Bioaccessibility of Amino acids and Antioxidant properties in Heat- and Lactase-treated Milk during in *Vitro* digestion

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Introduction

Heat treatment is one of important aspects to maintain the self-life of milk, which modifies several biochemical features of milk. Due to heat treatment of milk, beside the agglomeration of proteins, protein can bind with milk sugar – lactose – by Maillard reaction. Non-enzymatic lactosylation (Figure 1) of proteins is the first stage of Maillard reaction, where a reducing sugar (lactose) attaches to the ε-NH_2 group of lysine and produce lactosyl-lysine. The nutritional quality of milk decreases with an increase of the degree of lactosylation in milk proteins.

Physiologically important aspects of milk proteins are accessible after in vitro digestion of milk proteins. Different proteolytic enzymes in gastrointestinal tract are responsible for digestion of proteins. Heat treatment alters the native structure of proteins in milk and influences the digestibility. Hence, physiological importance of protein is linked with structure of protein and processing of milk. In present investigation, in vitro digestion simulation was used to study such effects in pasteurized, ultra-heat treated and lactose-free milk samples (Figure 2).

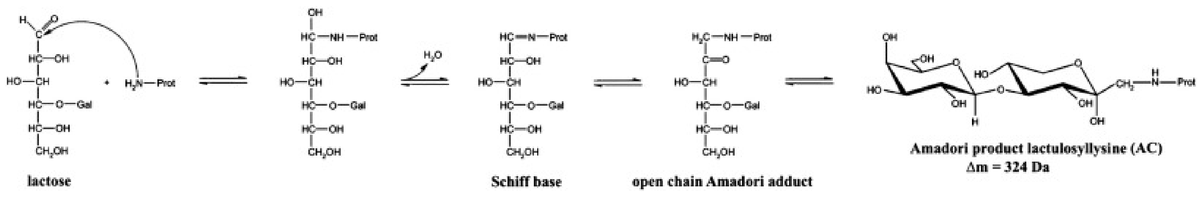

Figure 1. Biochemical mechanism of the lactosylation of proteins.

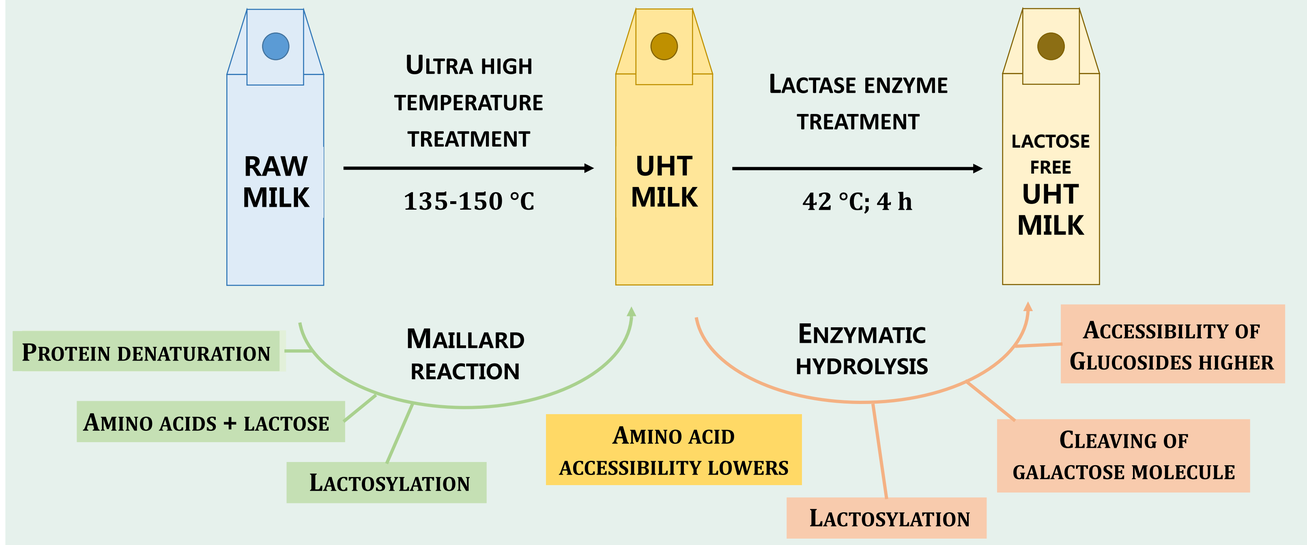

Figure 2. Sematic mechanism of UHT treatment and enzymatic hydrolysis on amino acid accessibility.

Protein identification

Milk samples were subjected to LC-ESI-QTOF-MS analysis, to identify the active peptides and occurrence of lactosylation based on their molecular weights in pasteurized milk, UHT and lactose free UHT milk. Using their monoisotopic respective masses the native proteins in the milk samples were identified as α-casein, β-casein and β-lactoglobulin.

In **pasteurized milk (Figure 3. A, B)** the proteins identified after deconvolution were β-lactoglobulin and β-casein. No lactosylation was observed in this spectrum, as expected from pasteurized milk due to low heat treatment.

In **UHT milk (Figure 3. C)** lactosylation of all proteins occurred, however at different ratios and the species had only one lactose unit attached to the protein.

Lactase enzyme treated UHT milk (Figure 3. D) showed similar peaks as UHT treated milk however there were smaller additional peaks between major peaks. These smaller peaks are indicating signs of glycosylated protein forms in lactose free UHT milk.

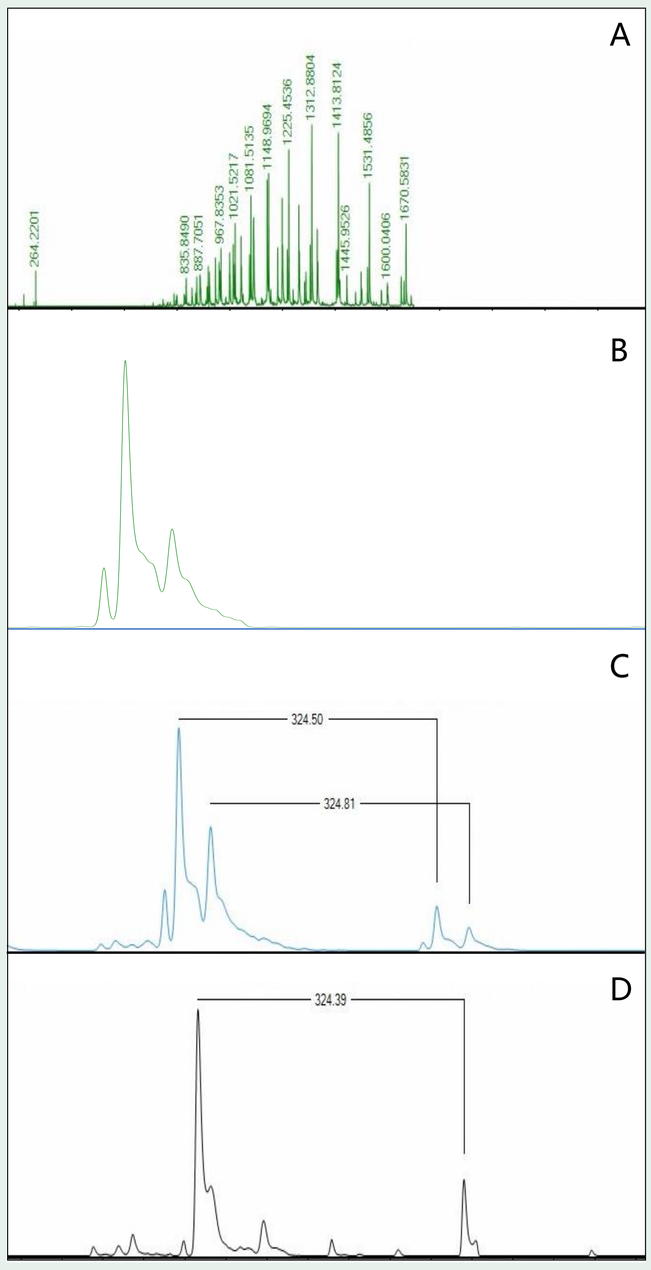

Figure 3. MS spectrum of β-casein in pasteurized milk (A: raw; B: deconvoluted), UHT milk (C) and lactose free UHT (D) milk.

Materials and Methods

Pasteurized milk with 2.8% fat and ultra-heat-treated milk (UHT) with 2.8% fat were collected from local dairy plant (Szeged). Lactose in UHT milk was hydrolysed with commercially available lactase at 42 ˚C for 4 h.

In vitro digestion

In vitro digestion simulation was according to Minekus et al., 2014[1]. Samples were collected from gastric phase at 30, 60, 90 and 120 min, and of the end of intestinal phase. Aliquots from gastric phase were heated at 100 °C for 15 min, precipitated protein were separated with centrifugation (10000 rpm, 30 min), supernatants were filtered with 0.45 µm syringe filter for antioxidant measurement. Bioaccessible protein fraction from intestinal phase aliquots were separated with methanolic extraction (80%), precipitates were separated with centrifugation (6000 rpm, 20 min, 4 °C) supernatants were use for amino acid analysis.

Analytical methods

A LC-ESI-TOF mass spectrometric method was set up using deconvolution of acquired intact protein mass spectra for detecting lactosylation of milk proteins in milk. Mass shift of +324 (characteristic for lactose attachment) and +162 (characteristic for glucose or galactose attachment) with respect to the unmodified casein, α-lactalbumin and β-lactoglobulin were considered for assessing the change of molecular mass of casein due to lactosylation. Bioaccessible (free) amino acid profile was analysed after AQC derivatization with UHPLC-UV method. Antioxidant capacity was measured by the FRAP method[3].

Amino Acid accessibility

Purpose of the in vitro study was to determine the effect of lactosylation and subsequent lactase treatment on bioaccessibility of amino acids.

Valine, lysine and histidine recorded the highest reduction in bioaccessibility (Figure 4) due to **UHT treatment of milk** with 59%, 45% and 41% bioaccessibility, respectively. The significant reduction in bioaccessibility is probably due to cross linking of amino acids with each other in the presence of and through lactose.

The recovery in bioaccessibility of amino acids in **lactose free UHT milk** was likely the effect of the proteolytic activity of lactase and the hydrolytic cleavage that was evident for aliphatic and aromatic amino acids. The lactose bridges between amino acid were cleaved by lactase eliminating the steric hindrance caused by structural changes due to said cross links.

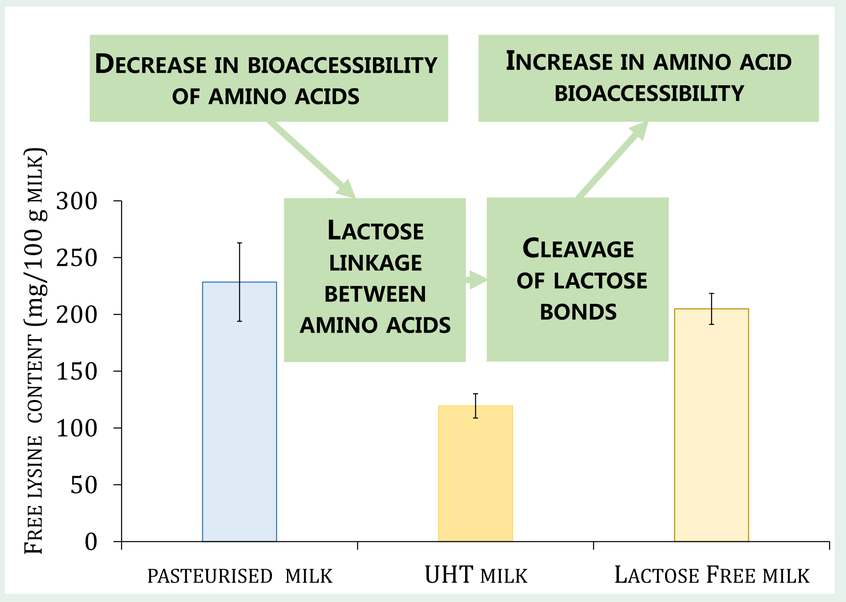

Figure 4. Free lysine content of pasteurized milk, UHT milk and lactose free milk.

Antioxidant properties

Antioxidant properties of milk proteins is associated with its amino acid composition. Hydrophobic amino acids are responsible for the increase in the antioxidant properties of the milk. Heat treatment increases the surface hydrophobicity of protein globules, which increase the antioxidant capacity. Enzymatic digestion produces peptides with hydrophobic amino acids in -C and -N terminal positions. Hydrophobic amino acids donate electrons to neutralize free radicals.

Pasteurized milk, UHT milk, and lactose free UHT milk had different antioxidant capacity at 0 min in the gastric phase (3.93 mg, 7.87 mg and 6.75 mg (equivalent to ascorbic acid·L^{-1}), respectively). The antioxidant activity continued to increase during digestion with the final concentration after intestinal digestion of 14.28 mg, 14.84 mg and 14.28 mg (equivalent to ascorbic acid·L^{-1}), respectively. The results indicate that the antioxidant properties of UHT milk are not only affected by temperature and time during processing but also enzymatic activity during digestion.

[1]**Minekus et al., 2014.** A standardised static in vitro digestion method suitable for food—An international consensus. Food & Function, 5(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

[2]**LAEMMLI, 1970** U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227, 680–685, doi:10.1038/227680a0.

[3]**Benzie, I. F. F., & Strain, J. J. (1996).** The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Analytical Biochemistry, 239(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Acknowledgements

Financial support of OTKA K135294 is kindly acknowledged. A. Nath acknowledges Hungarian State Postdoctoral Scholarship. J. Tormási acknowledges the Food Science Doctoral School of MATE.