

Isolation of Endoplasmic Reticulum Fractions from Mammary Epithelial Tissue

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Abstract In the mammary glands of lactating animals, the mammary epithelial cells that surround the lumen of the acini produce and secrete copious amounts of milk. Functional differentiation of these mammary epithelial cells depends on the development of high-efficiency secretory pathways, notably for protein and lipid secretion. Protein secretion is a fundamental process common to all animal cells that involves a subset of cellular organelles, including the endoplasmic reticulum and the Golgi apparatus. In contrast, en masse secretion of triglycerides and cholesterol esters in the form of milk fat globules is a unique feature of the mammary epithelial cell. Cytoplasmic lipid droplets, the intracellular precursors of milk fat globules, originate from the endoplasmic reticulum, as do most milk-specific proteins. This organelle is therefore pivotal in the biogenesis of milk components. Fractionation of the cell into its subcellular parts is an approach that has proven very powerful for understanding organelle function and for studying the specific role of an organelle in a given cell activity. Here we describe a method for the purification of both smooth and rough microsomes, the membrane-bound endoplasmic

reticulum fragments that form from endoplasmic reticulum domains when cells are broken up, from mammary gland tissue at lactation.

Keywords Biosynthesis · Casein · Endoplasmic reticulum · Lactation · Lipids · Mammary gland · Proteins · Secretory pathway

Abbreviations

cLDs	Cytoplasmic lipid droplets
ER	Endoplasmic reticulum
MEC	Mammary epithelial cells
MFG	Milk fat globules
PNS	Post-nuclear supernatant

Introduction

Functional differentiation of the mammary gland during pregnancy relies on the development of its epithelial tissues and terminal differentiation of the alveolar mammary epithelial cells (MECs) into cells specialized for secretion at the end of gestation (for review see [1]). Starting at parturition and during lactation, MECs secrete large quantities of milk-specific proteins and other components such as lipids, carbohydrates and ions in a polarised fashion, from their apical surface into the alveolar lumen, which they surround. In the protein biosynthetic pathway, membranous proteins and water-soluble secretory proteins are synthesized on electron-dense ribosomal particles docked on an extended network of parallel lamellar cisternae and branching tubules, the rough endoplasmic reticulum (ER). In his pioneering work, George Palade (Nobel Prize, 1974) used, among other techniques, a subcellular

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fractionation approach, to show that secreted proteins are subsequently transported to and through the sequential cisternae of the Golgi apparatus, and from the last Golgi compartment, the trans-Golgi network, to the plasma membrane using transport vesicles [2]. These vesicular carriers bud from the donor membrane and fuse with the acceptor membrane, thanks to the SNARE protein family [3], to deliver their contents to the next cellular compartment or to the outside world. Caseins, the main milk proteins synthesized by MECs, are a family of four acidic phosphoproteins. They are found in milk as aggregates, i.e., casein micelles. The primary interactions between caseins obviously take place in the ER of MECs [4, 5]. The subsequent phosphorylation of caseins in the Golgi apparatus is a key step in casein micelle formation. Indeed, this post-translational modification triggers calcium phosphate binding and further self-association of caseins (for review see [6, 7]). Casein aggregates progressively grow and become denser during the formation of secretory vesicles at the trans side of the Golgi apparatus and their transport to the apical plasma membrane, and structures with the characteristic honeycomb texture of casein micelles seen in milk are observed in mature distal secretory vesicles. As for lactose and the water fraction of milk, they both appear to enter the protein secretory pathway of MECs from the late Golgi compartment. These components are then delivered to milk via exocytosis of Golgi-derived secretory vesicles together with the caseins and skim milk proteins, like β -lactoglobulin or α -lactalbumin (see [8] and references therein). In line with this, morphological analysis has shown that these vesicles contain filamentous structures and casein micelles, similar to those found in milk, in an electron-lucent fluid [9].

Fat is present in milk as a supra-molecular structure made of a triglyceride core and cholesterol esters surrounded by MEC membrane-derived phospholipids, referred to as milk fat globules (MFGs). Due to the unique process of milk lipid secretion, the lipid core of MFGs is sequentially bound with a phospholipid monolayer and a standard bilayered phospholipid membrane [10–12]. The intracellular precursors of MFGs are the cytoplasmic lipid droplets (cLDs) that form in any cell type [13]. These structures are believed to be involved in lipid homeostasis, cell signalling and intracellular vesicle trafficking. Morphological and biochemical data are consistent with an ER origin for cLDs. Indeed, triacylglycerols are initially synthesized by enzymes that are associated with the ER. The newly synthesized lipids would be inserted between the two leaflets of the ER membranes, causing the cytoplasmic leaflet to bulge and then to bud off with its lipid core into the cytoplasm as a lipid microdroplet [13]. The cLD is therefore surrounded by a monolayer of phospholipids. In MECs, the cLDs grow by further triacylglycerol incorporation and/or by homotypic fusion during transport to the apex of the cell. During the process of milk fat secretion, the larger cLD is enveloped by a plasma membrane-derived phospholipid

bilayer as it buds through the apical membrane, and is finally pinched off into the lumen of the acini as a MFG [10]. Given the size of the MFG (mean diameter is 4 μ m in bovines), this secretion mechanism obviously requires considerable quantities of membrane. However, a large amount of membrane is provided to the apical membrane of the cell when secretory vesicles fuse with it in the context of milk protein secretion. One hypothesis, however, is that part of the MFG membrane may directly originate from the membranes of secretory vesicles that would simultaneously fuse with each other and with the apical plasma membrane. Such a process would result in the simultaneous release of both milk fat and proteins from the cell. An additional hypothesis is that the ER compartment may provide complementary amounts of membrane. These various mechanisms and hypotheses have been previously described [11, 14].

Considering the above, it is clear that the ER plays a key role in the biosynthesis and secretion of the main components of milk. It is also a historical fact that subcellular fractionation contributed extensively to the understanding of organelle structure and function. To make progress in our understanding of the role of the ER in the biogenesis of milk proteins and lipids, their secretion, and the mechanisms involved in the regulation of these secretory activities, it is therefore highly desirable to be able to work *in vitro* with purified fractions of the ER. Here, we describe a subcellular fractionation procedure for the purification of structures derived from both the smooth and the rough ER, the so-called microsomes, from mammary gland cells. We adapted this technique from the method initially developed for obtaining microsomes from rat liver [15, 16]. This procedure was successfully applied to the mammary gland tissue of both rat and goat [5].

Methods

General

The first step in subcellular fractionation is to break the cell so as to liberate the cellular organelles, membranes and soluble cytosolic components (homogenization). The second step consists of the separation of the various organelles contained in the homogenate (fractionation).

A major goal during the homogenization step is to liberate the cellular organelles as intact as possible while preserving the maximum of their biological and physical properties, the latter serving as the basis for their separation during the fractionation step. However, the extended membrane network of the ER also breaks up upon homogenization of the cells, and vesicle-like structures, the so-called microsomes, reform from the pieces of the ER. Organelles within the homogenate are usually separated by differential centrifugation and/or density gradient centrifugation with continuous or discontinuous

(step) gradients, using various density gradient media (e.g., sucrose, Ficoll™, Percoll™). The procedure below is described for rat tissue, and the few changes that are used for the preparation of microsomes from goat mammary gland tissue are pointed out in notes.

Materials and Instrumentation

All media and solutions described below are prepared with Milli-Q-purified water or equivalent. Unless otherwise indicated, chemicals are from Sigma-Aldrich (La Verpillère, France).

Preparation of Tissue Homogenate

Rats at mid-lactation (10–11 days, note 1).

Ice to maintain samples at low temperature.

Stock solutions: 2.31 M sucrose (80 % w/v), 200 mM PMSF.

0.25 M sucrose.

Protease inhibitor cocktail.

Surgical blades, scalpel and scissors.

Homemade multi-mounted razor blade device (note 2).

Aluminium plate (2.5 mm thick).

PVC cutting pad.

Forceps.

100 mL glass beaker.

Plastic or metal tea strainers.

Potter-Elvehjem tissue grinder (20 mL capacity) with ribbed Teflon® pestle (note 3).

Heidolph® overhead stirrer motor or equivalent.

Polypropylene mesh (150 µm mesh, ZBF, Rüslikon, Switzerland).

Glass Pasteur pipettes, blunt ended rods and funnels.

Graduated 50 mL conical tubes.

Rotary tube agitator.

Safety gloves.

Subcellular Fractionation

Stock solutions: 2.31 M sucrose (80 % w/v), 1 M imidazole pH 7.4.

Sucrose solutions: 0.25 M, 0.86 M, 1.0 M, 1.38 M, and 2.0 M solutions.

Sucrose/imidazole buffer: 0.25 M sucrose/3 mM imidazole.

Graduated 50 and 15 mL conical tubes.

Loose-fitting 7 and 15 mL Dounce homogenizers (with Wheaton type B piston).

Small plastic spatula.

Lint free tissue.

Parafilm.

Glass Pasteur pipettes and rods.

Glass centrifuge tubes (see note 4) and adaptors.

Centrifuge tubes for Beckman 50 Ti, 50.2 Ti, and SW 55 Ti rotors.

Low-speed centrifuge and ultracentrifuge with swinging-bucket and fixed-angle rotors (e.g., Beckman JA-20 and Beckman ultracentrifuge or equivalent with JS 13.1, SW 55 Ti and 50.2 Ti, 50 Ti rotors).

Refractometer.

Methods

Total rough and smooth ER microsomes were prepared from mammary gland homogenate by differential centrifugation followed by sucrose density gradient (see Fig. 1).

Preparation of Tissue Homogenate

1. Sacrifice a rat by decapitation (note 4) and excise the posterior mammary gland lobes (inguinal, weight between 7 and 11 g; note 5) and place in a 50 mL plastic tube containing 20 mL of ice-cold 0.25 M sucrose.
2. Unless otherwise indicated, all subsequent steps will be performed at 4 °C.
3. Place sample on a PVC cutting pad placed on an aluminium plate resting on ice, and cut into several about 2–3 cm-long pieces using surgical blades and a scalpel.
4. Dissect pieces of tissue to remove any potential remaining connective tissue, muscles and lymph nodes (note 6). Place cleaned tissue pieces in a 50 mL plastic tube containing 20 mL of ice-cold 0.25 M sucrose.
5. Drain the tissue pieces in a tea strainer and transfer to a pre-weighed beaker or plastic tube containing ice-cold 0.25 M sucrose and weigh.
6. Drain the tissue pieces in a tea strainer, transfer to the cutting pad and chop them finely with surgical scissors (1–2 mm³ fragments).
7. Transfer tissue fragments into two 50 mL plastic tubes containing 25 mL of ice-cold 0.25 M sucrose and subject them to gentle mixing on a rotary tube agitator for 10 min at 4 °C to remove any contaminating milk components (note 7). Let tissue fragments sediment and aspirate or pour off supernatant. Add 25 mL of fresh ice-cold 0.25 M sucrose to the tube and repeat the washing procedure twice.
8. Drain the tissue pieces in a tea strainer, transfer to the cutting pad and spread them out in a thin layer. Further chop tissue pieces using a homemade multi-mounted razor blade device (note 2) to obtain ≤1 mm³ pieces. Turn the pad 45° between each cutting action.
9. Transfer 1/4 (note 8) of the tissue fragments into the tube of the tissue grinder and add 12.5 mL (≥5

times the weight of the tissue) of ice-cold 0.25 M sucrose containing 0.5 mM PMSF and a protease inhibitor cocktail.

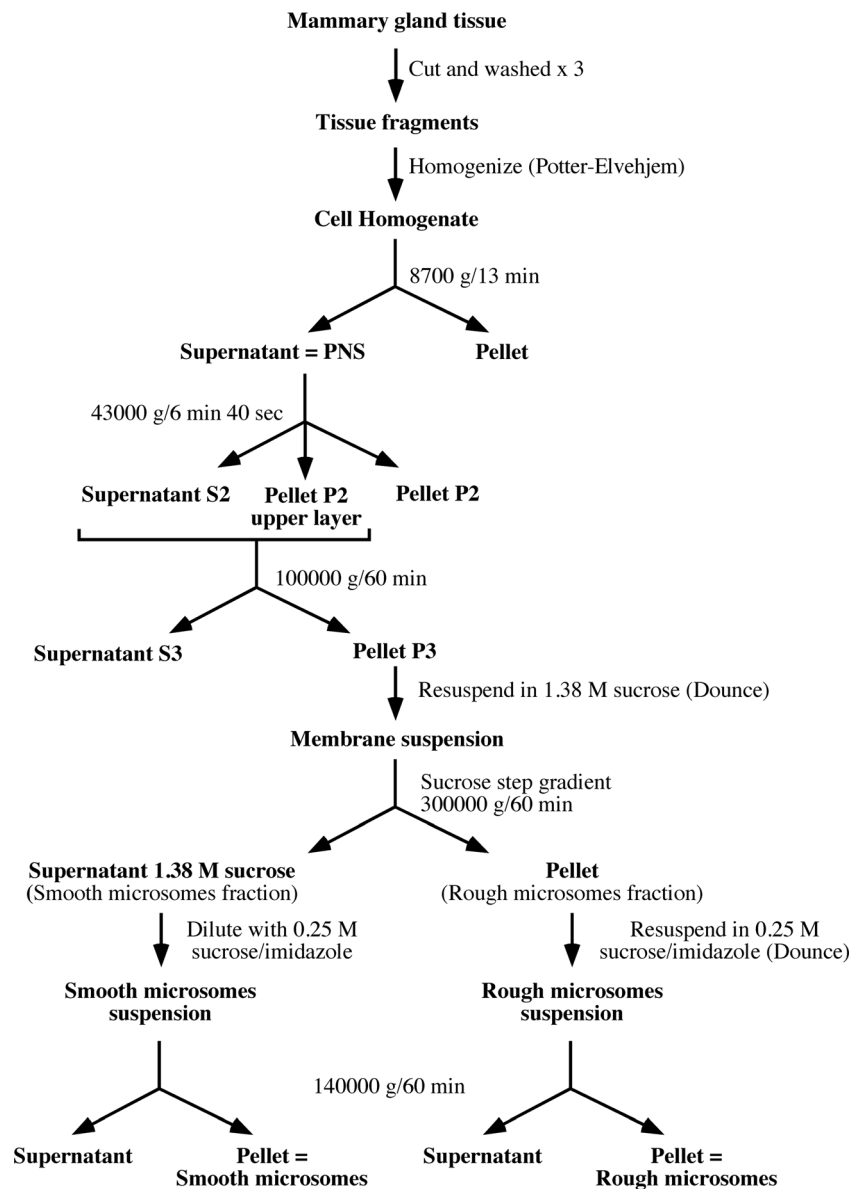
10. Homogenize using the ribbed Teflon pestle driven by the overhead stirrer motor at the lowest speed for 3–5 strokes (note 9).
11. Pour the homogenate into a piece of 150 μm polypropylene mesh folded over 4 times in a glass funnel sitting on a 50 mL plastic tube, and filter it, helping the liquid to drain with the rounded end of a blunt ended glass rod (note 10).
12. Discard debris in the tube of the tissue grinder, rinse and repeat steps 9 to 11 with the remaining tissue fragments, and pool the homogenates. Total homogenate should be ≈ 50 mL.

Subcellular Fractionation

All steps will be performed at 4 °C.

1. Distribute the homogenate into 6 \times 15 mL tubes (note 11) and centrifuge in a swing out rotor at 8700 $\times g$ (note 12) for 13 min with the break on to pellet cellular debris and nuclei. The resulting supernatant (S1) is referred to as the post-nuclear supernatant (PNS). At this point, a thin layer of cream floats above the supernatant.
2. Remove cream as much as possible using a small plastic spatula and collect the PNS with a glass Pasteur pipette through the remaining cream layer, if any. Transfer the PNS into a 50 mL graduated plastic tube and gently mix the liquid by inverting the tube. Withdraw an aliquot of

Fig. 1 Flow diagram for the subcellular fractionation of microsomes from mammary gland. See the [Methods](#) section for details



- the PNS for further analysis (note 13). The pellet (P1) can be discarded.
3. Distribute the PNS into 2 to 3 centrifuge tubes for the 50.2 Ti fixed angle rotor and centrifuge at $43,000\times g$ (19,000 rpm) in a TI 50.2 rotor for 6 min and 40 s (time starts once maximal speed is reached); stop the centrifuge with the break. The total centrifugation time should be approximately 10 min 40 s.
 4. Collect the supernatant (S2) and the flocculent upper layer (note 14) of the pellet (P2) with a Pasteur pipette. Pool the supernatants in a 50 mL plastic tube and mix the liquid by inverting the tube.
 5. Distribute the membrane suspension into 2 to 3 centrifuge tubes for the 50.2 Ti fixed angle rotor and centrifuge at $110,000\times g$ (30,000 rpm) for 1 h to pellet all membranes.
 6. Discard the supernatant (S3, note 15).
 7. Resuspend the pellet (P3) in sucrose at a final concentration of 1.38 M as follows:
 - Add 500 μL of 2.0 M sucrose to each tube and resuspend membrane pellet using a small plastic spatula. Transfer the membrane suspensions into the glass tube of the 7 mL Dounce homogenizer with a 1 mL pipette tip (note 16).
 - Add 500 μL of 2.0 M sucrose to one of the above centrifuge tubes, rinse and collect the remaining materials with a 1 mL pipette tip and transfer the suspension to the subsequent tube. After the rinsing of all centrifuge tubes is complete, transfer the membrane suspension to the 7 mL Dounce homogenizer. Repeat rinsing as needed (usually at least four times) but do not exceed a volume of 5 mL in the 7 mL Dounce homogenizer.
 - Homogenize the membrane suspension using ten gentle strokes of the Dounce piston (use a loose-fitting piston). Transfer the suspension to a 15 mL graduated plastic tube.
 - Rinse the Dounce homogenizer with 500 μL of 2.0 M sucrose and pool the wash solution with the membrane suspension. Cap the tube and gently mix by inverting the tube several times. Rinse all centrifuge tubes and the Dounce homogenizer again with 500 μL of 0.25 M sucrose, collect the wash solution and mix with the membrane suspension as above.
 - Adjust the density of the suspension to 1.38 M sucrose using a refractometer by adding 0.25 M or 2.0 M sucrose. If necessary, adjust the volume of the membrane suspension to 6 mL by adding 1.38 M sucrose stock solution as needed.
 8. Prepare four sucrose step-gradients in 5 mL tubes for the Beckman SW55 Ti rotor as follows (note 17).
 - Place 1.5 mL (note 18) of the 1.38 M sucrose membrane suspension in each tube.
 - Very carefully layer the membrane suspension with 0.9 mL of 1.0 M sucrose.
 - Very carefully layer the above sucrose layer with 0.9 mL of 0.86 M sucrose.
 - Gently cover the preceding layer with 0.6 mL of 0.25 M sucrose.
 9. Centrifuge gradients in a Beckman SW55 Ti rotor at $300,000\times g$ for 1 h.

From here, either proceed with step 10 to collect smooth ER microsomes, or go to step 11 to directly collect rough ER microsomes.
 10. Collect smooth microsomes.
 - Very carefully introduce a Pasteur pipette just below the interface between the bottom layer (load) and the 1.0 M sucrose layer and collect the top half of the 1.38 M sucrose fraction.
 - Place recovered fractions in centrifuge tubes for a Beckman 50 TI rotor and fill up the tube (to 2–3 mm from the top) with 0.25 M sucrose/imidazole buffer. Cap the tube with a piece of Parafilm and gently mix by inverting the tube several times. Proceed with step 12.
 11. Collect rough ER microsomes.
 - Discard gradient liquid layers by pouring out the contents of the tube and placing it upside down on a lint-free tissue to allow the residual liquid to drip out. Wipe the edges of the tubes above the pellet using a lint-free tissue wrapped around a glass rod.
 - Resuspend one of the pellets in 500 μL of sucrose/imidazole buffer using a small plastic spatula. Transfer the membrane suspension into the next centrifuge tube with a Pasteur pipette and resuspend the membrane pellet. Repeat steps in order to treat all membrane pellets. Transfer membrane suspension into the tube of a 15 mL Dounce homogenizer.
 - Rinse each tube with 500 μL of sucrose/imidazole buffer with a 1 mL tip. Collect and pool with the membrane suspension in the Dounce homogenizer.
 - Adjust the volume to ≈ 10 mL of sucrose/imidazole buffer and homogenize the membrane suspension using ten gentle strokes of the Dounce piston (use a loose-fitting piston).
 - Transfer the membrane suspensions into a tube for a Beckman 50 TI rotor and rinse the tube of the Dounce homogenizer with the sucrose/imidazole buffer. Add the sucrose/imidazole buffer until it reaches 2–3 mm from the top of the centrifuge tube

(note 19). Cap the tube with a piece of Parafilm and gently mix by inverting the tube several times.

12. Centrifuge smooth and/or rough ER microsomes at 140,000×*g* in a Beckman 50 TI rotor for 1 h.
13. Discard supernatant by pouring out the contents of the tube and placing it upside down on a lint-free tissue to allow the residual liquid to drip out. Wipe the edges of the tube above the pellet using a lint-free tissue wrapped around a glass rod.
14. Resuspend the final microsomal pellets in 2 mL of the sucrose/imidazole buffer with a 1 mL pipette tip.
15. Aliquot the microsomal fractions and store at -80°C .

Analytical Methods

The various fractions obtained during differential centrifugation, and notably the microsomal fractions, can be characterized using standard analytical methods. These include determination of the protein concentration by protein assay, characterization of the protein content by SDS-PAGE, 2D-PAGE, Western blotting or mass spectrometry, analysis of the membrane-bound compartments by protease protection assay, permeabilisation assay and, of course, by electron microscopy. Looking at enzymatic markers for the ER (glucose-6-phosphatase) and markers of other subcellular compartments (e.g., galactosyl transferase for Golgi membranes) and of the plasma membrane (5'-nucleotidase) will confirm the enrichment of endoplasmic membranes in the microsomal fractions and give a good estimate of the level of contaminating membranes. The presence of the ER markers calnexin, calreticulin, GRP78/BiP and protein disulfide isomerase can also be analysed by Western blot (see [5] Fig. 1), as well as mannosidase II and GM130 for Golgi-derived membranes and cadherin (pan) for plasma membrane-derived contaminants. Finally, electron microscopy analysis is particularly well suited for visualizing the rough microsomes in the preparation and estimating its purity (Fig. 2).

Discussion

Subcellular fractionation of mammary gland tissue was pioneered by T. Keenan's laboratory in the early 1970s (see [17] and references therein). They notably developed a procedure for the isolation of Golgi and rough ER-enriched fractions [18] based on an earlier method for the purification of such compartments from liver tissue. On the basis of morphological observations and specific enzymatic assays, the authors concluded that the procedure yielded a rough ER

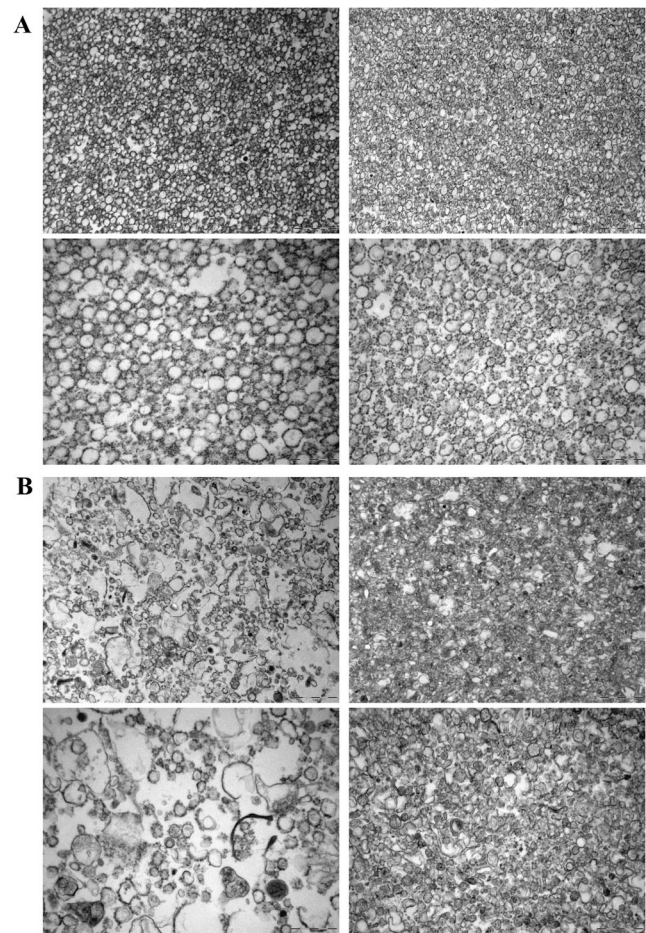


Fig. 2 Electron micrographs of microsomal fractions from mammary gland tissue. Rough microsomal (**a**) and smooth microsomal (**b**) fractions from two independent experiments (left vs. right panels) were fixed and processed for transmission electron microscopy. Representative images at two magnifications (top vs. bottom panels in **a** and **b**, see the length of the bar) show that the smooth microsomes have a more heterogeneous appearance, in agreement with their multiple cellular origins. We also observed heterogeneity in the smooth microsomal fraction between experiments

fraction in which rough ER membranes represented at least 85 % of total membrane bound structures. The major membranous contaminants were mitochondria. The fraction was also noticeably contaminated with casein micelles. When we started focusing our project on the molecular events occurring in the ER lumen, we decided to use a homogenization procedure that would cause the minimum physical and biological damage, while preserving the *in vivo* interactions as much as possible. Our goal was also to reach a high degree of purity in order to specifically monitor ER-specific molecular interactions. The method developed by the groups of JJ Bergeron and J Paiement for rat liver [19] appeared to be suitable to achieve these objectives. However, direct application of the procedure to mammary gland tissue yielded a rough ER microsomal fraction that was slightly contaminated by

mitochondria, but more importantly by a substantial amount of vesicular-free casein micelles (data not shown). The original method was therefore specifically modified to reduce these contaminants (see notes 7 and 12).

Although much longer and complicated than the method reported by Keenan's laboratory, the procedure described here is entirely satisfactory in terms of purity of the rough ER fraction. Both morphological and biochemical controls have demonstrated that the isolated rough ER fraction obtained here is almost pure (see [5], Fig. 1). We obtain a similar quality of the preparation with both rat and goat mammary gland tissues. Measurement of specific enzymatic activities is a standard approach for estimating the purity of subcellular fractions. Alternatively, Western blot analysis of compartment specific markers can be used (see [5] Fig. 1). However, the purity can be highly underestimated when measured on the basis of proteins, due to the high protein content of MECs. We therefore believe that electron microscopic observation of the fraction is the method of choice for estimating the purity of the preparation. By quantifying vesicles decorated with ribosomes, we estimated that the rough microsomal fraction contains greater than 90 % rough ER-derived vesicles. We also showed that the rough microsomes have a mean diameter of 150 ± 30 nm [5], which is in agreement with the size observed for rough microsomes prepared from rat liver [15]. Moreover, this fraction was barely contaminated with casein micelles. This allowed us to focus our analysis on the immature ER forms of the caseins, i.e., molecular forms that are neither phosphorylated nor glycosylated, and to specifically observe these forms within the ER lumen ([5] Fig. 1). These immature forms of proteins that are subjected to post-ER post-translational modifications provide a convenient internal control to monitor the purification of ER-derived membranes.

One important consideration with the present procedure is the time required for organelle purification. Indeed, the multiple steps of centrifugation take a long time, and mammary gland tissue sample preparation and homogenization also take time. In the course of our study, we modified the procedure, introducing a stopping point after the preparation of the PNS. The PNS is quick-frozen and stored at -80 °C. These frozen samples were quick-thawed and then fractionated by the above procedure. As far as we could observe, the fresh and stored PNS behaved similarly in the fractionation procedure and the rough ER fractions were similar in quality as evaluated by electron microscopy, protein patterns and the presence of ER markers by Western blot.

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Notes

- 1- Goats were at \approx 110 days lactation.
- 2- We use a homemade multi-mounted razor blade device consisting of 20 standard double-edged stainless steel razor blades separated by a 1 mm-thick metal plate. The assembly is attached to a holder comprising two handles, allowing a vigorous vertical slicing action. This step is not mandatory but obtaining smaller fragments greatly facilitates the homogenization step with the tissue homogenizer (see note 6).
- 3- We use a Thomas[®] Teflon pestle tissue homogenizer, size BB (Thomas Scientific, Swedesboro, NJ 08085, USA).
- 4- Goats are euthanized using captive bolt gun followed by exsanguination in an accredited abattoir.
- 5- From goats, collect 20–30 g of mammary parenchyma deep to the dermis so that 10 g can be used for subcellular fractionation after elimination of the fibrous portions and the large visible connective tissue pieces.
- 6- The presence of large pieces of connective tissue or other remaining tissue will interfere with the homogenization step and the specificity of the preparation.
- 7- Milk is present in the acini of the mammary gland tissue. Casein micelles are huge supramolecular structures with a high density, especially in rodents. Therefore, micelles sediment at rather low g force, e.g., 15 min at 16,000 \times g is sufficient to completely sediment casein micelles in rat milk. It is therefore advisable to wash tissue fragments to reduce contamination by milk components prior to the subcellular fractionation by differential centrifugation step. Although casein micelles have a lower density in goats, the washes are not dispensable.
- 8- With goat, we use 1/6 of the fragments at a time, as the tissue is harder and more difficult to homogenize. The volume of 0.25 M sucrose can accordingly be reduced to 7.5 mL. Of note, however, for an easier homogenization, it is better to keep a ratio of 5 to 10 between the volume of buffer and the weight of tissue. With both rat and goat tissue, however, it is not possible to achieve complete homogenization, and large tissue debris remain.
- 9- Use low speed to avoid warming of the suspension. This step requires a certain physical strength because the mammary tissue is quite fibrous and difficult to tear apart. Use safety gloves to manipulate the glass tube of the tissue grinder. Although we did not test this, if direct homogenization with the Teflon pestle is too difficult, a pre-homogenization with a Polytron homogeniser could be used. The probe of the Polytron could be inserted directly into the glass tube of the tissue grinder. The Polytron must be set at a rather low power and used for a short time in order to avoid complete disintegration of the tissue.
- 10- In order to facilitate the homogenate passing through the mesh. The homogenate should not be squeezed through the nylon mesh.
- 11- We use 15 mL borosilicate Corex[®] centrifuge tubes.
- 12- In the course of our study, we slightly increased the centrifugation force from 6000 \times g to 8700 \times g for this step in order to pellet more contaminating particles, including the remaining casein micelles from milk.
- 13- The complete procedure will take approximately 12 h. However, it is possible to freeze the PNS before subsequent processing of the sample.
- 14- Collect about 1/3 of the supernatant. Then, spray a gentle stream of supernatant onto the pellet with the Pasteur pipette in order to resuspend its flocculent upper thin layer, and collect this resuspended part of the pellet and supernatant.
- 15- Samples of pellet P2 and supernatant S3 can be taken for further analysis and characterization of the subcellular fractionation procedure.
- 16- The pelleted membranes have a tendency to stick to the plastic. If this is the case, keep the tip and use it for the washing steps in order to recover these membranes.
- 17- Do not decrease the volume of the gradients in order to load more sample. Rather, use more gradients for a given condition. Overloading gradients promotes membrane aggregation and causes a decrease in resolution and an increase in contamination of a given band by other organelles.
- 18- The sample volume and each of the following sucrose solutions are loaded one half at a time using a 1 mL pipette tip.
- 19- For electron microscopy analysis of the microsomal fractions, take an aliquot of the relevant sample and centrifuge as in step 13. Discard the supernatant and add fixative. Alternatively, microsomes can be fixed in suspension and pelleted afterwards.