Solubilization methods and reference 2-DE map of cow milk fat globules

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ABSTRACT

Milk fat globules (MFGs) are secretory vesicles assembled and secreted by mammary epithelial cells during lactation. They consist of fat globules surrounded by a lipid bilayer membrane which is derived from the apical membrane of the lactating cells. MFGs contain, besides lipids, proteins from the apical plasma membrane and from the cytoplasmatic material. Their peculiar vesicle nature makes them a suitable and easily available source of biological material in monitoring the physiopathological state of the mammary gland. Unfortunately, the conspicuous lipidic component of MFGs consistently limits protein extraction and purification for MFG proteomic investigations.

This work deals with the development of a suitable procedure for protein extraction from the cow MFGs in order to qualitatively and quantitatively improve 2-D electropherograms of the MFG. MFGs were purified from raw milk by centrifugation and then delipidated/precipitated. The resulting protein pellets were solubilised using four different 2-D SDS PAGE compatible lysis buffers. Applied methodological procedures for protein extraction and evaluation of the resulting 2-D protein-pattern are presented and discussed. Using these procedures a reference 2-D map of cow milk fat globules is also reported. The majority of the obtained identifications was represented by proteins involved in lipid synthesis or in fat globule secretion.

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1. Introduction

Milk is the first and unique foodstuff for all mammal newborns. Its nutritional importance mostly resides in lipids which provide a large proportion of offspring calories and precursors for membrane synthesis. Cow milk also represents a common alimentary source for adult people and its fat component greatly contributes to the organoleptic characteristics of dairy products.

The synthesis and secretion of lipids by mammary lactating cells is an interesting and highly ordered process that involves several distinct steps, not yet completely understood [1–6]. Triglycerides are the primary fat component of milk; they are synthesized in the smooth endoplasmic reticulum (SER) and incorporated into microlipid droplets, where they form a polar core coated by proteins and polar lipids. Microlipid droplets may fuse with each other to form the
larger cytoplasmic lipid droplets (CLD); both of them contact and become progressively enveloped by the apical plasma membrane via not yet entirely clarified protein–protein interactions [6–8]. The resulting budding process of the plasma membrane in the alveolar lumen leads to the secretion of milk fat globules (MFG), consisting of fat droplets compactly and completely surrounded by a membrane bilayer. On occasion, the closure of membrane behind the lumen projecting fat-droplets appears to occur by a route through the apical cytoplasm rather than along the droplet surface. This results in cytoplasmic portion presence in MFGs, known as cytoplasmic crescent. Depending on species, genetic and diurnal factors, crescent dimensions can vary from thin layers to large amounts of cytoplasm, and may contain, with the only exception of nuclei, all of the different membrane systems and organelles of lactating cells [3,7,9,10].

Up to date, the majority of studies on the MFG has been directed to characterize the protein and lipidic composition of the milk fat globule membrane (MFGM) [11–15], a complex tripartite structure allowing fat globule solubilization within milk plasma. Actually, many researchers are persuaded that the characterization of proteins associated with the MFGMs might help in understanding lipid synthesis and secretion process in milk. Moreover, being that MFGMs originated from SER and plasma membrane they can be considered as “cellular segments” representing the in vivo lactating cell. Consequently, they may vehicle some protein marker indicative of the physiopathological state of the secreting epithelial cells.

Based on the same concepts, cytoplasm crescents may provide, together with MFGMs, precious cellular material to value the physiopathological state of the mammary gland during lactation. For this reason we performed proteomic analysis (2-DE/MS) on MFG in its wholeness: MFGM, lipid droplet and cytoplasm crescent.

Unfortunately, the massive lipid amount and the high hydrophobicity of numerous MFG proteins interfere with protein extraction by standard solubilization procedures for 2-D electrophoresis. This causes quantitatively and qualitatively inadequate visualization of MFG protein expression profile. Sample preparation is a critical and fundamental step in proteomics and we decided to firstly direct our efforts to design and establish an appropriate method for MFG protein extraction and solubilization. Actually, a universally applicable method for sample preparation does not exist, and conventional protocols for protein extraction, disaggregation, solubilization, denaturation and reduction should be each time adapted with proteins or biological systems of interest [16,17].

Here we evaluated sample preparation efficiency of five different methods for 2-DE analysis of MFGs. To minimize lipid interference, a delipidation procedure was applied. The obtained precipitates were then resuspended using four different lysis buffers containing different detergents and chaotropes. All the applied sample preparation protocols implied a relatively poor sample manipulation which may limit protein losses and artefacts [17].

The obtained results were compared in terms of protein yield, number of detected spots, spot intensity, resolution quality and reproducibility. With this technological improve-

2. Materials and methods

2.1. Cow MFG purification

Fresh unpasteurised bovine milk for MFG purifcation was obtained from 25 different healthy animals during mid-lactation. The collected milk samples were classified, according to the Somatic Cell Count (SCC), as high or UHT quality milk, and pooled together, all them equally contributing to the pool. MFGs were prepared from 500 ml of the resulting pool.

Milk was centrifuged at 5000 × g for 15 min at 25 °C, as described by Basch [18], to obtain: a pellet containing milk cells, skim milk, and a floating lipid layer, known as cream, containing MFGs. In order to reduce protein contaminants from the aqueous phase, the recovered supernatant cream layer was washed three times in phosphate saline buffer (PBS: 0.5 M NaCl, 8.6 mM NaH₂PO₄–H₂O, pH 7.4) at 25 °C. Each washing step was followed by centrifugation at 5000 × g for 15 min to allow MFG separation from the washing aqueous phase. Finally, fat globules were collected and stored at –80 °C.

Washed globules were thawed out at room temperature and divided into five aliquots. One aliquot was directly resuspended in a conventional 2-DE PAGE lysis buffer, containing 8 M urea, 65 mM DTE and 4% (w/v) CHAPS, while the other four aliquots were subjected to delipidation/precipitation according to Wessel [19], with few modifications (we used a 1:1 methanol/chloroform mixture instead of the originally proposed 2:1 ratio). The obtained pellets were then resuspended using four different denaturating solutions compatible with 2-D electrophoresis. SOLUTION A: 8 M urea, 65 mM DTE and 4% (w/v) CHAPS; SOLUTION B: 2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 15 mM DHPC; SOLUTION C: 2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 2% (w/v) ASB-14; SOLUTION D: 2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 4% (w/v) CHAPS. To assist MFG solubilization and improve protein denaturation, samples were left 1 h at room temperature and repeatedly vortexed. Then they were centrifuged in a Micro Centrifuge 4214 (ALC, Milton Keynes, UK) at 12,000 rpm for 2 min at room temperature to remove the unsolubilised material. Protein concentration was estimated by the Bradford method [20], using BSA as standard. Finally, sample aliquots were stored at –80 °C until use.

2.3. 2-D SDS PAGE

All the MFG samples obtained were separated by 2-D electrophoresis performed using the Immobiline–polyacrylamide system as described [21,22].
IEF was carried out on preformed immobilized non-linear pH gradient, from pH 3 to 10, of 18 cm length (GE Healthcare, Uppsala, Sweden), and achieved using Ettan IPGphor system (GE Healthcare). For analytical runs the IPG strips were loaded with 60 µg of protein in 350 µl of lysis buffer and 0.2% (v/v) carrier ampholyte. The rehydration step was carried out at 0 V for 1 h and at 30 V for 8 h at 16 °C. The strips were then focused according to the following electrical conditions at 16 °C: 200 V for 1 h, from 300 to 3500 V in 30 min, 3500 V for 3 h, from 3500 V to 8000 V in 30 min and 8000 V until a total of 80,000 V h was reached. MS-preparative IPG strips were rehydrated with 350 µl of lysis buffer and 2% (v/v) carrier ampholyte for 12 h at room temperature. Sample load was performed at the cathodic end with 900 µg of protein for strip by cup loading in the IPGphor Cup Loading Strip Holders (GE Healthcare). The following IEF voltage was applied: 30 V for 30 min, 200 V for 4 h, from 200 to 3500 V in 7 h, 3500 V for 16 h, 5000 V for 4 h, from 5000 to 8000 V in 1 h and 8000 V for 6 h until a total of 116,000 V h was reached. Analytical and preparative focused strips were equilibrated in 6 M urea, 2% (w/v) SDS, 2% (w/v) DTE, 30% (v/v) glycerol and 0.05 M Tris-HCL pH 6.8 for 12 min and for further 5 min with a solution containing 6 M urea, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide, 30% (v/v) glycerol, 0.05 M Tris-HCL pH 6.8, and a trace of bromophenol blue. The second dimensional separation was performed on 9–16% SDS gradient polyacrylamide gels (18 cm x 20 cm x 1.5 mm) at 40 mA per gel constant current at 10 °C until the dye front reached the bottom of the gel, according to Hochstrasser et al. [23]. Analytical gels were stained by ammoniacal silver nitrate as described [24,25]. MS-preparative gels were stained by two different protocols: silver staining compatible with MS [26] and SYPRO Ruby (Bio-rad headquarters, Hercules, California) performed according to the manufacturer’s instruction. Bind-Silane (γ-methacryloyloxypropyltrimethoxysilane) (LKB-Produkter AB, Brommo, Sweden) was used previously to covalently attach polyacrylamide gels to a glass surface for gels subjected to SYPRO Ruby staining.

Analytical and preparative gel images stained by silver nitrate were digitized using a Molecular Dynamics 300S laser densitometer (4000×5000 pixel, 12 bits/pixel; Sunnyvale, CA, USA). Instead, preparative gel images stained with SYPRO Ruby were digitized with a Typhoon 9400 laser densitometer (GE Healthcare). Computer-aided 2-D image analysis was carried out using ImageMaster 2-D Platinum 6.0 software (GE Healthcare).

The comigration with human serum as internal standard had permitted us to experimentally determine pI and Mr (Da) values [27,28].

2.4. Protein spot identification by MS

Protein identification was performed by PMF on Ettan MALDI-ToF Pro mass spectrometer (GE Healthcare) as previously described [29,30].

After visualization with MS-compatible silver staining protocol, electrophoretic spots were manually excised, destained [31] and acetonitrile (ACN) dehydrated. Spots from SYPRO Ruby stained gels were instead mechanically excised by Ettan Spot Picker (GE Healthcare), destained in 2.5 mM ammonium bicarbonate and 50% acetonitrile, and finally dehydrated in acetonitrile. Subsequently, the resulting spots were rehydrated with trypsin solution and tryptic digestion was carried out overnight at 37 °C. Each protein digest was spotted on to the MALDI instrument target (0.75 µl) and allowed to dry. Then 0.45 µl of matrix solution of α-cyano-4-hydroxycinamic acid in 50% ACN and 0.5% (v/v) trifluoroacetic acid (TFA) was applied to the dried sample, dried again and tryptic peptide masses were acquired. PMF searching was carried out in NCBI and Swiss Prot database using MASCOT (Matrix Science Ltd, London, UK, http://www.matrixscience.com).
Fig. 2 – Silver stained 2-D electrophorograms of MFG samples obtained applying five different methodological procedures. (A) MFG solubilised in SOLUTION A (8 M urea, 65 mM DTE and 4% (w/v) CHAPS). MFG subjected to delipidation/precipitation and successively solubilised in: (B) SOLUTION A; (C) SOLUTION B (2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 15 mM DHPC); (D) SOLUTION C (2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 2% (w/v) ASB-14); (E) SOLUTION D (2 M thiourea, 7 M urea, 65 mM DTE, 0.5% (w/v) TritonX100 and 4% (w/v) CHAPS). Rectangles (a–d) show the gel areas which resulted more affected in protein presence and resolution in consequence of the different solubilizing buffers used.
com) on-line available software and limited by the following searching criteria: taxonomy was limited to Mammalia, mass tolerance allowed was 100 ppm, the number of accepted missed cleavage sites was set to one. Alkylation of cysteine by carbamidomethylation was assumed as fixed modification, while oxidation of methionine was considered as a possible modification. The extent of sequence coverage, number of matched peptides and probabilistic score, as detailed in Table 1, were other used criteria to accept the identification.

Identifications with values out of these ranges were confirmed by ESI-IT MS/MS peptide-sequencing on a LCQ DECA IT mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Tryptic peptide mixture was acidified with 2 µl of 1% TFA solution, equilibrated in 50% ACN solution, abundantly washed in 0.5% TFA and then concentrated by ZipTipC18 devices (Millipore, Billerica, MA, USA). Peptide elution was achieved with a 70% methanol, 0.5% formic acid solution. Nanospray method was employed to inject 3 µl of concentrate samples into the spectrometer. MS/MS database searching was performed by TurboSEQUEST (Thermo) and MASCOT MS/MS ion search software (http://www.matrixscience.com).

3. Results and discussion

According to their nature, MFG proteomic characterization is hindered by the very high presence of lipids and by the hydrophobicity of membrane proteins, which are the principal protein component of MFGs. To our knowledge most studies to date have focused on protein characterization of the milk fat globule membrane (MFGM) rather than in the MFGs themselves. The aim of this work was to set up a methodological procedure to optimize MFG protein extraction for 2-D SDS PAGE analysis. All the methodologies applied here showed high reproducibility, as proven by analysing three gel replicates for each sample preparation procedure with each gel resolving a sample from distinct single preparations. Each applied sample preparation protocol also implied a relatively

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Fig. 3 – Silver stained MFG reference map. Protein acrostic names match those present in Table 1 and are used to indicate spot identities.
poor sample manipulation which may limit protein losses and artefacts.

All the MFG prepared samples were isoelectrofocused on preformed non linear 3–10 pH gradient strips to obtain a broad-spectrum visualization of the MFG. Moreover, as preliminary analysis demonstrated (data not shown), the overwhelming majority of the MFG polypeptides are present in a pH range of 4.5–7.5 where the non linear pH gradient is expanded.

MFGs were obtained from cow milk according to well established purification methodology, as described in the materials and methods section, using raw milk fractionation by centrifugation. Depending on their lower buoyant density, MFGs fractionate at the top of centrifuged milk, in the milk cream, up to aqueous phase in which the primary milk proteins, caseins and whey proteins, are dissolved. Due to the conspicuous presence of these proteins in raw milk and to their potential adsorption onto the fat globule surface, the obtained milk cream was thoroughly washed in PBS to minimize the MFG contamination from aqueous phase proteins. A classical electropherogram of cow milk aqueous phase is shown in Fig. 1A. The applied MFG purification procedure was validated comparing milk aqueous phase with MFG 2-D protein patterns (Fig. 1A and B). Actually, only the really most abundant proteins from soluble phase were detected in the MFG gels where their presence is marginal.

After MFG purification, a direct protein extraction procedure was performed using a 2-D PAGE conventional lysis buffer, Solution A: 8 M urea, 65 mM DTE and 4% (w/v) CHAPS. As shown in Fig. 2A, it resulted in inadequate protein extraction and electrophoretic resolution, caused mainly by high lipid content and therefore suggesting the necessity of sample delipidation. Delipidation for 2-DE analysis is classically achieved by protein precipitation [32]. Unfortunately, it may be problematic and difficult to resolubilise proteins after their precipitation, especially proteins with large hydrophobic domains, such as several intrinsic-membrane proteins. We performed MFG protein delipidation/precipitation according to the protocol of Wessel [19], with some minor modifications, such as the methanol/chloroform ratio of 1:1 instead of 2:1. Based on our experience, this method was presumed appropriate in providing sufficiently-delipidated pellets whose resuspension and solubilisation, in presence of chaotropes and detergents, were not as difficult as for the precipitates obtained with other stronger precipitants. Fig. 2B shows the 2-D protein pattern of delipidated cow MFGs after solubilization in the same lysis buffer used for MFG shown in Fig. 2A. Evidently, protein extraction and resolution were largely improved by the applied delipidation procedure.

In relation to the several chemophysical differences occurring between different proteins in a sample, and to the biological variations between different biological systems, all the existing sample preparation methods for 2-DE have some qualitative and quantitative limitations in protein extraction and solubilization efficiency. Severe protein losses may actually result from the failure of detergents and chaotropes to solubilise and to maintain proteins in a soluble state throughout the entire analytical process, as well as from different protein solubilibilities in organic solvents, i.e.: during precipitation [16,32,33].

Even if alternative/complementary successful methods have been developed to overcome 2-DE limitation in protein analysis [34–39], 2-D PAGE still remains the technique of choice in simultaneous visualization of protein isoforms and variants from complex protein mixtures [17,40]. According to this concept and with the intent to proceed in cow MFG proteomic characterization, another three different lysis buffers were tested to resuspend and denature the precipitates. All the applied denaturing agents have been already demonstrated to improve hydrophobic protein solubilization and resolution in 2-DE analysis [41–49]. The applied solubilizing buffers contained a 7 M/2 M urea/thiourea combination [41,50], DTE as reducing agent, a surfactant and, as common practice in membrane protein analysis, a small amount of TritonX100 non ionic detergent. CHAPS (3-[3-cholamidopropyl]dimethyl-aminol-1-propanesulfonate), ASB-14 (myristic amidosulfobetaine), and DHPC (1,2-dihexanoyl-sn-glycero-3-phosphatidylcholine) were the tested surfactants. Fig. 2C, D and E show the 2-D electrophoretic maps from MFG solubilised by this denaturing buffer in which the surfactant was either DHPC (Solution B), ASB-14 (Solution C), or CHAPS (Solution D), respectively.

The different employed solubilizing buffers evidently resulted in a different quality of protein extraction and 2-DE resolution. According to the silver staining sensitivity, essentially four main gel areas were found most affected in protein presence and they have been pointed out in Fig. 2 by rectangles (a–c). As mentioned above, a method does not exist to extract the total protein amount from a sample: different methods may visualize different protein patterns from the same sample. In fact, different solubilizing buffers with different chemophysical properties perform best with stability and solubility of some different sample proteins thus resulting in presence improvement (or reduction, on the contrary) of such proteins in the corresponding gels. Consequently, comparing the obtained 2-D gels of delipidated and differentially solubilised MFGs, Solution B, ASB-containing buffer, came out to mainly improve solubilization of proteins resolved in the “a” area (Fig. 1A, B, C and D “a” area), while Solution C, DHPC-buffer, abundantly enriched some proteins localised in the “b” rectangle (Fig. 1A, B, C and D “b” area) and some others present in the “c” area. On the other hand, Solution D, CHAPS solution, resulted in more efficient solubilizing of some basic proteins resolved in the “c” region (Fig. 1A, B, C and D “c” area) and various proteins localised in the “d” rectangle (Fig. 1A, B, C and D “d” area), even if some acidic proteins of this last area were more abundant after MFG solubilization in Solution A, the applied 2-D conventional lysis buffer.

Notes to Table 1:

*Proteins whose presence was exclusive or enhanced using precipitation and solubilization in urea/thiourea and CHAPS/TritonX100 buffer.
* Protein acrostic names match those present in Fig. 3.
* Measured pl and Mr values were experimentally determined using human serum as internal standard.
* Supposed fragments in relation to the discrepancy occurring between theoretical and experimental Mr values and to the pmf sequence coverage.
* MS analysis detected both proteins in the same spot.
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Despite the quite similar overall quality of the delipidated-MFG obtained patterns, higher resolution, lesser streaking and higher qualitative and, in particular, quantitative protein recovery were achieved using the solubilizing buffer D. In conclusion, delipidation, urea/thiourea combination and CHAPS/TritonX100 application as surfactants gave a proper MFG protein pattern visualization (Fig. 2D), allowing us to obtain about 1200 well separated protein spots.

After improvement in MFG protein solubilization, we proceeded to 2-DE characterization of MFGs by mass spectrometry. Spots with several different protein-abundance were selected for MS identification. In some cases, the same protein spot obtained using different sample preparations was analysed and identified with little differences in Mascot search results. MS was also applied to identify some protein spots showing a protein abundance increase after sample precipitation and resuspension in buffer D, and, interestingly, the majority of those protein spots turned out to be membrane associated.

MALDI–TOF MS and ESI-ion trap MS/MS elicited the unambiguous identification of 62 protein spots which correspond to 42 different polypeptides. The obtained identifications are reported in Table 1 where they are catalogued in 7 functional classes according to biological activity. Protein name abbreviations listed in the table are used to indicate spot identities in the reference MFG map, shown in Fig. 3. An asterisk placed next to table protein-abbreviations points out protein spots whose presence was exclusive or enhanced using precipitation and solubilization in buffer D. Table 1 also reports for each identification SwissProt/TrEMBL or NCBInr accession number, theoretical and experimental pI/Mr values, and the Mascot search results: number of experimentally-measured peptide-masses matching the theoretical ones, percentage of protein sequence covered by the matching peptides, and the probabilistic score.

The majority of the obtained identifications were, as expected, proteins directly or indirectly involved in membrane and vesicular trafficking which have been previously described in MFGMs. Among them Butyrophilin (BT1A1), Adipophilin (ADFP) and Lactadherin (MFGM), which represent the major MFG proteins [51] and that turned out to be the most abundant in gel-resolved proteins (Fig. 3), were identified. Butyrophilin and Adipophilin, along with Xanthine dehydrogenase/oxidase (XDH), have been profusely suggested to be implicated in MFG secretion, even if, as recently reported, only Butyrophilin seems to mediate milk fat secretion directly [6,8]. Lactadherin is a peripheral membrane glycoprotein expressed in many mammary tissues and up regulated in lactating mammary epithelium. Numerous functions have been attributed to or were supposed to be mediated by this peculiar protein: it has been described to opsonize phosphatidylserine-exposing particles and cells [52–55], to contribute in exosome targeting and in cell–cell and/or cell–matrix interactions [56,57], and to absolve inhibitory role, not proven in cow, in pathogen infectivity [58,59]. Lactadherin was also proposed to play a specific role in MFG secretion, even if its contribution still remains to be clarified [60].

As well documented, microtubules play key roles in several membrane dynamics [61]. The MFG transport mechanism to apical cell regions is still unknown but it is supposed to involve cytoskeletal components such as microtubules and actin [7].

The cytoplasmatic calcium-binding protein p22 (CHP1), described as exerting pleiotropic biological activities [62], is a Ca2+ binding protein which was supposed to stabilize microtubules and endoplasmic reticulum organization, to regulate microtubule/membrane interactions [63]. Interestingly, p22 is required for targeting, docking and fusion of membrane vesicles to the apical plasma membrane [64]. Besides p22, we identified the translationally-controlled tumor protein (TCTP), another protein involved in calcium binding and microtubule stabilization. p22 and TCTP identification in MFG suggests their involvement in milk fat secretion process. Intracellular trafficking also relies on actin filaments [65]. Our analyses evidenced the presence not only of cytoplasmatic actin (ACTB) but also of the actin-modulating protein Gelsolin (GELS) in the MFG. Gelsolin is a calcium-regulated protein suggested to operate in membrane trafficking according to its activity in enhancing disassembly of actin filaments and so promoting reorganization of the cytoskeleton [66]. Interestingly, we also identified the cytoplasmatic molecular chaperone T-complex protein 1 subunit alpha (TCFA) which is known to operate in the folding of actin and tubulin [67]. Finally, Annexin A2 (ANXA2) identification further underlines the supposed correlation between MFG secretion process and the cytoskeleton. ANXA2 is actually essential in actin-dependent vesicle transport crosslinking, in presence of Ca2+, membrane phospholipids to the cytoskeletal actin [68,69].

Besides all these proteins, we also identified several other proteins associated to membrane and vesicular trafficking. Vesicle budding from donor compartments is mediated by protein coats, which are dynamic structures cycling on and off membranes. Their proper recruitment and assembly is effected by the Arf family, of the Ras superfamily, members which are regulatory GTP-binding proteins [70,71]. Arfs may be involved in microlipid droplet budding from the endoplasmatic reticulum, as suggested by our identification of ADP-ribosylation factor 1 (ARF1) and GTP-binding protein SAR1b (SAR1B) in the MFG.

Among the known regulatory factors in vesicular budding, transport, tethering and fusion we also identified Rab-1b (RAB1B), Rab 18 (RAB18) and the Rab regulatory protein GDP dissociation inhibitor beta (GDIb). Rabs are members of the Ras superfamily of small GTPases and represent the largest family of the known membrane trafficking proteins in eukaryotes [72–74]. Rabs associate/dissociate reversibly with target membranes as a consequence of GTP/GDP-binding active/inactive state. This GTP/GDP switch is under the control of guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP) and GDP dissociation inhibitors (RabGDI). Interestingly, RabGDIs, in addition to Rab escort proteins (REP), are involved in Rab delivery to their proper target membranes and thus solving a critical role in Rab activation and function [75]. Moreover, as recently described, Rab1b modulates the membrane association/dissociation of Arf1 at the Golgi [76]. Consequently it is reasonable that their concomitant presence in MFG may regulate the MFG exocytic process to other better known vesicular trafficking events where Rab and Arf family members have been reported to cooperate. Furthermore, we also detected in MFG the alpha soluble NSF attachment protein (SNAA), which is an ubiquitous and essential component of membrane fusion machinery [77,78], and the
transitional endoplasmic reticulum ATPase (TERA), from the AAA ATPase family, that is known to function in vesicle budding and fusion [79].

Rap proteins are small GTPases of the Ras superfamily implicated in several cellular functions such as signal transduction, Ras regulation, cell adhesion and cytoskeletal rearrangements [80]. This protein family is represented here by Rap-1b (RAP1B) protein spots, and probably its implication in MFG secretion process, in the event it has one, may rely on its cytoskeletal activity. Besides Rap, we identified some heterotrimeric G proteins (GNAS2, GB1/2, GNA11), usually known as modulators or transducers in several transmembrane signaling pathways.

Several proteinic and non-proteinic components of the cow MFGM have been reported as health-beneficial factors [81]. Among them we identified Lactotransferrin (TRFL), a secreted protein with broad-spectrum antimicrobial activity. Lactotransferrin has been described to reduce pathogen proliferation, by iron-chelating, and to directly function as a bactericidal by damaging surface virulence proteins as well as by altering pathogen permeability [82–84].

Reinhardt and Lippolis recently detected [14] the monocyte differentiation antigen CD14 in cow MFG. This is an early innate immune molecule abundantly described in milk as sCD14 soluble isoform. sCD14 is involved in recognition of gram-negative lipopolysaccharide (LPS), in enhancing host cell activation and detoxification from LPS, and, probably, in neonatal immune system education [85–87]. Interestingly, sCD14 was in vitro proven to enable intestinal epithelial cells to respond to LPS [88]. We identified three CD14 (CD14) protein spots in MFG and we speculate that the several immune functions ascribed to sCD14 may also be performed, at least in part, by this membrane counterpart.

We also detected serum albumin (BSA) in MFG. Albumin has been reported to cross from plasma to the mammary part, by this membrane counterpart. Functions ascribed to sCD14 may also be performed, at least in the first time in 2-DE analysis of bovine milk fat globules. Among them we inter-estedly found almost all the proteins classified in protein folding and enzymatic activity functional classes, as we expected from the crescent presence in MFGs. Besides these, also some proteins implicated in membrane and vesicular trafficking (TERA, GELS, GDB, SNA1, TCTP, TCPA, FDIA3, FP1A), in signal transduction (GNAS2, GB1/2, GNA11) and related to health-beneficial properties of milk (CD14) are reported here for the first time in 2-DE analysis of bovine milk fat globules.

4. Concluding remarks

Proteomic characterization and differential analysis of MFG may introduce important advancement in physiopathological evaluation of the lactating epithelium, in food control quality, as well as in understanding MFG formation and secretion process.

In this work we designed five different strategies of cow MFG preparation for 2-DE analysis. Sample delipidation and its subsequent resuspension in a lysis buffer, containing urea/thiourea and CHAPS/TritonX100 surfactants, appreciably limited problems relative to protein recovery. Numerous protein spots, including minor protein components, were actually detected by software image analysis, and 82 protein spots, representing 42 different polypeptides, were successfully identified by MS. The corresponding 2-D reference map will be available in the next update of the SIENA-2DPAGE [http://www.biomol.unisi.it/2d/2d.html]. Several identified proteins were found to be membrane associated, so proving the validity of the applied methodologies in hydrophobic protein extraction and purification. Our work provide advancements in MFG 2-DE visualization and characterization.

Acknowledgment

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REFERENCES

Krendel M, Mooseker MS. Myosins: tails (and heads) of
Ono S. Mechanism of depolymerization and severing of actin
864
Nakatani H, Aoki N, Nakagawa Y, Jin-No S, Aoyama K, Oshima
K, et al. Weanling-induced expression of a milk-fat globule protein, MFG-E8, in mouse mammary glands, as
demonstrated by the analyses of its mRNA, protein and
phosphatidylserine-binding activity. Biochem J Apr 1 2006;395
Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB,
Papworth GD, et al. Endocytosis, intracellular sorting, and
processing of exosomes by dendritic cells. Blood Nov 15
Andersen MH, Berglund L, Petersen TE, Rasmussen JT.
Functional analyses of two cellular binding domains of bovine
Bojesen A, Buesa J, Montava R, Kvistgaard AS, Kongsbak MB,
Petersen TE, et al. Inhibitory activities of bovine
macromolecular whey proteins on rotavirus infections in
Shahriar F, Ngeleka M, Gordon JR, Simko E. Identification by
mass spectrometry of F44c-fimbrial-binding proteins in
porcine milk and characterization of lactadherin
as an inhibitor of F44c-positive Escherichia coli
attachment to intestinal villi in vitro. Dev Comp Immunol
2006;30(8):723–34.
Oshima K, Aoki N, Kato T, Kitajima K, Matsuda T. Secretion of
a peripheral membrane protein, MFG-E8, as a complex with
Caviston JP, Holzbaur EL. Microtubule motors at the
intersection of trafficking and targeting. Trends Cell Biol Oct
Andrade J, Pearce ST, Zhao H, Barroso M. Interactions among
p22, glyceraldehyde-3-phosphate dehydrogenase and
Andrade J, Zhao H, Titus B, Timm Pearce S, Barroso M. The
EF-hand Ca2+-binding protein p22 plays a role in microtubule
and endoplasmic reticulum organization and dynamics with
distinct Ca2+-binding requirements. Mol Biol Cell Feb 2004;15
Barroso MR, Bernd KK, DeWitt ND, Chang A, Mills K, Sztul ES.
A novel Ca2+-binding protein, p22, is required for constitutive
–
10189.
Elevated milk soluble CD14 in bovine mammary glands challenged with
–
2388.
Lee JW, Paape MJ, Elsasser TH, Zhao X. Elevated milk soluble
Vidal K, Donnet-Hughes A. CD14: a soluble pattern
Bannerman DD, Chockalingam A, Paape MJ, Hope JC. The
bovine innate immune response during
experimentally-induced Pseudomonas aeruginosa mastitis.
Lee JW, Paape MJ, Elsasser TH, Zhao X. Elevated milk soluble
Innate recognition of bacteria in human milk is mediated by a
milk-derived highly expressed pattern recognition receptor,
Tuma PL, Hubbard AL. Transcytosis: crossing cellular barriers.
Physiol Rev Jul 2003;83(3):871–932.
Monks J, Neville MC. Albumin transcytosis across the
epithelium of the lactating mouse mammary gland. J Physiol
children allergic to cow’s milk and epiluphia. Pediatr Allergy
cell accumulation and accelerated atherosclerosis in mice.
[54] Nakatani H, Aoki N, Nakagawa Y, Jin-No S, Aoyama K, Oshima
K, et al. Weanling-induced expression of a milk-fat globule protein, MFG-E8, in mouse mammary glands, as
demonstrated by the analyses of its mRNA, protein and
phosphatidylserine-binding activity. Biochem J Apr 1 2006;395