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Lipid composition of peroxisomes from the yeast *Pichia pastoris* grown on different carbon sources

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Abstract

Highly purified peroxisomes from the yeast *Pichia pastoris* grown on methanol or oleic acid, respectively, were used to characterize the lipid composition of this organelle. For this purpose, an isolation procedure had to be adapted which yielded highly purified *P. pastoris* peroxisomes. When peroxisome proliferation was induced by growth on methanol, alcohol oxidase was the predominant peroxisomal protein. Cultivation of *P. pastoris* on oleic acid led to induction of a family of peroxisomal enzymes catalyzing fatty acid β-oxidation, whose most prominent members were identified by mass spectrometry. On either carbon source, phosphatidylcholine and phosphatidylethanolamine were the major peroxisomal phospholipids, and cardiolipin was present in peroxisomal membranes at a substantial amount, indicating that this phospholipid is a true peroxisomal component. Ergosterol was the most abundant sterol of *P. pastoris* peroxisomal membranes irrespective of the culture conditions. The fatty acid composition of whole cells and peroxisomes was highly affected by cultivation of *P. pastoris* on oleic acid. Under these conditions, oleic acid became the predominant fatty acid in phospholipids from total cell and peroxisomal extracts. Thus, oleic acid was not only utilized as an appropriate carbon source but also as a building block for complex membrane lipids. In summary, our data provide first insight into biochemical properties of *P. pastoris* peroxisomal membranes, which may become important for the biotechnological use of this yeast.

Keywords: *Pichia pastoris*; Peroxisomes; Alcohol oxidase; Phospholipids; Sterol; Oleic acid

1. Introduction

Peroxisomes are versatile, single-membrane-bound organelles occurring ubiquitously in eukaryotic cells. However, the number, size and functions of these organelles are strongly determined by cell type and physiology [1,2]. A characteristic feature of peroxisomes is their large amount of matrix enzymes which catalyze various oxidative and biosynthetic reactions involved in hydrogen peroxide metabolism, β-oxidation, ether lipid and cholesterol biosynthesis, the glyoxylate cycle, photorespiration and glycolysis [1]. In yeast and plant cells, β-oxidation has been exclusively localized to peroxisomes, whereas in mammalian cells mitochondria also harbor enzymes of this pathway. A specific feature of yeast peroxisomes is their ability to be induced by growth on media containing alkanes, fatty acids or methanol [2]. The importance of peroxisomes in human metabolism and development is illustrated by numerous disorders associated with peroxisomal dysfunction [3].

The yeast *Pichia pastoris* has the ability to use methanol or fatty acids as the sole carbon and energy source [4–6]. These growth conditions lead to massive proliferation of peroxisomes and enzymes involved in methanol metabolism or fatty acid β-oxidation, respectively. The possibility to induce peroxi-

Abbreviations: AOX, alcohol oxidase; TLC, thin layer chromatography; GLC, gas–liquid chromatography; LP, lysophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdCho, phosphatidyl choline; PtdEtn, phosphatidylethanolamine; CL, cardiolipin; DM-PtdEtn, dimethylphosphatidyl ethanolamine

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some formation by nutrients has made *P. pastoris* a suitable model organism for studying peroxisomal biogenesis and related defects [3]. Another advantage of *P. pastoris* is its strong and strictly regulated alcohol oxidase AOX1 promoter which allows this yeast to be used as an efficient expression system for heterologous proteins [7–10].

Despite the extensive use of this microorganism in biotechnology, fundamental biochemical and cell biological investigations of *P. pastoris* organelles are rare. For this reason, we started in our laboratory a systematic study to establish standardized methods for *P. pastoris* organelle isolation and characterization of subcellular fractions with special emphasis on the biochemistry of organelle membranes. For the work presented here, we employed a method based on density gradient centrifugation to prepare *P. pastoris* peroxisomes at high purity. This procedure was the basis for protein and lipid analysis of peroxisomal fractions isolated from *P. pastoris* grown on different carbon sources.

2. Materials and methods

2.1. Strains and culture conditions

The strain *P. pastoris* X33 (MATa, Mut^+) was used throughout this study. YPD medium containing 1% yeast extract, 2% peptone and 2% glucose was used to pre-cultivate the strain for 48 h at 30 °C in Erlenmeyer flasks with baffles at 140 rpm. For the induction of peroxisome proliferation, cells were shifted to YPM medium containing 0.5% methanol, 1% yeast extract and 2% peptone; or YPO medium consisting of 0.2% oleic acid, 1% yeast extract, 2% peptone and 0.02% Tween 40. Cells were cultivated in 2-l Erlenmeyer flasks with baffles under shaking conditions.

Electron microscopic analysis of *P. pastoris* cells grown on YPD, YPM or YPO medium was performed as previously described by Müllner et al. [11]. Electron microscopic analysis of isolated peroxisomes was carried out according to Erdmann and Blobel [12].

2.2. Isolation of organelles

Peroxisomes were isolated from *P. pastoris* X33 cells grown on YPM or YPO media for 26 h to the late logarithmic phase. To obtain highly purified peroxisomes the isolation procedure of Faber et al. [13] was modified. In brief, cells were harvested by centrifugation and converted to spheroplasts using Zymolyase 20T [14]. Spheroplasts were homogenized in breaking buffer (5 mM MES, 1 M sorbitol, 1 mM KCl, 1 mM Na_2EDTA, 0.1% EtOH, pH 6.0, 1 mM PMSF) with 10 strokes in a Dounce Homogenizer. The homogenate was centrifuged for 5 min at 3000×*g*, and the resulting pellet was homogenized twice with 5 strokes, each. The cell-free supernatants were combined and centrifuged at 30,000×*g* for 30 min to yield a pellet containing mitochondria and peroxisomes. This pellet was suspended in 10–15 ml breaking buffer and loaded on top of a step gradient composed of 5 ml 50%, 7 ml 35%, 7 ml 30%, 7 ml 24% and 7 ml 17% Accudenz (w/v) in 5 mM MES, 1 mM KCl, 0.24 M sucrose, pH 6.0. The gradient was centrifuged at 122,000×*g* for 90 min in a Sorvall AH629 rotor, and the peroxisomal fraction forming a band at a buoyant density of ~1.18 g/ml was collected with a syringe, diluted with four volumes of breaking buffer and centrifuged at 30,000×*g* for 30 min. The resulting peroxisomal pellet was suspended in 1 ml Tris/HCl buffer, pH 7.4. Mitochondria were obtained from the same gradient (buoyant density of ~1.16 g/ml) and isolated by the same procedure as peroxisomes.

2.3. Protein analysis

Protein concentration was determined as described previously [15] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [16] using 10 or 12.5% SDS gels. Western Blot analysis was performed according to Haid and Suissa [17]. Primary antibodies used in this work were from rabbits and directed against alcohol oxidase, Pex3p, Por1p, Fox2p, Pma1p, Aac2p and GAPDH (glyceraldehyde-3-phosphate-dehydrogenase). Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection (SuperSignal™, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

For the identification of peroxisomal proteins induced by growth of *P. pastoris* on oleic acid polypeptides of interest were manually excised from SDS-gels and digested overnight at 37 °C with 100 μg/ml trypsin (Sigma) in 50 mM ammonium hydrogen carbonate. In-gel digested peptide fragments were extracted by incubation with 5% formic acid in 50% acetonitrile at 37 °C for 15 min. This step was repeated 3 times; the combined extracts were dried by vacuum centrifugation to a final volume of 25 μl and transferred to a micro-vial. Water/acetonitrile (98:2; v/v) containing 0.1% formic acid was added, and samples were analyzed with an Agilent 1100 Series LC/MSD SL mass spectrometer (Agilent Technologies, Inc., Waldbronn, Germany). The Mascot protein database search Software from Matrix Science Ltd. (http://www.matrixscience.com) was used for identification by homology to *Saccharomyces cerevisiae* proteins. Additionally, the identity of *P. pastoris* proteins was confirmed by comparison of DNA sequence to that of a fresh sample of *P. pastoris* NRRL Y-11430 obtained from the Northern Regional Research Laboratories, Peoria, IL, USA.

The endoplasmic reticulum marker enzyme NADPH-cytochrome *c* reductase [18] and the vacuolar marker enzyme α-D-mannosidase [19] were assayed as described previously.

2.4. Lipid analysis

Lipids were extracted using the procedure of Folch et al. [20]. Individual phospholipids were separated by two-dimensional thin layer chromatography (TLC) on Silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% NH_3 (65:35:5, per vol.) as the first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, per vol.) as the second developing solvent. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off and quantified by the method of Broekhuyse [21].

Individual sterols were analyzed by gas–liquid chromatography (GLC) after alkaline hydrolysis of lipid extracts [22]. GLC was performed on a Hewlett-Packard 5890 Gas-Chromatograph equipped with a mass selective detector (HP 5972), using a HP5-MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Helium was used as carrier gas, and 1 μl aliquots of samples were injected onto the column.

Fatty acids were also analyzed by GLC. Lipids extracted as described above were subjected to methanolysis using BF_3/methanol and converted to methyl esters [23]. Fatty acyl methyl esters were separated by GLC using a Hewlett-Packard 6890 Gas-Chromatograph equipped with a 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 commercial fatty acyl methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

3. Results and discussion

3.1. Growth of P. pastoris cells on different carbon sources

P. pastoris strain X33 can grow on different carbon sources. The growth rate of cells cultivated on methanol, oleic acid and glycerol was much lower than on YPD (data not shown). This fact had to be taken into account for cell fractionation experiments because cells were always harvested in the late logarithmic phase. At this stage, peroxisomes were fully developed in *P. pastoris* grown on methanol or oleic acid. As reported previously [24], induction with methanol resulted in large and clustered peroxisomes (Fig. 1A), whereas growth on