Quantitative proteomics of *Saccharomyces cerevisiae* vacuoles and stress responses in *Lactococcus lactis*

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II. CHAPTER

ISOLATION OF VACUOLES FROM SACCHAROMYCES CEREVISIAE

Ludovico: I congratulate you, sir.
Galileo: I have improved it.
Ludovico: Of course, sir. I see you have made the cover red, in Holland it was green.
Abstract
Any proteomics analysis starts with sample preparation. For organelle proteomics, where the aim is the identification of organelle-specific proteins, the sample preparation involves the isolation of an organelle of interest to a high degree of purity devoid of cross-contaminations from other organelles. Different organelles of the yeast *Saccharomyces cerevisiae* possess unique physical/chemical properties that can be exploited or taken into account during isolation. The vacuole has a low density (it is the 'lightest' organelle of yeast) and can easily be separated from other organelles by density gradient centrifugation, yielding highly pure vacuole preparations that can be used for proteomics studies.
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**Introduction**

The interior of eukaryotic cells is divided into multiple compartments, the organelles, with distinct properties and proteomes. The study of organelle-specific proteomes is called organelle proteomics, and generally consists of two experimental steps. First, pure organelles are isolated and, second, the proteins are extracted, and peptides are generated and subjected to the mass spectrometry analysis. The challenge in organelle proteomics is to obtain pure organelles that contain as many organelle associated proteins and as few contaminants as possible. Because mass spectrometry is a sensitive technique, even contaminations that are present at low levels, may be detected, identified and subsequently assigned as proteins located in the organelle of interest (false positives).

In this chapter we first describe in general how organelles from the yeast *S. cerevisiae* are isolated. Next an overview is presented of the properties of *S. cerevisiae* organelles that are relevant for their isolation such as density, size, and fragility. Finally, an optimization of the vacuole isolation procedure is presented and some important observations that were made during the isolation of vacuoles from *S. cerevisiae* cells are described. In the Chapter III the optimized procedure for vacuole isolation is used in a proteomics study.

**Isolation of organelles**

Three major steps are common for all organelle purifications from yeast cells: 1) the cell wall is removed; 2) cells are lysed in order to liberate the organelles; 3) the released organelles are separated into different populations according to their physical or biological properties.

**Breaking cells.** The yeast cells can be disrupted mechanically, or first converted to spheroplasts by enzymatic removal of the cell wall. During mechanical disruption cells are exposed to rough sheering forces either in liquid suspension by the means of glass beads, or frozen when cells are broken to powder by a steel ball. Alternatively, mechanical lysis can be performed using a polytron homogenizer, which produces a combination of ultra-sonic and mechanical (blades) forces. The consequence of any mechanical disintegration of cells is an inevitable fragmentation of intracellular organelles and organelle membranes to a mixture of small membrane vesicles. The disturbed organelle integrity complicates to separate the soluble proteomes of different organelles because they become mixed up. In addition, the separation of organelle membrane vesicles of different origins using density gradient centrifugation (see below) becomes difficult because the densities of membrane vesicles are relatively alike whereas the intact organelles possess different densities. Utilizing lytic enzymes e.g. gluclase, zymolyase, mutanase (Glucanex) for removal of the cell wall and the consequent mild lysis (see below) allows preservation of the organelle integrity. The partial removal of the yeast cell wall upon digestion with lytic enzymes is referred to as spheroplasting and results in a spherical cell shape. For enzymatic lysis it is important to realize that glucans together with chitin determine rigidity and strength of the cell wall, whereas mannoproteins limit the porosity of the cell wall (Cabib et al., 1982; Zlotnik et al., 1984). The porosity is responsible for sieving substances that come in contact with the plasma membrane (Zlotnik et al., 1984), whereas the rigidity prevents cells from lysis in hypotonic solutions. Zymolyase which cleaves glucans at β-1,3 positions is the most common lytic enzyme that is used for spheroplasting of yeast cells. Furthermore, mutanase which cleaves α-1,3 glucosidic bonds and gluclase with β-glucuronidase and sulfatase activities can be used additionally to facilitate the removal of the yeast cell wall. The osmotic stabilizer sorbitol is usually used at high concentrations during spheroplasting (1-1.4 M), which creates hypertonie conditions. Under such conditions the cells have tendency to shrink facilitating the mechanical separation of the plasma membrane from the rigid cell wall.

In order to liberate organelles, the spheroplasts can be lysed osmotically or in the presence of isotonic solution. Under isotonic conditions spheroplasts can be disintegrated mechanically (using polytron or Dounce homogenizer), metabolically (Indge, 1968a) or upon a specific attack on the plasma membrane by polybasic...
macromolecules such as DEAE-Dextran (Durr et al., 1975). The lysis method should be carefully chosen considering specific application of each method. Whereas the nuclei can survive mechanical cell disintegration due to structure-stabilizing filaments, lyzing spheroplasts mechanically results in breakage of fragile organelles such as vacuoles, mitochondria and peroxisomes. This is disadvantageous for the yield and purity when aiming at isolation of these organelles.

The choice of homogenization medium is determined by the application as well. The homogenization media are buffered solutions that mimic physiological conditions e.g. 10 mM Tris-HCl, pH 6-7.5, containing an osmotic stabilizer such as sorbitol or mannitol. In addition protease inhibitors, and either MgCl$_2$ or EDTA are included. Thereby following has to be considered: upon lysis organelles tend to aggregate if MgCl$_2$ is present because Mg$^{2+}$ ions bind to negatively charged phospholipids and cause their aggregation. On the other hand, the presence of MgCl$_2$ is beneficial to nuclear integrity because the nuclear envelope becomes fragile and starts leaking DNA in presence of EDTA, a chelating agent for Mg$^{2+}$ ions, which causes aggregation of organelles due to the tendency of DNA to bind to other particles via ionic interactions. It is noteworthy that EDTA is also a very efficient inhibitor of Mg$^{2+}$- and other metal-dependent proteases. Use of protease inhibitors is essential to protect organelle proteins from attack of endogenous proteases during isolation procedure.

**Purification**

**Differential centrifugation.** The crude cell lysates are usually first subjected to differential centrifugation to obtain enriched crude organelles (Fig. 1). The differential centrifugation can involve multiple subsequent centrifugation steps that are performed at increasing centrifugation speed. An initial low-speed centrifugation (usually performed at 2,000 - 5,000 x g for 10 min) results in a so-called post-nuclear supernatant containing most organelles, organelle vesicles, the cytosol and free ribosomes. The corresponding pellet contains the cell walls (after direct mechanical disruption of cells) or the cell wall remnants (after enzymatic spheroplasting), unlysed cells and a heavy population of nuclei. The post-nuclear supernatant is then subjected to the high-speed centrifugation (at 20,000 - 30,000 x g) to sediment organelles such as intact mitochondria, peroxisomes, a light population of nuclei, Golgi and RER. The lighter organelles such as SER, endosomes, vacuoles, and lipid particles, and membrane vesicles of the plasma membrane and other lysed organelles remain in the supernatant. Finally, the membrane vesicles and some light organelles are sedimented upon ultra-centrifugation at >300,000 x g. The lipid particles usually do not sediment even upon ultra-centrifugation, because of their low density.

**Density gradients.** Crude organelles can be further purified based on different physical properties by the means of centrifugation using density gradients or free-flow electrophoresis (Zischka et al., 2006); or based on different antigenic properties by immuno-techniques (Luers et al., 1998). Equilibrium density centrifugation still remains the method of choice for subcellular fractionation and has been successfully used for the isolation of different organelles from yeast (Ohsumi & Anraku, 1981; Aris & Blobel, 1991; Strambio-de-Castillia et al., 1995; Meisinger et al., 2000; Yi et al., 2002). Equilibrium density centrifugation can be performed in two ways: top-down movement (sedimentation) or bottom-up movement (floatation) of particles. Upon centrifugation, particles sediment or float through a solution of smoothly (continuous gradients) or step-wise (discontinuous gradients) changing densities. The centrifugation results in equilibration of a particle in the part of the gradient medium where the density is equal to the apparent density of the particle. Polyhydric alcohols such as sucrose and sorbitol, polysaccharides such as Ficoll (polysucrose) and dextran (polyglucose), iodinated compounds based on metrizoic acid such as Nycodenz and iodixanol, and colloidal silica media such as Percoll (polyvinylpyrrolidone-coated silica particles) can be used as density-gradient media (Graham, 2001). The application purpose
determines the choice of a density-gradient medium considering physical, chemical and biological parameters of the compounds such as viscosity, osmolality, convenient density range and reactivity. Usually, the density range for separation of biological particles, e.g. organelles and membrane vesicles, covers densities from 1.00 g/mL to 1.4 g/mL. Sucrose is the most widely used compound, as sucrose is highly soluble in water and gradient solutions of sucrose can be prepared with densities up to 1.4 g/mL (corresponds to approximately 2.9 M sucrose solution) (Chang et al., 2008).

Figure 1. CENTRIFUGATION FOR SUBCELLULAR FRACTIONATION
Differential centrifugation is usually the first step after cell lysis in subcellular fractionation protocols and is often followed by density gradient centrifugation. The fragments of the cell wall, unlysed cells and the nuclei can be sedimented at low speed (3,000 - 5,000 x g). The pellet can be subjected to density gradient centrifugation for further purification of the nuclei. The post-nuclear supernatant, containing all other organelles and the plasma membrane vesicles, is usually subjected to density centrifugation for further fractionation. The post-nuclear supernatant can be also subjected to a second round of differential centrifugation at higher g-force (20,000-30,000 x g). The mitochondria and peroxisomes are then further purified from the pellet using density gradient centrifugation, and the vacuoles, SER and PM vesicles can be purified from the supernatant. Alternatively, the supernatant can be subjected to ultra-centrifugation in order to sediment light organelles and organellar vesicles, and separate these from the cytosol and lipid particles that remain in the supernatant. We emphasize that the figure is an oversimplification, because of limitations to the designation of organelle names to crude differential fractions. Nonetheless, differential centrifugation is commonly used as a first (crude) separation step.

However, when separating osmotically active particles such as vacuoles or mitochondria, the osmolality of density gradient media is especially relevant. Nearly constant osmolalities for all gradient layers are advantageous for separation of osmotically active particles because nearly unchanged internal volumes of particles are ensured and therefore their apparent densities remain almost constant while passing through different gradient layers. Physiological osmolalities lie between 250-500 mOsm for fungal cells. Thus, sucrose solutions become hyperosmotic at concentrations above 10% (w/v), which equals 300 mM, corresponding to the
density of only 1.03 g/mL. Therefore higher concentration of sucrose and other polyhydric alcohols cannot be used for separation of osmotically active particles when iso-osmotic conditions during separation are required. Polymeric sugars, e.g. Ficoll, overcome the osmolality problem because they do not change the osmolality of gradient media as much as small molecules at the same densities. As a sucrose solution at the concentration of 22% (w/v) which has a density of 1.085 g/mL creates hypertonic conditions with an osmolality of >800 mOsm, but a 25% Ficoll solution has the same density and is still hypotonic at 50 mOsm (Graham, 2001). Moreover, the osmolality of sucrose and other gradient media increases linearly as a function of concentration. So, small changes of concentrations already cause significant alterations in the osmolalities of gradient solutions. In contrast, the osmolality of Ficoll increases in an exponential manner and rises shallowly from 0 to 50 mOsm at the concentrations between 0% - 25% (w/v) which allows creating gradient solutions with various densities while not changing the osmolality much. However, the viscosity of Ficoll is 25-fold higher than that of sucrose at the equal concentration of 25% (w/v) resulting in longer centrifugation times of Ficoll gradients.

**Organelle properties in respect to proteomics**

A yeast cell consists of the cell envelope and the cytoplasm. The cell envelope consists of the cell wall, periplasm and the plasma membrane. The cytoplasm is the part of the cell that is enclosed by the plasma membrane and contains the cytosol (cytoskeleton fibers dispersed in water, ions and other micromolecules, proteins and protein macro-complexes e.g. polyribosomes and proteasome, lipid particles) and lipid bilayer-enclosed organelles. The membrane-bound organelles are mitochondria, peroxisomes, the nucleus, and the secretory system including endoplasmic reticulum (ER), the Golgi apparatus, vacuoles, and endo- and exocytic vesicles. Each type of organelle has distinct physical and (bio)-chemical properties that can affect successful isolation and purification. Below relevant properties of ten different organelles are described, and some physical organelle properties such as density and useful organellar marker proteins are summarized in Chapter V, Table 1.

**Cell wall**

The cell wall of yeast cells is a thick, rigid structure of 100-200 nm which makes up 15-25% of the dry cell mass. It maintains the shape of the cells, works against the internal pressure, the turgor, and serves as a mechanical barrier between a cell and its environment. The structure and assembly of the yeast cell wall have been reviewed extensively (Klis, 1994; Lesage & Bussey, 2006). The structural compounds of the cell wall are polysaccharides such as glucans that are glucose moieties with ß-1,3 and ß-1,6 linkages, chitin which is N-acetylglucosamine units with ß-1,4 linkage, and mannans that are polymers of mannose with α-1,2, α-1,3 and α-1,6 linkages. Glucans make up to 80-90%, the rest consists of mannans and chitin (2-4%), which is mainly located at bud scars. Polymers of glucans and chitin bind covalently to each other forming a fibrillar network, whereas mannans bind to proteins of the cell wall, accordingly called mannoproteins. The so-called “soluble” mannoproteins (e.g. invertase, phosphatases, hydrolases) are loosely entrapped inside the glucan-network and interact with each other via hydrophobic interaction and disulfide bonds. In contrast, the gluco-manno-proteins are covalently bound to the glucan-chitin network via glycosidic bonds. They are mainly associated with the outer surface of the cell wall shielding the glucan-chitin network from the external environment.

There are different ways to release the cell wall proteins based on the nature of their linkages to the cell wall polymers. The “soluble” cell wall mannoproteins can be extracted by treatment with reducing agents such as DTT or ß-mercaptoethanol breaking the disulfide bonds (Duell et al., 1964; De Nobel et al., 1989; De Nobel et al., 1990). The structural proteins of the PIR (proteins with inverted repeats) family are integrated into the cell wall via covalent alkali-sensitive (presumably thioester)
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There are also proteins that can be released upon treatment with reducing agents and mild-alkali, indicating that they are linked to the glucan structures covalently and to other proteins via disulfide-bonds at the same time. Fifty-one proteins of the *S. cerevisiae* proteome are annotated cell wall proteins with predicted glycoprophosphatidyl-insitol (GPI) anchor modification (GPI-SOM predictions; (Caro et al., 1997; de Groot et al., 2003)). The GPI-anchored proteins can be only released upon harsh treatment with SDS and hydrofluoric acid or sodium hydroxide, indicating that those proteins are bound to glucans via their GPI-remnants or oligosaccharide chains.

The cell wall is usually isolated upon mechanical disruption of cells, and due to its high density the cell wall can be easily separated from the rest of the cellular compartments by low-speed centrifugation. The cell wall is then found in the pellet as cell debris.

**Plasma membrane**

The fraction of proteins embedded into the yeast plasma membrane accounts for approximately 25% of the total membrane protein content (Schibeci et al., 1973). The plasma membrane surrounds the cell and is about 7 nm thick lipid-bilayer containing lipid rafts. The protein and lipid components of the plasma membrane can be modified with sugars, mainly mannose. The glyco-proteins of the cell wall are physically associated with the plasma membrane.

The plasma membrane can be isolated either upon mechanical disruption of cells e.g. French press, glass beads or cryogenic lysis, or enzymatic removal of the cell wall and subsequent lysis (e.g. osmotic; s. below). The method used for cell disruption is important for the subsequent subcellular fraction. When cells are lysed mechanically, the carbohydrate components of the glyco-proteins and glyco-lipids remain intact (0.7-0.9 mg carbohydrates per mg of protein; (Nurminen et al., 1976)) and the plasma membrane is present mainly as sheets and unsealed vesicles (Menendez et al., 1995) with a distinctly high density of 1.24-1.26 g/mL (Fuhrmann et al., 1974; Welten-Verstegen et al., 1980). These high-density membranes can be easily depleted from the bulk of intracellular organelar membranes with approximate densities of 1.17-1.19 g/mL (Serrano et al., 1991). Because unsealed vesicles and sheets are obtained, the P-type ATPase transport assay is not applicable anymore if the purity of preparation has to be assessed (low ATPase activity and no proton pumping activity) (Menendez et al., 1995).

When cells are treated with lytic enzymes (such as glusulase, zymolyase and/or mutanase) to remove the cell wall, the plasma membrane compartment looses its carbohydrate components to a certain extent, which results in sealed vesicles (mainly inside-out) with lower densities of 1.12-1.22 g/mL (Hossack et al., 1973; Serrano et al., 1991; Menendez et al., 1995; Navarre et al., 2002) depending on remaining carbohydrates content. The carbohydrate-free vesicles of the plasma membrane have similar density as mitochondrial membranes (1.14-1.21 g/mL (Nurminen et al., 1976)). Although the mitochondrial membranes can be removed by acidic precipitation (Franzusoff & Cirillo, 1983), potential contaminations derived from other subcellular compartments may remain. Because the density of plasma membrane vesicles isolated from spheroplasts is largely undistinguishable from other organelle membranes, the use of density gradients might not be the optimal purification method. An alternative separation method for plasma membrane vesicles is aqueous polymer two-phase partitioning, possibly in combination with other separation techniques (Walter et al., 1985; Menendez et al., 1995).

**Mitochondria**

Mitochondria are organelles with two membranes. The outer membrane, which encloses the entire organelle, comprises about 50% phospholipids (similar to the yeast plasma membrane). The major protein of the outer membrane is porin, a channel, which restricts the passage of molecules into the intermembrane space to 10 kDa. The inner membrane has a very high protein to lipid ration of > 3:1 by weight (1 protein per 15 phospholipids) and is highly permeable to water (Garlid & Paucek, 2003), which keeps mitochondria in a state of osmotic equilibrium with their
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The intact mitochondria are osmotically active particles with the osmolality of about 300 mOsm *in vivo* and can be osmotically stabilized in a solution of 250 mM sucrose *in vitro*. The mitochondria can be spherical with the diameter ranging between 0.1 to 2 µm in size, or ellipsoidal with dimensions of 1 x 2 µm. The number and size vary with growth conditions. Under conditions of repressed respiration *e.g.* aerobical growth in glucose-rich medium or anaerobical growth (*Visser et al.*, 1995) cells contain a few large mitochondria, whereas many small mitochondria are found under conditions of activated respiration *e.g.* grown aerobically on non-fermentable carbon sources such as ethanol, lactate or glycerol. Despite differences in size and number under different conditions, the total volume occupied by mitochondria within the cell remains constant.

The inner and outer membranes can be separated from each other and from the content of the matrix and intermembrane space by the digitonin-method (*Schnaitman & Greenawalt, 1968*). The method employs the initial rupture of the outer membrane by osmotic swelling in hypotonic buffer whereas the inner membrane surrounding the matrix (mitoplast) still remains intact. The re-sealed outer membrane vesicles with a low buoyout density due to a low protein-to-lipid ratio separate from the dense mitoplast with a high protein-to-lipid ratio on sucrose density gradient.

**Peroxisomes**

Peroxisomes are spherical, single membrane bound organelles with diameter between 0.2-1.0 µm. Peroxisomes contain enzymes to detoxify hydrogen peroxide and other reactive oxygen species, and several oxidases for utilization of specific carbon and nitrogen sources. Furthermore, peroxisomes are the site for fatty acid degradation (*β*-oxidation). The marker enzymes are catalase, isocutrate lyase, flavin oxidase.

Peroxisomes are small when grown on glucose-containing medium. Peroxisomal proliferation in *S. cerevisiae* is induced upon growth in oleic acid-containing medium for instance in the presence of 0.5% oleic acids and 0.1% Tween (*Veenhuis et al.*, 1987). In higher eukaryotes and *Neurospora crassa*, two types of peroxisomes exist: one type containing catalase and sedimenting at 1.24 g/mL, another one lacking catalase but harboring fatty acid oxidation system sediments at 1.21 g/mL in sucrose density gradient. *Elgersma* and *Van der Leij* also observed two peroxisomal peaks at different apparent densities during peroxisome isolation from *S. cerevisiae*. However, *Van Roermund et al.*, found only one with a distinctly lower density then mitochondria (*van Roermund et al.*, 2001). The intact peroxisomes could be isolated from the 35-50% interface of the Nycodenz gradient. Peroxisomes are permeable to sucrose and other small molecules such as Nycodenz and iodixanol but not to polymers and colloidal silica (Percoll) which might be a better separation medium for peroxisomes.

**Lipid particles**

Lipid particles from yeast are an important storage compartment for steryl esters and triacylglycerols and appear in light microscope as very bright spherical structures of ~0.1-0.3 µm diameter with a strong tendency to attach to vacuoles. The number of lipid particles dramatically increases from only few when grown on YPD medium to dozens on oleic acid-containing medium. The lipid particle consists of the triacylglycerol core, surrounded by multiple layers of steryl esters and a monolayer of phospholipids. The phospholipids layer harbors some crucial enzymes of lipid biosynthesis, especially proteins involved in the metabolism of phosphatidic acid, sterols and triacylglycerols. Some of these enzymes are not confined to lipid particles, but are also present in the endoplasmic reticulum and mitochondria. Due to a very low specific density as a result of an extremely high lipid-to-protein ratio of lipid particles and their robustness against harsh cell disruption methods, the lipid particles can be enriched up to 700-800 fold over homogenate to a high purity (*Leber et al.*, 1994).

**Nucleus**

The yeast nucleus is a double membrane-surrounded, round organelle of approximately 1.5 µm in diameter. Despite the absence of intermediate filaments, the
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lamins, the yeast nuclei are robust structures that can endure mechanical shear of spheroplasts breakage with a polytron homogenizer. In contrast to other eukaryotes, the nuclear membrane of yeast cells does not disintegrate during mitosis, which allows the isolation of nuclei in any growth phase. Due to differences in DNA content and the size at different mitosis-phases, there are different populations of nuclei when isolated from a non-synchronized culture. The nuclear envelope, which surrounds the nucleus is a double membrane and is an extension of the endoplasmic reticulum (ER). Therefore, ER proteins are usually found in nuclear preparations.

**Secretory system and vacuoles**

The secretory system of yeast consists of different membrane-surrounded compartments that ensure trafficking of proteins within and out of the cell. Proteins that are destined for secretion or intracellular organelles enter the secretory system at the site of protein biosynthesis, the endoplasmic reticulum (ER). The synthesis occurs at the ribosomal clusters that are attached to the cytoplasmic surface of the ER making it appear “rough”. The synthesized proteins move then from “rough” to the “smooth” ER-subcompartment, which routes them to the Golgi via COP II-coated vesicles. From the Golgi the proteins are delivered to different destinations within the cell or out of the cell via different pathways using different secretory vesicles. These destinations include *i*) vacuole, either directly or via the pre-vacuolar compartment, *ii*) periplasm, *iii*) plasma membrane, and *iv*) the bud-region during mitosis. The vacuole is an integral component of the endocytotic system, by which substances are taken up into the yeast cells from the outside and plasma membrane proteins are internalized for degradation.

**Endoplasmic reticulum**

Although morphologically different, smooth ER (SER) and rough ER (RER) seem to possess the same set of proteins apart from the ribosomal clusters at the surface. Due to association with ribosomes, the RER has a higher density of 1.17-1.19 g/cm³ when compared to the ribosome-free SER with the density of 1.146 g/mL (Sanderson & Meyer, 1991). The density of RER overlaps with the mitochondrial membranes and the lighter (de-glycosylated) plasma membrane fraction. When ribosomes are stripped off the RER, the density is shifted towards a lower density of 1.14 g/mL comparable with SER, allowing separation from contaminating plasma membrane vesicles and mitochondria with densities about 1.17-1.20 g/mL (Sanderson & Meyer, 1991).

**Golgi**

The Golgi apparatus is the major organelle for sorting and processing of proteins with different destinations, e.g. secreted or organellar proteins. About 60% of the Golgi apparatus of *S. cerevisiae* appears as single discs-like structures dispersed through the cytoplasm rather than parallel stacks known from other organisms (Papanikou & Glick, 2009). ~30% appears as doubly stacked structures and ~10% has three stacks (Preuss et al., 1992). A typical yeast cell of 5 µm contains about 30 Golgi compartments of ~400 nm in diameter and 50 nm thick that occupy a volume of ~0.2 femtoliter (0.5% of the total cell volume of ~40 femtoliter) (Preuss et al., 1992; Jorgensen et al., 2002). Cells with mutations in the sec14 and sec7 genes accumulate cup-shaped organelles or stacks of discs in the cytoplasm (Novick et al., 1980). A temperature of 25°C and low levels of glucose (0.1%) induce Golgi-like structures consisting of 2-11 parallel stacks in the sec7 mutant strain (Novick et al., 1981; Svoboda & Necas, 1987). The Golgi compartments can be enriched as spherical and discoid vesicles and tubes of 50-300 nm in diameter or length by combination of differential centrifugation and two subsequent sucrose density gradients (Lupashin et al., 1996). In contrast to Golgi-derived vesicles and tubes, the intact cisternae can be more easily separated from other organelles due to their distinctly large size, therefore the cells have to be lysed gently (no mechanical rupture, no sonication).

While lacking distinct morphological structures such as stacks like mammalian cells the functional Golgi unit is clearly defined by a set of specific enzymes (Chapter V, Table 1). At least three different functional Golgi sub-compartments are
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Vacuoles of yeast *S. cerevisiae* are analogous to mammalian lysosomes and responsible for turnover of proteins and organelles, ion homeostasis, storage of various ions and small organic molecules, and detoxification of cells (Matile & Wiemken, 1967; Halachmi & Eilam, 1996; Shirahama et al., 1996; Gharieb & Gadd, 1998). Vacuoles of *S. cerevisiae* have been studied extensively and methodological reviews are available (Roberts et al., 1991; Conibear & Stevens, 2002). Entire vacuoles and vacuolar vesicles have been used, for instance, for studying proteases (Wiemken et al., 1979) and amino acids content of yeast vacuoles (Wiemken & Durr, 1974), (Urech et al., 1978), the process of vacuole fusion (Sattler & Mayer, 2000) and solutes transport activities across the vacuolar membrane (Anraku et al., 1989), (Boller et al., 1989). But the knowledge of the vacuolar proteome is not complete. The aim of our proteomics study described in Chapter III was to make an inventory of vacuolar proteins using mass spectrometry (see Chapter III). For a correct assignment of identified proteins to the vacuolar compartment highly pure material is a prerequisite.

In order to increase the yield and improve the purity of isolated intact vacuoles from *S. cerevisiae* for proteomics purposes, a spheroplasting procedure described in Kipper et al., (Kipper et al., 2002) was used in combination with subcellular fractionation technique reported by Ohsumi et al., (Ohsumi & Anraku, 1981). Below the critical steps that were involved in a successful recovery of intact organelles from the yeast strain W303 during the spheroplasting, the lysis and the subcellular fractionation are discussed.

**Spheroplasting**

As described above, during spheroplasting the cell wall is partially removed using lytic enzymes that break down the cell wall compounds. The cell wall is not a static structure; moreover its properties such as rigidity and porosity are growth stage dependent, so that the cultivation conditions were found to be a major determinant for successful spheroplasts formation. Efficient spheroplasting is essential for a high yield recovery of

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vacuoles because cell lysis is critically dependent on removal of the cell wall. In batch cultures yeast cells grow asynchronously and pass through lag, exponential and stationary growth phases in which the growth rates are different. An important factor for efficient spheroplasting appeared to be the time for which the culture was maintained at a constant growth rate. To maintain the culture at a constant growth rate and ensure a balanced nutrient supply, exponentially growing yeast cells were transferred to fresh medium and allowed to divide for 4-5 generations. This procedure was repeated for up to 5 times imitating a continuous growing culture and allowing cells to grow at a constant growth rate for 3 days. It was found that efficiency of spheroplasting was improved for such a culture because almost no old cells remained.

Furthermore, the growth phase at the moment of harvesting seemed to play an important role for an efficient spheroplasting. We observed that cells harvested in the early to mid exponential phase (0.4-2 x 10^7 cells/mL) could be spheroplasted much more easily than cells from the late exponential phase corresponding to cell density of 3-4 x 10^7 cells/mL. This observation is in agreement with the fact that in the early exponential phase the cell porosity of batch-grown *S. cerevisiae* is maximal and falls off rapidly to lower levels in later growth phases (De Nobel et al., 1990).

Besides cell growth-related factors there are other important aspects during the spheroplasting procedure. In the present study prior to digestion of the cell wall with lytic enzymes yeast cells were pre-treated with reducing agent DTT in the presence of Tris-HCl buffer at pH 9.4. If no pre-treatment was done, cells could not be converted to spheroplasts effectively even after 3 hours of incubation with lytic enzymes (Fig. 2). Therefore, organelles could not be released efficiently in the subsequent lysis step. This observation clearly indicated the importance of alkali treatment and reduction of the cell wall compounds for a successful spheroplasting and subsequent lysis of *S. cerevisiae* W303 cells.

**Figure 2. SPHEROPLASTING**

This phenomenon can be explained by removal of cell wall mannoproteins at this step, thereby increasing the porosity of the cell wall. Mannoproteins are mainly associated with the outer surface of the cell wall shielding the glucan-chitin network from the external environment. Because mannoproteins limit the porosity of the cell wall to compounds with a molecular mass of approximately 600 Da or less, lytic enzymes with sizes of >10 kDa have a restricted access to these polysaccharides that protect cells against lysis in hypoosmotic environment. Consequently, removal of mannoproteins will result in an increased accessibility of polysaccharides to lytic enzymes, an enhanced disintegration of the cell wall and facilitated cell lysis. Additionally, mild alkaline conditions have direct effect on mannans by depolymerisation of mannan-chains and on proteins by denaturating them, thereby
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enhancing the cell wall porosity which facilitates formation of spheroplasts. After treatment with mild alkali and DTT, cells were incubated in a mixture of lytic enzymes. Thereby the pH of the incubation solution was important. Whereas the glucluslase has an optimal activity at acidic pH (pH 4.8 is optimal for glucuronidase-activity), the zymolyase has its optimum at pH 7.5. The optimal spheroplasting could be obtained using buffered sorbitol solution at pH 6 as empirically determined. Because spheroplasts are devoid of the cell wall and therefore osmotically unstable, the spheroplasting was performed in a hypertonic solution. In the course of cell wall digestion with lytic enzymes cells tended to aggregate and precipitate at the bottom of the flask. Usually, aggregation started after 30 min of incubation of the cells with lytic enzymes (Fig. 2) and reached the maximal extent after 45-60 minutes. The extent of aggregation was seen to be related to the efficiency of spheroplasting, *i.e.* vigorous and ubiquitous aggregation provided an indication of an effective cell wall disintegration which subsequently resulted in an efficient lysis of spheroplasts. After aggregation, cells/spheroplasts separated again within 2 hours. The spheroplasting was regarded to be completed after approximately 3 hours (Fig. 2). To establish the exact moment for terminating the digestion the progress of spheroplasting was followed in the light microscope. A morphological criterion was applied to decide when to finish the spheroplasting procedure. Therefore, a small aliquot of spheroplasts was diluted 10-fold in a solution containing 1.1 M sorbitol which served as an osmotic stabilizer and thus prevented bursting of osmotically fragile spheroplasts.

Completed spheroplasting resulted in cells/spheroplasts that appeared as dissociated spheres with buds mostly detached (Fig. 2). A second criterion was based on the efficiency of lysis. The extent of lysis could easily be followed in the microscope upon 10-fold dilution of a small aliquot of cells in the hypotonic lysis buffer (12% Ficoll, 10 mM Tris-MES pH 6.9, 0.1 mM MgCl₂). The spheroplasting was considered complete when nearly all spheroplasts were seen to be lysed. However, it must be noted that it has never been possible to achieve 100% lysis of spheroplasts.

**Cell lysis and subcellular fractionation**

When spheroplasting was complete, the spheroplasts were washed by a gentle spin through a layer of 7.5% Ficoll, 1.1 M sorbitol in order to remove lytic enzymes from the suspension. Use of a dense Ficoll-sorbitol layer prevented fragile spheroplasts from mechanical damage during centrifugation. Thereby, Ficoll contributed to the density and viscosity without changing the osmolarity of the 1.1 M sorbitol solution significantly. Upon slow centrifugation the lytic enzymes were retained in the less dense sorbitol layer on top of the Ficoll-sorbitol layer, whereas the spheroplasts were pelleted to the bottom of the centrifuge tube. The spheroplast pellet was lysed by resuspension in hypotonic lysis buffer containing 12% Ficoll, 10 mM Tris-MES, pH 6.9, 0.1 mM MgCl₂ by gentle agitating with a glass rod. At this step the cytoskeleton, cytosolic proteins and other organelles still remained attached to vacuoles. In order to liberate vacuoles from the attached cellular components, the lysate was homogenized in a Dounce homogeniser. Subsequently, the lysate was fractionated by means of a discontinuous floatation density gradient centrifugation. For purification of vacuoles in the present study, density gradient centrifugation was performed in two steps (Fig. 3A). The cellular lysate was loaded at the bottom of a centrifugation tube and overlaid with lysis buffer (12% Ficoll, 10 mM Tris-MES, pH 6.9, 0.1 mM MgCl₂ the density of which was 1.041 g/mL). Vacuoles that have density below 1.041 g/mL floated up upon centrifugation and were collected from the top of the centrifuge tube. The obtained crude vacuoles still contained some contaminants such as plasma membrane and mitochondria (#3 in Fig. 3B). Therefore, the crude vacuoles were overlaid with two layers of 10 mM Tris-MES, pH 6.9, plus 0.5 mM MgCl₂ buffer containing 8% and 4% Ficoll, respectively. After centrifugation, vacuoles were collected from the top of the 4% Ficoll layer (#4 in Fig. 3B). The layer of 4% Ficoll solution was not used by Ohsumi et al., (Ohsumi & Anraku, 1981) but it
improved the purity of obtained vacuoles. The purity tests will be demonstrated below. Also the addition of an extra layer of 2% Ficoll solution with a density of 1.006 g/mL was tested but a large proportion of the vacuoles lysed in this buffer probably due to the low osmolality of the solution and due to absence of an osmotic stabilizer such as sorbitol.

The vacuoles obtained from the top of 4% Ficoll layer had an apparent density of 1.011 g/mL which was not in agreement with the published densities of vacuoles (Wiemken & Durr, 1974). The discrepancy in the observed (1.0110 g/mL) and reported (1.075 g/mL) apparent densities of vacuoles might be explained by the loss of small molecules such as amino acids during osmotic lysis resulting in a lower apparent density, or by the used of Ficoll instead of sucrose as the gradient medium. Because the vacuoles are osmotically active organelles, they have the tendency to shrink when passing through hypertonic sucrose solutions, which might have caused their apparent density to increase. Because the purification of the vacuoles by differential density centrifugation was based on the buoyant density of the intact organelles, it was important to prevent organelles from bursting. When aiming at isolation of intact organelles, the centrifugation time for the second gradient was also found to be crucial. The centrifugation time of 45-60 min at 50,000 x g in the second step was observed to provide a good separation of the organelles resulting in very sharp bands at the different interfaces of the density gradient. Centrifugation for 30 min was too short for obtaining a good separation probably due to high viscosity of the Ficoll-solution, whereas after centrifugation for >10 hours only bursted vacuoles could be recovered as observed by the light microscopy and the separation resulted in diffuse bands. However, the vacuolar membranes after 12 hrs of centrifugation were found to be not significantly more contaminated than of intact organelles after short centrifugation neither with plasma membranes nor with outer mitochondrial membranes (not shown). This result revealed in agreement with microscopic observations that vacuoles bursted after separation. Thus, vacuoles first separated from other organelles upon centrifugation for approximately 1 hr, then upon longer centrifugation vacuoles bursted and finally, vacuolar membranes started sedimenting because the density of membranes is higher that the density of intact organelles.

Purity analysis of isolated vacuoles

The yield and the purity of the obtained vacuoles were examined by Western blot analysis. The enrichment of vacuoles was demonstrated by analyzing the spheroplast (Fig. 3B, #1) and vacuole fraction (Fig. 3B, #4) by SDS-PAGE. Proteins were transferred to a PVDF membrane and the membrane was probed with two specific monoclonal antibodies raised against vacuolar marker proteins carboxypeptidase-Y, CPY of the vacuolar lumen and alkaline phosphatase PHO8, ALP of the vacuole membranes. The result shows that detected signal intensities for both, ALP and CPY, were significantly higher in the vacuolar fraction (Fig. 3B, #4) in comparison to the spheroplast lysate (Fig. 3B, #1). This result indicated that the organelles floating on top of 4% Ficoll layer were highly enriched in intact vacuoles. However, an exact quantification of enrichment from Western blot attempted in this study appeared to be inaccurate. Light microscope was also used for evaluation of the purity of the vacuolar fractions. Literature reports also indicated a high purity of recovered vacuolar vesicles when vacuoles were collected from the top of 8% Ficoll (Kakinuma et al., 1981; Ohsumi & Anraku, 1981). We used an additional Ficoll layer for organelle separation and collected vacuoles from the 4% Ficoll layer. The analysis revealed a highly homogeneous population of vacuoles in fraction #4 (Fig. 3A), whereas organelle debris and unlysed spheroplasts were seen in #5 (not shown). These contaminants probably contributed to impurities of the vacuole fraction if collected from 8% Ficoll but apparently were not detectable in Ohsumi et al., (1981; Kakinuma et al., 1981; Ohsumi & Anraku, 1981) because the purity was examined by measuring and comparing enzymatic activities of specific organelle marker proteins: α-mannosidase (vacuolar membrane), glucose-6-phosphate dehydrogenase (cytosol), chitin
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synthase (plasma membrane) and succinate dehydrogenase (mitochondria) in the lysate and the vacuolar fraction.

**Figure 3. ISOLATION OF VACUOLES**

**Panel A:** Intact vacuoles were isolated according to Ohsumi et al. using two steps of floatation density centrifugation. The crude vacuoles (indicated by circles in the left-hand panel) as collected from the top of the first gradient were contaminated with other cellular constituents. Crude vacuoles were subjected to a second density centrifugation step. The highly pure vacuoles were collected from top of the second gradient (right-hand panel). Bar 10 µm.

**Panel B:** The purity and the enrichment of the crude and pure vacuoles was assessed by means of Western blot analysis, using antibodies raised against vacuolar alkaline phosphatase PHO8, plasma membrane ATPase PMA1, later Golgi sorting receptor Vps10 and mitochondrial outer membrane porin Por1. Equal amounts of protein (20 µg) as determined by the BCA assay were loaded in each lane. Lane 1: total spheroplast lysate; lane 2: pellet after the first step of density centrifugation; lane 3: crude vacuoles, obtained from top of the first density centrifugation; lane 4: pure vacuoles, obtained from the top of the second density centrifugation.

To prove that obtained vacuoles were highly pure in this study, specific monoclonal antibodies raised against marker proteins of different organelle membranes were also used to analyze the fractions: Porin, of the outer mitochondrial membranes; P-type ATPase of plasma membranes PMA1; sorting receptor VPS10 of late Golgi membranes. As demonstrated in Fig. 3B, PMA1, Vps10 and Porin were not detectable in the pure vacuolar fraction #4 indicating that the obtained vacuoles contaminations from mitochondrial, late Golgi and plasma membrane proteins were below the detection limit.

Lipid bodies are known to be tightly associated with the vacuolar membrane and the most frequent contaminant of vacuoles isolated by floatation (Meyer & Matile, 1975). Using the separation method described above it was not possible to obtain intact vacuoles devoid of lipid bodies (Fig. 3A). However, after osmotic lysis of intact vacuoles, crude vacuole membranes were collected by ultracentrifugation. The vacuolar membranes were found in the pellet fraction whereas the vacuole luminal proteins and lipid bodies remained in the supernatant, the latter due to their high lipid-protein ratio resulting in a low density compared to vacuolar membranes.

**Conclusions**

In the present study the isolation procedure of vacuoles from yeast cells was optimized. Thereby the yield and especially the purity of the vacuolar membranes were of a primary interest because the obtained material was subjected to mass spectrometry-based proteome analysis (see Chapter III). The yields of isolated material were influenced at the spheroplasting, lysis and subcellular fractionation.
We described factors that influenced the integrity of vacuoles in the course of the isolation procedure and that had an impact on the yields of obtained material. A positive effect on spheroplasting efficiency was observed when cells were grown for approximately 3 days at a constant growth rate (obtained by repeated inoculations of a fresh batch culture with an exponentially propagating pre-culture). Furthermore, the reduction and mild alkali treatment of cell wall components facilitated spheroplasting. Usually, for spheroplasting of yeast cells zymolyase only is used. Here we incubated cells in a mixture of three lytic enzymes facilitating the formation of spheroplasts. To ensure a mild washing of spheroplasts from lytic enzymes after digestion, cells were centrifuged through a dense layer of sugars instead of slow differential centrifugation as regularly used. Spheroplasts were lysed very rapidly under osmotic shock liberating the cellular compounds. After the cell lysis vacuoles had to be liberated from attached cellular compounds by homogenization. The use of a Dounce homogenizer with a loosely fitting pestle largely prevented disintegration of vacuoles due to shearing forces. The major factor influencing the integrity of intact vacuoles was the centrifugation time of the gradient during the subcellular fractionation. Long centrifugation times e.g. for 10-12 hrs resulted in a complete disintegration of intact vacuoles, so that only vacuolar membranes could be obtained after subcellular fraction. The optimal separation time was 45-60 min at 50,000 x g. Although the distinctive low density of vacuoles allows for a relatively easy separation from other organelles, our results indicated that the vacuolar fraction could be purified even further than has been reported previously (Kakinuma et al., 1981; Ohsumi & Anraku, 1981). A vacuole fraction with increased homogeneity was obtained when vacuoles were allowed to pass through an additional layer of Ficoll. These vacuoles were devoid of plasma membranes, mitochondrial membranes and late Golgi at the detectable levels of Western blot analysis. However, the vacuolar preparation was contaminated with lipid bodies. It was possible to remove lipid bodies after the lysis of vacuoles and pelleting the vacuole membranes. Lipid bodies were not disrupted under these conditions and remained floating upon centrifugation at 300,000 x g due to their very low density. The vacuole membranes prepared in the present study were regarded as pure and were subjected to proteomic analysis (Chapter III).

**Experimental procedures**

**Yeast strain and cell growth**

Haploid *Saccharomyces cerevisiae* W303 (MATα ade2-1 leu2-3,112 his3-22,15 trp1-1 ura3-1 can1-100) was used (Lawson & Douglas, 1988). All experiments were carried out as biological quadruplicates. For cell growth, 10 ml of YPD medium (0.3% yeast extract, 0.5% Bactopeptone, 1% glucose) was inoculated with a colony from a fresh agar plate, and incubated in a 100 mL Erlenmeyer flask for 15 hr at 30°C (shaking speed 160 rpm). 50 mL of fresh YPD medium was inoculated with 0.5 ml of the pre-culture. Cells were grown for 6–8 hr until the density was 1.5-2 x 10⁷ cells/mL. At least 4 subsequent 200-500 fold dilutions in fresh medium followed by growth to a density of 1.5-2 x 10⁷ cells/mL were carried out. Finally, for large-scale preparation, 12 L of medium in a fermenter was inoculated with 20 mL of the last pre-culture. Cells were grown aerobically (30% oxygen saturation, stirring speed 150 rpm) at a controlled pH of 6.3 in YPD medium. The doubling time was 1.5 hr. Exponentially growing cells (1.5 x 10⁷ cells/mL) were harvested by centrifugation at 4,000 x g for 5 min. Unless indicated otherwise, all steps were performed at Room Temperature (RT). The cells were washed with 1 L double distilled water and centrifuged again. The wet weight of cells from a 12 L culture was 20-30 g.

**Isolation of intact vacuoles**

Spheroplasting was carried out according to Kipper *et al.* (Kipper *et al.*, 2002) with a few alterations. 20 g cells were resuspended in 100 mL of 100 mM Tris-HCl, 10 mM DTT, pH 9.5 and incubated at 30°C for 10 min while shaking at 50 rpm. Cells were centrifuged at 4,000 x g for 5 min and washed with 100 mL water, followed by a wash with 100 mL 1.1 M sorbitol, and subsequently resuspended in 50 mL of 1.1 M sorbitol per 20 g cells. The cell wall was enzymatically digested with 1 mL glusulase, which contained glucuronidase (90,000 Units/mL) and sulfatase (19,000 Units/mL) (Cat.: NEE154001EA, Perkin Elmer), 3 mg zymolase T20, 3 mg Glucanex (Cat.:L1412, Sigma-Aldrich) per 10 ml of suspension in the presence of 5 mM DTT. The suspension was incubated for 2.5 hours at 30°C while shaking at 60 rpm. In addition, the suspension was swirled manually every 15 min to ensure homogeneous digestion.
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Light microscopy was used to evaluate the extent of spheroplasts formation. After digestion of the cell wall, the spheroplasts were cooled on ice. Each 25 mL of spheroplast suspension was pipetted on top of a layer of 25 mL ice-cold solution containing 7.5% Ficoll and 1.1 M sorbitol in a centrifuge tube. The spheroplasts were washed through this Ficoll-sorbitol layer by centrifugation at 4,000 x g for 20 min at 4°C. The spheroplasts were lysed by 6 x dilution of the spheroplast pellet with the ice-cold lysis buffer containing 10 mM Tris-MES, pH 6.9, 12% Ficoll, 0.1 mM MgCl₂ plus protease inhibitor cocktail (Cat.: 8215, Sigma-Aldrich). The suspension was homogenized on ice in a Dounce homogenizer (40 ml) by 15 strokes with a large clearance pestle “A” (Cat.: 885300-0040 Kontes Glassware, Vineland, NJ). The cells and solutions were kept at 4°C throughout.

For isolation of intact vacuoles, the protocol of Ohsumi et al. (Ohsumi & Anraku, 1981) was used, with the following modifications: samples of 20 mL of the spheroplast lysate were transferred to a centrifuge tube and overlaid with 10 mL lysis buffer. Centrifugation was performed in a swing-out bucket rotor (Beckman, rotor type SW32Ti) at 20,000 rpm (50,000 x g) for 30 min at 4°C. The fraction floating on top of the tube contained the crude vacuoles and was collected and resuspended in 10 mL of lysis buffer per 2-3 mL of crude vacuoles by homogenization with a loosely fitting Dounce homogenizer (5-6 strokes, pestle A). The homogenized crude vacuoles were overlaid in a centrifugation tube with a layer of 10 mL of 10 mM Tris-MES, pH 6.9, 8% Ficoll, 0.5 mM MgCl₂ plus proteinase inhibitor cocktail, and a second layer of 10 mL of the same buffer containing 4% Ficoll. Upon centrifugation at 20,000 rpm (50,000 x g) for 45 min, intact vacuoles were floating on top of the 4% Ficoll solution as a white wafer. Purified vacuoles were collected with a spoon-shaped spatula pre-wetted in 4% Ficoll-buffer.

**Preparation of vacuolar membranes**

The vacuoles - usually approximately 5 mL per 20 g of cells - were lysed osmotically in the same volume of buffer consisting of 20 mM Triethylammonium bicarbonate (TEAB), pH 8.0, 10 mM MgCl₂ and 50 mM KCl, and then diluted with 2 volumes of buffer containing 10 mM TEAB, pH 8.0, 5 mM MgCl₂ and 25 mM KCl. Without further incubation, the vacuolar membranes were recovered by centrifugation at 80,000 rpm (260,000 x g) for 20 min (Beckman, rotor type TLA100.3). To remove peripheral proteins, the pellet was resuspended in 100 mM sodium carbonate, pH 11.8, and subsequently incubated in the same buffer plus 2 mM EDTA for 15 min on ice. The membranes were recovered by centrifugation for 60 min at 80,000 rpm (Beckman, TLA-100.3, 260,000 x g). The protein concentration was determined by the BCA method (Pierce) after solubilization in 2% SDS.