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Targeted peptides for the quantitative evaluation of casein plasminolysis in drinking milk

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ABSTRACT

In addition to proteose peptones (PP), the extent of plasminolysis in different classes of drinking milk during storage has been evaluated by the quantification of the peptides α_{s2}-CN (f1–25) 4P and α_{s2}-CN (f1–21) 4P by UPLC/HR-MS. The rate of increase in the levels of all the studied peptides during storage depended on the heating process. The samples of drinking milk showed different levels of plasminolysis at their expiration dates, as revealed by α_{s2}-CN (f1–25) 4P and α_{s2}-CN (f1–21) 4P amounts. The different treatments applied during the manufacturing of extended shelf life (ESL) milk samples resulted in different levels of plasminolysis, confirming the heterogeneity of this class of drinking milk. The peptides from α_{s2}-CN accumulated faster than PP in all the samples with the exception of UHT milk. Therefore, these peptides can be considered as sensitive indices of early plasminolysis in pasteurised and ESL milk.

1. Introduction

Proteolysis, which occurs via both native and microbial enzymes, is the predominant factor that affects several of the technological properties of milk. Decreased stability of stored pasteurised milk and age gelation of UHT (Ultra High Temperature) milk are mainly dependent on enzymatic protein degradation. Casein breakdown in cheese milk can also significantly affect the milk properties. Shorter coagulation time, reduced curd firmness and lower cheese yield result from protease activity in milk (Crudden, Afoufa-Bastien, Fox, Brisson, & Kelly, 2005). In addition to lipolysis, the enzymatic degradation of proteins affects the sensory profile of milk (Santos, Ma, Caplan, & Barbano, 2003) and, consequently, its shelf life (De Noni, Pellegrino, Cattaneo, & Resmini, 2007). Moreover, the emulsifying and foaming properties of milk are strongly influenced by the degree of proteolysis of caseins and whey proteins (Caessens, Visser, Gruppen, van Aken, & Voragen, 1999).

Among the milk proteases, plasmin (PLM) (EC 3.4.21.7) plays a predominant role in milk quality. Plasmin activity in milk is regulated by a complex system that includes the zymogen plasminogen (PG), the inhibitors of PLM (PAI), the activators of PG and the inhibitors of the PG activators. The activity of each component of the PLM system in milk depends on several factors, including temperature maintenance, thermal treatment and the number of somatic cells (Ismail & Nielsen, 2010; Kelly, O'Flaherty, & Fox, 2006).

Beta-casein is the fraction of total casein that is most susceptible to PLM action and is rapidly hydrolysed to γ-casein and proteose peptones (PP5, PP5 fast, PP8 fast and PP8 slow) (Fox, 2003). α_{s2}-Casein is also promptly degraded by PLM, leading to the formation of several peptides that have been identified in both raw and heat-treated milk samples (Gaucher, Mollé, Gagnaire, & Gaucheron, 2008; Pinto et al., 2012; Wedholm, Moller, Lindmark-Mansson, Rasmussen, Andrén, & Larsen, 2008). While α_{s1}-casein is less prone to PLM action, peptides arising from this casein fraction have been identified in drinking milk (Gaucher et al., 2008; Meltretter, Schmidt, Humeny, Becker, & Pischetsrieder, 2008).

The effects of the heat treatments applied in milk sanitation on casein plasminolysis have mainly focused on pasteurisation and UHT sterilisation. In the last decade, extended shelf life (ESL) milk has gained increasing interest by consumers due to its both prolonged shelf life (usually up to 1 month) and limited heat damage. As no definition of ESL treatment exists in the EU legislation, ESL products can be described as products that are treated in a manner suitable to reduce the microbial count below that achieved by regular pasteurisation, that are packaged under strict hygienic (or *ultra-clean*) conditions and that have a defined and prolonged shelf life under refrigerated conditions (Rysstad & Kolstad, 2006). The manufacturing process of ESL milk may involve either direct or indirect heat treatment and more severe conditions than usual HTST (High Temperature Short Time) pasteurisation. Alternatively, microfiltration of the skimmed milk, heat treatment of the separated cream, and subsequent HTST pasteurisation of the two recombined parts can be applied (Hoffmann et al., 2006). Such a

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wide range of processing conditions that are currently in use for ESL milk production results in a variety of commercial milk products with very different levels of heat damage, as highlighted in some recently conducted surveys on the EU market (Lorenzen et al., 2011; Mayer, Raba, Meier, & Schimd, 2010; Schmidt, Kaufmann, Kulozik, Scherer, & Wenning, 2012).

Only a few studies (Meltretter et al., 2008; Pereda, Ferragut, Buffa, Guamis, & Trujillo, 2008) have dealt with the effect of ESL processing on plasminolysis in milk, and no PL activity was observed in HT (High Temperature)-pasteurised (peroxidase-negative) milk during refrigerated storage. De Noni et al. (2007) monitored PL activity in microfiltered ESL milk and compared it to that in pasteurised milk over a 14-day storage period at 4 °C. By measuring the accumulation of proteose peptone (PP), it was shown that microfiltration impairs plasminolysis possibly by removing the somatic cells from the milk. Although the effects of the storage conditions of heat-treated milk on PLM activity have been widely studied, conflicting evidence has been reported, especially with respect to UHT treatment (Chavan, Chavan, Khedkar, & Jana, 2011; Enright, Bland, Needs, & Kelly, 1999).

The current methods for the quantitative evaluation of PLM activity in milk are based on the use of either trinitrobenzene sulphonic acid or fluorescamine (Chove, Grandison, & Lewis, 2011). However, the corresponding increase in either the absorbance or fluorescence, which occurs as a result of the enzyme activity, does not allow for any qualitative evaluation of plasminolysis on the casein fractions (Chove et al., 2011). A further limitation of measuring PLM activity in model systems is the lack of information on the activity of PLM on caseins in a micellar status, as they are in milk. Electrophoretic techniques, based on either gel or capillary supports, are useful to directly highlight casein breakdown and the formation of large peptides (De Noni et al., 2007), while separation of the small peptides is not satisfactory (Meltretter et al., 2008). Among chromatographic techniques, RP-HPLC has been widely adopted for studying PLM activity in milk (Chove et al., 2011). However, the pattern of soluble peptides is usually very complex and the peak resolution is unsatisfactory. In these cases, the reliable identification and quantification of peptides can be achieved only with mass spectrometry (MS). Ultra performance liquid chromatography (UPLC) is an emerging technique for peptide separation that allows for higher resolution and sensitivity compared to HPLC. To our best knowledge, this technique has not been applied to evaluate the degradation of milk casein to date.

This work aimed to provide a reliable analytical tool for the quantitative evaluation of plasminolysis in milk by identifying and measuring selected markers of plasminolysis by UPLC/high resolution (HR)-MS. A further objective of the present work was to study the effects of different milk sanitation processes on the milk PLM activity. To this end, raw bulk milk was processed and packaged at industrial plants using heating conditions that are representative of the different processes that are currently in use for drinking milk manufacturing.

2. Materials and methods

2.1. Milk samples and storage conditions

Milk samples were provided by two industrial dairies. The conditions adopted for manufacturing of drinking milk are reported in Table 1. On the same day and using the same raw bulk milk (Standard Plate Count (SPC): 70,000 cfu $mL^{-1}$and Somatic Cell Count (SCC): 180,000 cells mL^{-1}) one manufacturer produced batches of pasteurised (sample A) and high-temperature (HT) pasteurised (sample B) milk. Using raw bulk milk with a similar hygienic quality (SPC: 82,000 cfu mL^{-1}; SCC: 210,000 cells mL^{-1}) and equal protein content (3.5 g 100 g^{-1}), the other manufacturer produced batches of microfiltered (MF) pasteurised ESL milk (sample C), steam infusion (SI) ESL milk (sample D) and direct UHT milk (sample E). All milk samples were full-fat (3.5 g 100 g^{-1}) and homogenised. Seven packages of each batch were kept at 5 °C or 8 °C for storage experiments. Seven packages of UHT milk were kept at ambient temperature (22 ± 2 °C). Packages from each batch were analysed at selected intervals (every 16 d and at 110 d for UHT milk; 1, 3, 5, 7, 10, 16 and 21 d for all other milk samples). After 21 days of storage at 5 °C or 16 days at 8 °C, the pasteurised milk samples coagulated and, therefore, were not sampled.

2.2. Determination of raw milk characteristics

The SCC was determined according to the ISO 13366-2 IDF 148-2 International Standard (2006). The SPC was determined according to the ISO 8553 IDF 131 (2004). The protein content was determined according to the ISO 8968-1 IDF 20-1 International Standard (2001).

2.3. Determination of heat-treated milk characteristics

The furosine level was determined according to the ISO 18329 IDF 193 International Standard (2004). The lactulose level was determined according to the ISO 11868 IDF 147 International Standard (2003). The level of soluble whey proteins was determined according to the ISO 13875 IDF 178 International Standard (2005). The qualitative determination of peroxidase was performed according to the EU Commission Decision 91/180/EEC (1991).

Table 1
Processing conditions, durability and levels of heat damage markers in drinking milk samples.

Sample code	Type of milk	Heating conditions (temperature/time)	Heating plant/process	Commercial durability (days)	Peroxidase	Furosine (mg 100 g^{-1} protein)	Lactulose (mg L^{-1})	Soluble whey proteins (mg L^{-1})	
								α-Lactalbumin	β-Lactoglobulin
A	Pasteurised	75 °C/18 s	Plate heat exchanger	7	Positive	6.0 ± 0.5	<20	1147 ± 23	3619 ± 43
B	HT-pasteurised	85 °C/18 s	Plate heat exchanger	10	Negative	9.1 ± 0.9	<20	960 ± 19	2682 ± 30
C	MF-pasteurised ESL	75 °C/18 s (cream: 95 °C/5 s)	Plate heat exchanger	16	Positive	8.2 ± 0.4	<20	1098 ± 23	3534 ± 45
D	SI-ESL	129 °C/1 s	Steam infusion	21	Negative	8.5 ± 0.3	<20	1025 ± 21	3219 ± 56
E	UHT	144 °C/4 s	Steam injection	110	Negative	75.0 ± 1.2	198 ± 5	450 ± 18	1271 ± 25

Data represent the average of triplicate analysis ± SD.