J. Dairy Sci. 96:75–88
http://dx.doi.org/10.3168/jds.2012-5946

Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes

C. B. Furlund,*[1] E. K. Ulleberg,* T. G. Devold,* R. Flengsrud,* M. Jacobsen,† C. Sekse,‡ H. Holm,§ and G. E. Vegarud*
*Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1432 Ås, Norway
†Oestfold Hospital Trust, 1603 Fredrikstad, Norway
‡Norwegian Veterinary Institute, PO Box 750, N-0106 Oslo, Norway
§Department of Nutrition, Institute for Basic Medical Science, University of Oslo, 0316 Oslo, Norway

ABSTRACT

Lactoferrin (LF) is a protein present in milk and other body fluids that plays important biological roles. As part of a diet, LF must survive gastrointestinal conditions or create bioactive fragments to exert its effects. The degradation of LF and formation of bioactive peptides is highly dependent on individual variation in intraluminal composition. The present study was designed to compare the degradation and peptide formation of bovine LF (bLF) following in vitro digestion under different simulated intraluminal conditions. Human gastrointestinal (GI) juices were used in a 2-step model digestion to mimic degradation in the stomach and duodenum. To account for variation in the buffering capacity of different lactoferrin-containing foods, gastric pH was adjusted either slowly or rapidly to 2.5 or 4.0. The results were compared with in vivo digestion of bLF performed in 2 volunteers. High concentration of GI juices and fast pH reduction to 2.5 resulted in complete degradation in the gastric step. More LF resisted gastric digestion when pH was slowly reduced to 2.5 or 4.0. Several peptides were identified; however, few matched with previously reported peptides from studies using nonhuman enzymes. Surprisingly, no bovine lactoferricin, f(17–41), was identified during in vitro or in vivo digestion under the intraluminal conditions used. The diversity of enzymes in human GI juices seems to affect the hydrolysis of bLF, generating different peptide fragments compared with those obtained when using only one or a few proteases of animal origin. Multiple sequence analysis of the identified peptides indicated a motif consisting of proline and neighboring hydrophobic residues that could restrict proteolytic processing. Further structure analysis showed that almost all proteolytic cutting sites were located on the surface and mainly on the nonglycosylated half of lactoferrin. Digestion of bLF by human enzymes may generate different peptides from those found when lactoferrin is digested by nonhuman enzymes. The degradation of LF in the GI tract should be taken into consideration when health effects are proposed, because LF has now been approved by the European Food Safety Authority as a dietary supplement in food products.
Key words: human gastrointestinal enzyme, model digestion, lactoferrin, lactoferricin

INTRODUCTION

Lactoferrin (**LF**) is an important iron-binding protein in many body fluids, including milk, where it is found in various concentrations among species. Human milk has a higher concentration of LF (1–2 g/L) compared with bovine milk (0.1–0.2 g/L) (Sánchez et al., 1992; Levay and Viljoen, 1995; Hamosh, 1998). However, the use and consumption of bovine milk and dairy products are generally very high in most countries. Recently, the European Food Safety Authority (EFSA) accepted and approved bovine lactoferrin (**bLF**) as a novel food ingredient (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012). Bovine lactoferrin may therefore soon be added as an ingredient in an increasing number of food products. Many infant formulas enriched with bLF are already available on the market. Orally administered LF in adults exerts several therapeutic effects in vivo, among them improvement of immune and antioxidative status (Mulder et al., 2008).

The antimicrobial properties of LF have been extensively studied, and LF displays antibacterial, antifungal, and antiprotozoal effects as well as effects against viruses (Orsi, 2004). Some LF fragments identified after degradation with commercial enzymes have been reported to have higher bactericidal activity than the mother protein (Hamosh, 1998). In particular, the peptide fragment lactoferricin [**LFcin**; f(17–41)], released

Received July 16, 2012.
Accepted September 23, 2012.
[1]Corresponding author: camilla.furlund@umb.no or camilla.furlund@hotmail.com

from bLF with bovine and porcine pepsin, has demonstrated strong antimicrobial potential (Bellamy et al., 1992) and antitumor activity (Freiburghaus et al., 2009). Another antimicrobial peptide detected in the N1-domain of LF is lactoferrampin [**LFampin**; f(268–284)], with residues adjacent to the LFcin sequence in the 3-dimensional structure of the molecule (van der Kraan et al., 2004).

It is largely unknown whether LF maintains its integrity and to what degree and at what time it is hydrolyzed into peptides in the gastrointestinal (**GI**) tract. In addition, whether bioactive peptides such as LFcin are actually formed in the GI tract and would be stable enough to exert the proposed effect is not clear; the few in vivo studies are inconclusive (Chabance et al., 1998; Kuwata et al., 1998a,b, 2001). However, factors such as gastric pH, age of the consumer, and enzyme:substrate ratios seem to be important (Kalantzi et al., 2006). Lactoferrin is only partly digested in neonates and may be absorbed in intact form from the gut of infants (Sánchez et al., 1992; Levay and Viljoen, 1995; Chatterton et al., 2004). Troost et al. (2001) showed that a major proportion (60–80%) of bLF administered in a high dose (15 mg/mL) survived passage through the stomach in adults, and that the transit time in the duodenum was 20 to 30 min after instillation. However, in a subsequent study in women with ileostomies, orally ingested human LF was totally degraded in the upper GI tract (Troost et al., 2002).

A challenge in all in vitro model digestions is the simulation of physiological parameters such as variation in enzyme, acid, and bile salt secretions; substrate availability; and transit time in the stomach and duodenum (Ekmekcioglu, 2002; Kalantzi et al., 2006). To date, most peptides have been obtained by in vitro studies using commercial proteases. Human gastrointestinal juices comprise a complex mixture of enzymes present in multiple isoforms, enzyme inhibitors, and bile salts that are important for the digestion process (Scheele et al., 1981; Dunn, 2002; Ekmekcioglu, 2002). Commercial digestive enzymes are purified in most cases from different animal species. In general, enzymes taken from the species to be studied might differ from commercial enzymes of other species concerning specificity, functional enzymatic parameters, and stability. In most in vitro digestion protocols reported, pH during simulated gastric digestion has been rapidly decreased to 2 or 2.5. Gastric pH has been shown to decrease gradually after ingestion of a bLF-containing test drink, illustrating the high buffering capacity of LF (Troost et al., 2001). Gastric pH will have an important effect on the activity of the pepsins as well as on the conformation of the substrate. This subsequently influences the release of peptides during the gastric phase, with regard to specific cleavage points, sequences, and quantity, and it will also affect the subsequent duodenal digestion (Eriksen et al., 2010).

In this study, we aimed to apply an in vitro digestion model using human gastrointestinal enzymes to study the difference in peptide generation of bLF caused by individual variations in gastric pH and in the amounts of gastric and duodenal juice. A limited in vivo experiment was applied to illustrate variations during in vivo bLF digestion.

MATERIALS AND METHODS

Lactoferrin

Bovine LF (95% purity) was supplied by DMV International (Veghel, the Netherlands) and stored at −20°C. Two commercial bLF fragments (lactoferricin, **bLFcin**) were used: bLFcin f(26–36) supplied by Sigma (St. Louis, MO) and bLFcin f(17–41) by GenScript (Piscataway, NJ), with molecular weights of 1,554.87 and 3,123.9, respectively. Both samples were >95% pure and stored at −20°C. The bLFcin from GenScript contained a Cys19–Cys36 bridge.

Description of Human Gastric and Duodenal Juices

Human gastric (**HGJ**) and duodenal (**HDJ**) juices were collected from 18 healthy volunteers with no previous history of health impairments according to Ulleberg et al. (2011). In brief, a three-lumen silicone tube developed especially for this purpose by Maxter Catheters (Marseille, France) enabled simultaneous instillation of a stimulation solution and aspiration of gastric and duodenal juices. Aspirates from the stomach and duodenum were collected on ice, with periodic pH measurements, to control reflux and possible mixing of duodenal and gastric juices. After centrifugation to remove mucus and cell debris (4,500 × *g* for 10 min at 4°C), aliquots were frozen at −20°C and stored at −80°C. Pooled samples of HGJ and HDJ from the 18 donors were used in the in vitro digestion experiments described herein.

The aspiration of human contents was approved by the Norwegian Ethics Committee, and all volunteers signed an agreement before participating in the study. Human gastric juice was analyzed for pepsin activity at pH 3.0 with hemoglobin (H2625, Sigma) as substrate within 2 mo after aspiration, and HDJ was analyzed for total proteolytic activity at pH 8.0 with casein (Hammarstein casein, Merck Co., Darmstadt, Germany) as substrate (Ulleberg et al., 2011).