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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], 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*

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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.

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].

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

* 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.
E-mail address: guenther.daum@tugraz.at (G. Daum).

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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 CGCGGATCCGCGTTTTTGTAGAAATGT CTTG GTGTCCTCGTCCAATCAGGTAGCCATCTCTG and GFP-rev ATAG TTTAGCGGCC GCCTCGAGCCCGGGATTTAAATACTTGTACAATTCATCCA TGCCATGTGTAATCCCAGCAGCAGT) 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′-CAATGCG GCCGCTTATTTGGCATCCAATGGTTTTC-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