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

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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

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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

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

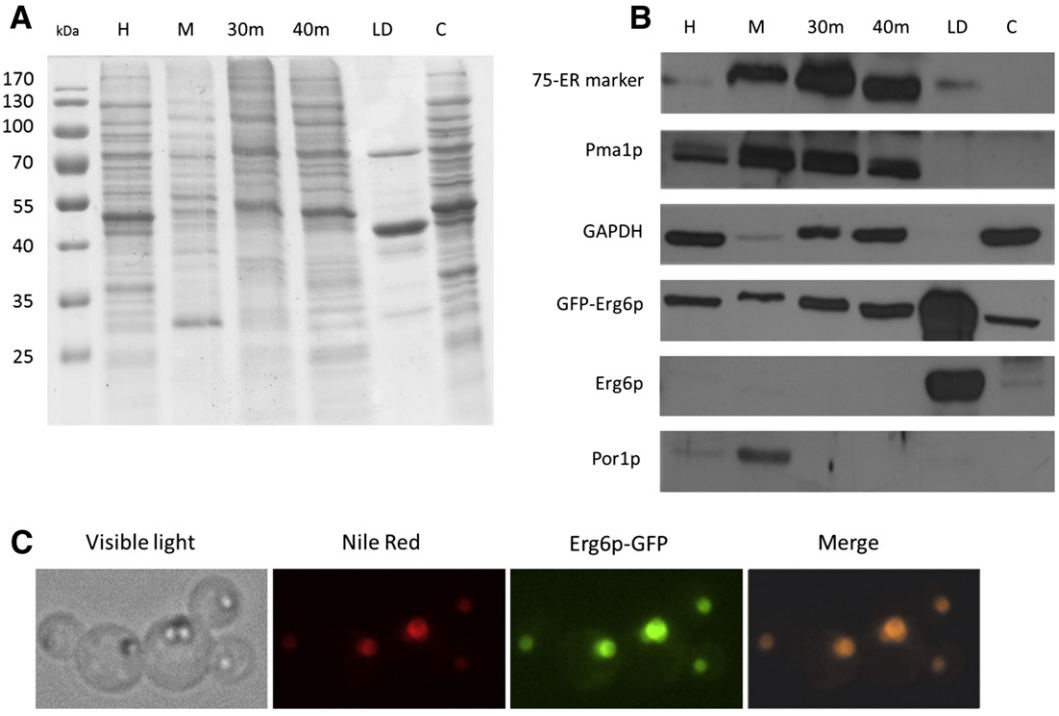

Fig. 1. Quality control of isolated lipid droplets. A—Protein pattern of lipid droplet fraction and control fractions (10 μg protein). Homogenate (H), lipid droplets (LD), 30,000 g microsomes (30 m), 40,000 g microsomes (40 m), cytosol (C) and mitochondria (M) fractions were loaded onto an SDS–polyacrylamide 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.

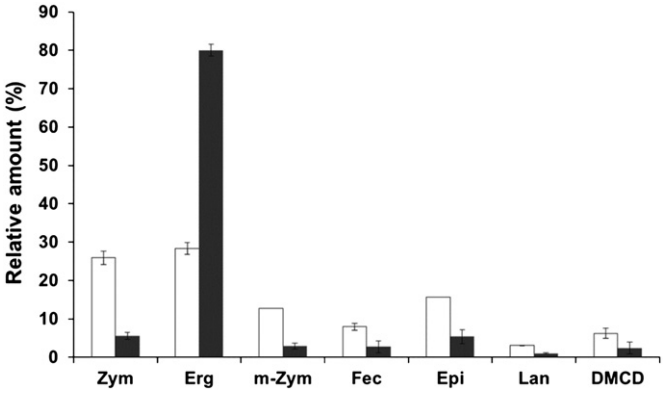

Fig. 2. Sterol composition of lipid droplets. Cells were grown on glucose at 30 °C to the stationary phase (26 h). Lipid extracts from LD (white bars) and homogenates (grey bars) were subjected to MS analysis of sterols. Amounts of individual sterols are shown as percentage of total sterols. Zym, Zymosterol; 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.

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

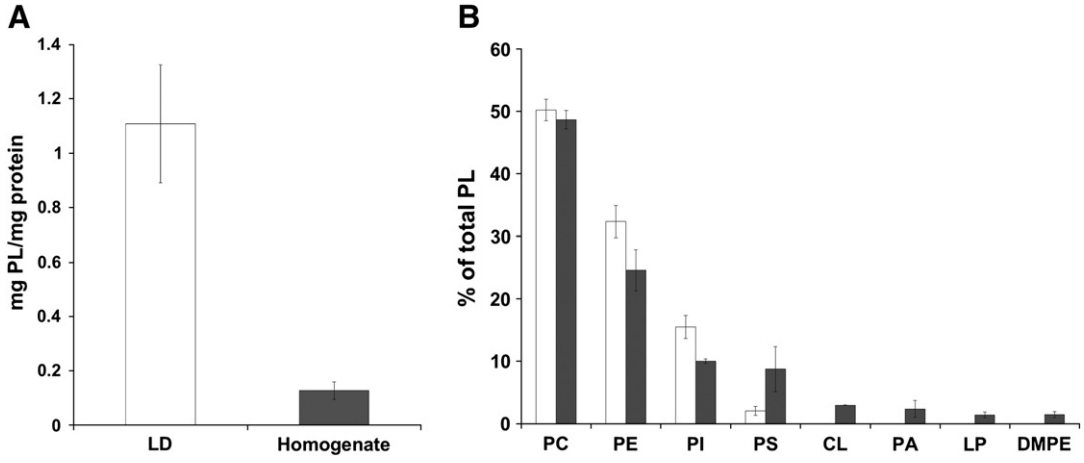

Fig. 3. Phospholipid pattern of lipid droplets. Cells were grown on glucose at 30 °C to stationary phase (26 h). Lipid extracts were analyzed for phospholipids as described in the experimental section. A—Total phospholipids in LD and homogenate. B—Relative distribution of individual phospholipids in 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.

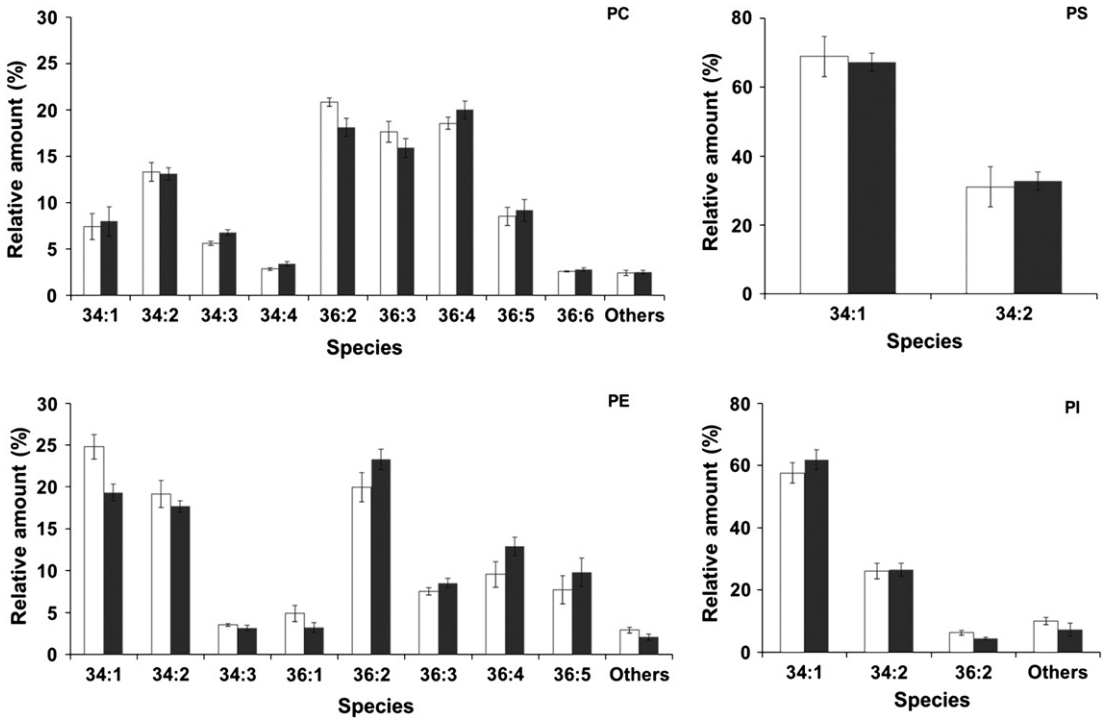

Fig. 4. Phospholipid species composition. Lipid extracts from homogenate (grey bars) and LD (white bars) were analyzed by LC–MS for phospholipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

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.

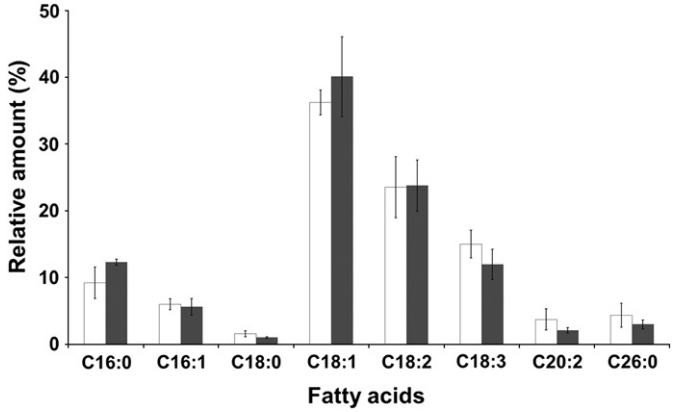

Fig. 5. Fatty acid pattern of lipid droplets. Lipid extracts from LD (white bars) and homogenate (grey bars) were analyzed by GC-FID for fatty acid composition. The amounts of individual fatty acids in homogenate and LD are shown as % of total fatty acids. Data are mean values of at least three independent experiments. Error bars indicate standard deviation.

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.

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
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
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
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

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