in the vacuolar membrane. Noteworthy, the sterol to PL ratio of vacuoles is similar to total cell membranes (see Table 2).

The analysis of individual phospholipids of vacuoles revealed that this organelle has a specific membrane phospholipid pattern compared to bulk cell membranes (Fig. 2B). The distinct feature of vacuoles from *P. pastoris* is the high abundance of phosphatidylinositol (PI) which turned out to be the major phospholipid. The fact that yeast vacuolar membranes are enriched in phosphorylated PI species and especially in phosphatidylinositol 3,5-bisphosphate (PtdIns[3,5]P_2) supports for this observation, although the quantities of these specific signaling phospholipids are low in the yeast cell. However, a more accurate mass spectrometry analysis of the individual phospholipids and particularly PI species in the vacuoles might be a more rational approach. Only traces of cardiolipin, phosphatidic acid and dimethylphosphatidylethanolamine were detected in vacuolar fractions. The fatty acid composition of vacuoles totally resembles the total cell extract (Fig.3.) suggesting that specific molecular species are not enriched there. Again, MS analysis of vacuolar lipidome will be required for closer inspection of the vacuolar lipid profile.

3.3. Perspectives

The results presented in the current study are preliminary and give an impetus for further characterization of *P. pastoris* vacuoles. The isolation procedure for vacuoles may still be modified to obtain a higher purity degree; and an improvement of the methodology for quality control will be necessary. However, vacuolar fractions obtained by the procedure described in this study are acceptable for further preliminary proteome and lipidome investigations. Analysis of vacuolar lipids and proteins from cells grown under different conditions may become a prerequisite for a broader overview of structure and function of this organelle.

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References

[1] S.C. Li, P.M. Kane, The Yeast Lysosome-like Vacuole: Endpoint and Crossroads, Biochim. Biophys. Acta. 1793 (2009) 650–663.

[2] Y. Kakinuma, Y. Ohsumi, Y. Anraku, Properties of H+-translocating adenosine triphosphatase in vacuolar membranes of Saccharomyces cerevisiae, J. Biol. Chem. 256 (1981) 10859–10863.

[3] K. Shirahama, Y. Yazaki, K. Sakano, Y. Wada, Y. Ohsumi, Vacuolar function in the phosphate homeostasis of the yeast Saccharomyces cerevisiae, Plant Cell Physiol. 37 (1996) 1090–1093.

[4] K.J. Indge, Polyphosphates of the yeast cell vacuole, J. Gen. Microbiol. 51 (1968) 447–455.

[5] E. Wiederhold, T. Gandhi, H.P. Permentier, R. Breitling, B. Poolman, D.J. Slotboom, The yeast vacuolar membrane proteome, Mol. Cell. Proteomics MCP. 8 (2009) 380–392.

[6] M. Horst, E.C. Knecht, P.V. Schu, Import into and degradation of cytosolic proteins by isolated yeast vacuoles, Mol. Biol. Cell. 10 (1999) 2879–2889.

[7] D. Rotin, O. Staub, R. Haguenauer-Tsapis, Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases, J. Membr. Biol. 176 (2000) 1–17.

[8] C.H. Moeller, W.W. Thomson, Uptake of lipid bodies by the yeast vacuole involving areas of the tonoplast depleted of intramembranous particles, J. Ultrastruct. Res. 68 (1979) 38–45.

[9] I. Kim, S. Rodriguez-Enriquez, J.J. Lemasters, Selective degradation of mitochondria by mitophagy, Arch. Biochem. Biophys. 462 (2007) 245–253.

[10] Y. Sakai, A. Koller, L.K. Rangell, G.A. Keller, S. Subramani, Peroxisome degradation by microautophagy in Pichia pastoris: identification of specific steps and morphological intermediates, J. Cell Biol. 141 (1998) 625–636.

[11] M. Oku, Y. Sakai, Pexophagy in Pichia pastoris, Methods Enzymol. 451 (2008) 217–228.

[12] R. Krick, Y. Mühe, T. Prick, M. Bredschneider, S. Bremer, D. Wenzel, et al., Piecemeal microautophagy of the nucleus: genetic and morphological traits, Autophagy. 5 (2009) 270–272.

[13] M. Ashburner, C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, et al., Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, Nat. Genet. 25 (2000) 25–29.

[14] R. Russnak, D. Konczal, S.L. McIntire, A Family of Yeast Proteins Mediating Bidirectional Vacuolar Amino Acid Transport, J. Biol. Chem. 276 (2001) 23849–23857.

[15] L. Eguez, Y.-S. Chung, A. Kuchibhatla, M. Paidhungat, S. Garrett, Yeast Mn2+ transporter, Smf1p, is regulated by ubiquitin-dependent vacuolar protein sorting., Genetics. 167 (2004) 107–117.

[16] Z.S. Li, M. Szczypka, Y.P. Lu, D.J. Thiele, P.A. Rea, The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump, J. Biol. Chem. 271 (1996) 6509–6517.

[17] J.-E. Sarry, S. Chen, R.P. Collum, S. Liang, M. Peng, A. Lang, et al., Analysis of the vacuolar luminal proteome of Saccharomyces cerevisiae, FEBS J. 274 (2007) 4287–4305.

[18] N.J. Bryant, T.H. Stevens, Vacuole biogenesis in Saccharomyces cerevisiae: protein transport pathways to the yeast vacuole, Microbiol. Mol. Biol. Rev. MMBR. 62 (1998) 230–247.

[19] W. Wickner, Yeast vacuoles and membrane fusion pathways, EMBO J. 21 (2002) 1241–1247.

[20] R. Schneiter, Brave little yeast, please guide us to Thebes: sphingolipid function in S. cerevisiae, BioEssays News Rev. Mol. Cell. Dev. Biol. 21 (1999) 1004–1010.

[21] E. Zinser, C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, G. Daum, Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae, J. Bacteriol. 173 (1991) 2026–2034.

[22] M. Bagnat, S. Keränen, A. Shevchenko, A. Shevchenko, K. Simons, Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3254–3259.

[23] E. Lauwers, B. André, Association of yeast transporters with detergent-resistant membranes correlates with their cell-surface location, Traffic Cph. Den. 7 (2006) 1045–1059.

[24] M. Kato, W. Wickner, Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion, EMBO J. 20 (2001) 4035–4040.

[25] R.A. Fratti, Y. Jun, A.J. Merz, N. Margolis, W. Wickner, Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles, J. Cell Biol. 167 (2004) 1087–1098.

[26] D.J. Klionsky, P.K. Herman, S.D. Emr, The fungal vacuole: composition, function, and biogenesis, Microbiol. Rev. 54 (1990) 266–292.

[27] R.A. Fratti, Y. Jun, A.J. Merz, N. Margolis, W. Wickner, Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles, J. Cell Biol. 167 (2004) 1087–1098.

[28] R. Nebauer, S. Rosenberger, G. Daum, Phosphatidylethanolamine, a limiting factor of autophagy in yeast strains bearing a defect in the carboxypeptidase Y pathway of vacuolar targeting, J. Biol. Chem. 282 (2007) 16736–16743.

[29] S.K. Dove, F.T. Cooke, M.R. Douglas, L.G. Sayers, P.J. Parker, R.H. Michell, Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis, Nature. 390 (1997) 187–192.

[30] T. Strahl, J. Thorner, Synthesis and function of membrane phosphoinositides in budding yeast, Saccharomyces cerevisiae, Biochim. Biophys. Acta. 1771 (2007) 353–404.

[31] S.K. Dove, R.K. McEwen, A. Mayes, D.C. Hughes, J.D. Beggs, R.H. Michell, Vac14 controls PtdIns(3,5)P(2) synthesis and Fab1-dependent protein trafficking to the multivesicular body, Curr. Biol. CB. 12 (2002) 885–893.

[32] S.K. Dove, R.C. Piper, R.K. McEwen, J.W. Yu, M.C. King, D.C. Hughes, et al., Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors, EMBO J. 23 (2004) 1922–1933.

[33] A. Yamamoto, D.B. DeWald, I.V. Boronenkov, R.A. Anderson, S.D. Emr, D. Koshland, Novel PI(4)P 5-kinase homologue, Fab1p, essential for normal vacuole function and morphology in yeast, Mol. Biol. Cell. 6 (1995) 525–539.

[34] J.A. Efe, R.J. Botelho, S.D. Emr, The Fab1 phosphatidylinositol kinase pathway in the regulation of vacuole morphology, Curr. Opin. Cell Biol. 17 (2005) 402–408.

[35] G. Bak, E.-J. Lee, Y. Lee, M. Kato, S. Segami, H. Sze, et al., Rapid Structural Changes and Acidification of Guard Cell Vacuoles during Stomatal Closure Require Phosphatidylinositol 3,5-Bisphosphate, Plant Cell Online. (2013) tpc.113.110411.

[36] J.-C. Farré, J. Vidal, S. Subramani, A cytoplasm to vacuole targeting pathway in P. pastoris, Autophagy. 3 (2007) 230–234.

[37] J.-C. Farré, R.D. Mathewson, R. Manjithaya, S. Subramani, Roles of Pichia pastoris Uvrag in vacuolar protein sorting and the phosphatidylinositol 3-kinase complex in phagophore elongation in autophagy pathways, Autophagy. 6 (2010) 86–99.

[38] J. Sinha, B.A. Plantz, M. Inan, M.M. Meagher, Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast Pichia pastoris: Case study with recombinant ovine interferon-τ, Biotechnol. Bioeng. 89 (2005) 102–112.

[39] L. Näätsaari, B. Mistlberger, C. Ruth, T. Hajek, F.S. Hartner, A. Glieder, Deletion of the Pichia pastoris KU70 Homologue Facilitates Platform Strain Generation for Gene Expression and Synthetic Biology, PLoS ONE. 7 (2012) e39720.

[40] J. Lin-Cereghino, W.W. Wong, S. Xiong, W. Giang, L.T. Luong, J. Vu, et al., Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris, BioTechniques. 38 (2005) 44, 46, 48.

[41] O.H. LOWRY, N.J. ROSEBROUGH, A.L. FARR, R.J. RANDALL, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[42] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature. 227 (1970) 680–685.

[43] A. Haid, M. Suissa, Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Methods Enzymol. 96 (1983) 192–205.

[44] Y. Ohsumi, Y. Anraku, Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of Saccharomyces cerevisiae., J. Biol. Chem. 256 (1981) 2079–2082.

[45] I.B. Kiššová, B. Salin, J. Schaeffer, S. Bhatia, S. Manon, N. Camougrand, Selective and Non-Selective Autophagic Degradation of Mitochondria in Yeast, Autophagy. 3 (n.d.) 329–336.

[46] M. Graef, J. Nunnari, A role for mitochondria in autophagy regulation, Autophagy. 7 (2011) 1245–1246.

[47] E. Kvam, D.S. Goldfarb, Nucleus-vacuole junctions in yeast: anatomy of a membrane contact site, Biochem. Soc. Trans. 34 (2006) 340–342.

[48] M. Graef, J.R. Friedman, C. Graham, M. Babu, J. Nunnari, ER exit sites are physical and functional core autophagosome biogenesis components, Mol. Biol. Cell. 24 (2013) 2918–2931.

Tables

Table 1. List of primers used in this study.

Primer name	Sequence from 5′ to 3′ end	Features
e-GFP_Fwd	AAT TAG AGC TCA TGG CTA GCA AAG GAG AAG AAC T	SacI restriction site
e-GFP_Rev	TAA TTC GCG GCC GCT TAC TTG TAC AAT TCA TCC ATG C	NotI restriction site
AliI bV Fw	ATT TCA CTT AAG TGG ATA GAG ACT GGT TTG AAG AC	AleI restriction site
Vac8_Rev	CCA TGA GCT CTA ATT TGA TCA TCT CCA AGA TTT G	SacI restriction site

Table 2. Sterol analysis of the *P. pastoris* vacuole.

Free sterols from cell homogenates and vacuoles were quantified and related to the phospholipid (PL) and protein content. Data are mean values of three independent experiments.

Cell fraction	mg Sterol/mg protein	mg Sterol/mg PL
Homogenate	0.023	0.165
Vacuole	0.148	0.162

Figures

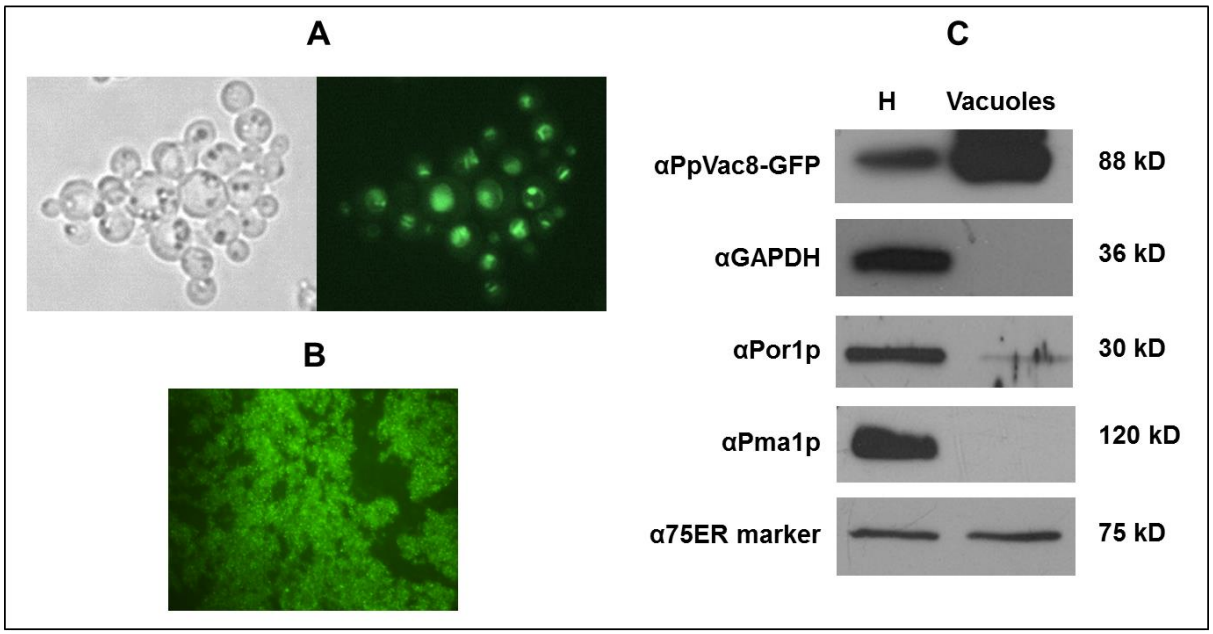

Fig.1 Quality control of isolated vacuoles.

A- GFP-Vac8p localizes to vacuolar membrane; B- isolated vacuoles are enriched in GFP-Vac8p; C–Western blot analysis. Antisera against the *Pichia pastoris* 75-ER marker protein (microsomal marker); Pma1p, plasma membrane H^+-ATPase (plasma membrane marker); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (cytosolic marker); GFP-Vac8p, green fluorescent protein fused to Vac8p (vacuole membrane marker); Por1p, mitochondrial porin (mitochondrial marker) were employed.

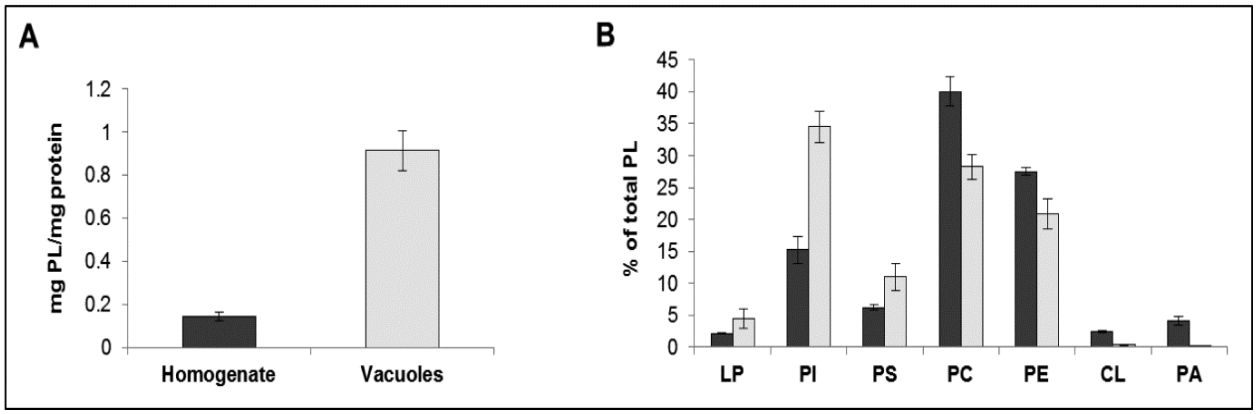

Fig.2 Phospholipid analysis of vacuoles isolated from *P. pastoris*.

Phospholipids were isolated and analyzed as described in the Experimental procedures. A–Total phospholipids in vacuole and homogenate. B–Relative distribution of individual phospholipids in the vacuole (light grey bars) and in homogenates (dark grey bars). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), phosphatidic acid (PA) are shown as percentage of total phospholipids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

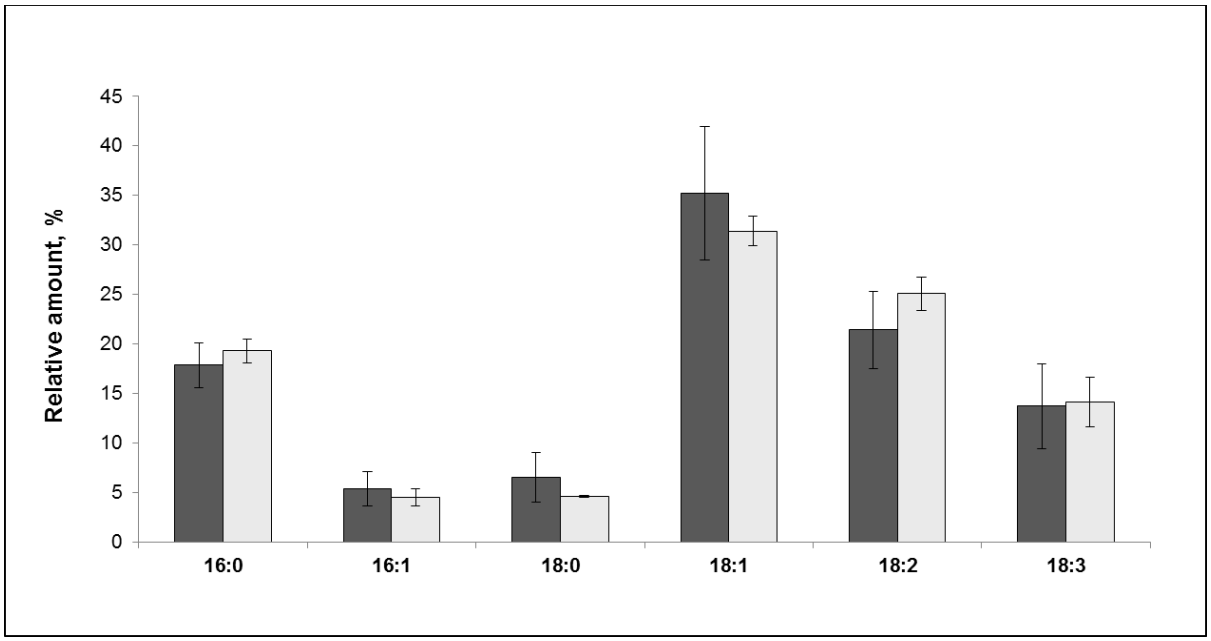

Fig.3 Fatty acid pattern of vacuoles from *P. pastoris*.

Lipid extracts from vacuoles (light grey bars) and homogenate (dark grey bars) were analyzed by GC-FID for their fatty acid composition. Amounts of individual fatty acids in homogenate and vacuoles are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate the standard deviation.

CHAPTER 6

GENERAL DISCUSSION

This study was performed in scope of a '*Pichia lipidomics* project' to broaden the fundamental knowledge of *Pichia pastoris* cell biology of lipids and characterization of organelles. This Thesis predominantly dedicated to the investigation of non-polar lipid storage and synthesis in this yeast. During this study two main objectives were addressed. First, we characterized molecular components of the non-polar lipid storage compartment, the lipid droplets (LD), in *P. pastoris* by means of lipidomics and proteomics. Secondly, we identified genes responsible for the formation of storage lipids, their role in this process and their contribution to the biogenesis of lipid droplets. Moreover, we summarized here the entire methodology employed in our laboratory for isolation of organelles and biomembranes from *P. pastoris* and the lipid analysis techniques required for their characterization. Additionally, we report the development of the reliable vacuole isolation procedure and discuss preliminary results of vacuole membrane lipid analysis. Finally, we report the approach initiated to identify *P. pastoris* genes responsible for non-polar lipid turnover and degradation.

1. Characterization of the lipid droplets from *Pichia pastoris*

The development of an appropriate isolation procedure was a prerequisite for the molecular analysis of LD. The method based on a combination of gradient centrifugation and floatation was adapted and optimized for the isolation of pure and highly enriched LD from *P. pastoris* cells. LD fractions were analyzed for their lipid and protein composition.

The major hallmarks of LD from *P. pastoris* and the differences to *S. cerevisiae* were shown in this study. Triacylglycerols (TG) comprise the vast majority of storage lipids deposited in *P. pastoris* LD, whereas amounts of steryl esters (SE) are minor. SE are enriched in ergosterol precursors whereas ergosterol is vastly prevalent in total cell extracts. Very small amounts of phospholipids (PL) detected in LD form its membrane monolayer with a profile that generally reflects the total cell PL composition, where phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major phospholipids. The high ratio of phosphatidylinositol (PI) to phosphatidylserine (PS) in LD is remarkable. Mass spectrometric species analysis of PL revealed that the vast majority of all PL is represented by C36 and C34 species with certain differences between classes, but generally resembling these of total cell

extracts. Unlike in *S. cerevisiae* [1], storage lipids of *P. pastoris* LD have an unspecific fatty acid profile similar to total cells with significant amounts of polyunsaturated fatty acids.

The proteomic study of *P. pastoris* LD revealed a small but distinct set of resident proteins. In total, mass spectrometry detected 49 proteins in LD. Thirty-five of these proteins have *S. cerevisiae* orthologs, whereas some are unassigned. Among the detected proteins, 13 localize to LD, 6 are dually localized to the endoplasmic reticulum (ER) and LD, and 5 are ER residents. Fourteen of these orthologs are known players of lipid metabolism, such as sterol, fatty acid, and sphingolipid biosynthesis, as well as lipolysis [1]. The obtained data provide a comprehensive view on the molecular structure of LD in *P. pastoris*.

2. Identification of non-polar lipid synthases in *P. pastoris*

To get a deeper insight into the synthesis of storage lipids in *P. pastoris* we aimed to identify genes and their gene products involved in the formation of non-polar lipids in this yeast. The general approach was based on UniProtKB/TrEMBL annotations of the *Pichia* proteome together with a blast analysis which resulted in the identification of three candidate genes annotated as *LRO1*, *DGA1* and *ARE2*. We generated a set of deletion strains deficient in these genes and characterized their ability to form and store lipids in LD. Our results suggest that there are two TG synthases in *P. pastoris*, namely gene products of *LRO1* and *DGA1* which synthesize the majority of TG. Only one SE synthase, gene product of *ARE2* was found to synthesize total SE. Moreover, we showed that Lro1p is the major TG synthase in *P. pastoris* in contrast to the model yeast *S. cerevisiae* [2]. We report that the presence of at least one of TG synthases is indispensable for the formation of LD. Strains lacking TG are not able to form LD solely from SE, which is in contrast to *S. cerevisiae* [2]. Interestingly, the absence of TG and hence LD leads to significant changes in the cellular fatty acid pool resulting in highly elevated amounts of polyunsaturated fatty acids such as linoleic and linolenic acids. The observation is noteworthy that *P. pastoris* strains unable to store an excess of exogenous fatty acids in LD do not exhibit serious growth defects, elevated levels of phospholipids and membrane proliferation as was reported for *S. cerevisiae* [3]. Additionally, we addressed the *in vivo* fatty acid substrate specificity of the identified three non-polar lipid synthases. Dga1p has a slight preference for saturated palmitic and stearic acid; Are2p has a certain preference for the low abundant stearic acid; and Lro1p is rather unspecific. These results provide novel

insight to the molecular enzymological mechanisms which govern non-polar lipid synthesis and LD biogenesis in *P. pastoris*.

3. Isolation of vacuoles from *P. pastoris*

In this Thesis, and as a part of the 'Pichia lipidomics' project we report preliminary results of isolation and characterization of vacuoles from *P. pastoris*. Here we describe a procedure for the isolation of intact enriched vacuoles and render preliminary results of the analysis of vacuolar lipids. The optimized procedure yields highly enriched intact vacuolar fractions which are largely devoid of other subcellular compartments. However, there is space for improvement of the applied method. The preliminary lipid analysis shows that *Pichia* vacuoles have a high PL to protein ratio and a specific phospholipid pattern where PI along with PC and PE was identified as the major phospholipid molecule. The fatty acid composition of vacuoles reflects total cell extracts. A more detailed proteome and lipidome analysis of vacuoles is currently in progress.

4. Identification of *P. pastoris* non-polar lipid hydrolases

To investigate non-polar lipid mobilization in *P. pastoris* we initiated the study aimed for the identification of non-polar lipid hydrolases. The approach relies on the generation of mutant strains deficient in the annotated candidate genes *TGL1*, *TGL3*, *TGL4* and *YEH1*. Here we report the procedure used for the generation of several single and double deletion strains and preliminary lipid analysis of the obtained mutants. All mutants showed similar TG amounts as wild type strain suggesting that none of them may be an active TG lipase *in vivo*. The mutants lacking *TGL1* showed elevated SE levels supporting the idea that this gene product may be a SE hydrolase in *P. pastoris*. Further search for candidate genes, and *in vivo* and *in vitro* experiments will be required to identify genes encoding non-polar lipid hydrolytic enzymes

5. Conclusion

In this Thesis, organelles, membranes and lipid metabolic enzymes from *Pichia pastoris* were studied. These investigations shed more light on *Pichia pastoris* cell biology of lipids and may become useful for fundamental lipid research in eukaryotes. This knowledge will be important for a better understanding the cell physiology of *Pichia pastoris* in line with its industrial applications.

References

[1] K. Grillitsch, M. Connerth, H. Köfeler, T.N. Arrey, B. Rietschel, B. Wagner, et al., Lipid particles/droplets of the yeast Saccharomyces cerevisiae revisited: lipidome meets proteome, Biochim. Biophys. Acta. 1811 (2011) 1165–1176.

[2] T. Czabany, A. Wagner, D. Zweytick, K. Lohner, E. Leitner, E. Ingolic, et al., Structural and biochemical properties of lipid particles from the yeast Saccharomyces cerevisiae, J. Biol. Chem. 283 (2008) 17065–17074.

[3] J. Petschnigg, H. Wolinski, D. Kolb, G. Zellnig, C.F. Kurat, K. Natter, et al., Good fat, essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast, J. Biol. Chem. 284 (2009) 30981–30993.

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List of Publications

1. V. A. Ivashov, K. Grillitsch, H. Koefeler, E. Leitner, D. Baeumlisberger, M. Karas, G. Daum, Lipidome and proteome of lipid droplets from the methylotrophic yeast Pichia pastoris, Biochim. Biophys. Acta 1831 (2013) 282–290.

2. V. A. Ivashov, G. Zellnig, K. Grillitsch and G. Daum, Identification of triacylglycerol and steryl ester synthases of the methylotrophic yeast Pichia pastoris, Biochim. Biophys. Acta, 1831 (2013) 1158–1166.

3. V. Ivashov, L. Klug, K. Grillitsch and G. Daum, Isolation of Pichia organelles, in Pichia protocols (Methods in Molecular Biology), Springer protocols. Third edition (2013) manuscript submitted

4. L. Klug, V. Ivashov, K. Grillitsch and G. Daum, Pichia pastoris lipidomics, in Pichia protocols (Methods in Molecular Biology), Third edition (2013) manuscript submitted

Presentations at International Conferences

Dates (M-Y)	Title of Conference	Type of presentation
06-2013	FEBS-EMBO lecture course "Biomembranes: Molecular Architecture, Dynamics and Function", Cargèse, France	Poster
05-2013	"11th Yeast Lipid Conference", Halifax, Canada	Poster
12-2012	"Enzymes and Signals" joint research summit with the DK Molecular Mechanisms in Cell Signaling (Vienna), Graz, Austria	Talk
09-2012	4th Annual Meeting 2012 of the Austrian Association of molecular Life Sciences and Biotechnology, Graz, Austria,	Poster
05-2012	Second European Symposium on Microbial Lipids (FEBS Workshop), "Microbial Lipids: Diversity in Structure and Function", Bern, Switzerland	Talk
03-2012	"4th International Graz Symposium on Lipid and Membrane Biology", Graz, Austria	Poster
02-2012	"Pichia 2012" Conference, Alpbach, Austria	Poster
09-2011	9th Euro Fed Lipid Congress, "Oils, Fats and Lipids for a Healthy and Sustainable World", Rotterdam, The Netherlands	Poster

CURRICULUM VITAE

Personal Information	
Name	**Vasyl A. IVASHOV**
Address	Riegelgasse 18/1, 8010 Graz, Austria
Telephone	+43 680 2420993
E-mail	ivashov@tugraz.at
Nationality	Ukraine
Date and place of birth	17.01.1986, Simferopol, Ukraine
Professional experience	
Dates (from – to)	2010-2014
Name of organisation	Institute of Biochemistry, Graz University of Technology, Graz, Austria
Position	University Project Assistant
Dates (from – to)	03-07. 2007
Name of organisation	Palladin Institute of Biochemistry, Dept. Molecular Biology, Kiev, Ukraine
Position	Internship student

Education	
Dates (from – to)	2010-2014
Name of organisation	Graz University of Technology, Graz, Austria, and Karl-Franzens-University Graz, Austria within the frame of the International PhD Program "DK Molecular Enzymology"
Occupation	Doctoral studies in cell biology
Project title	"Pichia lipidomics"
Title awarded	Dr. rer. nat.
Dates (from – to)	2007-2008
Name of organisation	Taurida National V. I. Vernadsky University, Simferopol, Ukraine
Occupation	Master study of biochemistry
Project title	"Biochemical indices of erythrocytes of chronic osteomyelitis patients"
Title awarded	MSc in biology
Dates (from – to)	2003-2007
Name of organisation	Taurida National V. I. Vernadsky University, Simferopol, Ukraine
Occupation	Bachelor study of biology
Title awarded	BSc in biology
Teaching activities	
Dates (from – to)	09-11.2009
Name of organisation	Taurida National V. I. Vernadsky University, Simferopol, Ukraine
Position	Assistant tutor
Type and title of course	University course: "Human carbohydrate and lipid metabolism"

Dates (from – to)	10.2009 – 04.2010
Name of organisation	Gymnasium № 7, Simferopol, Ukraine
Position	High school teacher
Type and title of course	High school classes: "General biology"
Languages	
Mother tongue	Russian
Languages	English – fluent, Ukrainian – fluent, German - basic