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

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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.

Keywords: UHT milk; AprX; plasmin; gelation; destabilization; hydrolysis; shelf life

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 et al., 2014b).

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, Sederevičius, Želvytė, Monkevičienė, Beliavska-aleksiejūnė & Stankevičius, 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 et al., 2014b).

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, Lidolt, Stressler, Fischer, Wenning & Hinrichs, 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, 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, physico-chemical 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℃ 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 ℃. The bacteria were then harvested by centrifugation (4,000 g, 10 min, 20 ℃). 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} $MgSO_4.7H_2O$, 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 ℃ 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 ℃ (rotor JA 16.250, Avanti Centrifuge J-26 XP, Beckman Coulter, USA). The supernatant was first concentrated by centrifuging at 4,000 g for

20 min at 4 ℃ 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 ℃ 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 ℃, 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 ℃, respectively. All the samples were analysed at 1 week intervals over 6 weeks of storage.

125 2.3. Dynamic light scattering (DLS)

126 The average hydrodynamic particle size was determined using a Zeta-sizer Nano ZS (Malvern
127 Instruments Ltd., Malvern, UK) and the associated DST1070 disposable folded capillary cell.
128 Samples were diluted 1/50 in milliQ water prior to the measurement. All the measurements
129 were performed in triplicate at 25 ℃. The refractive index was set to 1.45 for particles and to
130 1.354 for the dispersant. Since the AprX-induced gelled samples could not be mixed
131 homogeneously by vortexing, the size distribution of AprX-containing samples upon and after
132 gelation was not determined.

133 2.4. Characterization of stability by Turbiscan

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

140 2.5. Determination of protein degree of hydrolysis

141 DH values were determined in triplicate using o-phthaldialdehyde (OPA, Sigma) assay. The
142 OPA reagent was prepared as described by Wierenga, Meinders, Egmond, Voragen & de Jongh
143 (2003). Samples were diluted 2 times in a 2% (w/v) SDS solution, stirred for 20 min, and stored
144 at 4 ℃ overnight to fully solubilise the peptides and the possibly present intact protein. The
145 samples were then diluted 5 times in milliQ water aliquots (50 µL) for adjusting the
146 concentration within the linear range of the standard curve, and were added to 1500 µL of the
147 reagent solution in a cuvette and equilibrated for 10 min at room temperature. The presence of
148 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 6M HCl, 110℃ for 24h; 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 of SDS could sufficiently dissolve plasmin-induced gelled samples, but not the AprX-induced gelled samples. Therefore the AprX–containing samples were only analysed until 1 week before gelation happened.

2.6. Protein Profile Analysis

2.6.1. RP-HPLC

The intact protein composition was measured by Reversed Phase High Pressure Liquid Chromatography (RP-HPLC, Thermo Scientific™ UltiMate 3000) equipped with an Aeris Widepore 3.6 μm XB-C18 column, 250 × 4.6 mm (Phenomenex, Utrecht, the Netherlands), according to the method described by de Vries et al. (2015). Protein standards (β-casein, α_S-casein, κ- casein, α-lactalbumin and β-lactoglobulin; purities 70–85%, all from Sigma-Aldrich) were used to validate the elution times of milk proteins. The resulting chromatograms were

analysed through the software Chromeleon 7.1.2. We determined the total peak areas of β-, α_{s1} and κ-casein, and assume the peaks with same retention time are intact caseins. However, we could not quantitatively describe the development in α_{s2}-casein due to the co-elution of the peaks of this protein with other breakdown products, as also explained by Rauh et al. (2014b). The buffers in the presence of DL-dithiothreitol and urea could sufficiently dissolve plasmin-induced gelled samples, but not the AprX -induced gelled samples, so again the AprX -containing samples were analysed until 1 week before gelation happened.

2.6.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed to complement RP-HPLC results. The sample buffer, reducing agent, gels, running buffer and antioxidant agent were all purchased from Invitrogen (Carlsbad, USA). Two µL sample was diluted in 5 µL 4× concentrated NuPAGE® LDS sample buffer, 2µL NuPAGE® sample reducing agent and 15 µL MilliQ water. Then the mixture was centrifuged at 2000 rpm for 1 min and heated at 70 ℃ in a heating block (Labtherm Graphit, Liebisch, Germany) for 10 min. Samples were then loaded onto NuPAGE® Novex® 4–12% Bis–Tris Gels. The BlueRay Prestained Protein Marker 10-180 kDa (Jena, Germany) was applied as a reference. The running buffer was NuPAGE® MES running buffer, 0.5 mL NuPage® antioxidant was added to the running buffer. Electrophoresis was performed using an XCell SureLock™ unit (Invitrogen, Paisley, UK) at constant voltage (120 V). The gels were then Coomassie-stained.

3. Results

3.1. Physical changes during storage

3.1.1. Appearance and stability

The stability of the samples was monitored both visually and with a Turbiscan analysis. During 6 weeks of storage, the blank UHT milk remained liquid and homogeneous, and no sign of destabilization was observed (Fig. 1a). Visible differences in the structure of gels were observed between AprX and plasmin containing UHT milk samples, the gelled samples containing 20 µg/mL AprX and 1.6 µL/mL plasmin are shown as examples (Fig. 1a, 1b).

In AprX containing gelled samples, the gel-like sediment became more solid along with the liquid phase becoming less opaque (Fig 1a). The formed coagulation would remain intact at the bottom when inverting the tube (Fig. 1b), showing that the structure of the gel was compact and firm.

In plasmin containing samples, the destabilization appeared as a floating white soft gel (Fig. 1a). Unlike the firm gel induced by AprX, the plasmin-induced gel was soft and fragile, it would not stay at the bottom when inverting the tube (Fig. 1b), and the flowy gel can be easily dispersed in the samples therefore allowed the subsequent physical and chemical measurements after gelation. In addition, the clarification was more obvious in plasmin-containing gelled samples, as revealed by the lower backscattering intensity by Turbiscan (Fig. 1c).

Gelation could be visually distinguished when the thickness of the sediment was higher than 8 mm in the backscattering profile by Turbiscan, and the onset of gelation was thus determined accordingly (Fig. S1). The samples with the addition of 20 µg/mL AprX and 1.6 µL/mL plasmin both gelled in the fourth week, when stored at room temperature. With increasing enzyme

concentration, and increased storage temperature, the time necessary to observe visible milk gelation shortened.

3.1.2. Size distribution

Along with data from the Turbiscan, the physical stability of protease containing UHT milk samples was also characterised by the size distribution of the casein micelles and the agglomerates that may be generated. The change in size distribution was quicker with higher concentrations and temperatures, but the trend was the same for the individual enzymes. The results of samples with the addition of 20 µg/mL AprX and 1.6 µL/mL plasmin at room temperature are shown as examples (Fig. 2a, 2b).

The skim UHT milks (week 0) without incubation were determined to have a monomodal particles size distribution with an average particle size of around 200 nm, which corresponds to the normal casein micelle size (McMahon & Oommen, 2013) (Fig. 2a, 2b). For the UHT milks containing AprX (Fig. 2a), the peak corresponding to regular casein micelles diminished gradually until disappearance during storage at room temperature. After 1 week of storage, the distribution became bimodal. The size distribution of the second peak ranged from 500 nm to microns, and the size distribution further broadened to larger sizes with increased storage time. Similar trends in size distribution were found in all the one-week-before-gelled AprX containing samples, as shown in Fig. S2a.

The example of changes in size distribution in plasmin containing samples is shown in Fig. 2b. After a slight shift to a larger mean size in week 2, the main peak below 1,000 nm split into two parts in week 3, which is one week before gelation, and a new peak at micron size arose. Soon after this, the size distribution became even wider at gelation, with the peaks of small particles moving to smaller sizes, and at the same time peaks of particles at micron size further developing to larger sizes. The particles larger than 10 µm in size might also exist, but were

outside the detection range of the equipment. Gelation and clarification could be observed along with these changes of size distribution. Similar trends were found in all the other gelled plasmin containing samples; the comparisons of size distribution before and at the onset of visible gelation are shown in Fig. S2b, S2c. After gelation (week 5 and 6 in Fig. 2b), the peaks of small particles below 1,000 nm shifted to smaller size over time and the peak at microns lowered.

3.2. Chemical changes during storage

3.2.1. Changes in degree of hydrolysis

To provide an overall description of the activity of these two proteases on milk proteins, we studied the DH by OPA. Upon incubation, DH increased in the presence of AprX and plasmin. For both proteases, a specific DH was reached at the onset of gelation. When the DH in AprX containing samples increased beyond 1.3%, the samples gelled, irrespective of storage time or temperature (Fig. 3a, 3b). Likewise, in plasmin containing samples, there is also a "limit DH" of 2.1%, again irrespective of storage time or temperature (Fig. 3c, 3d).

3.2.2. Correlation of casein hydrolysis and UHT instability

Fig. 4 illustrates the comparison of protein profiles by RP-HPLC in both the blank UHT milk and the samples showing gelation due to the action of AprX or plasmin. As shown in the chromatograms, the gelled samples in both model systems went through extensive proteolysis, and many breakdown products were generated, as reflected by the increase of various unidentified peaks in the HPLC profiles during incubation. What also stands out in Fig. 4 is that for the respective model system with the addition of the same enzyme, the chromatograms of the gelled samples looked identical independent of temperature and protease concentration. Comparing the hydrolysis patterns between AprX and plasmin, the most apparent differences are in κ-casein, α_{s2}-casein, and differences in peaks 1-5. The two κ-casein peaks almost completely disappeared during the incubation with AprX, whereas these peaks did not decrease

markedly in the samples gelled by plasmin. Besides, plasmin led to more peaks of breakdown products of α_{s2}-casein than AprX. In addition, we observed significant differences in the peak development of α_{s1}-casein with 8 and 9 phosphorylations (8P & 9P, peak 1and 2 in Fig.4) as well as for A^1 and A^2 β-casein (peak 3 and 4 in Fig.4). In plasmin containing samples, peaks of A^1β -casein and α_{s1}-casein 9P disappeared at the onset of gelation, indicating that these had been almost completely hydrolyzed when gelation occurred. AprX, on the other hand, doesn't show such preference towards α_{s1}-casein 8P & 9P, and A^1 & A^2 β-casein. Moreover, a unique peak at a retention time of 31 min was found only in the AprX- hydrolyzed samples, the nature of this peak will be studied in future studies.

To understand how the hydrolysis of certain caseins can quantitatively affect the stability of UHT milk during storage, we determined the changes of the peak areas of β-, α_{s1} and κ-casein in AprX- and plasmin-containing samples during storage at room temperature (Fig. 5). As shown in Fig. 5a-c, AprX hydrolyzes κ-> β-> α_{s1}-casein, gelation occurred after more than 45% of β-casein, 35% of α_{s1}-casein and 95% of κ-casein was hydrolyzed, respectively. While, as shown in Fig. 5d-f, plasmin mainly hydrolyzes β-, α_{s1}-caseins, but hardly κ-casein. The intact β-casein and α_{s1}-casein fraction decreased in an exponential manner during storage. At the onset of gelation, more than 60% of both β-and α_{s1}-casein were hydrolyzed. The protein degradation at the optimal temperature of the enzymes was similar with that at room temperature (Fig. S3).

Hydrolysis of caseins was also analysed by SDS-PAGE (Fig. S4). The band intensities of β- and κ-casein diminished faster than the band of α_s–casein, indicating κ-casein and β-casein were more rapidly hydrolyzed. These results are in line with our RP-HPLC results. At the same time, many new bands at lower molecular weight appeared, as can be expected during proteolysis.

4. Discussion

This study compared the destabilization of skim UHT milk by AprX and plasmin during 6-weeks storage at two temperatures. The findings showed that different specificities towards cleaving the caseins of AprX and plasmin on milk proteins are the main cause of different gelation behaviours during the storage of UHT milk.

4.1. Proteolysis and gelation

4.1.1. AprX-induced gelation

AprX can not only cleave the peptide bond Phe_{105}-Met_{106} of κ-casein like chymosin (Recio, García-risco, Ramos & López-fandiño, 2000), but also non-specifically cleave around the region of soluble hydrophilic glycomacropeptide that is normally present as the "hairy layer" (Gaucher et al., 2011). As a result, the cleavage of the hydrophilic tails on the surface of the micelles reduces both the steric and electrostatic repulsion, which may promote the formation of a gel network (Matéos et al., 2015). Additionally, the ability of hydrolyzing β- and α_s-casein, even though to a lower extent than plasmin (Fig. 4, 5, S3, S4), may also help destabilize the internal part of casein micelles, further stimulating gelation.

Comparing the changes in size distribution in AprX- and plasmin-containing UHT milk samples, we can see that, different from plasmin, under the action of AprX the size distribution immediately started to shift to larger sizes (Fig. 2a, S2a). This is probably because AprX can easily access and breakdown κ-casein. Once a sufficient amount of κ-casein has been hydrolyzed, the collisions between the particles will lead to adhesion and the formation of casein aggregates (Sandra, Alexander & Dalgleish, 2007). The level of hydrolysis of κ-casein at the onset of gelation is found to be more than 95% in our study (Fig. 5c, S3c, S4a), which is similar to cheese making, where extensive (80–90%) hydrolysis of κ-casein needs to occur before visible coagulation (Sandra et al., 2007). The AprX-induced gels could not be physically

resuspended in the samples, which made analysis of these samples difficult. Further studies on the gel itself may aid in further understanding the mechanism of AprX-induced gelation.

4.1.2. Plasmin-induced gelation

Plasmin-induced gelation in UHT milk is more complicated compared with the AprX-induced gelation. According to our results, β-casein is most susceptible to plasmin action, followed by α_{s1}-casein and α_{s2}-casein (Fig. 4). Although κ-casein contains several lysine and arginine residues, it appears to be resistant to plasmin as shown before (Rauh, Johansen, Ipsen, Paulsson, Larsen & Hammershoj, 2014a; Fox, Uniacke-Lowe, McSweeney & O'Mahony, 2015). The final effect of plasmin depends on both the preference towards specific caseins as well as the dynamics of proteolysis. Combining our results with previous studies, the process of plasmin-induced gelation can be inferred as the following steps:

1) Penetrating phase: in our study, this phase corresponded to the first week for samples with 1.6 μL plasmin/mL at room temperature. During this phase, the size distribution hardly changed (Fig. 2b), but the β- and α_s-caseins were rapidly hydrolyzed (Fig. 5d, 5e), along with an increase in DH (Fig. 3c). Plasmin will first hydrolyze the easily accessible β- and α_s-caseins, after which plasmin needs some time to penetrate the casein micelles for further hydrolyzing β- and α_s-caseins.
2) Loosening phase: this phase is characterised by a slight increase in the mean particle size (week 2 in Fig. 2b), due to loosening of the micellar structure (Rauh et al., 2014a). Rauh et al. (2014a) found that plasmin could hydrolyze around the regions that are essential for the internal integrity and stabilization of the casein micelle, thereby weakening the hydrophobic interactions between caseins, the interactions of caseins with calcium phosphate, and the ionic or salt interactions between caseins.

3) Disassembling, rearranging and aggregation phase: in this phase, sample becomes polydisperse; some particles increased in size, whereas others dropped, and at the same time a new peak at micron size appeared (week 3-4 in Fig. 2b). The larger particles might be underestimated due to their settling in the sample cell during DLS measurement. The increasing size corresponds to further loosening of the casein micelles, while the decrease is due to hydrolysis causing the interactions to be insufficient to maintain an intact micellar arrangement. During the disassembly, some generated amphiphilic and charged polypeptides tend to rearrange and aggregate into a gel network (Rauh et al., 2014a). Visible gelation can be observed when enough aggregates are formed.

4) Clarification phase: in this period, the peaks of aggregates decreased (week 5-6 in Fig. 2b) and the particles smaller than 1,000 nm further reduced to smaller sizes. Both the gel and the caseins in micellar structures will be further hydrolyzed by plasmin until a translucent peptide solution is obtained.

Upon gelation, A^1 β-casein and α_{s1}-casein 9P had been almost completely hydrolyzed by plasmin (Fig. 5c). Our finding is in accordance with Rauh et al. (2014b), who showed that more than 95% of A^1 β-casein and α_{s1}-casein 9P had been hydrolyzed upon gelation. Besides, Kelly et al. (1997), Kohlmann et al. (1991) and Newstead et al. (2006) also observed that β-casein was almost completely hydrolyzed when gelation happened, based on gel electrophoresis. Likewise, we also found that all the bands of β- and α_s-caseins disappeared upon gelation (Fig. S4b, S4c).

The structure of the plasmin-induced gel was soft and fragile. Similar physical properties of plasmin induced gels have been reported by Kohlmann, Nielsen & Ladisch (1988), Kelly et al. (1997), Newstead et al. (2006) and Rauh et al. (2014b), but different from the re-dissolving of gel and the final translucent peptide solution found in our study.

356 4.1.3. Relationships between DH and the onset of gelation

357 Regarding the critical level of protein breakdown at which gelation occurs, we found the DH
358 for AprX- and plasmin-containing samples to be ~1.3% and ~2.1%, respectively, irrespective
359 of enzyme concentration or storage temperature. This means that there is a direct correlation
360 between the time of onset of gelation and the level of proteolysis expressed as DH. A strong
361 correlation between the extent of proteolysis and gelation time was also found by Keogh &
362 Pettingill (1984), Newstead et al. (2006) and Rauh et al. (2014b), i.e. the onset of sedimentation
363 and gelation occurred earlier in milk with greater enzyme activity. However, Kohlmann,
364 Nielsen & Ladisch (1988), Manji et al. (1988) and Auldist et al. (1996) reported that the extent
365 of proteolysis in UHT milk, especially long-term stored UHT milk, was not always related to
366 the onset of gelation. In addition to the differences in protein matrix resulting from different
367 processing conditions, these inconsistent findings may be the result of different dominating
368 reactions in each study, i.e. the enzymatic proteolysis or the non-enzymatic physicochemical
369 reactions. Glycation (or Maillard reaction) is the most important non-enzymatic reaction that
370 can retard gelation. On the one hand, the crosslinking between proteins and reducing sugars can
371 prevent the release of gel-forming peptides (McMahon, 1996); on the other hand, the enzymatic
372 cleavage involving lysine residues is inhibited by the casein–lactose interactions. Therefore,
373 glycation can influence the enzyme-induced gelation in UHT milk during long storage.

374 In the cases with high enzyme activities, like ours, and the study of Rauh et al. (2014a), gelation
375 was observed within a relatively short period. During this period, the enzymatic hydrolysis is
376 the leading reaction, and the slow non-enzymatic physicochemical changes would be
377 insignificant; no obvious browning within 6 weeks indicated there was no advanced Maillard
378 reaction (Fig. 1). In such a situation, the level of enzymatic hydrolysis is crucial for the critical
379 point of gelation. In long-term stored UHT milk with low proteolytic activity, on the other hand,

the non-enzymatic physicochemical changes, which are mainly influenced by the storage temperature (Manji et al., 1988), would become more important.

4.1.4. Comparison between AprX- and plasmin-induced gelation

The DH for the control samples also increased slightly with storage, although less than those with added AprX or plasmin (Fig. 3), indicating that the blank samples presumably contained a low level of indigenous plasmin activity that survived the direct UHT heating. The residual enzymatic activity should not be from AprX because no κ-casein cleavage is seen in the control sample (Fig. 5f, S3f). Comparing the destabilization of UHT milk by AprX and plasmin, a lower critical DH (~1.3%) was found in AprX containing samples than plasmin containing samples (~2.1%) (Fig. 3). With AprX, the combination of hydrolyzing κ-casein together with β-casein can dissociate the hairy layer and allow AprX to penetrate micelles, thereby quickly destabilizing the micelles leading to a compact gel. By contrast, β- and α_s-casein are less responsible for the micellar stability than κ-casein. Therefore, a higher DH is required for plasmin to induce gelation by hydrolyzing β- and α_s-caseins, and the breakdown of many interaction sites prevent the formation of a strong gel.

4.2. Identification of the enzyme causing age gelation

To identify the responsible enzyme for age gelation, some approaches can be proposed based on the above results. The action of AprX is indicated by:

1) A compact and strong gel that cannot be physically resuspended;
2) Only increase, but no decrease in particle size distribution compared to normal milk;
3) A fast specific hydrolysis of κ-casein detected by chromatographic or electrophoretic approaches;

On the other hand, the action of the native milk protease, plasmin, is indicated by:

1) A soft gel that can be physically resuspended, or a translucent appearance;
2) Both increase and decrease in particle size distribution compared to normal milk;
3) The selective hydrolysis of $A^1\beta$ -casein and α_{s1}-casein 9P, as detected by HPLC.

The work of Datta & Deeth (2003) on diagnosing the cause of proteolysis in UHT milk showed the non-specific cleavage of caseins by AprX can produce smaller peptides, that are soluble in 12% TCA, compared to the lysine/ arginine specific protease plasmin (Datta & Deeth, 2003).

In addition to identification of the responsible enzyme, the stability of UHT milk can be predicted by measuring the enzyme activity after heat treatment. Previous research recommended limits for protease activity (Adams, Barach & Speck, 1975; Ewings, O'Connor & Mitchell, 1984) in differently defined units, or in enzyme amount in weight (Mitchell & Ewings, 1985). But differences in assay techniques make comparisons inaccurate, and it is difficult to detect low amounts of enzymes in milk using these approaches. Therefore, developing an easy-to-use immunological assay that uses a combination of antibody affinity ELISA plates and spectrophotometric quantification of the enzyme activity may be a more suitable indicator for the stability of UHT milk. The shelf life can also be adjusted depending on the enzyme activity and environmental conditions, where shorter shelf life should be set for the products with higher enzyme activity and products stored at increased ambient temperature.

5. Conclusion

This study has shown that different forms of destabilization in skim UHT milk were caused by the protease AprX from *Pseudomonas fluorescens* and plasmin during storage. Higher concentrations of proteases and higher storage temperature lead to faster gelation, although the critical degrees of hydrolysis of specific caseins determines the onset of gelation, irrespective of protease concentration or storage temperature. The strong AprX-induced gelation in UHT

milk is mainly caused by the hydrolysis of κ-casein, whereas the soft plasmin-induced gelation in UHT milk is mainly caused by the hydrolysis of β- and α-casein; AprX can thereby induce gelation at lower DH than plasmin.

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Figure Captions

Figure 1. Images of the blank sample, the gelled samples containing 20 μg/mL AprX and 1.6 μL/mL plasmin (a); the image of the inverted tubes of gelled samples containing 20 μg/mL AprX and 1.6 μL/mL plasmin (b); the corresponding backscattering intensity profiles of samples in (a), the horizontal axis represents the position along the tube (c).

Figure 2. Intensity based particle size distribution of samples containing 20 μg/mL AprX stored from 0 to 3 weeks at room temperature (a); and samples containing 1.6 μL/mL plasmin from 0 to 6 weeks at room temperature (b).

Figure 3. Degree of hydrolysis values of skim UHT milk hydrolyzed by 0, 10, 20, 50 μg/mL AprX at room temperature (a) and 42 °C (b); and by 0, 0.8, 1.6, 2.4 μL/mL plasmin at room temperature (c) and 37 °C (d). The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH values. Error bars represent the standard deviations.

Figure 4. The comparison of RP-HPLC chromatograms between the AprX-induced gelation samples and plasmin-induced gelation samples. Peak 1, 2 stands for α_{s1}-casein with 8 or 9 phosphorylations (8P & 9P), respectively; peak 3, 4 stands for β-casein A1 & A2, respectively; peak 5 stands for the unique peak in AprX-hydrolyzed samples.

Figure 5. Hydrolysis of β-casein (a), α_{s1}-casein (b) and κ-casein (c) in samples containing 0, 10, 20, 50 μg/mL AprX during storage at room temperature; and in samples containing 0, 0.8, 1.6, 2.4 μL/mL plasmin at room temperature (d, e, f), expressed as the relative change in peak

area of UV absorption at 214 nm. The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH relative peak area values. Error bars represent the standard deviations.

Supplementary materials

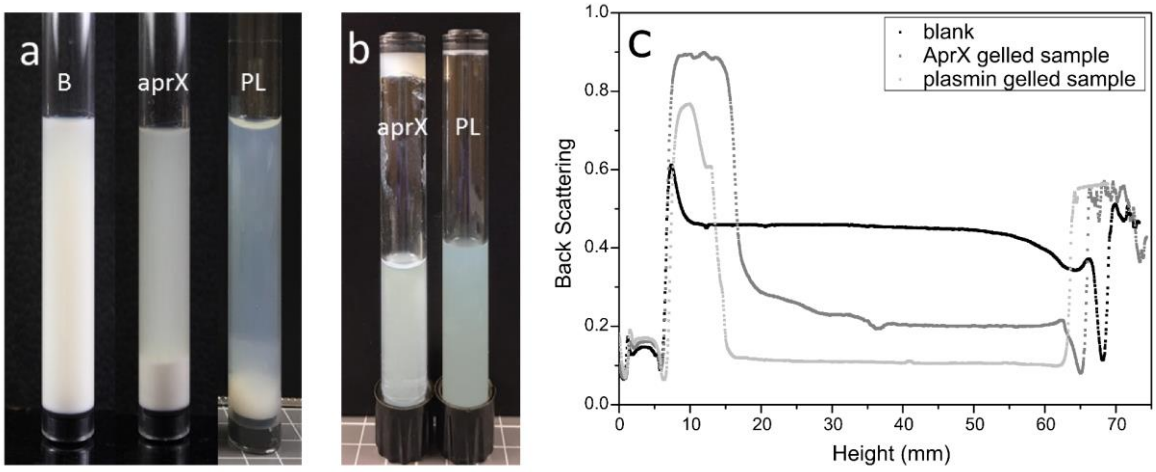
Figure S1. The correlation between the AprX (a) and plasmin (b) concentration in UHT milk and the gelation time.

Figure S2. Intensity based particle size distribution of samples containing 50 µg/mL AprX stored at room temperature and samples containing 20 µg/mL AprX stored at 42 °C before gelation (a); and samples containing 0, 0.8, 1.6, 2.4 µL /mL plasmin in 1 week before gelation and at the onset of gelation at room temperature (b) and 37 °C (c).

Figure S3. Hydrolysis of β-casein (a), α_{s1}-casein (b) and κ-casein (c) in samples containing 0, 10, 20, 50 µg/mL AprX during storage at 42 °C; and in samples containing 0, 0.8, 1.6, 2.4 µL/mL plasmin at 37 °C (d, e, f), expressed as the relative change in peak area of UV absorption at 214 nm. The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH relative peak area values. Error bars represent the standard deviations.

Figure S4. SDS-PAGE gels of UHT milk hydrolyzed by AprX at room temperature (a); by plasmin at room temperature (b) and 37 °C (c). α_s-casein: α_{s1}+ α_{s2} casein; Std: molecular mass standards; blank: skim UHT milk without addition of protease; W: storage time (week); AprX 10, 20, 50: UHT milk containing 0, 10, 20, 50 µg/mL AprX; plasmin 0.8, 1.6, 2.4: UHT milk containing 0, 0.8, 1.6, 2.4 µL/mL plasmin. The arrows indicate the gelled samples.

567 Figure 1



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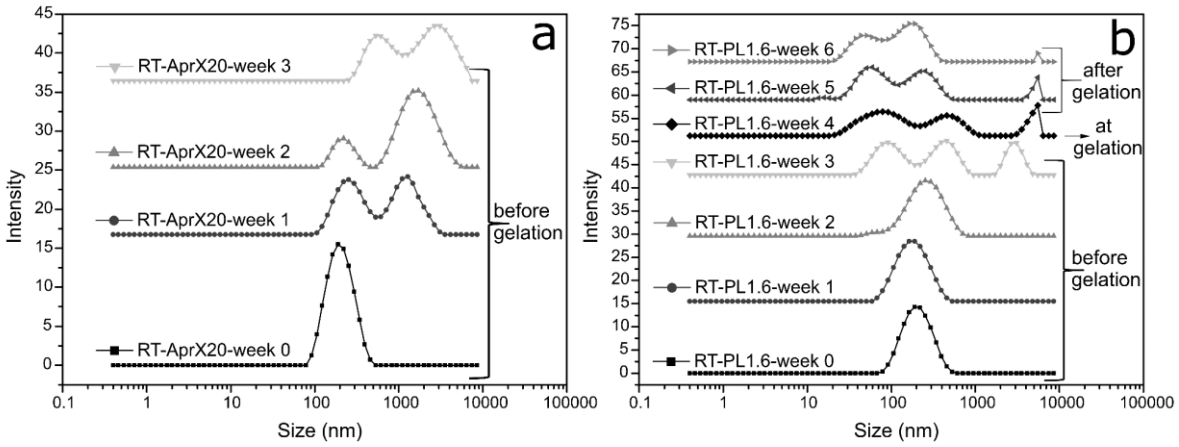
569 Figure 2



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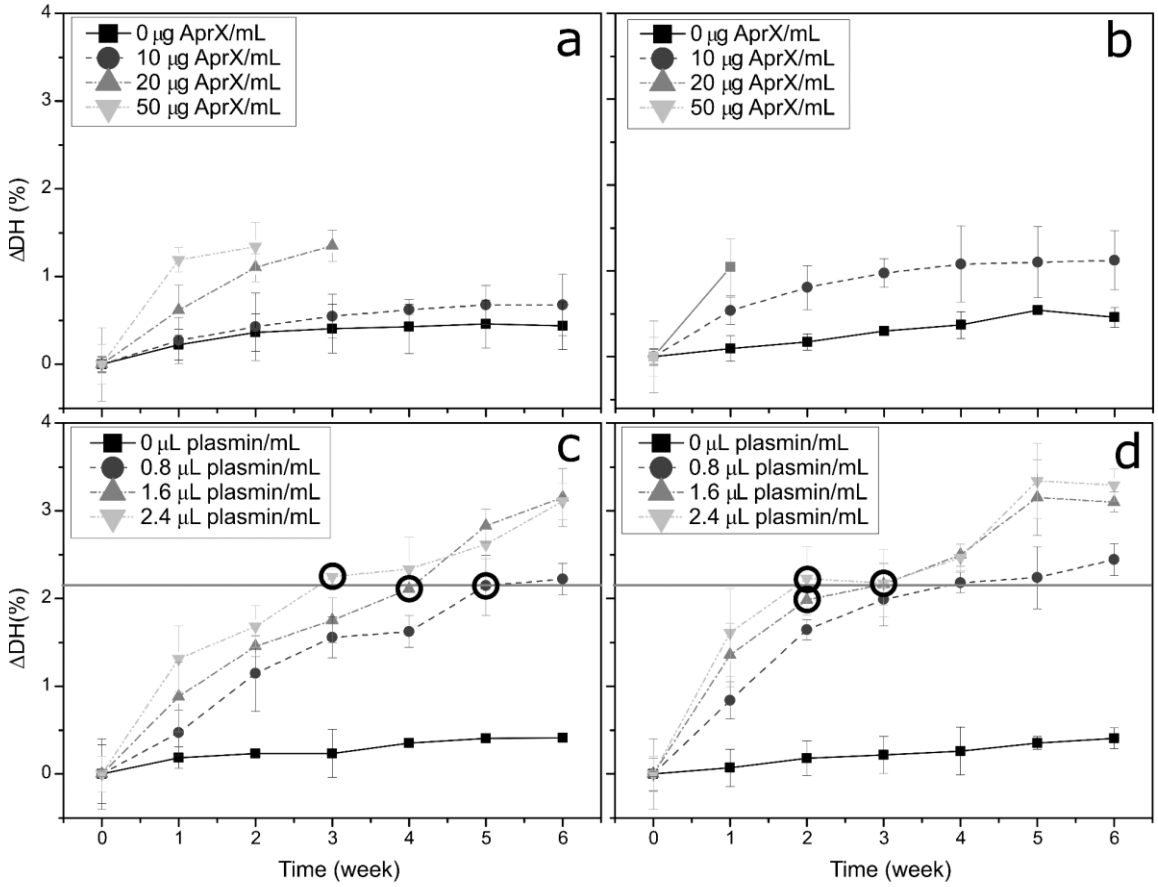

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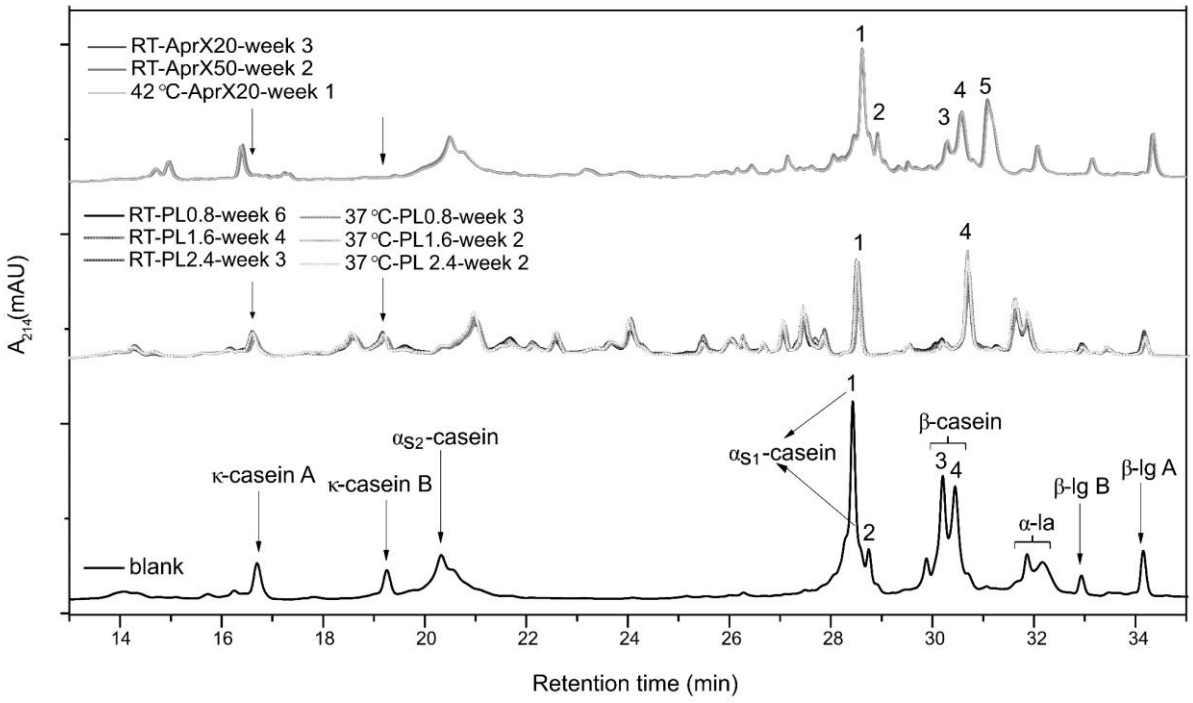

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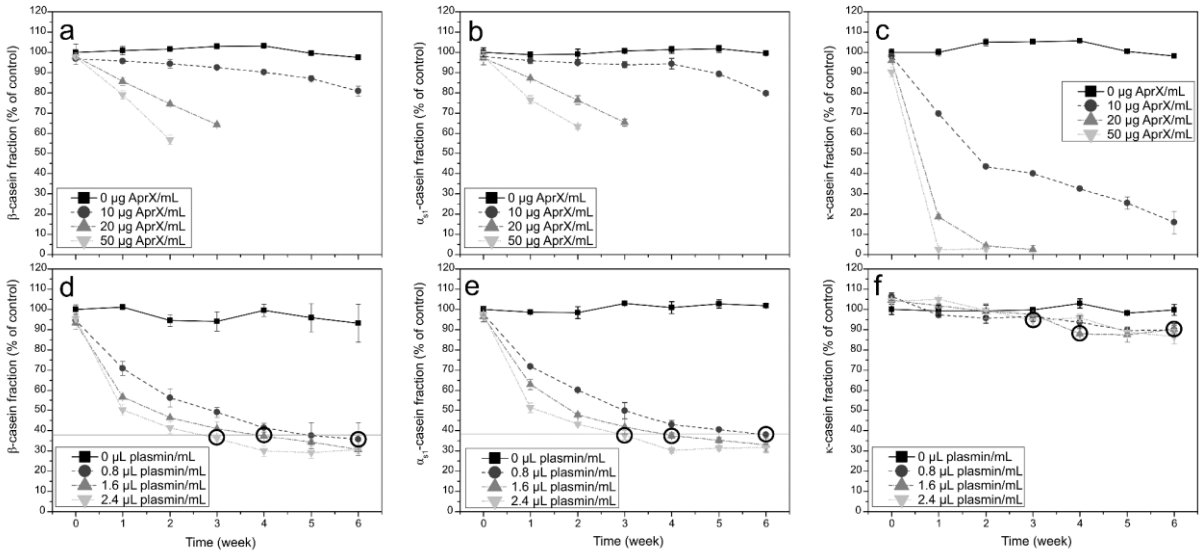

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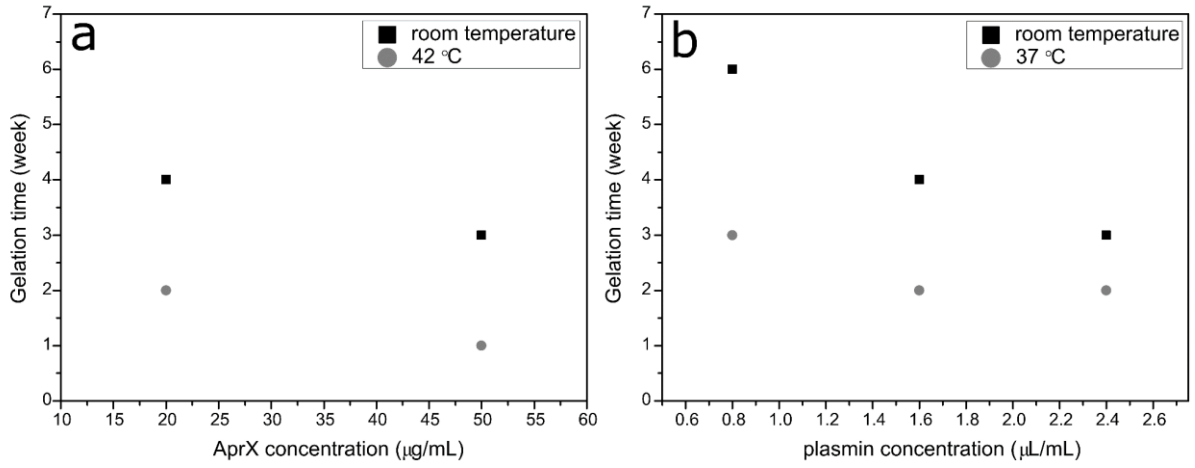
577 Figure S1



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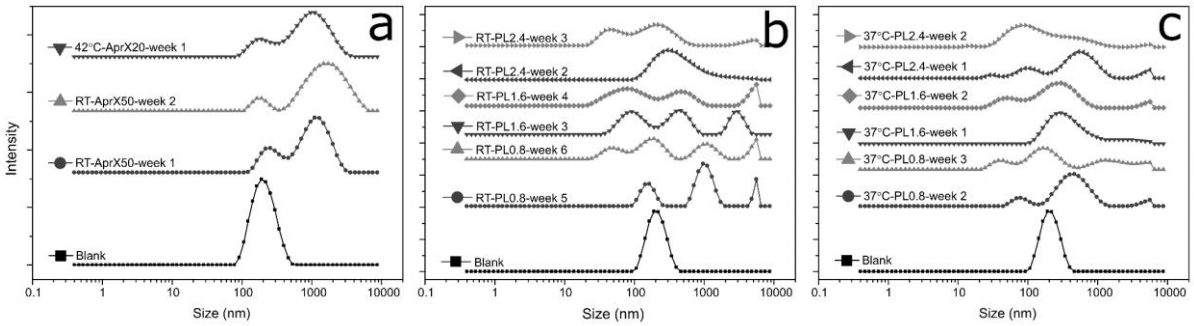

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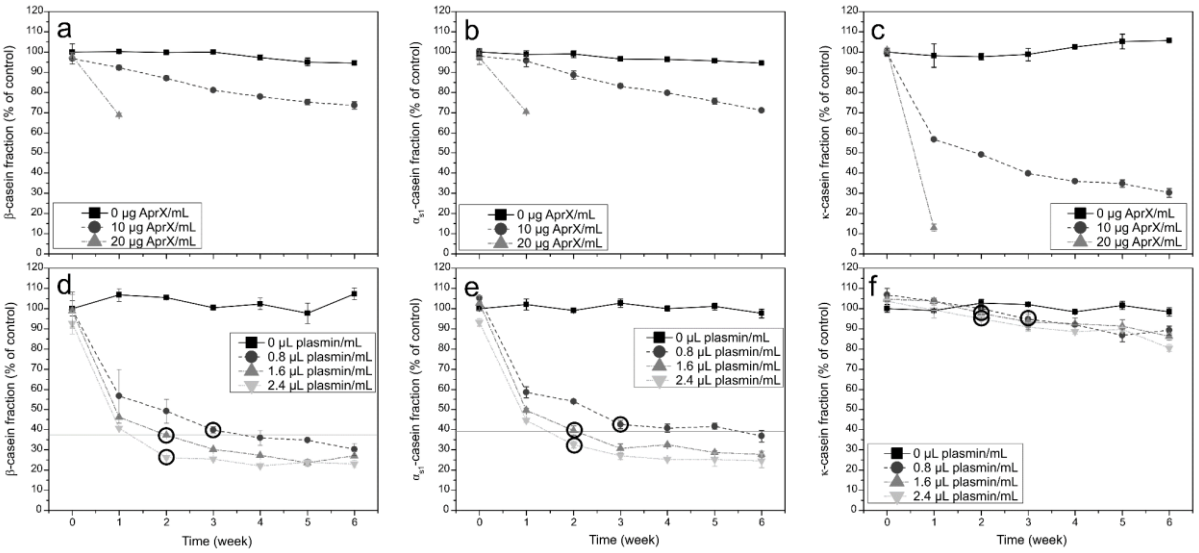

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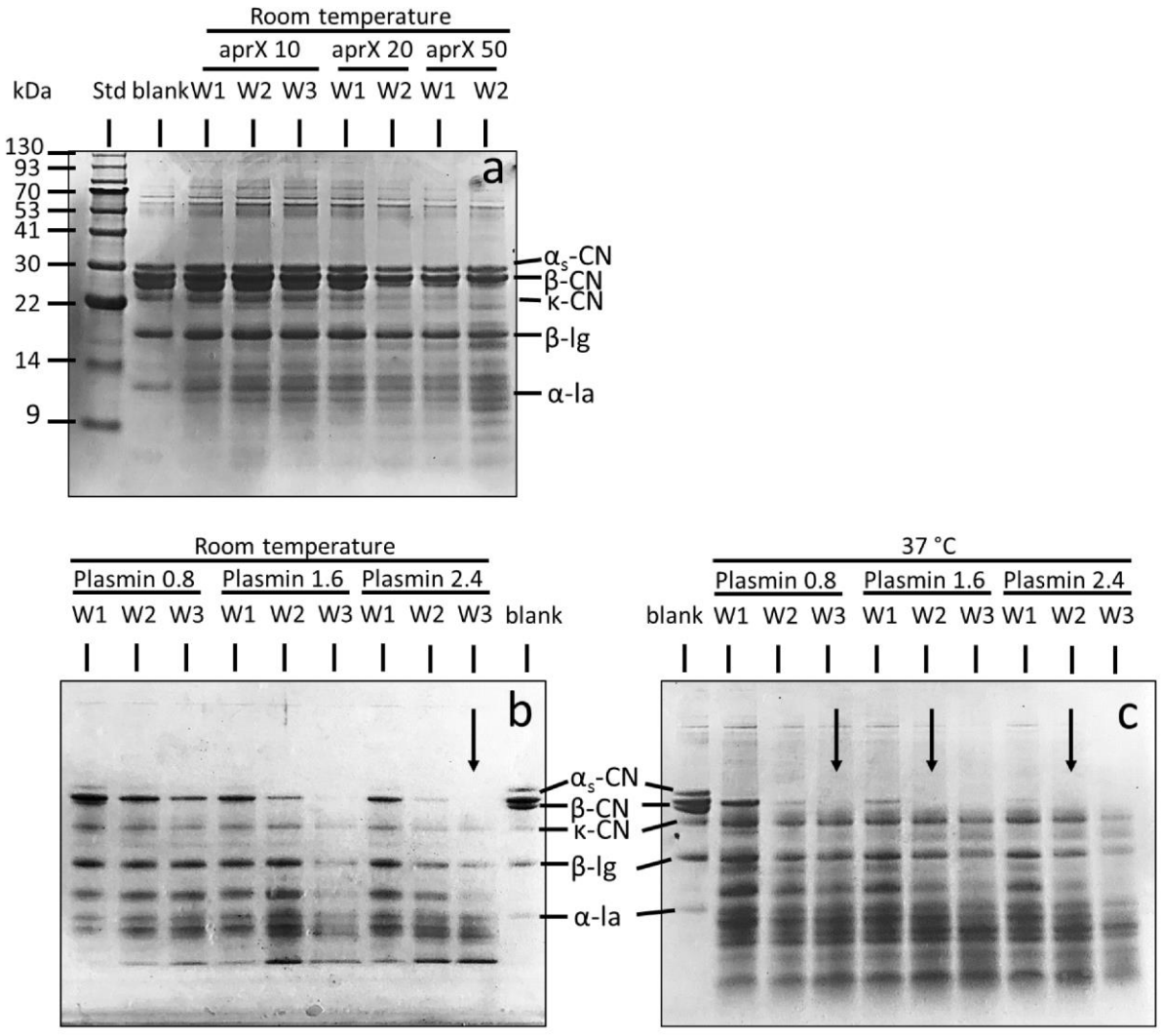

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