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Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder

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ABSTRACT

This study aims to evaluate the impact of heat treatment on the hydrolysis kinetics of cow milk proteins and on the peptide release during in vitro dynamic gastric digestion. SDS-PAGE and ELISA techniques were employed to assess the hydrolysis of proteins over time of digestion. The evolution of the peptidome generated through dynamic digestion of heated and non-heated milk was studied at different times, using MS-based techniques (ion trap and MALDI-TOF/TOF) coupled to liquid chromatography. The peptide homology value between both samples at the end of digestion (48%) confirmed the impact of heat treatment on the identity of peptides generated during digestion, despite their identical initial protein content and being the same matrix in both cases. Heat treatment produced an increased resistance to hydrolysis by pepsin in the casein fraction. However, β-lactoglobulin was found to be more susceptible to hydrolysis. Although differences on the pattern of peptide release were found between both samples, also some common traits after digestion were observed. The regions comprised between the residues 76–93 of β-casein, where several binding epitopes are included, as well as the β-casein domains 126–140 and 190–209 were found to be resistant to pepsin.

1. Introduction

The study of the digestion process has gained importance in the last years, since the released products may elicit subsequent health effects. The evaluation of the peptides generated during digestion may be relevant to understand the behavior of proteins upon this process, and also for nutritional, pharmacological and toxicological concerns. The formation of bioactive peptides derived from milk protein digestion has been recently reviewed (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014a), pointing out the MS-based techniques as indisputable tools to perform targeted or untargeted analyses of digestion products. Several in vitro static digestion models have been developed and widely employed over the years (Guerra et al., 2012; Hur, Lim, Decker, & McClements, 2011; Kopf-Bolanz et al., 2012). These models intend to mimic physiological conditions, however, they lack mechanisms to control the sequential secretion of enzymes, the removal of digestion products, the appropriate mixing and the continuous changes in pH. Therefore, dynamic digestion models have been proposed as more realistic with respect to physiological parameters (Guerra et al., 2012; Ménard et al., 2014; Wickham, Faulks, & Mills, 2009). The gastric phase plays an important role in digestion, as the peptides produced during this step are delivered to small intestine, where they can either interact in situ with gastrointestinal receptors or be absorbed, mainly in the duodenum and upper jejunum (Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011; Shimizu, 2010).

However, it is increasingly evident that food processing can influence the behavior of proteins upon digestion, and therefore it may affect the release of peptides. Milk products are often thermally treated in food industry in order to lengthen their self-life, to reduce pathogen risk, or to modify their functional properties (Guo, Fox, Flynn, & Kindstedt, 1996). The modifications that proteins usually undergo upon these technological processes include unfolding, protein aggregation, oxidations and, Maillard reaction that leads to a blockage of Lys. Heat treatment may also produce the formation of non-natural amino acids, i.e., isopeptide bonds in lysinoalanine or lantionine, which are no longer substrate of digestive proteases. Wada and Lönnerdal (2014) reported high content of lactulosyl-lysine after in vivo and in vitro digestion of milk subjected to heat treatment at high temperature i.e., sterilized and ultra high temperature (UHT). The formation of heat-induced aggregates of casein and whey protein can occur (Jean, Renan, Famelart, & Guyomarc'h, 2006; Patel, Singh, Anema, & Creamer, 2006). Likewise, modifications on β-lactoglobulin (β-Lg) structure produced by heat treatment have been evaluated under

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different conditions, for instance different values of pH, ionic strength, or protein concentration (Aymard, Durand & Nicolai, 1996; Iametti, Cairoli, De Gregori, & Bonomi, 1995; Loveday, Wang, Rao, Anema & Singh, 2012). Thermal modifications of proteins affect the allergenic response by increasing or decreasing digestibility and by hiding epitopes. Caseins and whey proteins from raw and heat treated cow milk induce different immunological responses. For instance, sterilization of cow milk affects the allergenicity by reducing the humoral and cell-mediated responses in mice, compared with pasteurized and raw cow milk (Shandilya, Kapila, Haq, Kapila & Kansal, 2013). The formation of bioactive peptides through digestion could also be affected by heat treatment, as different protein bonds will be available for enzymes in the gastrointestinal tract (Meisel, 1998). The heat-induced changes in food proteins influence their stability to in vitro static digestion as in the case of bovine and caprine milk (Almaas et al., 2006), or infant formula (Dupont, Boutrou, Ménard, Jardin, Tanguy, et al., 2010). These modifications induced by heat also affect the kinetics of protein digestion in vivo, in mini-pigs (Barbé et al., 2013). Although it has been reported that β-Lg is resistant to pepsin digestion (Miranda, & Pelissier, 1983), there is also evidence that under certain conditions it can be susceptible to hydrolysis by this enzyme. For instance, heat treatment, the presence of alcohols, esterification and heating under high pressure or at low pH (Bateman, Ye, & Singh, 2010; Chobert, Briand, Grinberg, & Haertle, 1995; Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995; Zeece, Huppertz, & Kelly, 2008). Heating temperatures above 90 °C, and especially 100 °C induce conformational changes in this protein that provoke the exposure of the hydrophobic regions, increasing its susceptibility to hydrolysis by pepsin (Guo, Fox, & Flynn, 1995; Peram, Loveday, Ye, & Singh, 2013). In this regard, the assessment of the peptidomic profile generated through dynamic digestion of milk products subjected to different heat treatments can help to understand the protein behavior during gastric digestion. Using a dynamic model represents a step forward in terms of mimicking physiological conditions. The pH is an important parameter that will affect the optimum activity of the enzyme. The model used in this work followed a pH curve previously used in TIM-1 by Minekus, Marteau, Havenaar, and Huis in Veld (1995), based on human trials conducted by Marteau et al. (1991), and that showed good correlation. The units of pepsin used were chosen from a pig model (Chiang, Croom, Chuang, Chiou, & Yu, 2008), and the gastric emptying rates have been evaluated on an in vivo trial in mini-pigs fed the same milks used in this work (Barbé et al., 2013). The pig is a suitable model to predict differences among dietary protein digestibility in men, showing good inter-species correlation in terms of true N and amino acid digestibility (Deglaire, Bos, Tomé, & Moughan, 2009). Therefore, the aim of this work is to evaluate the impact of the heat treatment on the hydrolysis kinetics of milk proteins and on the evolution of the peptidome generated at different times of gastric dynamic digestion under these conditions, in terms of comparative analysis between heated and non-heated milk samples by using two mass spectrometry analyzers (ion trap and MALDI TOF/TOF).

2. Materials and methods

2.1. Samples

Two milk samples (unheated and heat treated skim milk) were used for the experiments. Cow skimmed milk powder was rehydrated in water as described by Barbé et al. (2013). Briefly, to obtain the skim milk powder, a skimming step at 50 °C was applied, then it was subjected to microfiltration (1.4 μm, in cold), and finally it underwent evaporation at low temperature and spray drying. The powder contained 4% of humidity, 34% of protein, 54% of lactose and 8% of ash. The powder was reconstituted in distilled water to reach a final concentration of 50 g/L of protein, using a stirrer for 10 min to achieve full solubilization. Half of the milk was subjected to heat treatment (90 °C, 10 min) to obtain the heat-treated milk sample, and the other half did not undergo any further treatment after reconstitution of the powder (unheated milk). For digestion process, 200 mL of each sample was used.

2.2. Dynamic digestion

A dynamic digester available at STLO (DIDGI®, INRA Rennes, France) was used to perform digestions on the two samples (unheated and heated milk) in triplicate. The digestion system and the software (SToRM: stomach regulation and monitoring) were previously described by Ménard et al. (2014). The half-time of gastric emptying used in the present study was set at 191 and 283 min for unheated and heated milk, respectively, as estimated by Barbé et al. (2013) in mini-pigs fed the same milks. An exponential equation as described by Elashoff, Reedy, and Meyer (1982) was used to monitor the gastric emptying and the coefficient (β) has been set up at 0.8. The acidification curve occurring in the gastric compartment was controlled by the software, using the pH data previously reported (Minekus et al., 1995). Porcine pepsin (P-6887, Sigma) was diluted in simulated gastric fluid (SGF) (NaCl 150 mM, adjusted to pH = 6.5) in order to reach 1000 units/mL (Chiang et al., 2008), and added to the gastric compartment at a flow rate of 0.5 mL/min as previously described (Minekus et al., 1995), controlled by the software. Samples were collected at 4, 10, 20, 50, 105, 165, 225, 315 and 405 min of digestion.

2.3. SDS-PAGE

SDS-PAGE analyses were performed using 4–12% Bis–Tris polyacrilamide precast gels (1.5 mm × 15 wells; NuPAGE Novex, Invitrogen). Aliquots of the triplicates of digestion at different sampling times, as mentioned above, were submitted to electrophoretic analysis. The milks (heated or unheated) were also loaded on gels. These analyses were carried out as described by Bouzerzour et al. (2012). The molecular marker used for the experiments was Mark 12 Unstained Standard NuPAGE 4–12% (Invitrogen). The image analyses of the gels were performed using Image scanner III (GE Healthcare Europe GbmH, Velizy-Villacoublay, France). Densitometry analyses of the gel images were carried out. The relative quantification of the β-lactoglobulin (β-Lg) over time of digestion was performed by measuring the colored area volume of the band on the SDS-PAGE gel image. Both milk samples, previous to digestion, present in each run of SDS-PAGE experiments, were used to establish the initial amount of protein. The volume of their colored area was measured and considered as 100%. The hydrolysis of this protein through digestion was estimated by referring the colored areas at different digestion times to that of the initial amount.

2.4. ELISA

Inhibition ELISA was performed on the triplicates of digestion from each sample (unheated and heat treated milk) at different times of digestion (0, 4, 10, 20, 50, 105, 165, 205, 315 and 405 min). ELISA was carried out as previously described (Dupont, Mandalari, Molle, Jardin, Role-Répécaud, et al., 2010) using caseins-specific polyclonal antibodies to estimate the residual immunoreactivity of this protein during digestion process. Samples were first homogenized with a thurrax (Ultra Thurrax T8 IKA, Fischer Scientific, 20,000 tr/min–5 min). Triplicate analysis of each digestion time point has been performed.

2.5. LC–MS/MS-based analysis by ion trap and MALDI TOF/TOF

The digests from unheated and heated milk were individually analyzed by the two MS-based techniques: RP-HPLC-ion trap and nanoLC–MALDI-TOF/TOF at three digestion times (4, 50 and 405 min).

The RP-HPLC–MS/MS analyses of the triplicates of digestion were carried out on a HPLC-ion trap described by Sánchez-Rivera, Recio, Ramos, and Gómez-Ruiz (2013). The samples were eluted at 0.2 mL/min. A linear

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