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and plasmin

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1 Title :

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

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16 **Abstract:**

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

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

29

30 **1. Introduction:**

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

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

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

50 In addition to the well-known detrimental effects of plasmin, enzymes originating from
51 psychrotrophic bacteria can also be a serious problem in UHT milk because these bacteria are
52 inevitable in raw milk and some of them can produce heat-resistant proteases and lipases during
53 cold storage, that can withstand the UHT process. Among all the psychrotrophic bacteria,

54 *Pseudomonas* species are particularly incriminated in the destabilisation of UHT milk
55 (Vithanage, Yeager, Jadhav, Palombo & Datta, 2014). A single specific extracellular alkaline
56 metallo-protease belonging to the AprX enzyme family has been discovered in genus
57 *Pseudomonas*, which is responsible for milk spoilage (Vithanage et al., 2014; Matéos et al.,
58 2015). The heat-stable proteases have been reported to be produced by pseudomonads during
59 the late exponential/early stationary growth phase of the bacteria, generally at bacterial cell
60 counts of 10^7 - 10^8 cfu/mL (Stoeckel, Lidolt, Stressler, Fischer, Wenning & Hinrichs, 2016). This
61 means that the production of AprX is determined by the storage time, temperature, and the
62 count of pseudomonads. Therefore, the AprX level indirectly reflects the hygienic management
63 of the farm and the storage history of the milk.

64 Cow health, hygiene management and storage history of milk are all crucial links in the dairy
65 chain that can influence the stability of UHT milk. To better trace back which links needs to be
66 improved to prevent instability, we need knowledge of the responsible enzymes, especially with
67 regard to the differences between them in their proteolytic activities on milk proteins, and the
68 changes they induce in milk. However, to date, such systematic understanding of these enzymes
69 systems is still lacking.

70 The mechanisms for age gelation of UHT milk have been mainly described by two theories
71 (Kocak & Zadow, 1985; McMahon, 1996; Datta et al., 2001). The first involves the enzymatic
72 degradation of the milk proteins, and the promotion of age gelation by the formation of peptides.
73 The second mechanism is often referred to as “non-enzymatic” or “physicochemical” age
74 gelation in which no protein degradation is observed (McMahon, 1996; Anema, 2017). Age
75 gelation through the physicochemical mechanism is slow for unconcentrated milk samples, in
76 which it usually takes longer than 12 months (Anema, 2017). Therefore, the focus of this study
77 is the enzyme-induced destabilization. To reduce the interference from the non-enzymatic

78 physicochemical changes, visible destabilization was induced within a relatively short time by
79 the addition of high concentrations of enzymes.

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

86 **2. Materials and Methods:**

87 2.1. Enzymes

88 The AprX-producing bacterial strain *Pseudomonas fluorescens* Migula 1895 (DSM 50120) was
89 obtained from Deutsche Sammlung von Mikroorganismen (DSM). This strain has been
90 reported to be able to grow at 4-37°C and have proteolytic, lipolytic and pectinolytic activities.
91 The AprX sequence was found in the gene with an 15 bp insertion between bp position 395 and
92 410 on the sequence, proving the dairy origin of this isolate (Caldera et al., 2015). For
93 cultivation, the strain was cultured in a nutrient broth (VWR International B.V.) to the end of
94 its log phase (around 26 h) at 25 °C. The bacteria were then harvested by centrifugation (4,000
95 g, 10 min, 20 °C). To purify the extracellular enzymes, we inoculated the bacteria in minimal
96 medium (7 g L⁻¹ K₂HPO₄, 2 g L⁻¹ KH₂PO₄, 0.2 g L⁻¹ MgSO₄·7H₂O, 1 g L⁻¹ (NH₄)₂SO₄, 4 g L⁻¹
97 glycerol, pH 7.0) containing 2% (v/v) UHT milk as protease inducer for 24 h at 25 °C with
98 stirring at 160 rpm (Matéos et al., 2015). After 24 h of culturing, cells were removed by
99 centrifugation at 10,800 g for 30 min at 4 °C (rotor JA 16.250, Avanti Centrifuge J-26 XP,
100 Beckman Coulter, USA). The supernatant was first concentrated by centrifuging at 4,000 g for

101 20 min at 4 °C in Amicon Ultra filters (10 kDa cut-off, Millipore), after which the concentrate
102 obtained was dialysed against sterile 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for
103 48 h (3.5 K, Thermo Scientific Slide-A-Lyzer™ Dialysis Cassette) and lyophilised. No further
104 purification of the extracellular bacterial enzymes were performed, because the crude AprX
105 extract is more similar to the real situation in milk, which facilitates our simulation of the real
106 destabilization caused by pseudomonads.

107 Plasmin from bovine plasma was purchased from Roche, 5 units of plasmin were suspended in
108 3.2 M ammonium sulfate solution, where the unit is defined at 25 °C with Chromozym PL as
109 the substrate.

110 2.2. Milk sample preparation

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

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

149 absorbance of the sample at 340 nm. To calculate the amount of free NH₂ groups, a calibration
150 curve was made using leucine as a reference compound, the number of free NH₂ groups per
151 gram samples was expressed as h. The total number of peptide bonds per gram of protein
152 substrate was obtained by complete hydrolysis of the blank skim UHT sample in 6M HCl, 110°C
153 for 24h; h_{tot} was 8.68 mmol/g in this case. The detected DH value of the blank UHT milk was
154 7.0%, this value originated from native lysines and the N-termini of the milk proteins, as well
155 as the hydrolysis that had happened in the milk prior to our experiment. This value was
156 considered as the “blank” DH in milk and was subtracted from all the other data. The additional
157 amino groups from the added AprX and plasmin were also subtracted accordingly, but the
158 number of peptide bonds originating from the autolysis of proteases were neglected because the
159 level of autolysis differs in a system without caseins as substrates, and can thus not be properly
160 assessed. In this way, the DH discussed here refers to the hydrolysis caused merely by the added
161 proteases, and was calculated as $DH = (h/h_{tot} * 100\%) - 7.0\%$. The OPA reagent in the presence
162 of SDS could sufficiently dissolve plasmin-induced gelled samples, but not the AprX-induced
163 gelled samples. Therefore the AprX-containing samples were only analysed until 1 week before
164 gelation happened.

165 2.6. Protein Profile Analysis

166 2.6.1. RP-HPLC

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

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

180 2.6.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

181 SDS-PAGE was performed to complement RP-HPLC results. The sample buffer, reducing
182 agent, gels, running buffer and antioxidant agent were all purchased from Invitrogen (Carlsbad,
183 USA). Two μ L sample was diluted in 5 μ L 4 \times concentrated NuPAGE[®] LDS sample buffer,
184 2 μ L NuPAGE[®] sample reducing agent and 15 μ L MilliQ water. Then the mixture was
185 centrifuged at 2000 rpm for 1 min and heated at 70 °C in a heating block (Labtherm Graphit,
186 Liebisch, Germany) for 10 min. Samples were then loaded onto NuPAGE[®] Novex[®] 4–12%
187 Bis–Tris Gels. The BlueRay Prestained Protein Marker 10-180 kDa (Jena, Germany) was
188 applied as a reference. The running buffer was NuPAGE[®] MES running buffer, 0.5 mL
189 NuPage[®] antioxidant was added to the running buffer. Electrophoresis was performed using an
190 XCell SureLock[™] unit (Invitrogen, Paisley, UK) at constant voltage (120 V). The gels were
191 then Coomassie-stained.

192 3. Results

193 3.1. Physical changes during storage

194 3.1.1. Appearance and stability

195 The stability of the samples was monitored both visually and with a Turbiscan analysis. During
196 6 weeks of storage, the blank UHT milk remained liquid and homogeneous, and no sign of
197 destabilization was observed (Fig. 1a). Visible differences in the structure of gels were observed
198 between AprX and plasmin containing UHT milk samples, the gelled samples containing 20
199 $\mu\text{g}/\text{mL}$ AprX and 1.6 $\mu\text{L}/\text{mL}$ plasmin are shown as examples (Fig. 1a, 1b).

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

204 In plasmin containing samples, the destabilization appeared as a floating white soft gel (Fig.
205 1a). Unlike the firm gel induced by AprX, the plasmin-induced gel was soft and fragile, it would
206 not stay at the bottom when inverting the tube (Fig. 1b), and the flowy gel can be easily
207 dispersed in the samples therefore allowed the subsequent physical and chemical measurements
208 after gelation. In addition, the clarification was more obvious in plasmin-containing gelled
209 samples, as revealed by the lower backscattering intensity by Turbiscan (Fig. 1c).

210 Gelation could be visually distinguished when the thickness of the sediment was higher than 8
211 mm in the backscattering profile by Turbiscan, and the onset of gelation was thus determined
212 accordingly (Fig. S1). The samples with the addition of 20 $\mu\text{g}/\text{mL}$ AprX and 1.6 $\mu\text{L}/\text{mL}$ plasmin
213 both gelled in the fourth week, when stored at room temperature. With increasing enzyme

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

216 3.1.2. Size distribution

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

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

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

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

243 3.2. Chemical changes during storage

244 3.2.1. Changes in degree of hydrolysis

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

251 3.2.2. Correlation of casein hydrolysis and UHT instability

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

262 markedly in the samples gelled by plasmin. Besides, plasmin led to more peaks of breakdown
263 products of α_{s2} -casein than AprX. In addition, we observed significant differences in the peak
264 development of α_{s1} -casein with 8 and 9 phosphorylations (8P & 9P, peak 1 and 2 in Fig.4) as
265 well as for A¹ and A² β -casein (peak 3 and 4 in Fig.4). In plasmin containing samples, peaks of
266 A¹ β -casein and α_{s1} -casein 9P disappeared at the onset of gelation, indicating that these had
267 been almost completely hydrolyzed when gelation occurred. AprX, on the other hand, doesn't
268 show such preference towards α_{s1} -casein 8P & 9P, and A¹ & A² β -casein. Moreover, a unique
269 peak at a retention time of 31 min was found only in the AprX- hydrolyzed samples, the nature
270 of this peak will be studied in future studies.

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

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

284 4. Discussion

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

289 4.1. Proteolysis and gelation

290 4.1.1. AprX-induced gelation

291 AprX can not only cleave the peptide bond Phe₁₀₅-Met₁₀₆ of κ -casein like chymosin (Recio,
292 García-risco, Ramos & López-fandiño, 2000), but also non-specifically cleave around the
293 region of soluble hydrophilic glycomacropeptide that is normally present as the “hairy layer”
294 (Gaucher et al., 2011). As a result, the cleavage of the hydrophilic tails on the surface of the
295 micelles reduces both the steric and electrostatic repulsion, which may promote the formation
296 of a gel network (Matéos et al., 2015). Additionally, the ability of hydrolyzing β - and α_s -casein,
297 even though to a lower extent than plasmin (Fig. 4, 5, S3, S4), may also help destabilize the
298 internal part of casein micelles, further stimulating gelation.

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

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

310 4.1.2. Plasmin-induced gelation

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

- 319 1) Penetrating phase: in our study, this phase corresponded to the first week for samples
320 with 1.6 μ L plasmin/mL at room temperature. During this phase, the size distribution
321 hardly changed (Fig. 2b), but the β - and α_s -caseins were rapidly hydrolyzed (Fig. 5d,
322 5e), along with an increase in DH (Fig. 3c). Plasmin will first hydrolyze the easily
323 accessible β - and α_s -caseins, after which plasmin needs some time to penetrate the casein
324 micelles for further hydrolyzing β - and α_s -caseins.
- 325 2) Loosening phase: this phase is characterised by a slight increase in the mean particle
326 size (week 2 in Fig. 2b), due to loosening of the micellar structure (Rauh et al., 2014a).
327 Rauh et al. (2014a) found that plasmin could hydrolyze around the regions that are
328 essential for the internal integrity and stabilization of the casein micelle, thereby
329 weakening the hydrophobic interactions between caseins, the interactions of caseins
330 with calcium phosphate, and the ionic or salt interactions between caseins.

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

341 4) Clarification phase: in this period, the peaks of aggregates decreased (week 5-6 in [Fig.](#)
342 [2b](#)) and the particles smaller than 1,000 nm further reduced to smaller sizes. Both the
343 gel and the caseins in micellar structures will be further hydrolyzed by plasmin until a
344 translucent peptide solution is obtained.

345 Upon gelation, A¹ β -casein and α_{s1} -casein 9P had been almost completely hydrolyzed by
346 plasmin ([Fig. 5c](#)). Our finding is in accordance with [Rauh et al. \(2014b\)](#), who showed that more
347 than 95% of A¹ β -casein and α_{s1} -casein 9P had been hydrolyzed upon gelation. Besides, [Kelly](#)
348 [et al. \(1997\)](#), [Kohlmann et al. \(1991\)](#) and [Newstead et al. \(2006\)](#) also observed that β -casein
349 was almost completely hydrolyzed when gelation happened, based on gel electrophoresis.
350 Likewise, we also found that all the bands of β - and α_s -caseins disappeared upon gelation ([Fig.](#)
351 [S4b, S4c](#)).

352 The structure of the plasmin-induced gel was soft and fragile. Similar physical properties of
353 plasmin induced gels have been reported by [Kohlmann, Nielsen & Ladisch \(1988\)](#), [Kelly et al.](#)
354 [\(1997\)](#), [Newstead et al. \(2006\)](#) and [Rauh et al. \(2014b\)](#), but different from the re-dissolving of
355 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,

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

382 4.1.4. Comparison between AprX- and plasmin-induced gelation

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

395 4.2. Identification of the enzyme causing age gelation

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

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

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

- 403 1) A soft gel that can be physically resuspended, or a translucent appearance;
- 404 2) Both increase and decrease in particle size distribution compared to normal milk;
- 405 3) The selective hydrolysis of A¹ β -casein and α_{s1} -casein 9P, as detected by HPLC.

406 The work of Datta & Deeth (2003) on diagnosing the cause of proteolysis in UHT milk showed
407 the non-specific cleavage of caseins by AprX can produce smaller peptides, that are soluble in
408 12% TCA, compared to the lysine/ arginine specific protease plasmin (Datta & Deeth, 2003).

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

420 5. Conclusion

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

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

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433 assistance in performing the experiments. The authors report no conflict of interest in this paper.

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525 **Figure Captions**

526 Figure 1. Images of the blank sample, the gelled samples containing 20 $\mu\text{g}/\text{mL}$ AprX and 1.6
527 $\mu\text{L}/\text{mL}$ plasmin (a); the image of the inverted tubes of gelled samples containing 20 $\mu\text{g}/\text{mL}$
528 AprX and 1.6 $\mu\text{L}/\text{mL}$ plasmin (b); the corresponding backscattering intensity profiles of
529 samples in (a), the horizontal axis represents the position along the tube (c).

530 Figure 2. Intensity based particle size distribution of samples containing 20 $\mu\text{g}/\text{mL}$ AprX stored
531 from 0 to 3 weeks at room temperature (a); and samples containing 1.6 $\mu\text{L}/\text{mL}$ plasmin from 0
532 to 6 weeks at room temperature (b).

533 Figure 3. Degree of hydrolysis values of skim UHT milk hydrolyzed by 0, 10, 20, 50 $\mu\text{g}/\text{mL}$
534 AprX at room temperature (a) and 42 $^{\circ}\text{C}$ (b); and by 0, 0.8, 1.6, 2.4 $\mu\text{L}/\text{mL}$ plasmin at room
535 temperature (c) and 37 $^{\circ}\text{C}$ (d). The circled spots indicate the samples at the onset of gelation,
536 the sublimes crossing the circled spots are for guiding the eyes to the corresponding DH values.
537 Error bars represent the standard deviations.

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

542 Figure 5. Hydrolysis of β -casein (a), $\alpha_{\text{s}1}$ -casein (b) and κ -casein (c) in samples containing 0,
543 10, 20, 50 $\mu\text{g}/\text{mL}$ AprX during storage at room temperature; and in samples containing 0, 0.8,
544 1.6, 2.4 $\mu\text{L}/\text{mL}$ plasmin at room temperature (d, e, f), expressed as the relative change in peak

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

548 **Supplementary materials**

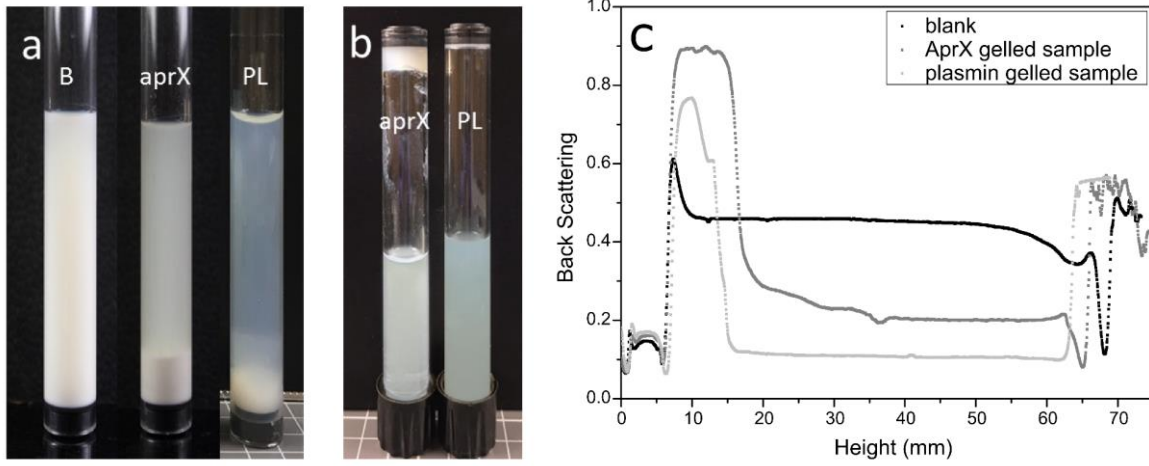
549 Figure S1. The correlation between the AprX (a) and plasmin (b) concentration in UHT milk
550 and the gelation time.

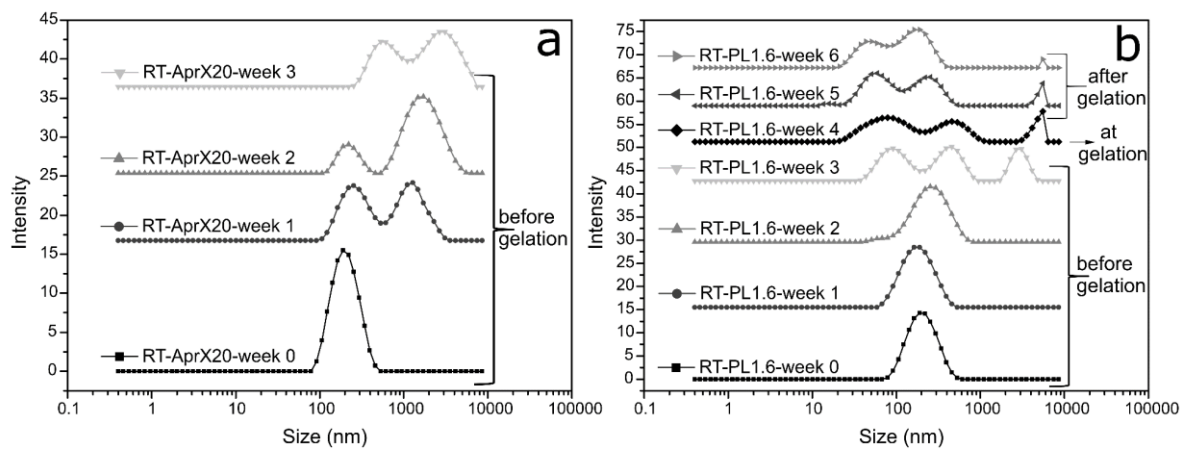
551 Figure S2. Intensity based particle size distribution of samples containing 50 $\mu\text{g/mL}$ AprX
552 stored at room temperature and samples containing 20 $\mu\text{g/mL}$ AprX stored at 42 °C before
553 gelation (a); and samples containing 0, 0.8, 1.6, 2.4 $\mu\text{L/mL}$ plasmin in 1 week before gelation
554 and at the onset of gelation at room temperature (b) and 37 °C (c).

555 Figure S3. Hydrolysis of β -casein (a), α_{s1} -casein (b) and κ -casein (c) in samples containing 0,
556 10, 20, 50 $\mu\text{g/mL}$ AprX during storage at 42 °C; and in samples containing 0, 0.8, 1.6, 2.4
557 $\mu\text{L/mL}$ plasmin at 37 °C (d, e, f), expressed as the relative change in peak area of UV absorption
558 at 214 nm. The circled spots indicate the samples at the onset of gelation, the sublines crossing
559 the circled spots are for guiding the eyes to the corresponding DH relative peak area values.
560 Error bars represent the standard deviations.

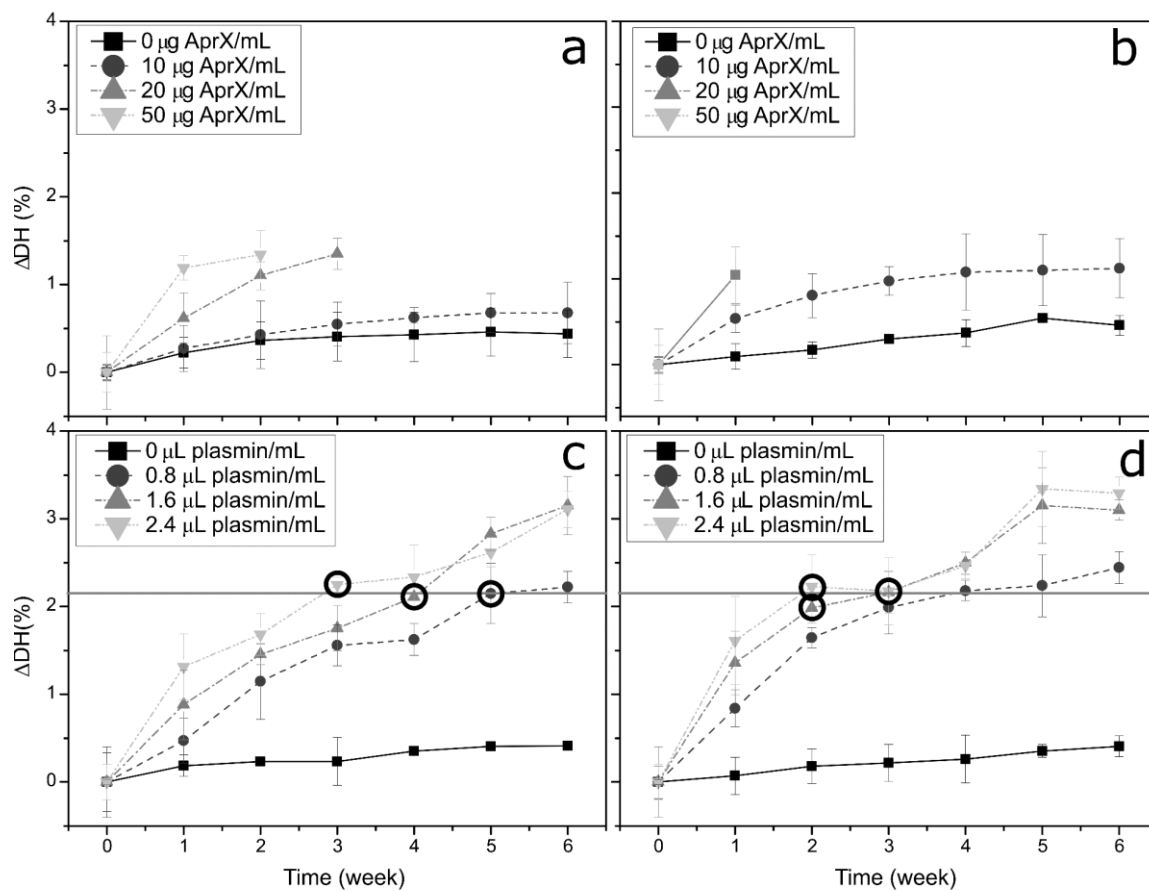
561 Figure S4. SDS-PAGE gels of UHT milk hydrolyzed by AprX at room temperature (a); by
562 plasmin at room temperature (b) and 37 °C (c). α_s -casein: $\alpha_{s1} + \alpha_{s2}$ casein; Std: molecular mass
563 standards; blank: skim UHT milk without addition of protease; W: storage time (week); AprX
564 10, 20, 50: UHT milk containing 0, 10, 20, 50 $\mu\text{g/mL}$ AprX; plasmin 0.8, 1.6, 2.4: UHT milk
565 containing 0, 0.8, 1.6, 2.4 $\mu\text{L/mL}$ plasmin. The arrows indicate the gelled samples.

566



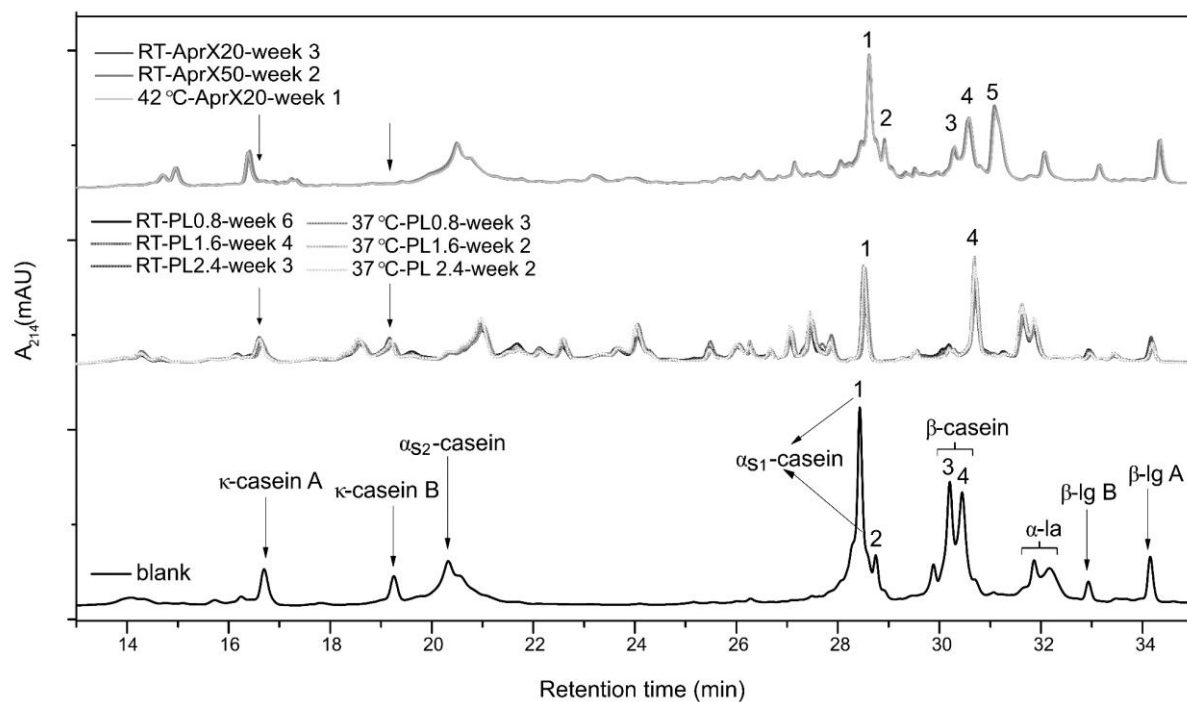


571 Figure 3



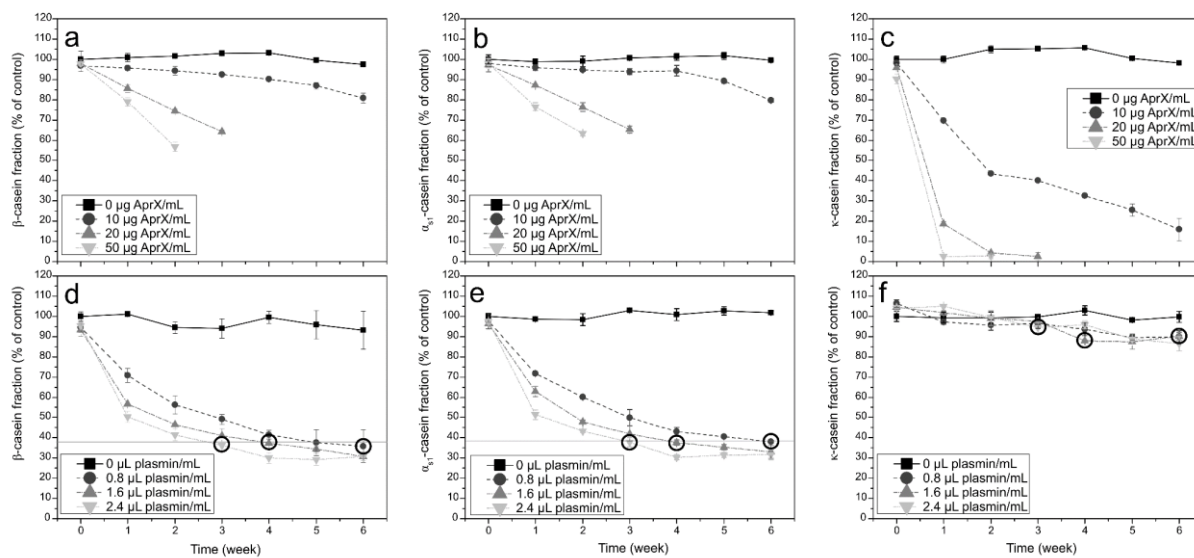
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573 Figure 4

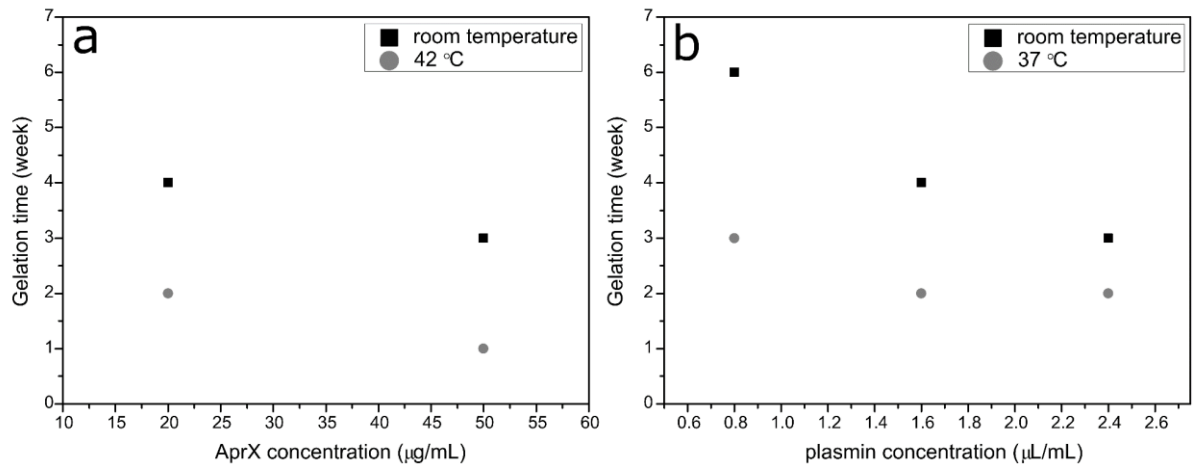


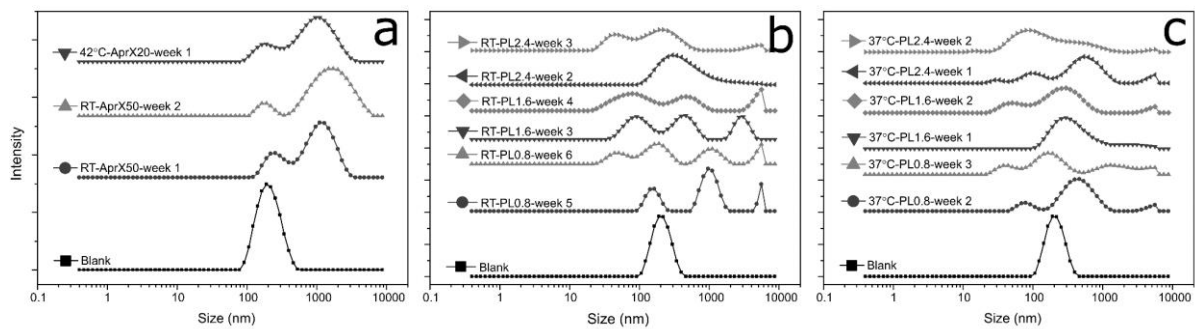
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575 Figure 5



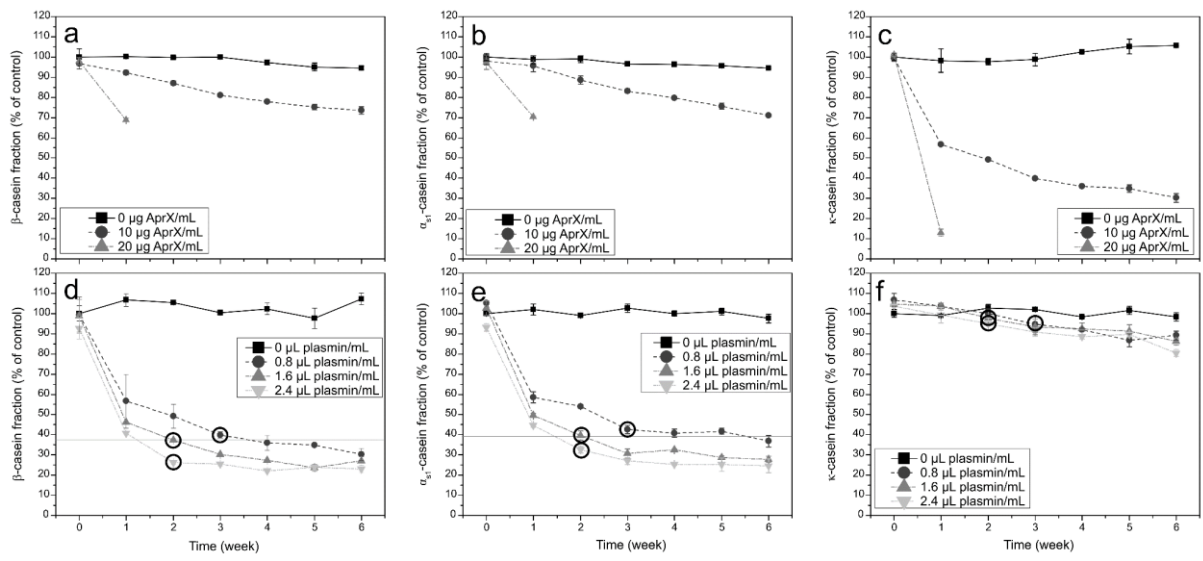
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580

581 Figure S3



582

