



Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis



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ABSTRACT

The digestion behaviour of unheated milk and heated milk (90 °C for 20 min) was investigated in a human gastric simulator (HGS). Unheated milks formed a clot after 10 min digestion, but the structure of the clot formed from unheated milk was different from that formed from heated milk. The clot obtained from unheated milk showed a closely knitted network with numerous small pores interspersed throughout the matrix, while a network structure with larger voids was observed in the clot of the heated milk. With increasing digestion time, as the pH decreased further, the structures of the clots tightened and became less permeable to serum and solutes. These changes apparently affected the hydrolysis of casein by pepsin in the gastric system. In unheated milk, casein hydrolysis was much slower than that in the heated milk. Whey proteins, β -lg and α -la, remained largely intact during the whole digestion period in unheated milk but in heated milk whey proteins were readily hydrolysed by pepsin. In heated milk, both casein and whey protein are involved in the formation of the clot. The present results indicate the clot formation is initially driven by the action of the milk-clotting enzyme pepsin on κ -casein and suggest that the formation of clots with different structures due to the different previous treatments affects the rate of protein proteolysis in the stomach.

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1. Introduction

Milk is an important source of protein in the human diet, and the digestion of milk proteins has been studied both *in vitro* and *in vivo*. The different proteins in a milk protein diet may cause differences in gastric emptying and may have different rates of proteolysis by pepsin. It has been shown that dietary caseins delay the delivery of amino acids to the upper intestinal lumen in humans (Mahé *et al.*, 1996). In contrast, whey proteins remain soluble and pass rapidly through the stomach, leading to a faster delivery of amino acids to the circulation (Boirie *et al.*, 1997). These observations have led to the concept of “slow” digested caseins and “fast” digested whey proteins. It has been suggested that the difference in digestion rate between caseins and whey proteins occurs because the caseins form a clot in the acid stomach environment and thus

are retained in the stomach for longer, whereas the whey proteins are soluble in the stomach and pass through to the duodenum without being hydrolysed by pepsin.

In milk, the caseins, together with colloidal calcium phosphate, form the particles known as casein micelles, which contain thousands of individual protein molecules (Dalgleish & Corredig, 2012). It is expected that the digestion behaviour of casein micelles in the gastrointestinal tract will be different from that of the individual caseins, because casein micelles are coagulated both by pepsin (Tam & Whitaker, 1972) and by low pH (Dalgleish & Corredig, 2012), both of which are present in the stomach, whereas caseinate is clotted only by acid and not by pepsin. Miranda and Pélissier (1981) reported that, *in vivo*, the clotting of skim milk in the rat stomach greatly reduced the rate of gastric emptying, compared with the rate of gastric emptying of a mixture of individual caseins. The proteolysis of caseins in skim milk and of the mixture of individual caseins, as observed by gel electrophoresis, appeared to follow different pathways; there was considerably greater proteolysis of the caseins in the mixture of individual caseins compared to that in milk.

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However, not all studies appear to support the concept of “slow” digested caseins and “fast” digested whey proteins. Some studies report a significantly slower rate of gastric emptying after casein ingestion compared with whey protein ingestion, whereas others report no difference (Hall, Millward, Long, & Morgan, 2003; Mahé et al., 1996). It has been suggested that these inconsistent results arise from the different methods used in measuring the gastric emptying rate (Calbet & Holst, 2004). Furthermore, differences in the previous processing of the ingested protein (whether or not heat treatment was applied, and the strength of the heat treatment), may have caused the inconsistent experimental outputs. These pre-treatments can alter the physical properties of the dietary proteins (e.g. viscosity, osmolality, particle size and degree of hydrolysis by the enzyme), because of changes in their structures. It has been demonstrated that these factors affect the gastric emptying rate (Hunt & Stubbs, 1975; Malagelada & Azpiroz, 1989; Vist & Maughan, 1995).

It has been shown that the processing treatment can affect the digestion of milk proteins, particularly whey proteins, by enzymes (Chobert, Briand, Grinberg, & Haertlé, 1995; Dalgalarondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995; Guo, Fox, Flynn, & Kindstedt, 1995; Li, Ye, Lee, & Singh, 2013; Peram, Loveday, Ye, & Singh, 2013; Zeece, Huppertz, & Kelly, 2008). Native β -lactoglobulin (β -lg), comprising 50% of the total whey protein in bovine milk, is resistant to some proteases, particularly to pepsin, because of its unique structural stability at low pH (Miranda & Pélissier, 1983; Reddy, Kella, & Kinsella, 1988). Most of the hydrophobic amino acids, which are potential cleavage sites for pepsin, are buried inside the hydrophobic core and are not readily accessible. Heating, the addition of alcohols and esterification and adsorption at an interface have been reported to increase the susceptibility of β -lg to hydrolysis by pepsin (Chobert et al., 1995; Dalgalarondo et al., 1995; Guo et al., 1995; Li et al., 2013; Peram et al., 2013; Zeece et al., 2008). These treatments induce conformational changes in β -lg, resulting in increased exposure of peptic cleavage sites and thus increased susceptibility to pepsin action. Also, the hydrolysis of α -lactalbumin (α -la) by pepsin is related to the conformation of the protein (Schmidt & Van Markwijk, 1993). In contrast, the caseins from sodium caseinate are easily hydrolysed by pepsin action (Guo et al., 1995). However, Juvonen et al. (2011) have reported that casein cross-linked with transglutaminase affects the plasma glucose, insulin, cholecystokinin, glucagon-like peptide 1 and peptide YY in the blood. They suggested that the food structure is more effective in modulating the postprandial responses than the type of dairy protein used.

In milk, heat treatment causes the denaturation of whey protein and the association of whey protein with the casein micelles (Oldfield, Singh, & Taylor, 1998; Anema & Li, 2003), as well as the formation in the serum of small complexes of whey proteins with κ -casein (Guyomarc'h, Law, & Dalglish, 2003). The inclusion of a high level of denatured whey protein can adversely affect the coagulation of milk by clotting enzymes (Anema, Lee, & Klostermeyer, 2007; Guinee, Pudja, & Farkye, 1993; Kethireddipalli, Hill, & Dalglish, 2010; Mulvihill & Grufferty, 1995). Thus, the casein clots formed during the digestion of milk under gastric conditions may differ between heated and unheated milk. Miranda and Pélissier (1987) have reported that heat treatment results in accelerated gastric emptying in milk digestion and appears to increase the rate of hydrolysis of the caseins *in vivo*.

As dietary milk proteins are treated under various conditions before ingestion into the human body, it is important to understand the digestion behaviour of the proteins in milk after different pre-treatments. Therefore, the objectives of this study were to investigate the coagulation/clotting behaviours of milk under *in vitro* gastric conditions and the influence of the coagulation/clotting on

the proteolysis of the milk proteins by pepsin. A stomach simulator was used as a tool in the study, in which milk and heated milk were compared.

2. Materials and methods

2.1. Materials

Skim milk was purchased from a local supermarket, and was used without further treatment. The heated milk samples were skim milk that had been heated at 90 °C for 20 min; this treatment is known to fully denature the whey proteins (Singh & Havea, 2003). Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalogue no. P7000, Sigma Chemical Co., St. Louis, MO, USA) had an enzymatic activity of 800–2500 units/mg protein, as stated by the manufacturer. All other chemicals were obtained from BDH Chemicals (BDH Ltd, Poole, England) unless otherwise specified. All solutions were prepared from analytical grade chemicals. Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) was used for the preparation of all solutions.

Simulated gastric fluid (SGF) was prepared by adding 3.8 mL of 37% HCl and 8.775 g of NaCl to 500 mL of water and then making up to 1 L with Milli-Q water; 3 g of pepsin was added to this solution before use, with stirring for 30 min, and then the pH of the SGF was adjusted to 1.5 using 1 N HCl/NaOH (Ye, Cui, & Singh, 2011).

2.2. Methods

2.2.1. Human gastric simulator (HGS)

An HGS, developed by Kong and Singh (2010), was used for gastric digestion (Fig. 1). The driving system of the HGS, consisting of 12 rollers, four belts, driving shafts and a pulley system, was installed to create peristaltic contractions on four sides of the latex stomach chamber. The rollers were screwed on belts distributed along the four equally spaced sides of the stomach. Each roller consisted of two side wheels that were 9 mm in length and 12 mm apart from each other. Each belt (length 81 cm) had three equally spaced rollers. A pair of opposite rollers was placed 30 mm higher than the pair at right angles to it. The gap between two opposite rollers was 10 mm when they contracted. A thin polyester mesh bag (pore size ~1 mm) was placed inside the latex stomach chamber (Fig. 1B) to mimic human gastric sieving, which allows particles only of size <~1 mm to pass through to the duodenum (Meyer, MacGregor, Gueller, Martin, & Cavalieri, 1976; Schulze, 2006).

A 200 g milk sample was fed into the HGS and was warmed at 37 °C for 2 min, and then the SGF was added at a rate of 2.5 mL/min. Because of the biphasic nature of gastric emptying, especially for high calorie fat and protein foods, the digesta were emptied out after 30 min (Collins, Horowitz, Cook, Harding, & Shearman, 1983; Siegel et al., 1988). For accurate control of the gastric emptying, the digesta were removed from the bottom of the stomach at 45 mL/15 min, equalling the gastric emptying rate of 3.0 mL/min. The contraction frequency was 3 times/min, simulating the actual contraction of the stomach. The temperature of the HGS was maintained at 37 °C by a heater and thermostat. The maximum digestion time was 240 min, but most experiments were terminated at shorter times so that the clot of coagulated milk could be collected for further analysis. For each time interval, the total sample was removed from the HGS and then was filtered through a mesh with pore size of 0.5 mm in diameter to obtain both solid mass and liquor phase for further analysis. In addition, to observe the effect of mechanical processing alone on the digestion of milk, experiments were carried out without the addition of pepsin and served as control experiments. The samples were prepared in duplicate. All tests were replicated twice.

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