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Proteomic profiling of camel and cow milk proteins under heat treatment

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ABSTRACT

Cow and camel milk proteins before and after heat treatment at 80 °C for 60 min were identified using LC/MS and LC–MS/MS following monodimensional electrophoresis. The database used for the identification of camel and cow proteins was set from http://www.uniprot.org/. The obtained results showed that, after heating, camel milk at 80 °C for 60 min, camel α-lactalbumin (α-la) and peptidoglycan recognition protein (PGRP) were not detected while camel serum albumin (CSA) was significantly diminished. When heating cow milk at 80 °C for 60 min, α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were not significantly detected. Moreover, 19 protein bands from SDS-PAGE were analyzed and a total of 45 different proteins were identified by LC–MS/MS. Casein fractions were kept intact under a heat treatment of 80 °C during 60 min of both camel and cow milks. Camel and bovine whey proteins were affected by a heat treatment of 80 °C for 60 min.

1. Introduction

Cow milk is composed of all nutrient components, mainly proteins, fat, lactose and minerals. The protein fraction of bovine milk consists essentially of whey proteins, mainly β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins (Ig) and bovine serum albumin (BSA), and caseins, assembled in micelles and accounting for about 80% of the total bovine milk protein content (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013). Heat treatment is included in most dairy industries to obtain bacteriologically safe final products and to extend their shelf life. A number of structural modifications have been recognized in the milk protein component depending on time, temperature and rate of heating. Singh (1995) showed that a range of large heterogeneous protein aggregates of milk proteins occurred in heat-treated milk. The heat-induced milk protein association occurring under different heating conditions has been extensively investigated (Donato & Guyomarc'H, 2009). Previous studies have shown that both caseins and whey proteins are engaged in protein aggregates found in heat-treated milk and that the formation of intermolecular disulfide bonds is mostly responsible for heat-induced protein association in milk (Manzo, Nicolai, & Pizzano, 2015). The thermal protein denaturation has been acknowledged as the primary step of the reactions leading to the aggregation of disulfide-linked milk proteins. Thiol groups of cysteine residues, appearing in unfolded proteins, can initiate thiol-disulfide exchange reactions within hydrophobically-linked protein aggregates. Self-aggregation of heat-denatured β-lg in water (Roefs & De Kruif, 1994), heat-induced association of whey proteins and/or their aggregates with caseins (Corredig & Dalgleish, 1999) have been explained according to this mechanism.

Camel milk is an important nutrition source for inhabitants in arid and semi-arid areas (Farah, 1996). Camel milk has been shown to have nutritional and therapeutic properties which are widely exploited for human health in several countries (Mal, Sena, Jain, & Sahani, 2006). It contains higher amounts of essential fatty acids and antimicrobial agents compared to other species' milk (Shamsia, 2009). The main components of whey proteins in camel milk are similar to those in bovine, except for the lack in β-lg. Currently, most of camel milk is consumed in the fresh state. Therefore, to extend its shelf life, different heat treatments such as pasteurization may be applied to camel milk. However, heat processing as a means of preserving milk is applied to camel milk in some regions, mainly in gulf countries and in Central Asia, and up to now only a few studies have investigated the effect of heat treatment on camel whey proteins (El-Agamy, 2000; Farah, 1986). Recently, Felfoul et al. (2016) have investigated the deposit generation during camel and cow milk heating and evaluated the microstructure and the chemical composition of the obtained

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deposits. They have demonstrated that the deposit obtained after heating camel milk at 80 °C for 60 min contained 57% *w/w* proteins and 35% *w/w* minerals. Proteomic techniques are used to obtain information about the changes in the protein fraction of heat-treated milk. Although not many studies have examined non-bovine milks using the proteomic approach (Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012; Pappa et al., 2008), to the best of our knowledge, a few data are available in the literature on camel milk (Zhang et al., 2016).

In the present work, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) were used to evaluate the fundamental differences in proteins aggregation after heat treatment of camel and cow milks. This study also aims to identify camel and cow proteins involved in the formation of the aggregates in both heat treated camel and cow milks.

2. Materials and methods

2.1. Milk samples

Camel milk was collected from reared camels by been directly milked into a sterile milking can. The camel milk used was pooled milk samples assembled from 20 different healthy camels (*Camelus dromedarius*) that ranged between 2 and 12 months in lactation. Cow milk was collected from 20 different Holstein cows. Both milk samples were derived from a local breeding located in the south of Tunisia. The milk samples were immediately transported to our laboratory using an ice box within 4 h. Once reaching the laboratory at 4 °C, pH (744-pH meter, Metrohm SA CH-9101, Herisau, Switzerland) was determined. Subsequently, both milks were skimmed by centrifugation at 3000*g* during 20 min at 4 °C (Gyrozen 1580MGR, Multi-purpose Centrifuge, Daejeon, Korea).

2.2. Heat treatment experiments

Heat treatment experiments of camel and bovine milks were conducted in a stainless steel recipient (316L; Total volume = 500 mL) at 80 °C for 60 min. Heat treatment consisted in heating over a hot plate without agitation (Felfoul, Lopez, Gaucheron, Attia, & Ayadi, 2015). The experiments were reproduced at least 3 times.

2.3. LC/MS analysis

100 μL of fresh and heat-treated skimmed milk samples were diluted in 400 μL buffer Urea 4 M/Tris 25 mM pH8, and then placed at 37 °C for 1 h 15 min in a water bath with regular manual agitation. All solutions were diluted 2 × in 0.5% TFA solution, afterwards, filtered through Millipore® Millex®filters HVPVDF membrane, 0.45 μm Pore Size before injection.

Mass spectrometry (MS) experiments were performed using an on-line liquid chromatography mass spectrometry setup *via* an Agilent 1100 HPLC system coupled to an AB Sciex QSTAR XL Quadrupole-Time Of Flight (QTOF) mass spectrometer. The separation of proteins was performed with a separation column C4 VYDAC Grace, reference 214TP5215, 150 mm length × 2.1 mm inner diameter (i.d.) using solvent A (0.106% TFA in deionized water) and solvent B (80% (v/v) acetonitrile and 0.1% (v/v) TFA in deionized water). A linear gradient from 37 to 60% of solvent B in 40 min was applied for the elution at a flow rate of 0.25 mL/min. Protein signal was recorded by both UV detection at 214 nm wavelength and electrospray mass spectrometry. Eluted proteins were directly electrosprayed into the mass spectrometer operated in positive mode. Mass spectra were collected in the selected mass range from 800 to 3000 Thomson. The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein, β-CN (193–209). The quantification data of the studied camel and cow milk protein fractions (expressed in μg/μL) was estimated basing on the peak areas of the chromatographic profiles as well as both milks compositional data. Measurements were performed in duplicate with independent samples.

2.4. SDS-PAGE

Skimmed camel and cow milks, before and after heat treatment at 80 °C for 60 min (fresh camel, heat-treated camel, fresh cow and heat-treated cow milks), were diluted with deionized water. Protein concentrations of skimmed milks were determined by dint of the Bradford method (1976) and adjusted to be the same among samples equal to 5 g/L. The proteins were then separated by polyacrylamide gel electrophoresis containing 0.1% sodium dodecyl sulphate (SDS-PAGE) in non-reducing conditions. Electrophoresis experiments were carried out using a Bio-Rad apparatus (Mini-Protean Tetra Cell, Bio-Rad Laboratories, Hercules, USA) of gels in vertical slabs. 50 μg of protein was loaded for each sample onto 12% acrylamide resolving gel. As described by Laemmli (1970), electrophoresis was run at 120 V/20 mA until the marker color (bromophenol blue, Sigma-Aldrich Chemie S.a.r.l., Saint-Quentin Fallavier, France) was 0.5 cm from the anode end of the block (approximately 3 h). The molecular weight of the different protein fractions were estimated by comparing their electrophoretic mobilities with those of marker proteins having known molecular weights. The electrophoresis experiment was repeated 3 times. All gels were photographed and the most significant one was presented.

2.5. Protein bands identification by tandem mass spectrometry

After separation by SDS-PAGE, the proteins were identified by mass spectrometry (MS) after an in-gel trypsin digestion according to Shevchenko, Wilm, Vorm, and Mann (1996). Gel pieces were excised from the gel, placed in eppendorf tubes of 0.5 mL, washed with 100 μL acetonitrile and NH_4HCO_3 solution (50%/50%) 1–3 times based on their coloration intensity. Subsequently, the supernatants were removed using a small benchtop centrifuge. Finally, the gel pieces were dried under vacuum in a SpeedVac concentrator (SVC100H-200; Savant, Thermo Fisher Scientific, Waltham, MA) for 15 min. In-gel trypsin digestion was performed overnight at 37 °C and stopped with spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich Chemie S.a.r.l., Saint-Quentin Fallavier, France). The supernatants containing peptides were then vacuum dried in a SpeedVac concentrator for mass spectrometry analysis.

Mass spectrometry (MS) experiments were performed by means of an on-line liquid chromatography tandem mass spectrometry (MS/MS) setup using a Thermo Scientific™ Dionex™ Ultimate™ 3000 RSLC nano-liquid chromatography (nano-LC) system fitted to a Qexactive (Thermo Scientific™, San Jose, USA) equipped with a nano-electrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). The instrument was externally calibrated according to the supplier's procedure. The samples were first concentrated on a PepMap 100 reverse-phase column (C18, 5 μm, 300-μm i.d by 5-mm length) (Dionex, Amesterdam, The Netherlands). Peptides were separated on a reverse-phase PepMap column at room temperature, using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water). A linear gradient from 5 to 50% of solvent B in 10 min was applied for the elution at flow rate of 0.3 μL/min. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full