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Isolation and characterization of the plasma membrane from the yeast *Pichia pastoris*

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ABSTRACT

Despite similarities of cellular membranes in all eukaryotes, every compartment displays characteristic and often unique features which are important for the functions of the specific organelles. In the present study, we biochemically characterized the plasma membrane of the methylotrophic yeast *Pichia pastoris* with emphasis on the lipids which form the matrix of this compartment. Prerequisite for this effort was the design of a standardized and reliable isolation protocol of the plasma membrane at high purity. Analysis of isolated plasma membrane samples from *P. pastoris* revealed an increase of phosphatidylserine and a decrease of phosphatidylcholine compared to bulk membranes. The amount of saturated fatty acids in the plasma membrane was higher than in total cell extracts. Ergosterol, the final product of the yeast sterol biosynthetic pathway, was found to be enriched in plasma membrane fractions, although markedly lower than in *Saccharomyces cerevisiae*. A further characteristic feature of the plasma membrane from *P. pastoris* was the enrichment of inositol phosphorylceramides over neutral sphingolipids, which accumulated in internal membranes. The detailed analysis of the *P. pastoris* plasma membrane is discussed in the light of cell biological features of this microorganism especially as a microbial cell factory for heterologous protein production.

1. Introduction

The yeast *Pichia pastoris* is widely accepted as an excellent expression system for the production of recombinant proteins for biotechnological applications [1,2]. It has already been successfully employed as a microbial cell factory for hundreds of proteins [3] with increasing interest in the production of biopharmaceuticals and reagents for biomedical research [4]. The beneficial applicability of this methylotrophic yeast is characterized by reaching high cell densities [5] at low costs, the presence of an efficient secretion machinery [6] and the availability of highly inducible promoter systems [2,3,7–10]. Genetic manipulation of *P. pastoris* by a well-established tool box recently benefited of the release of the entire genome sequence information [11–13]. Compared to other popular unicellular expression hosts, *P. pastoris* turned out to be of specific value for the production of heterologous eukaryotic proteins. The requirement of post-translational modifications such as glycosylation and phosphorylation, the absence of an N-terminal methionine and correct assembly and formation of disulphide bonds are fully met by *P. pastoris*, thus providing appropriate conditions for expression and secretion of eukaryotic proteins in large quantities [14].

Over the last decades, many efforts have been made to improve biotechnological applications of *P. pastoris*, but the general interest to study fundamental aspects of membrane and cell biology was low. This view is surprising because properties of cellular membranes play a major role for protein formation and secretion [15,16]. For this reason, work in our laboratory was initiated to study organelles of *P. pastoris*, such as peroxisomes [17], mitochondria [18], lipid droplets [19] and the endoplasmic reticulum [20] in more detail and at the molecular level. Here, we present studies extending these investigations to the plasma membrane from *P. pastoris*.

The structure of a typical eukaryotic plasma membrane is based on a membrane bilayer composed of glycerophospholipids with different head groups and fatty acids. Sterols and sphingolipids are further characteristic structural components of eukaryotic plasma membranes.

Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; IPC, inositolphosphorylceramide; MIPC, mannosyl-inositolphosphorylceramide; $M(IP)_2C$, mannosyl-diinositolphosphorylceramide; Cer, ceramide; HexCer, hexosylceramide

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They arrange asymmetrically in the two membrane leaflets, where phosphatidylcholine (PC) and sphingolipids are mostly found in the outer aspect of the membrane, while negatively charged phospholipids such as phosphatidylserine (PS) and phosphatidylinositol (PI) are enriched in the inner membrane leaflet. In addition to lipids, the plasma membrane contains a well-defined set of proteins, which can either span the entire bilayer or become attached to one side of the membrane [21]. The sub-compartmentalization of the plasma membrane in lateral domains has been a long term matter of dispute, but is now largely accepted. With the formation of local domains, often referred to as lipid rafts or fixed islands, the cell is assumed to get spatiotemporal control of plasma membrane related processes [22–25]. Noteworthy, the function of the plasma membrane goes far beyond being only a barrier shielding the cell interior from the environment. It rather represents a complex, flexible organelle that acts as a mediator and effector for bidirectional processes of cell communications [22]. It is mostly the protein equipment of the plasma membrane which facilitates sensing of signals or transport of substrates in addition to fulfilling structural functions [26,27]. Upon signal reception, transduction and transcriptional response, the uptake of nutrients gets tightly regulated by affecting transporters in the plasma membrane [28].

Although membrane proteins account for most of the plasma membrane mediated processes, the role of membrane lipids must not be underestimated [29]. Lipids provide a matrix for accommodating proteins which is very important for the functionality of the plasma membrane, but also serve themselves as signaling molecules [30]. Lipid biosynthetic, metabolic and degrading processes as well as vesicular fusion and fission events ensure permanent turnover of plasma membrane lipids [31]. These processes are of specific importance for protein secretion and a challenge for recombinant protein production in *P. pastoris*. Further important functions of the plasma membrane include the anchoring of the cytoskeleton and a direct interaction between these compartments [32]. Finally, several eukaryotic membrane proteins are associated with the plasma membrane bilayer through glycosylphosphatidylinositol (GPI) anchors [33].

In the present study we describe a standardized protocol for the isolation of highly purified plasma membrane from *P. pastoris*. Work presented here was focused on lipid analysis of the *P. pastoris* plasma membrane combining classical biochemical and mass spectrometric methods. This strategy allowed us to get insight into typical properties of the plasma membrane from this yeast. These features are compared to other yeasts and eukaryotic organisms, and specific properties of the plasma membrane from *P. pastoris* are highlighted.

2. Materials and methods

2.1. Yeast strains and culture conditions

The *P. pastoris* strain CBS7435 (MATa, Mut$^+$; kindly provided by Anton Glieder, ACIB c/o Institute of Molecular Biotechnology at Graz University of Technology) was used throughout this study. For cultivating the cells, complex medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (YPD), was used. The strain was precultivated at 25 °C in YPD for 48 h using baffled flasks with shaking at 150 rpm. Main cultures were grown in 2 l Erlenmeyer flasks with baffles with shaking at 25 °C.

2.2. Electron microscopy

Cells were cultivated at 25 °C with shaking at 150 rpm in baffled flasks using YPD until reaching the late exponential phase. Washed cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with double distilled water and fixed again in a 1% aqueous solution of $KMnO_4$ for 20 min. 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, in a graded series of 50%, 70%, 90% and 100% ethanol, each. 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 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.

2.3. Isolation of organelles

Plasma membrane was isolated from *P. pastoris* CBS7435 grown on YPD for 26 h until reaching the late exponential phase. Protocols published previously [34–36] were slightly modified to obtain highly pure plasma membrane fractions. In brief, yeast cells were suspended in breaking buffer (20 g cell wet weight in 84 ml dist. water; 5 ml 0.5 M TrisCl, pH 8.5; 1 ml 0.5 M EDTA, pH 8.0; 0.25 ml 0.2 M PMSF) and disintegrated with glass beads by vigorous shaking for 3 min in a Merckenschlager Cell Homogenizer under CO_2-cooling. Cell extracts were cleared of unbroken cells and glass beads by centrifugation at 1000 ×*g* for 10 min. The resulting supernatant representing the homogenate was centrifuged at 35,000 ×*g* for 30 min to sediment bulk membranes. The resuspended pellet was dissolved in TEDG-buffer (20% glycerol in 10 mM TrisCl, pH 7.5; 0.2 mM EDTA; 0.2 mM DTT) and homogenized with 10 strokes in a Dounce homogenizer using a loose-fitting pestle. This sample was loaded onto a discontinuous density gradient consisting of 1 volume 53% sucrose in TED-buffer (10 mM TrisCl, pH 7.5; 0.2 mM EDTA; 0.2 mM DTT) and 2 volumes of 43% sucrose in TED-buffer. After ultracentrifugation in a swing-out rotor (Sorvall AH629) for at least 3.5 h at 100,000 × g at 4 °C, crude plasma membrane was collected from the gradient at the 43/53% sucrose interface. After three-fold dilution with ice-cold water, membranes were sedimented at 45,000 × g for 20 min in an SS34 rotor. The pellet was suspended in MES-buffer (5 mM MES, pH 6.0; 0.2 mM EDTA) and homogenized in a Dounce homogenizer with 10 strokes using a loose-fitting pestle. The top interphase at 43% sucrose was harvested as well and used as an additional loading control in Western Blot experiments when testing the quality of isolated samples. The crude plasma membrane was loaded on top of another discontinuous sucrose density gradient consisting of 1 volume 53%, 43% and 38% sucrose, each, in MES-buffer. After 2.5 h of centrifugation at 100,000 ×*g* at 4 °C, the purified plasma membrane was harvested at the 43/53% sucrose interface, diluted in Tris buffer (10 mM TrisCl, pH 7.4) and sedimented as described above. The resulting plasma membrane (PM) pellet was suspended in Tris buffer and stored at −70 °C.

To obtain microsomes from *P. pastoris* cells were harvested and converted to spheroplasts using Zymolyase 20 T [37]. Spheroplasts were homogenized in breaking buffer (0.6 M mannitol; 10 mM TrisCl, pH 7.4) containing 1 mM PMSF in a Dounce Homogenizer with 15 strokes using a tight-fitting pestle. Homogenates were centrifuged at 3000 ×*g* for 5 min at 4 °C. Homogenization of the resulting pellet is repeated twice. Combined supernatants (homogenate) were centrifuged at 12,000 ×*g* for 10 min. The resulting supernatant was centrifuged at 20,000 ×*g* and then at 30,000 ×g for 30 min at 4 °C, each. The pellet (M30) was suspended in TrisCl, pH 7.4, and stored at −70 °C.

Bulk membranes (BM) were isolated from *P. pastoris* CBS7435 grown to the late exponential growth phase. The homogenate was prepared as described above for the isolation of plasma membrane and centrifuged at 200,000 ×*g* for 45 min at 4 °C using a T865 fixed-angle rotor (Sorvall). The resulting pellet represented bulk membranes (BM) of the cells.

Prior to mass spectrometric analysis, PM, M30 and BM samples were washed once with deionized water and sedimented in an Eppendorf tube using a Beckman Coulter Optima TLX table top ultracentrifuge with a TLA100.4 rotor. Organelles were stored at −70 °C.

2.4. Protein analysis

Proteins from obtained fractions were precipitated with trichloroacetic acid (TCA) at a final concentration of 10% and quantified by the method of Lowry et al. [38] using bovine serum albumin as a standard. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out as described by Laemmli [39] using 12.5% separation gels. Western blot analysis was performed according to Haid and Suissa [40] using in-house raised rabbit primary antibodies against *Saccharomyces cerevisiae* Pma1p, Por1p and GAPDH. Rabbit antisera against *S. cerevisiae* Gas1p and *P. pastoris* Pex3p were kindly provided by B. Glick and R. Erdmann, respectively. Commercially available mouse monoclonal HDEL-antibody (2E7, Santa Cruz Biotech) was immunoreactive against a synthetic HDEL peptide corresponding to the C-terminus of yeast BiP. Antisera against *S. cerevisiae* proteins described above were immunoreactive against the corresponding *P. pastoris* proteins. HRP-conjugated secondary antibodies and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce, Rockford, IL, USA) were used to visualize immunoreactive bands following the manufacturer's instructions.

2.5. Lipid analysis

Glycerolipids and non-polar lipids from *P. pastoris* fractions were extracted as described by Folch et al. [41]. For phospholipid analysis, lipid extracts from homogenate and PM were loaded manually onto silica gel 60 plates (Merck, Darmstadt, Germany). Individual phospholipids were separated by two dimensional 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 dimension solvent. Lipids were stained with iodine vapor, scraped off the plate and quantified by the method of Broekhuyse [42].

Fatty acids were converted to methyl esters by methanolysis using 2.5% sulfuric acid in methanol and heating at 85 °C for 90 min. Fatty acid methyl esters were extracted in a mixture of light petroleum and water (3:1; v/v.) and analyzed by gas liquid chromatography (Hewlett-Packard 6890 Gas-chromatograph) using an HP-INNOWax capillary column (15 m × 0.25 mm i.d. × 0.50 μm film thickness) with helium as carrier gas. Fatty acids were identified by comparison to the fatty acid methyl ester standard mix GLC-68B (NuCheck, Inc., Elysian, MN, USA) and hexacosanoic acid methyl ester standard (Sigma Aldrich, Vienna).

For quantification of ergosterol, lipid extracts were applied to Silica Gel 60 plates (Merck, Darmstadt, Germany), and chromatograms were developed in an ascending manner to one third of the plate using the solvent system light petroleum/diethyl ether/acetic acid (25:5:1; per vol.). Then, plates were briefly dried and further developed to the top using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of ergosterol was carried out by densitometric scanning at 275 nm with a Shimadzu dual wavelength chromatoscanner CS-930 using ergosterol as standard.

Individual sterols were analyzed by gas–liquid chromatography/mass spectrometry (GLC/MS) after alkaline hydrolysis and subsequent lipid extraction [43]. GLC/MS was performed on a Hewlett-Packard 5690 Gas-Chromatograph equipped with a mass selective detector (HP 5972) using an HP5-MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Sample aliquots of 1 μl were injected in the splitless mode at 270 °C injection temperature with helium as carrier gas and with a flow rate set to 0.9 ml/min in constant flow mode. The temperature program was 100 °C for 1 min, 10 °C/min to 250 °C, and 3 °C/min to 310 °C. Sterols were identified by their mass fragmentation pattern.

2.6. Sphingolipid analysis

Bulk membranes, microsomal membranes and plasma membrane samples containing 300 μg protein were spiked with 30 μl of a sphingolipid 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), 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 [44]. During the incubation, samples were briefly vortexed and sonicated after 0, 10, 20 and 30 min. Then, the extracts were cleared from cell debris by centrifugation, dried under a stream of nitrogen, dissolved in 800 μl tetrahydrofuran/methanol/water (4:4:1; per vol.) [45] and stored under argon at −20 °C. For analysis, samples were solubilized by gentle heating and sonication.

UPLC-nanoESI-MS/MS molecular species analysis was started by ultra performance liquid chromatography (UPLC) using an ACQUITY UPLC® system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 Column (100 mm × 1 mm, 1 μm; Waters Corp., Milford, MA, USA). Aliquots of 2 μl were injected in partial loop with needle overfill mode. The flow rate was 0.12 ml/min, and the separation temperature was 35 °C. Inositol containing sphingolipids were separated by linear gradient elution 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) were 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 was tetrahydrofuran/methanol/20 mM ammonium acetate (6:3:1; per vol.) containing 0.1% (v/v) acetic acid; and solvent A was methanol/20 mM ammonium acetate (3:7; per vol.) containing 0.1% (v/v) acetic acid.

Analysis of sphingolipid classes was achieved with an ACQUITY UPLC® BEH HILIC Column (100 mm × 1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) at a flow rate of 0.2 ml/min and separation temperature of 40 °C. Sphingolipid classes were resolved by linear gradient elution as follows: 100% solvent B held during 0.5 min, linear decrease to 92%, 70% and 20% solvent B in 0.6, 1.9 and 0.5 min respectively, 20% solvent B held during 5.5 min and 100% solvent B held during 3 min. Solvent B was acetonitrile containing 0.1% (v/v) formic acid and solvent A was water/methanol 1:1 (v/v) containing 200 mM ammonium acetate and 0.1% (v/v) formic acid.

Chip-based nanoelectrospray ionization was achieved with a TriVersa Nanomate® (Advion, Ithaca, NY, USA) in the positive ion mode with 5 μm internal diameter nozzles. Sphingolipid molecular species were detected with a 4000 QTRAP® tandem mass spectrometer (AB Sciex, Framingham, MA, USA) 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 [46,47]. Dwell time was 30 ms and MS parameters were optimized to maximize detector response.

3. Results

3.1. Isolation of plasma membrane from P. pastoris

P. pastoris cells were cultivated on complex media containing glucose as the sole carbon source at 25 °C until they reached the late exponential phase. Under these conditions cell organelles such as the nucleus, mitochondria, the endoplasmic reticulum (ER) and lipid droplets were fully developed (Fig. 1). The white arrow in Fig. 1 points to the plasma membrane with the peripheral ER in close vicinity.

Protocols for the isolation of highly purified plasma membrane from *S. cerevisiae* had been described before [34,35]. Slight modifications (see Materials and methods section) were required to obtain highly enriched plasma membrane fractions from *P. pastoris*. As can be seen from Fig. 2A the protein pattern of isolated plasma membrane (lane 3) was completely different from the total cell extract (lane 1) and from a mitochondria enriched fraction collected from the first sucrose gradient (lane 2). The quality of plasma membrane fractions was tested by Western Blot analysis using antibodies directed against marker proteins of different organelles. In plasma membrane samples, a high enrichment of the

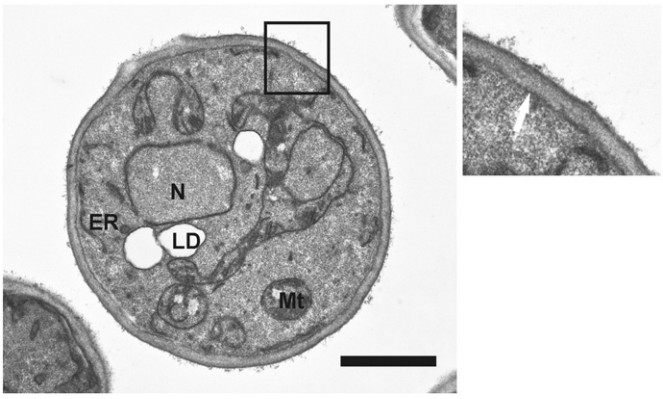

Fig. 1. Electron microscopy of *P. pastoris* CBS7435. Cells were grown in glucose containing medium until they reached the late exponential growth phase. N, nucleus; Mt, mitochondria; ER, endoplasmic reticulum; LD, lipid droplet. The white arrow in the image enlargement points to the plasma membrane. Scale bar: 1 μm.

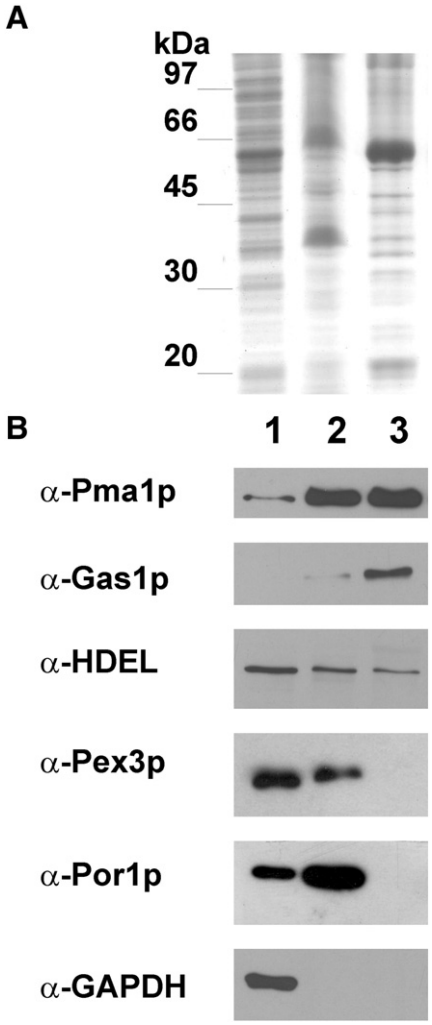

Fig. 2. Protein analysis of *P. pastoris* cell fractions and quality control. A: Protein patterns of the homogenate (lane 1); a mitochondria enriched fraction collected after the first density gradient centrifugation at the interphase 43% sucrose and TE-buffer (lane 2); and purified plasma membrane (lane 3) are shown. Molecular mass standards are indicated in the lane on the left (kDa). Lanes were loaded with 15 μg total protein, each. Proteins were stained with Coomassie blue. B: Quality control of subcellular fractions was performed by Western blot analysis. Antisera were directed against plasma membrane H^+-ATPase Pma1p, plasma membrane bound 1,3-beta-glucanosyltransferase Gas1p; HDEL, C-terminal microsomal retention signal of yeast BiP; peroxisomal membrane protein Pex3p; mitochondrial porin Por1p; and cytosolic glyceraldehyde-3-phosphate-dehydrogenase GAPDH.

plasma membrane marker proteins Pma1p and Gas1p was found (Fig. 2B). Peroxisomal, mitochondrial and microsomal marker proteins Pex3p, Por1p and HDEL, respectively, were present in homogenates and also in an intermediate fraction, which was collected after the first sucrose density gradient centrifugation. However, plasma membrane samples were largely devoid of cross-contamination as indicated by the absence of the above mentioned marker proteins from other organelles. The strong enrichment of the plasma membrane markers over the homogenate and the low degree of cross-contamination confirmed the reliability of the newly designed isolation procedure.

3.2. Phospholipid composition of the P. pastoris plasma membrane

The lipid composition of yeast membranes generally depends on environmental conditions such as temperature [48], carbon source [49] or oxygen supply to the culture [50]. Under standardized cultivation conditions as described above with glucose as the sole carbon source, the phospholipid composition of isolated plasma membrane samples exhibited characteristic features. Plasma membrane fractions were enriched in PA, phosphatidylserine (PS) and PE, mainly at the expense of PC compared to bulk membranes (Table 1). These changes, especially the increased PS to PC ratio, are characteristic for plasma membrane samples from many types of cells including other yeasts [51]. The strong reduction in PC accompanied by a slight increase in PE made the latter phospholipid the most abundant of the *P. pastoris* plasma membrane. All changes described above contribute to the specific biophysical properties of the yeast plasma membrane. Depletion of cardiolipin in plasma membrane samples served as a further indicator for the purity, especially for the absence of mitochondria.

3.3. Fatty acid composition of the P. pastoris plasma membrane

Fatty acids mainly incorporated into complex lipids are further important structural components of biological membranes. Fatty acid analysis of total cell extracts showed that monounsaturated oleic acid (C18:1; cis-9), polyunsaturated linoleic acid (C18:2; cis-9,12) and α-linolenic acid (C18:3; cis-9,12,15) and saturated palmitic acid (C16:0) were the most abundant fatty acids in *P. pastoris* (Table 2). The fatty acid pattern of isolated plasma membrane fractions was very characteristic and clearly distinct from homogenates. Oleic acid was found to be still predominant in plasma membrane samples, but the amounts of the polyunsaturated fatty acids linoleic and linolenic acid were strongly decreased compared to the homogenate. Noteworthy, a strong increase in the levels of saturated fatty acids C16:0 and C18:0 was observed in plasma membrane fractions which led to a marked shift of the ratio of unsaturated to saturated fatty acids. The increase of cerotic acid (C26:0) in the plasma membrane further contributed to this effect.

3.4. P. pastoris plasma membrane is enriched in ergosterol

Homogenates and plasma membrane samples were analyzed for their content of ergosterol, the final product of yeast sterol biosynthesis. In total cell extracts from *P. pastoris* an amount of 14.7 ± 1.8 μg ergosterol per mg protein was determined. Plasma membrane samples showed an approximately 4-fold enrichment of ergosterol (52.6 ± 10.2 μg ergosterol per mg protein) over the homogenate. Besides ergosterol, sterol precursors such as zymosterol, ergosta-5,8,22,24-tetraenol, 4-methylzymosterol, ergosta-5,8-dienol, fecosterol, episterol and lanosterol were found at low abundance in total cell extracts comprising less than 20% of the total sum of sterols. Sterol precursors found in plasma membrane samples were mostly fecosterol, episterol and lanosterol, but only at ~5% of total plasma membrane sterols.

Table 1
Phospholipid composition of total cell extracts and plasma membrane fractions of *P. pastoris*. PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; Lyso-PL, lysophospholipids; DMPE, dimethylphosphatidylethanolamine.

Fraction	% of total phospholipids							
	PA	PI	PS	PC	PE	CL	Lyso-PL	DMPE
Homogenate	2.17 ± 1.29	6.73 ± 2.91	6.41 ± 0.31	50.30 ± 6.36	30.86 ± 3.02	2.54 ± 0.64	1.0 ± 0.52	n.d.
Plasma membrane	8.23 ± 0.82	4.29 ± 1.67	20.42 ± 3.37	27.48 ± 4.42	36.15 ± 0.9	1.06 ± 0.04	1.86 ± 0.31	0.52 ± 0.73

3.5. Effect of different carbon sources on the lipid composition of P. pastoris plasma membrane

Carbon sources different from glucose which are of interest for biotechnological and industrial use of *P. pastoris* were also used to cultivate cells for plasma membrane preparation. Methanol is an interesting alternative to glucose due to the highly efficient and tightly regulated alcohol oxidase promoter system used for inducing production of heterologously expressed proteins [52]. Glycerol, a non-fermentable multicarbon source, has recently been shown to be advantageous in mixed feeding strategies [53–55]. Both carbon sources, however, had only minor effects on the composition of fatty acids, phospholipids and sterols of the plasma membrane from *P. pastoris* (Supplemental Tables 1 and 2). The only class of lipids which responded to different growth conditions were sterols. Growth on glycerol led to a 4.5-fold enrichment of ergosterol in the plasma membrane over the corresponding homogenate, whereas plasma membrane samples of cells cultivated on methanol were enriched in ergosterol only 2.1-fold.

3.6. Sphingolipids of the P. pastoris plasma membrane

Besides glycerophospholipids and sterols, sphingolipids are of special interest as components of the plasma membrane. Two major classes of complex sphingolipids have been identified in *P. pastoris*, namely the phosphosphingolipids inositolphosphorylceramide, IPC; mannosyl-inositolphosphorylceramide, MIPC; mannosyl-diinositolphosphorylceramide, $M(IP)_2C$ and the neutral sphingolipids ceramide, Cer; and hexosylceramide, HexCer [56]. These sphingolipid classes vary in their polar head groups as well as their ceramide backbones [57,58].

In the present study, sphingolipids of bulk membranes and plasma membrane fractions from *P. pastoris* cells cultivated on glucose containing media were analyzed by nanoESI-MS/MS. The patterns of both sphingolipid classes consisted of a number of species showing class specific distributions (Fig. 3). Differences in the species pattern of sphingolipids from bulk membranes and plasma membrane were mainly found for IPCs (see below). Most IPC-species carried 4-hydroxysphinganine (phytosphingosine; 18:0;3), which is a trihydroxylated amino alcohol with a straight C18-aliphatic chain as the long chain base (LCB). By contrast, ceramides displayed a broader spectrum of long chain bases (LCB) with sphinganine (18:0;2), sphing-4-enine (18:1;2), sphingadienine (18:2;2) and 4-hydroxysphinganine (18:0;3) as the most abundant representatives. HexCer which carry a hexose residue linked to the molecule through a glycosidic bond contained only dihydroxylated LCBs with sphingadienine (18:2;2) and its C9 methylated variant (19:2;2) with remarkable abundance. Besides obvious differences in the LCB composition of the two classes of the investigated sphingolipids, the amide-linked acyl chains also showed sphingolipid dependent distribution patterns. IPC, MIPC and $M(IP)_2C$ showed highest abundance of 42:0;4; 42:0;5; 44:0;4 and 44:0;5 species. Taking into account 18:0;3 for LCB leaves lignoceric acid (C24:0) and cerotic acid (C26:0), both present in a mono- or dihydroxylated form, as acyl chains in these molecules. Comparison between bulk membranes and plasma membrane fractions clearly showed that molecular species 42:0;5 (18:0;3/24:0;2) as well as 44:0;5 (18:0;3/26:0;2) were enriched in phosphosphingolipid classes of the plasma membrane, although the enrichment was lowest for $M(IP)_2C$. Only few molecular species were found in bulk membranes and plasma membrane samples which contained a dihydroxylated LCB instead of a trihydroxylated LCB, namely 34:0;2; 36:0;2; 38:0;2 and 40:0;2.

Ceramides showed the highest abundance for monohydroxylated lignoceric and cerotic acid but also contained palmitic (C16:0) and stearic acid (C18:0) in both mono- and unhydroxylated form. Hexosylceramides displayed a strong preference for α-hydroxy stearic acid. Species patterns of bulk membranes and plasma membranes were almost identical.

Results described above were complemented by a HILIC-UPLC-nanoESI-MS/MS analysis which enabled us to estimate the relative abundance of sphingolipid classes in plasma membrane samples compared to microsomes, representing a major portion of internal membranes. As can be seen from Figs. 4A and B, respectively, ceramides and hexosylceramides were clearly enriched in microsomes and only present at low amounts in the plasma membrane. Noteworthy, plasma membrane fractions contained five times more hexosylceramides (1.4 pmol/μg protein) than ceramides (0.3 pmol/μg protein). Inositol containing sphingolipids (IPC, MIPC, $M(IP)_2C$) were enriched in plasma membrane fractions over microsomes (Fig. 4C). However, the observed enrichment of IPCs did not account for all molecular species. Only IPC, MIPC and $M(IP)_2C$ ranging from C42 to C46 were clearly enriched in the plasma membrane compared to microsomes (Fig. 4C). *P. pastoris* plasma membrane phosphosphingolipids almost exclusively contained very long chain acyl residues, giving rise to C42–C46 backbones, whereas in microsomal fractions also substantial amounts of acyl chains ranging from C16 to C18 were found. Ceramides and hexosylceramides almost exclusively contained C34–36 backbones, which were several-fold enriched in the microsomal fraction compared to the plasma membrane (Fig. 4A and B). Thus, the plasma membrane signature of *P. pastoris* in contrast to that of internal membranes is defined by a clear preference for phosphosphingolipids containing very long chain fatty acids.

4. Discussion

During the last decades it has been recognized that biological membranes do not only serve as intracellular boundaries which compartmentalize organelles and shield the interior of the cell from the surrounding milieu, but also create an indispensable environment for certain enzymes and biosynthetic processes, contribute actively to the anchoring of constituents, and serve as reservoir for signaling molecules. In addition, the plasma membrane plays an important role

Table 2
Fatty acid composition of total cell extracts and plasma membrane fractions from *P. pastoris*.

Fraction	% of total fatty acids									Unsaturated/saturated fatty acids
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C26:0	Others	
Homogenate	0.3 ± 0.1	12.4 ± 0.8	3.0 ± 0.6	3.6 ± 0.4	30.5 ± 4.0	27.1 ± 2.6	21.2 ± 2.0	0.6 ± 0.3	1.2 ± 0.2	4.9
Plasma membrane	0.3 ± 0.1	17.0 ± 0.8	2.2 ± 0.3	5.6 ± 0.9	30.6 ± 3.7	21.3 ± 2.2	12.1 ± 1.6	3.6 ± 1.6	7.2 ± 0.9	2.7

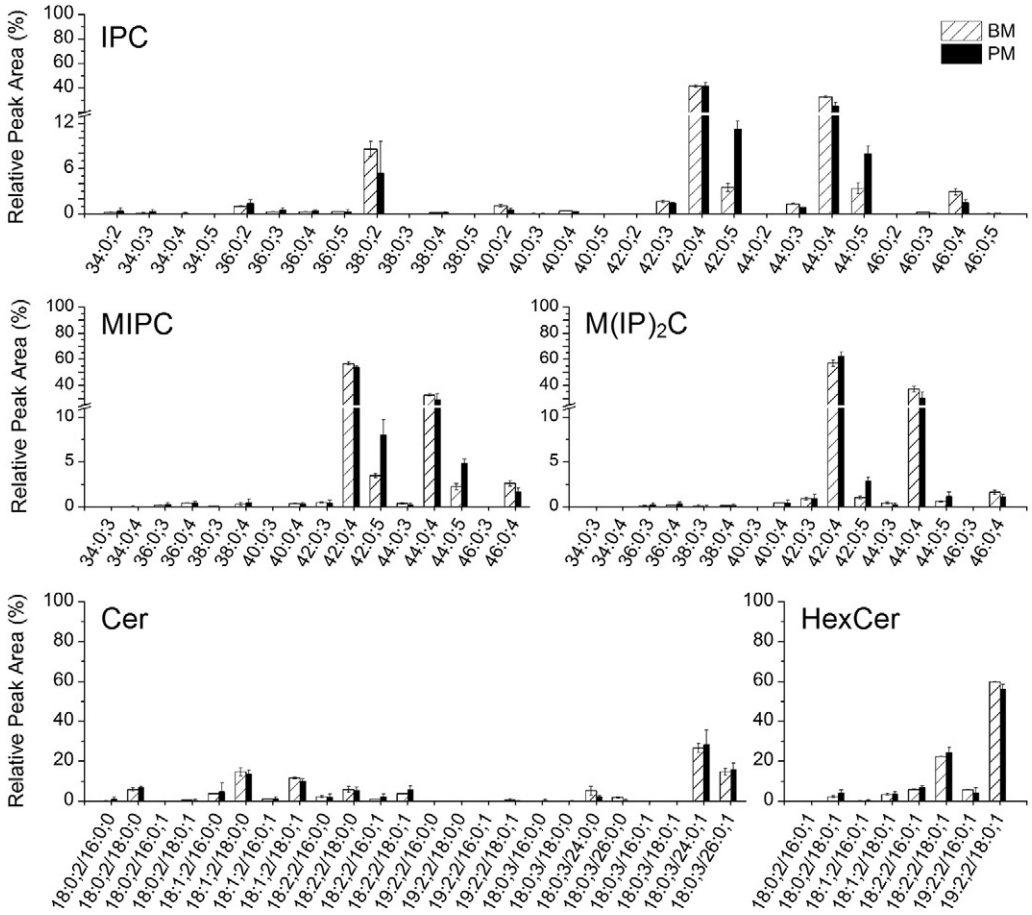

Fig. 3. Sphingolipid analysis of *P. pastoris* plasma membrane. Composition of molecular species of sphingolipids from *P. pastoris* bulk membranes (BM) and plasma membrane (PM) are shown. Cells were cultivated on glucose containing media until they reached the late exponential phase. UPLC-nanoESI-MS/MS was used to characterize inositolphosphorylceramide (IPC), MIPC (mannosyl-inositolphosphorylceramide), $M(IP)_2C$ (mannosyl-diinositolphosphorylceramide), Cer (ceramide) and HexCer (hexosylceramide). Sphingolipid molecular species are expressed as "Sphingolipid-class (Long-chain-base/Fatty-acyl)". Long chain bases and fatty acyls are expressed as XX:YY;Z (XX: number of carbons; YY: number of C – C double bonds, Z: number of hydroxyl groups).

in the import of components into the cell and the export of molecules to the surrounding environment. Whereas information about plasma membranes from many cell types including the baker's yeast *S. cerevisiae* [22,51] is available, the biochemistry and cell biology of the plasma membrane from the Crabtree-negative yeast *P. pastoris*, which is an important tool for various biotechnologically relevant processes, has so far been virtually ignored. For this reason, a biochemical characterization of the *P. pastoris* plasma membrane with emphasis on the lipid profile was included in the efforts of our laboratory to study organelles from this yeast in some detail.

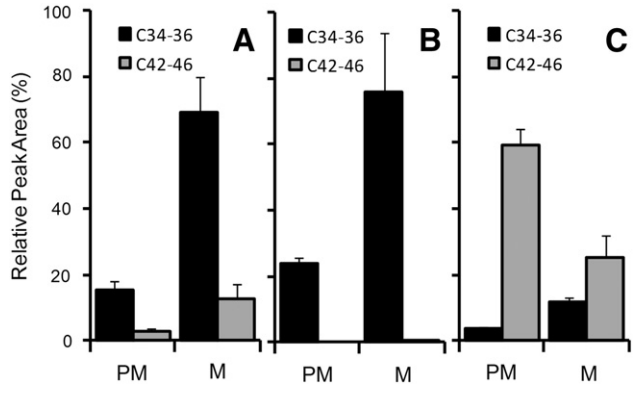

Fig. 4. Sphingolipid classes in the plasma membrane of *P. pastoris*. Microsomal membrane (M, 30,000 ×g) and plasma membrane (PM) fractions from *P. pastoris* were analyzed by HILIC-UPLC-nanoESI-MS/MS. Identical amounts of sample (0.3 mg of total protein) were analyzed. Peak intensities represent the relative abundance of (A) ceramides, (B) hexosylceramides and (C) inositol containing sphingolipids.

Not unexpectedly, a strong enrichment of PS and PA was found in the plasma membrane of *P. pastoris*. Similar results were obtained before with *S. cerevisiae* [51,59,60]. Surprisingly, also an enrichment of PE was found in the *P. pastoris* plasma membrane. All these enrichments were at the expense of PC whose concentration in the plasma membrane (25–27% of total phospholipids) was markedly lower than in bulk membranes (see Table 1). It has to be taken into account that the resulting pattern of phospholipids in the plasma membrane dramatically influences its biophysical properties. The negatively charged phospholipids PA and PS were reported to play a prominent structural role in biological membranes [61], generating a negative surface potential which is, e.g., essential for proteins containing pleckstrin homology domains (PH domain) [62], and influencing the activity of certain membrane proteins directly [63,64]. PS which is usually found enriched at the cytosolic side of the plasma membrane is also able to recruit proteins containing PS-binding domains. Translocation of PS to the outer leaflet of the plasma membrane results in a loss of bilayer asymmetry and triggers apoptosis [65]. PA which is an essential intermediate for the formation of other glycerophospholipids can be produced rapidly by stimulated hydrolysis of PC catalyzed by phospholipase D (PLD)

[66,67]. Zeniou-Meyer et al. [68] showed in mammalian cell cultures that activation of PLD led to increased levels of PA in the plasma membrane. Enhanced production of PA was proposed to cause alterations in membrane curvature which in turn promoted hemi-fusion during exocytosis. Besides PA also PE, present at high amount in the plasma membrane of *P. pastoris*, is highly important for fission and fusion events. Both phospholipids have smaller head groups than typical bilayer-forming lipids such as PC, which enables them to modulate membrane curvature [69,70] during membrane fusion and fission events. Thus, the presence of phospholipids with conical shape in the plasma membrane may be of special importance for *P. pastoris* because of its highly active protein secretory machinery where frequent fusion events of secretory vesicles with the plasma membrane are required. Furthermore, the small head group of PE may prevent dense packing of the entire membrane thereby lowering the lateral pressure and stabilizing membrane proteins in their favored conformation [71].

The low level of polyunsaturated fatty acids in the *P. pastoris* plasma membrane compared to bulk membranes and the simultaneously increased amounts of all saturated fatty acids with a carbon chain length of 16 or higher leads to a remarkably high degree of fatty acid saturation in the membrane (see Table 2). Consequently, plasma membranes exhibit a specific fatty acid pattern which is markedly different from total cell extracts. A reason for this finding may be the specific nature of plasma membrane proteins, because their function largely depends on a transition between conformational states [72]. Especially intrinsic membrane proteins such as molecular pumps or ion channel proteins are susceptible to changes in the lipid environment [73,74]. However, the presence of polyunsaturated fatty acids in the *P. pastoris* plasma membrane must not be ignored. These fatty acids may contribute to a membrane environment which is favorable for exocytotic/secretory processes with higher efficiency than in *S. cerevisiae* which can only produce monounsaturated fatty acids. With that respect, *P. pastoris* membranes rather resemble membranes from higher eukaryotes. Although mammalian cells are not able to synthesize polyunsaturated fatty acids such as linoleic acid (18:2) and alpha-linolenic acid (18:3) themselves de novo, these fatty acids are essential and have to be taken up from the diet [75]. Thus, the presence of certain amounts of polyunsaturated fatty acids in the *P. pastoris* plasma membrane may be regarded as another human-like factor of this yeast. The positive effect of polyunsaturated fatty acids on protein secretion was, as an example, shown recently by Wei et al. [76]. These authors showed that in islets of transgenic mice expressing an n − 3 fatty acid desaturase from *Caenorhabditis elegans* the increased level of n − 3 polyunsaturated fatty acids was paralleled by improved insulin secretion.

Surprisingly, the ergosterol level found in the plasma membrane of *P. pastoris* (~50 μg per mg protein) was rather low compared to the baker's yeast *S. cerevisiae* (~400 μg per mg protein) [60]. This finding is in line with the observation that steryl esters, the storage form of sterols present in excess, are present in *P. pastoris* at a much lower amount than in *S. cerevisiae*. Ivashov et al. [19] showed recently that steryl esters only account for 10% of total non-polar lipids in *P. pastoris*, whereas amounts of triacylglycerols and steryl esters in *S. cerevisiae* are almost equal. Reasons for the low sterol production and/or requirement of *P. pastoris* can be partially explained by genetic properties. Whereas *S. cerevisiae* contains enzyme pairs of sterol metabolism such as *HMG1*/*HMG2* or *ARE1*/*ARE2*, only single forms of the respective genes are present in *P. pastoris*. As sterols are by nature hydrophobic and stereochemically rigid, they can effectively influence the biophysical properties of biological membranes and thus their function [77]. These requirements may be different in *P. pastoris* compared to other cell types, or mechanisms to maintain and regulate membrane fluidity may vary. As sterols are known to decrease the fluidity of membranes, the low level of ergosterol in the *P. pastoris* plasma membrane in combination with the presence of polyunsaturated fatty acids may cause relatively high membrane fluidity. The negative impact of high amounts of sterols on protein secretion was most recently reported for mammalian cells. Bogan et al. [78] showed that upon accumulation of cholesterol in insulin-containing secretory granules from pancreatic β-cells the regulated exocytosis of the cargo was impaired. The excess of cholesterol reduced at least to a certain extent docking and fusion of insulin granules at the plasma membrane.

Analysis of sphingolipids from *P. pastoris* identified not only the pattern of this lipid class in the plasma membrane but also the specific subcellular distribution. Whereas *S. cerevisiae* mainly produces inositol containing phosphorylceramides, *P. pastoris* can also form hexosylceramides. Neutral sphingolipids and phosphosphingolipids were found to be different in their long chain base (LCB) composition as well as the length of the amide-linked acyl chains. Similar to *S. cerevisiae*, where C26:0 or hydroxylated C26:0 are the major fatty acids of IPCs [79,80], very long chain C24:0 and C26:0 fatty acids, hydroxylated or non-hydroxylated, were incorporated into *P. pastoris* IPC classes. Whereas *S. cerevisiae* has a strong preponderance for C26:0 fatty acids, in *P. pastoris* almost equal amounts of C24:0 and C26:0 were found. Compared to bulk membranes, IPCs of the *P. pastoris* plasma membranes were highly enriched in fatty acid residues with a high degree of hydroxylation. Interestingly, most characteristics of inositol phosphorylceramides were not observed with ceramides and hexosylceramides. In contrast to phosphosphingolipids which exclusively contained phytosphingosine as the LCB, neutral sphingolipids showed a broader spectrum of LCBs. Especially in ceramides a strong variation of LCBs was found whereas hexosylceramides were restricted to dihydroxylated LCB variants. As *S. cerevisiae* mainly accumulates phosphosphingolipids, its ceramides contain either dihydrosphingosine or phytosphingosine [81]. In contrast to *S. cerevisiae*, *P. pastoris* accumulates hexosylceramides, which is in agreement with the broader spectrum of LCBs found in its ceramides [56].

Interestingly, the different sphingolipid classes of *P. pastoris* accumulate in different subcellular compartments. Whereas internal membranes (microsomes) are enriched in ceramides and hexosylceramides, IPCs are preferentially present in the plasma membrane. This finding may be due to the fact that ceramide synthesis occurs in the endoplasmic reticulum [82], whereas IPC synthase activity was found in the Golgi [83]. Furthermore, the enzymatic equipment responsible for generating the specific structural features of IPCs such as the high degree of hydroxylation or the occurrence of very long chain fatty acids is also associated with the Golgi [84,85]. However, transport routes connecting the steps of sphingolipid metabolism are not well defined. It is still a matter of dispute whether ceramides follow the secretory route or a vesicle-independent pathway [81]. Since ceramides, hexosylceramides and IPCs differ in post-ER modifications such as hydroxylation or fatty acid elongation, it can be speculated that their supply to the plasma membrane might involve different transport routes.

Last but not least the possible role of microdomains in the plasma membrane named lipid rafts and their influence on the secretory process may be taken into account. In these specialized membrane domains sphingolipids associate laterally with each other, and open spaces are filled by sterols [24]. The existence of rafts in yeast has been a matter of dispute for a long time. Only recently, Toulmay and Prinz [25] convincingly reported the existence of such microdomains in *S. cerevisiae*. No such experiments have been performed with *P. pastoris*, and we can only speculate at present by analogy that rafts may also exist in this yeast. However, differences in the nature of potential raft components from *P. pastoris*, *S. cerevisiae* and mammalian cells have to be mentioned. First, sphingolipids found in the plasma membrane from *P. pastoris* and higher eukaryotes have little in common. Mammalian cells contain almost exclusively sphingomyelin and to a lesser extent glycosphingolipids such as gangliosides depending on the tissue [86]. By contrast, the plasma membrane from *P. pastoris* harbors almost exclusively inositolphosphoryl-containing ceramides and only small amounts of hexosylceramides. The structural difference between these sphingolipid species may have an influence on the raft formation. A further characteristic feature of plasma membranes from higher eukaryotes is

the occurrence of so-called caveolae, invaginations of sphingomyelin-cholesterol enriched microdomains harboring caveolin-1 [24,87]. So far, however, no evidence for the existence of caveolae in *P. pastoris* has been presented.

Thus, despite certain functional overlaps of *P. pastoris* and mammalian plasma membranes caused by structurally related components, properties of this compartment in the microorganism remain somehow unique. Most likely, a well-balanced composition of all lipid constituents creates the ideal membrane environmental for protein production and secretion. Our data provided here may help to improve our knowledge of *P. pastoris* as microbial cell factory, especially for recombinant protein production and secretion. As the plasma membrane is the final barrier of the secretory route its properties may have a marked influence on the whole process. Therefore, modification of membrane properties by "membrane engineering" may be considered as a useful strategy to manipulate *P. pastoris* as a biotechnological tool.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.03.012.

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