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Distribution and Carbohydrate Structures of High Molecular Weight Glycoproteins, MUC1 and MUCX, in Bovine Milk*

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ABSTRACT

High molecular weight glycoproteins, MUC1 and MUCX, originating from bovine milk, were compared with regard to their distribution in milk fat and skim milk fractions and for presence of carbohydrate structures. Polymorphic MUC1, which migrated into 6% resolving SDS-PAGE gels, was found in both milk fat globule membrane and skim milk phases of bovine milk. In contrast, MUCX, appearing as a non-polymorphic single band in 3% polyacrylamide stacking gels, was present only in the skim milk fraction. Peptide-N-glycosidase F digestion studies indicated that MUC1 and MUCX possessed N-glycans with MUC1 containing more N-glycans than MUCX. Exoglycosidase digestion studies revealed the existence of abundant terminal sialic acid residues in both MUC1 and MUCX. Lectin-binding studies showed that MUCX likely possessed more complex carbohydrate structures than MUC1. The complex carbohydrate structures carried by both MUC1 and MUCX suggest that they may have potential to bind a wide spectrum of pathogenic microorganisms. If that proves to be the case in vivo, such structures could have a role in preventing or reducing some infectious diseases.
(**Key words:** bovine milk, MUC1, MUCX, carbohydrate structures)

Abbreviation key: Gal = galactose, **MFGM** = milk fat globule membrane, **PAS** = periodic acid-Schiff's, **PNGase F** = Peptide-N-glycosidase F, **PBS-TW** = PBS containing 0.05% Tween 20, **PVDF** = polyvinylidene difluoride, **WGA** = wheat germ agglutinin.

INTRODUCTION

Mucins are a diverse family of heavily glycosylated proteins. Three different mucins, MUC1, MUCX, and MUC15, have been described in the milks of a number of species including bovine (Patton et al., 1995; Pallesen et al., 2002). All of these mucins originate from the apical plasma membrane of lactating cells and are components of milk fat globule membranes (**MFGM**). As one of periodic acid-Schiff's (**PAS**) reagent-stainable glycoproteins in MFGM (Mather, 2000), bovine milk MUC1, also known as PAS I, has been described as polymorphic bands on SDS-PAGE gels (Patton and Patton, 1990; Patton and Muller, 1992; Huott et al., 1995). This is mainly due to the existence of variable numbers of tandem repeats domain in its core protein (Pallesen et al., 2001). Bovine MUC1 carries both O- and N-glycans that are composed of N-acetylgalactosamine, N-acetylglucosamine, galactose (**Gal**), mannose, sialic acid, and fucose (Snow et al., 1977; Pallesen et al., 2001). Bovine MUCX has been observed as a single nonpolymorphic band on SDS-PAGE gels (unpublished data; Patton, 2001). Limited information is available for bovine MUCX with regard to the structure of either its core protein or its carbohydrate moiety. Another bovine MFGM protein, PAS III, has also been identified as a mucin-type glycoprotein and named as MUC15 in a recent study (Pallesen et al., 2002). In the current study, we report the results comparing the distribution and carbohydrate structures of MUC1 and MUCX from bovine milk.

MATERIALS AND METHODS

Materials

Polyvinylidene difluoride (**PVDF**) membrane Immobilon-P, PVDF membrane Immobilon-P, and 10 K Minicon Concentrator were purchased from Millipore (Bedford, MA). *Vibrio cholerae* neuraminidase and β-galactosidase were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Horseradish peroxidase-streptavidin conjugate was purchased from Pierce Biotechnology (Rockford, IL). Biotinylated lectins and a 3,3′-diaminobenzidine substrate kit were obtained from

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Vector Laboratories (Burlingame, CA). Schiff's reagent, alcian blue, glutaraldehyde, bovine serum albumin, Peptide-N-glycosidase F (**PNGase F**), and Ponceau S were obtained from Sigma-Aldrich Corp. (St. Louis, MO). The other chemicals were purchased from Fisher Chemicals (Fair Lawn, NJ).

Methods

Milk sample preparation. Individual bovine (Holstein) milk samples were obtained from the South Dakota State University Dairy Research Unit. All milk samples were taken at the beginning of the second milking of the day. Milk fat globules were isolated according to the method described by Patton and Huston (1986). Sucrose was added to a 30-mL fresh whole milk sample to yield a final sucrose concentration of 5 g/100 mL. The treated milk was delivered under a layer of distilled water (15 mL) in a 50-mL plastic centrifuge tube. The tube was centrifuged ($2000 \times g$, 20 min, room temperature). The top layer, mainly consisting of washed milk fat globules, was transferred into a new tube. The original tube was then centrifuged ($3000 \times g$, 2 h, room temperature) and frozen at –20°C. The water layer of the frozen tube was cut off to obtain the skim milk. Skim milk whey was obtained by adjusting the skim milk to pH 4.6 with addition of acetic acid followed by removal of the casein precipitate by centrifugation ($3000 \times g$, 10 min, room temperature). Milk fat globule membranes were prepared by the addition of 0.2% Triton X-100 to the washed milk fat globules followed by centrifugation ($100{,}000 \times g$, 2 h, 4°C; Patton, 1982).

SDS-PAGE and Western blotting. Protein concentrations were estimated by the procedure described by Peterson (1983). Samples were solubilized in an equal volume of 2× sample buffer [125 m*M* Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue] and separated by SDS-PAGE gels (Laemmli, 1970). Gels were either stained directly or electrotransferred to PVDF membrane Immobilon-P according to the procedure described by Towbin et al. (1979).

Protein staining on gels. The PAS reagent staining procedure was modified from the method described by Neville and Glossmann (1974). Gels were sequentially soaked in fixing solution (40% methanol, 7% acetic acid), oxidation solution (1% periodic acid, 7% acetic acid), wash solution (7% acetic acid), and Schiff's reagent. Incubation times for each solution were 30 min. The stained gels were finally washed in a large volume (>1 L) of water until the background was clear.

Silver staining was performed as described by Morrissey et al. (1981) with a final-step modification. At first, proteins were fixed in the gel by incubating the gel in 40% methanol and 7% acetic acid for 30 min and in 10% glutaraldehyde for 10 min. Gels were rinsed in a large volume (>1 L) of water for >2 h and then soaked in 5 μg/mL dithiothreitol for 30 min. Gels were incubated in 0.1% silver nitrate for 30 min. After being rinsed with water for 2 min, gels were then incubated in 3% sodium carbonate containing 0.019% formaldehyde until the desired color developed. Finally, the gel was washed in 5% acetic acid to stop the reaction.

Protein staining on polyvinylidene difluoride membranes. Alcian blue staining of PVDF membrane was performed as described previously (Akiba et al., 2000) with some minor modifications. The membrane was blocked with 2% BSA for 10 min followed by incubation in 0.5% alcian blue, 3% acetic acid (pH 2.5 for 2 min) and then a water rinse to clear the background.

For lectin binding, the membranes were washed with phosphate-buffered saline (15 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, 137 m*M* NaCl, and 2.6 m*M* KCl, pH 7.4) containing 0.05% Tween 20 (**PBS-TW**) 3 times for 10 min each and incubated with biotinylated lectins (10 μg/mL) in PBS-TW for 30 min. The membranes were washed with PBS-TW 3 times for 10 min and incubated with horseradish peroxidase-streptavidin conjugate (10 μg/mL) in PBS-TW for 30 min. The membranes were washed 3 times for 10 min each with PBS-TW, and the bound horseradish peroxidase-streptavidin was detected using 3,3′-diaminobenzidine substrate kit for peroxidase according to the manufacturer's protocol.

Peptide-N-glycosidase F digestion. Skim milk whey samples were first denatured by incubating in 0.5% SDS and 1% 2-mercaptoethanol at 100°C for 10 min. Then, samples were digested in reaction buffer [50 m*M* sodium phosphate (pH 7.5); 1% NP-40] supplemented with PNGase F (5 U/mg of sample protein) at 37°C for 16 h. Buffer exchanges were accomplished using 10 K Minicon Concentrator according to manufacturer's guide.

Exoglycosidase treatment. Skim milk whey samples were digested with *Vibrio cholerae* neuraminidase (0.15 mU/mL of whey) in digestion buffer (50 m*M* sodium acetate, 150 m*M* NaCl, 9 m*M* $CaCl_2$; pH 5.5) at 37°C for 16 h or with β-galactosidase (0.05 mU/mL of whey) in digestion buffer [50 m*M* sodium phosphate, 1 m*M* dithiothreitol, and 100 m*M* NaCl; pH 7.3] at 37°C for 16 h. Buffer exchanges were accomplished with 10 K Minicon Concentrator according to the manufacturer's guide.

Amino acid compositional analysis. Skim milk whey samples (50 μg per lane) were electrophoresed on SDS-PAGE (3 and 6% polyacrylamide for stacking and resolving gels, respectively) and transferred to PVDF membrane Immobilon-P as just described. The membrane was stained with Ponceau S (0.2% in 1% acetic acid) to visualize lanes. To determine the location of