JOURNAL OF PROTEOMICS 75 (2012) 1486–1492

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Bovine milk exosome proteome

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ARTICLE INFO

Article history:
Received 21 September 2011
Accepted 15 November 2011
Available online 23 November 2011

Keywords:
Milk exosome proteins
Milk proteomics
MFGM

ABSTRACT

Exosomes are 40–100 nm membrane vesicles of endocytic origin, secreted by cells and are found in biological fluids including milk. These exosomes are extracellular organelles important in intracellular communication, and immune function. Therefore, the proteome of bovine milk exosomes may provide insight into the complex processes of milk production. Exosomes were isolated from the milk of mid-lactation cows. Purified exosomes were trypsin digested, subjected offline high pH reverse phase chromatography and further fractionated on a nanoLC connected to tandem mass spectrometer. This resulted in identification of 2107 proteins that included all of the major exosome protein markers. The major milk fat globule membrane (MFGM) proteins (Butyrophilin, Xanthine oxidase, Adipophilin and Lactadherin) were the most abundant proteins found in milk exosomes. However, they represented only 0.4–1.2% of the total spectra collected from milk exosomes compared to 15–28% of the total spectra collected in the MFGM proteome. These data show that the milk exosome secretion pathway differs significantly from that of the MFGM in part due to the greatly reduced presence of MFGM proteins. The protein composition of milk exosomes provides new information on milk protein composition and the potential physiological significance of exosomes to mammary physiology.

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1. Introduction

Exosomes are 40–100 nm membrane vesicles of endocytic origin secreted by most cells. Exosomes are found in blood, urine, amniotic fluid, and bronchoalveolar lavage fluid as well as human and bovine milk [1–5]. Exosomes form from inward budding of early endosomes to form multivesicular endosomes (MVB) containing intralumenal vesicles. These enclosed vesicles containing cytoplasmic proteins, RNA and are then released from the MVB lumen into the extracellular environment via exocytosis [3,6,7].

Exosomes were initially described in 1983 [7,8] and were named exosomes in 1987 [9]. However, these extracellular vesicles were isolated and partially characterized in bovine milk in 1973 [10] and described as 100 nm membrane vesicles with a plasma membrane origin. Researchers noted some enzymatic and transport differences [10–12] between these milk "exosomes" and milk fat globule membranes (MFGM), which also have a plasma membrane origin [10–14].

There is emerging data that exosomes are extracellular organelles important in intracellular communication/signaling, immune function and as a source of biomarkers for disease [6,7,15]. Exosomes contain proteins, mRNA, miRNA, and lipids that can be transferred to cells to confer new functions or cell signaling events [2,4,16]. More recently both human and bovine milk exosomes have been shown to contain mRNA and miRNA that can be transferred to immune cells to potentially modulate immune cell function [4,17]. Exosomes have been shown to package and present antigen to immune cells and have other immune modulators roles [18,19]. The need for exosome proteomics has been well stated by Raimondo et. al. [5] and are paraphrased here: 1. The Enrichment in low abundance and membrane proteins that are usually underrepresented in proteomic lists, arising from cell lysates, cell

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1874-3919/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jprot.2011.11.017

fractions or unfractionated biological fluids; 2. The presence of a conserved set of common proteins that are essential for vesicle biogenesis, structure and trafficking, and therefore allow one to clarify the mechanism by which they originated which in this instance is the lactating mammary gland and milk somatic cells; 3. The presence of proteins that are specific for the experimental setting in which they are isolated and that presumably allow one to recognize the cell-type from which they are originated. Together all of these observations suggest the need to study milk exosome proteome as a means to better understand lactation physiology, milk composition and in future studies how exosomes change and provide clues to mastitis in lactating cows.

The MFGM proteome has been examined numerous times under different physiological conditions [13,20–28] but these membranes represent primarily lipid secretion pathways. The exosome proteome may reflect other functions of the secretory cells as well as mechanisms to influence immune cells in milk. Continual expansion of Omics databases related to mammary function and milk components will be keys to a greater understanding of lactation physiology and disease resistance [29–32]. In this manuscript we examine in detail the proteome of the bovine milk exosome.

2. Materials and methods

2.1. Animals

Mid-lactation Holstein cows at the National Animal Disease Center were used for this study. The National Animal Disease Center animal care and use committee approved all procedures used in this study. Prior to the study, all cows were healthy.

2.2. Milk exosome preparation

Milk was collected from Holstein cows in mid-lactation on three consecutive days. Milk somatic cell numbers averaged 150,000 cells/ml with no detectable bacteria. The milk was cooled to 4 C and centrifuged at 10,000 g for 15 min at 4 C. The floating milk fat pellet was removed and the skim milk + complete protease inhibitor (PI) cocktail (complete mix of serine and cysteine protease inhibitors) from Boehringer Mannheim (Indianapolis, IN) is then centrifuged in a 50.2 Ti rotor 100,000×*g* (29,000 rpm) at 4 C for 60 min. The supernatant is carefully removed and the loose pellet (crude exosomes) above the firm casein pellet is removed and diluted in PBS + PI. The crude exosomes are then overlaid onto 4 ml of 0.3 M (10 mM–Tris, pH = 7.5) sucrose + PI and centrifuged in a 50.2 Ti rotor 200,000×*g* (40,500 rpm) at 4 C for 120 min and the loose pellet crude exosomes are separated from residual casein pellet. Seven ml of 80% sucrose (10 mM–Tris, pH = 7.5) + PI is mixed with 6 ml of crude exosomes in 10 mM–Tris, pH = 7.5 for a final concentration of 43% sucrose. This 12 ml is loaded in the bottom of a centrifuge tube, followed by 13.5 ml 35% sucrose (10 mM–Tris, pH = 7.5) + PI and then the tube is filed with 5% sucrose (10 mM–Tris, pH = 7.5) + PI. The gradients are centrifuged in a SW-28 rotor at 25,000 rpm for 16 h at 4 C. The exosomes are collected from the 43%/35% interface (average density of 1.7 g/ml). The collected exosomes were diluted in 4 vol of PBS + PI and pelleted by centrifugation at 200,000×*g* for 60 min. The pellets were resuspended in PBS + PI. Final purification was filtration of exosomes through a low protein binding 0.2 μm Pall Acrodisc filter (Pall Corp, Ann Arbor, MI). Exosomes were assayed for protein using the BioRad Protein Assay Kit using a bovine serum albumin (BSA) standard. Purified exosomes were store at −20 C until use.

2.3. Preparation of trypsin digests

Pelleted and washed exosomes (200 μg) were resuspended in 50 μl 20 mM, triethylammonium bicarbonate buffer pH 8.5, 6 μl of 10% sodium deoxycholate acid, 2 μl of 2% SDS, vortexed, capped and heated to 90 C for 20 min. Samples were cooled on ice to room temperature. Four ul of freshly prepared 50 mM tris-(2-carboxyethyl)phosphine was added to the exosomes, mixed and incubated at room temperature for 10 min. Next 3 μl of freshly prepared 1 M iodoacetamide was added mixed and the exosomes proteins were incubated for 1 h in the dark. Fifty μl of 20 mM, Triethylammonium bicarbonate buffer pH 8.5 was added to the sample. One hundred μg of Promega Trypsin Gold Mass Spectrometry Grade trypsin was resuspended in 100 μL of 50 mM acetic acid. Sixteen ul of trypsin was mixed with the sample followed by the addition of 100 μl of methanol. The sample was then incubated in a shaking water bath at 37 C overnight. Finally the digestion was stopped and detergent precipitated by the addition of 20 μl of formic acid [33]. The sample was vortexed and centrifuged at 16000×*g* for 10 min to pellet detergent. The supernatant was transferred to a new 1.5 ml tube and dried in a Speedvac. The digest was stored at −20 C until used.

2.4. Off Line First Dimension Peptide Chromatography

A Waters 1525 μ Binary HPLC (Waters Corporation, Milford, MA) with a manual injector was used. Two hundred μg of digest was resuspended in 80 μl mobile phase A (72 mM Triethlyamine in H_2O, pH 10 with acetic acid) by incubating for 1 h in a sonic water bath at 37 C. The sample was then injected onto a Waters XBridge C18, 3.5um, 1×100 mm column in mobile phase A (72 mM Triethlyamine in H_2O, pH 10 with acetic acid). The gradient 0–60% mobile phase B (72 mM Triethlyamine in acetonitrile, pH 10 with acetic acid), over 60 min at 200 μl/min [34]. One min fractions were collected and dried in a SpeedVac.

2.5. On line second dimension peptide chromatography and mass spectroscopy

The second dimension chromatography used a Proxeon Easy-nLC (Thermo Fisher Scientific, West Palm Beach, FL). The sample was chromatographed on a Proxeon Easy-Column C18, 3 um, 75 μm×100 mm column in mobile phase A (95% H_2O: 5% acetonitrile and 0.1% formic acid) and mobile phase B (5% H_2O: 95% acetonitrile and 0.1% formic acid) gradient, 0% B for 9 min, 6%–15% B from 10–80 min, 15–30% B from 80–88 min, 30–90% B from 88–90 min, at 300 nl/min. The analytical column was connected to a Proxeon Nanospray Flex Ion Source (Thermo Fisher Scientific, West Palm Beach, FL)

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