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Technical note: Quantification of caseins from a crude extract of mammary epithelial cells

Eric Chanat*†[1] and Frédéric Dessauge*†
*INRA, UMR1348 PEGASE, F-35590 Saint-Gilles, France
†Agrocampus Ouest, UMR1348 PEGASE, F-35000 Rennes, France

ABSTRACT

En masse secretion of milk proteins, notably the caseins in the form of casein micelles, is a unique feature of the milk-secreting mammary epithelial cell. Caseins are therefore specific markers of these cells and constitute an ideal tool to monitor their differentiation, as well as functional status, during the development of the gland. To use them as such, a reliable method for quantitative analysis of the caseins from mammary cells or tissue is needed. Here we show that the caseins are heat-stable, a feature that leads to their complete extraction from a complex cellular extract by boiling. This allows for high enrichment and direct analysis of the caseins, even when they are poorly expressed in the starting material.
Key words: casein, lactation, mammary gland, proteins, secretory pathway

Technical Note

During lactation, mammary epithelial cells (**MEC**) secrete large quantities of milk proteins, notably caseins. Caseins (α_{S1}-, α_{S2}-, β-, and κ-CN in most species) are the main milk-specific proteins (for review see (Huppertz, 2013), and therefore specific markers of functional MEC. Quantitation of the expression of casein genes has been essential to the understanding of the cellular and molecular events that underlie the differentiation of the mammary gland. It appears, however, that mRNA levels do not systematically reflect the amount of the corresponding proteins within the tissue. Alternatively, estimating the degree of differentiation of the mammary cells via the amount of caseins in crude cell extracts could be challenging, especially at the onset of their expression. Having a simple and reliable biochemical method for assaying caseins from a tissue or cell preparation would be highly valuable, especially at the time they are expressed at a low level. With this aim, and knowing that caseins have been described as unfolded (or rheomorphic) proteins (Thorn et al., 2015) and therefore are most likely heat-stable, a method was developed to enrich the caseins from total cell extracts.

A heat-stable protein fraction (**HSF**) can be prepared either directly from cells in culture (cell lines or primary cultures), from a cell suspension, or from tissue samples (fresh or frozen). The first step, when starting from tissue, is to disrupt the HSF with a tissue homogenizer before the extraction of the proteins of interest. After homogenization of fresh tissue samples, it is advisable to prepare a postnuclear supernatant (**PNS**).

For the experiments described in the present report, mammary gland tissue samples were collected from either lactating bovine (Normande Holstein crossbreed, mid-lactation) mammary tissue or from nonlactating mid-gestation Alpine goats. Goats were raised at the experimental farm of Méjusseaume INRA-Rennes (France). They were slaughtered under general anesthesia with subsequent euthanasia (Rompun, i.v. 1 mL; Dolethal, i.v. 25 mL, Vetoquinol, Lure, France) and the mammary gland was removed from animals at autopsy for sampling. Cows were euthanized at the slaughterhouse of Gallais Viande (Montauban-de-Bretagne, France) following standard commercial practices. The mammary glands were collected at time of slaughter and immediately transported on ice to the laboratory to be sampled according to further analysis. Wistar rats were raised in our institute (research unit "Nutrition et Régulation Lipidiques des Fonctions Cérébrales, INRA, Jouy-en-Josas, France) and females at mid-lactation were euthanatized by decapitation. Animal welfare and handling were in accordance with the guidelines of the European Community for the care and use of animals in research (Directive no. 86/609/EEC). These experiments were approved by the Ministry of Research and Technology (agreement no. A78725). The principal investigator owns an accreditation for animal experimentation (license no. 78-62).

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[1]Corresponding author: eric.chanat@inra.fr

For the preparation of tissue powder from frozen tissue samples stored at −80°C, tissue pieces were first transferred to liquid nitrogen. Tissue fragments (≈10 g) were then crushed for ≈10 s using a tissue grinder (tissue grinder IKA model A 11 basic analytical mill, Staufen, Germany). The resulting powder was immediately stored in liquid nitrogen.

For the preparation of tissue homogenate from fresh tissue, all steps were performed at 4°C. Tissue pieces (≈10 g) taken from euthanatized animals were immediately put in ice-cold 0.25 *M* sucrose. Samples were cut into several 5 to 10 mm^3 pieces, and any remaining connective tissue, muscle, and lymph nodes were removed. Tissue pieces were weighed and finely chopped with surgical scissors (1–2 mm^3 fragments). Tissue fragments were washed twice with ice-cold 0.25 *M* sucrose for 10 min at 4°C and once with ice-cold 0.25 *M* sucrose in HMg buffer {10 m*M* Hepes-KOH pH 6.8, 5 m*M* $MgCl_2$ [or $Mg(OAc)_2$]} supplemented with phenylmethylsulfonyl fluoride (**PMSF**; 40 μL 250 m*M* PMSF for 100 mL). Tissue fragments were then resuspended in 30 mL of 0.25 *M* sucrose-HMg-PMSF supplemented with 100 μL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Tissue suspension (1/4 to 1/3 at a time) was transferred into the tissue grinder (Potter-Elvehjem tissue grinder with Teflon pestle type AA, 10 mL, or B, 30 mL capacity; Thomas Scientific, Swedesboro, NJ) and homogenized using the ribbed Teflon pestle driven by an overhead stirrer motor at the lowest speed for 3 to 5 strokes (use low speed to avoid warming of the suspension; if direct homogenization with the Teflon pestle is too difficult, short low power prehomogenization with a Polytron homogenizer could be performed). The resulting homogenate was filtered and its volume measured.

For preparation of a PNS, the homogenate was distributed into 2 centrifuge tubes, carefully layered with 2 mL of HMg buffer, and centrifuged in a swing-out rotor at 1,200 × *g* for 10 min at 4°C with the brake on to pellet cellular debris and nuclei. The resulting supernatant is referred to as PNS. If required, the thin layer of cream that might float above the supernatant was removed as much as possible by aspiration with a Pasteur pipette. The PNS was poured into a 50-mL tube, gently mixed by inverting the tube, and aliquoted for storage at −80°C.

For preparation of HSF from cultured cell lines or primary cell cultures, culture dishes were placed on an ice-chilled surface (the rapid cooling of the cells stops intracellular transport and secretion, slows metabolic activity, and potential protein degradation after cell lysis), the culture medium was removed and cells were washed 3 times with ice-cold Tris-buffered saline, the last wash containing 0.5 m*M* PMSF. Cells were layered with 1 mL (volume for a 3.5-cm diameter dish or well) of 1× Tris NaCl Tween EDTA (**TNTE**; 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 0.3% Tween-20, 20 m*M* EDTA from 0.5 *M* EDTA, pH 8.0) supplemented with 0.5 m*M* PMSF and incubated on ice for 15 to 30 min. The cell lysate was collected into a microtube or centrifuge tube.

For the preparation of a HSF from tissue powder, an appropriate amount of powder was taken into a microtube, 20 mg, and 2 mL of 1× TNTE with PMSF was added. The suspension was homogenized using a plastic pestle for microtubes or a small volume Potter-Elvehjem tissue grinder driven by an overhead stirrer motor at the lowest speed. Samples were incubated with gentle mixing for 30 to 45 min at 4°C.

For preparation of HSF from mammary tissue homogenate or PNS, the relevant volume of tissue lysate was distributed into microtubes or centrifuge tubes and diluted if required (it is recommended 10 mg of tissue/mL final volume). In concentrated homogenates, organelles can aggregate, which may result in modified protein precipitation during boiling. To decrease the salt concentration and improve the yield, it is recommended to dilute the cell lysate 5 to 10 times in low-salt buffer or with H_2O. Two times concentrated TNTE was added (1:1, vol/vol) and samples were incubated with gentle mixing for 30 to 45 min at 4°C.

To proceed to preparation of the HSF, a small hole was first made in tube caps. The tubes were then boiled for 5 min and centrifuged for 15 min at top speed in a microtube centrifuge (≈13,000 × *g*) or equivalent, at room temperature. The supernatant, referred to as HSF, was stored on ice. The pellet was resuspended in 1× TNTE (1/2 – 1/1 of starting volume), vortexed vigorously at room temperature and centrifuged as above. The resulting supernatant was pooled with the HSF (this washing step is not mandatory). The pellet (heat-unstable protein fraction; **HUF**) was solubilized in SDS-PAGE lysis buffer (as most cellular proteins are recovered in the HUF after boiling, analyzing the complete HUF might lead to saturation of the gel; it is therefore recommended to analyze only 10–50% of the HUF). In standard conditions and using mini-gels, a maximum of 20 μg of protein was loaded per lane. Proteins in the HSF were precipitated using trichloroacetic acid, washed with ice-cold ethanol/ether solution (1/1, vol/vol), resuspended in SDS-PAGE lysis buffer, and boiled immediately for 5 min. Samples were either stored at −20°C or immediately subjected to SDS-PAGE followed by Coomassie Blue (Bio-Rad, Marnes-la-Coquette, France) staining or Western blot, as previously described (Le Parc et al., 2010). Note that for the