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Assessing the heat induced changes in major cow and non-cow whey proteins conformation on kinetic and thermodynamic basis

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ABSTRACT

In this study, the RP-HPLC technique was used as the main analytical method to measure the residual native β-lactoglobulin and α-lactalbumin concentration after heat treatment in raw milk from three different species (goat, sheep and cow). Further, a detailed comparative kinetic study of β-lactoglobulin and α-lactalbumin denaturation was carried out at temperature ranging from 72.5 to 90 °C.

Kinetic studies showed that the thermal denaturation of β-lactoglobulin followed biphasic behavior, resulting in activation energy of $91.68 \pm 13.18\ kJ\ mol^{-1}$, $137.13 \pm 25.25\ kJ\ mol^{-1}$ and $62.11 \pm 3.26\ kJ\ mol^{-1}$ for the denaturated fraction in goat, sheep and cow milk and $307.91 \pm 61.29\ kJ\ mol^{-1}$, $158.99 \pm 23.64\ kJ\ mol^{-1}$ and $170.18 \pm 43.61\ kJ\ mol^{-1}$ for the native fraction in milk samples. α-Lactalbumin denaturation followed the first-order kinetics, resulting in activation energy values of $202.65 \pm 1.42\ kJ\ mol^{-1}$, $155.56 \pm 5.53\ kJ\ mol^{-1}$ and $140.44 \pm 6.14\ kJ\ mol^{-1}$ respectively in goat, sheep and cow milk. The heat-induced changes in protein structure were outlined after running molecular dynamics simulations at different temperatures, supporting the experimental observations. These experiments were conducted only for cow and goat α-lactalbumin and were limited by the lack of protein structures from databases.

1. Introduction

In the dairy industry, cow milk is frequently heated for pasteurization and sterilization. The heating process may induce oxidative losses of proteins, unsaturated lipids, vitamins, active enzymes, and immunological factors (Fidler et al., 1998). β-Lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulin, serum-albumin, and glycomacropeptide are the major milk whey component. In cow milk, β-LG accounts for 50–55% of the total whey proteins and α-LA is the second most abundant protein, its rate concentration being of 1.2 g/L of milk.

β-LG is a small globular protein with 162 amino acids in monomer form and a molar mass of 18.4 kDa (de la Hoz and Netto, 2008). The tertiary structure possesses a pocket (β-barrel calyx built from eight antiparallel β-sheets), where hydrophobic ligands can easily bind (Brownlow et al., 1997). Native β-LG has 40–50% β-sheet, 10–15% α-helix and 40% random coil structure (Qi et al., 1997; Stănciuc et al., 2012). β-LG has two tryptophans (Trp^{19} and Trp^{61}) located in different environments. Trp^{19} is located within the protein core (hydrophobic region), at the bottom of the binding pocket, close to Lys^{70} that is believed to be one of the specific binding places present in the protein chain.

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The other residue (Trp^{61}) is placed at the exterior surface of β-LG, in close contact with two strong quenchers: an aspartate residue and a disulfide bond (Cys^{66}–Cys^{160}) (Uhrínová et al., 2000).

At the pH and protein concentration of milk, β-LG is present in the form of dimers interacting by hydrophobic forces and extensive hydrogen bonding involving a small β-strand not included in the β-barrel and the single long α-helix (Brownlow et al., 1997). Trp^{61} and Cys^{121} are located at this interface and have been suggested to be involved in stabilization of the dimer (Burova et al., 1998).

α-LA performs some important function in mammary secretory cells (Hill and Brew, 1975) and it is a 14 kDa protein whose native structure is divided into two domains: one is largely helical (the α-domain) and the other has a significant content of β-sheets (the β-domain), which are connected by a calcium binding loop. The β-sheet domain is composed of a series of loops, a small three-stranded anti-parallel β-pleated sheet (residues 41–44, 47–50, and 55–56) and a short 3_{10} helix (h2: 77–80). The two domains are divided by a deep cleft. At the same time, the two domains are held together by the disulfide bridge between residues Cys^{73} and Cys^{91}, forming the Ca^{2+} binding loop. Overall, the structure of α-LA is stabilized by four disulfide bridges: Cys^{6}–Cys^{120}, Cys^{61}–Cys^{77}, Cys^{73}–Cys^{91}, and Cys^{28}–Cys^{111} (Permyakov and Berliner, 2000).

When compared to the cow milk, goat and sheep milk are very different in composition. The average values for fat, protein and total solids are 3.6%, 3.3% and 12.7 in cow milk, 4.0%, 3.5% and 13% in the case of goat milk and 7.5%, 5.5%, and 19% respectively in the case of sheep milk (Raynal-Ljutovac et al., 2007).

For any protein, the expression of biological activity is critically dependent on the thermodynamic stability of its native three-dimensional folded structure (Price, 2000). Many studies have focused on the kinetics of whey proteins, and especially on cow β-LG thermal denaturation process in a wide range of physicochemical environments, most of them at or about neutral pH, condition of practical relevance in dairy industrial processes (Roefs and de Kruif, 1994; Anema and McKenna, 1996; Galani and Owusu Apenten, 1997, 1999; Verheul et al., 1998; Sava et al., 2005; Stănciuc et al., 2012). Several models have been proposed to describe the mechanism of whey proteins denaturation, especially of β-LG, which implies some consecutive reactions involving dissociation of the dimer, unfolding and aggregation. Both Parris and Baginski (1991) and Pellegrino et al. (1995) used RP-HPLC to monitor the effects of heat treatments on whey protein denaturation and found new peaks eluting ahead of β-LG A and B in the samples heated at higher temperatures. These changes have been ascribed to modification of the protein by heat induced aggregation and lactosylation. However, the mechanism of the effect of heat on whey proteins has yet to be fully determined. The complex nature of the reactions occurring in whey protein solutions during heating and the importance of various types of chemical bonding is not easy to establish.

Considering the complexity of reactions that occur in milk during heat treatment, in direct correlation with milk composition, the results obtained with cow milk cannot be easily extrapolated to small ruminant counterparts. The approach in this study is due to the growing markets for dairy products containing goat, sheep or buffalo milk, which may increase the research interests to this area. Moreover, data regarding the heat induced structural changes of whey proteins in non-cow milk are limited.

Therefore, to extend the understanding of the relevance of conformational changes at the secondary and tertiary structure levels that are involved in the denaturation pathway of β-LG and α-LA induced by different time–temperatures combination, samples of milk from different species (cow, sheep and goat) were analyzed using RP-HPLC technique. In addition, the kinetic and thermodynamic parameters of protein denaturation were evaluated. In order to add valuable information about thermal dependent behavior of the proteins the molecular modeling approach was further employed. This part of the study was limited to the comparative evaluation goat and cow α-LA molecules because only these 3D models are available on the worldwide protein data banks.

2. Material and methods

2.1. Milk samples collection

Bulk milk samples of indigenous goat (55 individuals, White Banat Goats), sheep (75 individuals, Merino Sheep) and cow (45 individuals, Romanian Simmental Cows) breeds were purchased from different local farms (Galati, Romania). The samples were collected within September and October 2011. Each sample of raw milk was obtained from a batch of 10 L. Milk composition was determined using Portable Milk Analyzer (Milk-Lab Ltd, Odham, Lancashire, UK). The milk was divided in small portions (2 mL) and stored frozen at −20 °C until use.

2.2. Heat treatment

Thermal denaturation kinetics of β-LG and α-LA experiments in raw milk were conducted using the test tube method. Aliquots (5 mL) of milk were placed in test tubes and immersed in a water bath (Digibath-2 BAD 4, RaypaTrade, Spain) at temperatures ranging between 72.5 °C and 90 °C for different holding times (0–5 min). Different heating up times were considered at each tested temperature in order to reach the desired value. After thermal treatment, the test tubes were immediately immersed in ice water to allow rapid cooling.

2.3. Preparation of acid whey filtrates

The milk samples were 1:1 diluted with ultra-pure water and then, casein in the milk phase was precipitated at pH 4.5 using 33% acetic acid (Sigma–Aldrich, Germany) at 20 °C, followed by centrifugation at 2000 × *g* for 10 min at 4 °C. The supernatant was filtered through Whatman No 40 filter paper and stored at −23 °C until analysis.

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

The acid whey samples along with individual whey proteins (Sigma–Aldrich, Germany) as standards were analyzed by RP-HPLC method as described by (Moatsou et al., 2005). The HPLC equipment consisted of the pump WATERS 600E (Waters, USA), a photodiode array detector (Waters 996), a helium degasser, a Rheodyne injector (model 7125) and the Millenium32 software v. 3.05.01 (Waters). A Vydac C4 214 TP 5415 (Separations Group, Hesperia, CA, USA) column was used at room temperature. The flow rate was 1 mL min^{-1}. Solvent A was 0.1% trifluroacetic acid (TFA, Serva Electrophoresis, Heidelberg, Germany) in ultra-pure water (v/v) and Solvent B was 0.1% TFA, 80% acetonitrile (Lichrosolv grade, Merck, Darmstadt, Germany) in ultrapure water (v/v). The percentage of Solvent B changed in the elution mixture as follows: 0–15 min, 27–40% B; 15–55 min, 40–56% B; 55–57 min, 56–80% B; 57–60 min, 80% B; 60–62 min, 80–27% B.