Food Chemistry 211 (2016) 329–338

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Temporal alterations in the bovine buttermilk glycome from parturition to milk maturation

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 3 May 2016
Accepted 5 May 2016
Available online 6 May 2016

Keywords:
Milk fat globule membrane
Glycosylation
Glycoconjugates
Lectin microarray
Buttermilk

ABSTRACT

The bovine milk fat globule membrane (MFGM) has many associated biological activities, many of which are linked with specific carbohydrate structures of MFGM glycoconjugates. Bovine buttermilk is a commercially viable source of MFGM and is an under-valued by-product of butter making. However, the changes in buttermilk glycosylation over the course of lactation have not been extensively investigated. In this study, buttermilk was generated from three individual multiparous cows at 13 time points over the first three months of lactation. Buttermilk glycosylation was profiled using lectin microarrays and lectin blotting. Suggested differences in glycosylation, including N-glycosylation, sialylation and fucosylation, were observed between early and late time points and between individual animals. Overall, these data suggest temporal changes in the glycosylation of buttermilk proteins which may have an important impact on commercial isolation of glycosylated ingredients.

1. Introduction

The milk fat globule membrane (MFGM) is a heterogeneous membrane which surrounds and stabilises milk fat droplets (Evers et al., 2008) and is a rich source of proteins and lipids, many of which are glycosylated. The glycoconjugates of MFGM include glycoproteins such as mucins (Muc), butyrophilin (BTN), and Pas6/7, and glycolipids such as monosialogangliosides (GM), disialogangliosides (GD) and trisialogangliosides (GT) (Berglund, Petersen, & Rasmussen, 1996; Hvarregaard, Andersen, Berglund, Rasmussen, & Petersen, 1996; Pallesen et al., 2001; Ross, Lane, Kilcoyne, Joshi, & Hickey, 2015; Seok, Shimoda, Azuma, & Kanno, 2001).

Many potential health benefits imparted by MFGM glycoconjugates have been demonstrated *in vitro* and *in vivo*. For example, MFGM can inhibit pathogenic colonisation and subsequent infection in the gut and MFGM glycoconjugates are likely to contribute to this anti-infectivity (Ross, Lane, Kilcoyne, Joshi, & Hickey, 2016). Muc1 from human MFGM demonstrated prevention of *Escherichia coli* adhesion to buccal epithelial cells *in vitro* (Schroten et al., 1992) and Muc1 from bovine MFGM demonstrated inhibition of neuraminidase-sensitive rotavirus infection in MA104 cells (Kvistgaard et al., 2004). Specific carbohydrate structures on MFGM glycoconjugates have been demonstrated to play a role in the inhibition of microbial attachment. For example, sialylation of bovine Muc1 was suggested to reduce binding of *E. coli* and *Salmonella enterica* serovar Typhimurium to Caco-2 cells *in vitro* (Parker et al., 2010). In addition, deglycosylation of a complex of human milk mucin led to a reduction in rotavirus inhibitory activity which indicated the potential importance of glycosylation in imparting the anti-rotaviral activity (Yolken et al., 1992).

Changes in milk glycosylation and protein abundance occur as lactation progresses, with concentrations and specific structures differing between colostrum and mature milks (Takimori et al., 2011; Wilson et al., 2008). For instance, Muc1, Muc15, adipophilin and BTN are upregulated in bovine MFGM 7 days post-partum compared to colostrum (Reinhardt & Lippolis, 2008) while the abundance of immunoglobulin G (IgG) and lactoferrin are higher in whole milk colostrum than in samples taken later in lactation. Additionally, concentrations of sialylated and highly fucosylated glycoproteins in bovine whole milk are highest in colostrum compared to milk sampled at later time points and the ratio of *N*-glycolylneuraminic acid (Neu5Gc) to *N*-acetylneuraminic acid (Neu5Ac) is significantly higher in colostrum, and decreases gradually thereafter (Takimori et al., 2011). Glycosylation profiles of

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http://dx.doi.org/10.1016/j.foodchem.2016.05.027
0308-8146/

individual bovine milk glycoproteins have also demonstrated changes in glycosylation over the course of lactation (O'Riordan et al., 2014; Ujita et al., 1993) as have MFGM glycoprotein components. For example, MFGM glycoproteins from one Holstein cow's milk at days 0, 1, 3 and 5 post-parturition was analysed for glycosylation changes by soybean agglutinin (SBA) blotting which revealed that binding to a glycoprotein, believed to be CD36, increased over the six day period assessed (Ujita et al., 1993).

While changes in MFGM glycosylation over the course of lactation are expected, few investigations have been undertaken to date. Previous studies have focused on MFGM from milk sampled at just a few time points (Reinhardt & Lippolis, 2008) or from a single animal using a small number of lectins (Ujita et al., 1993). Other studies have compared bovine milk from early time points and late time points but did not monitor the potentially critical changes in between (Wilson et al., 2008). Since glycosylation of bovine MFGM components is an important factor contributing to their health-promoting activities, further knowledge on how lactation affects this glycosylation may be of commercial importance. Identification of lactation time points associated with the most glycosylated components may aid in generation of bovine milk fractions best suited for use as functional ingredients. For instance, since glycoproteins such as BTN are known to be conserved across MFGM from human and bovine milk (Lu et al., 2016), it may be of benefit to utilise highly glycosylated bovine milk ingredients to enrich infant formula since infant formula often has less free oligosaccharide and glycoconjugate content than human milk. This may help to narrow the gap between formula composition and breast milk. Buttermilk is a viable commercial source of MFGM and its associated functional glycoconjugates which have the potential to further benefit human health if used as nutritional food additives. To the best of our knowledge, changes in bovine buttermilk glycosylation over an extended lactation period in multiple animals have not been investigated.

In this study, buttermilk was generated from milk sampled from three animals at 13 time points (days 1–10 (D1–D10) and days 30, 70 and 90 (D30, D70 and D90) post-partum) from colostrum to mature milk. The glycosylation of the individual buttermilk samples was profiled using lectin microarrays featuring a panel of 43 lectins, electrophoretic analysis and lectin blotting.

2. Materials and methods

2.1. Materials

The bicinchoninic acid (BCA) Protein Assay Kit and the SuperSignal Pico kit were from Pierce Biotechnology (Thermo Fisher Scientific Inc., Dublin, Ireland). NuPAGE 4–12% Bis-Tris gels, MOPS buffer, and carboxylic acid succinimidyl ester Alexa Fluor® 647 (AF647) were purchased from Life Technologies (Carlsbad, CA). Molecular mass ladder (Mark12 Unstained Standard), LDS sample buffer, antioxidant and Coomassie Brilliant Blue (SimplyBlue SafeStain) were from Thermo Fisher Scientific (Carlsbad, CA). Pure, unlabelled lectins were acquired from EY Laboratories, Inc. (San Mateo, CA) or Vector Laboratories, Ltd. (Orton Southgate, UK) (Table S1). Biotinylated lectins (RCA-I, MPA, LTA, WGA, WFA, LEL and AIA) were from EY Laboratories, Inc. Avidin-D horseradish peroxidase (HRP) conjugate was from Vector Laboratories, Ltd. Nexterion® Slide H microarray slides were from Schott AG (Mainz, Germany). Glycoprotein Detection Kit, streptavidin horseradish peroxidase conjugate, bovine serum albumin (BSA), bovine asialofetuin (ASF), bromochloroindophosphate nitroblue tetrazolium (BCIP/NBT) were from Sigma-Aldrich Co. (Dublin, Ireland). Neu5Ac monosaccharide was obtained from Dextra Ltd. (Reading, U.K.). Polyvinylidene fluoride (PVDF) membranes (0.2 μm) were from Merck-Millipore Corp., (Dublin, Ireland). Unless otherwise noted, all other reagents were from Sigma-Aldrich Co. and were of the highest grade available.

2.2. Sample collection

Morning milk was collected daily from three multiparous Holstein-Friesian cows (animals 1, 2 and 3) at Teagasc Research Centre, Moorepark, Fermoy, Co. Cork. Samples were collected from D1 to D10 and at D30, D70, and D90 post-parturition per animal. Milk from D9 for animal 1 and D2 for animal 3 was not collected due to scheduling conflicts.

2.3. Buttermilk generation

Buttermilk generation was adapted from Morin, Britten, Jiménez-Flores, and Pouliot (2007). Briefly, samples were incubated at 45 °C for 1 h immediately after collection, followed by cream separation from whole milk using an FT15 disc bowl centrifuge (Armfield Ltd., Ringwood, England). The fat content was adjusted to 40% using Milkoscan FT120 (FOSS, Denmark) and cream was stored at 4 °C for 24 h. Buttermilk and butter were generated by agitating the cream using a food mixer. The buttermilk was passed through glass wool twice to remove butter granules. Samples were frozen at −20 °C, freeze-dried and stored in a desiccator at room temperature (RT) until further use.

2.4. Characterisation of buttermilk samples

Protein concentration was determined using the BCA Protein Assay Kit (Smith et al., 1985), with BSA as the standard. Carbohydrate content was assayed by the Monsigny method (Monsigny, Petit, & Roche, 1988) using glucose (Glc) as the standard. Total sialic acid content was determined using the periodate-resorcinol assay (Jourdian, Dean, & Roseman, 1971), using Neu5Ac as the standard. Assays for all samples were carried out in triplicate and the mean value is reported.

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Dithiothreitol-reduced buttermilk samples were electrophoresed in 4–12% Bis-Tris gels. Briefly, 65 μg protein was loaded for each sample with a molecular mass ladder in a single lane and all samples were diluted 1:10 with LDS sample buffer. The gel was resolved at 200 V for 50 min using NuPAGE MOPS buffer. NuPAGE MOPS buffer containing 0.25% NuPAGE antioxidant was used in the upper chamber. Protein bands were visualised on the gels using Coomassie Brilliant Blue following the manufacturer's procedure. Glycoprotein bands were visualised using a Glycoprotein Detection Kit. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used for relative quantitation of gel bands by densitometric analysis.

2.6. Fluorescent labeling of MFGM and glycoproteins

The bovine MFGM samples and the ASF standard for lectin microarrays were labelled with AF647 (λ_{ex} 650 nm, λ_{em} 665 nm) in 100 mM sodium bicarbonate, pH 8.3. Briefly, 10 μL of AF647, dissolved in DMSO, was added to 500 μL of sample (2 mg/mL) and incubated for 1 h in the dark at RT. Samples were kept in the dark after this point. Excess dye was removed from the labelled bovine buttermilk samples on a Bio-Gel P-6 column (1 × 12 cm) (Bio-Rad Laboratories, Ltd., Hertfordshire, U.K.) eluted with phosphate buffered saline (PBS), pH 7.4. Absorbance at 647 and 280 nm for each sample was measured and the protein concentration and degree of