Impact of plasmin activity on the shelf life and stability of UHT milk

PhD thesis by
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Preface

This PhD thesis concludes three years’ work at Arla Strategic Innovation Center and the Department of Food Science, Aarhus University. This industrial PhD project was a collaboration between Aarhus University, Arla Foods, Copenhagen University and Lund University. As part of the PhD study, a three months stay at Copenhagen University, Department of Food Science was carried out.

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I would like to thank my family and friends in Germany, Denmark and Sweden for a great time, for motivating me and showing me that there is a life outside the PhD. Thank you Vera and Thilo for your hospitality and always making me feel at home when I was in Stockholm and Copenhagen.

Valentin Rauh, August 2014
Abstract

Milk production in Europe is increasing, while the markets for dairy products are saturated and milk consumption is decreasing. The production of high quality long shelf life products will enable the dairy industry to distribute products into new markets and increase export into existing growth markets. Ultra high temperature (UHT) treatment of milk aims to give a commercially sterile product with a shelf life of three months or more without refrigeration. The shelf life of UHT milk is determined by deterioration of flavour and functional properties, which can be caused by either heat induced chemical changes or the activity of enzymes in milk. The principal enzyme in good quality milk which can survive UHT treatments is the indigenous protease plasmin. Plasmin is the active part of an enzyme system consisting of the inactive precursor plasminogen and several activators and inhibitors, which can interact with different milk constituents.

The main objective of this PhD project was to investigate the effect of residual plasmin activity on physical-chemical changes in UHT milk.

Due to the complexity of the plasmin and milk system, interpretation of plasmin activity measurement results can be difficult. In the first part of this project, a spectrophotometric assay for plasmin and plasminogen derived activity was optimized and extended to turbid samples and allowed measurement of plasmin activity in fat containing milk samples. The measured plasmin activity was largely affected by the sample preparation and assay conditions. The developed assay reduces the risk of underestimation of plasmin activity in milk and can be modified for different samples and purposes.

The effect of residual plasmin activity on physical-chemical changes in milk during storage at 20 ºC were studied in direct steam infusion heat treated milk with ultra-short holding times. Plasmin activity resulted in extensive proteolysis of caseins and a bitter off flavour appeared after six weeks of storage at which time more than 60 % of αS- and β-caseins were hydrolysed. After 10 weeks of storage more than 90 % of αS- and β-caseins were hydrolysed and the milk began to form a gel. Bitterness was found to be the most important shelf life limiting factor for milk with residual plasmin activity.

Identification of peptides in this UHT milk with residual plasmin activity allowed the exploration of the proteolytic pathway of plasmin in a milk system. 66 casein derived peptides were formed by the plasmin, of which 23 could have been responsible for the observed bitterness. Plasmin showed the
highest affinity for the hydrophilic regions in the caseins and the least affinity for hydrophobic and phosphorylated regions.

Protein lactosylation, the initial stage of the Maillard reaction, was studied in different UHT milks over a storage period of six months by liquid chromatography-mass spectrometry (LC-MS) and the common marker furosine. The initial furosine concentration in direct steam infusion heat treated milk was found to be 10-15 times lower as compared to an indirectly processed UHT milk, but no significant difference in the increase of furosine concentration during storage was found between the milks. A quantitative correlation lactosylation measured by LC-MS and furosine concentration was established, allowing for a simpler and faster analysis of protein lactosylation.

The results presented in this thesis expand our understanding of the plasmin system in milk and can help to control plasmin activity in UHT milk. This gives a direct way to improve the quality and shelf life of UHT milk products. Furthermore, the fundamental findings of this thesis can be transferred and applied to other dairy products.
Sammendrag (abstract in Danish)

Mælkeproduktion i Europa er stigende, samtidig er markedet for mejeriprodukter mættet og forbruget af mælk er faldende. Fremstilling af højkvalitetsprodukter med lang holdbarhed vil gøre det muligt for mejerindustrien at lancere mejeriprodukter på nye markede og samtidig øge eksporten til eksisterende vækstmarkeder.


Hovedformålet med dette PhD projekt var at undersøge effekten af restplasminaktivitet på fysisk-kemiske ændringer af UHT mælk.


Effekten af restplasminaktivitet på fysisk-kemiske ændringer i mælk opbevaret ved 20 °C blev undersøgt. Mælken blev varmebehandlet med direkte dampinfusion i ultrakort tid. Aktiviteten af plasmin resulerede i omfattende proteolysis af kaseiner og dannelse af bitter afsmag efter seks ugers opbevaring, hvor mere end 60% af αS- og β-kasein var hydrolyseret. Efter 10 ugers opbevaring var mere end 90% af αS- og β-kasein hydrolyseret og mælken begyndte at gelere. Bitter smag blev fundet at være den vigtigste holdbarhedsbegrænsende faktor for mælk indeholdende restplasminaktivitet.

Identifikation af peptider i UHT mælk med restplasminaktivitet gjorde det muligt, at udforske...
plamins proteolytiske nedbrydningsmønster i mælk. 66 kaseinafledte peptider blev dannet af plasmin, hvoraf 23 kan være årsag til den observerede bitterhed. Plasmins affinitet overfor spaltningsteder i kasein er anderledes i UHT mælk sammenlignet med kaseinmodelssystemer. Plasmin viste højest affinitet overfor de hydrofile regioner i kasein og mindst affinitet overfor de hydrofobe og fosforylerede regioner.

Laktosylering af proteiner, der er den indledende fase af Maillard-reaktionen, blev undersøgt i forskellige UHT mælkeprøver over en lagringsperiode på seks måneder ved anvendelse af væskekromatografi koblet med massespektrometri (LC-MS) og markøren furosin. Den indledende furosinkoncentration i mælk varmebehandlet med direkte dampinfusion blev målt til at være 10-15 gange lavere end indirekte UHT behandlet mælk, men der blev ikke påvist en signifikant forskel i stigningen af furosin under lagring. En kvantitativ korrelation mellem laktosylering målt ved LC-MS og furosinkoncentrationen blev etableret.

Resultaterne præsenteret i denne afhandling har udvidet forståelsen af plasminsystemet i mælk og kan bidrage til at kontrollere plasminaktivitet i UHT mælk. På denne måde kan der opnås en forbedret kvalitet samt holdbarhed af UHT produkter. Yderlig kan de fundamentale resultater fra denne afhandling overføres og anvendes på andre mejeriprodukter.
Zusammenfassung (abstract in German)


Das Hauptziel dieses Projektes war die Untersuchung des Einflusses der Plasminaktivität auf physikalische und chemische Veränderungen in UHT Milch.


Die erste Phase der Maillard Reaktion, die Laktosylierung von Proteinen, wurde in verschiedenen UHT Milchtypen über einen Zeitraum von sechs Monaten mit Hilfe von Flüssigchromatographie mit Massenspektrometrie-Kopplung (LC-MS) sowie der Analyse der Furosinkonzentration untersucht. Die anfängliche Furosinkonzentration in Milch, die durch direkte Dampfinfusion erhitzt wurde, war 10-15 mal niedriger im Vergleich zu indirekt erhitzter UHT Milch. Es wurden jedoch keine signifikanten Unterschiede in der Zunahme der Furosinkonzentration während der Lagerung in den UHT Milchtypen gefunden. Eine quantitative Korrelation zwischen der mit LC-MS gemessenen Laktosylierung und der Furosinkonzentration konnte hergestellt werden, was die Analyse der Proteinlaktosylierung vereinfacht und verkürzt.

Die Ergebnisse in der vorliegenden Arbeit erweitern das Verständnis des Plasminsystems in Milch und können dazu beitragen, Plasminaktivität in UHT Milch zu kontrollieren. Dadurch kann sowohl die Qualität als auch die Haltbarkeit von UHT Milchprodukten verbessert werden. Darüber hinaus können die grundlegenden Erkenntnisse dieser Arbeit auf andere Milchprodukte übertragen und angewendet werden.
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**List of included publications**

This thesis is mainly based on the papers listed below and are referred to in the text by their roman numerals. The papers are included in the back of the thesis.

**Paper I**

**Paper II**

**Paper III**

**Paper IV**

XII
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AU</td>
<td>arbitrary unit</td>
</tr>
<tr>
<td>α-la</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>β-lg</td>
<td>β-lactoglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cn</td>
<td>casein</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EACA</td>
<td>ε-amino caproic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>FAST</td>
<td>fluorescence of advanced Maillard products and soluble tryptophan</td>
</tr>
<tr>
<td>HMF</td>
<td>5-hydroxymethyl-2-furfural</td>
</tr>
<tr>
<td>(RP-) HPLC</td>
<td>(reverse phase) high performance liquid chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NCN</td>
<td>non-casein nitrogen</td>
</tr>
<tr>
<td>MALDI MS</td>
<td>matrix assisted laser desorption mass spectrometry</td>
</tr>
<tr>
<td>MFGM</td>
<td>milk fat globule membrane</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>PA</td>
<td>plasminogen activator</td>
</tr>
<tr>
<td>PL</td>
<td>plasmin</td>
</tr>
<tr>
<td>PP</td>
<td>proteose peptone</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>UHT</td>
<td>ultra high temperature</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
</tbody>
</table>
Almost all consumption milk is subjected to heat treatment during processing to inactivate pathogenic and spoilage microorganisms and to prolong the shelf life of the milk. Heat treatment of milk is always a balance between the inactivation of undesired components, i.e. microorganisms and enzymes, the preservation of desired properties and negative heat induced chemical effects. Depending on the severity of the heat treatment, the shelf life of milk is therefore limited by a decrease in organoleptic and functional properties, which can be caused by either spoilage microorganisms and enzymes or heat induced changes in milk. Low pasteurized milk (72 °C for 15-30 s) has an excellent flavour, however, the shelf life is only one to two weeks when refrigerated due to deterioration by spoilage microorganisms. On the other hand, UHT milk (135-150 °C for 1-10 s) is commercially sterile and has a shelf life from two months up to one year without refrigeration, but the flavour of the milk is significantly changed and further deteriorates during storage resulting in a low acceptance for UHT milk by consumers.

The quality of UHT milk can be improved by optimisation of the heat treatment process. Direct UHT processes use steam to rapidly heat up the milk to the desired temperature. Thereafter the milk is rapidly cooled in a vacuum chamber. In indirect UHT processes, milk is heated in a tubular or plate heat exchanger by hot water and/or steam, which is separated from the milk by a metal surface. Milk from direct steam UHT processes has an improved flavour compared to milk from indirect UHT processes. The fast heating and cooling rates in direct steam processes reduce the severity of the heat treatment in regards to chemical changes (Datta, et al., 2002).

Furthermore, the inactivation of microorganisms in the temperature range between 110-150 °C is mainly dependent on the temperature, whereas chemical changes are mainly dependent on the holding time when applying direct steam systems. Consequently, the heat treatment can be optimized by increasing the temperature while decreasing the heating time. This leads to the development of UHT processes outside the classic time temperature range operating at temperatures > 150 °C combined with ultra-short holding times of < 0.2 s (Holst, et al., 2010; Holst, et al., 2012; Huijs, et al., 2004). These processes enable the production of commercially sterile products with minimum chemical changes, but are not able to sufficiently inactivate heat resistant enzymes to ensure a long shelf life.

Enzymes in milk are either indigenous enzymes already present in milk or are produced by bacteria in milk before processing. Of these enzymes, heat resistant proteolytic and lipolytic enzymes from psychotrophic bacteria and the heat resistant major indigenous protease plasmin have the largest impact on product deterioration during storage of this type of UHT milk, causing
off flavours and gelation (Datta & Deeth, 2003; McKellar, et al., 1984). While the problem of bacterial enzymes can largely be avoided by using high quality raw milk and minimizing the growth of bacteria in milk before processing, there is no possibility to eliminate plasmin in milk. An additional pre or post-heat treatment is usually applied to inactivate plasmin, which on the other hand will decrease the organoleptic quality of the milk (van Asselt, et al., 2008).

While the heat inactivation of plasmin has been studied intensively, the effect of plasmin activity and proteolysis on UHT milk quality and the underlying mechanisms are only known to a limited extent. The main hypothesis of this project is that the shelf life of UHT milk with residual plasmin activity can be controlled, when proteolysis and quality defects by plasmin can be correlated. The second hypothesis is that the shelf life of the UHT milk can be predicted and optimised by correlating plasmin activity measured in an assay with the occurring proteolysis.

2 Project overview

2.1 Aim and objectives

The main objective of the project is to investigate plasmin mediated proteolysis and quality defects during the storage of UHT milk. The second objective is to correlate the proteolysis with plasmin activity measurements. Since plasmin is the active part of a complex enzyme system in a complex milk system, the measurement of plasmin activity depends largely on the used assay. The first objective is therefore to investigate the factors influencing plasmin activity in an assay and to develop a new or to improve existing assays.

2.2 Experimental outline

It was initially planned to study plasmin activity in whey and micellar casein besides milk. For this, a diafiltration experiment was conducted to obtain whey and casein fractions and to investigate plasmin activity during diafiltration. Due to lack of time and considerable process variation, it was decided to not further pursue this and the focus was put on milk only. The whey and casein fractions were used for initial testing of the plasmin and plasminogen derived activity assay. Different factors influencing the plasmin activity in different sample matrices were investigated during the method development of the plasmin activity assay.

A pilot scale UHT experiment was carried out at the Arla Foods pilot plant in Stockholm. In total, five UHT milks, four processed with direct steam infusion and ultra-short holding times and varying pre-heat treatment and one indirectly heat treated UHT milk as reference were produced. The trial was performed in triplicate on three consecutive weeks. The milk was transported to the
Arla Foods Strategic Innovation Center in Denmark and the milk was stored at 20 °C and analysed on a weekly basis for 14-16 weeks. It was aimed for that the 4 directly processed UHT milk samples should contain four different levels of plasmin activity. However, the UHT treatment resulted in two UHT milks with similar plasmin activity and two UHT milks without plasmin activity. A correlation of proteolysis and plasmin activity was therefore not possible. The UHT milks without plasmin activity were further analysed on a monthly basis up to a storage time of six months to investigate chemical changes and protein modification during storage.

2.3 Overview of methods

A summary of the methods used in this project is given in Table 1. The detailed descriptions of the methods can be found in the included publications. The paper in which the method is described is annotated in the table.
### Table 1: Overview of methods used in the project with the aim of the analysis and a short description of the principle. The paper in which the methods are described in detail are indicated in the table.

<table>
<thead>
<tr>
<th>Analysed property</th>
<th>Aim</th>
<th>Method</th>
<th>Principle</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin activity</td>
<td>Determination of plasmin activity</td>
<td>Activity assay</td>
<td>Plasmin activity is measured by the release of p-nitroaniline upon hydrolysis of the substrate S-2251 over time using a spectrophotometer.</td>
<td>I &amp; II</td>
</tr>
<tr>
<td>Plasminogen derived activity</td>
<td>Determination of plasminogen derived activity</td>
<td>Activity assay</td>
<td>Plasminogen derived activity is measured after activation of plasminogen by uPA in the same way as plasmin activity.</td>
<td>I &amp; II</td>
</tr>
<tr>
<td>Colour</td>
<td>Measurement of colour changes in milk during storage</td>
<td>Colorimeter</td>
<td>Colour was measured using the Lab colour space. It consists of a lightness value L (scale ranges from 0 to 100, where higher L values equals whiter colour), the a value for the red-green axis (scale ranges from -100 to 100, where positive a value equals red and negative a values equals green) and the b value for the yellow-blue axis (scale ranges from -100 to 100, where positive b value equals yellow and negative b value equals blue).</td>
<td>II</td>
</tr>
<tr>
<td>Particle size</td>
<td>Investigation of particle size changes</td>
<td>Static light scattering</td>
<td>Static light scattering uses the diffraction pattern of light from single particles into different directions by multiple detectors in a dilution. Particle size can be deduced from the diffraction pattern and the refractive index</td>
<td>II</td>
</tr>
<tr>
<td>Microscopy</td>
<td>Characterisation of gel structure</td>
<td>Confocal laser scanning microscopy (CLSM)</td>
<td>Gel particles are stained with the fluorophores FITC for protein and nile red for fat which fluoresce green and red, respectively. The confocal microscope allows high resolution images of the gel microstructure at different depths of the sample and differentiation of fat and protein.</td>
<td>II</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Determination of viscosity in relation to gelation</td>
<td>Rheometer</td>
<td>Viscosity of milk was measured in a double cup system at 25 °C with a constant shear rate of 100 s⁻¹ for 120 s after a 15 s pre-shearing step.</td>
<td>II</td>
</tr>
<tr>
<td>Furosine</td>
<td>Analysis of initial Maillard reaction and protein lactosylation</td>
<td>HPLC</td>
<td>The protein attached Amadori product lactulosyllysine is converted to furosine by acid hydrolysis at 115 °C and analysed by HPLC and diode array detection.</td>
<td>IV</td>
</tr>
<tr>
<td>Analysed property</td>
<td>Aim</td>
<td>Method</td>
<td>Principle</td>
<td>Paper</td>
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<tr>
<td>-------------------</td>
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</tr>
<tr>
<td>Protein analysis</td>
<td>Measurement of protein composition</td>
<td>LC-MS</td>
<td>Milk proteins are dissociated by urea and sodium citrate, and reduced by dithioerythritol. The proteins are separated by HPLC and detected by MS and UV.</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Analysis of whey protein denaturation</td>
<td>LC-MS</td>
<td>Caseins are precipitated by acidification to pH 4.6, and the soluble whey proteins are separated by HPLC and detected by UV and MS. Denaturation of whey proteins is measured by the difference in concentration of pH 4.6 soluble whey proteins before and after heat treatment.</td>
<td>II</td>
</tr>
<tr>
<td>Peptide analysis</td>
<td>Identification of peptides</td>
<td>LC-MS and tandem MS</td>
<td>The pH 4.6 soluble peptides are separated by HPLC and analysed by tandem MS. Both fixed fragmentation energies and dynamic fragmentation energies based on charge state and mass were used for peptide identification.</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Identification of polypeptides</td>
<td>LC-MS</td>
<td>Polypeptides are identified by mass in the pH 4.6 soluble peptide fraction and protein composition after deconvolution of the mass spectra. Besides mass, phosphorylation patterns and genetic variants are used for identification.</td>
<td>II</td>
</tr>
<tr>
<td>Protein modification</td>
<td>Analysis of protein lactosylation</td>
<td>LC-MS</td>
<td>Proteins are dissociated, reduced and separated by RP-HPLC. Lactosylation is either detected by deconvolution of the summed mass spectra of a protein by a mass shift of +324 Da or directly in the summed mass spectra as a mass shift of +324 Da divided by the charge state of the investigated ion.</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Analysis of thiol-disulphide rearrangements by cysteine</td>
<td>LC-MS</td>
<td>Proteins are dissociated by urea and sodium citrate, but not reduced. Rearrangement or thiol-disulphide interchange is characterized by the appearance of monomeric protein and/or appearance of cysteine attachment to proteins by a mass shift of +121 or +242 Da.</td>
<td>-</td>
</tr>
</tbody>
</table>
3 Summary of included publications

3.1 Paper I: The determination of plasmin and plasminogen-derived activity in turbid samples from various dairy products using an optimised spectrophotometric method

The objective of this study was to optimize and extend a spectrophotometric assay for plasmin and plasminogen-derived activity to enable measurement of turbid samples and different dairy products. The method was validated by assessing reproducibility, repeatability, level of detection and recovery of plasmin activity in different sample matrices. The level of detection and repeatability of this method were improved compared with previous spectrophotometric assays. The second objective was to explore the effect of sample preparation and dissociation of plasmin from caseins on plasmin activity. Skimming of the sample resulted in a decrease of plasmin activity by 30% in pasteurised and homogenised whole milk, while skimming did not affect plasmin activity in raw milk. Removal of the cream phase in homogenised milk samples by skimming lead to an underestimation of plasmin activity. Comparison of pasteurised milk with a micellar casein solution showed that the dissociation of plasmin and caseins by adding ε-amino caproic acid (EACA) and NaCl decreased interference by caseins in the assay, but increases inhibition of plasmin with serum-based inhibitory components.

3.2 Paper II: Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion

In this study, the effect of plasmin activity in direct steam infusion heat treated milk with ultra-short holding times (>150 °C for <0.2 s) on physical-chemical changes in milk during storage was investigated. Preheating at either 72 or 95 °C for 180 s was performed. Milk pre-heated at 72°C showed extensive proteolysis, but no proteolysis of κ-casein was detected. Plasmin was identified as active protease and activation of plasminogen was observed as an increase in the rate of casein hydrolysis during storage. A bitter off flavour appeared after six weeks of storage and the milk contained less than 40% intact αS- and β-caseins when bitterness was noticed. Proteolysis in the stored samples correlated with a decrease in pH and with changes in colour. Gelation occurred after 10 weeks along with an increase in viscosity and almost complete proteolysis of αS- and β-caseins. In conclusion, plasmin mediated proteolysis was involved in the occurrence of age gelation and bitter off flavour. Bitterness preceded age gelation and was the shelf-life limiting factor.
3.3 Paper III: Plasmin Activity in UHT Milk: Relationship between Proteolysis, Age Gelation, and Bitterness

A peptidomic study on the directly heated UHT milk (>150 °C for <0.2 s) preheated at 72 °C for 180 s was performed to gain a deeper insight in the specificity and proteolytic pathway of plasmin in a milk system in relation to age gelation and the formation of bitter peptides. Sixty-six peptides from αs- and β-caseins could be attributed to plasmin activity during the storage period, of which 23 were potentially bitter. Plasmin exhibited the highest affinity for the hydrophilic regions in the caseins that most probably were exposed to the serum phase and the least affinity for hydrophobic or phosphorylated regions. The proteolytic pattern observed suggests that plasmin destabilizes the casein micelle by hydrolysing casein–casein and casein–calcium phosphate interaction sites, which may subsequently cause age gelation in UHT milk.

3.4 Paper IV: Protein lactosylation in UHT milk during storage measured by liquid chromatography-mass spectrometry and furosine

The initial stage of the Maillard reaction, protein lactosylation, occurs during the heat treatment of milk and continues during subsequent storage. We compared the initial lactosylation and rate of lactosylation during storage of milk proteins in directly and indirectly heated UHT milk using liquid chromatography (LC) coupled with electron spray injection mass spectrometry (ESI-MS). Furosine was used as an overall marker to allow a quantitative correlation of lactosylation measured by LC-ESI-MS in the UHT milks. Protein lactosylation increased during the storage period of 6 months at 20°C. The extent of protein lactosylation in the directly heated UHT milk was approximately 10-15 times lower compared to indirectly heated UHT milk. The rate of protein lactosylation during storage however was not significantly different for indirect and directly heated UHT milk. Both the initial extent and the rate of lactosylation positively correlated with the amount of lysine residues in the different proteins. A linear or exponential correlation with furosine concentration could be established for minor and major lactosylated proteins, respectively.
4 The plasmin system in milk

4.1 Indigenous enzymes in milk

Indigenous enzymes in milk have been subject to research for over a century and by now, about 70 enzymes have been identified in bovine milk (O’Mahony, et al., 2013). Indigenous enzymes, as opposed to exogenous enzymes, originate from the cow itself. The indigenous enzymes in milk originate from different sources. One source of enzymes is the blood plasma, and enzymes can enter the milk by leaky junctions between mammary cells. Another source is the mammary epithelial secretory cell cytoplasm. The milk fat globule membrane contains a large number of enzymes as it originates from the apical membrane of the mammary cell, which itself originates from the Golgi membranes. The last source of indigenous enzymes are somatic cells, which enter the milk from blood during bacterial infections (O’Mahony, et al., 2013). While most of the indigenous enzymes in milk do not have an obvious physiological role in milk, many of them can have a significant effect on the quality and properties of dairy products (Kelly & Fox, 2006).

The enzymes most responsible for the deterioration of dairy products and relevant for the dairy industry are lipases and proteases. Milk contains two protease systems, plasmin and cathepsin, of which the first one is the principal protease in good quality milk, it has been extensively studied due to its high heat stability and impact on dairy products (Kelly, et al., 2006). In the following chapter, an overview of the plasmin system, factors affecting its action and its impact on dairy products are given.

4.2 The plasmin system in milk

4.2.1 Components and localisation of the plasmin system in milk

The major indigenous milk protease plasmin (EC 3.4.21.7) is a serine protease with a pH optimum of 7.5 at 37 °C (Grufferty & Fox, 1988b). Plasmin in milk is mainly present in its inactive form, plasminogen, which can be activated by tissue-type (tPA) or urokinase type (uPA) plasminogen activator (Bastian & Brown, 1996). The plasminogen concentration in fresh milk is 0.18-2.8 mg L⁻¹ and 2-30 times higher compared to plasmin (0.1-0.7 mg L⁻¹) (Benfeldt, Connie, et al., 1995; Ozen, et al., 2003; Richardson, B. C. & Pearce, 1981). Furthermore, the system is regulated by the presence of inhibitors for plasmin and plasminogen activators (Figure 1). Plasminogen is not expressed in the mammary gland and comparison of the amino acid sequence of plasmin and plasminogen from blood and milk show these to be identical from the two sources and it is thus demonstrated that plasmin originates from the blood (Benfeldt, Connie, et al., 1995; Berglund, et al., 1995).
The physiological role of plasmin in blood is fibrinolysis, i.e. the solubilisation of fibrin clots, a precisely regulated process that mainly depends on the binding of the enzymes to their substrate fibrin (Cesarman-Maus & Hajjar, 2005). In milk, plasmin, plasminogen and tPA are mainly bound to the casein micelles (Baer, et al., 1994; Benfeldt, Connie, et al., 1995; Haissat, et al., 1994; Politis, et al., 1992; Politis, et al., 1994; Wang, L., et al., 2006; White, et al., 1995). uPA, on the other hand, has been shown to be associated with somatic cells by uPA receptors (Heegaard, Rasmussen, et al., 1994) and upon release from the somatic cells associates with the casein micelles as well (Heegaard, Rasmussen, et al., 1994; Lu, D. D. & Nielsen, 1993a; White, et al., 1995). The inhibitors of the plasmin system are located in the serum phase of milk (Christensen, S., et al., 1995; Precetti, et al., 1997).
4.2.2 Molecular features of plasmin and plasminogen

Bovine plasminogen has a calculated molecular mass of 88,092 Da comprising 786 amino acid residues and is glycosylated at two positions (Marti, et al., 1988; Schaller, et al., 1985). Plasminogen consists of an N-terminal pre-activation peptide, 5 homologous kringle structures and a C-terminal catalytic centre (Figure 2). The overall structure is stabilized by disulphide bonds. The kringle structures contain lysine binding sites with different affinities for lysine, by which plasminogen and plasmin bind to lysine residues in the casein micelles (Bastian & Brown, 1996; Benfeldt, Connie, et al., 1995). The amino acid composition of human and bovine plasminogen differs, but the position of the cysteine residues is the same and based on the sequences the kringles are homologous, indicating their structure is conserved (Bastian & Brown, 1996; Schaller, et al., 1985).

![Figure 2: Schematic representation of plasminogen with the pre-activation peptide (PAP), kringle structures (K1-K5) and light chain containing the catalytic center. The cleavage sites and enzymes involved in the activation of plasminogen are indicated in red. Disulphide bonds are shown as dashed lines. Modified from Benfeldt et al. (1995).](image-url)
The crystal structure of human plasminogen kringle 4 in complex with the lysine analogue ε-aminocaproic acid (EACA) is shown in Figure 3. As can be seen, the ε-amino group of lysine or EACA is stabilized by two aspartic acid residues. Additionally, the hydrophobic C₄ chain is stabilized by two phenylalanine and one tryptophan residue (Ponting, et al., 1992; Wu, et al., 1991). The binding of lysine or EACA to the kringle structures further results in a conformational change of the kringle structures (Wu, et al., 1991). Binding to lysine residues or lysine analogues also affects the entire plasminogen molecule; from a compact and folded to a looser, unfolded one (Hayes, K. D., et al., 2003; Mangel, et al., 1990; Markus, 1996). The electrostatic nature of the lysine binding sites can explain why plasmin is not dissociated from the casein micelles at low temperatures. On the other hand, addition of 1 M NaCl or at pH values < 4.6 plasmin dissociates from the casein micelles which is the cause for the higher plasmin activity in acid whey compared to sweet whey (Crudden & Kelly, 2003; Grufferty & Fox, 1988b; Kelly & McSweeney, 2003).

![Figure 3](image-url)

**Figure 3: The refined structure of the ε-aminocaproic acid complex of human plasminogen kringle 4. The amino acids of plasminogen kringle 4 involved in the complexion of ε-aminocaproic acid are annotated (Wu, et al., 1991).**

The catalytic centre of plasmin is located in the C-terminal region and contains the serine protease specific catalytic triad consisting of His-Asp-Ser located at the amino acid residues 598, 641 and 736, respectively (Hedstrom, 2002; Schaller, et al., 1985).
4.2.3 Activation of plasminogen

Plasminogen is activated by conversion from a single chain zymogen to a two chain active enzyme connected by disulphide bonds by cleavage of the peptide bond Arg<sub>557</sub>-Ile<sub>558</sub> in plasminogen (Bastian & Brown, 1996). Plasmin is able to cleave plasminogen at Lys<sub>77</sub>-Arg<sub>78</sub> which yields the so called pre-activation peptide. This cleavage results in a conformational change of plasminogen in which the Arg<sub>557</sub>-Ile<sub>558</sub> bond is exposed. The rate of activation of plasminogen is therefore increased in the presence of even small amounts of plasmin (Bastian & Brown, 1996; Cesaran-Maus & Hajjar, 2005).

Both uPA and tPA are serine proteases and both show some homology with plasmin. In the human system, both activators exist in both a single and a two-chain form, with different activation efficiency (Cesarman-Maus & Hajjar, 2005). Activation of plasminogen by tPA in blood is weak in absence of fibrin. The activation of plasminogen by tPA increases by several orders of magnitude when both are bound to fibrin in blood or casein in milk, respectively (Cesarman-Maus & Hajjar, 2005; Markus, et al., 1993). Especially the α<sub>S2</sub>-casein dimer and κ-casein multimers are shown to accelerate the activation of plasminogen by tPA (Heegaard, Rasmussen, et al., 1994; Heegaard, et al., 1997). In contrast to tPA, uPA does not bind to fibrin and the rate of activation by uPA is not affected by the presence of fibrin (Lu, D. D. & Nielsen, 1993a; White, et al., 1995). Casein has been shown to enhance the activation of human plasminogen by uPA, which is most likely caused by the conformational change in plasminogen upon occupation of lysine binding sites as described above (Baer, et al., 1994; Markus, et al., 1993; Politis, et al., 1995). In model studies, both native and denatured α-la and denatured β-lg stimulated the activation of bovine plasminogen by human uPA. The enhanced rate of plasminogen activation was suggested to be caused by a faster release of plasmin from the plasminogen-uPA complex in the presence of whey proteins (Rippel, et al., 2004).

Benfeldt et al. (1995) purified and characterised a low molecular weight plasmin/plasminogen with a molecular weight of 50 kDa. This so-called midi-plasminogen/midi-plasmin was hydrolysed between Arg<sub>343</sub>-Met<sub>344</sub> and consisted only of kringle 4 and 5 and the light chain. The cleavage was suggested to be autocatalytic by plasmin or uPA. This midi plasminogen was especially found in the serum phase, suggesting that the loss of kringle 1-3 reduced the binding to caseins. Whether the full or midi form of plasmin and plasminogen are the predominant form in milk is unknown since the concentration of midi plasminogen could not be determined, but it was suggested that the midi-plasmin form was the major form of the bovine plasmin molecule in milk (Benfeldt, Connie, et al., 1995).
4.2.4 Inhibitors of plasmin and plasminogen activators

There are at least six inhibitors of plasmin present in milk: α2-antiplasmin, α2-macroglobulin, antithrombin III, C1-inhibitor, inter-α-trypsin inhibitor and bovine plasma-trypsin inhibitor (Christensen, S., et al., 1995). The two most important milk inhibitors are the plasmin specific α2-antiplasmin and the non-specific α2-macroglobulin (Kelly & McSweeney, 2003). The α2-antiplasmin belongs to the inhibitor family of serpins. All serpins form an irreversible complex with serine proteases, which is followed by a cleavage of the inhibitor, and both the protease and inhibitor lose their activities (Cesarman-Maus & Hajjar, 2005; Christensen, S. & Sottrup-Jensen, 1992; Wiman & Collen, 1978). The function of α2-antiplasmin in blood is the inhibition and inactivation of potentially harmful plasmin being released from fibrin clots. The formation of the plasmin-α2-antiplasmin complex requires a free lysine binding site on plasmin. Christensen et al. (1992) found that α2-antiplasmin inhibits both bovine plasmin and midi plasmin, suggesting that α2-antiplasmin binds to the lysine binding site in kringle 4 of plasmin. The reaction involves a very fast first step in which α2-antiplasmin binds to plasmin and a slower second step in which the irreversible complex is formed (Christensen, U., et al., 1996; Wiman & Collen, 1978). Prado et al. (2006) found a competitive inhibition of plasmin in a plasmin inhibitor extract from milk containing α2-antiplasmin. Since the inhibitor was added to a mix of plasmin and substrate, the measured inhibition was most likely only the first step of α2-antiplasmin binding to plasmin. Based on their molecular weights, plasmin and plasmin inhibitor are present in an equimolar concentration in fresh milk (Prado, et al., 2006; Precetti, et al., 1997). Two types of fast reacting plasminogen activator inhibitors (PAI-I and PAI-II) have been identified in milk (Kelly & McSweeney, 2003).

Besides specific protease inhibitors, plasmin can be inhibited by whey proteins. Both native and denatured forms of β-lactoglobulin act as inhibitors of plasmin activity towards a synthetic substrate and casein (Bastian, et al., 1993). Besides β-lg, also α-lactalbumin (α-la) and bovine serum albumin (BSA) inhibited plasmin as well. The variant β-lg A is shown to have a higher inhibitory effect than β-lg B (Politis, et al., 1993). This difference is surprising since the two variants differ only in two amino acid residues. Whether this finding is genuine or an artefact of the assay still needs verification. The mechanism of inhibition of plasmin by whey proteins, however, is still unknown. In case of a competitive inhibition of plasmin by β-lg, even small changes in the amino acid sequence could affect the binding of β-lg to the substrate binding site and explain the findings of Politis et al (1993), which has been shown on the catalytic efficiency of human plasmin towards similar peptide sequences (Hervio, et al., 2000). It is important to note
that the inhibitory effect of the whey proteins is different when synthetic substrates or casein is used as a substrate (Hayes, M. G., et al., 2002).

4.2.1 Specificity of plasmin

Plasmin shows the same specificity as trypsin and hydrolyses peptide bonds on the C-terminal side of lysine and, to a lesser extent, after arginine (Kelly & McSweeney, 2003). The primary substrate for plasmin in milk is β-casein. Plasmin hydrolyses β-casein primarily at Lys\textsubscript{28}-Lys\textsubscript{29}, Lys\textsubscript{105}-His\textsubscript{106} and Lys\textsubscript{107}-Glu\textsubscript{108} which results in the so-called proteose peptones and γ-caseins, derived from C- and N-terminal part of the β-casein molecule (Eigel, et al., 1984). The nomenclature and sequence of these polypeptides are given in Table 2.

**Table 2: γ-caseins and proteose peptones (PP) of β-casein by plasmin (Eigel, et al., 1984).**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP 8-fast</td>
<td>1-28</td>
</tr>
<tr>
<td>PP 8 slow</td>
<td>29-105, 29-107</td>
</tr>
<tr>
<td>PP component 5</td>
<td>1-105, 1-107</td>
</tr>
<tr>
<td>γ\textsubscript{1}-casein</td>
<td>29-209</td>
</tr>
<tr>
<td>γ\textsubscript{2}-casein</td>
<td>106-209</td>
</tr>
<tr>
<td>γ\textsubscript{3}-casein</td>
<td>108-209</td>
</tr>
</tbody>
</table>
Cleavage occurs also at Lys\textsubscript{113}-Tyr\textsubscript{114} and Arg\textsubscript{183}-Asp\textsubscript{184} (Kelly & McSweeney, 2003). In Paper III, we did not find any cleavage by plasmin at Arg\textsubscript{183}-Asp\textsubscript{184} in β-casein, but rather at Lys\textsubscript{169}-Ala\textsubscript{170} and Lys\textsubscript{176}-Ala\textsubscript{177}. Furthermore, the peptides identified in Paper III showed that plasmin hydrolysed β-casein in UHT milk at Lys\textsubscript{29}-Ile\textsubscript{30}, Lys\textsubscript{32}-Phe\textsubscript{33}, Lys\textsubscript{48}-Ile\textsubscript{49}, Lys\textsubscript{97}-Ala\textsubscript{98} and Lys\textsubscript{99}-Glu\textsubscript{100} (Figure 4), suggesting that the proteose peptone fraction is more heterogeneous than previously reported.

Figure 4: Primary structure of the bovine β-casein variant A\textsuperscript{2}5P with plasmin cleavage sites described in literature and Paper III indicated by arrows (Kelly & McSweeney, 2003). The cleavage site at Arg\textsubscript{183}-Asp\textsubscript{184} was not found in Paper III and is indicated red.

The storage time after which the peptides were identified and the rate of peptide formation indicated that the plasmin initially hydrolysed at Lys\textsubscript{105}-His\textsubscript{106} and Lys\textsubscript{107}-Glu\textsubscript{108} and sequentially continued to hydrolyse β-casein further around these cleavage sites at Lys\textsubscript{97}-Ala\textsubscript{98}, Lys\textsubscript{99}-Glu\textsubscript{100} and Lys\textsubscript{113}-Tyr\textsubscript{114} (Paper III). The only potential cleavage site in β-casein, which is hydrolysed by trypsin, but not plasmin, seems to be Arg\textsubscript{202}-Gly\textsubscript{203} (Bumberger & Belitz, 1993).
Plasmin hydrolyses $\alpha_{S2}$-casein at approximately the same rate as $\beta$-casein (Kelly & McSweeney, 2003). The major previously reported cleavage sites include Lys$_{21}$-Gln$_{22}$, Arg$_{114}$-Asn$_{115}$, Lys$_{149}$-Lys$_{150}$, Lys$_{150}$-Thr$_{151}$, Lys$_{181}$-Thr$_{182}$, Lys$_{188}$-Ala$_{189}$ and Lys$_{197}$-Thr$_{198}$ (Le Bars & Gripon, 1989; Visser, et al., 1989). By now, several additional cleavage sites have been identified from peptides occurring in the indigenous peptide profile of milk (Baum, Fedorova, et al., 2013; Dallas, et al., 2013). In Paper III, apart from known cleavage sites, we identified six additional plasmin specific cleavage sites in $\alpha_{S2}$-casein that have not been described before (Figure 5). The reason for the relatively scarce reports on cleavage sites in the literature for $\alpha_{S2}$-casein may be attributed to its relatively low concentration in milk and the dimeric nature of $\alpha_{S2}$-casein, which complicates peptide analysis of $\alpha_{S2}$-casein hydrolysates. The high and varying degree of phosphorylations on $\alpha_{S2}$-casein further impedes mass spectrometry analysis of $\alpha_{S2}$-casein peptides due to a reduced concentration of identical peptides varying in phosphorylations and the reduced ionisation and fragmentation ability of phosphorylated peptides (Baum, Ebner, et al., 2013; Tauzin, et al., 2003).

![Figure 5: Primary structure of bovine $\alpha_{S2}$-casein A 11P with plasmin cleavage sites reported in literature and Paper III are indicated by arrows (Baum, Fedorova, et al., 2013; Dallas, et al., 2013; Le Bars & Gripon, 1989). The novel plasmin cleavage sites in $\alpha_{S2}$-casein reported in Paper III are indicated as grey arrows.](image)

The activity of plasmin towards $\alpha_{S1}$-casein is lower compared to $\beta$-casein (Andrews & Alichanidis, 1983; Gazi, et al., 2014), which was also observed in Paper II. The major cleavage sites found for isolated $\alpha_{S1}$-casein are Arg$_{22}$-Phe$_{23}$, Arg$_{90}$-Tyr$_{91}$, Lys$_{102}$-Lys$_{103}$, Lys$_{103}$-Tyr$_{104}$, Lys$_{105}$-Val$_{106}$, Lys$_{124}$-Glu$_{125}$ and Arg$_{151}$-Gln$_{152}$ (Le Bars & Gripon, 1993; McSweeney, et al., 1993). In Paper III, we could not find indications for a cleavage at Arg$_{151}$-Gln$_{152}$ (Figure 6).
which is most likely due to a difference in the accessibility of cleavage sites on isolated and micellar casein, respectively. The region of α_{S1}-casein, which includes Arg\textsubscript{151}-Gln\textsubscript{152}, is hydrophobic and could be inaccessible for plasmin in a micellar system. The importance of hydrophobic domains of the caseins was also shown in Paper III for the N-terminal peptide of α_{S1}-casein formed by plasmin α\textsubscript{S1}-casein f(1-22). Relative quantification of peptide concentrations measured by UV and corrected for their calculated molar absorption coefficient further showed that Arg\textsubscript{22}-Phe\textsubscript{23} is not a major cleavage site in micellar casein, but that the initial hydrolysis of α_{S1}-casein is in the region 90-105 and Lys\textsubscript{124}-Glu\textsubscript{125} (Paper III). This is in line with the findings in Paper II, where α\textsubscript{S1}-casein f(1-124) and α\textsubscript{S1}-casein f(125-199) were the two large polypeptides present early in the storage period.

Plasmin has little activity on κ-casein compared to the other caseins. Reports on κ-casein hydrolysis by plasmin vary and the specificity has not been determined (Bastian & Brown, 1996; Dalsgaard, et al., 2007; Kelly & McSweeney, 2003). The low activity could be caused by the presence of carbohydrate moieties on κ-casein and its polymeric nature similar to α_{S2}-casein (Bastian & Brown, 1996).

Only few studies have looked into plasmin activity on whey proteins. In general, whey proteins are rather resistant to plasmin hydrolysis, although hydrolysis of BSA, lactoferrin, β-lg and α-la has been reported (Dalsgaard, et al., 2007; Hayes, M. G., et al., 2002; Kelly & McSweeney, 2003). In the peptidomic study conducted in Paper III, we identified 6 peptides originating from...
β-lg. The cleavage sites were Lys8-Gly9, Lys71-Thr72, Lys75-Ile76, Lys71-Thr72, Lys83-Ile84 and Lys91-Val92, which were described previously by (Caessens, et al., 1999). Plasmin hydrolysis of α-la has been reported in model studies (Dalsgaard, et al., 2008). The resistance of whey proteins towards hydrolysis is most likely due to their globular structure. The β-lg hydrolysed in the UHT milk of Paper II and Paper III was partially denatured and thus the structure was altered and easier to hydrolyse. The low levels of α-la denaturation in the UHT milk could explain the absence of α-la peptides.

4.2.2 Factors affecting the plasmin system

4.2.2.1 Environmental factors

Activity of the plasmin system in milk can be affected by either changes in the transport of its components from blood to milk or differences in the activation of plasminogen (Kelly & McSweeney, 2003). Furthermore, presence of inhibitors of either plasmin or plasminogen activator inhibitors will affect the net activity of plasmin present. Different breeds of cattle show variations in plasmin activity with milk from Jersey cows having a lower plasmin activity than milk from Friesian and Montbeliard cows (Benslimane, et al., 1990; Richardson, B. C., 1983b). Plasmin activity has also been found to be increased in milk from older cows (Kelly & McSweeney, 2003).

The stage of lactation can largely affect plasmin activity. Plasmin activity decreases and plasminogen activity increases in milk from the early lactation phase after calving (Benslimane, et al., 1990; Kelly & McSweeney, 2003). In late lactation milk, both plasmin, plasminogen and plasminogen activator activity are increased (Baldi, et al., 1996; Richardson, B. C., 1983b). The increased levels of plasmin and activation of plasminogen in late lactation milk have been linked to involution of the mammary gland (Politis, Lachance, et al., 1989; Politis, 1996).

Plasmin, plasminogen and plasminogen activator levels are increased in mastitic milk (Kelly & McSweeney, 2003). This could be caused by an increased transfer of plasmin system components from the blood into the milk during mastitis due to compromised barrier between milk and blood as well as elevated plasminogen activator levels attributed to increased levels of somatic cells (Heegaard, Christensen, et al., 1994; Heegaard, Rasmussen, et al., 1994; Politis, Ng Kwai Hang, et al., 1989). In general, there is a positive correlation of plasmin activity with the somatic cell count (Albenzio, et al., 2004; Kennedy & Kelly, 1997; Politis, Ng Kwai Hang, et al., 1989).

Besides mastitis, bacteria present in milk can affect the plasmin system as well. Proteases from psychotrophic bacteria are able to release plasmin and plasminogen from the casein micelles into the serum phase and are capable of inactivating plasmin (Fajardo-Lira & Nielsen, 1998; Frohbieter, et al., 2005). While most bacteria do not activate plasminogen, a protease from
*Bacillus polymyxa* shows plasminogen activator like activity (Larson, et al., 2006; Nielsen, S. S., 2002; Verdi & Barbano, 1991). A protease from *Pseudomonas fluorescens* does not activate plasmin itself, but is capable of increasing the catalytic efficiency of uPA by converting it into its two chain form (Guinot-Thomas, et al., 1995).

4.2.2.2 Effect of milk processing on the plasmin system

Plasmin and plasminogen activators can be active during cold storage of milk resulting in increased proteolysis and levels of plasmin activity (Ismail & Nielsen, 2010; Somers, et al., 2002). Storage of milk at 5 °C, on the other hand, results in decreased levels of plasmin activity caused by autolysis of plasmin compared to milk stored at room temperature (Crudden, Fox, et al., 2005). Autolysis has previously been observed for isolated plasmin fractions and autolysis can be reduced by addition of lysine analogues or providing a protein network for plasmin (Saint-Denis, et al., 2001b; Ueshima, et al., 1996). The plasmin concentration used in these studies however is much higher compared to concentration of plasmin in milk and the casein in milk provides a suitable protein network to stabilize plasmin against autolysis. Most studies reporting autolysis of plasmin in milk use raw milk (or pressure treated milk) in their experiments (Crudden, Fox, et al., 2005; Guinot-Thomas, et al., 1995; Huppertz, et al., 2004). The binding of plasmin to casein is a reversible process and the decrease in plasmin activity could also be attributed to irreversible inhibition of released plasmin by plasmin inhibitors. No evidence for autolysis or irreversible inhibition exists in literature, but could help to understand the dynamics of the plasmin system during the storage of milk. Identification of either plasmin and plasminogen fragments or the presence of the 10 kDa peptide originating from the N-terminal part of α2-antiplasmin upon inhibition/inactivation of plasmin in stored raw milk could solve this question.

Release of uPA from somatic cells could occur during storage of raw milk and prolonged storage could result in increased levels of uPA in milk. The effect of milk separation, bactofugation and spore filtration on uPA release from somatic cells has not been studied yet, but is expected to significantly affect the levels of uPA in milk.

Heat treatment of milk greatly affects the plasmin system. Plasmin, plasminogen and plasminogen activators are generally considered as heat stable, while the inhibitors of plasmin and plasminogen activators are relatively heat labile. Low-pasteurisation conditions (72-75°C for 15 s) do not significantly affect plasmin, plasminogen and plasminogen activators (Ismail & Nielsen, 2010). In fact, pasteurisation results in an increased plasmin activity due to the inactivation of plasmin inhibitors and plasminogen activator inhibitors (Prado, et al., 2006;
Richardson, B. C., 1983a). Plasminogen activation during cold storage of pasteurized milk is thus increased compared to raw milk (Lu, R., et al., 2009). Plasmin inhibitors have been shown to have a higher heat stability than plasminogen activator inhibitors (Prado, et al., 2006).

We examined the effect of heat treatment at 72 °C on plasmin activity in a small experiment as part of the method development for the plasmin assay (Paper I). Plasmin activity in the milk and the serum phase decreased slightly (Figure 7). While the recovery rate of added plasmin in the milk was constant during the heat treatment, the recovery rate in the serum increased by almost 10 % with increasing holding time. The increase in recovered activity most likely represented the inactivation of plasmin inhibitors during the heat treatment. The change in recovered activity fitted well to a first order reaction, indicating that this method could be used for a kinetic description of inactivation of plasmin inhibitors. Since the focus of the PhD project was on UHT milk, the inactivation of plasmin inhibitors was not pursued further in the project.

The increased plasmin activity after pasteurisation could also be attributed to the denaturation of plasminogen (temperature range 50.1-61.7 °C), which increases the activation by uPA (Burbrink & Hayes, 2006). Inactivation of plasmin and plasminogen during heat treatment mainly occurs via thiol-disulphide interchange reactions with denatured β-lg (Rollema & Poll, 1986). The thermal inactivation of plasmin, plasminogen and plasminogen activators will be covered in detail in section 6.3.2.

![Figure 7: Effect of heat treatment at 72 °C for up to 195 s on plasmin activity (A) and recovery rate of added plasmin (B) in skimmed raw milk (○) and the serum phase (●) obtained by ultracentrifugation (10^5 rcf for 60 min) after the heat treatment. Bars indicate standard deviation, n = 3;](image_url)
High pressure homogenisation has been shown to inactivate plasmin and plasminogen (Hayes, M. G. & Kelly, 2003). On the other side, more recent studies have indicated that these first findings were an artefact of the plasmin assay used in this study, which included a centrifugation step to remove the cream (and plasmin attached to it). High pressure homogenisation seems to have only a little effect on plasmin activity itself at elevated pressure (20 % plasmin inactivation at 200 MPa; Iucci, et al., 2008).

High pressure treatment on the other hand has been shown to significantly affect the plasmin system. High pressure treatment at room temperature up to 400 MPa does not inactivate plasmin (García-Risco, et al., 2003; Huppertz, et al., 2004) and treatment at 300-400 MPa even promotes plasmin activity (Huppertz, et al., 2004). Plasmin activity decreases after high pressure treatments at 600 MPa. The increase in plasmin activity could be attributed to disruption of casein micelles and transfer of plasmin system components into the serum phase, while the inactivation of plasmin at higher pressures could be caused by pressure induced denaturation of β-lg (García-Risco, et al., 2003; Hinrichs & Rademacher, 2004; Huppertz, et al., 2004; Moatsou, et al., 2008). Studies on the pressure-temperature stability of isolated plasmin indicated a stabilizing effect of plasmin at pressures >600 MPa and temperatures <50 °C (Borda, et al., 2004).

Cheeses made from milk concentrated by ultrafiltration have reduced plasmin activity and reduced rates of proteolysis (Benfeldt, C., 2006). The decrease is partially ascribed to the retention of whey proteins and inhibitors of plasmin and plasminogen activators during the ultrafiltration and subsequent inclusion in the cheese curd. The time-temperature conditions and presence of air could have affected the plasmin system, i.e. the inactivation of plasminogen activators (Benfeldt, C., 2006). During microfiltration and diafiltration, most plasmin and plasminogen remain in the retentate and exhibit an increased activity since whey proteins and inhibitors are removed by the microfiltration (Aaltonen & Ollikainen, 2011). We found similar trends in a pilot scale extended diafiltration (10 diafiltration steps, 20°C, pasteurised skim milk).
Plasmin activity varied during the diafiltration process, which could mostly be due to varying casein concentrations in the casein retentate (Figure 8A). After correction for casein concentration, plasmin activity increased by 35-37 % after five diafiltration steps. More than 95 % of whey proteins were removed from the casein retentate after four diafiltration steps. The plasmin activity in the permeate was much lower compared to the retentate and stayed constant during the filtration. The recovery rate in the permeate on the other hand decreased with increasing whey protein concentration, indicating that small amounts of plasmin dissociated form casein micelles and permeated. The recovery rate in the casein retentate increased rapidly (Figure 8B) until the whey proteins were removed. The further increase in recovery rate could be attributed to a slower permeation of plasmin inhibitors with a higher molecular weight than whey proteins. As reported by Aaltonen & Ollikainen (2011), we also found a linear correlation between whey protein concentration and plasmin activity.

**Figure 8:** Effect of diafiltration of pasteurized milk on plasmin activity (A) and recovery rate of plasmin activity (B) in the casein/retentate (□) and serum whey/permeate fraction (●). Bars indicate standard deviation, n = 3.
5 Measurement of the plasmin system activity

There are several possibilities to measure enzymes in milk. Enzyme concentration is usually measured by immunoassays such as enzyme-linked immune sorbent assay (ELISA), which are (in some cases) resistant to inhibitory and interfering compounds. A second possibility are activity measurements which aim to measure the effective enzyme activity in samples and take inhibitory effects into account. A third possibility is the measurement of products produced by the enzyme (Kelly & Fox, 2006). The most optimal method depends on the aim of the measurement. ELISA gives the concentration of the enzyme, but might not differentiate between active and inactive enzymes. Activity measurements usually use synthetic substrates and are largely affected by the buffers in use and the sample preparation. Analysis of enzyme reaction products might give the most unadulterated view of the effect of enzyme activity in a product, but the analysis of these products can be complex and, in the case of low enzyme activity, very time consuming. In the following, different ways of measuring the plasmin system will be presented and discussed.

5.1 Activity assays

5.1.1 Plasmin and plasminogen

Activity assays for plasmin use a synthetic substrate, i.e. small peptides containing a plasmin sensitive bond. Upon cleavage, a fluorescent or coloured product is released, which then can be quantified by measuring fluorescence or light absorbance (Figure 9). Synthetic substrates for plasmin are N-Suc-Ala-Phe-Lys-7-amido-4-methyl coumarin (fluorescence), D-Val-Leu-Lys-p-nitroanilide (S-2251, colorimetric) and HD-Norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide (Spectrozyme-PL, colorimetric).

![Figure 9: Schematic representation of the release of yellow p-nitroalnine by cleavage of S-2251 by plasmin.](image-url)
Plasminogen (derived) activity is measured as plasmin activity after activation of plasminogen. The difference between plasmin and plasmin plus plasminogen derived activity is used to calculate plasminogen activity.

Besides the different substrates, a variety of buffers and sample preparations are used, which can affect the outcome of the measurement. It is obvious that there are difficulties in comparing results obtained by different assays and, maybe even more important, to infer to the characteristics of the plasmin system in milk from these measurements. This problem was already addressed by Kelly et al. (2006), who recommended a standardisation and comparison of plasmin activity assays.

A considerable part of this PhD study dealt with the effect of buffer, sample composition and preparation on the outcome of plasmin activity measurements. Some of the key results were published in Paper I.

5.1.1.1 Effect of sample preparation on plasmin activity
Spectrophotometric and fluorescence analyses generally require a clear sample with as little scattering and quenching as possible. In milk, the main components that interfere with these measurements are casein micelles and fat. Accordingly, most described assays use sodium citrate or ethylenediaminetetraacetic acid (EDTA) to dissociate the casein micelles by complexion of calcium and calcium phosphate on skimmed samples. These additions do not dissociate the casein micelles completely, but results in small particles with sizes between 15 and 50 nm (McMahon & Oommen, 2013). Plasmin and plasminogen are most likely still bound to these particles, since the pH values of sodium citrate and EDTA solutions are usually above pH 4.6. In a preliminary study in the present work, no significant differences between the treatment with ε-amino caproic acid (EACA) or sodium citrate on plasmin activity were observed (results not shown), concluding that both reagents can be used.

A different approach is the incubation of the sample with substrate and the use of Clarifying Reagent to remove the turbidity before measurement (Saint-Denis, et al., 2001b). Politis et al. (1993) used EACA to release plasmin and plasminogen from the casein micelles and removed them by ultracentrifugation, resulting in a clear sample containing only plasm in and plasminogen. In order to avoid turbidity caused by fat, plasmin analysis is either carried out on skim milk samples (Rollema, et al., 1983) or includes a centrifugation step to remove the fat (Richardson, B. C. & Pearce, 1981). Using the assay described in Paper I, we were able to measure plasmin activity without removal of the fat phase. This was possible by correcting for turbidity using a second wavelength that represented the background (Prado, et al., 2006). The results showed that removal of the fat did not affect plasmin activity in unhomogenised raw milk, but the plasmin
activity decreased, when the fat was removed from pasteurized, homogenized milk. Upon homogenisation, the milk fat globule membrane (MFGM) becomes to consist to a considerable degree of caseins and whey proteins (Cano-Ruiz & Richter, 1997; Kessler, 2002). Removal of the cream phase thus results in the removal of plasmin associated with caseins on the milk fat globules. The same effect was observed when the surfactant Tween was added prior to the removal of fat of homogenized milk (Iucci, et al., 2008). As a result, the decrease in plasmin activity during high pressure homogenisation was found to be an artefact of the sample preparation rather than a real inactivation of plasmin (Hayes, M. G. & Kelly, 2003; Iucci, et al., 2008). This shows how important the consideration of interaction of plasmin, milk components and milk processing are for the design of an appropriate plasmin activity assay.

In milk samples subjected to extreme heat treatment at 90 °C for 5 -30 min, large protein aggregates were formed and interfered with the assay presented in Paper I. These aggregates sedimented during the performance of the assay within 2 h and due to the correction for turbidity, an apparent increase in absorbance was observed in these samples. Although no plasmin activity is to be expected in these samples, the use of Tween and centrifugation of the sample would be a more appropriate sample preparation. At the time of the assay development, we were unfortunately not aware of the work of Iucci et al. (2008).

5.1.1.2 Effect of sample composition and buffers on plasmin activity

In addition to the previously discussed inhibition of plasmin by whey proteins and plasmin inhibitors, the plasmin activity in an assay is also affected by the caseins. Caseins are the natural substrate for plasmin in milk and thus compete with the synthetic substrate for the active site of plasmin. They have been shown to act as competitive inhibitors against the synthetic substrate, which can be overcome by increasing the substrate concentration (Bastian, et al., 1991). The state of caseins (monomeric, small aggregates or intact micelles) might affect the interfering effect due to the differences in their mobility, diffusion rate and accessibility. Saint-Denis et al. (2001a) conclude that the main inhibitory components in the plasmin assay are the caseins. This is based on a linear decrease of plasmin activity in a dilution of pasteurized milk (containing native whey proteins and inhibitors) with UHT milk (denatured whey proteins, no inhibitors) compared to a non-linear decrease of plasmin activity, when pasteurized milk is diluted with buffer, similar to the findings reported in Paper I. Our results in Paper I suggest that the whey proteins are the main inhibitory components and that the difference in inhibition by native and denatured whey protein might not be significant, when analysing milk samples. Additionally, both native and denatured whey proteins have been shown to inhibit plasmin activity (Bastian, et al., 1993; Politis, et al., 1993). This together with our results
More recent studies indicate that the inhibitory effect of whey proteins on plasmin activity is affected by the choice of substrate, as some substrates may interact with the whey proteins (Rippel, et al., 2004). The substrate, S-2251, is shown to be able to bind to whey proteins by hydrophobic interactions, while another substrate for plasmin activity, Spectrozyme-PL, is not affected by whey proteins due to its more hydrophilic nature (Rippel, et al., 2004). Using the fluorescent substrate N-Suc-Ala-Phe-Lys-7-amido-4-methyl coumarin, the whey proteins have either an inhibitory or promoting effect at pH 6.5 or pH 5.2, respectively (Hayes, M. G., et al., 2002). These studies were carried out in model systems with plasmin and isolated whey proteins. For the milk and casein system used in our study, it could be expected that the casein particles, which have considerable hydrophobic domains, would be able to adsorb the substrate S-2251 due to their higher concentration. However, the dilution of a casein solution with buffer in Paper I did not show a significant concentration dependent decrease in plasmin activity, suggesting that the inhibitory effects measured in our study reflect real inhibition rather than substrate binding of whey proteins.

![Figure 10: Measured activity of added plasmin to skimmed raw milk (○), pasteurized skim milk (●) and UHT milk (□). Activity of added plasmin was measured in the assay buffer described in Paper I containing gelatine to stabilize plasmin. Error bars indicate standard deviation, n = 3.](image-url)

Apart from these factors, the buffers used for plasmin analysis have a large impact on the results. Buffers for plasmin analysis often contain NaCl and lysine derivatives like EACA to dissociate plasmin from the caseins and to enhance plasmin activity (Bastian, et al., 1991). We showed in
**Paper I** that dissociation of plasmin from caseins resulted in a decreased interference from the caseins in the assay, but on the other hand increased the inhibition of plasmin by inhibitory components. Since EACA reduces the inhibition of plasmin by α₂-antiplasmin, the major inhibitory components were most likely the whey proteins. An inhibitory effect of plasmin inhibitors cannot be completely excluded, as can be seen in Figure 10. Addition of plasmin to different milk samples showed that for raw and pasteurized milk, a certain amount of plasmin activity disappeared, when added in low concentrations, while a constant inhibition/interference was found for UHT milk (Figure 10). This disappearance could be caused by inhibition/inactivation of added plasmin by plasmin inhibitors.

### 5.1.1.3 Comparison of activity assays

Table 3 gives an overview of plasmin and plasminogen derived activity assays described and used in literature. The early plasmin assay by Richardson & Pierce (1981) and by Rollema et al. (1983) uses a simple sample preparation, i.e. dissociation of caseins and addition of substrate in a buffer. The methods differ only by the used substrate, N-Suc-Ala-Phe-Lys-7-amido-4-methyl coumarin and S-2251 in the assays described by Richardson & Pearce (1981) and Rollema et al. (1983), respectively, and dissociation of plasmin from caseins EACA in the assay used by Rollema et al (1983). The fluorescence assay by Richardson & Pearce (1981) shows a higher sensitivity and better level of detection compared to other colorimetric assays. Fluorescence assays, on the other hand, require a more careful sample handling (i.e. light exposure, differences in pH and turbidity). Both assay types require a skimming of the sample and are affected by inhibitors of plasmin and interference from caseins.
Table 3: Overview of different plasmin and plasminogen derived activity assays described in literature. If not indicated otherwise, the sample preparation refers to milk samples.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Measurement</th>
<th>Buffer</th>
<th>Sample preparation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin, plasminogen</td>
<td>Fluorescence</td>
<td>0.4 M sodium citrate</td>
<td>• Dissociation of caseins by sodium citrate&lt;br&gt;• Centrifugation/skimming to obtain clear sample&lt;br&gt;• Mixing with buffer and addition of substrate</td>
<td>Richardson &amp; Pearce (1981)</td>
</tr>
<tr>
<td>Plasmin, plasminogen</td>
<td>Spectrometric,</td>
<td>40 mM Tris-HCl, pH 7.4, 100 mM EDTA, 50 mM EACA</td>
<td>• Addition of skimmed milk to buffer containing substrate</td>
<td>Rollema et al. (1983)</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Spectrometric,</td>
<td>50 mM Tris, 110 mM NaCl, 3 mM EACA, pH 7.4</td>
<td>• Model study&lt;br&gt;• Mixing plasmin with buffer and reagents</td>
<td>Bastian et al. (1991)</td>
</tr>
<tr>
<td>Plasmin plus</td>
<td>Spectrometric,</td>
<td>50 mM Tris-HCl, pH 8, 110 mM NaCl, 50 mM EACA</td>
<td>• Centrifugation to obtain casein/plasmin pellet&lt;br&gt;• Resuspending pellet in extraction buffer&lt;br&gt;• Centrifugation to remove caseins&lt;br&gt;• Mixing with substrate buffer</td>
<td>Politis et al. (1993)</td>
</tr>
<tr>
<td>Plasmin, plasminogen</td>
<td>Fluorescence,</td>
<td>100 mM Tris-HCl, pH 8, 0.4 M NaCl, 8 mM EACA</td>
<td>• Mixing of sample with extraction buffer (EACA and NaCl)&lt;br&gt;• Mixing of sample with substrate buffer&lt;br&gt;• Removal of turbidity and stopping of enzymatic reaction by Clarifying Reagent&lt;br&gt;• No information on fat content of milk</td>
<td>Saint-Denis et al. (2001a)</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Spectrometric,</td>
<td>50 mM Tris, 0.1 M NaCl, Tween 80, pH 7.6</td>
<td>• Model study&lt;br&gt;• Mixing plasmin with buffer and reagents&lt;br&gt;• Correction for turbidity</td>
<td>Prado et al. (2006)</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Fluorescence</td>
<td>0.4 M sodium citrate</td>
<td>• Dissociation of caseins by sodium citrate&lt;br&gt;• Centrifugation/skimming to obtain clear sample in presence of Tween</td>
<td>Iucci et al. (2008)</td>
</tr>
<tr>
<td>Plasmin, plasminogen</td>
<td>Spectrometric,</td>
<td>0.4 M sodium citrate, pH 9; 100 mM Tris-HCl, pH 8, 0.4 M NaCl, 8 mM EACA</td>
<td>• Dissociation of caseins by sodium citrate&lt;br&gt;• Dissociation of caseins and plasmin by EACA and NaCl&lt;br&gt;• Addition of substrate</td>
<td>Paper I</td>
</tr>
</tbody>
</table>
Politis et al. (1993) proposed an assay in which both inhibition and interference are eliminated. The assay includes a first ultracentrifugation step to remove serum based inhibitory components. The casein pellet is treated with EACA and NaCl to release plasmin, which is separated from the caseins in a second ultracentrifugation step before plasmin activity is measured. By this, plasmin and plasminogen derived activity can be measured without the interference and inhibition of milk components. The assay is very time consuming and it is questionable, if the assay is applicable for different sample matrices and processed products. Furthermore, the number of sample preparation steps will introduce sources of variation.

The highest sensitivity and lowest level of detection is achieved by Saint-Denis et al. (2001) using high substrate and sample concentrations. Plasmin is dissociated from the casein micelles and incubated with the substrate. Turbidity is removed by addition of Clarifying reagent. The drawback of this assay is the point wise measurement compared to a continuous measurement used in the assays previously described. The increase in fluorescence is measured at three time points, which increases the number of measurements, time intensity and a poorer precision of the increase in fluorescence. Saint-Denis et al. (2001) also propose a correction factor for the interference of caseins referring to apparent and real activity. Since the whey proteins also do have an impact on plasmin activity, this recovery rate correction includes not only the interference by caseins, but also inhibitory effects. Since the inhibition and interference cannot be distinguished, the term recovery rate may be more correct than real activity.

The assay used by Prado et al. (2006) and Rippel et al. (2004) is used in model studies, but includes a second wavelength measurement to correct for turbidity, which increases the sensitivity and LOD of colorimetric assays compared to Rollema et al. (1983).

Iucci et al. (2008) use Tween to recover plasmin associated to fat globules in homogenized milk samples, which enables the measurement of plasmin activity in homogenized samples.

The assay presented in Paper I allows for measurement of plasmin activity in turbid samples and does not require a centrifugation step. This enables analysis of plasmin activity in different dairy products and processed milk samples.

All the described assays measure plasmin activity, but the term itself is ill defined. It is used to describe the concentration of active enzyme in a sample (Politis, et al., 1993), the activity corrected for interfering effects of caseins (Bastian, et al., 1991; Saint-Denis, et al., 2001b), or the activity of plasmin including interfering and inhibitory effects (Richardson, B. C. & Pearce, 1981; Rollema, et al., 1983). Due to the complexity of the plasmin system and the changes during activity assays (dissociation of casein micelle, release of plasmin, removal of components), conclusions on plasmin in milk based on activity measurement should be considered carefully.
5.1.2 Plasminogen activators

Plasminogen activator assays are based on the addition of plasminogen and the resulting increase in plasmin activity due to activation of plasmin by plasminogen activators. Both fibrin and synthetic substrates for plasmin have been used in literature to measure plasminogen activator activity (Korycha-Dahl, et al., 1983; Lu, D. D. & Nielsen, 1993c). Most assays use plasminogen activator fractions isolated from milk. This has the advantage that a differentiation between uPA and tPA is possible. uPA, but not tPA is inhibited by amiloride, while tPA activity on the other hand is extremely low in the absence of fibrin or presence of EACA (Lu, D. D. & Nielsen, 1993c; Vassalli & Belin, 1987; White, et al., 1995). Saint-Denis et al. (2001) describe an assay to measure plasminogen activator activity directly in milk, but no differentiation between uPA and tPA is possible. Compared to the analysis of extracts, the assay by Saint-Denis et al. (2001) is closer to the actual situation in milk. Addition of amiloride to the assay could allow for a distinction between tPA and uPA. It is however unclear, if the caseins could enhance tPA sufficiently in milk to measure tPA activity (Prado, et al., 2007).

Model studies with human plasminogen showed that synthetic substrates containing lysine residues can promote plasminogen activation by uPA. At the same time, the substrate can bind to lysine binding sites on plasminogen, resulting in a conformational change in plasminogen and releasing plasminogen from a protein matrix. This would greatly decrease activation of plasminogen by tPA, (Kolev, et al., 1995).

Plasminogen of various origins (human, bovine or ovine) is used for plasminogen activator assays, making a comparison between different assays almost impossible. Additionally, the available plasminogen preparations are composed of mixtures of different forms and purities (plasminogen with or without preactivation peptide, preparations containing EACA or lysine). The heterogeneity of bovine plasminogen (midi or full) and the potential effect on activation kinetics of bovine plasminogen has not been investigated yet and should be subject of future studies.

5.2 Immunoassays

ELISA with mono- and polyclonal antibodies has been used to quantify plasminogen and plasmin (Baer, et al., 1994; Benfeldt, Connie, et al., 1995; Dupont, et al., 1997; Haissat, et al., 1994; Politis, et al., 1992). Differentiation between plasmin and plasminogen was achieved by using an antibody specific for plasminogen and plasmin and an antibody specific for plasminogen only, although a complete quantification was not possible (Dupont, et al., 1997). Benfeldt et al. (1995) report a poor response of midi plasminogen using ELISA.
ELISA and western blotting have also successfully been used for the identification of plasmin inhibitors in milk (Christensen, S., et al., 1995; Precetti, et al., 1997).

### 5.3 Other assays

A special form of activity assay used a ferrocenyl peptide with a plasmin sensitive bond bound to a gold electrode (Ohtsuka, et al., 2009). Cleavage of the peptide by plasmin resulted in a redox signal, which could enable the direct measurement of plasmin in a sample.

Fourier-transform infrared spectroscopy has been used to differentiate plasmin and plasminogen (Ozen, et al., 2003). Subtraction of caseinate and whey protein spectra coupled with multivariate data analysis allows for quantification of both plasmin and plasminogen in the presence of these proteins. The lowest concentration of the enzymes in the calibration range is 1000-fold higher than the natural concentration in milk, limiting its application for dairy products.
5.4 Proteolysis

Plasmin activity can also be measured by its action on the caseins. Measurement of proteolysis gives a real measurement of plasmin activity in milk, but underlying mechanisms such as activation of plasminogen and inhibitory effects can often not be differentiated. Furthermore, generation of proteolysis products is also influenced by substrate folding, accessibility and substrate concentration.

5.4.1 Hydrolysis of caseins

Plasmin activity is usually measured by the hydrolysis of β-casein. Proteolysis can be measured by different techniques. The most commonly used techniques include gel electrophoresis and liquid chromatography. Gel electrophoresis has the advantage that the caseins and its larger cleavage products are separated very well, but it is mainly used for qualitative purposes. Compared to liquid chromatography, the analysis is more time intensive and shows a lower dynamic range (Chove, et al., 2011). Reverse phase HPLC coupled with MS was used for the analysis of casein hydrolysis in Paper II. Figure 11 shows a comparison of UHT milk protein composition after 0 and 12 weeks of storage from the present study.

![Figure 11: Reverse phase HPLC chromatogram of UHT milk with plasmin activity after 0 (bottom) and 12 weeks (top) of storage. Caseins (cn) and plasmin hydrolysis products are indicated. A detailed description for the HPLC method is given in Paper II.](image)

After 12 weeks of storage, almost no residual intact β- and αS1-casein was present in the UHT milk with plasmin activity. Quantification of casein hydrolysis was only possible for β-casein A\textsuperscript{1} and αS1-casein 9P due to substantial overlap of the proteolysis products with the other variants and αS2-casein; a complication also reported by Gazi et al. (2014). The use of the MS signal for the quantification of intact casein is impaired by day-to-day variation of the signal intensity,
limited linearity of the signal, potential ion suppression of overlaying peaks and casein modification during storage (Paper IV). Capillary electrophoresis has been used for the analysis of caseins, but has the same limitation in terms of overlay of eluting proteins (Recio, et al., 1996).

5.4.2 Peptide formation

Instead of measuring the hydrolysis of intact caseins, proteolysis products can also be used to measure plasmin activity. Measurement of peptide formation can be divided into unspecific and specific methods. Unspecific methods include e.g. measurement of free amino terminals, like fluorescamine assay or the OPA (o-phthaldialdehyde) method (Nielsen, P. M., et al., 2001), and the measurement of pH 4.6 or TCA soluble material. Specific methods determine the concentration of plasmin specific proteolysis products.

Unspecific methods and hydrolysis of caseins can be used for analysis of plasmin activity, when the presence of other proteases can be ruled out. In many dairy products with active plasmin, other proteases are likely to be present as well. In cheese, chymosin and peptidases from starter cultures are present, while in UHT milk proteases from psychotrophic bacteria can be present. In these cases, plasmin specific peptides should be chosen to evaluate plasmin activity.

![Figure 12: Reverse phase HPLC chromatogram of UHT milk with plasmin activity after 0 (bottom) and 12 (top) wk of storage. Some plasmin specific proteolysis products are indicated. A detailed description for the HPLC method is given in Paper II.](image)

Most plasmin proteolysis products are precipitated in trichloroacetic acid (TCA) extracts, while they are soluble at pH 4.6 (Datta & Deeth, 2003). The main plasmin hydrolysis products found in pH 4.6 soluble extracts are the proteose peptones (Figure 12). In contrast, γ-caseins associate with intact caseins and precipitate at pH 4.6. Quantification of the large proteose peptones β-casein f(1-105) and f(1-107) is difficult due to their similar retention time and overlay of different
genetic variants. Additionally, these proteose peptones are further hydrolysed by plasmin. The β-casein f(1-28) is formed fast and does not show genetic variation compared to β-casein f(1-105) and f(1-107). The presence of β-casein f(1-25) in **Paper III** indicates that β-casein f(1-28) can be further hydrolysed, although at a relatively low rate. Recent studies suggest that \( \alpha_{S2}\)-casein f(1-21) and f(1-24) are suitable peptides to evaluate plasmin activity in milk (Cattaneo, et al., 2014). Both peptides are formed fast by plasmin, but \( \alpha_{S2}\)-casein f(1-24) is rapidly hydrolysed to f(1-21) (**Paper III**). Thus, \( \alpha_{S2}\)-casein f(1-21) is maybe the most suitable peptide for the evaluation of plasmin activity in milk, since it is formed quickly and is not hydrolysed further by plasmin.

### 5.4.3 Kinetic aspects of proteolysis during storage

Measurement of plasmin activity in an assay is relatively easy. Simple hydrolysis usually follows first order reaction kinetics and the substrate is present in a high concentration, which ensures that only the linear part of the kinetic is measured during the assay. The assay time is relatively short (from 10 min to 3 h); hence, changes in activity due to activation of plasminogen are insignificant. When following plasmin activity and proteolysis during storage of UHT milk activation of plasminogen, substrate affinity and substrate depletion need to be taken into account for a kinetic evaluation.

It was initially aimed for that the UHT milks used for **Paper II-IV** would contain four different plasmin activity levels, which would enable a comparison and correlation of plasmin activity measured by the plasmin activity assay of **Paper I** with proteolysis. A combination of the two analytical methods would allow for a reliable prediction of plasmin activity and proteolysis in UHT milk during storage. Unfortunately, the experiment resulted in two UHT milks without measurable plasmin activity and two UHT milks with identical plasmin activities. Kinetic modelling of proteolysis and plasmin activity in milk with different plasmin activities was therefore not possible. The UHT milk with plasmin activity used in **Paper II** and **III** permitted a basic comparison and modelling of proteolysis and plasmin activity in this specific milk.
As discussed in Paper II, plasmin activity increased during storage while plasminogen derived activity decreased. Both the increase in plasmin and decrease of plasminogen could be fitted well to a first order kinetic model (Figure 13).

The kinetic parameters for plasmin increase and plasminogen decrease were not identical. The rate of plasminogen decrease was higher than the increase in plasmin activity. As can be seen from Figure 13, all plasminogen was activated after approximately six weeks of storage. The kinetic fit for the increase in plasmin activity therefore most likely also included changes in the inhibition/interference factors in the assay, such as a decreased level of intact caseins.
The decrease in β-casein (Paper II) and increase in β- and αS2-casein N-terminal peptides (Paper III) showed a distinct sigmoidal shape. Figure 14 reveals the decrease of intact β-casein and the development of β-casein f(1-28) during storage.

![Figure 14: Development of β-casein f(1-28) (A) and decrease of intact β-casein (B) during storage. Lines represent kinetic fits using a polynomial and first order reaction kinetic.](image)

The sigmoidal shape originates from the activation of plasminogen and thus increased rate of proteolysis in the initial part of the storage period and a decrease in the rate of proteolysis during the final part of the storage period due to substrate depletion. The kinetic of peptide formation or hydrolysis of caseins should therefore include an additional factor to represent the change in enzyme concentration. The activation of plasminogen follows a first order reaction kinetic. A normal first order reaction kinetic for product formation is:

\[
C(t) = C_{\text{max}} \cdot (1 - e^{-k \cdot t}) + c
\]  

(1)

In the case of plasminogen activation, the rate constant \( k \) itself changes in a first order reaction as well (Figure 14) and could be described as:

\[
k(t) = k_{\text{max}} \cdot (1 - e^{-k_{\text{act}} \cdot t}) + c
\]  

(2)

\( k_{\text{max}} \) represents the maximum velocity of the reaction, similar to \( C_{\text{max}} \) in (1), and is dependent on the rate of plasminogen activation depicted as \( k_{\text{act}} \). This results in complex reaction kinetics, which are almost impossible to model due to the presence of two exponential functions.

Plasminogen activator assays use high amounts of added plasminogen, which allows a kinetic interpretation of plasminogen activation using a polynomial expression rather than a first order kinetic as indicated in Figure 15:

\[
y = a \cdot x^2 + b \cdot x + c
\]  

(3)

The factor \( a \) in equation (3) represents the change in reaction rate due to the activation of plasminogen, while factor \( b \) represent the initial plasmin activity present in the sample. The
proteolysis could therefore be described by using equation (3) for the initial proteolysis and equation (1) for the remaining storage time (Figure 14).

A different approach is to assume a linear kinetic for proteolysis in combination with a first order kinetic to include the increase in plasmin activity:

\[ C(t) = k \cdot (1 - e^{-k_{act}t}) \cdot t + c \]  

(4)

Both peptide formation and casein hydrolysis can be described by equation (4) by changing the prefix of \( k \). Applying this fit allows an accurate description of the reaction until the substrate depletion phenomenon appears (Figure 15).

Figure 15: Development of β-casein f(1-28) (A) and decrease of intact β-casein (B) during storage. Lines represent kinetic fits using equation (4).

This approach might be more suitable for prediction purposes since the quality of the milk would be unacceptable when substrate depletion occurs. Bitterness and gelation occurred after 70 % and > 90 % of β-casein, respectively, was hydrolysed (Paper II). When sufficient kinetic data is provided, a correlation of the plasmin and plasminogen derived activity measured by the activity assay with the kinetic of proteolysis could be established, allowing the prediction of plasmin activity and proteolysis in UHT milk.

The kinetics of αS1-casein hydrolysis and the αS1-casein N-terminal peptide f(1-22) in Paper II and III showed a different kinetic behaviour compared to β-casein and β-casein f(1-28) respectively. The decrease in intact αS1-casein in the UHT milk showed an extended “lag” phase of approximately two weeks. This could have been caused by a reduced affinity and accessibility of αS1-casein for plasmin. A similar reduced affinity was seen for αS1-casein f(1-22), but in comparison to αS1-casein f(106-124), the lag phase was identical even though αS1-casein f(106-124) requires two cleavages by plasmin to be formed. This suggests that plasmin has a reduced affinity for the cleavage at Arg22-Phe23 and a different peptide might be more suitable for the
modelling of the proteolysis. The C-terminal peptide $\alpha_{S1}$-casein f(194-199) was formed slowly and was only present in low concentrations and could not be used for modelling either. The peptide $\alpha_{S1}$-casein f(106-124) would thus be most suitable to be used for modelling, but the precursor formation would have to be included into the kinetics, resulting in one or two additional variables, hence making the modelling complex.

The decrease in intact $\alpha_{S2}$-casein could not be assessed due to overlap with proteose peptones eluting at the same time (Paper II) and a quantitative assessment using the MS signal is impaired by the heterogeneity in phosphorylations of $\alpha_{S2}$-casein. As discussed before, the N-terminal peptides of $\alpha_{S2}$-casein formed by plasmin hydrolysis are present in high concentrations and $\alpha_{S2}$-casein f(1-21) can be used for modelling.
6 UHT milk

6.1 Heat treatment of milk

The main aim of heat treatment of milk is to partially or fully inactivate microorganisms and enzymes to guarantee consumer safety and prolong the shelf life of milk. Increasing the time and/or temperature of the heat treatment results in increased inactivation of microorganisms and enzymes, but can negatively affect other milk constituents. Heat treatment of milk is therefore often a balance between inactivation of undesired components and preservation of desired properties.

Heat treatments can be categorized by their time temperature combinations, which are defined by norms and legislation. Pasteurisation can either be high temperature, short time (HTST) with heating at 72-75 °C for 15-30 s, or low temperature, long time as e.g. thermisation at 62-65 °C for 30 min. Pasteurisation results in the inactivation of pathogen microorganisms and gives a negative result when tested for alkaline phosphatase. Any other time temperature combination resulting in the same effect can be applied as well. Milk subjected to high temperature pasteurisation is heat treated at temperatures > 85 °C and should give lactoperoxidase negative test results (Kessler, 2002). Ultra high temperature (UHT) treatment is defined as high temperature (>135 °C) for a short time, usually 135-150 °C for 1-10 s. The aim of UHT treatment is to achieve commercial sterility, i.e. free from microorganisms that can grow under storage conditions (EC, 2005). The effect of UHT treatment on relevant milk constituents will be discussed later in this section.

6.2 UHT milk processing

6.2.1 Design of UHT processes

The inactivation rate of microorganisms at a given temperature is usually measured by the decimal reduction time, D, which is the time necessary to reduce the number of microorganisms by a factor of 10. The temperature effect of the inactivation of microorganisms can be expressed by the z value and the $Q_{10}$ value. The z value is defined as the change in temperature necessary to increase D by a factor of 10. The $Q_{10}$ value on the other hand is defined as the change in the inactivation rate by a temperature increase of 10 °C (Kessler, 2002). Chemical effects occurring during heat treatment can be described using similar parameters as described above. Based on these relationships, lines of equal effects, i.e. time temperature combinations resulting in the same inactivation of microorganisms or chemical changes can be defined to design UHT processes.
Kessler & Horak (1981) introduced the biological effect $B^*$ and the chemical effect $C^*$ to determine time temperature regions for UHT milk processing. A $B^*$ value of one equals the 9 decimal reduction of natural thermophilic spores, which results in a commercial sterile product (Kessler & Horak, 1981; Kessler, 2002). The negative effect of heat treatment on milk constituents can be characterized by the chemical effect or $C^*$ value. A $C^*$ value of one equals the chemical effect of boiling the product for 1 min. Similar to the $B^*$ value, a $C^*$ value can be used to define the acceptable limit of chemical changes. In milk, this limit correlates with a loss of 3 % thiamine and corresponds to a $C^*$ value of one (Kessler, 2002). Based on these two factors, the optimal time temperature conditions for UHT processes are in an area where $B^* > 1$ and $C^* < 1$ (Figure 16).

In the last decade, UHT processes with ultra-short holding times have been developed that are beyond these time temperature regimes concerning the holding time. It has been shown that UHT treatment $> 150 \degree C$ with holding times of 0.2 s result in a sufficient spore reduction to obtain a commercially sterile product (Huijs, et al., 2004; van Asselt, et al., 2008). The UHT process used in Paper II-IV ($> 150 \degree C$, $< 0.2$ s) has been validated by Arla on full scale and gave a commercially sterile product.

![Figure 16: Bacteriological $B^*$ and chemical $C^*$ effect and UHT processing area. Adapted from Kessler (2002).](image-url)
6.2.2 UHT processing systems

UHT processing systems can be divided into direct and indirect systems. In indirect systems, the product is heated by counter flow of cold milk and hot water or steam in a tubular or plate heat exchanger. In direct systems, the product is heated by directly mixing it with steam under pressure, which is later removed using vacuum cooling (Burton, 1994a; Kessler, 2002). A comparison of a direct, indirect and the direct UHT process used in the trials described in Paper II-IV is shown in Figure 17.

![Figure 17: Schematic representation of time temperature profiles of indirect (dotted line), direct (dashed line) and direct/ultra-short time (solid) UHT processes. Holding time prior (indirect) and after (direct) UHT treatment represent the homogenisation step.](image)

As can be seen from Figure 17, a large advantage of direct systems is the very fast heating rate, which is only a fraction of a second and that results in minimal chemical changes to the product compared to indirect systems. One of the disadvantages of using steam to directly heat the product are that high quality steam is required, and so far the energy recovery in the process is poor. The product is therefore usually heated to 60-80 °C by indirect heat exchangers. While in indirect systems, the energy recovery is approximately 90 %, it is only 45 % when using direct systems, making direct UHT systems costlier to operate (Burton, 1994a; Kessler, 2002; Malmgren, 2007).

The homogenisation step can be placed before or after the UHT treatment in indirect systems, while it is always placed after the UHT treatment in direct systems to avoid the formation of aggregates (Burton, 1994a; Datta, et al., 2002).
Direct UHT systems can be further distinguished into steam injection and steam infusion systems (Figure 18). In steam injection systems, the steam is “injected” as small bubbles into the product. In steam infusion systems, the product falls as a thin stream or film through a steam pressured chamber. Steam infusion is generally considered to be gentler than steam injection. During steam injection, steam bubbles condensate and collapse leading to cavitation and a homogenisation effect, whereas the steam condensates on the surface of the falling product in steam infusion systems. A homogenisation effect can be observed in direct steam infusion systems as well, most likely caused by cavitation occurring during the vacuum cooling (Burton, 1994a; Datta, et al., 2002; Hougaard, et al., 2009). Fouling is reduced in steam infusions systems since in contrast to steam injection systems the product does not come into contact with surfaces hotter than itself (Datta, et al., 2002).

Figure 18: Tetra Pak steam injector nozzle (left) and APV steam infusion chamber (right). Pictures from www.tetrapak.com and www.apv.com.

The UHT trials described in Paper II-IV were performed in the UHT pilot plant (APV/SPX, Silkeborg, Denmark) at the Arla Strategic Innovation Center in Stockholm. The pilot plant (Figure 19) is able to run indirect, steam infusion and steam injection UHT processes with a capacity of 150 l/h. Both tubular and plate heat exchangers can be used to heat or cool the product. Holding times can be controlled by an external holding cell (15-300 s). The pilot plant is equipped with a two-stage homogenizer with a maximum pressure of 40 MPa. In the direct UHT process, the temperature for the flash vacuum cooling was set to 8 °C lower than the pre-heat temperature to obtain the same dry matter content. Bottling of the milk samples was done in a sterile bench and every batch was checked for sterility according to (ISO-4833, 2003).
6.3 Heat-induced changes in milk

6.3.1 Proteins

6.3.1.1 Whey proteins

The whey proteins are a heterogeneous group of proteins that are found in the serum phase after precipitation of caseins by acidification or renneting. The effect of heat treatment on the two major whey proteins, β-lg and α-la, has been researched intensively in milk and isolated proteins under different conditions.

When looking at the denaturation of whey proteins, a distinction between reversible and irreversible denaturation has to be made. Differential scanning calorimetry measurements show that β-lg and α-la unfold and lose their tertiary globular structure at 75-80 °C and 65 °C, respectively (Paulsson, et al., 1985; Tolkach, 2007). This denaturation of the whey proteins is highly reversible. β-lg contains five cysteine residues of which four form disulphide bridges and one free cysteine residue. In its native form, the free cysteine residue is buried in the globular structure of β-lg. Upon unfolding of β-lg at higher temperatures (> 75-80 °C), the free cysteine residue and hydrophobic residues become exposed and are able to react via thiol-thiol, thiol-disulphide and hydrophobic interactions with other proteins; as a result, b-lg denatures irreversibly and forms aggregates (Anema, 2008; Sawyer, 2003). Isolated, α-la is very resistant to irreversible heat induced denaturation (Wang, Q., et al., 2006). However, it contains 4 disulphide
bonds and the irreversible denaturation is accelerated in the presence of β-lg (Anema, 2008; Tolkach, 2007). The kinetics of irreversible heat induced denaturation of α-la and β-lg consist of two reactions with different activation energies. The Arrhenius plots for α-la and β-lg show a distinct discontinuity at 80 and 90 °C, respectively. Below 80 and 90 °C, the activation energies are high, indicating that a large number of bonds are broken and the rate limiting factor for irreversible denaturation is the unfolding of the whey proteins. Above 80 and 90 °C, the activation energy is much lower and the whey proteins are completely unfolded and the speed limiting factor is the aggregation of the whey proteins (Anema, 2008; Dannenberg & Kessler, 1988; Tolkach, 2007). Besides the formation of aggregates between whey proteins, β-lg can react with κ-casein on the surface of the casein micelle via thiol-disulphide interchange reactions (Anema, 2007). Irreversibly denatured whey proteins precipitate at pH 4.6 and the degree of denaturation of whey proteins is usually measured as the concentration of pH 4.6 soluble whey proteins before and after heat treatment. While whey protein denaturation is almost negligible during HTST pasteurisation at 72 °C for 15 s, UHT treatments can result in almost complete denaturation of whey proteins. β-lg denaturation is lower in UHT milk processed with direct systems (35-80 %) than indirect systems (79-100 %) due to the faster heating rate in direct systems (Burton, 1994a; Datta, et al., 2002). Association of whey proteins with the casein micelles is lower in direct systems compared to indirect systems. This can also be attributed to the fast heating rate, i.e. short holding time. Basically, all whey proteins are instantly unfolded during direct UHT processing, giving the reactive monomers a greater opportunity to react with themselves than associating with the casein micelles (Anema, 2008). The whey protein denaturation in the UHT milks produced for Paper II-IV is shown in Table 4. At a pre-heat temperature of 72 °C, the direct steam infusion step alone resulted in 34 % β-lg and less than 10 % α-la denaturation. Prolonging the pre-heat treatment to 180 s at 72 °C increased the degree of whey protein denaturation only slightly. However, increasing the pre-heat temperature to 95 °C drastically increased the degree of whey protein denaturation. Analysis of the process showed that the main reason for the increase was caused by the difference in flash cooling temperature (8 °C lower than the pre-heat temperature) and the higher temperature in the following homogenisation step, resulting in an increased heat load (approximately 87 °C for 15-20 s). In the indirect UHT milk and the direct UHT milk 95°C/180 s + > 150°C /< 0.2 s, β-lg is almost completely denatured (Table 4). There is, however, a significant difference in the α-la denaturation, indicating that the heat damage was still lower in the direct UHT compared to the indirect.
Table 4: Whey protein denaturation in direct (pre-heat treatment + direct steam infusion step) and indirect UHT milk used for Paper II-IV. Whey protein denaturation was measured as the difference in pH 4.6 soluble whey proteins before and after heat treatment. Values for β-lg are an average of β-lg A and B. Different superscripts indicate significant differences (P < 0.05).

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Whey protein denaturation [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-lg</td>
</tr>
<tr>
<td>72°C/5 s + &gt; 150°C /&lt; 0.2 s</td>
<td>33.9 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>72°C/180 s + &gt; 150°C /&lt; 0.2 s</td>
<td>36.7 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>95°C/5 s + &gt; 150°C /&lt; 0.2 s</td>
<td>86.6 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>95°C/180 s + &gt; 150°C /&lt; 0.2 s</td>
<td>94.1 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Indirect 140 °C/4 s</td>
<td>93.6 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

6.3.1.2 Caseins

Compared to the whey proteins, the caseins and casein micelles are very heat stable and the micellar conformation is preserved even during severe heat treatments (Walstra, et al., 2006). During severe heat treatments, polymerisation of caseins can occur, which could be caused by the formation of lysinoalanine, isopeptide formation or the Maillard reaction (Zin El-Din & Aoki, 1993). The most important heat induced change of caseins during heat treatment is an increase in the casein micelle size due to the association of denatured whey proteins (Anema & Li, 2003a; Anema, 2007; Crudden, Oliveira, et al., 2005a). The increase in casein micelle size depends largely on the degree of whey protein denaturation. Only small changes are observed when less than 80 % of the whey proteins are denatured, while a fast and larger increase in micelle size is observed at higher degrees of denaturation (Anema & Li, 2003b).

6.3.2 Heat inactivation of the plasmin system

Inactivation by heat of plasmin and plasminogen in milk has been closely linked to the denaturation of β-lg and thiol-disulphide interchange reactions. Plasmin inactivation in the absence of β-lg is decreased (Rollema & Poll, 1986) and a positive correlation between the concentration of β-lg and the rate of plasmin activation has been found (Saint-Denis, et al., 2001a). Proof of the thiol-disulphide reaction as the main driving force for plasmin inactivation was provided by the increased inactivation of plasmin upon addition of cysteine (Durkee & Hayes, 2008; Lu, R., et al., 2009; Metwalli, et al., 1998; Rollema & Poll, 1986) and decreased plasmin inactivation upon blocking the free thiol group of β-lg by addition of KIO₃ (Kelly & Foley, 1997). The heat inactivation kinetics of plasmin and plasminogen in various dairy systems has been investigated by several authors (Alichanidis, et al., 1986; Crudden, Oliveira, et al.,
A selection of kinetic parameters is summarized in an Arrhenius plot in Figure 20.

![Arrhenius plot](image)

**Figure 20:** Arrhenius plot for the heat inactivation of plasmin in milk (red line) from Saint-Denis et al (2001) and plasmin-plasminogen in milk (green line) and a casein micelle solution (black line) from Rollema et al. (1986). The Arrhenius plot for heat denaturation of β-lg is indicated (dashed line) using the kinetic parameters of Dannenberg & Kessler (1988).

In milk, inactivation of plasmin and plasminogen shows a similar kinetic pattern to that of β-lg denaturation, with a discontinuity at around 95 °C, indicating that the inactivation of plasmin and plasminogen consists of two reactions with different activation energies as well. Older studies report the discontinuity at 77 °C (Driessen & van der Waals, 1978; Metwalli, et al., 1998). The difference in the kinetic reported by Rollema et al. (1986) and Saint-Denis et al. (2001b) above 90 °C could be attributed to the different assays used and the fact that the kinetics of Rollema et al. (1986) are a combined kinetic for plasmin and plasminogen. Plasminogen exhibited a lower heat stability compared to plasmin below 90 °C, but a higher stability above 90 °C is found by Rollema et al. (1986), while Saint-Denis et al. (2001b) show comparable heat stabilities of plasmin and plasminogen. Comparing the rate of β-lg denaturation with plasmin inactivation, it becomes obvious that the rate for plasmin inactivation below 90 °C is much faster compared to the denaturation rate of β-lg. Above 90 °C, the rates are very similar and, depending on the applied inactivation kinetic, β-lg denaturation is actually faster than plasmin inactivation above 120 °C according to Saint-Denis et al. (2001b). As previously discussed, interaction of β-lg with the casein micelle is less pronounced at higher temperatures. Since plasmin and plasminogen are
associated with the casein micelle, the same mechanism could apply for the inactivation of plasmin and plasminogen. A reported comparison of plasmin inactivation and β-lg association with the casein micelle suggested that the interaction of plasmin with β-lg and the β-lg association with the casein micelle was faster than aggregation of β-lg in the temperature range between 65-80 °C (Crudden, Oliveira, et al., 2005a). Direct UHT treatment with a pre-heat at 72 °C for 180 s and direct steam infusion at >150 °C for < 0.2 s resulted in an almost equal degree of β-lg denaturation and plasmin activation (Paper II).

In comparison to the inactivation kinetic of plasmin in milk, the activation energy for the inactivation of plasmin and plasminogen in a casein micelle solution is constant and the inactivation rate is decreased by a factor of 5-10 in the casein micelle solution (Rollema & Poll, 1986). The same effect has been observed for the denaturation kinetic of α-la in absence of β-lg (Tolkach, 2007; Wang, Q., et al., 2006). Based on the two step reaction kinetic consisting of an unfolding and aggregation reaction, Tolkach (2007) concluded that the unfolding of α-la should still result in a slight discontinuity of the activation energy in absence of β-g. While this is not the case for isolated α-la, Metwalli et al. (1998) found a discontinuity in the inactivation kinetic of isolated plasmin at 77 °C, indicating that the unfolding of plasmin has an effect on the inactivation of plasmin. The difference to the results of Rollema et al. (1986) could be due to the presence of caseins, which affect the inactivation of plasmin as well.

Figure 21 gives a good overview of the factors influencing plasmin inactivation in a milk system. Plasmin inactivation in the presence of sodium caseinate is reduced compared to isolated plasmin. Caseins are also able to protect plasmin against inactivation in the presence of β-lg, but not against inactivation by cysteine (Metwalli, et al., 1998). The protective effect of caseins against inactivation of plasmin is not fully understood yet. In absence of free thiol groups, caseins could bind to the lysine binding sites of plasmin, which has been shown to enhance the heat resistance of plasmin against inactivation by EACA (Grufferty & Fox, 1988a). Caseins have also been shown to act as chaperones and could reduce the heat induced denaturation and inactivation or assist in a refolding after heat treatment (He, et al., 2011; Morgan, et al., 2005; Trewick, et al., 2011; Zhang, et al., 2005). This could especially be the case for the sodium caseinate used by Metwalli et al. (1998). Metwalli et al. (1998) on the other hand showed that temperature-activity of plasmin is not affected by caseins, suggesting that caseins do not protect plasmin from unfolding. However, the unfolding and inactivation of plasmin in the absence of free thiol groups does not necessarily include the unfolding of the light chain containing the active centre, but could also involve aggregation or structural changes in the kringle structures.
In the presence of free thiol groups, a different protective effect of caseins seems to take place. The fact that caseins are able to protect plasmin against inactivation by β-lg, but not by cysteine implies a protection via a steric hindrance of the reaction between β-lg and plasmin by the caseins. In this project, an experiment was planned to study the heat stability of plasmin associated with caseins and plasmin released by NaCl and EACA. Using the protocol by Politis et al. (1993) to dissociate plasmin, we could not detect any significant difference between the inactivation rate of dissociated and associated plasmin at 80 °C in skim milk. Ultracentrifugation of the milk, however, showed that approximately 50 % of plasmin was still attached to the caseins. Due to lack of time, this could not be investigated further. Clarification of the protective effect of caseins on plasmin inactivation could help to develop processes with improved plasmin inactivation.

The fat content of milk influenced the heat inactivation of plasmin using lenient steam infusion heat treatment (Dickow, 2011). A possible explanation for this could be the relatively high number of free thiol groups on the MFGM, which could catalyse and enhance thiol-disulphide interchange reactions during heat treatment (Lee & Sherbon, 2002). Plasmin inactivation in whey was found to be very dependent on the type of whey. Plasmin inactivation in acid whey was much slower compared to sweet whey, which could partially be attributed to the difference in pH (Crudden, Oliveira, et al., 2005b). Interestingly, neither the inactivation kinetic of plasmin nor of
β-lg in acid whey showed the characteristic discontinuity found for milk (Crudden, Oliveira, et al., 2005b).

Results on the heat inactivation kinetic of plasminogen activators are inconsistent. Overall, it is known that plasminogen activators show slightly higher heat stability compared to plasmin (Lu, D. D. & Nielsen, 1993b; Saint-Denis, et al., 2001a). While Saint-Denis et al. (2001b) find the same kinetic for plasminogen activators compared to plasmin with a discontinuity at 90 °C, whereas Lu & Nielsen (1993) report a single reaction with constant activation energy. While Saint-Denis et al. (2001a) assay the plasminogen activators directly in milk, whereas Lu & Nielsen (1993) use a milk model system containing buffer and casein. The absence of β-lg can explain the difference in the inactivation kinetic. uPA and tPA contain 12 and 17 disulphide bonds respectively, indicating that the inactivation mechanism is most likely similar to plasmin and plasminogen. Heat inactivation of tPA and uPA shows that the latter is more heat stable (Prado, et al., 2007). This could be due to the higher number of disulphide bonds in tPA compared to uPA.

Similar to plasmin activity, the term ‘inactivation’ of plasmin system components is ambiguous. Thiol-disulphide interchange reaction of β-lg with the kringle structure of tPA would result in a largely decreased activity since tPA would no longer be able to bind to the caseins. The kringle structure of uPA on the other hand is not important for the binding to caseins and a reaction with β-lg would most likely not affect its activity. An assay using fibrin to enhance tPA activity (Prado, et al., 2007) thus could lead to false assumptions regarding inactivation or reduced activity of tPA. The same applies for the inactivation of plasmin. Disruption of the kringle structures by thiol-disulphide interchange reactions could release plasmin and inhibit its action on caseins, similar to the effect of EACA on plasmin activity towards fibrin. The activity in an assay using a small synthetic substrate would however not be affected.

6.3.3 Effect of cysteine on plasmin and milk proteins during heat treatment

The addition of cysteine to milk has been proposed in several studies to improve the inactivation of plasmin, however these studies do not specify the impact of cysteine addition on flavour and milk proteins (Durkee & Hayes, 2008; Rollema & Poll, 1986). The effect of cysteine or other reducing agents on milk proteins during heat treatment is largely unknown and has only been studied in relation to acid gel formation after severe heating conditions (Goddard, 1996; Nguyen, et al., 2012; Nguyen, et al., 2013). In order to evaluate the practical use of cysteine for improved plasmin inactivation, the potential changes in milk proteins need to be considered. In an initial study, low concentrations of cysteine up to 1.5 µM were added to pasteurized skim milk and heat
treated at 80 °C with holding times up to 60 s in a water bath. Plasmin inactivation increased with increasing cysteine concentration as expected (Figure 22A).

**Figure 22:** Effect of addition of 0 (●), 0.5 (○), 1 (▼) and 1.5 (△) µM cysteine on the inactivation of plasmin (A), monomeric κ-casein (B) and denaturation of β-lg (C) and α-la (D) during heat treatment at 80 °C. The sample needed approximately 10 s to heat up and the unheated sample is marked as -10 s.

A considerable inactivation of plasmin already occurred during heating up of the sample in the first 10 s. In milk with 0.5 µM cysteine, a shift in reaction kinetic was observed after 30 s, which indicated that the cysteine available for plasmin inactivation had reacted; thereafter inactivation occurred at a similar rate as in the control sample without cysteine. The protein composition was analysed as described in **Paper II**, with the exception that no reducing agent was added to the urea and sodium citrate buffer. In samples containing cysteine, a peak appeared at the retention time of unreduced κ-casein and was confirmed by MS-analysis as a monomeric κ-casein with a mass shift of -2 Da compared to the reduced κ-casein, indicating an intramolecular disulphide bond. The concentration hereof depended both on the holding time and on the cysteine concentration (Figure 22B). This indicated a decrease of multimeric κ-casein by increased formation of intramolecular disulphide bonds catalysed by cysteine as reported by Nguyen et al (2012). In the sample with 1.5 µM cysteine, the concentration of monomeric κ-casein decreased
after 60 s holding time and a different peak appeared which was identified to be κ-casein with two cysteine residues attached, suggesting a time and cysteine concentration dependent formation of monomeric κ-casein and/or monomeric κ-casein with two cysteine residues.

Surprisingly, the degree of β-lg denaturation was only slightly affected by the presence of cysteine (Figure 22C). Milk heat treated with higher cysteine concentrations showed slightly increased β-lg denaturation, but no difference could be seen for 0.5 μM cysteine addition compared with the control milk. In contrast to β-lg, the degree of α-la denaturation increased with increasing cysteine addition (Figure 22D). The difference in the denaturation of β-lg and α-la could be related to their structure and number of cysteine residues. The main mechanism for α-la denaturation in milk is the thiol-disulphide interchange of α-la with the free thiol group of β-lg and the presence of additional free thiol groups accelerates the denaturation (Tolkach, 2007). The relatively small change in the denaturation of β-lg could be attributed to the free thiol group of β-lg that might act as a catalyst to regain the native structure by releasing cysteine. Reaction of cysteine with the free thiol group of β-lg resulted in denaturation of β-lg since β-lg with an attached cysteine was present in total protein, but not in the pH 4.6 soluble material.

The differently treated milks were not evaluated sensorically, but all milk samples with added cysteine had a distinct sulphuric smell. An immediate repetition of the trial was not possible since the analysis of unreduced heat treated milk samples rendered the HPLC column unusable and no appropriate column was available. The trial showed that the addition of cysteine significantly affects milk proteins. In regards to a practical use of cysteine for the heat inactivation of plasmin in milk products, these effects need to be further studied and characterized. While the modifications and denaturation of whey proteins might not be a crucial factor for UHT milk, the modifications of κ-casein could affect the stability of the casein micelle and result in sedimentation or other quality defects.
6.3.4 Maillard reaction

The Maillard reaction is a chemical reaction of reducing sugars and amino groups. In milk, the Maillard reaction occurs mainly during the heat treatment, but also during subsequent storage, although at a much lower rate (see section 6.4.3). The main reducing sugar in milk is lactose and it reacts mainly with lysine residues on proteins since the level of free amino acids in milk is relatively low (Van Boekel, 1998). The Maillard reaction is sometimes divided into an early, an advanced and a final stage.

![Formation of the Amadori product lactulosyllysine by the reaction of a protein bound lysine with lactose. From Siciliano et al. (2013).](image)

The early stage of the Maillard reaction, depicted in Figure 23, is the condensation of the reducing sugar with the amino group and the formation of a Schiff’s base, which following Amadori rearrangement gives the Amadori product lactulosyllysine (Van Boekel, 1998).

In the advanced stage of the Maillard reaction, the Amadori product breaks down into numerous products. The final stage consists of the condensation of amino and sugar compounds to polymerized proteins and brown pigments.

The Maillard reaction can significantly affect the quality of milk and milk products. Blockade of the lysine residues reduces the nutritional value and digestibility (Dalsgaard, et al., 2007; Van Boekel, 1998). Various flavour compounds and compounds with either beneficial (antioxidative, antimicrobial) or harmful (mutagenic, allergenic) properties can be formed (Siciliano, et al., 2013; Van Boekel, 1998). Colour changes occur when brown pigments are formed. Additionally, the functionality of proteins can be changed by lactosylation, such as solubility and thermal stability (Wang & Ismail, 2012). The heat load applied during UHT processing is usually not sufficient to produce the final Maillard reaction products and colour changes. The main Maillard reaction product found in UHT milk is the Amadori product. Some advanced Maillard reaction products, such as 5-hydroxyl-2-methylfurfural (HMF), are present in UHT milk, but only in a limited amount (Birlouez-Aragon, et al., 1998; Claeys, et al., 2003, 2004).
Direct analysis of the Amadori product is difficult, as it can only be measured after enzymatic breakdown of the lactosylated proteins. The most common way to measure protein lactosylation is to measure furosine, a product that is formed by acid hydrolysis of the Amadori product (Erbersdobler & Somoza, 2007; Van Boekel, 1998). A different approach is the boiling of the lactosylated proteins in oxalic acid to induce the formation of HMF. Both methods have the disadvantage that only a part of the Amadori product is actually converted to furosine and HMF, and HMF itself is already present in milk. In addition, the sample preparation is a very time intensive process.

The formation of lactulosyllysine results in a mass change of the unmodified protein of +324 Da and can be measured by mass spectrometry (Figure 23). Several studies investigated the lactosylation of whey proteins and caseins (Czerwenka, et al., 2006; Meltretter, et al., 2009; Scaloni, et al., 2002; Siciliano, et al., 2013). While a qualitative assessment of protein lactosylation can easily be achieved, quantification of the extent of lactosylation using MS can be challenging. The quantification of protein lactosylation will be discussed in detail in section 6.4.3.

6.3.5 Lactulose

Besides whey protein denaturation, furosine and HMF, lactulose is often used as marker for the heat treatment of UHT milk (Datta, et al., 2002). Lactulose is an isomeric form of lactose and is formed during heat treatment. The reaction is catalysed by either amino groups of the milk proteins or by inorganic salts (Burton, 1994b; Datta, et al., 2002). Lactulose is a stable product and not present in raw milk. However, the formation of lactulose is very pH dependent (Burton, 1994b).
6.4 Shelf life development of UHT milk

6.4.1 Age gelation of UHT milk

The term ‘age gelation’ has been used in literature to describe the phenomena of physical instability of sterilized milk. Age gelation observed by different researchers differs greatly in gels appearance and the cause and/or mechanism by which they are formed (Nieuwenhuijse & van Boekel, 2003). The mechanisms of age gelation have been divided into a proteinase-induced, non-enzymatic-induced and a two stage process consisting of a combination of proteolysis followed by physical chemical changes (Aroonkamonsri, 1996; Harwalkar, 1992; Manji & Kakuda, 1988). An overview of causes and mechanisms of age gelation by Nieuwenhuijse & van Boekel (2003) is shown in Figure 24.

![Figure 24: Simplified scheme of various pathways of protein destabilisation in milk and milk products. From Nieuwenhuijse & van Boekel, 2003.](image)

Non-enzymatic age gelation in normal UHT milk is extremely slow, while fast in concentrated milks (Manji & Kakuda, 1986; Nieuwenhuijse & van Boekel, 2003). The proposed mechanism for age gelation at medium and high storage temperatures in concentrated UHT milks is the
reaction between β-lg and κ-casein during the heat treatment. This complex can dissociate from the casein micelle during subsequent storage and form a gel network (McMahon, 1996). In age gelation of concentrated UHT milk, protein dissociation and casein micelle crosslinking are regarded as factors causing age gelation. The hypothesis that proteolysis merely facilitates the release of β-lg-κ-casein complexes from the casein micelle is untenable (Datta & Deeth, 2001, 2003; Malmgren, 2007; McMahon, 1996). Various studies, including Paper II, show that protease induced age gelation is actually impaired by increasing heat treatment. Furthermore, Paper II showed that age gelation is possible with almost no β-lg associated to κ-casein, findings in accordance to (Kelly & Foley, 1997; Manji & Kakuda, 1988).

Protease induced age gelation can be caused both by plasmin and heat stable proteases originating from psychotrophic bacteria. In contrast to plasmin, bacterial proteases are able to hydrolyse κ-casein and can, in a similar fashion as chymosin, result in a rennet-curd like gel (Nieuwenhuijse & van Boekel, 2003; Snoeren, et al., 1979). Recent studies showed that bacterial proteases, besides hydrolysing κ-casein, also are able to destabilize casein micelles by proteolysis in general (Gaucher, et al., 2009; Gaucher, et al., 2011). Bacterial proteases show a more unspecific proteolysis pattern, which usually results in small peptides and can be distinguished from plasmin peptides by different RP-HPLC preparation methods. While both peptides formed by bacterial proteases and plasmin are soluble at pH 4.6, the larger plasmin derived peptides are not soluble in TCA (Datta & Deeth, 2003).

The role of plasmin in age gelation has been subject of intensive research, but it has not been unequivocally proven that plasmin can cause age gelation (O’ Mahony, et al., 2013). Although plasmin has been shown to be involved in age gelation, the conditions under which gelation occurs vary considerable and so far there is no direct correlation between the onset time of gelation and level of proteolysis (Manji & Kakuda, 1988). As the study described in Paper II shows, age gelation can occur in UHT milk with residual plasmin activity. No age gelation takes place when heat treatment conditions result in complete inactivation of plasmin and plasminogen, or when plasmin activity is inhibited (de Koning, et al., 1985; Kohlmann, et al., 1988; Manji & Kakuda, 1986). Age gelation in relation to plasmin therefore occurs more frequent when using direct UHT systems compared to indirect systems (Datta, et al., 2002; Manji & Kakuda, 1986). A first crucial factor for age gelation caused by plasmin is the rate of proteolysis. A high rate of proteolysis, either by high residual plasmin activity or addition of plasmin, plasminogen or somatic cell extracts results in a solution of polypeptides, but not in gelation. Addition of different concentrations of plasmin shows that high levels of plasmin activity result in 2-phase separation with clarification of milk and creaming (Crudden, Afoufa-Bastien, et al., 2005; Kohlmann, et al., 1991). Addition of low concentration of plasmin or plasminogen on the other
hand results in gel formation (Kohlmann, et al., 1991). Similar effects are shown, when somatic cell extracts are added to milk (Kelly & Foley, 1997). A second impact factor is the storage temperature of UHT milk. Gelation is slow at low storage temperatures, most rapid at 20-25 °C, and does not or very late occur at storage at > 30 °C (Harwalkar, 1992; Malmgren, 2007; Manji & Kakuda, 1986; Nieuwenhuijse & van Boekel, 2003). Since proteolysis was on-going in these UHT milks, it is assumable that proteolysis by plasmin was too fast to allow gel formation at storage temperatures > 30 °C (Nieuwenhuijse & van Boekel, 2003). Manji & Kakuda (1988) tried to separate the effect of proteolysis from the effect of storage temperature and time by pre-hydrolysing milk with plasmin to different degrees before storage. They found that gelation occurred faster at higher storage temperatures, but did not find a correlation between the time of gelation and the degree of hydrolysis.

Plasmin induced age gelation is therefore most likely a two-step process, consisting of the proteolysis itself and physical-chemical changes in the casein structure. In the UHT trial conducted for Paper II and III, two UHT milks with plasmin activity were produced: The UHT milk described in Paper II with a pre-heat treatment of 72 °C for 180 s and a UHT milk pre-heat treated at 72 °C for 5 s. While two replicates of the latter UHT milk were identical and showed comparable plasmin activities and proteolysis compared to the milk used for Paper II and III, one replicate showed decreased plasmin activity and reduced proteolysis of caseins and peptide formation (Figure 25).
Figure 25: Hydrolysis of αS1-casein (A), development of β-casein f(1-28) (B), plasmin activity (C) and viscosity development (D) in three replicate UHT milks (●, ○, □) heat treated at >150 °C for > 0.2 s with a pre-heat treatment of 72 °C for 5 s during storage.

The time point of gelation, analysed as viscosity increase, in this replicate was also delayed by 2-3 weeks compared to the other two replicates (Figure 25D) and occurred when the same level of αS1-casein had been hydrolysed (Figure 25A). This indicates that at least in a narrow range, the extent of proteolysis does have an effect on the time point of gelation.

In conclusion, the plasmin induced age gelation depends mainly on the following critical parameters at a given storage temperature:

- **Minimum degree of proteolysis** to sufficiently destabilize the casein micelle.
- **Minimum storage time** for changes in casein micelle structure to form a gel which mainly depends on the storage temperature.
- **Maximum degree of proteolysis** so that the peptides are no longer able to form a gel matrix any longer.
Although this is a very simplified model, these three factors could explain the different findings in relation to storage time and temperature and degree of proteolysis upon gelation. A schematic illustration of the model is shown in Figure 26:

![Figure 26: Schematic illustration of the critical parameters for plasmin induced age gelation as indicated by dashed lines: Maximum degree of proteolysis (1), minimum degree of proteolysis (2) and minimum storage time (3).](image)

Other factors could influence these parameters as well. Proteolysis correlated well with a decrease in pH, which could affect the calcium equilibrium in milk and the stability of the casein micelle. Whether such changes are sufficient to affect casein micelle stability is unknown and the effect of calcium on age gelation by plasmin is limited (Nieuwenhuijse & van Boekel, 2003). Another factor could be the activation of plasminogen and the previously discussed change in the rate of proteolysis, which makes the comparison of UHT milk systems and model systems with added plasmin difficult. Destabilisation of the casein micelle could also change the proteolytic pattern of plasmin, as indicated in Paper III, and proteolysis and destabilisation could have large influences on each other.

In contrast to age gelation by bacterial proteases, the mechanism of casein micelle destabilisation and gel formation as a consequence of plasmin activity is largely unknown. Electron micrographs of plasmin induced gels showed partially disintegrated casein micelles and the gel was made of threads and knots of protein (Aroonkamonsri, 1996; de Koning, et al., 1985). The presence of homogenized fat globules with a size distribution similar to casein micelles interfered with the particle size measurement of casein micelles in the study presented in Paper II. We could therefore not investigate changes in the particle size distribution of casein micelles, which could indicate disintegration caused by proteolysis. An attempt was made to interpret the observed decrease in Lab-colour values with changes in casein micelle structure. Dilution of UHT milk
without proteolysis with water resulted in a decrease of the b-value, i.e. less yellow, while the L- and a-values remained constant. Dilution with sodium citrate and dissociation of the casein micelles resulted in a decrease of L-, a-, and b-values similar to the UHT milk with plasmin activity, which could indicate a disruption and dissociation of casein micelles due to proteolysis.

The initial gel is usually formed at the bottom of the container and has been described as fragile and custardy (Aroonkamonsri, 1996; Datta & Deeth, 2003; Kelly & Foley, 1997). The gel found in the UHT milk described in Paper II showed similar features as can be seen in Figure 27.

![Figure 27: Effect of Storage time on gel formation in the bottom of the bottles in 3 replicates of UHT milk in Paper II. Ages of milk: 13, 12 and 11 weeks (from left to right). Notice the increase in gel volume over time.](image)

The gel at the bottom of the bottle increased in volume during the storage time and after approximately 20 weeks, the entire bottle was gelled. Although the UHT milk was not analysed further, some bottles were kept for visual inspection and after 25 weeks, a clear phase separation between the gel and the serum phase was observed. According to Nieuwenhuijse & van Boekel (2003), gelation is dependent on the dissociation and aggregation of peptides since non-casein nitrogen (NCN) increases upon proteolysis by plasmin. Analysis of pH 4.6 soluble peptides showed that main polypeptides found in the serum were proteose peptones. The only large polypeptide deriving from αS1-casein was f(125-199) and it was associated with caseins. Comparison of protein and peptide compositions of gel and liquid phases showed a similar composition, but the gel contained lower concentrations of κ-casein. The main bond type to stabilize a plasmin induced gel seems to be hydrophobic interaction (Aroonkamonsri, 1996; Nieuwenhuijse & van Boekel, 2003).
In Paper II, we compared the gelation mechanism of plasmin induced gelation with acid gelation of milk, which is caused by reduction of charge repulsion and solubilisation of colloidal calcium phosphate (Lucey, et al., 2001; Lucey & Singh, 2003). The plasmin induced gel found in the UHT milk exhibited a similar microstructure as found in acid gels (Figure 28). The proteolytic pattern of plasmin on the other hand showed that plasmin hydrolysed caseins preferably in hydrophilic domains and at the border of hydrophilic-hydrophobic or hydrophilic-phosphoserine regions (Paper III).

Figure 28: CLSM micrographs of gel particles extracted from gelled UHT milk after 13 weeks of storage at different magnifications. Protein is stained green, fat is stained red. Fat globule clusters and their incorporation in the gel network can be seen. From Paper II.

This indicates that plasmin could destabilize the casein micelle by hydrolysing around regions, which stabilize the casein micelle by calcium phosphate and hydrophobic interactions. It could be hypothesized that this proteolysis pattern could result in a disruption of the casein micelle, when enough stabilizing regions are hydrolysed. Loss of the casein micelle structure could subsequently result in a rearrangement of the polypeptides to a gel like network.

The exact mechanism of plasmin induced age gelation still remains unknown. The UHT trial conducted in this project aimed to investigate the effect of plasmin on UHT milk in general and it was not known beforehand, whether gelation actually would occur. In order to investigate the
relation between proteolysis and gelation, UHT milks with a broad range of residual plasmin activities should be produced. The level of residual plasmin activity could be easier controlled by choosing one pre-heat temperature and changing the holding time to create appropriate residual plasmin activities. To study gelation mechanisms, especially destabilisation of casein micelles, the use of skim milk as feed would allow to observe changes in particle size. The release of caseins or peptides in relation to proteolysis could be achieved by separating the serum phase from casein micelles by ultracentrifugation and analysis of both fractions for peptides and intact caseins. A deeper investigation of the gel by either addition of citrate or cooling or transferring the gel into a hydrophobic medium could allow insight to, whether calcium or hydrophobic bonds stabilize the gel.

6.4.2 Flavour development

6.4.2.1 Chemical changes affecting flavour development

Freshly prepared UHT milk has a distinctive cooked, sulphurous flavour, which disappears during storage. At the same time, an aged and stale flavour develops (Figure X). In addition, flavour attributes such as ‘heated’ and ‘oxidized’ develop. The flavour of UHT milk is commonly perceived as poor and is a problem for the dairy industry since the customer acceptance for UHT milk is low (Colahan-Sederstrom & Peterson, 2004; Perkins, M. L. & Deeth, 2001).

![Figure 29: Diagrammatic representation of flavour changes in UHT milk during storage. Adapted from Burton (1994).](image)

The initial cooked flavour arises from volatile sulphur components, which are formed during the UHT treatment. The major source of these sulphur components are free thiol groups, particularly of β-lg. The MFGM is also rich in free thiol groups and the concentration of volatile sulphur
components increases with increasing fat content (Al-Attabi, et al., 2009; Vazquez-Landaverde, et al., 2006). In addition, several volatiles can be formed during the Maillard reaction with methionine and cysteine (Al-Attabi, et al., 2009). The main components responsible for the cooked flavour are methanethiol, dimethylsulphide and dimethyldisulphide. Hydrogen sulphide itself has a relatively high odour activity value, but can react further to other sulphur volatiles (Vazquez-Landaverde, et al., 2006; Zabbia, et al., 2011). Disappearance of cooked flavour is dependent on the concentration of oxygen in UHT milk. In the presence of oxygen, volatile sulphur components are rapidly oxidized and the cooked flavour decreases (Al-Attabi, et al., 2009; Burton, 1994a). Directly processed UHT milk usually has a lower initial cooked flavour compared to indirectly processed UHT milk. This is due to the lower overall heat load in directly processed UHT milk and some of the volatile components can be removed during the vacuum cooling step (Datta, et al., 2002). On the other hand, most of the oxygen is removed during vacuum cooling as well and the cooked flavour disappears slower compared to indirectly processed UHT milk (Datta, et al., 2002; Valero, et al., 2001).

High levels of oxygen accelerate the appearance of stale and oxidized flavour. Stale and oxidized flavours have been linked to the presence of lipid oxidation products, such as methyl ketones and aldehydes (Calvo & de la Hoz, 1992; Perkins, Melinda L., et al., 2005; Valero, et al., 2001; Vazquez-Landaverde, et al., 2005). Aldehydes are regarded to have a higher impact on flavour than methyl ketones due to their lower sensory threshold (Datta, et al., 2002). Oxidation of proteins can also lead to an unpleasant oxidized or ‘activated flavour’ (Burton, 1994a). Methyl ketones also form in the Maillard reaction and contribute to the heated flavour of UHT milk. Other Maillard reaction products contributing to the flavour are lactones, diacetyl, maltol, vanillin and benzaldehyde (Calvo & de la Hoz, 1992; Contarini, et al., 1997; Jansson, Jensen, et al., 2014).

### 6.4.2.2 Effect of enzymes on flavour

Flavour development of UHT milk also depends on raw milk quality. Psychotrophic bacteria are able to produce heat resistant lipases, whose activity can lead to a rancid flavour (Burton, 1994a). Proteolysis can indirectly affect the flavour of UHT milk. Increased levels of free amino acids accelerate the Maillard reaction and lead to a higher concentration of Strecker degradation products (Jansson, Clausen, et al., 2014).

Several studies reported the occurrence of bitterness before gelation of milk in stored UHT milks (Burton, 1994a; McKellar, et al., 1984; Nieuwenhuijse & van Boekel, 2003). While many studies
focussed on age gelation of milk, studies on bitterness in milk are scarcer. Since bitterness occurs before gelation, it is the shelf life limiting factor and of higher importance for the dairy industry. Bacterial proteases are found to cause bitterness at very low levels of proteolysis (McKellar, 1981). These proteases cleave relatively unspecific and are able to hydrolyse hydrophobic domains of the caseins, which are the largest source of bitter peptides (Gaucher, et al., 2011; Lemieux & Simard, 1992). The appearance of bitterness thus has mostly been connected to the presence of bacterial proteases.

The appearance of bitterness in the UHT milk described in Paper II was linked to plasmin since the milk was as fresh as possible, of excellent microbial quality and proteolysis of bacterial proteases was not detected during storage (Paper III). Huijs et al. (2004) report plasmin as the cause for bitterness in directly processed UHT milk with ultra-short holding time, similar to the process used in Paper II. Bitterness is also observed in directly processed UHT milk with high somatic cell count and plasmin activity (Santos, et al., 2003; Topçu, et al., 2006). The milk also contained a high level of psychotrophic bacteria so the exact cause of bitterness cannot be differentiated.

The literature only describes one sensory evaluation of peptides produced by plasmin and it is done on isolated β-casein (Harwalkar, et al., 1993). Proteolysis of β-casein caused an astringent flavor and was linked to the formation of γ-caseins. Peptides from tryptic digests of β-casein were described as ’astringent, tickling, burning and bitter’, verifying that proteolysis of β-casein by plasmin could lead to bitter peptides (Bumberger & Belitz, 1993).

A common way to estimate the bitterness of a peptide is the Q-rule. Bitterness has for long been associated with the hydrophobicity of a peptide. The Q-value is an average value of the hydrophobicity of the amino acid side chains divided by the number of amino acids (Ney, 1971). Peptides with a Q-value < 1300 cal res\(^{-1}\) are not bitter while peptides with Q > 1400 cal res\(^{-1}\) are generally considered as bitter. No prediction of bitterness is possible for Q-values between 1300-1400 cal res\(^{-1}\) (Lemieux & Simard, 1992; Ney, 1971). In Paper III, we identified 23 potentially bitter peptides based on the Q-rule. With the exception of β-casein f(49-97), the potential bitter peptides originating from β-casein found in the UHT milk were not described as bitter in tryptic digests (Bumberger & Belitz, 1993). Interestingly, the most bitter peptide found in tryptic digests of β-casein, β-casein f(203-209), was not identified in UHT milk (Bumberger & Belitz, 1993).

The C-terminal region of β-casein is very hydrophobic and it was suggested that it is inaccessible for plasmin in a milk system. Most of the peptides, also previously proposed bitter peptides, derived from hydrophobic regions of α\(_{S1}\)- and α\(_{S2}\)-casein. (Habibi-Najafi, et al., 1996; Le Bars & Gripon, 1989; Maehashi & Huang, 2009).
Although the Q-rule allows for an initial assessment of potential bitter peptides, it was not possible to ascribe the observed bitterness in the UHT milk to certain peptides. An exception to the Q-rule is that it is only valid up to a molecular weight of 6000 Da (Lemieux & Simard, 1992; Ney, 1971). In order to identify the sensory properties of these potential bitter peptides, they would need to be either extracted from milk or synthesized and evaluated sensorically. This would have to be done in a milk system to include matrix effects, which could partially mask bitter taste (Toelstede & Hofmann, 2008). Determination of the formation rate and quantification of confirmed bitter peptides could allow a prediction of bitterness and consequently the shelf life of UHT milk.

6.4.3 Lactosylation of milk proteins during storage of UHT milk

As indicated in section 6.3.4, the Maillard reaction takes place during storage of UHT milk at ambient temperatures and results in an increase in protein lactosylation during storage. The rate of protein lactosylation depends on the storage temperature with increased rates of protein lactosylation at higher storage temperatures (Bosch, et al., 2008; Corzo, et al., 1994; Nangpal & Reuter, 1990). Nangpal & Reuter (1990) reported a linear increase in furosine concentration in UHT milk during storage at 20 and 30 °C, with an increase in furosine of 1 mg L\(^{-1}\) per week, similar to the rate of furosine increase reported for the UHT milks in Paper IV (Figure 30). The increase in furosine over time was found to be independent of the initial furosine concentration (Paper IV). At higher storage temperatures, the increase in furosine is not linear any more at prolonged storage times as lactulosyllysine reacts further and intermediate and late Maillard reaction products are formed (Le, et al., 2011; Nangpal & Reuter, 1990).

![Figure 30: Furosine development in directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 (●) or 180 s (○) and in indirect UHT milk (140 °C for 4 s, □) during storage at 20 °C. Error bars indicate standard deviation, n = 3; from Paper IV.](image)

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Assessment of protein lactosylation by measurement of furosine includes an 18 h acid boiling step to form furosine followed by analysis of furosine by HPLC, making this analysis expensive and time intensive. Lactosylation can also be observed by LC-MS using the protein composition method described in Paper II & IV. This method requires a one hour sample preparation step and a HPLC run time of 20 min per sample and can give a more detailed view on the Maillard reaction since the lactosylation of different proteins can be observed.

![Figure 31: Summed mass spectra (retention time 11.0-13.2 min) of unmodified and lactosylated α\textsubscript{S1}-casein 8 P in indirect UHT milk (140 °C for 4 s) after 6 months of storage at 20 °C. Inset is showing a zoom of the most abundant ions. The m/z shifts due addition of lactose are indicated. From Paper IV.](image)

Lactosylation renders a protein more hydrophilic and lactosylated proteins elute earlier in reverse phase HPLC than non lactosylated proteins (Czerwenka, et al., 2006; Losito, et al., 2007; Losito, et al., 2010). This results in a broadening of peaks in the UV chromatogram of proteins (Paper II & IV). A chromatographic separation of lactosylated proteins, which would allow for quantification of lactosylation using the UV signal, is hard to achieve since the change in hydrophobicity upon lactosylation is little. Analysis of summed mass spectra of a peak containing non lactosylated and lactosylated proteins on the other hand allows a relative comparison of the MS intensities of the different lactosylated proteins. The comparison can either be performed on specific ions (Figure 31) or on deconvoluted spectra (Figure 32).
Exploration of milk protein lactosylation has mainly been focused on whey proteins, especially pH 4.6 soluble whey proteins (Siciliano, et al., 2013). This limits the characterisation of protein lactosylation to milk products in which a sufficient amount of pH 4.6 soluble whey proteins are present (Meltretter, et al., 2009). In products where protein lactosylation actually could affect the overall product quality, the heat treatment is so severe that most or all of the whey proteins are denatured, such as the indirectly processed UHT milk in Paper IV or milk powders. Furthermore, denaturation and unfolding of whey proteins could affect the kinetic of lactosylation due to increased susceptibility of lysine residues (Losito, et al., 2010). Still for a limited range of heat treatments a quantitative relationship between lactosylation of whey proteins and heat markers could be established. The used heat markers were whey protein denaturation as well as the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index (Losito, et al., 2010; Meltretter, et al., 2009). While these markers reflect the severity of the heat treatment, they do not necessarily correlate with the extent of the Maillard reaction. The only direct correlation of milk protein lactosylation measured by MS and furosine concentration was established for the lactosylation of β-casein in processed cheese (Steffan, et al., 2006). Other studies on lactosylation of caseins measured by MS are only qualitative (Johnson, et al., 2011; Scaloni, et al., 2002).

Quantitative analysis of milk protein lactosylation can be challenging since reports on response factors of differently lactosylated protein species differ and quantitative comparison with furosine is lacking in most studies. For proteins in their native state, such as whey proteins and lysozyme, the response factor was found to be not affected by lactosylation of proteins when analysed with
both electrospray ionisation MS (ESI-MS) and matrix assisted laser desorption (MALDI) MS (Czerwenka, et al., 2006; Meltretter, et al., 2009; Yeboah & Yaylayan, 2001). For reduced caseins without any notable tertiary structure however, a lower response factor for lactosylated proteins was found compared to non lactosylated caseins (Scaloni, et al., 2002). In Paper IV, both lactosylated species of caseins and whey proteins exhibited a reduced response factor in MS intensity, although the UV chromatogram showed no loss of protein and was constant throughout the storage period. Although the response factors were not identical for different lactosylated protein species in Paper IV, a linear regression of the relative area distribution of the proteins was possible. The rate of lactosylation measured as decrease in unlactosylated protein correlated with the number of lysine residues in protein for both caseins and whey proteins. The decrease of unlactosylated proteins correlated well with the increase in furosine during storage, allowing a quantitative estimation of protein lactosylation by LC-MS measurements in the covered range of furosine.

The differences in response factors could be attributed to the structure of proteins. The globular structure of whey proteins results in an overall decreased ionisation. A comparison of mass spectra of pH 4.6 soluble whey proteins and reduced whey proteins in low pasteurized milk samples showed large differences. The most abundant charge state for reduced β-lg and α-la were 17 and 13 compared to 11 and 6 for pH 4.6 soluble whey proteins. With increasing charge state, a potential reduction in the ionisation efficiency of a lactosylated lysine residue would become more pronounced compared to lower charge states, where ionisable residues are present in excess. Analysis of the response factors of lactosylated pH 4.6 soluble whey proteins and comparison to the response factors after reduction of the same samples could give an insight in the underlying mechanism.

A different approach to determine the extent of lactosylation milk is the analysis of lactosylated peptides present in milk (Pinto, et al., 2012). Plasmin derived peptides and especially β-casein f(1-28) were found to be well suited to detect lactosylation in milk as they are already present in raw milk. A high concentration of lactosylated β-casein f(1-28) was detected in the peptidomic study of UHT milk with plasmin activity in Paper III, but the concentration of β-casein f(1-28) was not high enough in the UHT milks without plasmin activity in Paper IV to obtain reproducible results.
7 Conclusions

The studies presented in this thesis have provided insight into the measurement of plasmin activity and the effect of plasmin activity on the shelf life development of UHT milk.

The tested conditions for measuring plasmin activity in an assay showed that the measured plasmin activity is not only dependent on sample composition and treatment, but also on sample preparation and buffer systems used. The term activity has been used in literature to describe plasmin concentration, real activity and apparent activity including interfering components, but should actually be referred to as the rate of hydrolysis of a synthetic substrate under given conditions. There is no right or wrong way to measure activity, but one should be aware of all factors that influence the activity in an assay in order to take decisions on assay conditions to be used and to draw conclusions from activity measurements on changes in activity or the characteristics of plasmin in milk. Considering these factors allows the design of assays that measure an activity, which in the best way reflects the research aim.

Although it is more time and work intensive than an activity assay, following proteolysis by plasmin gives a direct insight to the effect of plasmin activity in UHT milk. Identification of peptides allowed identification of several marker peptides for plasmin activity, which were not further hydrolysed during the presently applied storage period. Furthermore, differences in affinity of plasmin for specific cleavage sites lead to a time dependent appearance of peptides, which can potentially be used to characterize different stages of proteolysis. It is possible to model the proteolysis of β-casein on a protein and peptide level, and of αs2-casein on a peptide level with the analytical tools available in this project. Unfortunately, we could only model the kinetic at one plasmin concentration and were not able to validate the model.

In terms of quality defects, plasmin activity could be linked to the occurrence of bitterness and age gelation in UHT milk in this study. The identification of (potential) bitter peptides provides a starting point for further studies on the sensory threshold of bitter peptides, which would allow the establishment of critical peptide concentrations that can limit the shelf life of UHT milk.

Proteolysis by plasmin can cause age gelation of UHT milk, but the underlying mechanisms are still not fully understood. The gelation mechanism is most likely a two-step process and the proteolysis pattern leading to gelation can provide a hypothetical mechanism of destabilisation of the casein micelle by proteolysis.

The quality defects in the UHT milk investigated in this study occurred only after substantial proteolysis with 60-70 % of αs1- and β-casein being hydrolysed for the milk to taste bitter and more than 90 % to cause gelation. While the extent of proteolysis necessary for gelation and bitterness could not be answered for different residual plasmin levels, this study gave an insight
in the effect of plasmin activity in milk and provided a setup to explore this further. The results show that low levels of plasmin activity in UTH milk can be acceptable, when the quality defects are matched with the shelf life of the UHT milk.
8 Perspectives

The research effort on plasmin and the plasmin system in milk is considerable, and with every published study, some questions are answered, but new and often more profound questions arise. The same applies for this study.

The presented results give an insight into the effect of proteolysis on UHT milk, but cannot answer the mechanism of age gelation or establish a direct connection between plasmin activity, proteolysis and quality defects. In retro perspective, the UHT trial conducted in this study could have been planned differently. Applying the same preheat temperature and only varying the holding time would have allowed a more specific control over residual plasmin activities in the UHT milk and maybe could have been predicted using kinetic data in literature. Sensory evaluation of the UHT milk by a trained sensory panel would have given a more detailed view on off flavour and bitterness caused by plasmin. Most potential bitter peptides were present in low concentrations and could not be quantified using the UV signal. Quantification of these peptides can be improved by optimizing the chromatographic separation or adapting the sample preparation for smaller hydrophilic peptides. An alternative is the use of an internal standard for the LC-MS analysis, which then allows quantification using the MS signal over a certain range.

In order to study the gelation phenomenon, a skim milk system and dynamic light scattering instead of static light scattering should be used in order to investigate changes in the casein micelle structure. Furthermore, release of peptides and caseins from the casein fraction could be used as indicators for casein micelle destabilisation. A repetition of the UHT storage experiment in this study under the conditions mentioned above could enable the correlation of plasmin activity, proteolysis and quality defects and allow the dairy industry to control the effects of plasmin activity better.

As indicated in Paper I, the dissociation of plasmin and caseins affect the interaction of plasmin with inhibitory components. A dissociation of plasmin and caseins during processing, for example by pressure, could result in an increased inhibition and/or inactivation of plasmin by whey proteins and inhibitors. Although we could not see differences in the heat inactivation of plasmin in the presence of NaCl and EACA, it could be hypothesized that the dissociation of plasmin and caseins could negate the protective effect of caseins towards the heat inactivation of plasmin.

Beside these follow up studies to this project, several more basic aspects of plasmin still need more detailed attention. The concentration of plasminogen activators and the higher heat stability of plasmin compared to plasminogen make them an important factor to limit proteolysis in milk and dairy products. The influence of storage conditions of raw or pasteurized milk on the
activation of plasminogen before further processing has only been partially studied and could be a starting point to reduce plasminogen activation. During the separation of milk into skim milk and cream, somatic cells and other large particles are separated as well. Most of the uPA is initially bound to somatic cells and the effect of the separation on the stability of somatic cells and release of uPA is unknown. As uPA is considered the major plasminogen activator and has a higher heat stability compared to tPA, a reduction of uPA concentration in milk would reduce proteolysis in milk and milk products. Pre-processing of UHT milk yields several possibilities, which could result in a minimisation of the plasmin system load in the final product, which could positively affect product quality.
9 References


Paper I


The determination of plasmin and plasminogen-derived activity in turbid samples from various dairy products using an optimised spectrophotometric method

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The determination of plasmin and plasminogen-derived activity in turbid samples from various dairy products using an optimised spectrophotometric method

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Abstract
A spectrophotometric assay for plasmin and plasminogen-derived activity in dairy products was optimised and extended to determine plasmin and plasminogen-derived activity in turbid samples of dairy products. The method was validated by assessing reproducibility, repeatability, level of detection and recovery of plasmin activity in different sample matrices. Plasmin activity in raw milk was not affected by skimming, but decreased by 30% in pasteurised and homogenised whole milk, leading to an underestimation of plasmin activity. The effects of dissociation of plasmin and caseins by ε-aminocaproic acid (EACA) plus NaCl on the plasmin activity were investigated. Comparison of pasteurised milk with a micellar casein solution showed that the dissociation of plasmin and caseins on adding EACA and NaCl decreases interference by caseins, but increases inhibition of plasmin with serum-based inhibitory components. The level of detection and repeatability of this method for plasmin activity analysis were improved compared with previous spectrophotometric assays.

1. Introduction

The major indigenous proteinase in milk, plasmin (PL; EC 3.4.21.7), is the active part of a complex enzyme system. Its zymogen, plasminogen (PLG), is present in raw, bovine milk at a 2–30 times higher concentration than PL (Ismail & Nielsen, 2010), and is activated by two families of plasminogen activators (PA), urokinase-type PA (uPA) and tissue-type PA (tPA). The activity of PL and PA is regulated by plasmin inhibitors (PIs), such as α2-antiplasmin, and plasminogen activator inhibitors (Grufferty & Fox, 1988; Ismail & Nielsen, 2010; Korycka-Dahl, Dumas, Chene, & Martal, 1983; Lu & Nielsen, 1993; Precetti, Oria, & Nielsen, 1997). The level of PL in milk can vary and depends on environmental factors, such as stage of lactation and somatic cell count (Larsen et al., 2010; Nicholas, Auldist, Molan, Stelwagen, & Prosser, 2002). PL readily hydrolys β- and γ2-casein, and, to a lesser extent, γ51-casein (Grufferty & Fox, 1988). PL activity may be linked to the formation of unclean and bitter off-flavours in milk, and age gelation of UHT milk (Harwalkar, Cholette, McKellar, & Emmons, 1993; Kelly & Foley, 1997). On the other hand, hydrolysis of caseins by PL plays an important role in the initial ripening of Swiss cheese types (Bastian & Brown, 1996).

Various methods to measure PL and PLG-derived activities have been described (Politis, Zavision, Barbano, & Gorewit, 1993; Richardson & Pearce, 1981; Rollema, Visser, & Poll, 1983; Saint-Denis, Humbert, & Gaillard, 2001). The determination of PL activity in these assays is based on the hydrolysis of a specific substrate, which upon cleavage releases a chromogenic (Rollema et al., 1983) or fluorogenic product (Richardson & Pearce, 1981; Saint-Denis et al., 2001). The different sample preparations used in these assays, however, cause varying results and different interfering effects have been reported. Two substrates (S-2251 and Spectrozyme PL) have been reported to enhance PLG activation (Kolev, Owen, & Machovich, 1995), while ε-aminocaproic acid (EACA), a lysine...
derivative often used to enhance PL activity, inhibits activation of human PLG (Alikjaersig, Fletcher, & Sherry, 1959; Cesarian-Maus & Hajjar, 2005). EACA dissociates PL and PLG from caseins by binding to the lysine-binding sites on PL and PLG (Korycka-Dahl et al., 1983).

Furthermore, it has been shown that the dissociation of human PLG by EACA also inhibits activation of PL by tPA and uPA (Cesarian-Maus & Hajjar, 2005; Collen, 1987; Sun et al., 2002). On the other hand, the natural substrate of PL in milk, the caseins, can act as competitive inhibitors towards a chromogenic substrate used in the assays (Bastian, Brown, & Ernstsm, 1991) and affect the activation of PLG (Heegaard, Andreassen, Petersen, & Rasmussen, 1997; Markus, Hitt, Harvey, & Tritsch, 1993). Whey proteins are also able to inhibit PL activity (Hayes, McSweeney, & Kelly, 2002; Politis et al., 1993), but the exact mechanism has not been described so far; β-lactoglobulin and bovine serum albumin have shown a higher inhibitory effect than α-lactalbumin (Politis et al., 1993).

Another consideration in terms of assay suitability and performance is that, especially for fluorescence assays, the turbidity of milk can severely interfere with the measurement. In most assays described, an extensive sample preparation protocol is needed to avoid these interferences (Politis et al., 1993; Rollema et al., 1983). In case of fluorescence methods, a high dilution of the sample (Richardson & Pearce, 1981) or an additional clarification treatment (clarifying reagent) before measurement (Saint-Denis et al., 2001) is required. These preparations may not only reduce the sensitivity of the assay, but could also complicate the comparison of PL activities measured by different sample preparations, substrates and detection methods (Kelly, O’Flaherty, & Fox, 2006).

The aim of this study was to combine the spectrophotometric assay for PL and PLG-derived activity described by Rollema et al. (1983) with the sample preparation proposed by Saint-Denis et al. (2001) and expand it to turbid, fat-containing milk types and different sample matrices including cheeses. A further aim was to study the effect of skimming on PL activity in different milk types and to characterise the inhibitory effects of casein and serum -based components on the assay, especially the effect of dissociation of PL and caseins by EACA and NaCl on PL and PLG-derived activity in different sample types.

2. Materials and methods

2.1. Materials

PL from bovine serum was obtained from Roche Diagnostics (Hvidovre, Denmark) and urokinase (Thrombolyisin) was from Immuno Danmark A/S (Copenhagen, Denmark). The ε-amino-caproic acid (EACA) was obtained from Sigma–Aldrich Denmark ApS (Brøndby, Denmark) and H-ω-valyl-ε-leucyl-ε-lysyl-4-nitroanilide (S-2251) from Haemochrom Diagnostica GmbH (Essen, Germany).

2.2. Origin of samples

The pasteurised (minimum of 72 °C for 15 s) skim milk (SM), homogenised and pasteurised whole milk with 3.5% fat (WM), and UHT milk (0.5% fat) used in this study were commercially available milk products obtained from a local supermarket, stored at 5 °C and analysed before expiry date. Raw bulk milk (RM) was obtained from Arla Foods (Brabrand Dairy Plant, Brabrand, Denmark). A whey-protein-free milk serum (UF-permeate) was prepared by ultrafiltration of SM using 5-kDa spiral wound membranes (Koch Membrane Systems HFK-328). A micellar casein solution (MCS) was prepared by ultracentrifugation of SM at 100,000 × g for 60 min at 25 °C. The casein pellet was reconstituted with UF permeate to original volume and gently stirred at 4 °C overnight. Samples of Swedish Herrgårds cheese (>28% fat, >6 months) and parmigiano Reggiano (>32% fat, >6 months) were obtained from a local supermarket. Danish Havarti cheese (>60% fat, 1 week) was produced in the pilot plant at Arla Foods Strategic Innovation Center (Brabrand, Denmark).

2.3. Basic sample preparation and measurement of PL and PLG-derived activity

Milk samples (1 mL) were mixed with 250 μL 0.4 M tri-sodium citrate buffer, pH 8.9, and shaken for 15 min to dissociate the casein micelles. Sample preparation for cheese was adapted from that of Richardson and Pearce (1981) with modifications as follows. A mass of 5 g of grated cheese was dissolved in 45 mL 0.4 M tri-sodium citrate buffer, pH 8.9, and stirred for 30 min at room temperature, followed by holding for 30 min at 45 °C, and finally, 15 min at room temperature to separate the fat phase. The solution was then centrifuged at 7500 × g for 15 min, and the supernatant below the cream phase was removed and centrifuged again under the same conditions.

The citrate-treated milk or cheese samples were diluted 1:1 (v/v) with an assay buffer as described by Saint-Denis et al. (2001) containing 0.1 M Tris–HCl, 8 mM EACA, 0.4 M NaCl, pH 8, and mixed for 15 min to dissociate PL and PLG from the caseins.

For determination of PLG-derived activity, PLG in the citrate-treated samples was activated by a 1:1 (v/v) dilution with an assay buffer containing 200 Plough units mL⁻¹ urokinase and incubated for 60 min at 37 °C (Korycka-Dahl et al., 1983; Rollema et al., 1983; Saint-Denis et al., 2001).

PL and PLG-derived activity were determined by measuring the rate of hydrolysis of the chromogenic substrate S-2251 (Rollema et al., 1983) and release of p-nitroaniline. To compensate for lower dilution of 1:5 v/v the sample in the final mixture compared with 1:10 (v/v) in the assay described by Rollema et al. (1983) and, to increase the sensitivity of the assay, the substrate concentration was increased from 0.6 mM S-2251 (Rollema et al., 1983) to 2 mM in the final mixture. The substrate, S-2251, was dissolved in 0.1 M Tris–HCl buffer, pH 8 to a concentration of 4 mM.

The sample solution (50 μL) was transferred to a microtitre plate and the reaction was initiated by addition of 50 μL of the substrate solution. Absorbance was read at 405 nm and 490 nm at 37 °C using an ELISA plate reader (Bio-Tek EL 808 and Bio-Tek Synergy MX, BioTek Instruments Inc., Wisconsin, IL, USA) at intervals of 2–10 min for 60–180 min depending on the level of PL activity in the sample. Substrate volumes per sample were minimised through use of Costar 3695 clear half-area polystyrene 96-well plates (Corning Inc., New York, NY, USA). To correct for turbidity, the background absorbance values (490 nm) were subtracted from the absorbance values at 405 nm, corresponding to colour development due to release of p-nitroaniline. Correction for turbidity using the absorbances at 540 and 630 nm was also tested. The increase in absorbance, indicated as ΔA405 nm–490 nm as a function of time, dA/ dt, was converted into PL units using a standard curve prepared with commercial PL solution from a PL stock solution (0.5 U in Tris buffer, pH 8) with assay buffer additionally containing 4 mg mL⁻¹ gelatin to stabilise PL. The correlation between PL activity and absorbance was linear in the range 15 μL mL⁻¹ to 1 μL mL⁻¹ (R² = 0.999). No increase over time in absorbance was observed in a blank sample containing assay buffer with 4 mg mL⁻¹ gelatine only.

2.4. Method validation

The linearity of the assay was evaluated by measuring PL activity in undiluted SM as well as 1:1 and 1:10 (v/v) dilutions of SM and 3...
different wavelength to correct for turbidity (490, 540 and 630 nm). The wavelength (490, 540 or 630 nm) used for turbidity correction had no significant effect on the slope, $dA/dt$, of the curve, for reference, 490 nm was thus selected. Linearity and turbidity of the assay for fat containing milks was assessed with WM and RM using absorbance at 490 nm to correct for turbidity.

Repeatability of the assay was evaluated by measuring PL and PLG-derived activity of a SM sample for a total of 14 times. Reproducibility was assessed by measuring PL and PLG-derived activity in frozen aliquots of SM, RM, Danish Havarti cheese, Swedish Herrgård cheese and Parmigiano Reggiano on 4 occasions (measured on 4 different plates on 4 different days).

Limit of detection was compared with that determined by Saint-Denis et al. (2001) by diluting SM with 0.1 M Tris–HCl, pH 8. The limit of detection was the highest dilution of SM with a significantly higher activity than a blank consisting of assay buffer with 4 mg mL$^{-1}$ gelatine.

### 2.5. Effect of skimming on plasmin activity in milk

The effect of fat removal by centrifugation on the development of absorbance in the PL assay was investigated using RM and WM with SM as control. Prior to skimming, a volume of 2.5 mL 0.4 M tri-sodium citrate buffer, pH 8.9 was added to 10 mL of RM, WM or SM and the solutions were mixed for 15 min. One mL of each solution was taken out and stored at 4 °C to ensure the same experimental conditions, while the remaining volume was further prepared for skimming by centrifugation at 10,000 × g for 10 min at 4 °C. The upper fat-containing layer was discarded and 1 mL of the clear liquid was used for further analysis. Both the un-skimmed and skimmed samples were further diluted 1:1 (v/v) 0.1 M Tris–HCl, 8 mM EACA, 0.4 M NaCl, pH 8 and PL activity was measured as described in Section 2.3. One independent batches of each milk (RM, WM and SM) were used for the experiment and sample preparation was performed in duplicate.

### 2.6. Recovery of activity of added PL and inhibitory effects of milk components

Recovered activity of PL was measured by addition of a defined amount of PL with known activity to samples, along with the assay buffer containing 0.1 M Tris–HCl, 8 mM EACA, 0.4 M NaCl, pH 8, to investigate possible inhibitory and interfering effects in the assay. Samples tested were RM, SM, UHT milk, MCS as well as Havarti, Herrgård and Parmigiano Reggiano cheese. Furthermore, to provide a reference system, which does not contain the PL inhibitors present in milk, a gelatine solution (4 mg mL$^{-1}$) containing 0.1 M Tris–HCl buffer, pH 8 was prepared. Gelatine has earlier been shown to be a suitable agent for stabilising PL (Saint-Denis et al., 2001). The measured activity of PL in the gelatine system was set as 100% recovered activity.

The effect of sample concentration on recovered activity and inhibitory effects was evaluated by diluting a UHT milk sample with 0.1 M Tris–HCl buffer, pH 8, to a final concentration of UHT milk of 100, 75, 50 and 25% (v/v) and a constant amount of added PL. PL activity in the UHT milk dilution was compared with PL activity in dilutions of a SM sample to which UHT milk had been added to a final proportion of SM in the mixture of 100, 75, 50 and 25% (v/v). PL activity was measured as described in Section 2.3.

### 2.7. Effect of dissociation of plasmin by EACA and NaCl in presence and absence of whey proteins on PL and PLG-derived activity

To further investigate the effect of milk components, PL was dissociated from casein by the use of EACA and NaCl (EACA + NaCl). EACA + NaCl at concentrations of 4 mM and 2 mM, respectively, have been shown previously to dissociate PL from casein micelles (Saint-Denis et al., 2001).

The effect of EACA + NaCl on PL and PLG-derived activities in RM and SM was evaluated by diluting the sodium-citrate-treated samples (1:1 v/v) with either 0.1 M Tris–HCl, pH 8, or 0.1 M Tris–HCl, pH 8, containing 8 mM EACA and 0.4 M NaCl. The effect of pre-incubation with EACA + NaCl on PL activity was compared for a SM and a MCS sample prepared from the same SM. A dilution series of SM and MCS was prepared by addition of 0.1 M Tris–HCl buffer, pH 8, to a final concentration of SM or MCS of 100, 80, 60, 40 and 20% (v/v). From each of these dilutions, 1 mL was taken and a volume of 250 μL 0.4 M tri-sodium citrate buffer, pH 8.9 was added. Each sample was shaken for 15 min before further dilution (1:1, v/v) with either 0.1 M Tris–HCl, pH 8, or 0.1 M Tris–HCl, pH 8, containing 8 mM EACA and 0.4 M NaCl. PL activity was measured as described in Section 2.3.

### 2.8. Statistical analysis

Analysis of variance (ANOVA) and separation of means was carried out using MATLAB (Mathworks, Natick, MA, USA). One-way-ANOVA was used to determine significant differences ($P < 0.05$) among groups. Differences between the means and mean separation were determined usingTukey–Kramer multiple means comparison tests ($P < 0.05$ and 0.01).

### 3. Results and discussion

#### 3.1. Method validation

The linearity of the assay was evaluated by measuring PL activity in undiluted SM as well as 1:1 and 1:10 (v/v) dilutions of SM with 0.1 M Tris–HCl buffer, pH 8. The reference wavelength measurements showed that the turbidity in undiluted and diluted SM samples was not constant throughout the assay. Within the first 20 min, the turbidity decreased rapidly in an exponential manner, which led to a non-linear increase in the ΔA$405-490$ nm in that time period (Fig. 1), and this increase appeared to be independent of sample concentration. The first 20 min of the measurements were therefore excluded from the calculations of the slope. Following this, the absorbance, ΔA$405-490$ nm increased linearly (20–120 min) in both the diluted and undiluted SM samples ($R^2 > 0.99$).

![Fig. 1. Plasmin activity measured as development in absorbance ΔA$405-490$ nm due to substrate hydrolysis and release of 4-nitroaniline in undiluted (○), 1:1 (●) and 1:10 (▲) dilution of pasteurised skim milk with 0.1 M Tris–HCl buffer, pH 8 (average of 3 determinations ± standard deviation). Lines are linear fits for the time period 20–120 min to guide the eye.](image-url)
The development of absorbance at 405 nm and at 490 nm in RM, WM and SM are shown in Fig. 2A. The assay with RM was only stable up to 2 h, after which time creaming occurred, and no further measurement was possible. Due to the presence of fat, the initial absorbance at 405 and 490 nm of RM and WM was 4–5 times higher compared with SM. The absorbance at 405 nm in SM and WM increased slightly during the incubation time, but decreased in RM, which may be due to the onset of creaming. At the same time, the absorbance at 490 nm decreased for both RM and SM, while it remained almost constant for SM. Fig. 2B shows the development in absorbance $A_{405} \text{nm} - 490 \text{nm}$ after correction for turbidity. As can be seen, the absorbance increased linearly in all milk samples, and there was only a minor difference in the initial absorbance. The correction for turbidity using a reference wavelength at 490 nm thus enables measurements in turbid samples.

Reproducibility of the assay and performance in different sample matrices was assessed by measurement of PL and PLG-derived activity in frozen aliquots of RM, SM, 1 week old Danish Havarti cheese, Herrgård cheese and Parmigiano Reggiano on 4 occasions (Table 1). SM showed a higher PL activity than RM, while the PLG-derived activity was similar in SM and RM. The PLG/PL ratio was 8.2 for RM and 7.1 for SM, which is in the reported range for milk (Ismail & Nielsen, 2010). Due to the fat content, the reproducibility in RM was poorer compared with SM, with a relative standard deviation of 12.1% for PL activity and 4.1% for PLG-derived activity, compared with 2.8% and 1.1% for SM. PLG-derived activity in the Havarti cheese was approximately 11.5 times higher than the PL activity and was approximately 2 times higher than the PL activity in the Herrgård cheese (Table 1). The PLG to PL ratio of the Havarti cheese is within the range of previously reported values of approximately 3 (Benfeldt, 2006) to 21 (Bastian, Hansen, & Brown, 1991b).

To our knowledge, PL and PLG-derived activity in Herrgård cheese has not been described previously. PLG-derived activity in Parmigiano Reggiano was similar to that in the Herrgård cheese, while PL activity was lower. The relative standard deviations of the PL and PLG-derived activity in the cheeses (Table 1) were from 3.0 to 4.1% for PL activity and from 1.3 to 3.7% for PLG-derived activity. In comparison, Richardson and Pearce (1981) reported a reproducibility of 9% for a Swiss-type cheese ($n = 9$). Repeatability of the assay was assessed by measuring a SM sample 14 times. The repeatability, measured as % relative standard deviation, for PL and PLG-derived activity was 2% and 1%, respectively, compared with 2% reported for PL activity by Saint-Denis et al. (2001) and 10% reported for PL activity by Rollema et al. (1983).

The lowest PL activity significantly higher than a blank sample ($P < 0.05$) was detected in a 100-fold diluted sample of SM, indicating an overall limit of detection of 1%. This is an improvement compared with previous chromogenic methods, where the level of detection was reported by Rollema et al. (1983) to be between 6 and 10% of PL activities in bulk milk, but does not reach the level of detection of fluorogenic methods (0.1% according to Saint-Denis et al., 2001).

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plasmin activity</th>
<th>PLG-derived activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>390 ± 47</td>
<td>3203 ± 130</td>
</tr>
<tr>
<td>Pasteurised skim milk</td>
<td>462 ± 13</td>
<td>3259 ± 37</td>
</tr>
<tr>
<td>Havarti (1 week old)</td>
<td>4095 ± 167</td>
<td>46,940 ± 600</td>
</tr>
<tr>
<td>Herrgård (&gt;6 months)</td>
<td>10,225 ± 309</td>
<td>25,841 ± 958</td>
</tr>
<tr>
<td>Parmigiano Reggiano</td>
<td>8579 ± 228</td>
<td>25,728 ± 255</td>
</tr>
</tbody>
</table>

* Results are shown as means ± standard deviation of 4 determinations on 4 occasions. PL and PLG derived activity are shown in μU mL$^{-1}$ for milk and in μU g$^{-1}$ for cheeses.

#### 3.2. Effect of skimming by centrifugation on plasmin activity in milk

Previous methods for measurement of PL activity have been based on measurements in skimmed milk (Rollema et al., 1983), and have either included a centrifugation step to remove the cream phase (Politis et al., 1993; Richardson & Pearce, 1981) or do not specify the fat content of the milk tested (Saint-Denis et al., 2001). To investigate the effect of skimming on the development of absorbance in the assay, samples of WM and RM were analysed before and after skimming, with SM as a control.

Table 2 shows the measured PL activities before and after skimming and the percentage difference in PL activity due to skimming. The PL activity in un-skimmed WM was ~25% higher compared with the SM, but decreased significantly by almost 30% after skimming ($P < 0.01$), to a similar level as in the SM, while skimming had no significant effect on the PL activity in RM and SM ($P > 0.05$). The measurements in RM provide further proof that the described method is suitable for measurement in turbid fat-containing samples.

The decrease in PL activity in WM after skimming could be attributed to the removal of PL associated with caseins on the milk fat globules. The native milk fat globule membrane fraction of raw milk contains a low level of PL activity, probably due to casein contamination (Benfeldt, Larsen, Rasmussen, Andreassen, & Petersen, 1995; Politis, Barbano, & Gorewit, 1992). Upon homogenisation, the composition of the milk fat globule membrane (MFGM) changes and up to 70% of the reformed membrane consists of caseins and casein micellar fragments (Cano-Ruiz & Richter, 1997). The high initial PL activity and the decrease in PL activity in the WM upon skimming indicates that a large portion of PL could be associated with the caseins in the MFGM after homogenisation.
and explain the similar PL activities of the skimmed WM and SM. The higher PL activity in WM compared with SM is in contrast to earlier findings, which reported that 40% of PL is inactivated during a standard single-stage homogenisation of milk (Hayes & Kelly, 2003). Initial experiments to confirm PL activity in the reformed MFGM of homogenised cream were not possible due to difficulty of dispersing the washed cream in the presently used buffer systems. In conclusion, skimming and centrifugation of the sample prior to analysis facilitates the measurement of PL in RM samples, but leads to an underestimation of the PL activity in homogenised milk.

### 3.3. Recovery of activity of added PL and inhibitory effects of milk components

Fig. 3A shows the PL activity in a dilution of SM with UHT milk and the activity of a fixed amount of PL added to a dilution of UHT milk with buffer. PL activity in the dilution of SM with UHT milk decreased linearly with increasing proportion of UHT milk added with the same casein and whey protein concentration. Lowering the whey protein and casein concentration increased measured PL activity, as can be seen from the increase in activity of the added PL in UHT milk with decreasing proportion of added UHT milk. The recovered activity of the added PL in UHT milk increased from 48.1 ± 1.3% in the sample containing UHT milk only to 71.2 ± 0.7% in the dilution containing 25% UHT milk (Fig. 3B), which shows the inhibitory and interfering effects of caseins and denatured whey proteins (Bastian, Hansen, & Brown, 1993; Hayes et al., 2002) on the PL activity.

### Table 2

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Before skimming (µU mL⁻¹)</th>
<th>After skimming (µU mL⁻¹)</th>
<th>Percentage activity after skimming</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM A</td>
<td>703 ± 33</td>
<td>509 ± 10</td>
<td>72.4 ± 5.1</td>
</tr>
<tr>
<td>B</td>
<td>683 ± 35</td>
<td>491 ± 5</td>
<td>71.9 ± 5.2</td>
</tr>
<tr>
<td>C</td>
<td>679 ± 24</td>
<td>482 ± 2</td>
<td>70.9 ± 3.6</td>
</tr>
<tr>
<td>RM A</td>
<td>558 ± 6</td>
<td>561 ± 3</td>
<td>100.4 ± 1.1</td>
</tr>
<tr>
<td>B</td>
<td>636 ± 9</td>
<td>694 ± 3</td>
<td>109.2 ± 1.9</td>
</tr>
<tr>
<td>C</td>
<td>712 ± 18</td>
<td>646 ± 10</td>
<td>90.8 ± 2.9</td>
</tr>
<tr>
<td>SM A</td>
<td>549 ± 15</td>
<td>523 ± 8</td>
<td>95.1 ± 3.2</td>
</tr>
<tr>
<td>B</td>
<td>534 ± 8</td>
<td>552 ± 9</td>
<td>103.3 ± 2.1</td>
</tr>
<tr>
<td>C</td>
<td>558 ± 15</td>
<td>563 ± 1</td>
<td>101.0 ± 2.7</td>
</tr>
</tbody>
</table>

* Results are means of 2 determinations ± standard deviation.

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recovered activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>38.8 ± 2.9b</td>
</tr>
<tr>
<td>Pasteurised skim milk</td>
<td>43.8 ± 5.3b</td>
</tr>
<tr>
<td>UHT milk</td>
<td>50.3 ± 2.7a</td>
</tr>
<tr>
<td>Micellar casein</td>
<td>66.9 ± 1.5a</td>
</tr>
<tr>
<td>Havarti</td>
<td>70.4 ± 1.8c</td>
</tr>
<tr>
<td>Herrgård</td>
<td>64.8 ± 0.8b</td>
</tr>
<tr>
<td>Parmigiano Reggiano</td>
<td>65.5 ± 3.3b</td>
</tr>
</tbody>
</table>

* Results are shown as means of 3 determinations ± standard deviation; values without common superscripts are significantly different (P ≤ 0.05).

The recovered activity of added PL in SM, RM, MCS and UHT milk, and Danish Havarti, Swedish Herrgård and Parmigiano Reggiano cheese, are shown in Table 3. The recovered activity in the milk samples was correlated with the heat treatment of the samples with RM showing the lowest recovered activity and UHT milk the highest. The increase in recovered activity with increasing heat treatment is most likely due to inactivation of PL, which has been shown to be heat-labile (Prado, Sombers, Ismail, & Hayes, 2006). The recovery rate in MCS was only approximately 67%, due to competitive inhibition of the synthetic substrate by caseins (Bastian et al., 1991a). Compared with the milk samples, MCS showed a 16–29% higher recovered activity, reflecting the inhibitory effect of serum-based PL and whey proteins on PL activity.

Bastian et al. (1991a) showed that activity of PL towards S-2251 is competitively inhibited by caseins and, by increasing the substrate concentration to 0.4 mM, eliminated the inhibitory effect of 5 mg mL⁻¹ casein. In this assay, a casein concentration of approximately 2.6 mg mL⁻¹ in the MCS and ~4 times higher substrate concentration compared with those used by Bastian et al. (1991a) had an inhibitory effect on the PL activity. The differences in these results may arise from the source of PL and higher PL concentrations used for the experiment. In this study, the activity of the indigenous PL associated with the casein fraction was measured, whereas Bastian et al. (1991a) used commercial PL from bovine plasma (final concentration 26.8 nm) at a concentration 3–17 times higher than that present in milk (1.6–8.2 nm).

Recovered activity in cheeses was similar to that for MCS, although the protein concentration in the final mixture was approximately double that of the milk samples. Protein content in the cheeses varied due to the removal of fat and the recovered activity in the cheese samples increased with increasing fat content. Although the fat content in the Danish Havarti cheese was approximately twice as high as for the other cheese types, the recovered activity was only slightly higher. The Havarti cheese was only one week old and the hydrolysis of caseins during the ripening...
of the cheeses could cause a reduced inhibitory effect on the synthetic substrate as they could be too small to block the active centre or not have a cleavage site for PL and thus not bind at all. Hydrolysed caseins and the resulting peptides may have a reduced inhibitory effect on the synthetic substrate.

The recovered activities for milk and micellar casein in Table 3 are in the same range as previously reported results. Saint-Denis et al. (2001) reported a recovery of 30–40% in diluted (1:4, v/v) pasteurised milk, while Richardson and Pearce (1981) reported a recovery of 40% in a 1:16 (v/v) diluted 3% solution of sodium caseinate. This shows that the described method has a similar recovery of 40% in a 1:16 (v/v) diluted 3% solution of sodium caseinate. This suggests that the described method has a similar sensitivity and sufficient substrate concentration to compensate for inhibitory effects compared with previously described methods, although a direct comparison is difficult due to the different buffers, dilutions and samples used for the determination.

3.4. Effect of dissociation of plasmin by EACA and NaCl in the presence and absence of whey proteins on PL and PLG-derived activity

PL in milk is known to be associated with caseins via lysine-binding sites that are not affected by addition of trisodium citrate (Politis et al., 1993; Rollema et al., 1983). EACA has previously been shown to dissociate PL and PLG from caseins by binding to the lysine-binding sites of PL and PLG, and enhance measured PL activity (Korycka-Dahl et al., 1983). The lysine-binding sites of PL are also of great importance for the physiological function of human PL in blood and the fibrinolytic system. Binding of PL to fibrin ensures a close proximity of the enzyme to its substrate and also protects PL from irreversible inhibition by α2-antiplasmin, a major indigenous PL inhibitor, which requires a free lysine-binding site and a free catalytic centre of PL to interact and inhibit PL (Cesarman-Maus & Hajjar, 2005; Collen, 1987). The dissociation of PL and caseins could therefore reduce the competitive inhibition of the synthetic substrate by caseins and affect the interaction of PL with PL and whey proteins.

The dissociation of PL and caseins by EACA + NaCl increased PL activity in SM significantly (P > 0.05) by approximately 9% and 11% for PL and PLG-derived activity, respectively (Table 4). The PLG/PL ratio of the free and bound SM was not significantly different, indicating that EACA + NaCl did not affect the activation of PLG by uPA under the chosen assay conditions. The dissociation of PL did not lead to a significant increase in PL activity in RM, suggesting that the dissociation increased inhibition of PL by serum-based inhibitors.

To further investigate the inhibitory effect of serum-based inhibitory components on free and bound PL, EACA and NaCl were added together to a dilution series of SM and MCS. Fig. 4 shows measured PL activity as a function of the percentage of SM or MCS in the dilutions in the presence or absence of EACA + NaCl.

The PL activity in the MCS did not decrease proportionally with the casein content. This suggests that the inhibitory effect of casein increases with concentration, as previously reported by Bastian et al. (1999a). Dissociation of PL and caseins in the undiluted MCS increased PL activity by 44%. This shows that the dissociation of PL and caseins greatly reduces the competitive inhibition of the synthetic substrate by caseins in the assay. The difference in PL activity between free and bound PL in MCS was constant in all dilutions, indicating that the same competitive inhibition applies at different casein concentrations.

Compared with MCS, the increase in PL activity upon dissociation of PL and caseins in SM was less pronounced. In contrast to the MCS, the difference in PL activity increased with increasing dilution from 9% in the undiluted SM to 24% in the sample containing 20% SM, showing that dilution of serum-based inhibitors increases PL activity. For the SM samples, on the other hand, the relatively small difference in PL activity compared with the MCS samples indicates that the interfering effect of the caseins is overshadowed by a change in the interaction of PL with serum-based inhibitory components (e.g., whey proteins). With increasing sample dilution, the percentage difference between samples with free and bound PL increased from an initial of 8% in the 100% SM to 19% in the 20% SM. This shows that the inhibitory effect of serum-based components decreases with increasing dilution of SM.

Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>PL activity (µU mL⁻¹)</th>
<th>Plasminogen-derived activity (µU mL⁻¹)</th>
<th>Plasminogen/plasmin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk + EACA and NaCl</td>
<td>438 ± 4*</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Raw milk – EACA and NaCl</td>
<td>443 ± 6*</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Pasteurised skim milk + EACA</td>
<td>459 ± 6*</td>
<td>3367 ± 87*</td>
<td>7.3 ± 0.2*</td>
</tr>
<tr>
<td>Pasteurised skim milk + EACA</td>
<td>422 ± 6*</td>
<td>3036 ± 47*</td>
<td>7.2 ± 0.2*</td>
</tr>
</tbody>
</table>

* Results are shown as mean 3 determinations ± standard deviation (n.d., not determined); values without common superscripts for each row are significantly different (P < 0.05).

containing 20% SM, showing that dilution of serum-based inhibitors increases PL activity. For the SM samples, the other hand, the relatively small difference in PL activity compared with the MCS samples indicates that the interfering effect of the caseins is overshadowed by a change in the interaction of PL with serum-based inhibitory components (e.g., whey proteins). With increasing sample dilution, the percentage difference between samples with free and bound PL increased from an initial of 8% in the 100% SM to 19% in the 20% SM. This shows that the inhibitory effect of serum-based components decreases with increasing dilution of SM.

Since the interaction of PL and α2-antiplasmin is reduced in presence of EACA (Christensen, Sorup-Jensen, & Christensen, 1995) and pasteurisation conditions largely inactivate Pts (Prado et al., 2006), the observed inhibition is most likely due to an enhanced inhibition of PL by whey proteins. This protective effect of caseins towards inhibition of PL by whey proteins in milk has not to our knowledge been documented previously, although this was suggested by Politis et al. (1993). Whether the dissociation of PL and caseins also enhances the inhibition of PL in a native milk environment and reduces proteolysis of casein was not the focus of this study, but should be further investigated to allow a correlation of proteolysis and PL activity.

**Fig. 4.** Effect of adding EACA + NaCl on the plasmin activity in SM (○,■) and a micellar casein solution (○,●). Milk and the micellar casein solution were diluted with Tris-HCl buffer, pH 8. The samples were mixed 1:1 with Tris-HCl buffer without (dashed line, filled symbols) or with 6 mM EACA and 0.4 mM NaCl (solid line, open symbols) and assayed for plasmin activity (average of 3 determinations ± standard deviation).
4. Conclusion

The method presented enables the measurement of PL and PLG-derived activity in turbid samples, such as homogenised whole milk, micellar casein solutions and cheese extracts, using a microtitre plate real-time kinetic method with high substrate. The performance of the assay was determined in relation to limit of detection, repeatability, reproducibility, recovery of added PL and the effect of milk concentration on the linearity of the assay. When pasteurised, homogenised milk was centrifuged, approximately 30% of the PL activity was removed, in contrast to raw, whole milk, where the level did not decrease after skimming. The dissociation of PL from the caseins by simultaneous addition of EACA and NaCl mainly affected interactions of PL with inhibitory components present in whey, e.g., Pls and whey proteins, which reduced PL activity by approximately 30–40%. Further studies will be required to clarify the effect of homogenisation on the PL system and PL activity and the mechanism of inhibition of PL by whey proteins.

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References


Paper II


Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion

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Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion

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A B S T R A C T

The effect of enzymatic activity in direct steam infusion heat treated milk with ultra-short holding times (>150 °C for <0.2 s) on age gelation during storage was investigated. Preheating at either 72 or 95 °C for 180 s was performed. Milk pre-heated at 72 °C showed extensive proteolysis and exhibited bitter off-flavour and contained <40% intact αs- and β-caseins after 6 weeks storage at 20 °C. No proteolysis of k-casein was detected. Plasmin was identified as active protease and activation of plasminogen was observed as an increase in the rate of casein hydrolysis. Proteolysis in the stored samples correlated with a decrease in pH and with changes in colour. Gelation occurred after 10 weeks along with an increase in viscosity and extensive proteolysis of αs- and β-caseins. In conclusion, plasmin activity was involved in age gelation and bitterness caused by proteolysis was the shelf-life limiting factor.

1. Introduction

Ultra-high temperature (UHT) treatment of milk is a heating process at very high temperatures for short holding times, which renders the milk commercially sterile and gives a product with a long shelf-life at ambient temperatures. UHT treatments are usually carried out at temperatures of 140 °C for 4–6 s. Both indirect and direct heating systems (steam injection or infusion) have been applied to increase the shelf-life of milk. More recently, UHT treatments with temperatures >150 °C and ultra-short holding times (<0.2 s) have been shown to result in a sufficient reduction of micro-organisms, while doing minimal heat damage to milk constituents, such as vitamins and whey proteins (De Jong, 1996).

A major limiting factor for the shelf life of UHT milk is proteolysis of caseins, which can be caused by residual heat-stable bacterial proteases, indigenous milk proteases or combinations of these. Such proteolysis can cause formation of bitter off-flavours, sedimentation and age gelation of UHT milk (Datta & Deeth, 2003; Kelly & Foley, 1997). These problems more often occur in directly heated UHT milk, since the heat load is reduced, when compared with indirect methods, due to the rapid heating and cooling of the milk (Newstead, Paterson, Anema, Coker, & Wewala, 2006). This may be solved by inclusion of a preheat treatment step (Newstead et al., 2006; Van Asselt, Sweere, Rollema, & de Jong, 2008) or by increasing the temperature during UHT treatment (Topcu, Numanguloglu, & Saldamlı, 2006).

The cause for age gelation in UHT milk is not known, but it has been suggested that release of the β-lactoglobulin–k-casein complex from the casein micelle is involved (McMahon, 1996). This complex is formed during heat treatment by the denaturation of β-lactoglobulin (β-lg) and subsequent aggregation with k-casein via disulphide bonds. The released complex aggregates and forms a gel. Proteolysis accelerates or can cause the release of the β-lactoglobulin–k-casein complex by hydrolysis of the caseins that anchor the β-lactoglobulin–k-casein in the casein micelle (Datta & Deeth, 2001; McMahon, 1996). While a certain level of proteolysis is required to induce age gelation, the relation between gelation time and extent of proteolysis is controversial (Manji & Kakuda, 1988; Newstead et al., 2006).

Plasmin (PL, EC 3.4.21.7), the major indigenous protease in milk, has been closely linked to the age gelation of UHT milk (Kelly & Foley, 1997; Kohlmann, Nielsen, & Ladisch, 1991; Manji & Kakuda, 1988; Newstead et al., 2006). PL readily hydrolysates β- and αs2-casein, and to a lesser extent, αs1-casein (Grufferty & Fox, 1988). The
level and activity of PL in milk can vary and depends on biological factors, such as stage of lactation and somatic cell count (Larsen et al., 2010; Nicholas, Auldist, Molan, Stelwagen, & Prosser, 2002).

PL is the active part of a complex enzyme system. Its zymogen, plasminogen (PLG), is present in raw, bovine milk at a 2–30 times higher concentration than PL (Ismail & Nielsen, 2010; Korycka-Dahl, Dumas, Chene, & Martal, 1983; Rauh et al., submitted for publication), and is activated by two families of plasminogen activators (PA), urokinase-type PA (uPA) and tissue-type PA (tPA). The activity of PL and PA is regulated by plasmin inhibitors (PIs), such as α2-antiplasmin, and plasminogen activator inhibitors (PAIs) (Grufferty & Fox, 1988; Ismail & Nielsen, 2010; Lu & Nielsen, 1993; Precetti, Oria, & Nielsen, 1997).

The components of the PL system show very different thermal stabilities. While PL, PLG and PA are heat-stable enzymes, the inhibitors of the PL system are generally considered as heat labile (Prado, Ismail, Ramos, & Hayes, 2007; Prado, Sombers, Ismail, & Hayes, 2006; Richardson, 1983). Due to this complexity it is difficult to predict the actual PL activity and the consequence of proteolysis for heat treatment of milk under novel and untreated temperature conditions.

The aim of the present study was to investigate the effect of plasmin activity on proteolysis and physical-chemical changes in milk treated with direct steam infusion heating and ultra-short holding times during subsequent storage.

2. Materials and methods

2.1. Materials

One day old low pasteurised milk (72 °C for 15 s) with 1.5% fat was obtained from Arla Foods Stockholm Dairy (Stockholm, Sweden) and processed in the UHT pilot plant (SPX, Silkeborg, Denmark) at Arla Strategic Innovation Centre (Stockholm, Sweden). The milk (200 L) was pre-heated at 72 °C (Milk72) or 95 °C (Milk95) for 180 s in a tubular heat exchanger before being subjected to the direct steam infusion heat treatment (> 150 °C for < 0.2 s). Pre-heat treatment conditions were chosen to obtain a milk with (Milk72) and without (Milk95) residual PL activity. After flash cooling, the milk was homogenised using a two-stage homogeniser (160/40 bar), further cooled down to 20 °C by a tubular heat exchanger and packed aseptically in 300 mL glass bottles. The trials were performed during three consecutive weeks. The milk was stored in the dark at 20 °C in a climate chamber for 14–16 weeks. Samples, i.e., one 300 mL bottle per treatment, were collected once every week for further analysis.

2.2. Analytical methods

Each batch of processed milk was tested for sterility according to ISO 4833 (ISO, 2003). The milk was visually inspected for sedimentation, gelation and colour. Milk72, Milk95 and fresh low pasteurised milk was tasted for perceived bitterness by five panellists. The pH of the milk was measured at room temperature by a pH meter (Knicker Elektronische Messgeräte GmbH & Co. KG., Berlin, Germany). Measurements were performed in duplicates. Colour development was measured using Hunters Lab colour space with a colorimeter Minolta CR-A70 (Minolta Camera Co., Ltd, Osaka, Japan). The L-, a- and b-values reflect lightness (0 = black, 100 = white), redness (−100 = green, 100 = red) and yellowness (−100 = blue, 100 = yellow), respectively. The instrument was calibrated with a Minolta standard plate (standard values: Y = 92.4; x = 0.3161; y = 0.3325). Measurements were performed in triplicate. Viscosity of the milk was measured using a double gap concentric cylinder geometry (inside cup diameter 40.0 mm, inside bob diameter 40.8 mm, cylinder height 59.5 mm, operating gap 2.0 mm, sample volume 6.8 mL) on a TA Discovery HR-2 rheometer (TA Instruments, New Castle, DE, USA). Measurements were performed at 25 °C. After the temperature was reached, a 15 s pre-shearing step was conducted before viscosity was recorded for 120 s at a shear rate of 100 s⁻¹. Measurements were performed in triplicate.

The volume based particle size distribution of the milk was obtained with a Master Sizer 3000 (Malvern Instruments Ltd., Malvern, UK). Distilled, degassed water was used as dispersant. The refractive index was set to 1.45 for particles and to 1.33 for the dispersant. Milk was added under agitation at 1000 rpm to the dispersion unit until laser obscuration was in the range of 5.5–8.0% and particle size distributions were recorded. Measurements were performed in duplicate.

After 10 weeks storage, visually detectable gel fragments were placed on a microscope slide and stained by adding 1 µL of a 0.02% fluorescein-5-isothiocyanate (FITC; Merck, Darmstadt, Germany) solution in acetone for proteins and a 0.02% Nile red (9-diethylamino-5H-benzophenoxazine-5-one; Merck) solution in acetone for fat. After the acetone was fully evaporated, the samples were examined using a 40 x and 100 x oil immersion objective on a Leica DMIRE2 inverted confocal laser scanning microscope (Leica Microsystems GmbH, Heidelberg, Germany) fitted with an Ar/Kr laser for FITC and a He 543/554 nm laser for Nile red. FITC was excited at 485 nm and emission was recorded between 500 and 545 nm. Nile red was excited at 543 nm and the emitted signal recorded from 560 to 700 nm.

PL and PLG derived activity were measured by the rate of hydrolysis of the chromogenic substrate S-2251 (Rollema, Visser, & Poll, 1983) and release of p-nitroaniline as described previously (Rauh et al., submitted for publication). PLG was activated by addition of uPA (Immuno Danmark A/S, Copenhagen, Denmark). The rate of increase in absorbance intensity during the assay is proportional to the PL or PLG derived activity.

Analysis of protein composition in the milk was performed as described by Bonfatti, Grigoletto, Cecchinato, Gallo, and Carnier (2008) with the following modifications. A sample of 200 µL of milk (w/w) was frozen at −20 °C until further use.

Disulphide bonds were reduced by the addition of 20 µL 1 M dithiothreitol and 1 mL 100 mM tri-sodium citrate, 6 M urea at 30 °C for 60 min. The samples were centrifuged at 9300 × g at 5 °C for 10 min. From the clear phase, 200 µL were used for the analysis. A volume of 5 µL was injected into the LC–MS system. The column was a BioSuite™ C18 PA-B (C18, 3.5 µm; 2.1 mm × 250 mm, Waters, Milford, MA, USA). Buffer A was Milli-Q water with 0.5% (v/v) tri-fluoroacetic acid (TFA) and buffer B acetonitrile with 0.1% (v/v) TFA. Column temperature was 47 °C and a linear gradient from 46.8% to 20% in 16 min. The flow rate was set to 0.35 mL min⁻¹ with UV detection at 214 nm. MS detection was performed with a quadrupole time-of-flight mass spectrometer with (Q-TOF-MS; Agilent 6530 Aquarate mass detector, Agilent Technologies, Santa Clara, CA, USA) and MS scans were continuously recorded between a mass to charge ratio (m/z) of 300 and 3500. Data analysis was made with Mass Hunter software (Agilent Technologies). Measurements were performed in duplicate.

For the analysis of pH 4.6 soluble peptides and native whey proteins, volumes of 20 mL of milk were adjusted to pH 4.6 with 1 M HCl and centrifuged at 11,000 × g for 10 min at 4 °C. From the clear supernatant, 1.5 mL was taken out and frozen at −20 °C until further use. Prior to analysis, 1 mL of the sample was centrifuged at 9300 × g at 5 °C for 10 min and the supernatant used for the analysis by injecting 10–20 µL of the LC system, depending on the peptide concentration. The column was a Xbridge BEH 300 (C18, 3.5 µm; 2.1 mm × 250 mm, Waters) operated at 45 °C Buffer A was Milli-Q water with 0.1% (v/v) TFA and buffer B acetonitrile with 0.5% (v/v) TFA. A linear gradient was applied with 100% buffer A from 3 min,
showed a higher degree of denaturation for and Milk95 were measured one day after the heat treatment and denaturation and residual PL and PLG derived activities for Milk72 activity was detected during storage. The degree of whey protein PLG derived activity could be detected in Milk95. Denaturation of 3.1. Influence of heat treatment

The heat treatment rendered the milk sterile as no microbial activity was detected during storage. The degree of whey protein denaturation and residual PL and PLG derived activities for Milk72 and Milk95 were measured one day after the heat treatment and are shown in Table 1. In comparison with other direct heat treatments applying ultra-short holding times reported earlier, Milk72 showed a higher degree of denaturation for β-lg. Previously reported values for β-lactoglobulin denaturation were 19–21% at 150–170 °C (Huijs, Van Asselt, Verdurmen, & De Jong, 2004) and 15% and 28% at 150 °C (Dickow, 2011) for skim and whole milk, respectively, both using a steam injection system. This difference can be explained by the additional β-lactoglobulin denaturation during the pre-heat treatment at 72 °C for 180 s amounting to approximately 2–4% (Tolkach & Kulowiz, 2007) and additionally approximately 4% during the homogenisation step following the direct heat treatment (Garcia-Risco, Ramos, & Lopez-Fandiño, 2002). Milk95 showed 90% denaturation of β-lg and denaturation of α-lactalbumin (α-la) in Milk95 was approximately 3.5 times higher than in Milk72. Residual PL and PLG derived activities in Milk72 were 30.9 ± 2.3% and 14.0 ± 2.7%, respectively, whereas no residual PL or PLG derived activity could be detected in Milk95. Denaturation of β-lg and inactivation of PL and PLG are known to be closely correlated (Ismail & Nielsen, 2010; Newstead et al., 2006). The higher heat load and degree of β-lg denaturation of Milk72 correlates with the lower residual PL and PLG derived activity compared to the residual levels of 50% and 37% PL and PLG derived activity, respectively, found after steam injection (150 °C, Dickow, Nielsen, & Hammershøj, 2012). Interestingly, it seems that in direct UHT treatments with ultra-short holding times, PLG appears to have lower heat stability than PL (Table 1). Kinetic studies on the heat inactivation of PL and PLG as well as direct UHT treatments show a similar or higher heat stability of PLG compared with PL at temperatures between 90 and 142 °C (Manji & Kakuda, 1986; Rollema & Poll, 1986; Saint-Denis, Humbert, & Gaillard, 2001). This suggests that a different heat inactivation kinetic for PL and PLG applies for direct UHT treatments with ultra-short holding times compared with other UHT treatments and previous kinetic studies.

Table 1 WHEY PROTEIN DENATURATION AND RESIDUAL PLASMIN (PL) AND PLASMINOGEN (PLG) ACTIVITY IN MILK72 AND MILK95 AFTER INDIRECT PRE-HEAT TREATMENT AND DIRECT STEAM INFUSION (150 °C FOR 0.2 s). (a)

<table>
<thead>
<tr>
<th>Milk</th>
<th>Indirect pre-heat treatment</th>
<th>Whey protein denaturation (%)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-lg</td>
<td>α-la</td>
<td>PL</td>
</tr>
<tr>
<td>Milk72 72 °C/180 s</td>
<td>36.7 ± 3.2</td>
<td>13.1 ± 2.2</td>
<td>30.9 ± 2.3</td>
</tr>
<tr>
<td>Milk95 95 °C/180 s</td>
<td>94.1 ± 1.6</td>
<td>45.3 ± 3.3</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

3.2. Appearance and taste

In Milk72 a bitter flavour was noticeable after 6 weeks that rapidly intensified, and the milk was extremely bitter after 7 weeks of storage. A similar development has been shown for direct UHT treatments with ultra-short holding time (Huijs et al., 2004). No bitterness was detected in Milk95.

After 10 weeks storage, a visible difference in the colours between Milk72 and 95 was observed, with Milk72 being less opaque than Milk95. After 10 weeks, a gel layer was found at the bottom of the bottle of Milk72, which increased in thickness in the following week. Milk95 showed no gelation or sedimentation during the storage period. The gel was soft and fragile. Along with this gel layer, floating gel particles appeared in Milk72 after 11 weeks storage. After 16 weeks storage, Milk72 was completely gelled and syneresis could be observed. The gel formation and appearance was similar to the description of a plasmin induced gel found in UHT milk by Kelly and Foley (1997). In contrast to other similar studies (Newstead et al., 2006; Topçu et al., 2006), Milk72 showed no sedimentation. This difference may arise from the shorter storage time needed for gelation in this study. Milk72 already gelled after 80 d of storage, while Newstead et al. (2006) and Topçu et al. (2006) reported gelation after 150–180 d of storage.

In addition to the gel formation, a creamy layer appeared on the top of Milk72 after 80 d of storage, a phenomenon that has been described for milk in connection with plasmin activity by Kohlmann et al. (1991). This was most apparent in the samples adjusted to pH 4.6, due to the homogenisation, small fat globules could be observed in the supernatant of Milk95 after centrifugation, while the supernatant of Milk72 was clear with a creamy layer on top.

3.3. Physical-chemical changes during storage

The pH of Milk72 and Milk95 (Fig. 1) decreased during storage, but the decrease was more pronounced in Milk72, by approximately 0.2 pH-units. The larger decrease in Milk72 can be related to the residual PL activity in the sample and proteolysis after lysine residues (Kelly & Foley, 1997; Kohlmann et al., 1991). Interestingly, the pH did not decrease further after the onset of gelation after 10 weeks storage.

The colour values of Milk95 only decreased slightly during the storage period, while the colour values for Milk72 decreased gradually, becoming less white (decreased L-value), more greenish (higher negative a-value), and less yellow (lower b-value). As can be seen in Fig. 2, Milk95 showed higher initial a and b colour values, which can be attributed to the higher extent of Maillard reaction due to the more severe heat treatment (Kneifel, Ulberth, & Schaffer,
Similar to the pH value, the colour values levelled out with the onset of gelation, and showed a tendency to increase again for the $a$ and $b$ colour values. The difference in the $L$ value of Milk72 and Milk95 correlates well with the observed visible difference in opacity and could indicate a change in the casein micelles properties. A reduction of the $L$ value has been correlated with an increase in casein micelle size after extensive hydrolysis with PL (Crudden, Afoufa-Bastien, Fox, Brisson, & Kelly, 2005) as well with a dissociation of casein micelles upon tri-sodium citrate addition (O’Sullivan, Kelly, & Fox, 2002). The latter could also be associated with PL mediated proteolysis as partially disintegrated casein micelles have been found in PL-treated milk (Aroonkamonsri, 1996).

The development in viscosity of Milk72 and 95 is shown in Fig. 2. Viscosity of both milks was constant between 2.0 and 2.5 mPa s during the first 9 weeks of storage. After 10 weeks storage, the onset of gelation caused the viscosity of Milk72 to increase and it further increased rapidly in the following week up to 13.6 \pm 2.6 mPa s. The viscosity of Milk95 did not change during the applied storage period. A similar observed increase in viscosity with the onset of gelation has been reported previously by Kohlmann et al. (1991) and Kelly and Foley (1997).

During the first 10 weeks storage, the particle size distribution did not change significantly (data not shown). Fig. 3 shows a representative volume based particle size distribution of particles in Milk72 between 0 and 14 weeks storage. Due to the homogenisation of the milk, the individual size distributions of casein micelles and fat globules could not be distinguished. The 10 week old milk shows a mono-modal distribution consisting of casein micelles and homogenised fat globules with an average diameter of approximately 190 nm. The change in casein micelles observed in the colour measurement could not be seen in the particle size distribution and is most likely overshadowed by the contribution of the fat globules to the particle size distribution. After 11 weeks storage, larger particles between 8 and 10 $\mu$m could be observed. In the following week, the population of the larger particles increased while the casein micelle/fat globule distribution became narrower and contributed to a relatively smaller amount of the particles. The large particles were most likely gel fragments. Due to the stirring during the measurement, the gel particles broke down to smaller fragments resulting in a relatively narrow size distribution. The broadening of the distribution towards higher sizes after 14 weeks reflects the increased firmness of the gel.
The microstructure of the gel found in Milk72 was visualised by confocal laser scanning microscopy (see Supplementary material). The gel appeared to have a loose, non-homogeneous structure with liquid filled cavities, similar to the microstructure of yoghurts and acid milk gels (Pereira, Matía-Merino, Jones, & Singh, 2006; Torres, Amigo Rubio, & Ipsen, 2012). The micrographs show small fat globules (0.5–2 μm), that are partially present in clusters and incorporated in the gel structure. The diameter of the fat globules is in line with the observed particle size distributions. The presence of agglutinated fat globules could account for the observed cream layer in Milk72.

3.4. Enzymatic activity and proteolysis during storage

3.4.1. Plasmin and plasminogen derived activity

The development in PL and PLG derived activities in Milk72 are shown in Fig. 4. As can be seen, PLG derived activity decreased rapidly, while PL activity increased. This indicates activation of PLG into PL during storage, and no PLG derived activity was detectable after 6 weeks storage. A similar development has been reported in direct heated UHT milk (Manji & Kakuda, 1986). The increase in PL activity could be fitted well to a first order reaction kinetic \( k = 0.1828, R^2 = 0.995 \), while PLG derived activity on the other hand decreased from 0 to 6 week in a linear fashion \( y = -104.7x + 603; R^2 = 0.997 \). The inhibitors of the PL system are heat labile: under pasteurisation conditions (74.5 °C for 15 s) PAI is completely inactivated, while about 36% of PI is inactivated (Prado et al., 2006). PA have been shown to have a similar or even higher heat stability than PL and PLG (Lu & Nielsen, 1993; Saint-Denis et al., 2001), suggesting that a significant proportion of PA remained in Milk72 after the heat treatment. This, together with the inactivation of PAI, explains the fast rate of PLG activation in Milk72. After 4 weeks storage, the PL activity in Milk72 was comparable to the PL activity found in the low pasteurised milk \( (632 \pm 28 \mu L -1) \) prior to heat treatment. The gelation of Milk72 had no apparent effect on PL activity.

The PL and PLG derived activities developed differently and PL activity continued to increase after depletion of PLG. This development can be ascribed to other factors affecting PL activity, such as a reduced competitive inhibition of PL by caseins towards the synthetic substrate in the analysed milk samples (Bastian, Brown, & Ernstrom, 1991) due to proteolysis.

3.4.2. Changes in protein profiles during storage

In Fig. 5, a comparison of the protein profiles of Milk72 and 95 after 0, 4, 8 and 12 weeks storage is shown. Milk72 showed very extensive proteolysis during the storage period, while the overall chromatographic pattern for Milk95 remained unchanged. From the UV chromatograms, no statement about the development in \( \alpha_2 \)-casein could be made due to overlay with upcoming polypeptides (Fig. 5 A–D). After 4 weeks storage, the \( \beta \)-casein \( A^1 \) peak decreased the most, followed by a slight decrease in \( \beta_\gamma \)-casein and \( \beta \)-casein \( A^2 \). The peak of \( \beta \)-casein \( A^3 \) disappeared almost completely after 8 weeks storage, along with a large decrease in the \( \alpha_\delta \)-casein peak. After 12 weeks storage, no intact \( \alpha_\delta \)-casein with 9 phosphorylations (P) and \( \beta \)-casein \( A^1 \) was present in the UV chromatogram (Fig. 5D). Over the storage period, the \( \kappa \)-casein peaks seemed to disappear, but developed similarly in both milk types, ruling out proteolysis as a cause for this change in \( \kappa \)-casein profile. Instead, the disappearance can be attributed to a broadening of the peaks due to modifications of the proteins, likely by the Maillard reactions induced by the heat treatment (Gaucher, Mollé, Gagnaire, & Gaucher, 2008). For Milk95, the same tendency could be seen for \( \alpha_2 \)-casein.

Interestingly, significant differences in the peak development of \( \beta \)-casein \( A^1 \) and \( A^2 \) as well as for \( \beta_\gamma \)-casein with 8P and 9P were observed. Therefore, the mass spectra of peaks 4–9 were analysed after 8 weeks storage to investigate the components present in the peaks. The results are summarised in Table 2 and show that proteose peptones 5 and 8 (slow) from \( \beta \)-casein \( A^1 \) are co-eluting with proteose peptone 5 and 8 along with a polypeptide from \( \alpha_\delta \)-casein f(1–124). The corresponding polypeptide \( \alpha_\delta \)-casein f(125–199) was co-eluting with \( \alpha_\delta \)-casein 8P. While the peaks of \( \alpha_\delta \)-casein 9P and \( \beta \)-casein \( A^1 \), either were not or only slightly contaminated with polypeptides, \( \gamma_1 \)- and \( \gamma_2 \)-caseins were co-eluting with \( \beta \)-casein \( A^2 \) and \( \gamma_1 \)-casein with \( \alpha \)-la. This explains the difference in the peak development of \( \alpha_\delta \)-casein 8 and 9P as well as \( \beta \)-casein \( A^1 \) and \( A^2 \). A comparison of the mass spectra (data not shown) showed that the rate of hydrolysis was similar for the two genetic variants \( A^1 \) and \( A^2 \) of \( \beta \)-casein and for the differently phosphorylated \( \alpha_\delta \)-caseins. For this reason, \( \alpha_\delta \)-casein 9P and \( \beta \)-casein \( A^1 \) were chosen for a relative quantification of the proteolysis based on the UV chromatograms.

Proteolysis in UHT milk can be caused by PL and heat resistant proteases from, e.g., psychrotrophic bacteria. The milk used in this study was one day old low-pasteurised milk, which makes it unlikely that the extensive proteolysis in Milk72 was caused by bacterial proteases. The appearance of proteose peptides and \( \gamma \)-caseins suggested that PL was the main actor of proteolysis. Bacterial proteases are more unspecific in their cleavage sites and are capable of hydrolysing \( \kappa \)-casein (Datta & Deeth, 2001). In Milk72 and Milk95 no proteolysis of \( \kappa \)-casein was observed, further strengthening the indications that PL was the main active protease in Milk72.

The hydrolysis of \( \alpha_\delta \)-casein 9P and \( \beta \)-casein \( A^1 \) in Milk72 during storage are shown in Fig 6. Both \( \alpha_\delta \)-casein 9P and \( \beta \)-casein \( A^1 \) decrease in an inverse sigmoidal manner. Hydrolysis of casein by PL can usually be described by an exponential decay function (Newstead et al., 2006). In Milk72, a lag phase in the first 2–3 weeks storage preceded the exponential decay. This lag phase can be attributed to the activation of PLG to active PL as shown in Fig. 4. With increasing PL activity, the rate of hydrolysis increased.

The \( \beta \)-casein \( A^1 \) was hydrolysed faster than \( \alpha_\delta \)-casein 9P, as also reported in the literature (Grifferty & Fox, 1988). After 9 weeks storage, more than 95% of \( \beta \)-casein \( A^1 \) was hydrolysed, while the same degree of hydrolysis for \( \alpha_\delta \)-casein 9P was reached after 11 week.

The rate of hydrolysis of \( \alpha_\delta \)-casein by PL has been shown to be similar to \( \beta \)-casein (Grifferty & Fox, 1988). The MS spectrum of the \( \alpha_\delta \)-casein peak at different storage times (data not shown) showed that \( \alpha_\delta \)-casein was almost completely hydrolysed after 9 weeks storage, indicating the same rate of hydrolysis as \( \beta \)-casein. Gellation

![Fig. 4. Plasmin (○) and plasminogen (△) derived activity in milk pre-heat treated at 72 °C for 180 s and subjected to direct steam infusion (>150 °C for >0.2 s) during storage at 20 °C. Results are shown as average ± standard deviation of three replicates.](image-url)
occurred after 95% β-casein A1 and αS1-casein 9P had been hydrolysed.

The protein composition of the gel formed had the same pattern as the liquid (Fig. 7). The relative concentration of residual intact α- and β-caseins and the polypeptides in the gel were higher than in the liquid, while the concentrations of κ-casein and whey proteins were similar in the gel and in the liquid. As the micrographs indicated, the gel had a relatively loose structure, which contained a high proportion of liquid phase. This shows that κ-casein and whey proteins were not incorporated into the gel network. To the authors’ knowledge, the composition of a plasmin induced gel has not been reported previously.

Bitterness in Milk72 was noticeable after 6 weeks storage, at a time point when approximately 70% of αS1-casein and approximately 75% of β-casein (and presumably αS2-casein) was hydrolysed. Bitterness caused by PL has been attributed to peptides from

![Fig. 5. Protein profiles of milk subjected to direct steam infusion (≥150 °C for <0.2 s) and pre-heat treated at 72 °C (Milk72, solid line) or 95 °C (Milk95, dashed line) for 180 s after 0 (A), 4 (B), 8 (C) and 12 (D) weeks storage at 20 °C. The observed proteins at week 0 are κ-casein (peaks 1–3), αS2-casein (4), αS1-casein with 8 (5) or 9 (6) phosphorylations, β-casein A1 (7) and A2 (8), α-lactalbumin (9), β-lactoglobulin B (10) and A (11).](image)

![Fig. 6. Hydrolysis of αS1-casein 9P (○) and β-casein A1 (△) in milk pre-heat treated at 72 °C for 180 s and subjected to direct steam infusion (≥150 °C for <0.2 s) during storage at 20 °C expressed as the relative change in peak area of UV absorption at 214 nm. The arrow indicates the onset of gelation. Results are shown as average ± standard deviation of three replicates.](image)

Table 2

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<th>Deconvoluted mass (Da)</th>
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<th>Mass error (ppm)</th>
<th>ID</th>
</tr>
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</tr>
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</tr>
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<td>β-CN f(108-209)</td>
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<td>14,187.00</td>
<td>14,187.12</td>
<td>8.3</td>
<td>α-lactalbumin</td>
<td></td>
</tr>
</tbody>
</table>

* Identity is based on mass fingerprinting using average mass and reduced proteins and peptides: CN, casein; P, phosphorylated.
3.4.3. Changes in peptide profiles during storage

The development in the peptide profile of Milk72 (Fig. 8) showed a rapid increase of peaks with 31–34 min retention time between 0 and 4 weeks storage. These peaks were identified as proteose peptones. After 8 weeks storage, more peptides appeared between 17 and 30 min. Most of the peptides eluted late in the RP-UHPLC, which has been reported previously to correlate with PL activity (Datta & Deeth, 2003). The peptide profile changed slightly between 12 and 14 weeks storage, although the total peak area of the peptide profile of Milk72 remained constant in the same time period. The most profound change in the peptide profile between 12 and 14 weeks storage was seen in the proteose peptone peaks. The proteose peptone β-casein A1 f(1–105) eluting at 33.0 min decreased and β-casein A1 f(29–105) eluting at 32.7 min increased (peptides are indicated by arrows in Fig. 8). This shows that PL was still active after gelation of Milk72, as already suggested by the PL activity measurement. The decreased rate of proteolysis was most likely due to a limited accessibility of remaining substrate for PL in the gel matrix and depletion of preferred cleavage sites.

In Fig. 9, the development in the total peak area of pH 4.6 soluble peptides during storage is shown. The peak area of Milk72 developed in a sigmoidal way and appeared to almost inversely mirror the hydrolysis of αS1-casein 9P and β-casein A1 (Fig. 8). After a 2–3 week lag phase, the peak area increased rapidly and levelled out after the onset of gelation. This is in line with the almost complete hydrolysis of αS1-casein 9P and β-casein A1 after 11 weeks, showing that no more material soluble at pH 4.6 was formed. The total peak area of Milk95 increased only slightly during the storage period, confirming that no significant proteolysis occurred.

3.4.4. Correlation of proteolysis and gel formation

Proteolysis mediated by PL can cause gelation and increases transparency of milk and the outcome of proteolysis seem to depend largely on the extent of PL activity and rate of proteolysis in the milk. Large amounts of added PL have earlier been reported to cause clarification of milk (Cruden et al., 2005), while low amounts or indigenous residual PL has caused gelation of milk (Kelly & Foley, 1997; Kohlmann et al., 1991; Newstead et al., 2006).

In this study, at the onset of gelation of Milk72 more than 95% of α- and β-caseins were hydrolysed. Previous studies (Kelly & Foley, 1997; Kohlmann et al., 1991; Newstead et al., 2006) showed that almost complete proteolysis of β-casein was reached before a gel was formed, while α-casein on the other hand was either still present to some extent (Kelly & Foley, 1997) or almost completely hydrolysed (Kohlmann et al., 1991). The low level of β-lg denaturation in Milk72, compared to other UHT treatments, and the low concentration of β-lg and κ-casein in the formed gel suggests that the formation and release of a β-lactoglobulin-κ-casein complex was not the main cause of gelation. Most studies on PL in milk have focussed on β-casein as an indicator of proteolysis. In relation to age gelation, it is important to consider both hydrolysis of αS2- and of β-caseins. A part of β-casein has been shown to be highly mobile in the casein micelle (Dalgleish, 2011) and hydrolysis of β-casein could be responsible for the reported increase in hydrophobicity (Aroonkamonsri, 1996), while the hydrolysis of αS1-casein could be responsible for loss of micellar integrity, which in combination could lead to the age gelation of milk. Manji and Kakuda (1988) suggested gelation to be a two-stage process, which requires some degree of proteolysis before time-dependent physical-chemical changes lead to the gelation of milk. This could be a
reason for the different observations on age gelation of milk caused by PL. If PL activity in the milk is high, the polypeptides could be further degraded and inhibit the formation of a gel, before physical-chemical changes will take place. In milk with only little residual PL activity, physical-chemical changes induced by proteolysis could occur prior to achieving a degree of proteolysis sufficient to cause a gelation, which could be the case for observed sedimentation prior to or instead of gelation (Kelly & Foley, 1997; Newstead et al., 2006). This may also explain why the degree of proteolysis and the onset of gelation have not been directly correlated yet.

The results obtained in the present study suggest a gelation mechanism that is similar to acid gelation, with the difference that proteolysis instead of acid causes a destabilisation of the casein micelle. Acidification of milk leads to a reduced repulsion of casein micelles due to a reduced negative charge of κ-casein, followed by a rearrangement of the casein micelles by solubilisation of calcium due to the pH drop that leads to the formation of a gel network (Lucey & Singh, 2003). While the gelation by plasmin depends mainly on the rate of hydrolysis, the gel properties of acid gels depend largely on the rate of acidification (Anema, 2008). Since the pH in Milk72 is only decreasing slowly, the repulsion by κ-casein is still present in Milk72 and inhibits aggregation of casein micelles, which explains the absence of κ-casein in the gel network.

To fully understand the mechanism of PL induced age gelation, these two stages would need to be investigated separately, e.g., by following changes in casein micelle structure in milk with different degrees of proteolysis.

4. Conclusions

Our results indicate that PL mediated proteolysis can be a likely cause for age gelation in UHT milk produced with direct steam infusion (> 150 °C, 0.2 s). Activation of PLG to PL could be observed as an increase in PL activity and an increase in the rate of casein hydrolysis and peptide formation. The observed changes in colour and pH correlated with the proteolysis. The main shelf life limiting factor for the UHT milk used was the appearance of bitterness, which preceded age gelation. Further studies are necessary to investigate the peptide formation by PL and to identify peptides related to the development of bitterness.

Acknowledgements

The Danish Agency for Science, Technology and Innovation (DASTI) is thanked for financial support of the study. Many thanks to Jens Röschmann and Lena Bergström for the help in the Arla Foods pilot plant and Ulf Andersen, Bettina Mikkelsen (Arla Strategic Innovation Center) and Isabel Celi gueta (Nestlé Product Technology Center) for the help with the confocal microscopy.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2013.12.007

References


S. 1. Microstructure of gel particles in milk pre-heat treated at 72 °C for 180 s and subjected to direct steam infusion (>150 °C for <0.2 s) after 13 wk of storage at 20 °C. Green areas indicate protein stained with FITC and red areas fat stained with Nile red (red). The scale is indicated in the micrographs.

Plasmin Activity in UHT Milk: Relationship between Proteolysis, Age Gelation, and Bitterness

Journal of Agricultural and Food Chemistry, 62, 6852-6860
Errata

First paragraph in materials and methods on page 6853, first column, should say:

Milk Processing. Three batches of 1 day old pasteurized (72 °C for 15 s) and homogenized milk (1.5% fat) were obtained from Arla Foods Stockholm Dairy (Sweden) during three consecutive weeks. The milk was preheat treated at 72 °C for 180 s in a tubular heat exchanger before being subjected to a direct steam infusion heat treatment at >150 °C for <0.2 s (UHT pilot plant, APV/SPX, Silkeborg, Denmark). After flash cooling to 67 °C, the milk was homogenized (pressure 160/40 bar), further cooled to 20 °C, and packed aseptically in 300 mL glass bottles. The UHT milk was stored in the dark at 20 °C in a climate chamber for 14 weeks. One bottle was used once every week of the storage period for analysis. The sterility of each batch of processed milk was tested according to ISO 483324.
Plasmin Activity in UHT Milk: Relationship between Proteolysis, Age Gelation, and Bitterness

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Supporting Information

ABSTRACT: Plasmin, the major indigenous protease in milk, is linked to quality defects in dairy products. The specificity of plasmin on caseins has previously been studied using purified caseins and in the indigenous peptide profile of milk. We investigated the specificity and proteolytic pathway of plasmin in directly heated UHT milk (>150 °C for <0.2 s) during 14 weeks of storage at 20 °C in relation to age gelation and bitter peptides. Sixty-six peptides from αs- and β-caseins could be attributed to plasmin activity during the storage period, of which 23 were potentially bitter. Plasmin exhibited the highest affinity for the hydrophilic regions in the caseins that most probably were exposed to the serum phase and the least affinity for hydrophobic or phosphorylated regions. The proteolytic pattern observed suggests that plasmin destabilizes the casein micelle by hydrolyzing casein—casein and casein—calcium phosphate interaction sites, which may subsequently cause age gelation in UHT milk.

KEYWORDS: age gelation, bitterness, casein, plasmin, proteolysis, UHT milk

INTRODUCTION

Proteolysis by plasmin (PL), the major indigenous milk protease, can greatly affect the quality of dairy products. Proteolysis of caseins by plasmin leads to the formation of bitter off-flavours in milk and has been linked to the age gelation of UHT milk, making it a critical factor for the shelf life of these products.1–3 While PL is an important factor for the ripening characteristics of some cheeses,4 PL-induced proteolysis can also reduce cheese yield and curd firmness.5,6 PL is the active part of a complex enzyme system in milk consisting of, apart from PL itself, its zymogen, plasminogen (PLG), which is present in a 6–10 times higher concentration than PL, as well as plasminogen activators (PA) and inhibitors of both PL and PA.7 In contrast to the inhibitors, both PL and PA exhibit a high thermal stability.8–10 Heat treatment of milk thus promotes PL-induced proteolysis due to both reduced inhibitory capacity for residual PL and to increased activation of PLG into PL. The caseins (CN) in milk are structurally present as colloidal particles (casein micelles), rich in calcium phosphate nanoclusters which are surrounded by the phosphorylated caseins. The casein micelles are internally stabilized by ionic interactions with calcium phosphate and hydrophobic interactions between caseins, while the protruding κ-casein on the surface sterically stabilizes the casein micelle externally against aggregation.11 In milk, PL is associated with the casein micelle, ensuring a close proximity to its substrate.12,13 The most susceptible casein for PL in milk is β-CN, giving rise to the well described γ-CN fraction and proteose peptones.14 PL also readily cleaves αs-CN and to a lesser extent αs1-CN, but has little or no activity toward κ-CN and the major whey protein, β-lactoglobulin (β-lg). The specificity of PL toward βs-, αs2-, and αs1-CN in model studies using purified caseins has been described15–17. The specificity of PL toward caseins in a micellar system has been studied in relation to the endogenous peptide profile of milk,18,19 but not in relation to extensive proteolysis and quality defects of dairy products. The proteolytic specificity of PL could give an insight into the mobility and interaction of PL with the casein micelles. Age gelation of UHT milk is previously suggested to be caused by complexation of κ-CN with denatured β-lg during the heat treatment of milk. A subsequent release and aggregation of this complex during storage of UHT milk could then result in gelation.20 Proteolysis is hypothesized to facilitate the release of the κ-CN–β-lg complex by hydrolyzing the caseins that anchor the complex on the surface of the casein micelle.21

In a recent study, we investigated the effect of PL activity on physicochemical changes in milk heated by direct steam infusion,3 where we found that proteolysis by residual PL resulted in a decrease in pH and whiteness of the milk during storage. Simultaneously, PL activity increased during the storage period, while PLG-derived activity decreased. This correlated with an increase in the rate of βs-, αs1-, and αs2-CN hydrolysis. Bitter off-flavor developed after 7 weeks of storage, and age gelation of the milk was observed after 11 weeks. At
Peptides with Q

The milk was preheat treated at 74 °C for 15 s and homogenized milk (1.5% fat) were obtained from Arla Silkeborg, Denmark. After one mL of the sample was centrifuged at 13200 g for 10 min at 4 °C, the clear supernatant was recovered and stored at 20 °C in the dark at 20 °C for 14 weeks. One bottle was investigated for potential peptides formed by plasmin, which were not identified by MS/MS. The MS spectra were recorded in positive mode with a resolution of 20000 in the Q-TOF MS, Agilent Technologies. The MS spectra were searched against a custom database containing human and bovine protein sequences.

Database Search. The obtained tandem mass spectra were analyzed with Mascot v2.4 (Matrix Science, London, UK). Tandem mass spectra were searched against a custom database containing human and bovine protein sequences.

### Table 1. Presence and Identity of pH 4.6 Soluble Peptides from αs1-CN Formed by Plasmin in Directly Heated UHT Milk; Peptides with Q Values >1400 Are Potentially Bitter and Are Shown in Bold

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mass (amu)</th>
<th>Day 1</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
<th>14 Weeks</th>
<th>Q Value</th>
</tr>
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<tbody>
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<td>αs1-CN f(1–7)</td>
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<td>2916.53</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
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<td>2675.35</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>1304</td>
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<tr>
<td>αs1-CN f(104–119) + P</td>
<td>1950.95</td>
<td>x</td>
<td>x</td>
<td>x</td>
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</tr>
<tr>
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<td>2547.26</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>1294</td>
<td></td>
</tr>
<tr>
<td>αs1-CN f(106–119) + P</td>
<td>1659.79</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>1218</td>
<td></td>
</tr>
<tr>
<td>αs1-CN f(106–124) + P</td>
<td>2256.10</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>1201</td>
<td></td>
</tr>
<tr>
<td>αs1-CN f(120–124)</td>
<td>614.32</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
<td>αs1-CN f(125–132)</td>
<td>909.47</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
<td>αs1-CN f(194–199)</td>
<td>747.36</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>1703</td>
<td></td>
</tr>
</tbody>
</table>

“Phosphorylations are indicated after the peptide. α-Q value was calculated according to Ney (1971) in cal mol⁻¹. Presence of peptides at different storage times is indicated by ‘x’.

MATERIALS AND METHODS

**Milk Processing.** Three batches of 1 day old pasteurized (72 °C for 15 s) and homogenized milk (1.5% fat) were obtained from Arla Foods Stockholm Dairy (Sweden) during three consecutive weeks. The milk was heat treated at 74 °C for 180 s in a tubular heat exchanger before being subjected to a direct steam infusion heat treatment at >150 °C for >0.2 s (UHT pilot plant, APV/SPX, Silkeborg, Denmark). After flash cooling to 67 °C, the milk was homogenized (pressure 160/40 bar), further cooled to 20 °C, and packed aseptically in 300 mL glass bottles. The UHT milk was stored in a dark at 20 °C in a climate chamber for 14 weeks. One bottle was used once every week of the storage period for analysis. The sterility of each batch of processed milk was tested according to ISO 4833.24

**Sample Preparation.** A volume of 15 mL of milk was taken from each bottle and adjusted to pH 4.6 with 1 M HCl followed by centrifugation at 11000g for 10 min at 4 °C. The clear supernatant was recovered and stored at −20 °C until further analysis. At the time of analysis, the samples were thawed for 60 min at room temperature. One mL of the sample was centrifuged at 13200g at 5 °C for 10 min and used for the analysis. Sample preparation was performed in duplicate.

**Peptide Analysis.** Ultra-high-performance liquid chromatography (uHPLC) was performed on an Agilent 1290 Infinity uHPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight mass spectrometer (Q-TOF MS, Agilent 6530 Accurate Mass Q-TOF LC/MS, Agilent Technologies) with a Jetstream interface. The Q-TOF MS was run in 4 GHz high resolution mode. The Jetstream interface added a sheath gas at 350 °C with a flow of 8 L min⁻¹. A nozzle voltage of 100 V was applied to improve the ionization. The drying gas temperature was set to 325 °C at a flow rate of 10 L min⁻¹. The capillary voltage was set to 2.5 kV in the first time segment (3.0–24.0 min) and to 4.0 kV in the second time segment (24.0–35.2 min) to achieve optimal ionization for both small and large peptides. Spectra in the mass range from 300 to 3200 m/z were recorded in positive mode with a resolution of 20000 in the Q-TOF. Tandem mass spectra of the two most abundant ions with a charge state of 1, 2, or 3 in each MS spectrum were obtained in collision-induced dissociation (CID) mode (collision gas nitrogen, isolation width 1.3 m/z (narrow) at 5 different fixed collision energies and flexible collision energies optimized for larger peptides. The fixed collision energies were 10, 20, 30, 50, and 100 V, and the flexible energy settings were based on charge state and m/z. Spectra and UV chromatograms were analyzed by MassHunter software (version B 6.01, Agilent Technologies). The MS spectra were investigated for potential peptides formed by plasmin, which were not identified by MS/MS, using the „Find by Formula“ feature in MassHunter. Matching ions and isotopic patterns were further evaluated with regard to their retention time.
known genetic and PTM (post translation modification) variants of major milk proteins. The following search parameters were applied: protease: unspecific; mass tolerance for precursor ions 15 ppm and 0.6 Da for product ions; variable modifications: phosphorylations serine, oxidation methionine. Only identified peptides (expect score <0.05) that were present in at least two of the three replicates were considered.

**Relative Quantification of Identified Peptides.** Peaks in the UV chromatogram were identified by their corresponding MS spectra and retention time of the identified peptides by MS/MS. The retention time shift between the UV and MS signal was approximately 0.25 min. Only peaks without or with only minor overlaps with other components in the MS spectra were considered. In order to compare the rate of peptide formation, the integrated UV peak area was divided by a calculated molar extinction coefficient (ε) for the peptide as described previously.\(^25\) Phosphorylation of serine was not taken into account. Peak areas were further normalized for the initial casein distribution to correct for the differences in peptide concentration. The casein composition was based on the UV peak area corrected for the calculated ε (αs1-CN: 30%, αs2-CN: 13%, β-CN: 44%, κ-CN: 13%). Calculated ε in M\(^{-1}\) cm\(^{-1}\) were 346843 (αs1-CN), 375032 (αs2-CN), 331417 (β-CN) and 279859 (κ-CN).

**Assessment of Bitterness.** Bitterness of peptides was evaluated by applying Ney’s Q-rule based on peptide hydrophobicity.\(^26\) An average hydrophobicity Q of the peptide is calculated as the sum of the amino acids side chains hydrophobicity divided by the number of amino acid residues of the peptide. Peptides with a Q value >1400 cal/res\(^{-1}\) are considered bitter.

### RESULTS AND DISCUSSION

A total of 84 different casein-derived peptides were identified by the uHPLC-Q-TOF analysis (Tables 1–4). In the 1 day old milk (analyzed 1 day after processing), only 11 peptides could be identified by MS/MS. This number is small compared to other recent studies, which used more profound peptide extraction and concentration methods.\(^18,19\) The main objective of this study, however, was to study the proteolysis and development of peptides during storage rather than the peptide profile of the milk used for the UHT treatment. The majority by number of the peptides identified were derived from αs1-CN (36 peptides), followed by αs3-CN (31) and β-CN (17).

During the entire storage period, no peptides derived from κ-CN were detected, indicating that indigenous proteases other than PL, such as cashepsin D, were probably heat inactivated as could be expected due to their lower heat stability compared with PL and also that the raw material had not contained heat stable proteases from psychrotrophic bacteria, which are known to be able to hydrolyze κ-CN.\(^27,28\) More than 75% of the peptides (66 peptides out of 79) could be assigned to be the result of PL action solely (Table 4). In the remaining 18 identified peptides, PL was responsible for either the N- or C-terminal cleavage in 11 of the 18 identified peptides.

**Identified Peptides Formed by Plasmin.** Identified peptides derived from αs1-CN and the storage time of their occurrence are in good agreement with previous studies using purified αs1-CN,\(^29\) with the exception of the cleavage sites at Arg151-Gln152, a proposed major cleavage site by McSweeney et al.,\(^30\) and at Lys58-Gln59. Relevant peptides and polypeptides containing the cleavage site Arg151-Gln152 (αs1-CN f(125–151), αs1-CN f(133–151), αs1-CN f(106–151), and αs2-CN f(152–199)) were not detected. The cleavage site Arg151-Gln152 is located in middle of the most hydrophobic region of αs1-CN (Figure 4A), which has been identified as the main polymerization and interaction region of αs1-CN via hydrophobic interactions.\(^30\) In contrast to purified αs1-CN, this region is likely to be inaccessible for PL and Arg151-Gln152 and was also not hydrolyzed by trypsin in a micellar system.\(^31,32\) The second not-present cleavage site, Lys58-Gln59 is placed between the two phosphoserine clusters of αs1-CN (residues 41–51 and 61–70). The phosphoserine residues tightly bind to the calcium phosphate clusters in the casein micelle, which makes this region inaccessible for enzymatic hydrolysis.\(^31,33\)

The largest numbers of peptides present as a result of PL activity derives from αs2-CN (Table 2). After 1 day of storage, peptides originating from the N- and C-terminal region of αs2-CN were present (Figure 2). The C-terminal region of αs2-CN has previously been shown to be exposed and easily accessible for hydrolysis,\(^34\) and the appearance of αs2-CN f(182–188) and αs2-CN f(174–181) after 4 weeks of storage suggests a sequential hydrolysis of the C-terminal region. Initial cleavage sites are Lys188-Thr189 and Lys188-Ala189 followed by Lys181-Thr182 and Lys173-Phe174. After the same storage time, peptides originating from the central region of αs2-CN were formed by hydrolysis of Arg144-Gln152, Lys136-Lys137, Lys137-Glu138, Lys140-

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**Figure 1.** Primary structure of bovine αs1-CN B-8P. Phosphoserine residues are indicated by circles. Open circle indicates a possible phosphorylation at Ser41. Identified pH 4.6 soluble peptides formed by plasmin during storage at 20 °C are indicated by arrows.
Lys150, and Lys 150-Thr151. Earlier studies suggest that the cleavage sites Lys149-Lys150 and Lys150-Thr151 are preferred over Arg114-Asn115, but the simultaneous appearance of αS2-CN f(115−136), αS2-CN f(137−149), and αS2-CN f(138−150) indicates that PL readily hydrolyzed all of these cleavage sites. After 8 weeks of storage peptides originating from the region 151−173 in αS2-CN were identified. The late appearance of these peptides indicates a sequential hydrolysis of this region from an N- and C-terminal direction after the preferred cleavage sites Lys150-Thr151 and Lys173-Phe174 are hydrolyzed. The least preferred cleavage sites in αS2-CN seem to be Arg125-Glu126 and Lys152-Leu153, which are either in close proximity to phosphoserine residues or to major cleavage sites (Figure 2).

Although the region 25−114 contains eight potential cleavage sites for PL, only αS2-CN f(71−80) and αS2-CN f(25−45) could be identified. Masses corresponding to peptides including the cleavage site Lys91-Phe92, Lys32-Glu33, and Lys41-Glu42 were not present in the milk. The not-present cleavage at Lys91-Phe92 is located in a hydrophobic region of αS2-CN (Figure 4B), which may be inaccessible for enzymatic hydrolysis. Cleavage of this bond can be additionally hindered by Pro93 which induces a change in the secondary structure of αS2-CN. The least preferred cleavage sites in αS2-CN seem to be Arg125-Glu126 and Lys152-Leu153, which are either in close proximity to phosphoserine residues or to major cleavage sites (Figure 2).

**Table 2. Presence and Identity of pH 4.6 Soluble Peptides from αS2-CN Formed by Plasmin in Directly Heated UHT Milk; Peptides with Q Values >1400 Are Potentially Bitter and Are Shown in Bold**

<table>
<thead>
<tr>
<th>peptide</th>
<th>mass</th>
<th>day 1</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>14 weeks</th>
<th>Q Value</th>
</tr>
</thead>
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<tr>
<td>αS2-CN f(1−21) + 4P</td>
<td>2745.99</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>851</td>
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<tr>
<td>αS2-CN f(25−45)</td>
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<td>αS2-CN f(71−80)</td>
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<td></td>
<td>x</td>
<td>x</td>
<td></td>
<td>1245</td>
</tr>
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<td>αS2-CN f(115−125)</td>
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<td></td>
<td>1331</td>
</tr>
<tr>
<td>αS2-CN f(115−136) + 2P</td>
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<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>939</td>
</tr>
<tr>
<td>αS2-CN f(115−137) + 2P</td>
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<td></td>
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<td>x</td>
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<td>963</td>
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<tr>
<td>αS2-CN f(115−149) + 3P</td>
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<tr>
<td>αS2-CN f(115−150) + 3P</td>
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<tr>
<td>αS2-CN f(137−149) + P</td>
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<td></td>
<td></td>
<td>x</td>
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</tr>
<tr>
<td>αS2-CN f(137−150) + P</td>
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</tr>
<tr>
<td>αS2-CN f(138−149) + P</td>
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<td>x</td>
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<tr>
<td>αS2-CN f(138−150) + P</td>
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<td></td>
<td></td>
<td>x</td>
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<td>αS2-CN f(151−165)</td>
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<td>αS2-CN f(151−166)</td>
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<td>αS2-CN f(151−173)</td>
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<td>αS2-CN f(153−165)</td>
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<tr>
<td>αS2-CN f(166−173)</td>
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<td>αS2-CN f(167−173)</td>
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<td>αS2-CN f(174−181)</td>
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<td>x</td>
<td>x</td>
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<td>αS2-CN f(174−188)</td>
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<td>αS2-CN f(182−188)</td>
<td>902.46</td>
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<td></td>
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<td>αS2-CN f(182−197)</td>
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<td>x</td>
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<tr>
<td>αS2-CN f(182−207)</td>
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<td></td>
<td>x</td>
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</tr>
<tr>
<td>αS2-CN f(189−197)</td>
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<td>x</td>
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</tr>
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<td>αS2-CN f(189−207)</td>
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<td>x</td>
<td>x</td>
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<td>αS2-CN f(198−205)</td>
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</tr>
<tr>
<td>αS2-CN f(198−207)</td>
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<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>1980</td>
</tr>
<tr>
<td>αS2-CN f(200−207)</td>
<td>1021.60</td>
<td></td>
<td>x</td>
<td>x</td>
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<td>2233</td>
</tr>
</tbody>
</table>

*Phosphorylations are indicated after the peptide. *Q value was calculated according to Ney (1971) in cal mol⁻¹. *Peptide was identified by MS only.

**Figure 2.** Primary structure of bovine αS2-CN A-11P. Phosphoserine residues are indicated by circles. The dotted line between Cys36 and Cys40 indicates the intramolecular disulfide bridge. Identified pH 4.6 soluble peptides formed by plasmin during storage at 20 °C are indicated by arrows.
cysteine residues of αS2-CN. The majority of the αS2-CN molecules is present in a form with an intramolecular disulfide bond, which induces a tight loop in the protein34,36 which may limit the accessibility of Lys41-Glu42 and Lys32-Glu33 for PL. To our knowledge, the cleavage of Arg45-Asn46, Lys70-Ile71, Lys80-Ile81, Arg183-Asp184 and Arg202-Gly203.

Table 3. Presence and Identity of pH 4.6 Soluble Peptides from β-CN Formed by Plasmin in Directly Heated UHT Milk; Peptides with Q Values >1400 Are Potentially Bitter and Are Shown in Bold

<table>
<thead>
<tr>
<th>peptideα</th>
<th>mass (Da)</th>
<th>day 1</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>14 weeks</th>
<th>Q_valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-CN f(1−25) + 4P</td>
<td>3121.26</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>3121.26</td>
<td></td>
</tr>
<tr>
<td>β-CN f(1−28) + 4P</td>
<td>3476.48</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>3476.48</td>
<td></td>
</tr>
<tr>
<td>β-CN f(1−29) + 4P</td>
<td>3604.58</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>3604.58</td>
<td></td>
</tr>
<tr>
<td>β-CN f(29−48) + P</td>
<td>2559.13</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
<td>β-CN f(33−48) + P</td>
<td>2060.82</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>2060.82</td>
<td></td>
</tr>
<tr>
<td>β-CN f(98−105)</td>
<td>872.48</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
<td>β-CN f(98−107)</td>
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<td>x</td>
<td>x</td>
<td>1137.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-CN f(100−105)</td>
<td>645.32</td>
<td>x</td>
<td>x</td>
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<td></td>
</tr>
<tr>
<td>β-CN f(100−107)</td>
<td>910.47</td>
<td>x</td>
<td>910.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-CN f(106−113)</td>
<td>1012.52</td>
<td>x</td>
<td>x</td>
<td>1012.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-CN f(108−113)</td>
<td>747.36</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>747.36</td>
<td></td>
</tr>
<tr>
<td>β-CN f(170−176)</td>
<td>779.49</td>
<td>x</td>
<td>x</td>
<td>779.49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

αPhosphorylations are indicated after the peptide. bQ value was calculated according to Ney (1971) in mol−1. Presence of peptides at different storage times is indicated by ‘x’.

Peptides Formed by Other Proteases. Peptides that were identified in the UHT milk samples and which cannot be assigned to be a result of PL activity alone are summarized in Table 4. Most peptides deriving from αS2-CN or β-CN indicate activity of cathepsins alone or in combination with PL.28,38,39 Peptides formed by cathepsins alone were identified after 1 day of storage or after 4 weeks of storage in the case of αS2-CN f(1−14). Peptides formed by the combined activity of cathepsins and PL on the other hand appear after 4–8 weeks of storage. This indicates that cathepsins were active in the raw or pasteurized milk, but inactivated by the UHT treatment and that the polypeptides formed by cathepsins were further hydrolyzed by residual PL to smaller peptides. With the exception of cathepsin D, the heat stability of cathepsins is largely unknown. Although cathepsin D in its active form can partially survive HTST pasteurization,40 it is unlikely that sufficient active cathepsins are present after the UHT treatment to significantly contribute to the observed proteolysis. The peptides deriving from αS2-CN originate from the C-terminal of the protein. With the exception of αS2-CN f(201−206), the peptides contained the PL cleavage site Lys179-Thr189. The apparently unspecific C-terminal cleavage site of the peptides suggests activity of carboxypeptidases in the raw milk or heat-induced hydrolysis.41,42

Peptide Development during Storage. The presence of identified peptides after different storage periods gives a good indication of preferred and uncleaved cleavage sites of PL in the UHT milk system, but only gives limited information on the actual concentration and development of the peptides. Semiquantitative analysis of the peptides using MS spectra was not possible because their response factors are unknown and their intensity might be affected by ion suppression of coeluting components. The UV chromatograms at 214 nm were therefore used for a relative quantification and comparison of the peptides. Due to substantial overlap of large areas in the chromatogram, only a limited number of peaks could be used for such relative quantification. Quantified peptides included αS2-CN f(1−22), αS1-CN f(106−124), αS2-CN f(1−21), αS2-CN f(1−24), αS2-CN f(115−150), as well as β-CN f(1−28) and β-CN f(29−48). A comparison of the UV peaks and the extracted ion chromatograms are shown in the Supporting Information together with MS spectra of the peaks.

Figure 3. Primary structure of bovine β-CN A2-SP. Phosphoserine residues are indicated by circles. Identified pH 4.6 soluble peptides formed by plasmin during storage at 20 °C are indicated by arrows.
A comparison of peptides originating from the N-terminus of their parent proteins is shown in Figure 5. The peptide β-CN f(1–28) had the highest formation rate. During the first 4–5 weeks of storage, the relative concentration increased exponentially, reflecting the previously described increase in PL activity and rate of peptide formation. From 5 weeks of storage and further on, the relative concentration of β-CN f(1–28) increased almost linearly and then leveled out after 12 weeks of storage. The decrease in the rate of formation could be linked to changes in the matrix induced by the starting gelation of the milk after 11 weeks of storage. Further degradation of β-CN f(1–28) to β-CN f(1–25) may also reduce the concentration. Since the protease peptones β-CN f(1–105) and β-CN f(1–107) were still present after 14 weeks of storage (data not shown), substrate depletion can be excluded as a cause for the reduced rate of formation. The peptide αs2-CN f(1–24) showed the same initial rate of formation as β-CN f(1–28) and leveled off after 6 weeks of storage. The peptide αs2-CN f(1–21) exhibited a lower rate of formation than αs2-CN f(1–24), but in contrast to αs2-CN f(1–24), continued to increase after 6 weeks of storage. This indicates a hydrolysis of αs2-CN f(1–24) to αs2-CN f(1–21) that may be caused by substrate depletion of the cleavage site Ly32-Asn33. The development of αs1-CN f(1–22) differed from the other N-terminal peptides. The initial lag phase was not observable for αs1-CN f(1–22), and the overall concentration was significantly lower compared to that of the N-terminal peptides derived from αs2- and β-CN. The cleavage sites for these peptides in αs2- and β-CN are in very hydrophilic domains of the proteins (Figure 4A–C), while the cleavage site Arg22-Phe23 in αs1-CN is in a hydrophobic domain involved in casein–casein interactions.44

Peptides being present as the result of two proteolytic cleavages are shown in Figure 6. They exhibited an extended lag phase compared to the N-terminal peptides, due to the time needed to form their precursors. As discussed above, αs2-CN f(115–150) originates from two of the most preferred cleavage sites in αs2-CN and showed the highest formation rate. The peptide αs1-CN f(106–124) developed almost in the same manner as αs3-CN f(1–22) within the first 6 weeks of storage but showed a higher rate of formation from 7 weeks of storage on. These findings further highlight the preference of PL for cleavage sites located in the hydrophilic regions of the caseins. Gagnaire et al. (1998) found that the protease peptones β-CN f(1–105/7) were retained in the casein micelle, while β-CN f(29–105/7) was released into the serum phase.45 This release limits further hydrolysis of these proteose peptones by the casein-associated PL, which explains why β-CN f(29–48) was only formed in very small amounts.

Destabilization of Casein Micelles by Proteolysis. PL enters the milk from the blood34 and associates itself with the casein micelle.45 Whether PL is able to enter the micellar structure or is located entirely on the surface of the casein micelle is unknown. It becomes obvious from Figure 3 that the majority of the major cleavage sites of PL within the caseins, similar to those of trypsin,31,32 are located at the most hydrophilic and serum-exposed regions of the proteins. β-CN is mainly present in the interior of the casein micelle46 and is proposed to stabilize water or serum channels within the casein micelle.47,48 αs2-CN is involved in the binding to calcium phosphate clusters, regions that are hypothesized to be surrounded by hydrophobic domains11 and likely to be inaccessible for PL. The rapid cleavage, within 4 weeks of storage, of the N-terminal regions of β-CN and αs2-CN implies that PL is able to penetrate the casein micelle. In relation to a destabilization of the casein micelle by proteolysis, it is notable that the minor cleavage sites, together with some of the major cleavage sites, are located at the boundary between hydrophilic, hydrophobic, or phosphorylated regions of the caseins (Figure 4A–C). Thus, PL hydrolyses around the regions essential for the internal integrity and stabilization of the casein micelle, i.e. hydrophobic interaction between caseins, interactions of
caseins with calcium phosphate, and ionic or salt interactions between caseins. Cleavage of these regions in the casein micelle would lead to a loosening of the micelle structure, which correlates with the reported increase in micelle size after incubation with PL.49 A looser structure is hypothesized to allow PL to reach previously inaccessible regions, which can explain the initial resistance of $\alpha_{S1}$-CN to hydrolysis.

Age gelation of UHT milk has been proposed to be a two-stage process of proteolysis and physicochemical changes that lead to the gelation.50 Proteolysis-induced age gelation of UHT milk by PL which does not involve the $\kappa$-CN−$\beta$-lg complex, could occur after a critical number of the casein−casein and casein−calcium phosphate interaction sites have been hydrolyzed and the remaining interactions are not sufficient to maintain a micellar arrangement. While at the point of gelation, more than 95% of the $\alpha_S$- and $\beta$-CN were hydrolyzed,1 the peptide development showed that far from all cleavage sites were hydrolyzed completely. This indicates that a large number of amphiphilic and charged polypeptides were present in the milk system that could form and stabilize a gel network. While some proteolysis is needed for gelation, the extent of proteolysis has not yet been related with gelation. The lack of consistency in degree of proteolysis in relation to age gelation and PL activity described in previous studies could also be attributed to differences in processing conditions and levels of PL activity (direct or indirect UHT treatments, addition of PL to UHT milk).2,50−53 Different processing conditions can greatly affect the protein matrix in milk, such as the degree of whey protein denaturation on casein micelles,22 thereby influencing the substrate accessibility and the proteolytic pattern of PL in milk. A high PL activity could result in a fast breakdown of polypeptides to small peptides, which are unable to form a gel. Our results suggest that the proteolytic...
pathway of caseins by PL is more important for the destabilization of the casein micelles than the overall decrease in the level of intact caseins.

**Potential Bitter Peptides.** Potential bitter peptides with a Q value >1400 cal mol\(^{-1}\) are highlighted in Table 1—4. Bitterness of peptides from β-CN was found in the γ-CN fraction after hydrolysis with PL, and analysis of tryptic digests of β-CN have shown that β-CN f(203–209) is extremely bitter.\(^3\) The potential bitter peptides found in the present study were β-CN f(108–113) and β-CN f(170–176), which have previously been described sensorially as tickling and burning.\(^3\) The bitter peptide β-CN f(203–209) was not identified in the UHT milk or was only present in a very low concentration. Previously described bitter peptides deriving from αs1-CN, that can be formed by PL, are αs3-CN f(23–34) and αs3-CN f(91–100).\(^5,6\) Besides αs3-CN f(91–100), all peptides from residue 91 to 100–105 found in the milk had Q values >1400, and were potentially bitter. The smaller peptides αs1-CN f(1–7) and αs1-CN f(194–199) were highly hydrophobic, and could have contributed to bitterness. The peptides αs2-CN f(182–207), αs2-CN f(189–207), and αs2-CN f(198–207) have been correlated with bitterness and were also present early in the storage period in the UHT milk.\(^6\) Besides αs2-CN f(182–188), all peptides deriving from the C-terminal region of αs2-CN from residue 171 and onward are potentially bitter. Cleavage of Arg205–Tyr206 in αs2-CN gives rise to the bitter dipeptide Tyr-Leu.\(^6\) The identified potential bitter peptides indicate that the bitterness caused by PL might mainly be caused by hydrolysis of αs1- and αs2-CN, rather than β-CN as previously reported.\(^3\) Several peptides formed by the combined action of PL and cathepsins were potentially bitter as well. Most of these peptides derive from the N-terminal region of αs2-CN and the C-terminal region of αs2-CN.

A bitter off-flavor was detected in the milk after 6 weeks of storage, which intensified rapidly within 1 week of storage.\(^1\) The concentration and sensory thresholds of the potential bitter peptides in the UHT milk are unknown, and the bitterness cannot be attributed to a specific peptide. We suggest that either the overall concentration of bitter peptides reached the sensory threshold after 7 weeks of storage or that one or several bitter peptides with a lower sensory threshold were formed very rapidly in the storage period between 5 and 7 weeks. Similar to trypsin, PL has the capability to form a range of potential bitter peptides, but further studies are necessary to confirm the bitterness of these peptides and their sensory threshold.

In conclusion, the special UHT process used allowed the investigation of specificity and effect of PL in a sterile milk system, without causing substantial changes to the milk by the heat treatment. PL showed a different specificity in the milk system compared to previous model studies, and 6 new cleavage sites of PL in αs2-CN could be identified. The relative quantification of peptides and time of their appearance allowed a direct comparison of formation rates of peptides to assess the affinity of plasmin to certain cleavage sites. We could show that PL is able to produce potentially bitter peptides and bitterness was most likely caused by peptides arising from αs1- and αs2-CN. The observed proteolytic pattern indicated that PL-mediated proteolysis can destabilize the casein micelle and explain proteolysis-induced age gelation of milk.

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**ASSOCIATED CONTENT**

**Supporting Information**

Spectra of peptides used for relative quantification and proteins in Mascot database used for identification. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Corresponding Author

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


6860 dx.doi.org/10.1021/jf502088u J. Agric. Food Chem. 2014, 62, 6852–6860
Supplementary data

1. Proteins in custom Mascot database used for identification of peptides from tandem MS

sp|P02662|CAS1A_BOVIN Alpha-S1-casein
sp|P02662|CAS1B_BOVIN Alpha-S1-casein
sp|P02662|CAS1C_BOVIN Alpha-S1-casein
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sp|P02754|LACBB_BOVIN Beta-lactoglobulin
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sp|P02754|LACBD_BOVIN Beta-lactoglobulin
sp|P02769|ALBU_BOVIN Serum albumin
sp|P02769|ALBU214_BOVIN Serum albumin
sp|P24627|TRFL_BOVIN Lactotransferrin
sp|P01239|PRL_BOVIN Prolactin
sp|P80025|PERL_BOVIN Lactoperoxidase
sp|P80025|PERLS_BOVIN Lactoperoxidase Ser394 variant
sp|Q29443|TRFE_BOVIN Serotransferrin
sp|P79345|NPC2_BOVIN Epididymal secretory protein E1
sp|Q9XSC9|TCO2_BOVIN Transcobalamin-2
sp|P18902|RET4_BOVIN Retinol-binding protein 4
sp|P15497|APOA1_BOVIN Apolipoprotein A-I
sp|P18892|BT1A1_BOVIN Butyrophilin subfamily 1 member A1
sp|P80457|XDH_BOVIN Xanthine dehydrogenase/oxidase
sp|P06868|PLMN_BOVIN Plasminogen
sp|Q95114|MFGM_BOVIN Lactadherin
sp|P31096|OSTP_BOVIN Osteopontin
sp|Q8WML4|MUC1_BOVIN Mucin-1
sp|Q8MI01|MUC15_BOVIN Mucin-15
sp|Q8MI01-2|MUC15S_BOVIN Isoform 2 of Mucin-15
sp|Q9TUM6|PLIN2_BOVIN Perilipin-2 isoform-1
sp|Q9TUM6-2|PLIN2_BOVIN_2 Isoform 2 of Perilipin-2
2. Supplementary spectra of peptides used for relative quantification

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<th>Peptide</th>
<th>UV and EIC chromatogram</th>
<th>MS spectrum at peak top</th>
<th>Comments/Contamination</th>
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<td><img src="image2.png" alt="MS spectrum at peak top" /></td>
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<td>2000</td>
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Absorbance 214 nm and Intensity (AU) as a function of Retention time (min).
Overlay from αS1 f(1-22)

αS2-CN f(1-21)
UV and EIC chromatogram | MS spectrum at peak top | Comments/Contamination
Overlay with 550.3334+1
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Paper IV


Protein lactosylation in UHT milk during storage measured by liquid chromatography-mass spectrometry and furosine

Manuscript intended for International Journal of Dairy Technology
Protein Lactosylation in UHT Milk during Storage measured by Liquid Chromatography-Mass Spectrometry and Furosine

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ABSTRACT

The initial stage of the Maillard reaction, protein lactosylation, occurs during the heat treatment of milk and continues during subsequent storage. We compared the initial lactosylation as well as the rate of lactosylation of milk proteins during storage in UHT milk subjected to directly or indirectly heat treatment using liquid chromatography (LC) coupled with electro spray injection mass spectrometry (ESI-MS). Furosine content was used as an overall marker to allow for a quantitative correlation of lactosylation measured by LC-ESI-MS in the UHT milks. Protein lactosylation increased during the storage period of 6 months at 20°C. Both the initial extent and the rate of lactosylation positively correlated with the number of lysine residues in the different proteins. An exponential or linear correlation with furosine concentration could be established for major and minor lactosylated proteins, respectively.
1. Introduction

During heat treatment and following storage of UHT milk, the Maillard reaction is one of the major quality deterioration factors. The formation of the Amadori product $\varepsilon$-lactulosyllysine between lactose and the free $\varepsilon$-amino group of lysine results in a decreased nutritional value of the milk as the lysine will no longer be bioavailable and extensive lactosylation can also reduce the digestibility of proteins (Dalsgaard et al., 2007). In the past, the extent of lactosylation has mainly been studied using indirect methods, such as the measurement of furosine, which is a product of the acid hydrolysis of lactulosyllysine (Erbersdobler and Somoza, 2007). More recently, mass spectrometry (MS) has been shown to be a powerful tool for the analysis of milk protein lactosylation in both wet and dry systems (Siciliano et al., 2013). Protein lactosylation in wet milk has been studied both in the casein fraction (Scaloni et al., 2002; Johnson et al., 2011), in the native whey fraction (Czerwenka et al., 2006; Meltretter et al., 2009; Losito et al., 2007) and in indigenous phospho-peptides (Pinto et al., 2012). Heat treatment of milk has a major impact on the whey protein fraction and severe heat treatments, such as e.g. UHT (136-140 $^\circ$C for 2-6 s), result in an almost complete denaturation of the whey proteins (Datta et al., 2002). In previous studies, the degree of lactosylation of native whey proteins could be correlated with whey protein denaturation and fluorescence (Meltretter et al., 2009; Losito et al., 2010). Despite the fact that caseins are the quantitatively major milk proteins, lactosylation has only been observed for the most abundant caseins: $\alpha_{s1}$-casein ($\alpha_{s1}$-CN) and $\beta$-casein ($\beta$-CN). A quantitative correlation in wet milk systems with furosine concentration could only be achieved for a singly lactosylated $\beta$-CN in processed cheese (Steffan et al., 2006). In dry milk systems, a quantitative relationship between furosine concentration and lactosylated peptides originating from whey proteins was established for milk powder (Le et al., 2013).

Furthermore, the Amadori product is continuously formed during storage of milk. The development of lactulosyllysine during the storage is therefore an important parameter for shelf life
estimation of UHT milks. To our knowledge the development of protein lactosylation during storage has not been studied using mass spectrometry before.

The aim of this study was to follow the lactosylation of the casein and whey fractions in directly and indirectly heat treated UHT milks by ESI-MS and furosine analysis during subsequent storage. In particular, the rate of lactosylation of different caseins and whey proteins during storage was compared.

2. Materials and Methods

2.1 Materials

One day old low pasteurized milk (72 °C for 15 s) with 1.5 % fat was obtained from Arla Foods Stockholm Dairy (Stockholm, Sweden) and processed in the UHT pilot plant (SPX, Silkeborg, Denmark) at the Arla Strategic Innovation Center (Stockholm, Sweden). The milk (200 L) was pre-heated at 95 °C for 5 s (Direct A) or 180 s (Direct B) in a tubular heat exchanger before being subjected to the direct steam infusion heat treatment ( >150 °C for <0.2 s). After flash cooling, the milk was homogenized using a two-stage homogenizer (160/40 bar), further cooled down to 20 °C by a tubular heat exchanger and packed aseptically in 300 mL glass bottles. For the indirect UHT process (Indirect), milk was heated to 80 °C, homogenized using a two-stage homogenizer (160/40 bar) and further heated at 140 °C for 4 s. The milk was cooled down to 20 °C by a tubular heat exchanger and packed aseptically in 300 mL glass bottles. These trials were repeated three times during three consecutive weeks and the milk was stored in the dark at 20 °C in a climate chamber for 6 months. One 300 mL bottle per treatment was collected once every month for analysis.

2.1 Analytical methods

Protein content was measured by a MilkoScan FT 120 (Foss, Hillerød, Denmark). Analysis of protein composition in the milk was performed as described by Bonfatti, Grigoletto, Cecchi-
nato, Gallo, and Carnier (2008) with the following modifications. A sample of 200 µL of milk (w/w) was frozen at -20 °C until further use. Disulphide bonds were reduced by the addition of 20 µL 0.5 M dithiothreitol and 1 mL 100 mM tri-sodium citrate, 6 M urea at 30 °C for 60 min. The samples were centrifuged at 9300 x g at 5 °C for 10 min. From the clear phase, 200 µL were used for the analysis. A volume of 5 µL was injected into the LC-MS system. The column was a BioSuite™ C18 PA-B (C\textsubscript{18}, 3.5 µm 2.1 mm x 250 mm, Waters, Milford, MA, USA). Buffer A was Milli-Q water with 0.05 % trifluoroacetic acid (TFA) (v/v) and buffer B acetonitrile with 0.1 % (v/v) TFA. Column temperature was 47 °C and a linear gradient from 34.8 % to 46.5 % buffer B from 2 to 16.5 min was applied at a flow rate of 0.35 mL min\textsuperscript{-1} with UV detection at 214 nm. MS detection was performed with a electrospray-time-of-flight mass spectrometer with (ESI-TOF-MS; Agilent 6230 Aquurate mass detector, Agilent Technologies) and MS scans were continuously recorded between a mass to charge ratio (m/z) of 300 and 3200 in 2 GHZ extended dynamic positive mode. The TOF-MS had a drying gas temperature at 325 °C at a flow rate of 10 L min\textsuperscript{-1} and a nebulizer pressure of 35 psi. Sheath gas temperature was 300 °C at a flow rate of 7 L min\textsuperscript{-1}. Fragmentor energy was set 200 V and the applied energy to the capillary was 3500 V. Data analysis was performed with Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA).

Mass spectra and UV chromatograms were analyzed by MassHunter (version B 6.01) and Profinder (pre-release version B.06.00 Service Pack 1) software (Agilent Technologies, Santa Clara, CA, USA).

Analysis of furosine was performed as follows: One mL milk sample was mixed with 3 mL of 10 M HCl in screw-cap tubes. Nitrogen was bubbled through the samples for 2 min to mix the samples. The tubes were closed and heated at 115 ± 1 °C for 18 h. After heating, the samples were cooled and filtered through a paper filter (pore size 20 µm). The filtrate was further filtered through a 0.45 µM disposable filter, diluted 5 times in 3 M HCl and transferred into HPLC vials. For furosine analysis, 10 µL of the filtrate were injected into a Dionex 300DX.
system (Dionex, Germering, Germany) with a DAD detector (UV at 280 nm) using a Supelcosil LC-8 column for isocratic separation with 0.06 M sodium acetate as buffer at a flow rate of 1 mL min\(^{-1}\).

All measurements were performed in duplicate. Standard deviation between replicates for all measurements was ranging from 5-10%.

2.1 Data analysis

In order to achieve the optimal results, the ESI mass spectra were analyzed by 3 different methods of data analysis for \(\alpha_S1\)-CN 8P. The \(\alpha_S1\)-CN 8P was chosen, as it is the most abundant casein and MS analysis of the milk showed that \(\alpha_S1\)-CN 8P was present in only one genetic in contrast to \(\beta\)-CN. Extracted ion chromatograms (EIC) of unmodified, mono- lactosylated and di-lactosylated \(\alpha_S1\)-CN were extracted using the most abundant ion as quantifier and the second abundant ion as qualifier (the used quantifier ions are shown in Figure 2). The mass error was set to 100 ppm. Mass spectra were deconvoluted in MassHunter using the maximum entropy setting (mass step 1 Da, signal to noise > 30 counts, minimum consecutive charge states 5). Finally, the mass spectra were analyzed using the Profinder software, a batch recursive deconvolution tool. A large molecular extractor algorithm (LMFE) was used. Retention time tolerance was set to 1 min and an m/z window of 50 ppm. All EIC from proteins having 5 to 35 charge states were smoothed using a Gaussian function before integration. Additionally, the UV signal at 214 nm was integrated. Due to a co-elution of \(\alpha_S1\)-CN 8P and 9P, both variants were integrated as one peak.

Results are shown as mean value ± standard deviation of the 3 replicates. Linear regression analysis and ANOVA was performed with Matlab software (Mathworks, Natick, MA). Model? Class variables were heat treatment (1, 2, 3), storage time points (1- 6 months)? Differences were regarded significant at 95%-level i.e. \((P<0.05)\).
3. Results and Discussion

3.1 Initial furosine concentration and whey protein denaturation

In order to evaluate the effect of heat treatment, the initial furosine concentration and degree of whey protein denaturation was analyzed in the different UHT milks after one day of storage (Table 1). As expected, the degree of whey protein denaturation increased with increasing severity of the heat treatment. As previously reported, the degree of denaturation of β-lg was higher compared to α-la (Dannenberg and Kessler, 1988). While there is a clear difference of the heat treatment in respect to α-la denaturation for all milks, the degree of β-lg denaturation in the Direct B and Indirect was not significantly different. This can be attributed to the almost complete denaturation of β-lg in the milk (Table 1). As expected, the initial furosine concentrations in Direct A and Direct B were approximately 10-15 times lower compared to Indirect and within the range of previously reported results (Datta et al., 2002). No furosine could be detected in the low pasteurized feed. The furosine content in Direct A was slightly higher than in Direct B and the pre heat treatment of 95 °C for 180 s of Direct B did not affect the furosine concentration. This trend continued during the entire storage period (Figure 1). The furosine content increased linearly in all UHT milks during the 6 months storage period and no significant difference in the rate of formation between the milks was observed. This is in line with previous findings (Corzo et al., 1994; Nangpal and Reuter, 1990) and indicates that the severity of the heat treatment affects the initial extent of the lactosylation but not the rate of formation during storage under similar conditions. The linear increase of furosine during storage in all milks further shows that the Maillard reaction was in its initial stage during the entire storage period and that degradation of lactulosyllysine to intermediate Maillard reaction products, such as 5-hydroxy-2-methylfurfural (HMF), most likely only occurred to a limited extent (Le et al., 2011).
3.2 Comparison of MS analysis

Analysis of protein lactosylation using ESI-MS was evaluated for α\textsubscript{S1}-CN 8 P using EIC, deconvolution and Agilent Profinder. A representative mass spectrum of α\textsubscript{S1}-CN 8 P in Indirect after 6 months of storage is shown in Figure 2. The most abundant ion for α\textsubscript{S1}-CN 8 P was at a charge state +22 and the 2 ions with a m/z shift of 14.6 and 29.2 represented the mono and di-lactosylated α\textsubscript{S1}-CN 8 P species. No ion for a tri-lactosylated α\textsubscript{S1}-CN 8 P could be found in the mass spectrum. An EIC of α\textsubscript{S1}-CN 8 P using the ions shown in the inset of Figure 2 are shown in Figure 3 A. As previously reported, the addition of lactose renders the protein more hydrophilic, which results in a shift to lower retention times and a broadening of the peaks (Czerwenka et al., 2006; Losito et al., 2007). The EIC of lactosylated and especially di-lactosylated α\textsubscript{S1}-CN 8 P are broader compared to the unlactosylated α\textsubscript{S1}-CN 8 P, which can be caused by the lactosylation of different lysine residues, which can result in a change in the retention time (Czerwenka et al., 2006). Figure 3B shows the corresponding UV signal of α\textsubscript{S1}-CN 8 P and 9 P of Indirect, seen as a shoulder on the right side of the α\textsubscript{S1}-CN 8 P peak. The comparison of the UV signal after 0 and 6 months of storage of Indirect shows a decrease in peak height and a broadening of the peak due to the changes in retention time observed in the EIC. The deconvoluted spectra of α\textsubscript{S1}-CN 8 P after 0 and 6 months of storage are shown in Figure 4. After 6 months of storage, a tri-lactosylated α\textsubscript{S1}-CN 8 P could be observed in the UHT milk and the intensity distribution shifted towards higher degrees of lactosylation (Figure 4B). The decrease in un-lactosylated α\textsubscript{S1}-CN 8 P intensity was not proportional to the increase in the intensity of the lactosylated α\textsubscript{S1}-CN 8 P species. The UV area of α\textsubscript{S1}-CN 8 P on the other hand was constant during the entire storage period in all UHT milks (± 6 %). This relationship was valid for all l reduced proteins and the summed MS area decreased by approximately 25-40 % after 6 months of storage. The decrease in intensity indicates a reduced ionizability for lactosylated proteins, which previously are reported for caseins by Scaloni et al. (2002) using a single quadrupole MS (Scaloni et al., 2002). For pH 4.6 soluble un-
lacostylated and lactosylated β-lg and α-la on the other hand, an identical ionizability is suggested for matrix assisted laser desorption (MALDI) TOF-MS and triple quadrupole MS (Czerwenka et al., 2006; Meltretter et al., 2009). A shift in the charge state distribution towards lower charge states occurred upon lactosylation of pH 4.6 soluble β-lg analysed with triple quadrupole MS, which might be caused by a hindrance of protonation by the added lactose to lysine residues (Czerwenka et al., 2006). The reported differences in the ionization of un-lactosylated and lactosylated milk proteins could be ascribed to the different MS instrumentation and analysis conditions used. It is likely that the ionizability of lactosylated proteins could depend on the charge state distribution of the protein. A reduced ionization of lactosylated proteins might be negligible for lower charge state distribution or singly charged proteins in MALDI-TOF-MS as there are other ionizable residues present in excess. However, the lactosylation might have a substantial impact on the ionization of reduced and unfolded proteins with a higher charge state distribution as observed in this study. A direct comparison of the lactosylation of denatured and pH 4.6 soluble whey proteins was not possible in this study due to the high degree of whey protein denaturation in the UHT milks (Table 1).

Because of the decrease and variation in MS area (measured as counts per second), the area distribution of the summed areas of un-lactosylated and lactosylated proteins was used for calculating the extent of lactosylation. Analysis using deconvolution and the Profinder software gave similar results, while analysis using EIC showed higher degrees of lactosylation due to a missing exclusion of background noise (see supplementary section). In comparison to the deconvolution algorithm, Profinder resulted in an improved extraction of the ion envelopes due to the recursive workflow and was thus used for further analysis.

3.2 Kinetics of protein lactosylation during storage

Di-lactosylated (for α-la and κ-CN) and tri-lactosylated proteins (for β-lg, αS1-CN, αS2-CN and β-CN) were not present in the one day old UHT milks and accounted for < 2% of the ar-
ea in the most severely lactosylated UHT milk samples, i.e. Indirect after 6 months of storage, and were not considered in the data analysis. A comparison of the absolute and relative area distribution of $\alpha_{S1}$-CN 8 P lactosylation during storage in the UHT milks is shown in Figure 5 (for other proteins see supplementary section). As discussed above, the overall area decreased during storage. In Indirect, both the area of un-lactosylated and mono-lactosylated $\alpha_{S1}$-CN 8 P decreased during storage. The relative change in intensity of un-lactosylated and lactosylated $\alpha_{S1}$-CN 8 P, on the other hand, fitted well to a linear regression in all milks (Figure 5 bottom row). On a closer look, both the relative area of un-lactosylated and mono-lactosylated $\alpha_{S1}$-CN 8 P seemed to level out due to the difference in ionization efficiency of the lactosylated $\alpha_{S1}$-CN 8 P and could also be fitted to an exponential function. In order to compare the lactosylation rates of different proteins and to reduce the number of variables, a linear regression was applied.

Table 2 shows the results of the linear regression of casein lactosylation during storage. For $\alpha_{S1}$-CN 8 P, $\beta$-CN A$^2$ and $\kappa$-CN, the degree of lactosylation was significantly higher in Direct B compared to Direct A, showing that the measurement of lactosylation by LC-MS is a very sensitive method for discrimination between heat treatments. As the initial furosine results already indicated, the initial degree of protein lactosylation in Direct A and Direct B was similar for most of the proteins. The degree of lactosylation measured by LC-MS after heat treatment in Indirect was only 2-4 times higher than in Direct A and Direct B. The lower degree of lactosylation found, compared to the furosine measurement (10-15 time higher; Figure 1), could be caused by the different response factors of the lactosylated proteins and the fact that the two lactose moieties in di-lactosylated proteins were not taken into account. The initial extent of lactosylation for the different caseins was $\alpha_{S2}$-CN $>$ $\alpha_{S1}$-CN $>$ $\beta$-CN $>$ $\kappa$-CN, which matches the number of lysine residues (24, 14, 11 and 9) and thus the available reaction sites of the caseins. The results do, to some extent, support previous findings by Scaloni et al. suggesting $\alpha_{S1}$-CN and $\beta$-CN being the most lactosylated caseins (Scaloni et al., 2002).
Similar to the initial casein lactosylation, the rate of lactosylation was alike for Direct A and Direct B with the exception of β-CN A\textsuperscript{2}. The decrease in un-lactosylated and increase in mono-lactosylated protein was more pronounced in the Direct A and Direct B than indirect. A higher degree of initial lactosylation resulted in a lower rate of lactosylation. This can be ascribed to the slightly exponential nature of the curves (see Figure 5). The increase in di-lactosylated proteins on the other hand was significantly higher in Indirect reflecting the more advanced degree of lactosylation in this UHT milk. The α\textsubscript{S1}-CN 8 P showed the highest rate of lactosylation in all UHT milks followed by β-CN, α\textsubscript{S2}-CN and κ-CN. The lower rate of lactosylation of α\textsubscript{S2}-CN was ascribed to a high initial degree of lactosylation and reduced response factors of the lactosylated α\textsubscript{S2}-CN. Additionally, the amount of phosphorylations of α\textsubscript{S2}-CN varies, which results in a massive overlay of the differently phosphorylated and lactosylated α\textsubscript{S2}-CN variants. This limits the extraction of ion envelopes and may cause ion suppression. This was also reflected in the lower R\textsuperscript{2}–value of the linear regression of α\textsubscript{S2}-CN. The rate of lactosylation for κ-CN was not significantly different between the UHT milks. This could be caused by the low initial degree of lactosylation and the absence of multi-lactosylated variants with accordingly lower response factors. To our knowledge, the lactosylation of α\textsubscript{S2}-CN and κ-CN has not been reported previously.

The lactosylation of whey proteins during storage is shown in Table 3. The initial lactosylation and rate of lactosylation of the reduced whey proteins was similar to the lactosylation of the reduced caseins. The initial lactosylation as well as the rate of lactosylation during storage of β-lg were higher compared to those of α-la, which correlate with the amount of available reaction sites on the proteins with 15 lysine residues in β-lg and 12 in α-la, and is in line with previous findings (Czerwenka et al., 2006; Losito et al., 2007). The initial degree of lactosylation of α-la was significantly different for all UHT milks, but as with κ-CN, there was no significant difference in the rate of lactosylation of α-la between the UHT milks. Previous studies indicate a difference in the extent of lactosylation of native and unfolded β-lg. The lysine...
residues in unfolded β-lg could be easier accessible to react with lactose, and di-lactosylated β-lg is the predominant form in the unfolded β-lg population (Losito et al., 2010). The rate of lactosylation for β-lg during storage showed a similar trend, with a positive correlation of rates of formation of dilatosylated β-lg and severity of the heat treatment and whey protein denaturation (Table 3). However, the same trend was found for the caseins as well with the exception of κ-casein. The caseins do not possess any notable tertiary structure, which would explain this phenomenon. Thus, it cannot be concluded, whether the higher degree of whey protein denaturation or simply a higher initial degree of lactosylation caused the higher rate of formation of di-lactosylated β-lg in this study.

3.3 Correlation of lactosylation measured by furosine and LC-MS

The correlation of furosine concentration and decrease in un-lactosylated reduced protein is shown in Figure 6 for β-CN, αS1-CN 8 P, α-la and β-lg. For less lactosylated proteins, such as β-CN, κ-CN and α-la linear correlations between decrease in un-lactosylated proteins and increase in furosine concentrations were found with correlation coefficients of $R^2=0.95$, 0.92, and 0.87, respectively. This is in line with Steffan et al. (2006), who found a linear correlation between unmodified β-CN and furosine in processed cheese, although only a singly lactosylated β-CN was detected (Steffan et al., 2006). For the higher lactosylated proteins αS1-CN 8 P and β-lg, the best correlations were achieved with an exponential fit resulting in $R^2=0.93$ and $R^2=0.95$, respectively. The difference was caused by the change in response factor and the higher concentration of di-lactosylated variants. Figure 6 shows that the area distribution of lactosylated milk proteins measured by LC-MS can be used to quantify the extent of protein lactosylation in milk during storage. Meltretter et al. (2009) previously showed that the lactosylation of pH 4.6 soluble whey proteins correlated with several heat markers, but the limiting factor for this method is the amount of pH 4.6 soluble whey proteins, which does not allow assessment of severely heat treated products (Meltretter et al., 2009). Furthermore, a potential
higher degree of lactosylation for denatured whey proteins compared to native whey proteins might lead to an underestimation of the degree of lactosylation when measuring on pH 4.6 soluble whey proteins. The analysis of reduced caseins and whey proteins in this study, on the other hand, did not have this limitation. For highly heat treated products with higher degrees of lactosylation compared to the UHT milk used in this study, the less lactosylated proteins (β-CN, κ-CN and α-la) could serve as marker proteins, since the effect of reduced intensity of lactosylated species would be less pronounced. An improved chromatographic separation could further facilitate the extraction of the MS spectra due to a reduced overlap of the different lactosylated species. The use of a relative area distribution of un-lactosylated and lactosylated protein species additionally makes this method robust against other protein modifications, such as oxidation, and limited proteolysis. It should be noted that the described method is only suited for analysis of the initial stage of the Maillard reaction. For assessment of products containing intermediate and advanced Maillard reaction products, the protein lactosylation is not proportional to the extent of the Maillard reaction due to a degradation of lactulopylysine and different methods, such as measurement of advanced glycosylation end products or fluorescence accumulation, should be applied (Le et al., 2013; Van Boekel, 1998; Bosch et al., 2007; Le et al., 2011).

4. Conclusion

In conclusion, we could show that LC-MS is a suitable analysis to investigate the initial stage of the Maillard reaction in UHT milk during storage. The results obtained by LC-MS allowed a quantitative correlation with the commonly used marker, furosine. In comparison to the furosine method, the LC-MS method allowed the analysis of the protein lactosylation of all major milk proteins, giving a more detailed view of the Maillard reaction in UHT milk during storage. The extent of protein lactosylation in the directly heated UHT milk was approximately 10-15 times lower compared to indirectly heated UHT milk. The rate of protein lactosyla-
tion during storage was constant for indirect and directly heated UHT milk and correlated with the number of lysine residues in the caseins and whey proteins.

5. Acknowledgements

The Danish Agency for Science, Technology and Innovation (DASTI) is thanked for financial support of the study. We thank laboratory technicians Vibeke Mortensen and Betina Hansen at the Arla Strategic Innovation Center for the assistance with the analysis and helpful discussions. Steve Madden and Steve Fischer at Agilent Technologies are thanked for expanding the Profinder software, which enabled the analysis of intact proteins in this study.

6. References


Table 1: Degree of whey protein denaturation and furosine content in UHT milks one day after processing. The values for β-lg are an average of β-lg A and B. Different superscripts in one column indicate significant difference ($P < 0.05$).

<table>
<thead>
<tr>
<th>Milk</th>
<th>Heat treatment</th>
<th>Whey protein denaturation [%]</th>
<th>Furosine (mg/100 g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β-lg</td>
<td>α-la</td>
</tr>
<tr>
<td>Direct A</td>
<td>95°C/5 s + &gt; 150°C /&lt; 0.2 s</td>
<td>86.6 ± 2.1$^a$</td>
<td>26.6 ± 4.0$^a$</td>
</tr>
<tr>
<td>Direct B</td>
<td>95°C/180 s + &gt; 150°C /&lt; 0.2 s</td>
<td>94.1 ± 1.6$^b$</td>
<td>45.5 ± 3.3$^b$</td>
</tr>
<tr>
<td>Indirect</td>
<td>140 °C/4 s</td>
<td>93.6 ± 1.5$^b$</td>
<td>70.0 ± 6.1$^c$</td>
</tr>
</tbody>
</table>
Table 2: Initial lactosylation and rate of lactosylation of reduced caseins in directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 s (Direct A) or 180 s (Direct B) and in indirect UHT milk (140°C for 4 s, Indirect) during storage at 20 °C. Results from linear regression (y=ax+b) shown as average ± 95 % confidence interval. Different superscripts within a row for ‘slope a’ or within a row for ‘initial lactosylation b’ indicate significant difference (P<0.05).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slope a (% month⁻¹)</th>
<th>Initial lactosylation b (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct A</td>
<td>Direct B</td>
<td>Indirect</td>
</tr>
<tr>
<td>αs1-CN</td>
<td>-4.210±0.209a</td>
<td>-3.837±0.216a</td>
<td>-2.730±0.491b</td>
</tr>
<tr>
<td>αs1-CN+lac</td>
<td>3.449±0.216a</td>
<td>3.110±0.221a</td>
<td>1.631±0.380b</td>
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<tr>
<td>αs1-CN+2lac</td>
<td>0.7609±0.055a</td>
<td>0.726±0.039a</td>
<td>1.099±0.181b</td>
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<tr>
<td>αs2-CN</td>
<td>-2.293±0.299a</td>
<td>-2.368±0.846a</td>
<td>-1.385±1.002b</td>
</tr>
<tr>
<td>αs2-CN+lac</td>
<td>1.715±0.210a</td>
<td>1.794±0.582ab</td>
<td>0.590±0.680b</td>
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<tr>
<td>αs2-CN+2lac</td>
<td>0.578±0.136a</td>
<td>0.575±0.295a</td>
<td>0.795±0.344a</td>
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<tr>
<td>β-CN A¹</td>
<td>-2.607±0.132a</td>
<td>-2.892±0.406a</td>
<td>-2.002±0.529a</td>
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<tr>
<td>β-CN A¹+lac</td>
<td>2.337±0.125a</td>
<td>2.537±0.334a</td>
<td>1.367±0.413b</td>
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<td>β-CN A¹+2lac</td>
<td>0.270±0.036a</td>
<td>0.355±0.082a</td>
<td>0.635±0.135b</td>
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<td>β-CN A²</td>
<td>-2.534±0.162abc</td>
<td>-2.076±0.176bc</td>
<td>-1.944±0.614abc</td>
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<tr>
<td>β-CN A²+lac</td>
<td>2.194±0.142a</td>
<td>1.828±0.159b</td>
<td>1.284±0.477b</td>
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<tr>
<td>β-CN A²+2lac</td>
<td>0.340±0.045a</td>
<td>0.247±0.036b</td>
<td>0.660±0.146c</td>
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<tr>
<td>κ-CN</td>
<td>-1.543±0.112a</td>
<td>-1.613±0.143a</td>
<td>-1.280±0.321a</td>
</tr>
<tr>
<td>κ-CN+lac</td>
<td>1.543±0.112a</td>
<td>1.613±0.143a</td>
<td>1.280±0.321a</td>
</tr>
</tbody>
</table>
Table 3: Initial lactosylation and rate of lactosylation of whey proteins in directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 (Direct A) or 180 s (Direct B) and in indirect UHT milk (140°C for 4 s, Indirect) during storage at 20 °C. Results from linear regression (y=ax+b). Different superscripts within a row for ‘slope a’ or within a row for ‘initial lactosylation b’ indicate significant difference (P<0.05).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slope a (% month⁻¹)</th>
<th>Initial lactosylation b (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct A</td>
<td>Direct B</td>
<td>Indirect</td>
</tr>
<tr>
<td>α-la</td>
<td>-2.626±0.580a</td>
<td>-2.352±0.199a</td>
<td>-1.686±0.491a</td>
</tr>
<tr>
<td>α-la +lac</td>
<td>2.626±0.580a</td>
<td>2.352±0.199a</td>
<td>1.686±0.491a</td>
</tr>
<tr>
<td>β-lg A</td>
<td>-4.032±0.461a</td>
<td>-3.313±0.188b</td>
<td>-2.533±0.477c</td>
</tr>
<tr>
<td>β-lg A+lac</td>
<td>2.893±0.462a</td>
<td>2.470±0.185c</td>
<td>1.256±0.287c</td>
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<tr>
<td>β-lg A+2lac</td>
<td>1.140±0.081a</td>
<td>0.842±0.081b</td>
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<tr>
<td>β-lg B</td>
<td>-4.004±0.257a</td>
<td>-3.257±0.328b</td>
<td>-2.408±0.447c</td>
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<tr>
<td>β-lg B+lac</td>
<td>3.355±0.281a</td>
<td>2.745±0.303b</td>
<td>1.479±0.310c</td>
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<tr>
<td>β-lg B+2lac</td>
<td>0.650±0.047a</td>
<td>0.512±0.064b</td>
<td>0.929±0.148a</td>
</tr>
</tbody>
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Figures

Figure 1: Furosine development in directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 (○) or 180 s (●) and in indirect UHT milk (140 °C for 4 s, □) during storage at 20 °C.

Figure 2: Summed mass spectra (retention time 11.0-13.2 min) of unmodified and lactosylated αS1-CN 8 P in indirect UHT milk (140 °C for 4 s) after 6 months of storage at 20 °C. Inset is showing a zoom of the most abundant ions. The m/z shifts due addition of lactose are indicated.
Figure 3: Extracted ion chromatogram of unmodified (solid line), mono-lactosylated (dashed line) and di-lactosylated (dash-dot line) $\alpha_{S1}$-CN 8 P in indirect UHT milk (140 °C for 4 s) after 6 months of storage at 20 °C using the ions shown in Figure 2 (A). Corresponding UV signal at 214 nm of $\alpha_{S1}$-CN 8 P and 9 P after 0 (solid line) and 6 (dashed line) months of storage at 20 °C (B).

Figure 4: Deconvoluted mass spectrum of $\alpha_{S1}$-CN 8 P in indirect UHT milk (140 °C for 4 s) after 0 (A) and 6 (B) months of storage at 20 °C. Mass shifts due to addition of lactose are indicated.
Fig 5: Absolute areas (top row) and area distribution (bottom row) of unmodified and lactosylated α\textsubscript{S1}-CN 8 P in directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 (A) or 180 s (B) and in indirect UHT milk (140 °C for 4 s, C) during storage at 20 °C.
Figure 6: Correlation of furosine content and % of unmodified protein for α-la (A), β-lg A (B), β-CN A^2 (C) and αS1-CN 8 P (D) from directly heated UHT milk (> 150 °C for < 0.2 s) pre-heated at 95 °C for 5 (●) or 180 s (○) and in indirect UHT milk (140 °C for 4 s, □). The results of a linear regression are shown.
1. Summed mass spectra of unmodified and lactosylated caseins in indirect UHT milk (140 °C for 4 s) after 6 months of storage at 20 °C. Inset is showing a zoom of the most abundant ions. The m/z shifts due addition of lactose are indicated.
2. Absolute and relative change in MS area of reduced α$_{s_2}$-CN during storage in Direct A (A), Direct B (B) and Indirect (C).

3. Absolute and relative change in MS area of reduced β-CN during storage in Direct A (A), Direct B (B) and Indirect (C).
3. Absolute and relative change in MS area of reduced κ-CN during storage in Direct A (A), Direct B (B) and Indirect (C).

4. Absolute and relative change in MS area of reduced α-la during storage in Direct A (A), Direct B (B) and Indirect (C).
5. Absolute and relative change in MS area of reduced β-lg during storage in Direct A (A), Direct B (B) and Indirect (C).

6. Development of summed intensity of unmodified and lactosylated reduced α\textsubscript{s1}-CN 8 P using EIC area, deconvolution intensity, profinder area and UV area at 214 nm of α\textsubscript{s1}-CN 8 P and 9 P in indirect UHT milk (140 °C for 4 s) during storage at 20 °C.