Bioactive Proteins in Human Milk: Mechanisms of Action

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Human milk contains a multitude of bioactive proteins, with very diverse functions. Some of these proteins are involved in the synthesis and expression of milk, but the majority appear to have evolved to provide physiological activities in the breast-fed infant. These activities are exerted by a wide variety of mechanisms and have largely been unraveled by in vitro studies. To be active in the gastrointestinal tract, these proteins must be able to resist proteolytic degradation, at least for some time. We have evaluated the human milk proteins lactoferrin, haptocorrin, α1-antitrypsin, and transforming growth factor-β in an in vitro digestion model, mimicking the conditions of the infant gastrointestinal milieu. These bioactive proteins are resistant against pro teaseolysis and can remain intact or as larger fragments through passage of the gastrointestinal tract. In vitro digestibility assays can be helpful to assess which human milk proteins can resist proteolysis and to what extent. (J Pediatr 2010;156:S26-30).

Most studies show that there are still considerable differences between breast-fed and formula-fed infants, even though the composition of infant formulas has undergone several changes in the past decades. The growth patterns, nutritional status, prevalence of infection, and gut microbiota of breast-fed infants are different. Most meta-analyses of long-term outcomes, such as obesity, diabetes, and cardiovascular disease, also show advantages of breast-feeding when controlling for confounders.1-3 Although many factors are likely to be responsible for these differences, it is apparent that breast milk provides a multitude of bioactive proteins that are capable of physiological activities in the newborn infant and therefore can affect short- and long-term outcomes. In this review, these bioactive proteins are presented along with their mechanisms of action. The necessary requirements for them to remain active in the gut are also discussed.

Bioactive Proteins

α-Lactalbumin is a major protein in human milk, comprising about 25% to 35% of total protein.4 It has a well-balanced amino acid composition and a high proportion of essential amino acids and is most likely well utilized, as no immunoreactive fragments are found in the stool of breast-fed term5 or preterm infants.6 However, some proteolytic fragments of α-lactalbumin have been shown to have prebiotic functions in vitro by stimulating the growth of beneficial microorganisms, such as Bifidobacteria.7 Further, bactericidal peptides have been found by in vitro digestion with proteolytic enzymes.8 It is possible that such fragments are also temporarily formed in the gastrointestinal tract and facilitate the establishment of a beneficial microflora.

Lysozyme is present in breast milk at relatively high concentrations and is known to degrade the outer cell walls of Gram-negative bacteria. Feeding rice expressing recombinant human lysozyme to chickens could serve as a natural antibiotic,9 suggesting that it could replace currently used antibiotic drugs. Lysozyme together with lactoferrin is able to kill Gram-positive bacteria.

Lactoferrin is also a major protein in human milk and has the capacity to exert several physiological functions.10 It has bacteriostatic activity, most likely by its very high affinity toward iron, thereby withholding iron from iron-requiring pathogens. Recently, growth of Enterobacter sakazaki, a food-borne pathogen known to cause diarrhea in infants, was shown to be inhibited by iron-unsaturated lactoferrin (apo-lactoferrin) but not by holo-lactoferrin,11 showing that the iron-sequestering capacity of lactoferrin was responsible for the activity. Lactoferrin in breast milk is predominantly (>90%) in the apo-form. Lactoferrin also has bactericidal activity, killing a variety of pathogens, such as Vibrio cholerae.12 In a recent study on young children with acute diarrhea, oral rehydration solution with recombinant human lactoferrin and lysozyme significantly reduced diarrhea duration, diarrhea volume, and recurrence of diarrhea.13 From this study, it cannot be ascertained that lactoferrin was responsible for the observed effect because there was no lactoferrin-only group. There is also a possible synergistic effect of lactoferrin and lysozyme as reported in an in vitro study by Ellison and Giehl.14 Lactoferrin has also been shown to have antiviral activity against hepatitis C virus, cytomegalovirus, Herpes simplex virus, rotavirus, adenovirus, and human immunodeficiency virus.15 Three recent studies support that lactoferrin may prevent infections in children.16-18

Haptocorrin, or transcobalamin II, is the major vitamin B12-binding protein in breast milk, but only a small fraction of its vitamin B12-binding capacity is utilized in breast milk, making it very unsaturated.19 Haptocorrin can also inhibit the growth of...
pathogens by withholding vitamin B₁₂ due to its high binding affinity. We have shown, however, that both apo- and holo-haptocorrin can inhibit the growth of bacteria, for example, EPEC. Haptocorrin is able to enhance uptake of vitamin B₁₂ by human intestinal cells in culture, and it is possible that this protein facilitates the absorption of vitamin B₁₂ in young infants because expression of intrinsic factor is low during early life.

Bile-salt–stimulated lipase aids in the digestion of milk lipids, particularly in preterm infants who have low lipase activity and poor lipid utilization. This has been shown by pasteurization of breast milk fed to preterm infants; the heat treatment inactivates this enzyme and results in decreased lipid utilization. It is also possible that bile-salt–stimulated lipase aids in lipid digestion in term infants because of its unusually wide substrate specificity; it hydrolyzes mono-, di-, and tri-acylglycerols as well as cholesterol esters and diacylphosphatidylglycerols.

β-Casein is the predominant casein in human milk, and it is highly phosphorylated. Several phosphorylated serine and threonine residues are located close to the N-terminal of the protein and are capable of binding Ca²⁺ as well as other cations, such as Fe³⁺ and Zn²⁺. During digestion, phosphopeptides are formed, which have been shown to keep Ca²⁺ soluble, thus facilitating its absorption. It is therefore likely that casein phosphopeptides contribute to the high bioavailability of calcium from human milk.

κ-Casein is a minor casein subunit in breast milk, and it is highly glycosylated, containing charged sialic acid residues. The heavily glycosylated κ-casein has been shown to inhibit the binding of Helicobacter pylori to human gastric mucosa in vitro, possibly explaining why breastfeeding appears to provide some protection against H pylori. κ-Casein has been shown to prevent the attachment of bacteria to the mucosal epithelium by acting as a receptor analogue. During digestion of κ-casein, a large glycosylated fragment called glycomacropeptide is formed. This glycomacropeptide will probably also serve as a receptor analogue, but it has also been shown to enhance zinc absorption in infant rhesus monkeys, possibly due to the negatively charged sialic acid residues chelating zinc.

Secretory IgA, the predominant immunoglobulin in breast milk, is present in very high concentrations during early lactation but remains in substantial concentrations during lactation. Maternal immunity can be transferred to the infant via antigen-specific secretory IgA in the mother’s milk and thereby prevent adherence and penetration of both bacterial and dietary antigens capable of provoking inflammation in the intestinal mucosa.

Growth factors and cytokines are also present in breast milk in physiologically relevant concentrations, especially during early lactation. Insulin-like growth factor (IGF) I and II are bound to IGF-binding proteins, which may help to protect them against digestion and modulate their interaction with intestinal receptors. After binding of IGFs to enterocytes, they may exert bioactivity locally but also systemically. Transforming growth factor-β (TGF-β)1 and 2 have been found in human milk and has effects on several cellular processes, such as cell proliferation and differentiation. It is an immunoregulatory cytokine, involved in the regulation of T-cell activation, but it also affects B-cells, NK-cells, macrophages, and dendritic cells. Studies in TGF-β1 knock-out mice show that TGF-β is important for immune modulation and inflammatory responses as TGF-β knock-out mice die at the age of 3 weeks of systemic autoimmune disease. TGF-β can also initiate IgA production locally, enhancing mucosal immunity. Recent studies suggest that TGF-β in milk can induce and maintain oral tolerance and therefore possibly can be involved in prevention of allergy. The concentration of TGF-β in colostrum samples from mothers of infants with IgE-mediated allergy was found to be significantly lower in than in samples from mothers of infants with non–IgE-mediated allergy. Several other cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and IL-10, have been found in free form in breast milk but may also be released from cells in breast milk, particularly in colostrum. Although all these factors are immunomodulatory, most of them are anti-inflammatory, possibly lessening the effects of infections.

α₁-Antitrypsin may represent a unique bioactivity in human milk. This protease inhibitor is present in relatively high concentration in human milk, particularly during early lactation. We have shown that this protein is present in intact form in significant quantities in the stool of breast-fed infants and is thus, during infancy, not indicative of protein-losing enteropathy. It binds tightly to trypsin and is thus possible that α₁-antitrypsin can help to limit protein digestion during early infancy when α₁-antitrypsin concentrations in milk are high and secretion of proteolytic enzymes is immature. In this capacity, α₁-antitrypsin may facilitate the action of other bioactive proteins.

### Proteolytic Fate of Bioactive Proteins

To exert their functions in the gastrointestinal tract of the infant, the bioactive milk proteins must survive the action of proteolytic enzymes in intact or partially intact form. Thus, they need to be resistant to the low pH and pepsin activity in the stomach and, at least for some time, activities of pancreatic enzymes. That this is possible was first shown for secretory IgA, which was found intact in the stool of breast-fed infants. The arrangement with 2 IgA molecules together with the secretory component and the J-chain renders this specific type of antibody resistance toward proteolytic attack. Subsequently, lactoferrin and α₁-antitrypsin were also found in physiologically significant quantities in fecal collections from healthy, exclusively breast-fed infants. That this is possible is most likely due to both the fact that some proteins are unusually resistant toward proteolysis and that conditions for effective digestion of proteins are not fully developed. Gastric pH of young infants is rarely down to the range of 1 to 2 of adults and is often more like pH 3 to 5, with a decreasing trend with increasing age and physiological maturity. Secretion of pancreatic enzymes is
also low at birth and develops slowly during infancy. Thus, not all proteins are effectively digested and can remain intact or partially intact throughout at least part of the gastrointestinal tract. It should be noted, however, that most proteins in breast milk are digested, and even the resistant proteins are digested to some extent, possibly in the lower part of the small intestine, after exerting their biological functions.

Only proteins that are present in comparatively high concentrations in breast milk can be detected in the stool of breast-fed infants due to the very large quantity of other proteins being present in the feces (bacterial proteins, endogenously secreted proteins, protein fragments, etc). To investigate the capacity of various breast milk proteins to resist proteolytic attack, we have developed an in vitro digestion model. This allows us to investigate breast milk components present at concentrations that are low but adequate for their presumed physiological activity. We have used this model in a series of studies on human milk proteins, and it is briefly discussed below.

**In Vitro Digestion Model**

For simulation of the conditions in the infant’s stomach, we use a pH of 5.0 or 3.5, the former being more representative of the pH in a newborn and the latter of an infant of perhaps 4 to 6 months of age. As a comparison, we use a pH of 2.0 to be representative of the conditions in adulthood. The protein is incubated with pepsin at 37°C at the chosen pH for 30 or 60 minutes (to allow for different transit times), and the reaction is stopped by the addition of sodium bicarbonate to pH 7. Aliquots of samples are analyzed as described below, and the remainder of the solution is incubated with porcine pancreatin (a mixture of pancreatic enzymes) for 30 minutes to 2 hours. The reaction is stopped by either freezing or heating, depending on the protein and the assays to be used.

Protein concentrations can be analyzed by ELISA methods and can be assayed for enzyme activity, binding properties, or biological activity. Polyacrylamide gel electrophoresis (PAGE) with regular protein staining (Coomassie blue) can be used to detect the molecular size of the protein to verify that it is intact, proteolytic fragments formed and presence of other components when digested in the presence of other proteins (eg, human milk, infant formula). For specific detection of a certain protein in a complex solution, Western blotting using a specific antibody can be used.

**Applications of the In Vitro Digestion Model**

We have used the in vitro digestion model to study the stability of native human lactoferrin and of recombinant human lactoferrin expressed in rice. This study showed that 50% of both native and recombinant lactoferrin remained after digestion with both pepsin (at pH 3.8, 30 minutes) and pancreatin (60 minutes), whereas human serum albumin (present in human milk) was completely digested. Western blot analysis showed that both lactoferrins predominantly remained in intact form (80 kD).

We have also applied the in vitro digestion model to assess the bioactivity of α1-antitrypsin with regard to enhancing the protection against proteolysis of other bioactive components in breast milk. Alpha-1 antitrypsin was remarkably resistant to proteolysis by pepsin at pH 4.5 for 60 minutes and by pancreatic enzymes for 120 minutes in both buffer and human milk. Serum albumin was completely digested under these conditions. Addition of α1-antitrypsin during in vitro digestion of human lactoferrin enhanced the survival of lactoferrin. This did not occur for human serum albumin; this protein was effectively digested, showing that α1-antitrypsin may protect some proteins that are resistant to digestion (eg, lactoferrin), whereas enough trypsin remains present to thoroughly digest other proteins.

**Figure.** Structural stability of apo-HC and holo-HC in human milk before and after exposure to proteolytic enzymes. An in vitro digestion experiment was carried out to mimic the conditions of the infant stomach and small intestine. A, SDS-PAGE (10% to 12% gel) showed a 68 kDa band corresponding to HC after exposure to proteolytic enzymes; B, Western blot analysis shows positive immunoreactivity to HC, suggesting that HC is structurally intact. Confirming that enough enzymes were used in this experiment, SDS-PAGE showed that human milk α-lactalbumin (14 kDa), caseins (~20 to 40 kDa), and serum albumin (64 kDa), which comigrate with HC, are virtually absent after proteolytic digestion (n = 5). Reproduced with permission from Am J Clin Nutr 2003;777:1234-40, American Society of Nutrition.
uncomplexed to α₁-antitrypsin, allowing for digestion of more easily digested proteins (eg, serum albumin). We subsequently showed that native human milk α₁-antitrypsin (as well as a recombinant form) could survive even when pepsin digestion was performed at pH 2 and that short heat treatment did not inactivate its trypsin-inhibiting activity. This “quenching” role of α₁-antitrypsin with regard to proteolytic activity may be crucial for survival of some bioactive proteins in the upper part of the small intestine.

In vitro digestion studies of human milk haptocorrin showed very clearly that haptocorrin can resist digestion by both pepsin at pH 3.5 and by pancreatic enzymes. As shown in the Figure, haptocorrin and lactoferrin are resistant against proteolysis (in the same position on the left gel, due to similar molecular size), whereas other human milk proteins such as caseins and α-lactalbumin are more or less completely digested at the end of the incubation. Western blotting (right side gel) shows that haptocorrin, in both its holo- and apo-form, remained largely intact, as judging from its molecular size. That this was the case was verified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). We also showed that haptocorrin exposed to in vitro digestion remained active, as evidenced by inhibition of growth of EPEC.

Recently, we exposed TGF-β, which is found both in human milk as well as in some powdered infant formulas, together with purified recombinant TGF-β, to our in vitro digestion model. Digestion with pepsin at pH 2 or 3.5 and by pancreatin substantially increased concentrations of TGF-β in both human milk and infant formula (powdered Enfamil LIPIL, Novartis, Basel, Switzerland), most likely as acidification and/or proteolysis released the latent peptide and possibly other milk proteins associated with TGF-β. The immunoreactive TGF-β was found to be in intact form and was biologically active as shown by a specific Smad2 translocation assay.

**Conclusions**

Human milk contains a multitude of proteins, some of which have biological activities in the breast-fed infant. Several of these bioactive proteins are resistant against proteolysis and can remain intact or in larger fragments through passage of the gastrointestinal tract. In vitro digestibility assays can be helpful to assess which of these proteins can resist proteolysis and to what extent.

**Author Disclosures**

Bo Lönnlerdal, PhD, is a recipient of grants from Mead Johnson Nutrition and Harvest Plus. Mead Johnson Nutrition sponsored the symposium and provided an honorarium for attendance, presentation, and manuscript preparation. This article is an overview of the presentation given by Dr Lönnlerdal at the above symposium, and was written by Dr Lönnlerdal. Dr Lönnlerdal has no financial interests in the production or sales of infant formula or nutritional supplements.

References


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