

## Identification of N-glycoproteins in human milk by mass spectrometry

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Milk contains a range of “protective factors” contributing to protect new born from disease. Milk immunoglobulins (Ig) not synthesized in the mammary epithelial cells are transported from the mother to neonate transferring humoral immunity. Ig are glycoproteins with carbohydrate groups linked to the constant regions of heavy chains. Combined with high concentration of lactoferrin and high activity of lysozyme human milk exerts a particularly effective antimicrobial activity. Furthermore a growing body of evidence is suggesting that milk oligosaccharides and glycoconjugates, such as glycoproteins, are involved in the protection of infants from pathogenic attack. Here we describe a proteomic approach to profile human milk N-glycoproteins aimed to establish factors involved in the immunity and antimicrobial activity. It consists of four steps: (1) Isolation of milk proteins by precipitation with 12% trichloroacetic acid (TCA), dephosphorylation with alkaline phosphatase and tryptic digestion of proteins; (2) Selective glycopeptide enrichment from a complex peptide mixture via Hydrophilic Interaction Liquid Chromatography (HILIC); (3) Deglycosylation of isolated glycopeptides by Peptide N-glycosidase F (PNGase F); and (4) Sequencing of native and deglycosylated peptides by complementary mass spectrometric techniques (MALDI-TOF/TOF MS/MS, MALDI-Q/TOF MS/MS and micro-LC-ESI-Q/TOF MS/MS) and identification of parent glycoproteins by correlation of tandem mass spectrometric data with sequence databases. The selectivity of the method was apparent from the finding that over 70% of the identified peptides derived from glycopeptides, the other being unglycosylated caseins and serum albumin. MALDI signals attributed to glycopeptides, on the base of the typical molecular masses differences, not shifting in molecular mass after PNGase F treatment, were assigned to O-glycosylated peptides. By this approach, on the whole 30 sites of N-glycosylation were identified at Asn residues of consensus triplets Asn-X-Ser/Thr (X being any amino acid) belonging to 15 different glycosylated proteins. We identified only one peptide with multiple glycosylation sites. Most of the proteins were of structural or secretory origin such as lactadherin or lactotransferrin, involved in the host defence against infection through sequestration of iron required for microbial growth. Protein glycosylation play also a main role in intermolecular interactions and in protecting the proteins from intra- and extra-cellular degradation. The study revealed the presence in the human milk of N-glycosylated proteins such as immunocompetent complexes, membrane fat globule enzymes, proteins involved in lipid degradation, specific receptors and proteins with a still unknown function. Our results contribute to define on molecular basis the protective role of human milk and correlate specific glycoconjugate components to justify the low incidence of symptomatic gastrointestinal infections observed in breast-fed infants. The described strategy for glycopeptide selective enrichment is suggested for the identification of oligosaccharide moieties of N-glycoproteins from colostrum and mature human milk using specific endo-glycosidases.