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Destabilization of UHT milk by protease AprX from *Pseudomonas fluorescens* and plasmin

Chunyue Zhang, Etske Bijl, Kasper Hettinga*

Dairy Science and Technology, Food Quality and Design Group, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

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ABSTRACT

Destabilization of UHT milk during its shelf life is mainly promoted by the residual proteolytic activity attributed to the psychrotrophic bacterial proteases and native milk proteases. In this study, we built skim UHT milk-based model systems to which either the major bacterial protease (AprX from *Pseudomonas fluorescens*), or the major native milk protease (plasmin) was added, to allow a direct comparison between the destabilization of skim UHT milk by both categories of enzymes. The physical and chemical properties were studied during 6 weeks. Our results showed AprX induced compact gels when almost all the κ-casein was hydrolyzed and the degree of hydrolysis (DH) exceeded 1.3%. Plasmin induced soft gels when around 60% of both β- and α_{s1}-casein were hydrolyzed and the DH reached 2.1%. The knowledge gained from this study may be used for developing diagnostic tests for determining the protease responsible for UHT milk destabilisation.

1. Introduction

The demand for ultra-high-temperature (UHT) processed and aseptically packaged milk is increasing worldwide. UHT milk is the best choice of liquid dairy products for many developing and tropical countries because it does not required cooled logistics and storage, and has a relatively long shelf life (≥6 months). These features also well facilitate its compatibility with the commercial exploitation in international trade for dairy exporting countries.

In spite of the broad market for UHT milk, it can be subject to a range of undesirable changes, such as age gelation and fat separation during its shelf life (Datta & Deeth, 2001). The onset of sedimentation, age gelation and, sometimes, a bitter taste is promoted by the proteolytic activity due to residual enzymes, which can survive the UHT treatment and remain active during storage (Manji & Kakuda, 1988; Datta et al., 2001; Rauh, Sundgren et al., 2014).

Milk contains a large number of native enzymes with differing specificity, stability and impact on product quality (Kelly & Fox, 2006). Plasmin (EC 3.4.21.7), with its zymogen plasminogen and other parts of the complex enzyme system, constitute the major native protease system in milk, and has been reported to be correlated with udder health, as indicated by the somatic cell count (SCC) (Ramos, Costa, Pinto, Pinto, & Abreu, 2015; Musayeva et al., 2016). This protease system exhibits a high thermal stability and can remain partially active after the UHT treatment; therefore, the plasmin system has been closely linked to physicochemical deterioration of UHT milk (Kohlmann, Nielsen, & Ladisch, 1991; Kelly & Foley, 1997; Rauh, Sundgren et al., 2014).

In addition to the well-known detrimental effects of plasmin, enzymes originating from psychrotrophic bacteria can also be a serious problem in UHT milk because these bacteria are inevitable in raw milk and some of them can produce heat-resistant proteases and lipases during cold storage, that can withstand the UHT process. Among all the psychrotrophic bacteria, *Pseudomonas* species are particularly incriminated in the destabilisation of UHT milk (Vithanage, Yeager, Jadhav, Palombo, & Datta, 2014). A single specific extracellular alkaline metallo-protease belonging to the AprX enzyme family has been discovered in genus *Pseudomonas*, which is responsible for milk spoilage (Vithanage et al., 2014; Matéos et al., 2015). The heat-stable proteases have been reported to be produced by pseudomonads during the late exponential/early stationary growth phase of the bacteria, generally at bacterial cell counts of 10^7–10^8 cfu/mL (Stoeckel et al., 2016). This means that the production of AprX is determined by the storage time, temperature, and the count of pseudomonads. Therefore, the AprX level indirectly reflects the hygienic management of the farm and the storage history of the milk.

Cow health, hygiene management and storage history of milk are all crucial links in the dairy chain that can influence the stability of UHT milk. To better trace back which links needs to be improved to prevent instability, we need knowledge of the responsible enzymes, especially with regard to the differences between them in their proteolytic activities on milk proteins, and the changes they induce in milk. However,

* Corresponding author.
E-mail addresses: chunyue.zhang@wur.nl (C. Zhang), etske.bijl@wur.nl (E. Bijl), kasper.hettinga@wur.nl (K. Hettinga).

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to date, such systematic understanding of these enzymes systems is still lacking.

The mechanisms for age gelation of UHT milk have been mainly described by two theories (Kocak & Zadow, 1985; McMahon, 1996; Datta et al., 2001). The first involves the enzymatic degradation of the milk proteins, and the promotion of age gelation by the formation of peptides. The second mechanism is often referred to as "non-enzymatic" or "physicochemical" age gelation in which no protein degradation is observed (McMahon, 1996; Anema, 2017). Age gelation through the physicochemical mechanism is slow for unconcentrated milk samples, in which it usually takes longer than 12 months (Anema, 2017). Therefore, the focus of this study is the enzyme-induced destabilization. To reduce the interference from the non-enzymatic physicochemical changes, visible destabilization was induced within a relatively short time by the addition of high concentrations of enzymes.

This study aims to provide insights in the differences of the hydrolytic process on milk proteins between AprX and plasmin. To assess this, skim UHT milk samples to which different concentrations of AprX or plasmin were added, and were stored for 6 weeks at both room temperature and the optimal temperatures for both proteases. During this period, the differences in the visual deterioration of milk, physicochemical modifications and hydrolysis patterns of caseins were studied.

2. Materials and methods

2.1. Enzymes

The AprX-producing bacterial strain *Pseudomonas fluorescens* Migula 1895 (DSM 50120) was obtained from Deutsche Sammlung von Mikroorganismen (DSM). This strain has been reported to be able to grow at 4–37 °C and have proteolytic, lipolytic and pectinolytic activities. The AprX sequence was found in the gene with an 15 bp insertion between bp position 395 and 410 on the sequence, proving the dairy origin of this isolate (Caldera et al., 2015). For cultivation, the strain was cultured in a nutrient broth (VWR International B.V.) to the end of its log phase (around 26 h) at 25 °C. The bacteria were then harvested by centrifugation (4000*g*, 10 min, 20 °C). To purify the extracellular enzymes, we inoculated the bacteria in minimal medium (7 g L^{-1} K_2HPO_4, 2 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1 g L^{-1} $(NH_4)_2SO_4$, 4 g L^{-1} glycerol, pH 7.0) containing 2% (v/v) UHT milk as protease inducer for 24 h at 25 °C with stirring at 160 rpm (Matéos et al., 2015). After 24 h of culturing, cells were removed by centrifugation at 10,800*g* for 30 min at 4 °C (rotor JA 16.250, Avanti Centrifuge J-26 XP, Beckman Coulter, USA). The supernatant was first concentrated by centrifuging at 4000*g* for 20 min at 4 °C in Amicon Ultra filters (10 kDa cut-off, Millipore), after which the concentrate obtained was dialysed against sterile 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for 48 h (3.5 K, Thermo Scientific Slide-A-Lyzer™ Dialysis Cassette) and lyophilised. No further purification of the extracellular bacterial enzymes were performed, because the crude AprX extract is more similar to the real situation in milk, which facilitates our simulation of the real destabilization caused by pseudomonads.

Plasmin from bovine plasma was purchased from Roche, 5 units of plasmin were suspended in 3.2 M ammonium sulfate solution, where the unit is defined at 25 °C with Chromozym PL as the substrate.

2.2. Milk sample preparation

Commercial skim UHT milk was purchased from a local supermarket, the milk was subjected to direct sterilization by steam infusion heat treatment. The protein and fat content was 3.76% and 0.07% (w/v) respectively, analyzed by MilkoScan 134A/B (Foss Electric, Hillerød, Denmark). To keep a low starting degree of hydrolysis, we used milk samples that were not older than 1 month after manufacture. To prevent spoilage during storage, 0.02% sodium azide and 0.0005% bronopol were added as preservatives.

In a preliminary study, the enzyme concentrations were determined, to allow both model systems to gel in around 1 month at room temperature. In the main experiment, the AprX extract was accurately weighed into skim UHT milk samples at the concentrations of 0, 10, 20, 50 μg/mL, the samples were aliquoted in flat-bottomed screw-top Turbiscan tubes and centrifugation tubes in an upright position, and incubated in dark without agitation at room temperature and 42 °C, respectively. Likewise, plasmin was added at the concentrations of 0, 0.8, 1.6, 2.4 μL/mL, and the samples were incubated at room temperature and 37 °C, respectively. All the samples were analysed at 1 week intervals over 6 weeks of storage.

2.3. Dynamic light scattering (DLS)

The average hydrodynamic particle size was determined using a Zeta-sizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) and the associated DST1070 disposable folded capillary cell. Samples were diluted 1/50 in milliQ water prior to the measurement. All the measurements were performed in triplicate at 25 °C. The refractive index was set to 1.45 for particles and to 1.354 for the dispersant. Since the AprX-induced gelled samples could not be mixed homogeneously by vortexing, the size distribution of AprX-containing samples upon and after gelation was not determined.

2.4. Characterization of stability by Turbiscan

Sample stability was monitored using the optical analyzer Turbiscan MA 2000 (Formulaction, Ramonville St. Agne, France) under gravity force. The back scattering intensity were measured as a function of height under a near-infrared light source at 880 nm. It was carried out in 100 mm tall borosilicate glass tubes with 12 mm inner diameter. The apparatus scanned at 2000 acquisitions/scan in 3 s (i.e. 1 acquisition each 40 μm) at room temperature. The thickness of sediment (mm) is the length where the sample's backscattering intensity is higher than the blank.

2.5. Determination of protein degree of hydrolysis

DH values were determined in triplicate using o-phthaldialdehyde (OPA, Sigma) assay. The OPA reagent was prepared as described by Wierenga, Meinders, Egmond, Voragen, and de Jongh (2003). Samples were diluted 2 times in a 2% (w/v) SDS solution, stirred for 20 min, and stored at 4 °C overnight to fully solubilise the peptides and the possibly present intact protein. The samples were then diluted 5 times in milliQ water aliquots (50 μL) for adjusting the concentration within the linear range of the standard curve, and were added to 1500 μL of the reagent solution in a cuvette and equilibrated for 10 min at room temperature. The presence of alkylisoindoles formed by the reaction of free amino groups with OPA was measured by the absorbance of the sample at 340 nm. To calculate the amount of free NH_2 groups, a calibration curve was made using leucine as a reference compound, the number of free NH_2 groups per gram samples was expressed as h. The total number of peptide bonds per gram of protein substrate was obtained by complete hydrolysis of the blank skim UHT sample in 6 M HCl, 110 °C for 24 h; h_{tot} was 8.68 mmol/g in this case. The detected DH value of the blank UHT milk was 7.0%, this value originated from native lysines and the *N*-termini of the milk proteins, as well as the hydrolysis that had happened in the milk prior to our experiment. This value was considered as the "blank" DH in milk and was subtracted from all the other data. The additional amino groups from the added AprX and plasmin were also subtracted accordingly, but the number of peptide bonds originating from the autolysis of proteases were neglected because the level of autolysis differs in a system without caseins as substrates, and can thus not be properly assessed. In this way, the DH discussed here refers to the hydrolysis caused merely by the added proteases, and was calculated as $DH = (h/h_{tot} * 100\%) - 7.0\%$. The OPA reagent in the presence