Stability of fat globules in UHT milk during proteolysis by the AprX protease from Pseudomonas fluorescens and by plasmin

Zhang, C., Bijl, E., Muis, K. E., & Hettinga, K.

This is a "Post-Print" accepted manuscript, which has been Published in "Journal of Dairy Science"

Stability of fat globules in UHT milk during proteolysis by proteases AprX from *Pseudomonas fluorescens* and plasmin. By Zhang et al. The shelf life of UHT milk can be shortened due to fat separation. In this study, we showed that enzymatic hydrolysis of fat surface-adsorbed proteins can enhance fat separation in UHT milk. Bacterial proteases and native proteases can lead to sedimentation and creaming of milk fat, respectively. Our results open new perspectives regarding the fat destabilisation mechanism in UHT milk, showing that the activity of both kinds of enzymes in the production chain of UHT milk should be reduced.

ENZYMATIC DESTABLIZATION OF FAT GLOBULES IN UHT MILK

Stability of fat globules in UHT milk during proteolysis by proteases AprX from *Pseudomonas fluorescens* and plasmin

Chunyue Zhang, Etske Bijl, Karinska Eunike Muis, Kasper Hettinga*

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

*Corresponding author:

Full name: Kasper Hettinga

Mailing address: Postbus 17, 6700AA, Wageningen, the Netherlands

Phone number: +31 6 14327822

E-mail address: kasper.hettinga@wur.nl

ABSTRACT

Fat separation is a limiting factor for the shelf life of UHT milk. It may be promoted by the proteolysis of the fat surface-adsorbed proteins (FSAP) by proteases that remain active after UHT treatment. The aim of this research was to explore the relationship between the proteolysis of FSAP and fat destabilization. In this study, we developed a full fat UHT milk-based model system to which either the major bacterial protease AprX from *Pseudomonas fluorescens* or the major native milk protease plasmin was added at high levels to induce fast destabilisation of the milk fat globules. The changes in physical properties, FSAP composition and the structural changes of the fat globules were monitored over 24 hours. Our results show that AprX induced sedimentation due to flocculation of fat globules, while plasmin induced cream to float due to coalescence of fat globules. This study confirmed that AprX and plasmin can indeed both lead to fat destabilization in full fat UHT milk, and provides insights in the underlying mechanisms.

Key Words. fat stability, UHT milk, proteolysis, AprX, plasmin

INTRODUCTION

The consumption of ultra-high temperature (UHT)-processed milk is increasing worldwide because of its long shelf life and the possibility of long distance transport and storage at room temperature. However, its shelf life can be compromised by unwanted changes like age gelation and sedimentation. For full-fat and semi-skimmed UHT milk, another possible detrimental change during its storage is fat separation; the agglomeration of the fat globules with the formation of a floating fat layer , or a fat-rich protein aggregate that is dense enough to sediment (Chavan et al., 2011; Hardham et al., 2000).

Fat globules in UHT milk are heated and homogenized. Homogenization causes the native milk fat globules to mechanically disrupt into smaller particles, after which the natural milk fat globule membrane material is insufficient to cover the larger fat globule surface area. Hence,

the surface of fat globules is covered and stabilized by adsorbed milk proteins, primarily caseins (Cano-Ruiz and Richter, 1997), which we will call fat-surface adsorbed proteins (**FSAP**). Fat separation during shelf life of UHT milk has been commonly recognized to be caused by insufficient homogenisation (Hardham et al., 2000; Ramsey and Swartzel, 1984). If fat globules do not achieve sufficient homogenization, a small number of fat globules will remain large and represent a significant volume of the total fat; these large globules can rise rapidly and cause a significant cream layer (Wilbey, 2011).

However, it is assumed that nowadays, adequate homogenization is used in most commercial UHT production processes, thus insufficient homogenisation should not be a major issue. In UHT milk that has been adequately homogenized, proteolysis of FSAP could be the main cause of fat instability during storage. Such proteolysis of UHT milk may be due to the residual activity of heat-stable proteases, which are mainly bacterial proteases, represented by AprX, and native milk proteases, represented by the plasmin system. AprX is a caseinolytic extracellular alkaline metalloprotease produced by *Pseudomonas* species (Zhang et al., 2019), with κ-casein being most susceptible to its action, followed by β- and α_{s1}-casein (Recio et al., 2000; Zhang et al., 2018). Plasmin hydrolyses in the order β->α_{s1}->α_{s2}-casein, but hardly hydrolyses κ-casein (Rauh et al., 2014a; Zhang et al., 2018). In view of the differences in preferences of AprX and plasmin towards caseins, differences in AprX- and plasmin-induced protein destabilization of UHT milk, such as gelation and sedimentation, have been frequently reported (Matéos et al., 2015; Rauh et al., 2014b; Zhang et al., 2018). However, the influence of protease on fat destabilisation has been studied much less. It has been observed that in full-fat UHT milk, bacterial proteases lead to the formation of sediment or a custard-like gel (Harwalkar, 1992; Visser, 1981), whereas native proteases cause a creamy surface layer (Hardham, 1998; Kohlmann et al., 1991; Rauh et al., 2014b). However, the underlying

mechanisms of how these two categories of proteases destabilize fat globules in UHT milk have not been unequivocally elucidated.

This study thus aims to compare the differences between AprX and plasmin in hydrolysing FSAP and the subsequent consequences for fat stability in UHT milk. To assess this, high levels of AprX or plasmin was added to full-fat UHT milk samples, in order to induce fast destabilisation of milk fat globules without the interference from non-enzymatic physicochemical changes.

MATERIALS AND METHODS

Enzymatic Hydrolysis

Commercial full-fat UHT milk was obtained from FrieslandCampina (Venecolaan, Belgium). The type of processing used on the UHT milk was direct sterilization by steam infusion heat treatment according to additional information obtained from the manufacturer. The protein and fat content of the UHT milk was 3.6% and 3.5% (w/v), respectively, as analysed by MilkoScan 134A/B (Foss Electric, Hillerød, Denmark). To have a low starting degree of hydrolysis, we used milk samples that were not older than 1 month after manufacturing. To prevent spoilage during storage, 0.02% sodium azide was added as preservative. The protease AprX was isolated from *Pseudomonas fluorescens migula* (DSM 50120, Deutsche Sammlung von Mikroorganismen) and purified as described by Zhang et al. (2018). Plasmin from bovine plasma was purchased from Sigma-Aldrich (Roche 10602370001), which was suspended in 3.2 M ammonium sulfate solution.

The enzyme concentrations were determined in a preliminary study. Activity of 1 µl plasmin/mL milk and the equivalent activity of AprX were found to be able to induce obvious changes to fat stability in the model systems within 24 hours of incubation at 37 ℃. Samples were stored at 37 ℃ in order to accelerate destabilization. Our previous study has shown that

storage at 37 °C will not change the hydrolysis patterns of milk proteins by AprX and plasmin, but only its rate (Zhang et al., 2018). In the main experiment, crude AprX extract was weighed into full-fat UHT milk samples at a concentrations of 84 µg/mL, whose activity matched 1 µl plasmin/mL milk. The activity of plasmin and AprX was determined using the azocasein assay as described by Dufour et al. (2008). Samples were collected at 4 hour intervals over 24 hours of incubation at 37 ℃, after which the samples were directly used for physical stability measurement and FSAP analysis.

Characterization of Physical Stability During Proteolysis

Measuring Fat Content in Different Layers. The samples for fat content measurement were placed in a standing position without disturbance. After incubation, as explained in 2.1, a pipette tip was slowly inserted along the inner wall of the tube until reaching the bottom without stirring the milk.

Then the same volume of the bottom, middle and top layer was collected successively from this vial. Lipid extraction from full-fat UHT milk samples was optimised based on the methods of Lu et al., (2017), using a mixture of hexane and isopropanol (3:2, v/v). The milk sample was mixed with the organic solvent mixture at a ratio of 1:5 (v/v) in a capped Eppendorf tube. The tubes were incubated at 60 ℃ for 15 min with occasional gentle shaking to aid lipid extraction (Baümler et al., 2010). Then the upper organic phase was collected in a weighed tube, dried under a stream of nitrogen and weighed again, with the increase in weight used to calculate the fat content.

Stability Analysis by LUMiFuge. The physical stability of the hydrolysed samples was examined with the LUMiFuge (L.U.M.GmbH, Berlin, Germany). LUMiFuge is an optical stability analyser which accelerates physical destabilization by centrifugal forces and exposes the sample cells to near infrared light while measuring the transmission continuously during the

centrifugation. High concentrations of particles yield low transmission profiles and vice versa. Prior to analysis, but after incubation, the samples were diluted 10 times with simulated milk ultrafiltrate (SMUF, pH 6.8) to reduce the turbidity of full-fat UHT milk and facilitate the optical measurement. SMUF was prepared according to Jenness and Koops (1962), with the following composition: 11.6 mM KH_2PO_4, 3.70 mM $C_6H_5K_3O_7 \cdot H_2O$, 6.09 mM $C_6H_5Na_3O_7 \cdot 2H_2O$, 1.03 mM K_2SO_4, 8.05mM KCl, 8.97mM $CaCl_2 \cdot 2H_2O$, and 3.20 mM $MgCl_2 \cdot 6H_2O$. The sample was then agitated by gently shaking from side to side, after which 400 µL sample was inserted to LUMiFuge sample cells (LUM 2mm, PC, Rectangular synthetic cell (110-131xx), Berlin, Germany). The LUMiFuge measurements were carried out at 2300 × g for 43 min at room temperature, and transmission was measured for 255 cycles with a duration of 10 s at a near-infrared wavelength of 865 nm. These parameters were set according to Sunds (2016).

To quantify the emulsion stability of the samples, the integrated transmission percentage against time was used. This will further be referred to as the "instability index" in this study. The instability index was automatically calculated with the LUMiFuge software SEPView 6.3. Lower instability index values indicate better emulsion stability.

Size Distribution of Milk Particles. Particle size distribution analysis was performed using laser light diffraction (Mastersizer 3000, Malvern Instruments, Ltd, Malvern, UK). Samples diluted 50 times with MilliQ water were injected directly into the dispersion cell under agitation at 1500 rpm. A higher stirring intensity was avoided to prevent disintegration of aggregates. The droplet size was calculated with a refractive index of 1.45 and 1.33 for the droplet and continuous phase (MilliQ water), respectively. The particle absorption index was set as 0.002.

Viscosity. The viscosity of the samples was measured in a stress controlled rheometer (MCR302, Anton Paar), using a double-gap concentric cylinder geometry (MCR 72). A solvent trap was used to prevent evaporation. The samples were stabilized for 2 min. Subsequently, the

viscosity was monitored by exponentially increasing the shear rate from 0.01 to 500 s^{-1}. Measurements were performed at 20 °C.

FSAP Isolation. The method was optimized based upon previously described methods by McCrae et al., (1994); McPherson et al., (1984) and Lu et al. (2016). The FSAP isolation method was divided into two parts, namely cream separating and washing. Cream was separated from full-fat UHT milk samples by ultracentrifugation at 60,000 × g for 1 hour at 20 ℃ using an L60 Beckman ultracentrifuge (Beckman, Fullerton, CA, 70 Ti rotor). To aid the separation of fat droplets from milk serum, 0.6 M sucrose was incorporated before centrifugation as density-increasing agent. Pre-experiments showed that after ultracentrifugation with the aid of sucrose, the cream layer of UHT milk samples was as thick as that of raw milk, indicating most milk fat ended up in the cream layer. Immediately after ultracentrifugation, the samples were immersed into an ice bath for 15 min to solidify the cream layer. The cream was then collected into a new tube and washed as described next. To wash away the proteins that are loosely attached or entrapped in the cream layer, the collected cream was washed in three steps: 1) submersion of the cream layer (at 1:10; w/v) in SMUF with added sucrose (35% w/v); 2) centrifugation at 21,000 × g for 10 min at 10 ℃; 3) discarding the washing solution, and collection of the cream layer. After repeating the 3-step washing procedure three times, the washed cream fraction was weighed, and the FSAP were analysed with RP-HPLC and SDS-PAGE.

Characterization of Protein Profile in FSAP During Proteolysis

RP-HPLC. The protein composition was determined 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 Zhang et al. (2018). Protein standards (β-casein, α_{S1}- casein, α_{S2}- casein, κ- casein, α-lactalbumin and β-lactoglobulin; all with purities

of 70–98 %, all from Sigma-Aldrich) were used to validate the elution times of milk proteins. The resulting chromatograms were analyzed by Chromeleon 7.1.2 software. The changes in α_{s2}-casein could not be quantitatively described due to the co-elution of the peaks of α_{s2}-casein with breakdown products of protein hydrolysis, as also explained by Rauh et al. (2014b).

SDS-PAGE. SDS-PAGE was performed under reducing conditions to complement RP-HPLC results. The sample buffer, reducing agent, gels, running buffer and antioxidant agent were all purchased from Invitrogen (Carlsbad, USA). The washed cream was diluted 30 times with SMUF, 5 µL diluted cream was mixed with 5 µL 4× concentrated NuPAGE® LDS sample buffer, 2 µL 10× concentrated NuPAGE® sample reducing agent and 10 µL MilliQ water. Then the mixture was centrifuged at 425 × g for 1 min and heated at 70°C in a heating block (Labtherm Graphit, Liebisch, Germany) for 10 min. Samples were loaded onto NuPAGE® Novex® 12% Bis-Tris gels. The BlueRay Prestained Protein Marker 10-180 kDa (Jena, Germany) was applied as a reference. NuPAGE®MES buffer with addition of 0.5 mL NuPage® antioxidant was used for running the gels. Electrophoresis was performed using an XCell SureLock™ unit (Invitrogen, Paisley, UK) at constant voltage (120 V). The gels were Coomassie-stained.

Characterization of Microstructure of Fat Globules

Confocal Laser Scanning Microscopy (CLSM). The distribution of lipid and protein and their aggregation were observed with a Zeiss LSM 510-META 18 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The stock solutions of protein-specific stain rhodamine B (Sigma-Aldrich, 10 mg/mL) and lipid-specific stain Bodipy 505/515 (Invitrogen Molecular Probes, Carlsbad, CA, USA, 5 mg/mL in 70% ethanol) were mixed and diluted in SMUF to a final concentration of 1 mg/mL. One µL of mixed stain was added to 99 µL milk sample, vortexed and dropwise transferred to a cavity slide.

The CLSM was connected to an inverted microscope (Axiovert 200M) with differential interference contrast (DIC). Bodipy 505/515 was excited with an Argon laser (488 nm) and emission wavelengths between 505 nm and 530 nm were detected. A He-Ne laser operated at a excitation wavelength of 543 nm and emission wavelengths of 560 nm and higher was used to detect rhodamine B. All images were acquired using a Plan-Apochromat 63× oil immersion objective (1.4 NA) with a resolution of 512×512 pixels, and the pinhole of the microscope was set to 84 μm. The samples stained with both dyes were analysed by multi-channel tracking to enable the visibility of the dyes.

Cryostation Scanning Electron Microscope (Cryo-SEM). To analyse the morphological changes of fat globules in UHT milk after enzymatic hydrolysis, Cryo-SEM images were taken at distinct times after incubation with proteases. A milk sample of 10 μL was transferred to a copper hollow rivet and quickly plunged into liquid nitrogen for snap-freezing. The sample holder was transferred to a cryopreparation system (MED 020/VCT 100, Leica, Vienna, Austria), in which the samples were fractured and freeze dried to remove water vapor contamination. Then the samples were sputter coated with a layer of 15 nm Tungsten. Samples were cryo-shielded transferred into the field-emission scanning microscope (FEI Magellan 400, FEI, Eindhoven, The Netherlands) equipped with a Leica cold stage for cryo-SEM. The analysis was performed at a working distance of 4.2 mm, with SE detection at an acceleration voltage of 2 kV. The particle size distribution was analyzed by Image J software (1.51f, National Institutes of Health, USA).

Statistical analysis. All the measurements were performed in biological triplicates and results were expressed as mean with standard deviations (SD). Data were analysed by one-way repeated measures ANOVA and Tukey as post-hoc test using SPSS version 24 (SPSS Inc., Chicago IL). Statistical significance was set as $p < 0.05$.

RESULTS AND DISCUSSION

Change of Physical Stability

Change of Fat Content in Different Layers. The fat redistribution was monitored by measuring the fat content in the top, middle and bottom layers of the sample tubes. As shown in Fig. 1a, the samples incubated with AprX, fat in the top layer started to decrease from 12 h onwards ($p<0.05$), whereas, the fat content in the middle and bottom layers increased from 12 h and 16 h ($p<0.001$), respectively, indicating fat started to sediment. On the contrary, in plasmin-hydrolysed samples the fat content in the bottom layer decreased after 0 h ($p<0.05$), along with an increase of fat content in the top layer ($p<0.05$, Fig. 1b), indicating the fat globules floated to the surface. Similar plasmin-induced redistribution of fat in different layers of UHT milk during storage has been reported by Malmgren et al. (2017), who observed an increase in fat content of the top layers and, at the same time, a reduction in the lower layers.

Stability Test by Lumifuge. Due to the high turbidity, fat separation was difficult to judge visually in full-fat UHT samples until the sample clarified. Therefore, LUMiFuge was used to aid in analysing the particle migration by calculating the instability index according to the transmission profiles under centrifugal forces. As shown in Fig. 2, the instability index increased during incubation in both AprX- and plasmin-containing samples, indicating that sample destabilisation happened in both systems. For the samples incubated with AprX, the instability index rapidly increased between 4 h and 12 h ($p<0.001$), wherein creaming gradually changed into sedimentation, as shown by the shape of transmission profiles (Supplementary Fig. 1). In plasmin-containing samples, the instability index increased at a slower pace compared to AprX-containing samples, with more creaming and less sedimentation formed, as inferred from the transmission profiles.

240 ***Size Distributions.*** The changes in the size distribution of all the colloidal particles in full-
241 fat UHT milk, which include casein micelles, fat globules, and the agglomerates that may form,
242 were monitored during hydrolysis by AprX and plasmin (Fig. 3). The blank full-fat UHT milk
243 at 0 h incubation had a pseudo-monomodal size distribution centred at approximately 0.2 μm,
244 which are actually two overlapping distributions with the casein micelles being smaller and the
245 fat globules being larger than the average. A similar pseudo-monomodal size distribution of
246 full-fat UHT milk has been reported by Matéos et al. (2015).

247 For the UHT milk containing AprX (Fig. 3a), the size distribution started to broaden at 8 h. A
248 new peak appeared in the micron size range at 12 h, and shifted to over 10 μm in the subsequent
249 incubation times. Meanwhile, the peak corresponding to regular particles in the sample before
250 incubation gradually diminished, indicating that the small particles were incorporated in the
251 larger aggregates. This observation is in agreement with earlier findings, where large aggregates
252 ranging from 2-100 μm have been reported in full-fat UHT milk (Matéos et al., 2015). However,
253 in skim UHT milk samples incubated with AprX, these aggregates have also been observed
254 (Baglinière et al., 2013; Zhang et al., 2018), indicating that the underlying mechanism for
255 aggregate formation could be similar. It can also be speculated that fat is entrapped in the
256 formed protein aggregate, similar to cheese production.

257 Compared to these AprX-containing samples, the size distribution changed to a lesser extent in
258 the plasmin-containing samples. No new peaks appeared, but the average size slightly increased
259 ($p<0.05$) and a population of particles with a size larger than 2 μm arose, indicating that larger
260 particles were formed during the hydrolysis by plasmin. Similar changes in the size distribution
261 have also been found in semi-skim UHT milk (1.5% fat, w/v) with residual plasmin activity
262 during storage (Rauh et al., 2014b). But the plasmin-induced changes of particle size
263 distribution in the skim UHT milk (0.07% fat, w/v) was slightly different, where the peak
264 corresponding to native casein micelles shifted, instead of broadening to a larger size (Zhang et

al., 2018), which may be due to the fact that in skim milk there are only casein micelles, making the peak sharper, whereas in non-skim homogenized milk, the peaks of casein micelles and fat globules overlap.

Viscosity. The flow behavior of milk as determined by a rheometer is closely related to the consumers' perception of viscosity. Fig. 4 displays the apparent viscosity curves for both AprX- and plasmin-hydrolysed samples. The viscosity of samples with AprX and plasmin both showed shear thinning behavior, and the viscosity increased with increasing incubation time.

In AprX-containing samples, after incubation for 8 hours, the viscosity sharply rose to more than 10 mPa·s (at 20 °C) (Fig. 4a), which is generally regarded as a manifestation of age gelation in UHT milk (Datta and Deeth, 2001; Deeth and Lewis, 2016). This coincided with the time that the sedimentation started (Fig. 1) and large agglomerates were formed (Fig. 3). Therefore we can conclude that a gel network formed after 8 h. In comparison, the viscosity in plasmin-containing samples rose steadily, indicating that the changes in the microstructure were gradual and progressive. In our study, none of the plasmin-containing samples reached a viscosity higher than 10 mPa·s (Fig. 4b). But fat floating was detected (Fig. 1), showing that creaming happened earlier than gelation in our plasmin-containing UHT milk.

Comparing these two systems, AprX induced a greater increase in viscosity and a less pronounced shear thinning behavior compared to plasmin, which both reflected that AprX induced the formation of stronger network structures than plasmin.

Change of FSAP Content and Composition

Change of FSAP Content. To explain how the fat stability may be influenced by the hydrolysis of FSAP, the samples with AprX or plasmin were further studied for their FSAP content and composition. Because the same ratio of milk fat ended up in the cream layer after ultracentrifugation, the differences in the weight of cream layer per unit milk was mainly

attributed to the different content of hydrated FSAP. As shown in Fig. 5, in the milk with AprX, the weight of cream started to increase rapidly after 8 h ($p<0.001$), and remained constant after 20 h. In milk with plasmin, the weight of the cream layer decreased in the first 12 hours ($p<0.005$), and remained constant afterwards. Similar trends as for weight of cream were seen in both systems when FSAP concentration in the washed cream was quantified using HPLC and expressed as the total HPLC peak area (Fig. 5). Both measurements showed that more protein was trapped in the cream layer along with the hydrolysis by AprX, whereas, part of the FSAP seems to get released to the serum when hydrolysed by plasmin.

Change of FSAP Composition. The changes of FSAP composition during incubation of the full-fat UHT milk samples with AprX or plasmin were studied using RP-HPLC and SDS-PAGE (Fig. 6). In AprX-hydrolysed samples, α_{s1}- and β-caseins remained constant in the first 4 hours, but started to increase sharply between 4 and 16 h ($p<0.001$), after which they declined slightly ($p<0.05$) (Fig. 6a). Meanwhile, almost all the κ-casein was rapidly hydrolysed within the first 4 hours ($p<0.001$). Besides that, the peak area of whey proteins increased gradually upon hydrolysis ($p<0.001$). On SDS-PAGE, the band corresponding to κ-casein diminished rapidly (Fig. 6c). It is known that AprX preferably cleaves the peptide bond Phe105-Met106 of κ-casein, like chymosin, generating *para*-κ-casein which remains in casein micelles and glycomacropeptide (GMP) that goes into serum (Zhang et al., 2019). But no band of *para*-κ-casein (located at ca. 15 kDa) was visible on the gel, possibly because AprX can further cleave the *para*-κ-casein molecule during storage (Matéos et al., 2015). The numerous bands exhibited after 12 h might correspond to the breakdown products of α- and β-caseins.

After the incubation with plasmin, α_{s1}- and β-caseins in FSAP significantly decreased ($p<0.005$), while κ-casein and whey proteins remained constant. Even though the decrease in α_s- and β-caseins are not obvious to see from the gel (Fig. 6d), some new bands can be observed after incubation for 8 hours. The bands located at approx. 25 kDa, 15 kDa, and 12 kDa may

correspond to the major breakdown products of β-casein by plasmin, which are f29–209 (γ1-CN), f69–209 (γ4-CN) and f106–209 (γ2-CN) & f108–209 (γ3-CN), respectively (Fig. 6d) (Petrella et al., 2015).

Microstructure of Fat Globules with Hydrolysed FSAP

The microstructure of the blank and full-fat UHT milk with AprX or plasmin was visualised by confocal laser scanning microscopy after incubation for 16h (Fig. 7). These samples were selected because the FSAP fraction in both milk samples seemed to not change further from this time onwards (Fig. 5, 6). Protein and lipid were stained red and green, respectively. In the blank full-fat UHT milk, both the casein micelles (Fig. 7a) and the fat globules (Fig. 7b) had uniform sizes and were homogeneously distributed. No aggregation was observed (Fig. 7a-c).

In the AprX-hydrolysed samples, on the other hand, a remarkably different state of the fat and protein was observed. There, a dense gel network was observed in the red protein channel (Fig. 7d), while the fat globules, which were shown as green spots, remained as individual droplets (Fig. 7e) that were embedded in the protein gel network (Fig. 7f). Compared to traditional dairy products, the AprX-induced network structure is more compact than the open gel in full-fat yoghurt where colloidal calcium phosphate is dissolved and the κ-casein brush collapses (Torres et al., 2012). But the network is similar to chymosin-induced milk gel (Ong et al., 2011), which agrees with the results discussed in size distributions.

In plasmin-hydrolysed samples, we observed some red circles in the protein channel (Fig. 7g) and large green spots in lipid channel (Fig. 7h), as indicated by the white arrows. The green spots, corresponding to fat globules, were larger than those in the blank and AprX-hydrolysed samples, which was in line with the increase of the particles in the size distribution results (Fig. 3b), and indicated fat coalescence in these samples. After combining the channels, we found the coalesced fat globules were covered by a thin continuous layer of proteins (Fig. 7i). The ascending or descending of these coalesced fat globules is determined by the protein to fat ratio.

In our case, the relative amount of protein associated with the fat globules decreased upon hydrolysis by plasmin (Fig. 5, 6). As a result, the average density of fat globules would drop and the density of fluid would increase. Therefore, according to Stokes' law, the coalesced fat globules would float to the surface, which is in agreement with our findings in Fig. 1b.

Cryo-SEM images were taken to investigate the morphologic changes of the fat globules in UHT milk, and meant as examples to further strengthen our theory. Fat globules could be easily distinguished from casein micelles because casein micelles were smaller than fat globules and present as uniform, not perfectly spherical-shaped particles. The sizes of casein micelles in UHT milk ranged from 50-200 nm, with an average diameter of about 100 nm (Supplementary Fig. 2). Kamigaki et al., (2018) reported a similar distribution of casein micelle sizes based on the observation of casein micelles in raw milk, with an average diameter of about 140 nm. the smaller average diameter observed in our samples may be explained by the disruption of the micellar structure during homogenization (Dalgleish et al., 2004).

The fat globules, in both the blank and hydrolysed samples, were much rounder and bigger than casein micelles, with the diameter of most fat globules ranging from 200 -500 nm, which is in agreement with Lopez (2005), who suggested that the volume-weighted average diameter of fat globules in homogenized milks is in the range 200-500 nm. In the blank full-fat UHT milk, as shown in Fig. 8a, the surface of the fat globules appears to be covered by woolly structures, protruding from the bulk of the fat globule. These structures with a diameter of about 10 and 20 nm are assumed to be casein micelle fragments, which originate from casein micelles that have been pulled apart, and have then spread on the fat globules during homogenization (Dalgleish et al., 2004). These woolly structures probably contain many κ-caseins on their surface, because they were absent after incubation with AprX, as shown in Fig. 8b. In addition, in AprX-hydrolysed samples, fat globules appeared to be embedded in an aggregated protein network (Fig. 8b). The two findings for the AprX-hydrolysed samples agree with the

interpretation of both the amount of cream layer and its protein content (Fig. 5a), as well as the CLSM pictures (Fig. 7d-f). The surface conformation of fat globules in plasmin-hydrolysed samples (Fig. 8c) was more difficult to interpret. Deduced from the FSAP hydrolysis by plasmin (Fig. 6b), and the "protein shell" observed in the CLSM pictures (Fig. 7g-i), the structure covering the surface of fat globules might consist of casein micelle fragments.

Comparison between Aprx- and Plasmin-induced Fat Destabilization

When comparing the physical and chemical changes in full-fat UHT milk samples hydrolysed by AprX and plasmin, different mechanisms of fat destabilization can be proposed, as schematically illustrated in Fig. 9. Our previous study has showed that the enzyme-induced physical-chemical changes and hydrolysis of milk proteins are independent of protease concentration or temperature (Zhang et al, 2018). Therefore, the finding from the fast destabilisation model systems in this study (storage at 37 °C with the addition of high concentrations of enzymes) should also apply to lower temperatures and biological levels of the enzymes.

AprX destabilizes fat globules in full-fat UHT milk by flocculation (Fig. 9). In AprX-containing samples, since AprX specifically cleaved the protruding κ-casein (Fig. 5a, c), casein micelles, together with the casein micelle fragments absorbed on the surface of fat globules, quickly destabilize. Consequently, a network is formed due to the reduced steric and electrostatic repulsion. Being part of the three-dimensional gel network, even though fat globules remain as individual entities, they are closely embedded in the continuous protein network (Fig. 7d-f, Fig. 8b). From the perspective of fat globules, more proteins, which are the micellar casein aggregated with the existing FSAP were absorbed (Fig. 5). As a result, the hydrodynamic diameter increased (Fig. 3a), as well as the viscosity (Fig. 4a). Furthermore, due to the adsorption of additional milk proteins with the fat globules, which increases their density, fat globules tended to sink under gravitational force (Fig. 1a).

By contrast, the mechanism responsible for the physical instability of fat globules in full-fat UHT milk induced by plasmin is fat globule partial coalescence (Fig. 9). In plasmin-containing samples, plasmin preferentially hydrolysed α- and β-caseins (Fig. 6b, d), probably causing collapse of the FSAP which mainly consists of casein micelle fragments (Fig. 8c). As a consequence, the fat globules did not longer repel each other, instead, several individual fat globules merged into a larger fat droplet (Fig. 7g-i). These coalesced fat droplets lead to an increase in the particle size (Fig. 3b) and viscosity (Fig. 4b). Because some of FSAPs were cleaved by plasmin, less protein was present on the surface of the fat droplets (Fig. 5), and the fat globules thus have a lower density and cream (Fig. 1b). It should also be noted that a creamy layer on the top is commonly accompanied by a gel on the bottom when plasmin is present (Kohlmann et al., 1991; Stoeckel et al., 2016; Visser, 1981).

CONCLUSIONS

This study elucidated the mechanisms of fat destabilization in UHT milk as a consequence of proteolytic degradation by bacterial protease AprX and native protease plasmin. Our results have shown that AprX induces sedimentation of milk fat, being embedded in a protein gel network, caused by the fast hydrolysis of κ-casein leading to flocculation of casein micelles and fat globules. Plasmin, on the other hand, can induce creaming in full-fat UHT milk, because the hydrolysis of α- and β-caseins disrupts the casein micelle fragments on the milk fat globule surface, causing the fat globules to coalesce and ascend. The knowledge gained from this study may be used for determining the protease responsible for the full-fat UHT milk destabilisation during storage. Overall, both enzymes can bring about destabilization in full-fat UHT milk, thus actions should be taken to decrease the activity of both enzymes in the production chain.

ACKNOWLEDGEMENTS

This research was funded by the Sino Dutch Dairy Development Center, which aims at improving dairy production, safety and quality levels throughout the entire dairy chain in China (www.sdddc.org). We acknowledge, Dr. Marcel Giesbers (Wageningen Electron Microscopy Centre) for the assistance in performing the Cryo-SEM experiments, and Dr. Norbert C.A. de Ruijter (Wageningen Light Microscopy Center) for the assistance in performing the CLSM experiments. The authors report no conflict of interest in this paper.

REFERENCES

Baglinière, F., Matéos, A., Tanguy, G., Jardin, J., Briard-Bion, V., Rousseau, F., Robert, B., Beaucher, E., Gaillard, J. L., Amiel, C. Humber, G., Dary, A., & Gaucheron, F. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. Int. Dairy J. 31:55-61. https://doi.org/10.1016/j.idairyj.2013.02.011

Baümler, E. R., Crapiste, G. H., & Carelli, A. A. 2010. Solvent extraction: kinetic study of major and minor compounds. J. Am. Oil Chem. Soc. 87:1489-1495. https://doi.org/10.1007/s11746-010-1637-3

Cano-Ruiz, M., & Richter, R. 1997. Effect of homogenization pressure on the milk fat globule membrane proteins. J. Dairy Sci. 80:2732-2739. https://doi.org/10.3168/jds.S0022-0302(97)76235-0

Chavan, R. S., Chavan, S. R., Khedkar, C. D., & Jana, A. H. 2011. UHT milk processing and effect of plasmin activity on shelf life: A review. Compr. Rev. Food Sci. F. 10:251-268. https://doi.org/10.1111/j.1541-4337.2011.00157.x

433 Dalgleish, D. G., Spagnuolo, P. A., & Goff, H. D. 2004. A possible structure of the casein
434 micelle based on high-resolution field-emission scanning electron microscopy. Int. Dairy J.
435 14:1025-1031. https://doi.org/10.1016/j.idairyj.2004.04.008

436 Datta, N., & Deeth, H. C. 2001. Age gelation of UHT milk - A review. Food Bioprod. Process.
437 Trans. Inst. Chem. Eng. Part C, 79:197-210. https://doi.org/10.1205/096030801753252261

438 Deeth, H., & Lewis, M. 2016. Protein stability in sterilised milk and milk products. In P. L. H.
439 McSweeney, & J. A. O'Mahony (Eds.), Advanced Dairy Chemistry (4th ed., Vol. 1B:
440 Applied aspects, pp. 247-286). New York, USA: Springer.

441 Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., Gaillard, J. L, &
442 Dary, A. 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from
443 milk and genetical and biochemical characterization of an extracellular protease produced
444 by one of them. Int. J. Food Microbiol. 125:188-196.
445 https://doi.org/10.1016/j.ijfoodmicro.2008.04.004

446 Hardham, J. F. 1998. Effect of protein standardisation of milk by addition of UF milk permeate
447 on the composition and storage stability of UHT processed milk. Aust. J. Dairy Technol.
448 53:22.

449 Hardham, J. F., Imison, B. W., & French, H. M. 2000. Effect of homogenisation and
450 microfluidisation on the extent of fat separation during storage of UHT milk. Aust. J. Dairy
451 Technol. 55:16-22.

452 Harwalkar, V. R. 1992. Age gelation of sterilized milks. In P. F. Fox (Ed.), *Advanced Dairy*
453 *Chemistry* (2nd ed., Vol. 1: Proteins, pp. 691–734). London: Elsevier.

454 Jenness, R. R., & J. Koops, 1962. Preparation and properties of a salt solution which simulates
455 milk ultrafiltrate. Neth. Milk Dairy J. 16:153-164.

456 Kamigaki, T., Ito, Y., Nishino, Y., & Miyazawa, A. 2018. Microstructural observation of casein
457 micelles in milk by cryo-electron microscopy of vitreous sections (CEMOVIS). Microscopy.
458 67:164-170. https://doi.org/10.1093/jmicro/dfy012

459 Kohlmann, K. L., Nielsen, S. S., & Ladisch, M. R. 1991. Effects of a low concentration of
460 added plasmin on ultra-high temperature processed milk. J. Dairy Sci. 74:1151-1156.
461 https://doi.org/10.3168/jds.S0022-0302(91)78267-2

462 Lopez, C. 2005. Focus on the supramolecular structure of milk fat in dairy products. Reprod.
463 Nutr. Dev. 45:497-511. https://doi.org/10.1051/rnd:2005034

464 Lu, J., Argov-Argaman, N., Anggrek, J., Boeren, S., van Hooijdonk, T., Vervoort, J., &
465 Hettinga, K. A. 2016. The protein and lipid composition of the membrane of milk fat
466 globules depends on their size. J. Dairy Sci. 99:4726-4738. https://doi.org/10.3168/jds.2015-
467 10375

468 Lu, J., Pickova, J., Vázquez-Gutiérrez, J. L., & Langton, M. 2017. Influence of seasonal
469 variation and ultra high temperature processing on lipid profile and fat globule structure of
470 Swedish cow milk. Food Chem. 239:848-857.
471 https://doi.org/10.1016/j.foodchem.2017.07.018

472 Malmgren, B., Ardö, Y., Langton, M., Altskär, A., Bremer, M. G., Dejmek, P., & Paulsson, M.
473 2017. Changes in proteins, physical stability and structure in directly heated UHT milk
474 during storage at different temperatures. Int. Dairy. J. 71:60-75.
475 https://doi.org/10.1016/j.idairyj.2017.03.002

476 Matéos, A., Guyard-Nicodème, M., Baglinière, F., Jardin, J., Gaucheron, F., Dary, A., Humbert,
477 G., & Gaillard, J. L. 2015. Proteolysis of milk proteins by AprX, an extracellular protease
478 identified in *Pseudomonas* LBSA1 isolated from bulk raw milk, and implications for the
479 stability of UHT milk. Int. Dairy. J. 49:78-88. https://doi.org/10.1016/j.idairyj.2015.04.008

McCrae, C. H., Hirst, D., Law, A. J. R., & Muir, D. D. 1994. Heat-stability of homogenized milk - role of interfacial protein. J. Dairy Res. 61:507-516. https://doi.org/10.1017/S0022029900028430

McPherson, A. V., Dash, M. C., & Kitchen, B. J. 1984. Isolation and composition of milk fat globule membrane material: II. From homogenized and ultra heat treated milks. J. Dairy Res. 51:289-297. https://doi.org/10.1017/S0022029900023554

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. 2011. Microstructure of milk gel and cheese curd observed using cryo scanning electron microscopy and confocal microscopy. LWT-Food Sci. Technol. 44: 1291-1302. https://doi.org/10.1016/j.lwt.2010.12.026

Petrella, G., Pati, S., Gagliardi, R., Rizzuti, A., Mastrorilli, P., La Gatta, B., & Di Luccia, A. 2015. Study of proteolysis in river buffalo mozzarella cheese using a proteomics approach. J. Dairy Sci. 98, 7560-7572. https://doi.org/10.3168/jds.2015-9732

Ramsey, J. A., & Swartzel, K. R. 1984. Effect of ultra high temperature processing and storage conditions on rates of sedimentation and fat separation of aseptically packaged milk. J. Food Sci. 49:257-262. https://doi.org/10.1111/j.1365-2621.1984.tb13721.x

Rauh, V. M., Johansen, L. B., Ipsen, R., Paulsson, M., Larsen, L. B., & Hammershøj, M. 2014a. Plasmin activity in UHT milk: relationship between proteolysis, age gelation, and bitterness. J. Agr. Food Chem. 62:6852-6860. https://doi.org/10.1021/jf502088u

Rauh, V. M., Sundgren, A., Bakman, M., Ipsen, R., Paulsson, M., Larsen, L. B., & Hammershøj, M. 2014b. Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion. Int. Dairy. J. 38:199-207. https://doi.org/10.1016/j.idairyj.2013.12.007

502 Recio, I., García-risco, M. R., Ramos, M., & López-fandiño, R. 2000. Characterization of
503 peptides produced by the action of psychrotrophic proteinases on κ-casein. J. Dairy Res.
504 67:625-630. https://doi.org/10.1017/S002202990000443X

505 Stoeckel, M., Lidolt, M., Achberger, V., Glück, C., Krewinkel, M., Stressler, T., von Neubeck,
506 M., Wenning, M., Scherer, S., & Hinrichs, J. 2016. Growth of *Pseudomonas*
507 *weihenstephanensis*, *Pseudomonas proteolytica* and *Pseudomonas* sp. in raw milk: Impact
508 of residual heat-stable enzyme activity on stability of UHT milk during shelf-life. Int. Dairy.
509 J. 59:20-28. https://doi.org/10.1016/j.idairyj.2016.02.045

510 Sunds, A. V. 2016. Evaluation of accelerated shelf life testing of UHT milk. Master Thesis.
511 Aarhus University, Department of Food Science, Denmark,

512 Torres, I. C., Rubio, J. M. A., & Ipsen, R. 2012. Using fractal image analysis to characterize
513 microstructure of low-fat stirred yoghurt manufactured with microparticulated whey protein.
514 J. Food Eng. 109:721-729. https://doi.org/10.1016/j.jfoodeng.2011.11.016

515 Visser, S. 1981. Proteoloytic enzymes and their action on milk proteins. A review. Neth. Milk
516 Dairy J. 35:65-88.

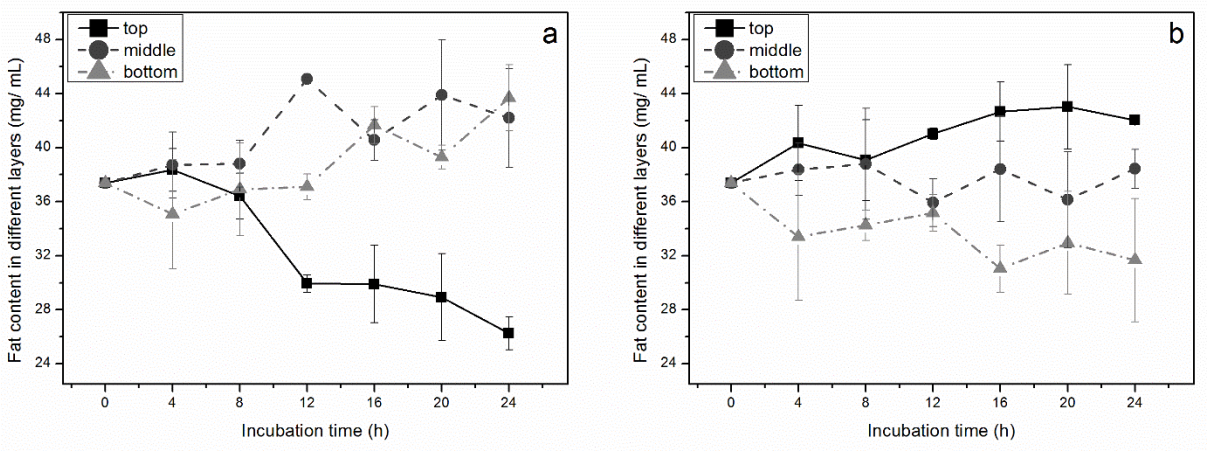
517 Wilbey, R. A. 2011. Homogenization of milk: principles and mechanism of homogenization,
518 effects and assessment of efficiency: valve homogenizers. In J. W. Fuquay (Ed.),
519 *Encyclopedia of dairy sciences* (2nd ed., pp. 750–754). San Diego: Academic Press.

520 Zhang, C., Bijl, E., & Hettinga, K. 2018. Destabilization of UHT milk by protease AprX from
521 *Pseudomonas fluorescens* and plasmin. Food Chem. 263:127-134.
522 https://doi.org/10.1016/j.foodchem.2018.04.128

523 Zhang, C., Bijl, E., Svensson, B., & Hettinga, K. 2019. The extracellular protease AprX from
524 *Pseudomonas* and its spoilage potential for UHT milk: a review. Compr. Rev. Food Sci. F.
525 18: 834-852. https://doi.org/10.1111/1541-4337.12452

526

527 Zhang et al., Fig. 1



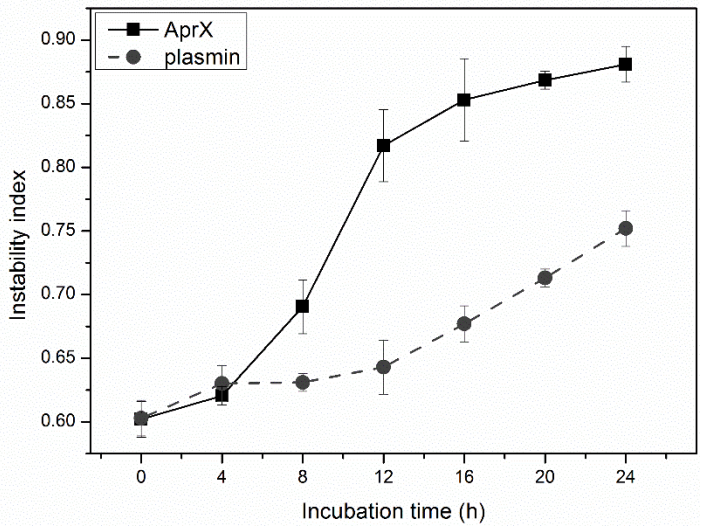
528

529 Fig. 1. Fat content in the top, middle and bottom layer of full-fat UHT milk samples incubated

530 with AprX (a) and plasmin (b). Data are presented as mean ± SD of triplicates.

531

532 Zhang et al., Fig. 2

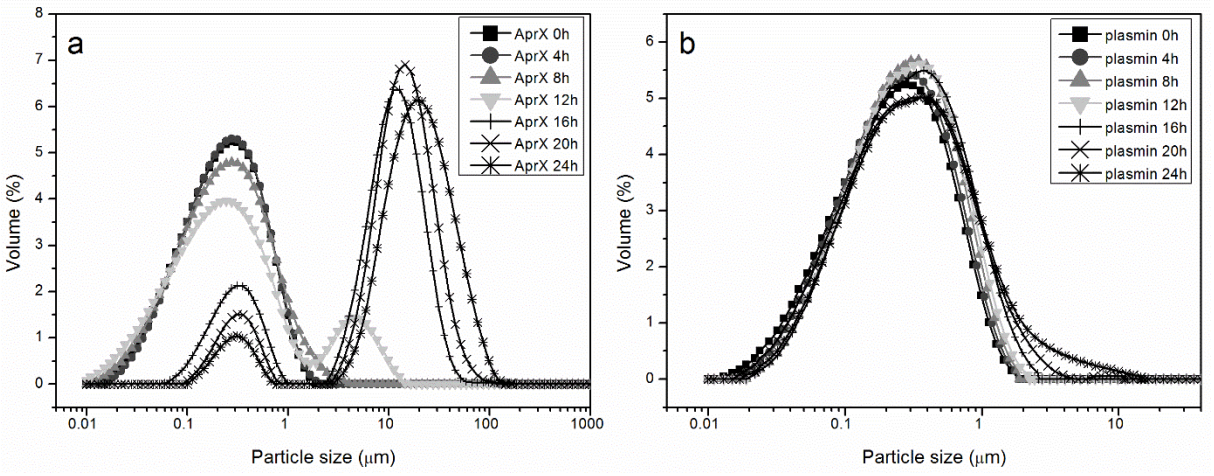


533

534 Fig. 2. Instability index obtained by LUMiFuge of full-fat UHT milk samples incubated with
535 AprX (a) and plasmin (b). All the samples were 10-fold diluted to facilitate the optical
536 measurement. Data are presented as mean ± SD of triplicates.

537

538 Zhang et al., Fig. 3

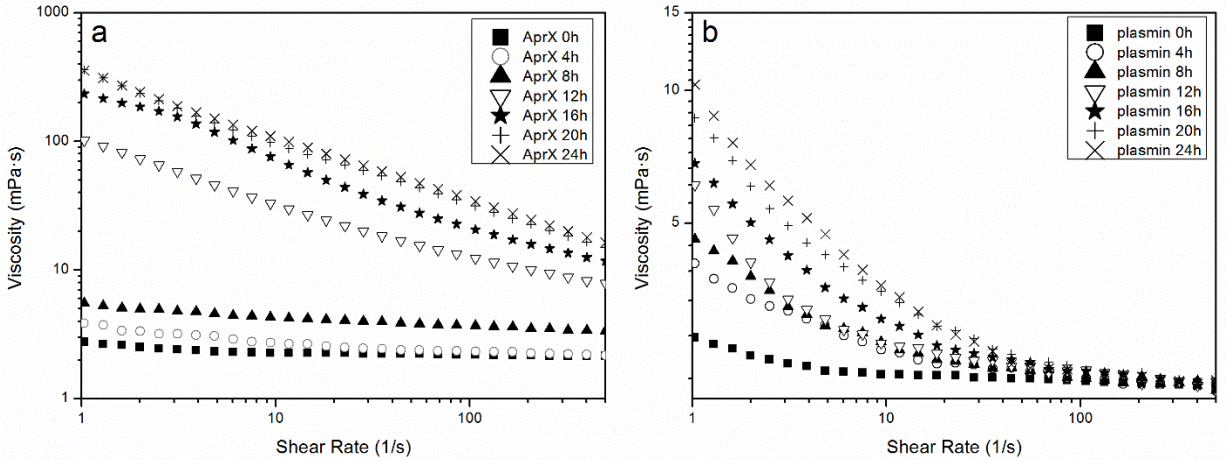


539

540 Fig. 3. Volume based particle size distribution in blank full-fat UHT milk samples incubated
541 with AprX (a) and plasmin (b).

542

543 Zhang et al., Fig. 4

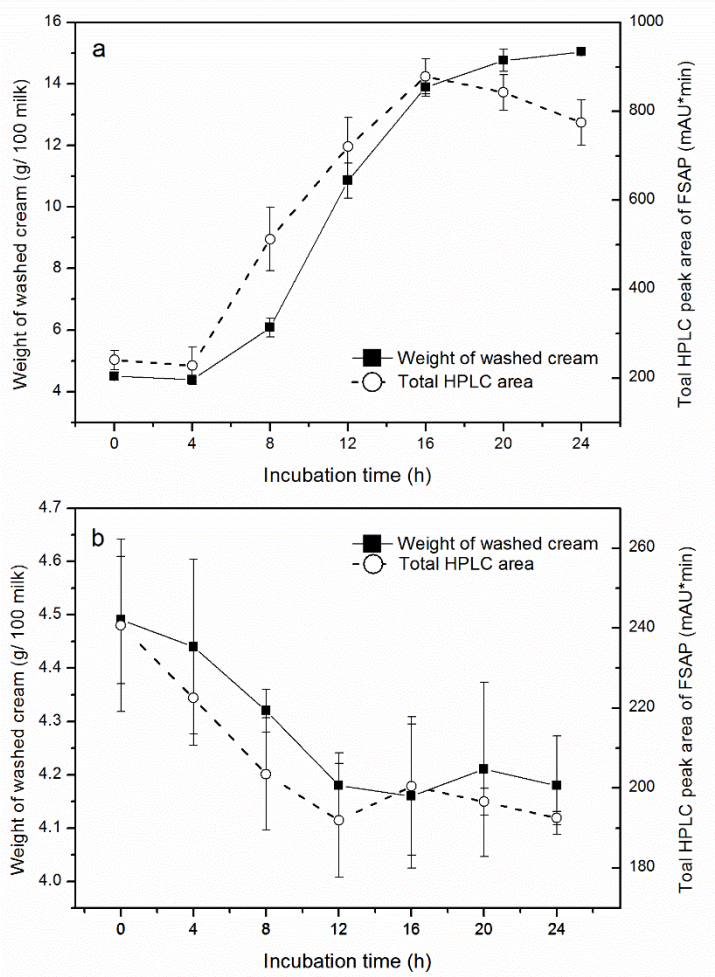


544

545 Fig. 4. Viscosity of full-fat UHT milk samples incubated with AprX (a) and plasmin (b) as a
546 function of shear rate.

547

548 Zhang et al., Fig. 5

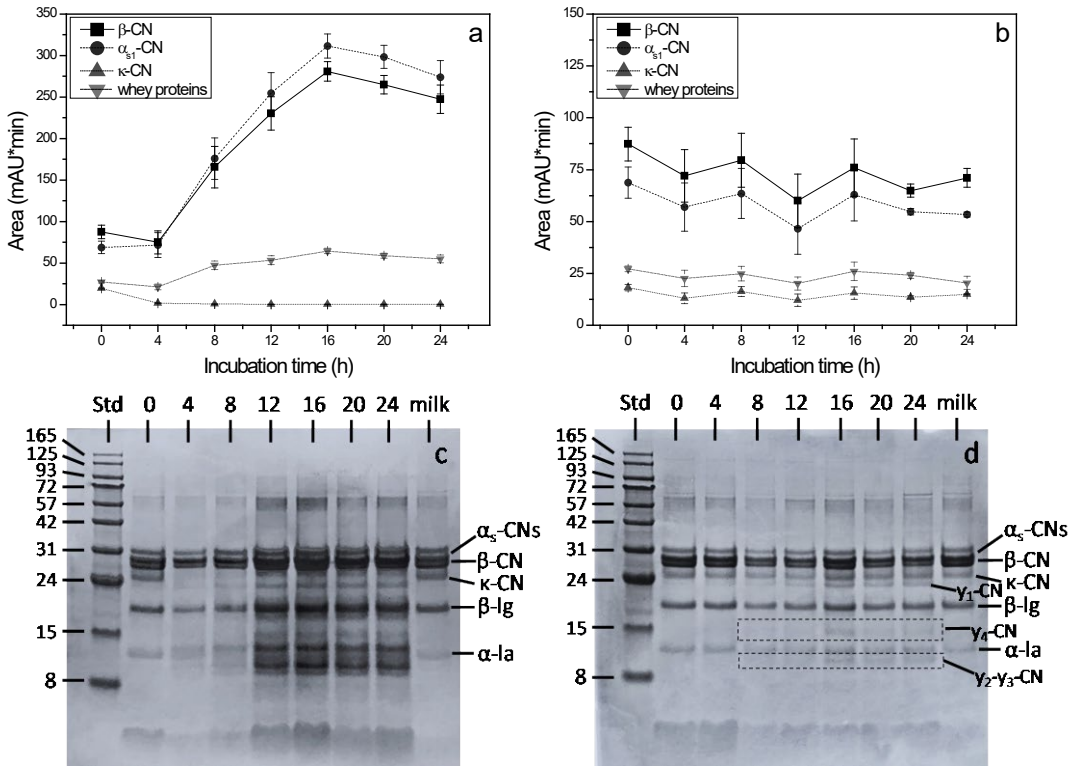


549

550 Fig. 5. The weight of washed cream and the total HPLC peak area of fat surface-adsorbed
551 proteins (FSAP) after integration of UV absorbance at 214 nm of full-fat milk samples
552 incubated with AprX (a) and plasmin (b). Data are presented as mean ± SD of triplicates.

553

554 Zhang et al., Fig. 6

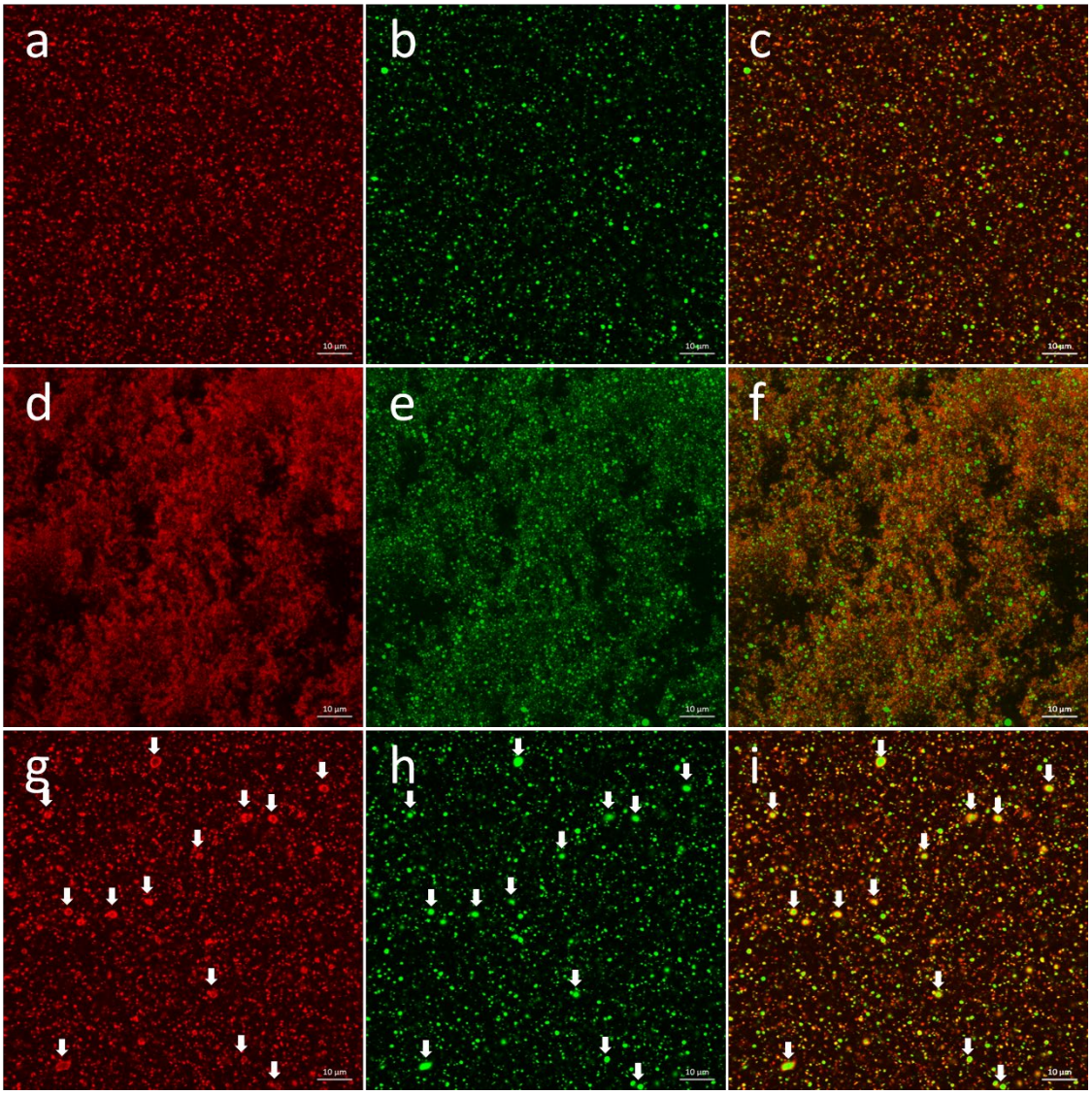


555

556 Fig. 6. Changes of α_{s1}-casein, β-casein, κ-casein and total whey proteins in the FSAP fraction
557 in full-fat UHT milk samples incubated with AprX (a) and plasmin (b), expressed as the total
558 peak area of UV absorption at 214 nm using RP-HPLC, data are presented as mean ± SD of
559 triplicates; SDS-PAGE patterns of full-fat UHT milk samples incubated with AprX (c) and
560 plasmin (d). For (c) and (d): Std: molecular mass standards; milk: full-fat UHT milk without
561 addition of protease; α_s-CNs: α_{s1}+ α_{s2} casein; the numbers above the gel indicate the incubation
562 time with protease in hours.

563

564 Zhang et al., Fig. 7

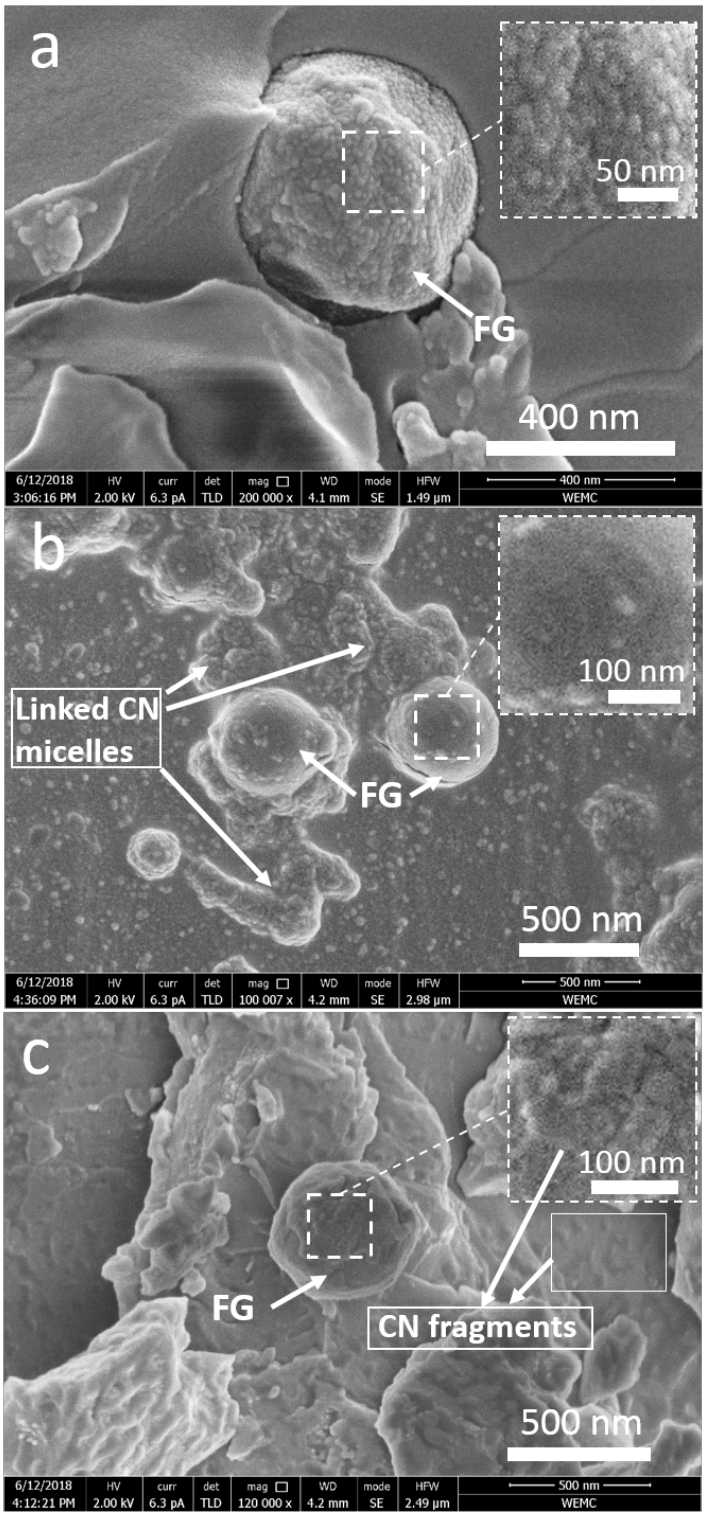


565

566 Fig. 7. Confocal micrographs showing the microstructure of blank full-fat UHT milk (a-c), full-
567 fat UHT milk samples incubated with AprX (d-f) or plasmin (g-i) at 37 ℃ for 16 hours. Protein
568 and lipid were stained with rhodamine B (color red, in Fig 7. a, d, g) and BODIPY 505/515
569 (color green, in Fig 7. b, e, h), respectively. Column 3 (Fig 7. c, f, i) are merged channels of
570 lipid and protein. White arrows indicate coalesced fat globules. The scale bars are 10 μm in
571 length.

572

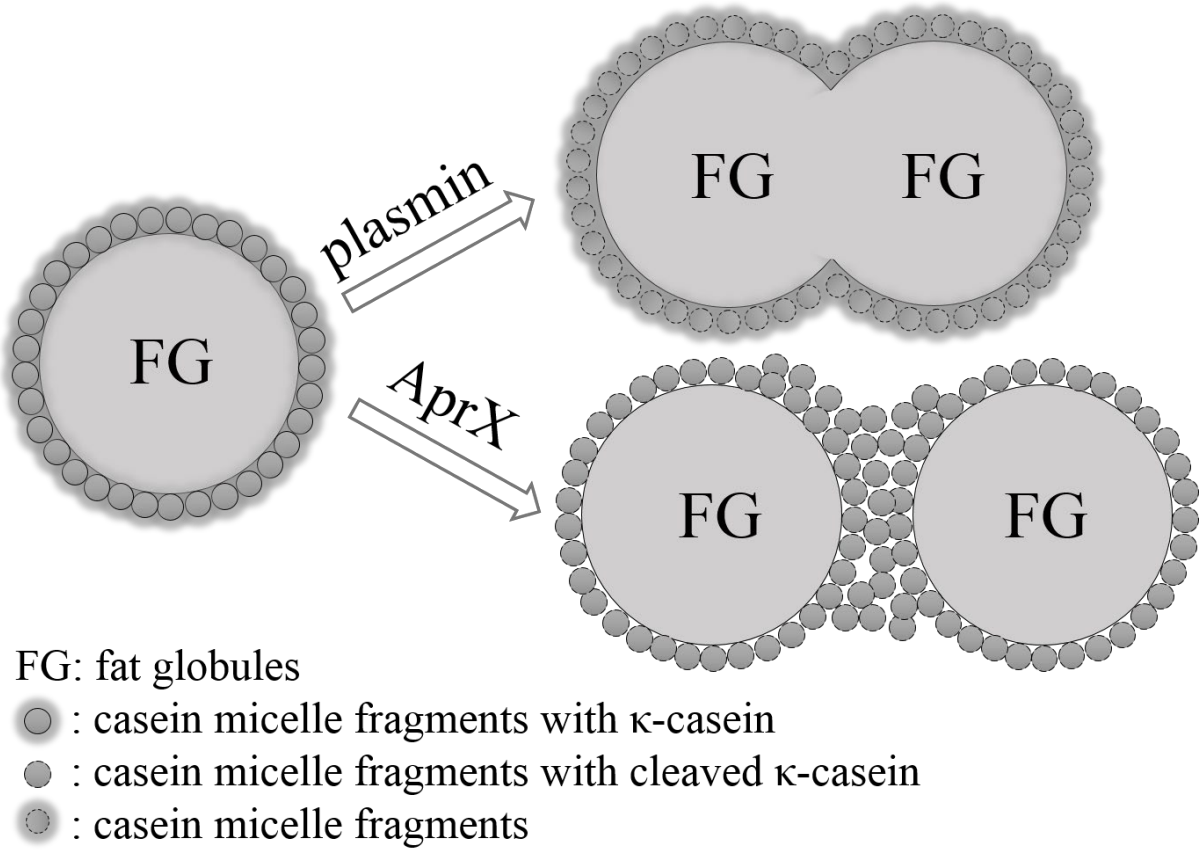
573 Zhang et al., Fig. 8



574

575 Fig. 8. Cryo scanning electron microscopy images of fat globule(s) in blank full-fat UHT milk
576 (a), full- fat UHT milk samples incubated with AprX (b) and plasmin (c) at 37 ℃ for 16 hours.
577 The inserts show the enlargement of the fat globule surface. The scale bars are shown in the
578 images. FG: fat globules. The linked casein micelles and casein micelle fragments as discussed
579 in the text are pointed out in (b) and (c), respectively.

580 Zhang et al., Fig. 9



581

582 Fig. 9. Simplified scheme describing the different mechanisms of fat destabilization in full-fat

583 UHT milk induced by AprX and plasmin.